

# Ectopic RNase E sites promote bypass of 5'-end-dependent mRNA decay in *Escherichia coli*

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## Summary

**In *Escherichia coli*, 5'-terminal stem-loops form major impediments to mRNA decay, yet conditions that determine their effectiveness or the use of alternative decay pathway(s) are unclear. A synthetic 5'-terminal hairpin stabilizes the *rpsT* mRNA sixfold. This stabilization is dependent on efficient translational initiation and ribosome transit through at least two-thirds of the coding sequence past a major RNase E cleavage site in the *rpsT* mRNA. Insertion of a 12–15 residue 'ectopic' RNase E cleavage site from either the *rne* leader or 9S pre-rRNA into the 5'-non-coding region of the *rpsT* mRNA significantly reduces the stabilizing effect of the terminal stem-loop, dependent on RNase E. A similar insertion into the *rpsT* coding sequence is partially destabilizing. These findings demonstrate that RNase E can bypass an interaction with the 5'-terminus, and exploit an alternative 'internal entry' pathway. We propose a model for degradation of the *rpsT* mRNA, which explains the hierarchy of protection afforded by different 5'-termini, the use of internal entry for bypass of barriers to decay, 'ectopic sites' and the role of translating ribosomes.**

## Introduction

Regulation of mRNA stability provides a potent mechanism for governing gene expression. In bacteria, mRNA decay not only expedites adaptation of patterns of protein synthesis to changing environmental conditions, but also enables ribonucleotide salvage and differential expression of genes encoded by polycistronic mRNAs (Grunberg-Manago, 1999; Rauhut and Klug, 1999; Régnier and Arraiano, 2000). In *Escherichia coli*, the degradation of many, if not most, mRNAs is initiated by an endonucle-

olytic cleavage catalysed by RNase E (Cohen and McDowall, 1997; Coburn and Mackie, 1999; Carpousis *et al.*, 1999; Steege, 2000). Products of this initial cleavage become substrates for additional RNase E cleavages as well as for digestion by the 3' → 5' exonucleases, polynucleotide phosphorylase (PNPase) and RNase II (Donovan and Kushner, 1986; Coburn and Mackie, 1999).

RNase E was initially characterized as an activity required for the processing of 9S rRNA (Ghora and Apirion, 1978). Inactivation of temperature-sensitive *rne* mutants leads not only to accumulation of improperly processed stable RNAs (Ghora and Apirion, 1978; Li *et al.*, 1999; Ow and Kushner, 2002), but also to increased lifetimes for individual mRNAs, as well as for bulk mRNA (Ono and Kuwano, 1979; Cohen and McDowall, 1997; Coburn and Mackie, 1999). Despite its established role in the initiation of mRNA decay, RNase E appears to lack strict sequence specificity and cleaves single-stranded RNA predominantly 5' to AU dinucleotides (Mackie, 1991; Mackie and Genereaux, 1993; McDowall *et al.*, 1994). Although mRNA half-lives vary significantly in *E. coli*, there is no correlation between mRNA length and half-life, suggesting that mechanisms other than dinucleotide recognition by RNase E dictate the range of stabilities exhibited by mRNAs (Cohen and McDowall, 1997). In this regard, the unusual stability of the *ompA* mRNA provides a striking example of how the initiation of mRNA decay can be modulated independently of RNA sequence (Belasco *et al.*, 1986; Melefors and von Gabain, 1988). The *ompA* mRNA contains a thermodynamically stable, conserved 5'-terminal stem-loop, which is necessary and sufficient to confer stability to the entire mRNA, or to heterologous labile mRNAs to which it is fused (Emory and Belasco, 1990; Hansen *et al.*, 1994). Interestingly, this stabilization is independent of the sequence of the 5'-stem-loop, as a synthetic hairpin structure is essentially equally effective (Bouvet and Belasco, 1992; Emory *et al.*, 1992; Arnold *et al.*, 1998). However, as few as four unpaired residues 5' to the stem-loop can abolish its protective effect (Emory *et al.*, 1992; Bouvet and Belasco, 1992; Arnold *et al.*, 1998). The intrinsic dependence of RNase E activity on a single-stranded 5'-terminus ('5'-end-dependence') offers a plausible explanation for the protection afforded by 5'-stem-loops or circularization (Mackie, 1998; 2000). Nonetheless, this property of RNase E does not fully explain the wide range of mRNA half-lives for mRNAs possessing no known 5'-barrier to

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decay, or provide a mechanism for the ultimate decay of mRNAs possessing a 5'-terminal stem-loop.

The functional association of mRNA with ribosomes necessarily implicates translation in modulating mRNA longevity and its accessibility to RNase E. Although protection of mRNA by ribosomes is not universal, numerous observations have correlated translational efficiency with mRNA stability, presumably through the masking of initiating cleavage sites by bound ribosomes (Yarchuk *et al.*, 1991; Petersen, 1993; Iost and Dreyfus, 1995; Braun *et al.*, 1998). Somewhat surprisingly, translation also appears to be required to stabilize mRNAs which contain intrinsic protective features. For example, in the *ompA* mRNA, a threshold level of ribosome passage through the protein-coding region is necessary for its stability (Arnold *et al.*, 1998). Furthermore, *lacZ* mRNA containing an artificial 5'-stem-loop is no longer protected against RNase E-initiated degradation in the absence of translation (Lopez and Dreyfus, 1996; Joyce and Dreyfus, 1998). This raises a conundrum: if initiation of mRNA decay by RNase E occurs via a rate-limiting 5'-end-dependent mechanism, how does translation influence the stability of mRNAs, and particularly those with 5'-terminal stem-loop structures?

The small, monocistronic *rpsT* mRNA whose properties are well characterized has provided many insights into the mechanisms of mRNA degradation (Coburn and Mackie, 1999; Goodrich and Steege, 1999). In particular, cleavage by RNase E between residues 300 and 301 in the RNA sequence (between codons 55 and 56) can initiate its decay (Mackie, 1991). Moreover, this mRNA demonstrates 5'-end-dependent decay *in vivo* (Mackie, 2000). In this report, we have placed a stable hairpin structure at the extreme 5'-terminus of the *rpsT* mRNA and have investigated how this barrier to decay can be overcome by RNase E *in vivo*. We show that the 5'-protective barrier is ineffective without efficient translational initiation or ribosome translocation past the rate-limiting RNase E site. Most importantly, we demonstrate that short, 12–15 nt insertions based on known RNase E cleavage sites ('ectopic sites'), can largely or fully overcome the otherwise efficient 5'-protective RNA structure. This provides clear evidence for two modes of initial substrate recognition by RNase E: one involving 5'-end recognition (Mackie, 1998; 2000), and a second which we term 'bypass' or 'internal entry'.

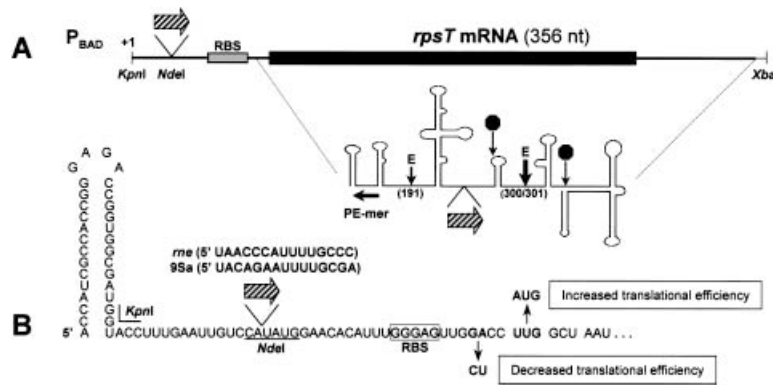
## Results

### *Regulated expression and stability of chimeric rpsT mRNA*

To facilitate the analysis of sequence or structural changes on the kinetics of *rpsT* mRNA decay, recombi-

nant *rpsT* mRNA was expressed from a modified low-copy-number vector based on pBAD28 which contains the arabinose-inducible promoter, P<sub>BAD</sub> (Guzman *et al.*, 1995). To avoid the inclusion of vector-encoded sequences in the expressed mRNA, pBAD28 was altered by site-directed mutagenesis to introduce a unique *KpnI* restriction site at the exact point of P<sub>BAD</sub> transcriptional initiation, creating pKEB106 (see *Experimental procedures*). Residues 91–447 of the endogenous *rpsT* gene, beginning at the site of initiation of the second (P2) of two tandem promoters, and including the native, rho-independent transcriptional terminator at the 3'-end, were cloned into pKEB106 to generate pKEB107, the parental plasmid for all further gene constructions. The *rpsT* mRNA expressed from P<sub>BAD</sub> in pKEB107 (Fig. 1A) was undistinguishable in size from the 356 nt chromosomally encoded *rpsT* P2 mRNA (data not shown; Mackie and Parsons, 1983). Recombinant mRNAs transcribed from the P<sub>BAD</sub> promoter will be designated according to their template; thus, *rpsT*(107) mRNA is the *rpsT* mRNA encoded by pKEB107. To confirm that expression of the cloned *rpsT* gene in pKEB107 was under catabolite repression by glucose and inducible by arabinose (Schleif, 1996), RNA was isolated from MG1693 (wild type) containing pKEB107 grown in the presence of glucose or arabinose. Plasmid-encoded *rpsT*(107) mRNA was not detectable in MG1693/pKEB107 grown in glucose; in contrast, it was induced two- to fivefold relative to the endogenous *rpsT* P2 mRNA after growth of cells in 0.05% arabinose for 60 min (Fig. 2A; compare ★ and P2 in lanes 1 and 2). The kinetics of decay of the *rpsT* mRNAs in MG1693/pKEB107 was evaluated by Northern blot analysis of RNA samples isolated after blocking transcriptional initiation with rifampicin. The half-lives of the coincident P2 and *rpsT*(107) mRNAs were 2 min (Fig. 2D), identical to that of the chromosomally encoded P2 *rpsT* mRNA in glucose-grown MG1693/pKEB107 or in untransformed MG1693 (Table 1, entries 1 versus 2; Mackie, 1987). In addition, the *rpsT* P1 mRNA transcribed from the endogenous, upstream promoter decayed with a half-life of 1.5 min, identical to published values (Mackie, 1987). Maintenance of wild-type half-lives suggests that transient expression of plasmid-encoded *rpsT*(107) mRNA does not alter the kinetics of *rpsT* mRNA decay during the experiment.

We created a stable 5'-terminal secondary structure in the *rpsT* mRNA (cf. Bouvet and Belasco, 1992) by inserting complementary oligodeoxynucleotides into the unique *KpnI* site in pKEB107 (see Fig. 1A and *Experimental procedures*). The resulting chimeric *rpsT* gene in pKEB110 should encode an mRNA with a 14 bp stem-loop closed by a GNRA tetra-loop at its extreme 5'-terminus (overall  $\Delta G \leq 30$  kCal mol<sup>-1</sup>; Fig. 1B). Formation of the desired secondary structure was confirmed by structure mapping (see Fig. 4 below, and data not shown). Northern blot



**Fig. 1.** Maps of plasmid-encoded *rpsT* mRNAs.

**A.** Schematic representation of the 356 nt *rpsT* mRNA expressed from the *P<sub>BAD</sub>* promoter in pKEB107, the parental plasmid for all *rpsT* gene constructions (see text and *Experimental procedures*). The ribosomal binding site (RBS; grey box), coding region (black box), single stranded 5'-UTR, rho-independent transcriptional terminator, and experimentally determined secondary structure (Mackie, 1992) are shown. Modifications introduced into the plasmid-encoded *rpsT* mRNA include: 'ectopic' RNase E cleavage sites in the 5'-UTR or open reading frame (ORF) (broad, striped arrow), and premature termination codons (black octagons). Positions of previously characterized major RNase E cleavage sites ('E' vertical arrows) and the region complementary to the oligonucleotide used in primer extension analysis (horizontal arrow labelled 'PE-mer') are also shown.

**B.** Nucleotide sequence of the 5'-region of the *rpsT* mRNA expressed from pKEB110. Residues encompassing either the *rne* or 9Sa 'ectopic sites' in their native orientation (broad, striped arrow) were ligated into the *NdeI* site of several pKEB110 derivatives (see Table 1). Sequence modifications that change the translational efficiency of the *rpsT* mRNA are shown.

analysis of RNA from MG1693/pKEB110 revealed an *rpsT* mRNA of intermediate size (*rpsT*(110) mRNA; 388 nt [★]) expressed from the plasmid (Fig. 2B), and, at reduced levels, the two chromosomally encoded *rpsT* transcripts of 447 nt and 356 nt (P1 and P2, respectively). The half-life of the *rpsT*(110) mRNA was 12 min (Fig. 2D; Table 1, entry 3), corresponding to a sixfold stabilization relative to *rpsT*(107) mRNA (Fig. 2D; Table 1, entry 2). This value is consistent with the extent of stabilization obtained with

similar heterologous fusions of stem-loop structures to other mRNAs (Emory and Belasco, 1990; Bouvet and Belasco, 1992; Hansen *et al.*, 1994). Unexpectedly, the *rpsT*(110) mRNA decayed with biphasic kinetics, with a second, more rapid decay phase commencing 8–9 min after addition of rifampicin (see *Discussion*). Henceforth, the half-lives of the mRNAs related to the *rpsT*(110) mRNA will be determined from the initial rates (i.e. the first phase) of disappearance of the mRNA.

**Table 1.** Decay rates of recombinant *rpsT* mRNA in *Escherichia coli*.

Entry	Plasmid	Recombinant <i>rpsT</i> mRNA feature				Translation <sup>b</sup>	Half-life (min) <sup>c</sup>
		Length (nt)	5'-stem-loop	'Ectopic site' <sup>a</sup>			
1	pBAD28	NA <sup>d</sup>	NA	NA	NA	2 <sup>e</sup>	
2	pKEB107	356	–	–	UUG	2.0 ± 0.2	
3	pKEB110	388	+	–	UUG	12 ± 2	
4	pKEB127	403	+	<i>rne</i> →	UUG	1.0 ± 0.2	
5	pKEB128	403	+	<i>rne</i> ←	UUG	13 ± 2	
6	pKEB152	403	+	9Sa →	UUG	3.0 ± 1	
7	pKEB154	403	+	9Sa ←	UUG	1.0 ± 0.5	
8	pKEB138	403	+	–	<i>rne</i> →	UUG	2.5 ± 0.5
9	pKEB142	403	+	–	<i>rne</i> ←	UUG	10 ± 1
10	pKEB145	403	+	–	<i>rne</i> →	–3, –4	1.5 ± 0.5
11	pKEB146	403	+	–	<i>rne</i> →	AUG	15 ± 1
12	pKEB143	403	+	–	–3, –4	1.5 ± 0.2	
13	pKEB144	403	+	–	AUG	>20	
14	pKEB136	388	+	–	Stop @285	2.0 ± 0.5	
15	pKEB147	388	+	–	Stop @337	7.0 ± 1.5	

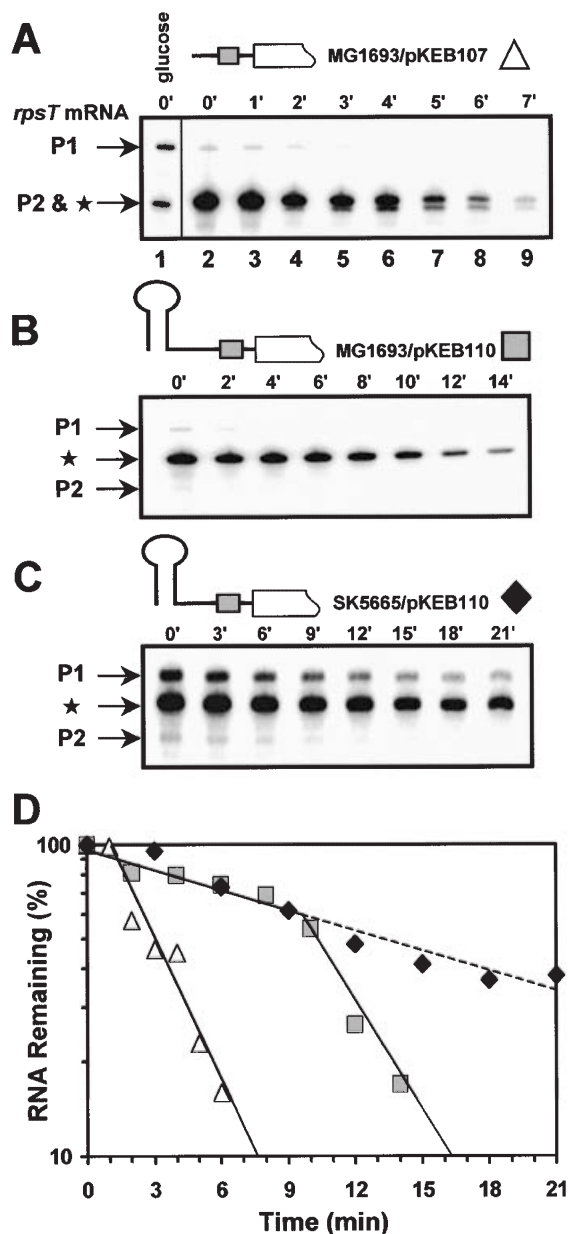
**a.** Sequence of 'ectopic sites': *rne* →, 5'-UAACCCAUUUUGCCC-3'; *rne* ←, 5'-UAGGGCAAAAUGGGU-3'; 9Sa →, 5'-UACAGAAUUUUGCGA-3'; and 9Sa ←, 5'-UAUCGCAAAAUUCUG-3'. The first column refers to sites in the 5'-UTR; the second to sites in the coding region.

**b.** UUG, wild-type translational initiation sequence; AUG, mutation increasing translational initiation; –3, –4, mutation decreasing translation initiation; stop, position of premature stop codon (nucleotide position based on Mackie, 1992).

**c.** Values represent the average of at least four measurements.

**d.** Empty pBAD28 vector.

**e.** Half-life of the chromosomally encoded *rpsT* P2 mRNA (Mackie, 1987).



**Fig. 2.** Stabilization of plasmid-borne *rpsT* mRNA by a 5'-terminal stem-loop. Northern blot analysis was performed on 5  $\mu$ g samples of RNA isolated from derivatives of MG1693 or SK5665 extracted at various times (in minutes; above each panel) after the addition of rifampicin (see *Experimental procedures*). The source of RNA and a schematic diagram of the 5'-end of the plasmid-encoded *rpsT* mRNA (5'-UTR, horizontal line; RBS, shaded box; ORF, open box) are given above each panel. Positions of the chromosomally encoded *rpsT* mRNAs, P1 (447 nt) and P2 (356 nt), or plasmid-encoded *rpsT* mRNAs (P2 and ★ respectively; lanes 2–9).

A. The decay of *rpsT* mRNA from MG1693/pKEB107 grown in the presence of glucose (lane 1) or arabinose (lanes 2–9). The plasmid-encoded *rpsT*(107) mRNA and the chromosomally encoded P2 *rpsT* mRNA comigrate (P2 and ★ respectively; lanes 2–9).

B. Decay of *rpsT* mRNA from MG1693/pKEB110. The plasmid-encoded *rpsT*(110) mRNA (★) is 388 nt.

C. Decay of *rpsT* mRNA from SK5665/pKEB110 (*rne-1*).

D. Plot of the first-order decay of plasmid-encoded *rpsT* mRNA versus time. Symbols used for each mRNA are indicated in A–C.

We determined the effect of several mutations affecting RNA metabolism on the stability of the *rpsT*(110) mRNA (see *Experimental procedures*) to confirm that this mRNA retains the same dependencies as the endogenous *rpsT* mRNAs. In strain SK5665, which is temperature-sensitive for RNase E activity, the endogenous *rpsT* P1 mRNA from SK5665 decayed with a half-life of 6 min, fourfold slower than the wild-type rate, demonstrating the sensitivity of the endogenous *rpsT* mRNA to RNase E (Fig. 2C; Mackie, 1991). The *rpsT*(110) mRNA decayed with a half-life of 13 min (Fig. 2C), a value not significantly different from the rate in MG1693 (Table 1, entry 3). However, unlike the situation in MG1693, the kinetics of decay of *rpsT*(110) mRNA in SK5665 were clearly monophasic, with no break in the decay curve even after 21 min of exposure to rifampicin. The kinetics of decay of *rpsT*(110) in isogenic strains with mutations in *pnp*, *rnb*, *pcnB* or *rng*, encoding PNPase, RNase II, poly(A) polymerase, and RNase G, respectively, were not altered from that observed in MG1693 (data not shown). Therefore, none of these enzymes can play a role in initiating the decay of the *rpsT*(110) mRNA.

#### *Degradation of 5'-protected rpsT mRNA is sensitive to translational efficiency*

As the *rpsT*(110) mRNA is relatively resistant to decay, we tested whether its stability is still influenced by translational efficiency, a major determinant of the lifetime of the endogenous *rpsT* mRNAs (Wirth *et al.*, 1982; Parsons *et al.*, 1988). Previously characterized mutations that vary the efficiency of translational initiation in the *rpsT* mRNA were introduced into pKEB110 (Fig. 1B). Altering the context three and four residues 5' to the UUG initiation codon (GA  $\rightarrow$  CU; pKEB110  $\rightarrow$  pKEB143) decreases translational efficiency sixfold, whereas changing the UUG initiation codon to AUG (pKEB110  $\rightarrow$  pKEB144) increases translational efficiency 2.5-fold (Parsons *et al.*, 1988). The half-life of the *rpsT*(143) mRNA was 1–2 min, effectively abolishing any protective effect of the 5'-stem-loop structure (Fig. 3A and E; Table 1, entry 12). In contrast, the half-life of the *rpsT*(144) mRNA was greater than 25 min (Fig. 3B and E; Table 1, entry 13). Interestingly, the rapidly decaying *rpsT*(143) mRNA displayed uniphasic rather than biphasic decay kinetics, whereas the onset of the rapid second phase of decay of the very stable *rpsT*(144) mRNA was delayed by 2–3 min in comparison to *rpsT*(110), presumably reflecting the increase in translational efficiency (see *Discussion*).

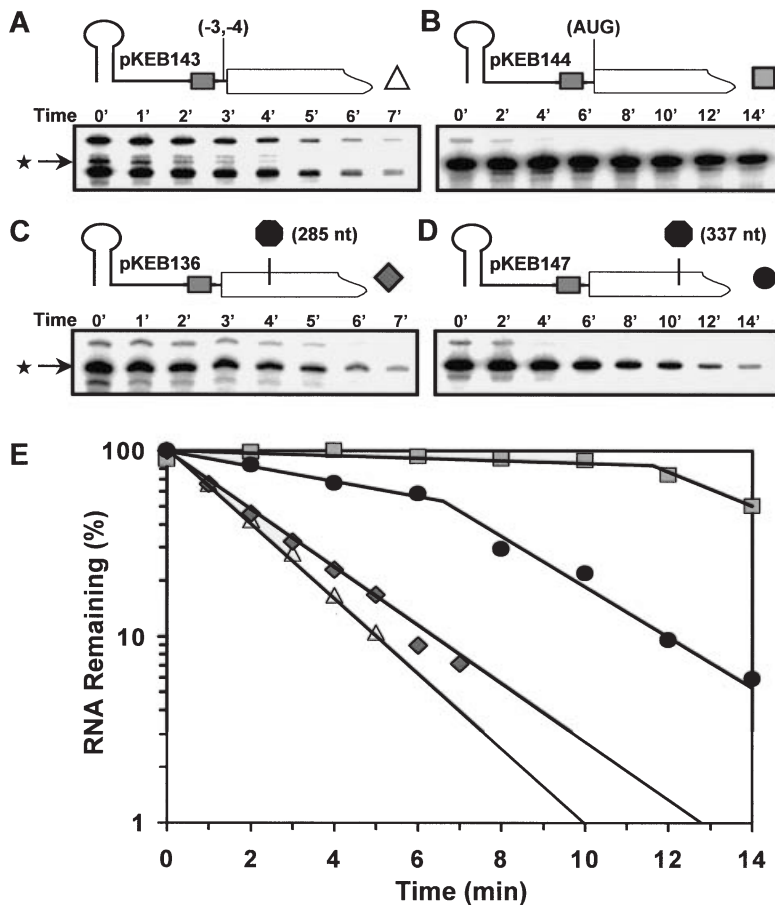
To determine whether translation initiation *per se*, or ribosome transit through the coding region governs the stability of *rpsT*(110) and its derivatives, we introduced premature termination codons into pKEB110 (Fig. 1A; see also *Experimental procedures*). A stop codon placed at

residue 285 (codon 52) in pKEB136 occurs 16 nt 5' to the major RNase E cleavage site at residues 300/301 (between codons 55 and 56). In pKEB147, the stop codon was introduced at residue 337 (codon 69), 36 nt 3' to the same major cleavage site. Neither nucleotide change was predicted to alter the secondary structure of the *rpsT* mRNA (Mackie, 1992). The half-life of the *rpsT*(136) mRNA with the proximal stop codon was 2 min (Fig. 3C and E; Table 1, entry 14), a sixfold decrease relative to the parental *rpsT*(110) mRNA. However, the half-life of the *rpsT*(147) mRNA (distal stop codon) was 7 min, a more modest reduction (Fig. 3D and E; Table 1, entry 15). Thus, the stability of stem-loop-protected *rpsT* mRNA is enhanced by passage of ribosomes beyond the major internal RNase E cleavage site in the *rpsT* mRNA; merely loading ribosomes does not suffice.

*'Ectopic' RNase E cleavage sequences destabilize 5'-terminal stem-loop-protected rpsT mRNA*

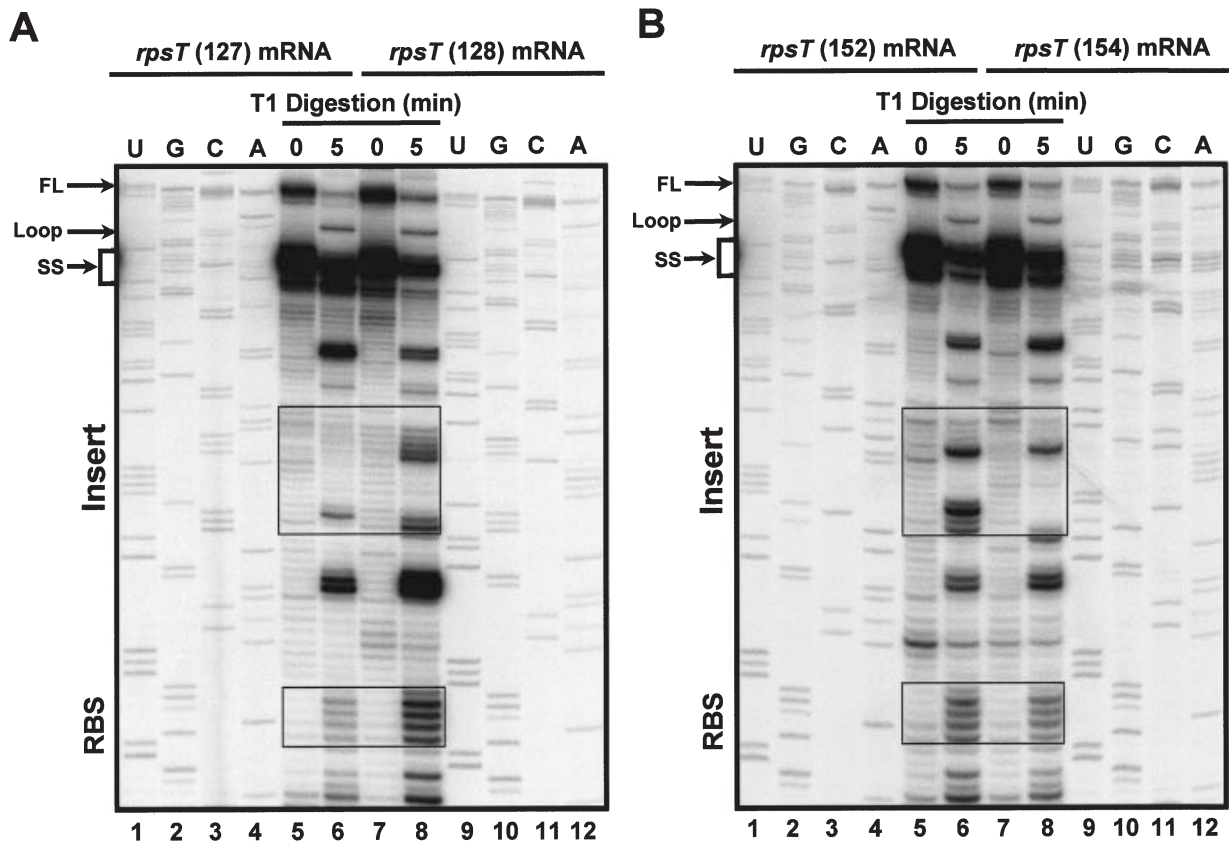
We postulated that a synthetic sequence containing a known or potential RNase E cleavage site might be suffi-

cient to destabilize a stem-loop-protected *rpsT* mRNA if inserted at a site available for recognition and cleavage. As prototypes for such 'ectopic sites', we used 15 nt sequences encompassing one of two well characterized RNase E cleavage sites: a defined cleavage site within the *rne* leader (Jain and Belasco, 1995; 'the *rne* recognition sequence') or the 9S ribosomal RNA 'a' site (Ghora and Apirion, 1978). Each site was inserted as complementary oligodeoxynucleotides into the *Nde*I site in the 5'-UTR of *rpsT* in pKEB110 (Fig. 1B), in both native or reverse-complement orientations. We thereby generated two plasmid pairs: pKEB127 (*rne*-native) and pKEB128 (*rne*-reverse-complement); and pKEB152 (9Sa-native) and pKEB154 (9Sa-reverse-complement). Structure mapping was used to evaluate the reactivity of residues in the 5'-UTR of *rpsT* mRNAs containing 'ectopic sites' to control for possible structural rearrangements. To eliminate interference from endogenous *rpsT* mRNA, strain MRA10, deleted for chromosomal *rpsT* (Rydén-Aulin *et al.*, 1993), was transformed with plasmids pKEB127, 128, 152 or 154, and RNA was isolated from arabinose-induced cultures. The resistance to cleavage of G-residues in the 5'-terminal stem-loop confirmed the formation of the pre-



**Fig. 3.** Effect of translational initiation efficiency and stop-codon position on *rpsT* mRNA stability. Total RNA (5 µg), extracted from MG1693 containing pKEB143 (A), pKEB144 (B), pKEB136 (C), or pKEB147 (D) was analysed on Northern blots as described in Fig. 2 and probed for *rpsT* mRNAs. The time of sampling (in min), the source of the mRNA and a schematic diagram of the 5'-end of the plasmid-encoded *rpsT* mRNA are indicated above each panel (also see Fig. 1). Mutations decreasing (-3, -4) or increasing (AUG) translational initiation, or the position (in nucleotides) of premature stop codons within the plasmid-encoded *rpsT* mRNAs are also shown. Position of the plasmid-encoded *rpsT* mRNA is indicated (★) to the left of the autoradiograms.

E. Plot of first-order decay of plasmid-encoded *rpsT* mRNA. Symbols used for each mRNA are indicated beside the mRNA schematics in A-D.



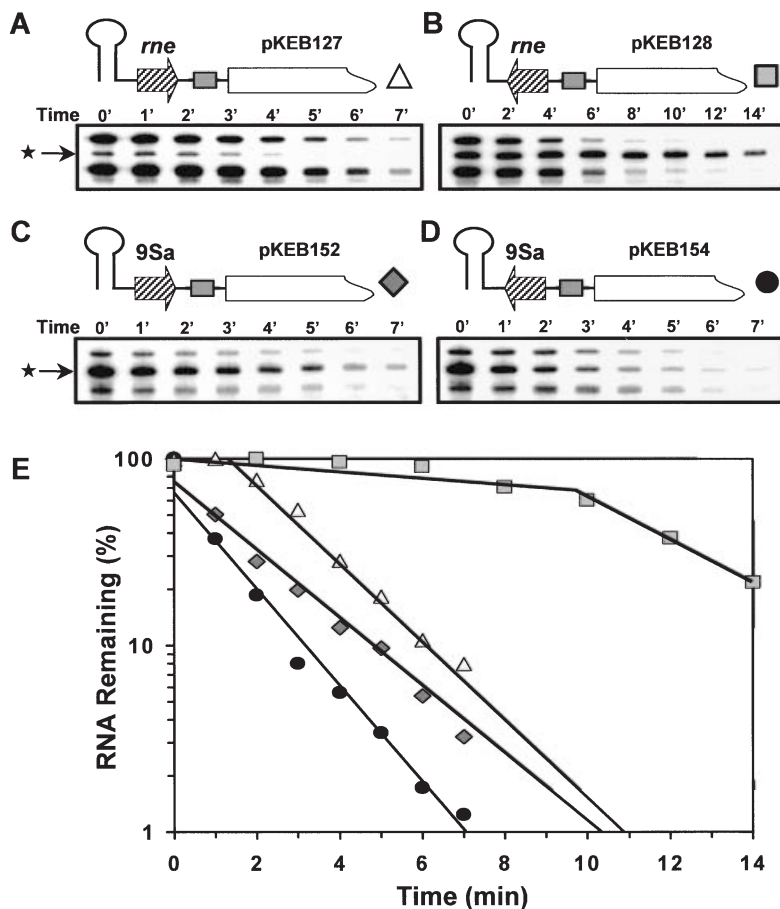
**Fig. 4.** Structure mapping of the 5'-leader in modified *rpsT* mRNAs. RNA was prepared from arabinose-induced cultures of MRA10 containing the appropriate plasmid and subjected to partial digestion with RNase T1. Primer extension analysis using radiolabelled PE-mer (see Fig. 1) was performed as described in *Experimental procedures*. Extension products are shown in lanes 5–8 of each panel alongside the corresponding sequence ladders in lanes 1–4 and 9–12. Regions of interest in lanes 5–8 are boxed and/or indicated in the left margin with the following symbols: FL, full length cDNA; loop, position of the GAGA tetraloop (see Fig. 1B); SS, truncated cDNAs due to a strong stop at the 3'-base of the terminal hairpin (see Fig. 1B); insert, 'ectopic' RNase E site; RBS, ribosome binding site (5'-GGGAG-3').

A. Partial T1 digestion of stem-loop-protected *rpsT* mRNA containing the *rne* cleavage sequence (see Fig. 1B) in the native (*rpsT*(127); lanes 5 and 6 or reverse-complement orientation (*rpsT*(128); lanes 7 and 8).

B. Partial T1 digestion of stem-loop-protected *rpsT* mRNA containing the 9Sa cleavage sequence (see Fig. 1B) in the native (*rpsT*(152); lanes 5 and 6 or reverse-complement orientation (*rpsT*(154); lanes 7 and 8).

dicted structure in *rpsT*(127), *rpsT*(128), *rpsT*(152), and *rpsT*(154) mRNAs (Fig. 4A, lanes 6 and 8 and Fig. 4B, lanes 6 and 8 respectively). The data also revealed that all G residues within the 'ectopic sites' were accessible to RNase T1 (Fig. 4A and B: box marked 'insert' in lanes 5–8). However, G residues in the ribosomal binding site (5'-GGGAG) of *rpsT*(127) mRNA (Fig. 4A, lane 6, box marked RBS) were less susceptible to T1 cleavage than the same residues in *rpsT*(128), *rpsT*(152) and *rpsT*(154) mRNAs. We conclude that the 5'-UTRs of *rpsT*(128), *rpsT*(152) and *rpsT*(154) mRNAs distal to the 5'-stem-loop are single-stranded, as is the equivalent region in the endogenous *rpsT* P2 mRNA (Mackie, 1992). In contrast, the 15 nt *rne* insertion in *rpsT*(127) mRNA subtly alters the secondary structure of the 5'-UTR to reduce the single-strandedness of residues encompassing its Shine-Dalgarno sequence.

The effect of the presence of 'ectopic sites' on the stability of the appropriate mRNA was measured by Northern blotting. The half-life of the *rpsT*(127) mRNA (*rne*-native) was 1 min, a 12-fold reduction compared with the parental *rpsT*(110) mRNA (Fig. 5A and E; Table 1, entry 4). In contrast, *rpsT*(128) mRNA (*rne*-reverse-complement) decayed with a half-life of 13 min, essentially identical to that of the parental *rpsT*(110) mRNA (Fig. 5B and E; Table 1, entries 5 versus 3). In the pair of strains containing plasmids with the 9Sa site, the *rpsT*(152) mRNA (native orientation) decayed with a half-life of 3 min (Fig. 5C and E; Table 1, entry 6), representing a moderate (fourfold) destabilization of the *rpsT*(152) relative to its parent, *rpsT*(110) mRNA. Somewhat surprisingly, the half-life for *rpsT*(154) mRNA (9Sa-reverse-complement) was 1 min (Fig. 5D and E; Table 1, entry 7). Inspection of the reverse-complement 9Sa sequence (5'-UAUCGCAAAA



**Fig. 5.** Effect of 'ectopic RNase E sites' on *rpsT* mRNA stability. Total RNA (5  $\mu$ g), extracted from MG1693 containing pKEB127 (A), pKEB128 (B), pKEB152 (C) or pKEB154 (D) was analysed on Northern blots as described in Fig. 2 and probed for *rpsT* mRNAs. The time of sampling (in min), the source of the mRNA and a schematic of the 5'-end of the plasmid-encoded *rpsT* mRNA are indicated above each panel (see also Fig. 1). The source and orientation of the 'ectopic site' (wide, hatched arrow) within the *rpsT* 5'-UTR are highlighted in the schematic representations. The position of the plasmid-encoded *rpsT* mRNA is indicated (★) at left of the autoradiograms. E. Plot of first-order decay of plasmid-encoded *rpsT* mRNA. Symbols used for each recombinant mRNA are indicated beside the mRNA schematics in A–D.

UUCUG) reveals two AU dinucleotides commonly found in consensus RNase E cleavage sites (Ehretsmann *et al.*, 1992; McDowall *et al.*, 1994). Moreover, RNase E can cleave antisense 9S rRNA *in vitro* at this sequence with high efficiency (Cormack and Mackie, 1992).

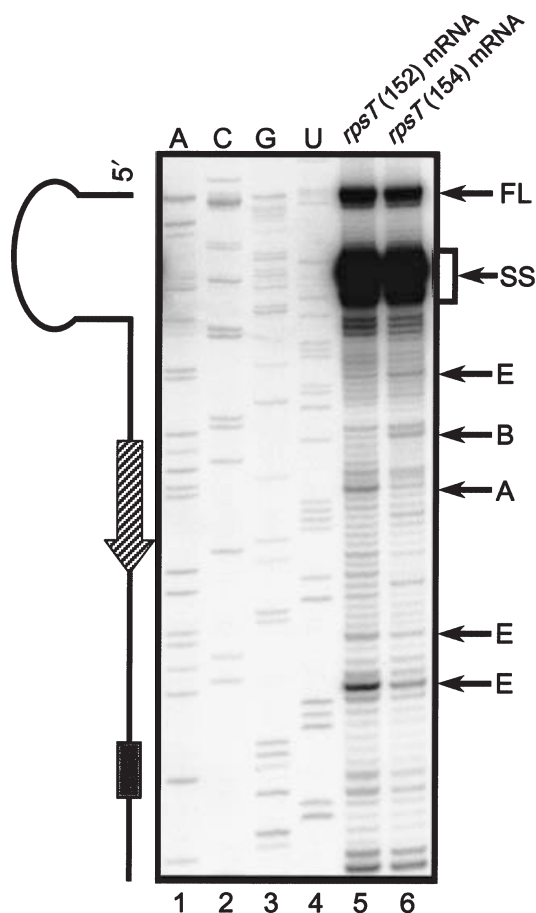
We attempted to detect if the inserted 'ectopic sites' are cleaved by RNase E by mapping the anticipated degradative intermediates using primer extension (see *Experimental procedures*). Analysis of *rpsT*(152) mRNA (9Sa-native orientation) revealed a 5'-terminus within the 9Sa site corresponding to the exact position of cleavage by RNase E in 9S RNA (AGA $\downarrow$ AUUUUG; Fig. 6, lane 5, arrow A; Ghora and Apirion, 1978). Likewise, examination of *rpsT*(154) mRNA showed an extension product corresponding to an endonucleolytic cleavage 5' to an AU dinucleotide in the reverse-complement 9Sa insertion sequence (Fig. 6, lane 6, arrow B). As expected, in addition to some minor products, several previously characterized RNase E cleavage sites within the *rpsT* 5'-UTR (Mackie, 1991) were also detected in both *rpsT*(152) and *rpsT*(154) mRNAs (Fig. 6, lanes 5 and 6; 'E' arrows). We also examined *rpsT* mRNAs containing the *rne* leader site by similar methods. A minor primer extension product

terminating at a position 5' to an AU dinucleotide within the site in *rpsT*(127) mRNA was weakly detectable (data not shown). In contrast, no unique products terminating within the *rne* site in the reverse-complement orientation (*rpsT*(128) mRNA) could be detected (not shown).

As an additional test of whether 'ectopic sites' mediate cleavage by RNase E, we measured the kinetics of decay of *rpsT*(152) and *rpsT*(154) mRNA in SK5665 (*rne*-1). After thermal inactivation of RNase E in SK5665, the half-lives of both the *rpsT*(152) and *rpsT*(154) mRNAs were approximately 9 min, representing a three- to sixfold stabilization (data not shown). Taken together, these data demonstrate that insertion of synthetic RNase E recognition sites into the 5'-UTR of the *rpsT* mRNA can overcome the stability conferred by a 5'-terminal stem-loop. This effect requires RNase E activity and correlates with observable cleavages within the 'ectopic sites'.

#### *mRNA destabilization by an 'ectopic site' in the rpsT open reading frame (ORF)*

We tested whether an 'ectopic site' can destabilize the *rpsT* mRNA when placed within its coding sequence



**Fig. 6.** Detection of RNase E cleavages by primer extension analysis. Primer extension was performed on RNA isolated from MRA10 containing either pKEB152 or pKEB154 (see Table 1) and grown in arabinose (see *Experimental procedures*). A schematic representation of *rpsT*(152) mRNA is shown in the left margin; the positions of the 5'-terminal stem-loop, 9Sa 'ectopic' cleavage sequence (broad, striped arrow), and ribosome binding site (RBS; black box) correspond to their positions in the sequence ladder. The position of full length cDNAs (FL) or cDNAs terminated at the base of the 5'-terminal stem (SS; strong stop) are indicated by corresponding arrows. 'E' arrows denote extension products corresponding to previously characterized RNase E cleavage sites (Mackie, 1991). Extension products obtained exclusively from *rpsT*(152) or *rpsT*(154) mRNA analysis are highlighted by an 'A' or 'B'-labelled arrow respectively. Lanes 1–4, sequence ladder; lane 5, cDNAs from *rpsT*(152) mRNA (9Sa native orientation); lane 6, cDNAs from *rpsT*(154) mRNA (9Sa reverse complement orientation).

rather than in the 5'-UTR. The *rne* 'ectopic site' was cloned into pKEB110 in both native (pKEB138) and reverse-complement (pKEB142) orientations, 145 residues downstream of the start codon into a single-stranded region of the *rpsT* mRNA (Fig. 1A; see *Experimental procedures*). At this position, it is well removed from all known RNase E cleavage sites, and does not disrupt the *rpsT* translational reading frame. The half-life of *rpsT*(138) mRNA containing this site in the native orientation was 3 min, a

3.5-fold reduction relative to *rpsT*(110) mRNA (Fig. 7A and E; Table 1, entries 8 versus 3). In contrast, the half-life of the *rpsT*(142) mRNA (reverse-complement orientation) was 10 min, essentially identical to that of the parental mRNA (Fig. 7B and E; Table 1, entries 9 and 3).

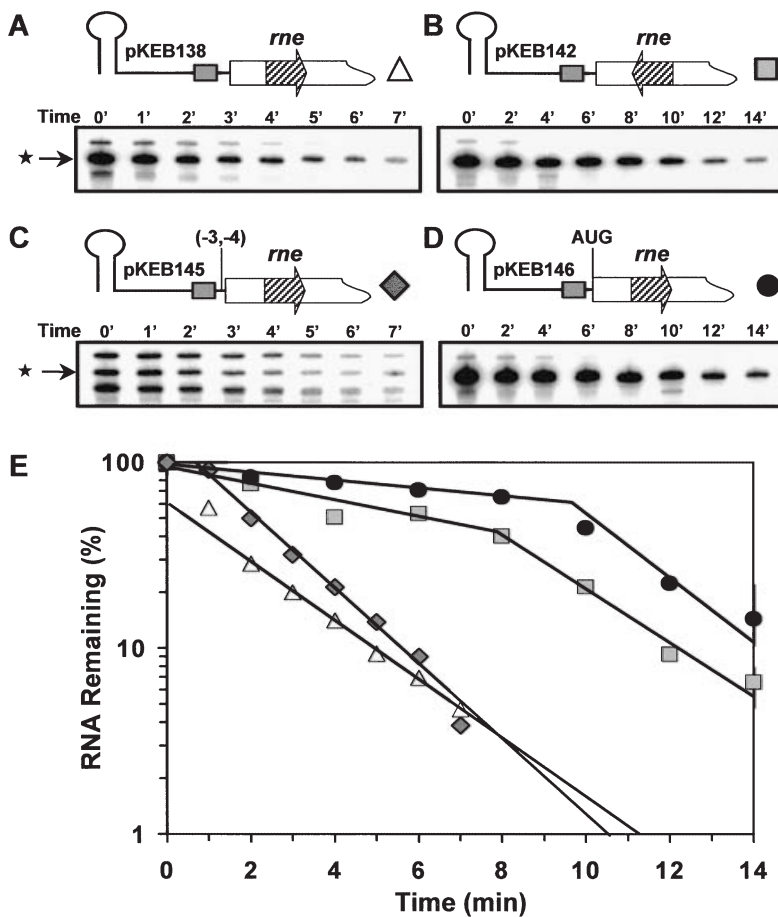
We also evaluated whether the action of an 'ectopic site' in the coding region was sensitive to translational efficiency. We introduced either the GA → CU or UUG → AUG mutations into pKEB138 to generate pKEB145 and pKEB146 respectively (Fig. 1B). The half-life of the *rpsT*(145) mRNA containing the former mutations that decrease translation was 1.5 min, half that of its parent (Fig. 7C and E; Table 1, entry 10). Conversely, the *rpsT*(146) mRNA exhibited a half-life of 15 min, a fivefold stabilization of the mRNA in comparison to the parental *rpsT*(138) mRNA (Fig. 7D and E; Table 1, entry 11). Thus, when placed within the ORF, an 'ectopic site' displays moderate efficiency and orientation-dependence. Moreover, the efficacy of such an insertion is highly sensitive to translational efficiency.

## Discussion

### *Ectopic RNase E cleavage sites can bypass stabilizing 5'-stem-loops*

The 5'-terminal hairpin in the *ompA* mRNA in several microorganisms is a major determinant of its atypical longevity (Belasco *et al.*, 1986; Melefors and von Gabain, 1988; Bouvet and Belasco, 1992; Emory *et al.*, 1992; Hansen *et al.*, 1994). The protection afforded by this 5'-stem-loop is not absolute, however, as a number of factors, including growth rate (Emory and Belasco, 1990) or a heterologous insertion (Meyer *et al.*, 1996), can modify this mRNA's stability. Our data show that the *rpsT* mRNA, whose structure and mode of decay are well characterized (Coburn and Mackie, 1999), can be also stabilized by a prosthetic stem-loop at its extreme 5'-terminus. However, we show here that this protective feature can be overcome by highly effective, relatively short, destabilizing elements ('ectopic sites'). The efficacy of these sites depends on RNase E activity and is sensitive to a number of modifying factors, most notably translation. These results document for the first time the possibility of increasing dramatically the sensitivity of an mRNA to RNase E by introducing small, well defined, destabilizing elements.

Our work defines several key features of such 'ectopic sites'. First, they can be as short as 12 nt, if not smaller. Second, although we did not undertake an exhaustive survey, each of the three effective destabilizers was based on a known site of RNase E cleavage and thus probably functions as a site of RNase E cleavage, rather than recognition. Although the products of cleavage at 'ectopic sites' should be ephemeral given their 5'-mono-



**Fig. 7.** Destabilization of *rpsT* mRNA by an 'ectopic RNase E site' is modulated by translation. Total RNA (5 µg), extracted from MG1693 containing pKEB138 (A), pKEB142 (B), pKEB145 (C), or pKEB146 (D) was analysed on Northern blots as described in Fig. 2 and probed for *rpsT* mRNAs. The time of sampling (in min), the source of the mRNA and a schematic of the 5'-end of the plasmid-encoded *rpsT* mRNA are indicated above each panel (also see Fig. 1). The sources and orientation of the 'ectopic site' (wide, hatched arrow) within the *rpsT* coding region, and mutations influencing translational initiation (see also Fig. 3) are highlighted in the schematic representations. The position of the plasmid-encoded *rpsT* mRNA is indicated (★) at left of the autoradiograms.

E. Plot of first-order decay of plasmid-encoded *rpsT* mRNA. Symbols used for each recombinant mRNA are indicated beside the mRNA schematics in A–D.

phosphorylated termini (Spickler *et al.*, 2001), our data show that the 9Sa sites function as direct targets for RNase E cleavage *in vivo* (as they do *in vitro*; Cormack and Mackie, 1992) and do not operate through simple expansion of or structural changes in the target RNA. Moreover, the destabilizing effect of 'ectopic sites' depends on an active *rne* allele. Interestingly, the 9Sa site destabilizes its host mRNA to different extents depending on its orientation, presumably due to differential recognition of primary sequences at the site of cleavage by RNase E (Cormack and Mackie, 1992). In the *rpsT*(127) mRNA, however, insertion of the *rne* leader site induced a subtle reorganization of RNA structure that probably reduces the efficiency of translation. In this case, we cannot distinguish whether the 'ectopic site' acts directly or indirectly to overcome the protective effect of the 5'-stem-loop (see below). Care is required, therefore, in the design of such elements to avoid creating alternative secondary structures that might preclude endonucleolytic cleavage or affect translation. Third, the efficiency of an 'ectopic' cleavage site depends on its position within the targeted mRNA, with positioning in the 5'-UTR being more effective for a given sequence than in the coding region. We were

unable to test the effect of 'ectopic sites' in the 3'-UTR of the *rpsT* mRNA, which is highly structured. Nonetheless, precedent suggests that 'ectopic sites' ought to function well in such a location as a 287 nt fragment from the 3'-UTR of the *cat* gene can be inserted into the 3'-UTR of the *ompA* gene to produce a dominant, orientation-dependent destabilization of the *ompA-cat* mRNA (Meyer *et al.*, 1996). Finally, perhaps the most critical and complex determinant of an 'ectopic site' is the efficiency with which the host mRNA is translated (see below). In particular, efficient translational initiation can virtually eliminate the effectiveness of an internal 'ectopic site' (e.g. compare *rpsT*(138) mRNA with *rpsT*(146) in Table 1).

#### The internal entry model

How 5'-terminal hairpin loops protect mRNAs from RNase E or an alternative decay pathway has not been clearly established (Arnold *et al.*, 1998). We believe that 5'-stem-loops block the 5'-end-dependent pathway (Mackie, 1998; 2000) and channel the *rpsT* mRNA into an inherently less efficient internal entry pathway. In the first pathway, an accessible, triphosphorylated, 5'-terminal residue is

thought to facilitate an initial interaction between RNase E and an mRNA substrate (Mackie, 1998; Tock *et al.*, 2000; Jiang *et al.*, 2000; Spickler *et al.*, 2001). This interaction would weakly tether RNase E to the RNA before cleavage. The enzyme–RNA complex would then rearrange by looping to permit endonucleolytic cleavage at a favourable downstream site (Fig. 8A). In stem–loop-protected or circular mRNAs, the 5'-terminus of the mRNA is sequestered, precluding interaction with the 5'-triphosphate. As such RNAs still decay slowly, dependent on RNase E (Bouvet and Belasco, 1992; Hansen *et al.*, 1994; Mackie, 1998; 2000), RNase E must bypass the 5'-end of the protected mRNA to interact directly with its substrate to catalyse the initiating cleavage (Fig. 8B). The efficiency of this 'internal entry' process is determined by the intrinsic susceptibility of the rate-limiting cleavage site and by external variables such as translation (see below). 'Ectopic sites' clearly favour use of the internal entry pathway. They can be visualized as special cases of resident RNase E cleavage sites that function with high efficiency so that the stimulatory effect of a free 5'-terminus is unnecessary.

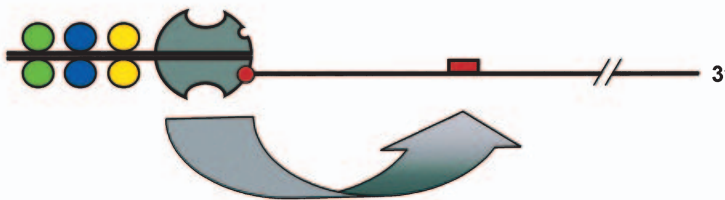
#### *The interplay between translation and decay of the rpsT mRNA*

The extent to which the *rpsT* mRNA can be stabilized by the protective 5'-stem–loop clearly depends on the efficiency of translational initiation. Generally similar outcomes have been obtained in other mRNAs. A 5'-hairpin

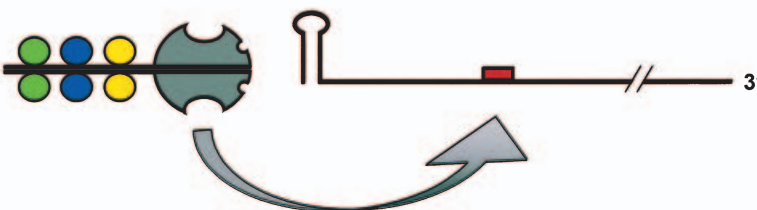
loop is unable to protect the *lacZ* mRNA from rapid decay in the complete absence of translation (Joyce and Dreyfus, 1998). Likewise, translation is required for the longevity of the *ompA* mRNA. In this regard, competition between 30S subunits and the growth-rate regulated protein, Hfq, for binding to the 5'-UTR of the *ompA* mRNA leads to reduced translational initiation and significantly decreased stability (Vytvytska *et al.*, 1998; 2000). Moreover, our data show that passage of ribosomes beyond an efficient site for RNase E cleavage (i.e. residues 300/301 within the *rpsT* coding region) is also required for the protective effect of the 5'-terminal stem–loop (cf. Nilsson *et al.*, 1987).

The simplest model to explain the protective effect of translating ribosomes in either the 5'-end-dependent or internal entry pathways is steric masking of cleavage sites resulting in competition between translation and RNase E. Ribosomes can protect at least 30 residues of an mRNA (Steitz, 1969). As RNase E is also large, especially when incorporated into the RNA degradosome (Py *et al.*, 1996), ribosomes would readily interfere with cleavage site recognition by RNase E acting in either a looping or internal entry mode (compare panels A and B, respectively, in Fig. 8). Masking can explain the negative correlation between the effectiveness of a 'ectopic site' and translational efficiency as ribosomes and RNase E effectively compete with each other. It also rationalizes the differential effect of premature termination codons (Fig. 3C and D). For these reasons, we believe that masking of cleavage sites better explains the correlation

#### **A Tethered Initial Cleavage (Unimolecular)**



#### **B Direct Initial Cleavage (Bimolecular)**



**Fig. 8.** A model for RNase E-mediated cleavage of RNA. The RNA degradosome is depicted according to Vanzo *et al.* (1998). The N-terminal catalytic domain of RNase E (grey half circle), and the C-terminal domain (horizontal line) form a dimer interacting with RhlB, enolase and PNPase (yellow, blue and green circles respectively). The endonucleolytic catalytic site and a putative phosphate binding pocket are shown as separate sites (oval and round cut-outs respectively).

A. Looping by RNase E from an accessible 5'-end. Interaction between the phosphate binding pocket of RNase E with a single-stranded 5'-triphosphorylated terminus (red circle; see text) would tether the RNA degradosome to its substrate. Looping of the mRNA–enzyme complex would facilitate recognition and cleavage (wide curved arrow) of a downstream cleavage site (red box).

B. Internal entry by RNase E into a 5' stem–loop-protected mRNA. Interaction between the 5'-terminus of an mRNA is precluded by the 5'-duplex structure (Mackie, 1998). Direct recognition of a cleavage site (red box) by RNase E would require bypass of the 5'-terminus through a slow, bimolecular interaction (thin, curved arrow).

between translational initiation and mRNA stability in *rpsT* mRNAs containing 5'-stem-loops than a model invoking competition between initiation of translation and recognition of the 5'-end of an RNA by RNase E (Rapaport and Mackie, 1994). Further support for steric masking of RNase E sites has been found in the *rpsO* mRNA (Braun *et al.*, 1998). Increasing the distance between the termination codon and the rate-limiting M2 cleavage site in the 3'-UTR of this mRNA destabilizes it. As the M2 site occurs 10 residues 3' to the termination codon, the normal delay in the release step would effectively mask the site (Braun *et al.*, 1998). A more complex alternative to steric masking postulates that RNase E initially binds non-specifically to an mRNA either during looping (in a 5'-end-dependent mode) or direct entry. It would then migrate to the first cleavage site. Translating ribosomes would compete with the migration step rather than the actual initiating cleavage. The principal argument against this model is that RNase E would have to migrate through both single and double-stranded RNA without unwinding or cleaving either.

Although the 5'-terminal stem-loop stabilizes *rpsT*(110) mRNA significantly, moderate expression of this mRNA leads to reduced steady state levels of the two chromosomally encoded *rpsT* mRNAs (e.g. Fig. 2B). In addition, the *rpsT*(110) mRNA decays with biphasic decay kinetics that are lost when RNase E is inactivated. Both observations probably reflect autogenous translational repression of *rpsT* mRNAs exerted by accumulated S20 protein (Wirth *et al.*, 1982), which is further exacerbated by inhibition of accumulation of 16S rRNA by rifampicin. During rifampicin treatment, the ratio of S20 to its ligands increases resulting in severe translational repression. This would decrease translational initiation, resulting in loss of ribosomal masking, and thus would enhance access of RNase E to the repressed *rpsT* mRNA. This would explain the reduced levels of the chromosomal *rpsT* P1 and P2 mRNAs in many of our experiments. Such behaviour is similar to that observed for the alpha operon and other ribosomal protein mRNAs (Singer and Nomura, 1985; Cole and Nomura, 1986). In addition, the ratio of RNase E to its substrates will increase as the rifampicin treatment persists. The time of onset of the second rapid phase of decay of the plasmid-borne *rpsT* mRNA should reflect both factors. In support, we observed that the presence of an AUG codon (*rpsT*(144) mRNA) that reduces translational repression (Parsons *et al.*, 1988) prolongs the first phase of decay. Similarly, in the absence of active RNase E (in SK5665), the second phase of decay is abolished. This interpretation of how translational control affects the stability of the *rpsT* mRNA differs from previous results in which gene dosage was much higher and where expression of *rpsT* mRNAs could not be regulated as tightly as in the present work (Mackie, 1987).

### Ectopic sites and the hierarchical model for RNase E action

The present and previous data show that there are three key determinants of RNase E action on the *rpsT* mRNA and, by implication, on other targets for RNase E. The first is the status of the 5'-end that defines a three level hierarchy of efficiencies by which RNase E recognizes substrates independently of translation (Mackie, 2000). In this hierarchy, monophosphorylated RNAs are most susceptible to RNase E, followed by triphosphorylated, single-stranded termini, and finally by stem-loop-protected or circular RNAs. The second is the efficiency with which the *rpsT* mRNA (or other mRNAs) is translated (this work; Parsons *et al.*, 1988; Petersen, 1993; Rapaport and Mackie, 1994; lost and Dreyfus, 1995; Joyce and Dreyfus, 1998; Mackie, 2000; Vytvytska *et al.*, 2000). The third determinant is the cleavage site itself. Potential cleavage sites span a continuum of efficiencies, ranging from ineffective (e.g. the *rne* leader site in the reverse-complement orientation in *rpsT*(128) mRNA), to those more efficient than the resident sites in the *rpsT* mRNA (e.g. the 9Sa site in *rpsT*(152) mRNA). Determinants of cleavage site efficiency are known to encompass primary sequence (Mackie, 1991; McDowall *et al.*, 1994), position relative to secondary structure (Cormack and Mackie, 1992; Mackie, 1992; Mackie and Genereaux, 1993; McDowall *et al.*, 1995), masking by ribosomes (Braun *et al.*, 1998; this work), and the presence of RNA binding proteins (Vytvytska *et al.*, 1998; Jerome *et al.*, 1999). We are currently employing a combinatorial approach to modify an 'ectopic site' and explore fully this continuum of efficiencies.

## Experimental procedures

### Bacterial strains and growth conditions

*Escherichia coli* K12 strains MG1693 (*thyA715*, *rph-1*; Arraiano *et al.*, 1988), and MRA10 (MG1655, *rpsT147*; Rydén-Aulin *et al.*, 1993) were routinely grown at 37°C in Luria-Bertani (LB) medium supplemented with glucose (0.2%), MgSO<sub>4</sub> (1 mM), thymidine (50 µg ml<sup>-1</sup>), ampicillin (50 µg ml<sup>-1</sup>) and chloramphenicol (25 µg ml<sup>-1</sup>), as needed. Induction of transcription from P<sub>BAD</sub> (see below) was achieved by filtering mid-exponential phase cells through 1.2 µ filters (Millipore), washing with unsupplemented medium, and resuspending in supplemented LB containing 0.05% arabinose in place of glucose, followed by further growth for 60 min. Cultures of strain SK5665 (*rne-1*, *thyA715*, *rph-1*; Arraiano *et al.*, 1988) were grown at 30°C, induced as described above, then shifted to 44°C for 15 min before the addition of rifampicin to 300 µg ml<sup>-1</sup>.

### Plasmid constructions

Restriction endonucleases, T4 DNA ligase, and polynucle-

otide kinase were obtained from either New England Biolabs (Beverly, MA, USA) or Life Technologies (Gaithersburg, MD, USA). Oligonucleotides were synthesized by either the NAPS Unit, University of British Columbia, or by Life Technologies. Manipulations of DNA followed standard procedures (Sambrook *et al.*, 1989). All recombinant plasmids were verified by restriction analysis and partial DNA sequencing. The vector pBAD28 (Guzman *et al.*, 1995) was obtained from Dr J. Beckwith (Harvard Medical School, Boston, MA, USA). It was linearized with *KpnI* and re-ligated after treatment with mung bean nuclease to eliminate the unique *KpnI* restriction site from the polylinker, generating pKEB105. A new unique *KpnI* site was introduced into the position of transcriptional initiation of P<sub>BAD</sub> in pKEB105 by site-directed mutagenesis (Stratagene QuikChange™, La Jolla, CA, USA) using the complementary oligonucleotides 5'-GCCCAAAAAACGGGTACCGAGAAACAGTAGAGAG-3' and 5'-CTCTCTACTGTTCTCGGTACCCGTTTTTTTTGGGC-3' (mutant sequences are underlined and the *KpnI* site italicized), creating pKEB106. Residues 92–447 of the *rpsT* gene from pGM79 (Mackie, 1991) were amplified using *Taq* DNA polymerase and the primers 5'-GGGGTACCTTTGAATTGTCCATATGG AACACATTTGGG-3' (the *KpnI* and *NdeI* sites are italicized) and 5'-GC7CTAGAGCATCACAAAAGCAGCAGGC-3' (*XbaI* site italicized). The product was ligated into *KpnI*- and *XbaI*-digested pKEB106 to generate pKEB107. Complementary oligonucleotides 5'-CATCGCCACCGGAGACCGGTGGCG ATGGTAC-3' and 5'-CATCGCCACCGGTCTCCCGGTGGCG ATGGTAC-3' were 5'-phosphorylated, annealed and ligated into the *KpnI* site of pKEB107 to generate pKEB110 encoding an *rpsT* mRNA with a stem-loop at its extreme 5'-terminus (see Fig. 1B).

Complementary oligonucleotide pairs 5'-TAACCCATTTTGCCC and 5'-TAGGGCAAATGGGT-3' or 5'-TACAGAA TTTTGCGA-3' and 5'-TATCGCAAATCTG-3' encoding the RNase E cleavage site at residues 46–56 in the *rne* gene (Jain and Belasco, 1995) or nucleotides encompassing the 9S rRNA 'a' cleavage site (Ghora and Apirion, 1978), respectively, were ligated into the unique *NdeI* site of pKEB110. Plasmids harbouring the sequence in the native and reverse-complement orientations (pKEB127, pKEB128 and pKEB152, pKEB154, respectively; see Table 1) were selected. The *rne* cleavage site (see above) was inserted into the *rpsT* coding region of pKEB110 through polymerase chain reaction (PCR) amplification of two partial *rpsT* sequence fragments. Sequences 5' to the position of insertion were amplified from pKEB110 DNA using oligonucleotides 5'-GATTAGCGGATCCTACCTGACGC-3' (*Bam*HI site italicized) and 5'-ACTCCGCTCGAGAGCAGCTTTGTGCGCAGCTTCG-3' (*Xho*I site italicized). The 3'-fragment encompassing the insertion sequence (the *Xho*I site is italicized and the *rne* insertion sequence is underlined) was amplified using as forward primers either 5'-TGAGGCCTCGAGACCATTTTGCCCAAAGCATTTAACGAAATGCAACCG-3' for the native orientation, or 5'-TGAGGCCTCGAGGGGCAAATGGTA AAGCATTTAACGAAATGCAACCG-3' for the reverse-complement orientation, coupled with 5'-GGTCGACTCTA GAGCATCAC-3' (*Xba*I site italicized) as reverse primer. The resultant DNA fragments were digested and ligated into pBAD28 digested with *Bam*HI and *Xba*I. Mutations in the translational initiation region of the *rpsT* gene were introduced by

site-directed mutagenesis and complementary oligonucleotides 5'-GGGAGTTGGACCATGGCTAATATCAAATC-3' and 5'-GATTTGATATTAGCCATGGTCCAACCTCCC-3' (altered residue(s) underlined), for increased translational efficiency, or 5'-CACATTTGGGAGTTGCTCCTTGGCTAATATC-3' and 5'-GATATTAGCCAAGGAGCAACTCCCAAATGTG-3', for decreased translational efficiency (Parsons *et al.*, 1988). A stop codon was introduced into the *rpsT* gene of pKEB110 at codon 69 to make pKEB147 by site-directed mutagenesis using oligonucleotides 5'-CCTCTGATCCACIAAAACAAAGCTGCACGTC-3' and 5'-GACGTGCAGCTTTGTTTTAGTGGATCAGACC-3' (mutant residue underlined). Codon 51 was mutated to a stop codon by PCR amplification of two *rpsT* partial sequences to create pKEB136 (details provided upon request).

#### RNA isolation and Northern blot analysis

Early log phase cultures grown as described above were poisoned by the addition of rifampicin to 300 µg ml<sup>-1</sup>. Aliquots (25 ml) were removed at various times and added to 12.5 ml ice-cold 12.5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> containing 200 µg ml<sup>-1</sup> of chloramphenicol. Bacteria were collected by centrifugation and RNA isolated as described previously (Method I in Mackie, 1989). Purified RNA was quantified spectrophotometrically. For RNA blots, 5 µg was separated on 5.5% polyacrylamide gels containing 8 M urea. RNAs were transferred electrophoretically to Hybond-NX (Amersham Pharmacia Biotech), fixed by UV crosslinking, and probed to detect the *rpsT* mRNAs with a radiolabelled RNA complementary to *rpsT* residues 91–441. Hybridization was performed at 55°C in 50% formamide, 5x SSC, 5x Denhardt's solution (Sambrook *et al.*, 1989), 40 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.0), 0.25 mg ml<sup>-1</sup> of yeast RNA, 0.06 mg ml<sup>-1</sup> of salmon sperm DNA, and 0.1% SDS. Membranes were washed (4 × 15 min) with 2x SSC and 0.1% SDS at 55°C. Signals were visualized and quantified using a Molecular Dynamics PhosphorImager. RNA loading was periodically verified by reprobing for 5S rRNA. Half-lives were deduced from the initial slope of a semilogarithmic plot of the fraction of mRNA remaining versus time after rifampicin addition.

#### Primer extension analysis

First, 10 µg of RNA isolated from MRA10 transformed with either pKEB127, 128, 152 or 154 (see Table 1) was concentrated by precipitation with ethanol and 100 µg ml<sup>-1</sup> of glycogen. RNA was dissolved in 10 µl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT and 2 pmol primer (PE-mer; 5'-[<sup>32</sup>P]-CGGCTTGCCTGTGCTTACGAG-3') complementary to *rpsT* residues 182–203 (see Fig. 1A; Mackie, 1992) and incubated at 65°C for 5 min. Synthesis of cDNAs was initiated by addition of (final concentrations) 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 5 mM dNTPs and 100 units of M-MLV Reverse Transcriptase in a final volume of 20 µl followed by incubation at 37°C for 45 min. Extension products were resolved alongside sequence ladders, using the same primer, on 6% sequencing gels containing 8 M urea and visualized by autoradiography.

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