A 5′-terminal stem–loop structure can stabilize mRNA in *Escherichia coli*

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The 5′-untranslated region of the long-lived *Escherichia coli ompA* transcript functions as an mRNA stabilizer capable of prolonging the lifetime in *E. coli* of a number of heterologous messages to which it is fused. To elucidate the structural basis of differential mRNA stability in bacteria, the domains of the *ompA* 5′-untranslated region that allow it to protect mRNA from degradation have been identified by mutational analysis. The presence of a stem–loop no more than 2–4 nucleotides from the extreme 5′ terminus of this RNA segment is crucial to its stabilizing influence, whereas the sequence of the stem–loop is relatively unimportant. The potential to form a hairpin very close to the 5′ end is a feature common to a number of stable prokaryotic messages. Moreover, the lifetime of a normally labile message (*bla* mRNA) can be prolonged in *E. coli* by adding a simple hairpin structure at its 5′ terminus. Accelerated degradation of *ompA* mRNA in the absence of a 5′-terminal stem–loop appears to start downstream of the 5′ end. We propose that *E. coli* messages beginning with a single-stranded RNA segment of significant length are preferentially targeted by a degradative ribonuclease that interacts with the mRNA 5′ terminus before cleaving internally at one or more distal sites.

[*Key Words*: mRNA stability; 5′-untranslated region; *Escherichia coli*; gene regulation; *ompA*; RNA structure]

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Degradation of mRNA is a cellular process that is highly important for controlling gene expression. The capacity to degrade mRNA is essential to the ability of cells to alter their patterns of protein synthesis rapidly in response to their changing needs. In addition, the steady-state cellular concentration of a continuously synthesized message is directly proportional to its half-life. The lifetimes of individual messages can vary widely within a single cell. For instance, mRNA half-lives in *Escherichia coli* range from seconds to nearly an hour, with an average lifetime of 2–4 min (Pedersen et al. 1978; Nilsson et al. 1984; Donovan and Kushner 1986; Emory and Belasco 1990). Despite the importance of mRNA instability to gene regulation, the mechanisms and structural determinants of prokaryotic mRNA decay are not well understood (Kennell 1986; Belasco and Higgins 1988).

Elements at the 5′ and 3′ ends of bacterial and phage messages have been implicated in mRNA stabilization. Most prokaryotic mRNAs end in a 3′-terminal stem–loop structure, which serves as a protective barrier against degradation by 3′ exoribonucleases (Belasco et al. 1985; Mott et al. 1985; Newbury et al. 1987; Chen et al. 1988). However, for bacterial mRNAs that end with a 3′ hairpin, there is hardly any evidence to indicate that stability differences in vivo result from differential rates of exonucleolytic penetration of these 3′ stem–loop structures (Belasco et al. 1986; Chen et al. 1988; Chen and Belasco 1990). Instead, it appears that the disparate lifetimes of most prokaryotic mRNAs are controlled by decay determinants located upstream of the 3′ end. Especially noteworthy are a small number of 5′-terminal mRNA segments that have been shown to stabilize heterologous messages to which they are fused. These 5′ mRNA stabilizers comprise the 5′-untranslated region (UTR) of certain stable bacterial and phage messages, such as the *ompA* transcript of *E. coli*, the *ermC* and *ermA* transcripts of *Bacillus subtilis* and *Staphylococcus aureus*, and the gene 32 message of phage T4, as well as a 5′ segment of the phage λ p_L transcript (Yamamoto and Imamoto 1975; Gorski et al. 1985; Belasco et al. 1986; Bechhofer and Dubnau 1987; Sandler and Weisblum 1988). Apparently, these 5′ elements are able to protect mRNA from attack by an important cellular ribonuclease that initiates mRNA degradation in vivo; the identity of this key degradative enzyme remains uncertain. Most of the known 5′ mRNA stabilizers function only under special conditions. For example, the gene 32 5′ UTR can increase message lifetimes only in T4-infected cells (Gorski et al. 1985), and stabilization of mRNA by the *ermC* and *ermA* 5′ UTRs requires ribosome stalling induced by an antibiotic that inhibits translation (Bechhofer and Dubnau 1987; Sandler and Weisblum 1988). Similarly, message stabilization by the 5′ portion of the λ p_L transcript is most pronounced only

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after prolonged λ infection (Yamamoto and Imamoto 1975).

In contrast, the *ompA* 5′ UTR can stabilize mRNA in *E. coli* under normal conditions of rapid cell growth (Belasco et al. 1986; Emory and Belasco 1990). This RNA segment is derived from the *ompA* gene transcript, which encodes a major *E. coli* outer membrane protein (OmpA). In cells growing rapidly at 30°C, the *ompA* message decays with a half-life of ~17 min, making it one of the most stable mRNAs in *E. coli* (von Gabain et al. 1983). The lifetime of *ompA* mRNA is growth rate regulated and falls by as much as a factor of 4 in slowly growing cells (Nilsson et al. 1984). Growth rate regulation of *ompA* mRNA stability, like the longevity of the *ompA* transcript under conditions of rapid growth, is determined by the 133-nucleotide *ompA* 5′ UTR, which can confer both of these properties onto other messages to which it is joined (Emory and Belasco 1990).

As the lifetimes of a variety of labile messages can be prolonged by fusion to the *ompA* 5′ UTR (Belasco et al. 1986; M. Hansen and J. Belasco, unpubl.), it appears that many *E. coli* mRNAs are degraded via a common pathway against which this 5′ RNA segment provides protection. Message stabilization by the *ompA* 5′ UTR is not an indirect consequence of close ribosome spacing, which theoretically might sterically hinder ribonuclease attack (Lundberg et al. 1988; Emory and Belasco 1990; M. Hansen and J. Belasco, unpubl.). Instead, this RNA segment seems to act directly to protect mRNA from degradation by a ribonuclease that responds to one or more elements near the mRNA 5′ end. Both the secondary structure of the *ompA* 5′ UTR and its ability to function as a growth-rate-regulated mRNA stabilizer are highly conserved among enteric bacteria (Chen et al. 1991). However, the identity of the specific *ompA* 5′ UTR structural features that are important for mRNA stabilization has not been explored previously.

To elucidate the mechanism of mRNA stabilization by the *E. coli ompA* 5′ UTR, we have dissected this RNA segment to identify the structural components that can protect mRNA from degradation in *E. coli*. Our studies reveal that the presence of a stem–loop at or very near the extreme 5′ terminus of the *ompA* 5′ UTR is essential for its function as a potent mRNA stabilizer. Furthermore, the half-life of a normally labile *E. coli* message can be prolonged by adding a simple stem–loop at its 5′ end. The 5′ stem–loop protects *ompA* mRNA from degradation that appears to begin downstream of the 5′ end. By defining important aspects of the substrate specificity of the *E. coli* mRNA degradation machinery, these findings provide key insights into both the structural basis of differential message stability in bacteria and the mechanism of mRNA decay.

Results

Two domains of the ompA *5′ UTR are important for mRNA stabilization*

We have shown previously that the *E. coli ompA* 5′ UTR comprises two imperfect stem–loop structures (hp1 and hp2) and two single-stranded RNA segments (ss1 and ss2) (Fig. 1; Chen et al. 1991). The *ompA* 5′ UTR was dissected to identify the structural domains responsible for its activity as an mRNA stabilizer in rapidly growing *E. coli* cells. Various 5′ UTR segments were deleted from a plasmid-borne copy of a pseudo-wild-type *ompA* gene (*ompA+4*) that encodes a transcript virtually identical in sequence and stability to the wild-type *ompA* message (Emory and Belasco 1990). These deleted segments corresponded to one or more RNA structural domains of the *ompA* 5′ UTR upstream of the ribosome-binding site (Fig. 1). Plasmids encoding the resulting mutant *ompA* messages were introduced into *E. coli* strain C600S, and total cellular RNA was isolated from rapidly growing cultures at time intervals after transcription inhibition with rifampicin. Degradation of each mutant message and of the endogenous wild-type *E. coli ompA* message was monitored simultaneously by S1 analysis of these RNA samples (Table 1). The parallel analysis of the decay of wild-type *ompA* mRNA provided an internal standard that facilitated interpretation of stability differences among the various mutant transcripts.

Two distinct structural domains appear to contribute to mRNA stabilization by the *ompA* 5′ UTR. One is the 5′-terminal stem–loop structure (hp1; Fig. 1), whose precise deletion reduces the half-life of *ompA* mRNA by a factor of 3 to just 5.7 ± 0.4 min (*ompAΔ64*; Fig. 2, left).

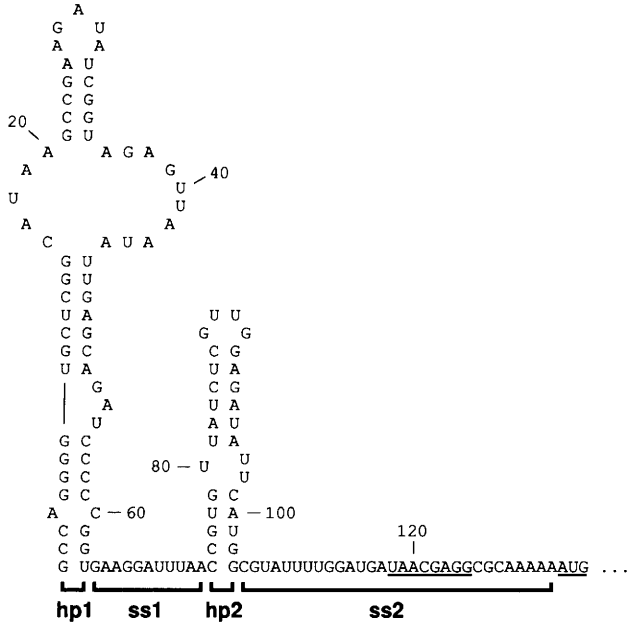


Figure 1. Secondary structure of the *ompA* 5′ UTR. Brackets delineate the boundaries of the four secondary-structure domains within this RNA segment (Chen et al. 1991): hp1 (nucleotides 1–63), ss1 (64–74), hp2 (75–103), and ss2 (104–133). The Shine-Dalgarno element and translation initiation codon are underlined. Precise 5′-end mapping by primer extension shows that roughly equal numbers of *E. coli ompA* transcripts begin with the indicated 5′-terminal nucleotide or with a cytosine residue 1 nucleotide upstream (data not shown).

The other is a single-stranded RNA segment (ss2; Fig. 1), the 3′ portion of which contains the ribosome-binding site. The stability of *ompA* mRNA is halved by deleting from ss2 an 11-nucleotide RNA segment (5′-ss2) located a few nucleotides upstream of the *ompA* Shine-Dalgarno sequence (*ompAΔ104-114*; Fig. 2, middle). Larger deletions that remove either of these two elements (hp1 or 5′-ss2) also destabilize the *ompA* message (*ompAΔ104*, *ompAΔ115*; Table 1). In contrast, deletion of the internal hairpin (hp2) within the 5′ UTR and of the single-stranded segment (ss1) between hp1 and hp2 (*ompAΔ65-104*, *ompAΔ74-103*) has little or no effect on the degradation rate of the *ompA* transcript (Fig. 2, middle; Table 1). All of these deletions leave the *ompA* ribosome-binding site intact, and none impair translation of *ompA* mRNA (Emory and Belasco 1990; Emory 1991).

Stabilization by the 5′-terminal stem–loop is independent of its sequence

The 5′ UTRs of *ompA* mRNA from *Serratia marcescens* and *Enterobacter aerogenes* function as effective mRNA stabilizers in *E. coli* despite extensive sequence differences in both hp1 and hp2 (Chen et al. 1991). To assess the importance of the sequence of hp1 to *ompA* mRNA stability, the degradation of variant *E. coli ompA* messages with altered 5′-terminal hairpins was examined (Fig. 3; Table 2). Truncation of hp1 by deleting 44 nucleotides from its top reduces the half-life of the *ompA* message by <28% (*ompAΔ9-52*). Moreover, replacement of hp1 with an entirely different and perfectly paired stem–loop structure (hp*: GAUCGCCCACCGGCAGCUGCCGGUGGGCGAUC) also has little effect on the stability of the *ompA* transcript (*ompAΔ64**). Perhaps surprisingly, deletion of the leading strand of hp1 does not accelerate degradation of the message (*ompAΔ29*). However, the longevity of this mutant *ompA* transcript can be explained by its ability to fold so as to create an alternative stem–loop within 2 nucleotides of the mRNA 5′ end. Formation of this alternative hairpin structure is supported by the results of RNA methylation analysis in vivo with dimethylsulfate (DMS) (Fig. 3), a membrane-permeating reagent that methylates most unpaired adenosine and cytosine residues, as well as some unpaired uridine nucleotides (methylation of guanosine residues by DMS is not sensitive to base-pairing) (Mayford and Weisblum 1989). The stability of three distinct *ompA* messages with 5′-terminal stem–loop structures strikingly different from that of the wild-type transcript shows that the presence of this terminal stem–loop is more important than its sequence to the activity of the *ompA* mRNA stabilizer.

The location of the 5′ stem–loop is crucial to message stability

To determine whether it is the simple presence of hp1 or its proximity to the 5′ end of the *ompA* transcript that is important for its contribution to mRNA stability, the effect of adding nucleotides upstream of this hairpin was examined. With the aid of a computer program for predicting RNA secondary structure (Jaeger et al. 1989), the sequences of the 5′ extensions were chosen to minimize the possibility that they would participate in base-pairing. Plasmids encoding mutant *ompA* transcripts with three different 5′ extensions (*ompA+12a*: AGACUUUACAUC . . . ; *ompA+16a*: GAUCAGACUUUACAUC . . . ; or *ompA+16b*: GAUCUAUACUAUAACC . . .) were constructed (Table 3). These 12- to 16-nucleotide extensions were expected to add 10–15 unpaired nucleotides to the *ompA* 5′ end and to augment *ompA* hp1 by 1–2 bp at its base, owing to pairing of the last 1–2 nucleotides of each extension (C or UC) with the first 1–2 nucleotides of segment ss1 (G or GA). [Base-pairing of these two ss1 nucleotides has no effect on *ompA* mRNA stability (Emory and Belasco 1990).] The half-lives of *ompA+12a*, *ompA+16a*, and *ompA+16b* mRNA were then measured in strain C600S (Fig. 4). In every case, the addition of 12–16 nucleotides to the 5′ end was found to reduce the *ompA* mRNA half-life to just 3–6 min. This acceleration of decay is similar to that resulting from deletion of the 5′-terminal hairpin.

Because *ompA* mRNA is destabilized by three different 5′ extensions, two of which (*ompA+12a* and *ompA+16b*) have entirely distinct sequences, the destabilizing influence of these 5′ extensions is likely to be the result of internalization of what is normally a 5′-terminal hairpin and not to possible disruption of the secondary structure of the *ompA* 5′ UTR by the base-pairing potential of the added nucleotides. This conclusion is supported by the finding that nearly full longevity is restored to these mutant messages by adding a new 5′-terminal hairpin (hp*) upstream of the 5′ extensions (*ompA+12a** and *ompA+16b**; Table 3; Fig. 4), as this stabilizing modification should not interfere with the base-pairing potential of the original 12- to 16-nucleotide 5′ extensions. Therefore, it is the 5′-terminal location of the single-stranded extensions, rather than their mere presence, that accelerates degradation of *ompA* mRNA. Further confirmation that these extensions do not alter the structure of the *ompA* 5′ UTR was obtained by chemically probing the secondary structure of *ompA+12a*, *ompA+16a*, and *ompA+16b* mRNA. This was accomplished in vivo by using dimethylsulfate (DMS). Also tested was the susceptibility of these messages to alkylation in vitro by DMS and by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT), which alkylates unpaired uridine residues and, to a lesser extent, unpaired guanosine residues (Moazed et al. 1986). When the DMS and CMCT alkylation sites within these 5′ UTRs were mapped by primer extension (Fig. 5) and compared with data from a similar analysis of wild-type *ompA* mRNA (Chen et al. 1991), it was evident that the *ompA+12a*, *ompA+16a*, and *ompA+16b* extensions do not perturb the overall secondary structure of the rest of the 5′ UTR and that, as expected, the first 14–15 nucleotides of these 5′ extensions apparently are single stranded.

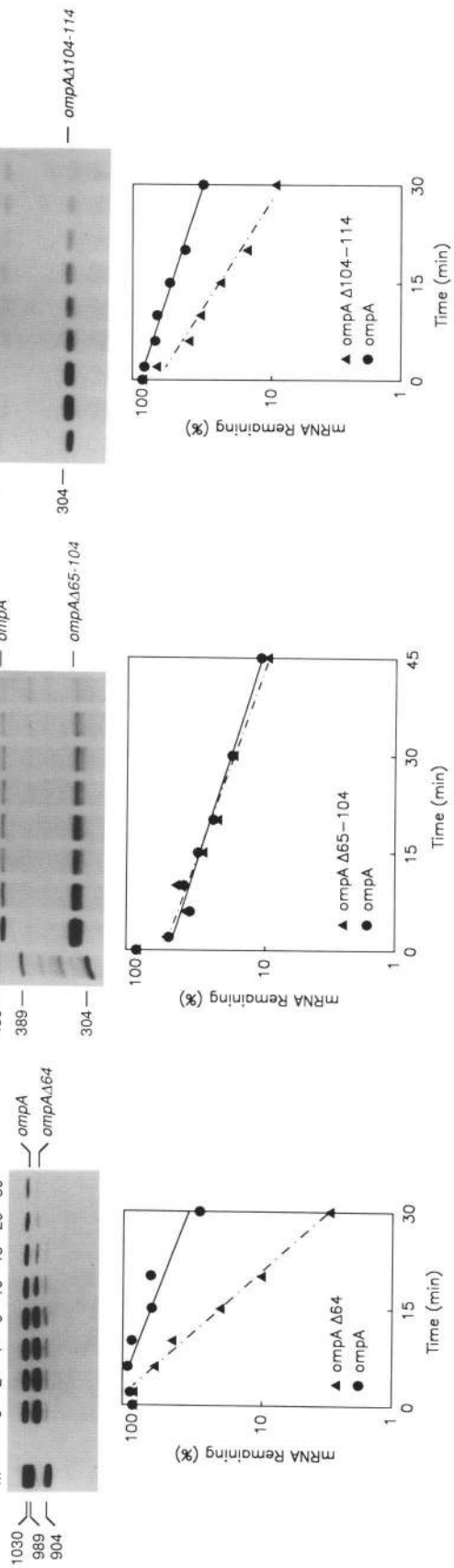


Figure 2. Degradation of mutant *ompA* transcripts with contiguous 5′ UTR deletions. At time intervals after transcription inhibition with rifampicin (200 μg/ml), total cellular RNA was isolated from a rapidly growing culture of *E. coli* strain C600S containing pOMPAΔ64 (*left*), pOMPAΔ65-104 (*middle*), or pOMPAΔ104-114 (*right*). Each mutant *ompA* transcript and the endogenous wild-type *ompA* message was detected by S1 analysis of RNA samples (2 μg) with either of two DNA probes. Bands that correspond to *ompAΔ64*, *ompAΔ65-104*, and *ompAΔ104-114* mRNA are indicated (protected probe fragment lengths of 0.96, 0.31, and 0.30 kb, respectively), as are the bands that represent wild-type *ompA* mRNA (protected probe fragment length of 1.02 or 0.42 kb). A set of molecular size standards (lane *M*) was generated by cutting the probe with each of three restriction enzymes. Calibration is in nucleotides. Beneath each autoradiograph is a semilogarithmic plot of mRNA concentration as a function of time. The half-life of wild-type *ompA* mRNA as measured by S1 analysis with either of these probes is essentially the same, as both probes extend past the 3′ boundary of the comparatively stable 5′ segment of *ompA* mRNA (von Gabain et al. 1983). (▲) *ompAΔ64*, (●) *ompA* (*left*); (▲) *ompAΔ65-104*, (●) *ompA* (*middle*); (▲) *ompAΔ104-114*, (●) *ompA* (*right*).

Table 1. *Half-lives of mutant* ompA *transcripts lacking 5′ UTR segments*

ompA allele	5′ UTR structure[a]	Mutant half-life (min)	Wild-type half-life (min)[b]
ompA+4[c] (pseudo-wild-type)		19 ± 2[d]	ND
ompAΔ64[e] (Δhp1)		5.7 ± 0.7	16 ± 2
ompAΔ104-114[c,f] (Δ5′-ss2)		9.5 ± 0.8	18 ± 1
ompAΔ74-103[c] (Δhp2)		14 ± 1	14 ± 1
ompAΔ65-104[g] (Δss1, hp2)		17 ± 1	19 ± 1
ompAΔ104[d,e] (Δhp1, ss1, hp2)		4.4 ± 0.2	17 ± 3
ompAΔ115[e,f] (Δhp1, ss1, hp2, 5′-ss2)		3.6 ± 0.6[d]	19 ± 4

[a]The expected secondary structure of each mutant 5′ UTR is represented diagrammatically (for a detailed secondary structure of the wild-type 5′ UTR, see Fig. 1). All were transcribed from the *bla* promoter, whose transcription initiation site was mapped precisely by primer extension (data not shown). (Solid line) Retained 5′ UTR segment; (dotted or absent line) deleted 5′ UTR segment; (solid rectangle) Shine-Dalgarno element.
[b]The half-life of the endogenous wild-type *ompA* message was measured simultaneously in the same strain as each mutant transcript. (ND) Not determined.
[c]The *ompA+4*, *ompAΔ74-103*, and *ompAΔ104-114* 5′ UTRs differ at the 5′ end from that of wild-type *ompA* mRNA in that the 5′-terminal G has been replaced by the sequence GAUCA. The last 3 nucleotides of this substituted pentanucleotide (UCA) are expected to augment hp1 by 2 bp by pairing with the last nucleotide of hp1 (U) and the first 2 nucleotides of ss1 (GA) (see Table 2).
[d]Emory and Belasco (1990).
[e]In addition to deletion of the number of 5′-terminal nucleotides indicated by the *ompA* allele name, 4 nucleotides (GAUC) have been added at the 5′ end of *ompAΔ64* mRNA, and 6 nucleotides (GAUCAG) have been added at the 5′ end of *ompAΔ104* and *ompAΔ115* mRNA.
[f]Segment 5′-ss2 comprises *ompA* nucleotides 104–114.
[g]In *ompAΔ65-104* mRNA, the G-U pair normally at the bottom of hp1 has been changed to an A-U pair, and hp1 has been augmented at its base by 4 additional nucleotide pairs (GAUC-hp1-GAUC). Furthermore, the deleted RNA segment (ss1-hp2) has been replaced by 2 nucleotides (AG).

Owing to the presence of the wild-type *ompA* transcript in strain C600S, it was unclear initially whether the rapid disappearance of the extended *ompA* messages represented mRNA degradation or mere removal of the single-stranded portion of the 5′ extension to generate a stable processing product resembling wild-type *ompA*

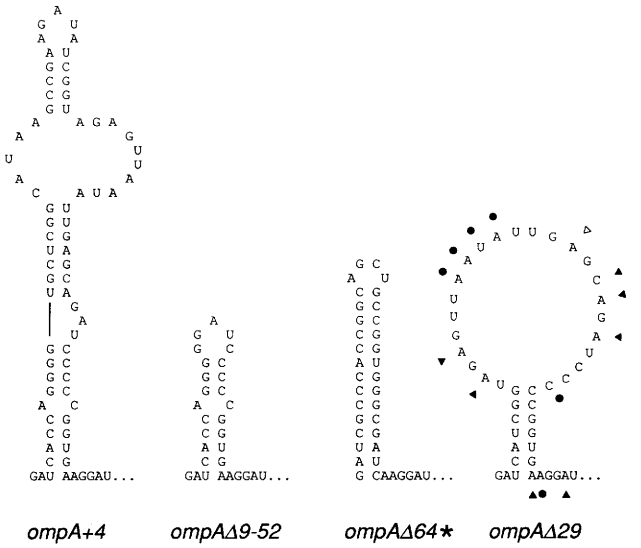


Figure 3. Variant 5′ stem–loop structures. For each *ompA* message with an altered 5′ hairpin, the sequence and expected secondary structure of a 5′-terminal RNA segment that extends into ss1 are shown. In every case, the remainder of the mRNA sequence is identical to that of the wild-type *ompA* transcript. Adenosine, cytosine, and uridine residues susceptible to significant methylation by DMS in *E. coli* are indicated for the 5′-terminal segment of *ompAΔ29* mRNA. (●) Heavy methylation; (▲) moderate methylation; (△) weak methylation.

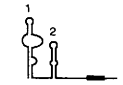
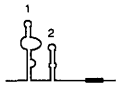
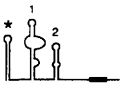
mRNA. If stable, such a processing product would be expected to accumulate in the cell to a steady-state concentration higher than that of its more labile precursors, the full-length *ompA* transcripts with 5′ extensions. To distinguish these possibilities, RNA was isolated from an isogenic host strain (SE600) that bore a chromosomal deletion of the *ompA* gene and carried plasmid pOMPA+16a or pOMPA+16b. S1 and primer-extension analysis of these steady-state RNA samples showed little or no detectable *ompA+16a* or *ompA+16b* mRNA processing product that was similar in length to the wild-type *ompA* message (Fig. 5, control lanes; Fig. 6). Therefore, addition of a short, single-stranded RNA segment to

Table 2. *Half-lives of mutant* ompA *transcripts with variant 5′ hairpins*

	Half-life (min)	
ompA allele	mutant message	wild-type message[a]
ompA+4	19 ± 2[b]	ND
ompAΔ9-52	13 ± 1	18 ± 2
*ompAΔ64**	12 ± 1	18 ± 3
ompAΔ29	15 ± 2	17 ± 2

[a]The half-life of the endogenous wild-type *ompA* message in each strain was measured simultaneously. (ND) Not determined.
[b]Emory and Belasco (1990).

Table 3. *Mutant* ompA *transcripts with or without destabilizing 5′ extensions*

ompA allele	5′ UTR structure[a]	Mutant half-life (min)	Wild-type half-life (min)[b]
ompA			17 ± 2
ompA+4		19 ± 2[c]	ND
ompA+12a		3.2 ± 1.1	15 ± 3
ompA+16a		5.8 ± 0.3	16 ± 2
ompA+16b		6.2 ± 0.8	12 ± 1
*ompA+12a**		13 ± 1	17 ± 1
*ompA+16b**		13 ± 1	15 ± 1
ompAΔ73a		6.0 ± 0.3	14 ± 1
ompAΔ73b		13 ± 1	15 ± 1
*ompAΔ104**		24 ± 3	19 ± 3

[a]The secondary structure of each mutant 5′ UTR is represented diagrammatically. Only 5′-terminal single-stranded extensions >2 nucleotides in length are shown. (Solid rectangle) Shine-Dalgarno element; (*1*) *ompA* hp1; (*2*) *ompA* hp2; (*) synthetic hp*.
[b]The half-life of the endogenous wild-type *ompA* message in each strain was measured simultaneously. (ND) Not determined.
[c]Emory and Belasco (1990).

the 5′ end of *ompA* mRNA destabilizes the entire transcript.

Five unpaired nucleotides at the 5′ end can destabilize ompA *mRNA*

An *ompA* deletion mutant (*ompAΔ73a*) lacking hp1 and ss1 and beginning instead with hp2–ss2 preceded by just 5 nucleotides (GAUCA) decays with a short half-life (6.0 ± 0.3 min) compared with wild-type *ompA* mRNA (14 ± 1 min) (Table 3; Fig. 7). As a 5′ hairpin of any kind and ss2 appear to be the only features of the *ompA* 5′ UTR that are necessary for mRNA longevity, the short lifetime of *ompAΔ73a* mRNA suggests that a 5′-terminal single-stranded segment only 5 nucleotides in length may be enough to accelerate *ompA* mRNA degradation. This hypothesis was confirmed by measuring the decay rate of a message (*ompAΔ73b*) that is identical to *ompAΔ73a* except that it has only 1 nucleotide (A) upstream of hp2–ss2 (Table 3). As expected, the half-life of *ompAΔ73b* mRNA (13 ± 1 min) proved to be about as long as that of the wild-type *ompA* transcript (15 ± 1 min) (Fig. 7). These findings indicate that as few as 5 unpaired bases at the 5′ end are sufficient to target *ompA* mRNA for rapid degradation in *E. coli*.

Addition of a 5′-terminal stem–loop can prolong the lifetime of a normally short-lived mRNA

As a final demonstration that a 5′ stem–loop and the single-stranded RNA segment encompassing the *ompA* ribosome-binding site are sufficient for the full efficacy of the *ompA* mRNA stabilizer, a minimal 5′ UTR comprising only a synthetic terminal hairpin (hp*) and the *ompA* ss2 segment was evaluated. As shown in Figure 7, an *ompA* transcript bearing this minimal 5′ UTR (*ompAΔ104**; Table 3) is as stable as the wild-type message. It is also five times more stable than a similar mes-

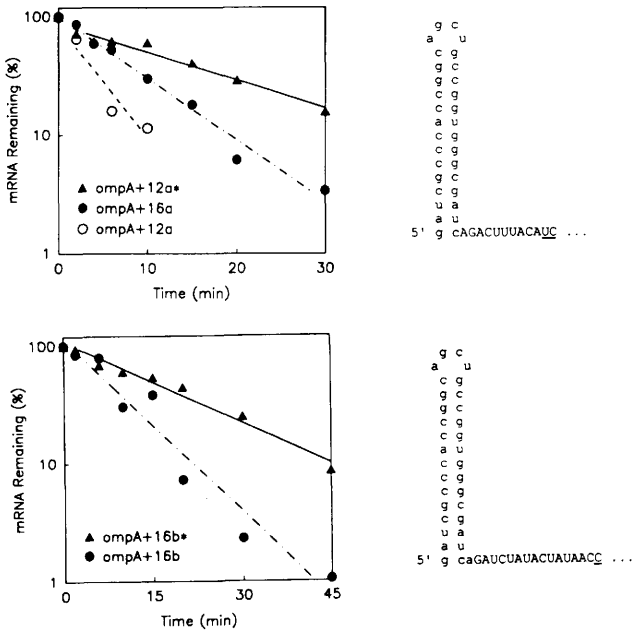


Figure 4. Decay of mutant *ompA* transcripts with 5′ extensions. After transcription inhibition, total cellular RNA was isolated periodically from *E. coli* strain C600S containing pOMPA+12a, pOMPA+16a, or pOMPA+12a* (*top*) or containing pOMPA+16b or pOMPA+16b* (*bottom*). The mutant transcripts and the endogenous wild-type *ompA* message were detected by S1 analysis, and mRNA concentration was plotted semilogarithmically as a function of time. Beside the graphs are drawn the 5′ extensions of *ompA+12a** (*top*) and *ompA+16b** (*bottom*) mRNA. The 5′ extensions of *ompA+12a* and *ompA+16b* mRNA are related to those of *ompA+12a** and *ompA+16b** mRNA, respectively, but include only those nucleotides shown in uppercase letters. The 5′ extension of *ompA+16a* mRNA is identical to that of *ompA+12a* except for 4 additional nucleotides (GAUC) at the 5′ end. Underlined nucleotides at the 3′ end of each extension are expected to base-pair with the first 1–2 nucleotides of 5′ UTR segment ss1. Transcription initiation sites for these messages were mapped precisely by primer extension (data not shown). The measured half-lives were 3.2 ± 1.1 min for *ompA+12a* mRNA (○) and 15 ± 3 min for wild-type *ompA* mRNA (*top*), 5.8 ± 0.3 min for *ompA+16a* mRNA (●) and 16 ± 2 min for wild-type *ompA* mRNA (*top*), 13 ± 1 min for *ompA+12a** mRNA (▲) and 17 ± 1 min for wild-type *ompA* mRNA (*top*), 6.2 ± 0.8 min for *ompA+16b* mRNA (●) and 12 ± 1 min for wild-type *ompA* mRNA (*bottom*), and 13 ± 1 min for *ompA+16b** mRNA (▲) and 15 ± 1 min for wild-type *ompA* mRNA (*bottom*).

sage (*ompAΔ104*) with a 5′ UTR consisting only of the ss2 segment (Table 1; Fig. 7). This finding confirms that a simple 5′-terminal hairpin and the *ompA* ss2 segment together are as effective as the complete *ompA* 5′ UTR at stabilizing mRNA. Moreover, when the same synthetic stem–loop (hp*) was introduced at the 5′ terminus of *bla* mRNA, which encodes β-lactamase, the resulting *bla202* messages were found to decay with a half-life of 6.8 ± 0.4 min (Fig. 7), about twice the lifetime of wild-type *bla* mRNA (3.7 ± 0.3 min) (Fig. 7; von Gabain et al. 1983). Thus, the ability of *bla* mRNA to resist degradation in *E. coli* can be enhanced simply by adding a stem–loop at its 5′ end.

Degradation in the absence of a 5′-terminal stem–loop appears to begin downstream of the ompA *5′ end*

In principle, accelerated degradation of *ompA* mRNA lacking a 5′-terminal hairpin might be initiated either by a 5′ exonuclease (defined here as a ribonuclease that removes single nucleotides sequentially from the RNA 5′ terminus) or by a 5′-end-dependent endonuclease that cuts internally but is sensitive to the presence or absence of secondary structure at the 5′ terminus of mRNA. Of these two possibilities, degradation by a single-strand-specific 5′ exonuclease seems less likely because no 5′ exoribonuclease has ever been detected in *E. coli* and because destabilizing single-stranded extensions added upstream of *ompA* hp1 are not preferentially removed from the 5′ end of *ompA+16a* and *ompA+16b* mRNA to generate wild-type-like *ompA* mRNA as a readily detectable processing product. As 5′ exoribonucleases, by definition, digest RNA from the 5′ end, the plausibility of 5′-exonucleolytic initiation of mRNA decay can be tested further by determining whether degradation of the 5′ mRNA segment is faster or slower than decay of the rest of the message.

Degradation of three different *ompA* messages without a 5′-terminal stem–loop (*ompAΔ64*, *ompA+16a*, and *ompA+16b* mRNA) was monitored by S1 analysis with a mixture of two DNA probes, one complementary to the 5′-terminal segment of these transcripts (the 5′ UTR and codons 1–37) and the other complementary to an internal *ompA* segment spanning codons 67–295. By combining these two probes in each S1 protection assay, differences in the relative decay rates of the two RNA segments could be measured with considerable accuracy. As observed previously for wild-type *ompA* mRNA (von Gabain et al. 1983), the internal segment of all three of these mutant messages was found to decay more rapidly than the corresponding 5′-terminal segment (Fig. 8). To demonstrate that the differential lifetimes measured for the 5′-terminal and internal *ompA* mRNA segments are independent of the relative lengths of the two DNA probes used simultaneously for S1 protection, the measurements were repeated with the same 5′-terminal probe and a much shorter internal probe complementary to *ompA* codons 249–295. Regardless of the length of either the internal probe or the RNA segment with which it hybridized, the same segmental difference in stability was observed for each of the three mutant *ompA* messages (Fig. 8). In every one of these six independent experiments, the relative concentration of the internal mRNA segment declined steadily to a level between one-third and one-half that of the 5′-terminal segment within 30 min after transcription inhibition.

Thus, it appears for three different labile *ompA* transcripts lacking a 5′-terminal stem–loop that degradation of an internal RNA segment precedes decay of the 5′ UTR. This conclusion is consistent with our finding, for a number of mutant *ompA* messages, that there is no correlation in rapidly growing cells between mRNA half-life and the relative steady-state concentration of the major products of endonucleolytic cleavage within the 5′ UTR (data not shown; Lundberg et al. 1990); only when *ompA* mRNA decay accelerates in slowly growing cells does its rate appear to be controlled by 5′ UTR cleavage (Melefors and von Gabain 1988). Together, our data suggest that under conditions of rapid bacterial growth, the function of the 5′-terminal *ompA* hairpin is to protect the message from degradation by a ribonuclease that initiates decay downstream of the 5′ end.

Discussion

A key to understanding the molecular basis for differential mRNA stability in bacteria is to identify the structural features of stable messages that are responsible for their unusual longevity in vivo. The studies reported here show that a 5′-terminal stem–loop is both crucial to the function of the *ompA* 5′ UTR as a potent mRNA stabilizer and sufficient to prolong the lifetime of a normally labile *E. coli* message. Remarkably, the 5′-terminal stem–loop shields *ompA* mRNA from attack by a cellular ribonuclease that appears to initiate degradation far from the 5′ end.

Our data also indicate that the *ompA* mRNA stabilizer is bipartite, with a second, distinct domain of functional importance. Both the 5′ stem–loop and a single-stranded RNA segment in the vicinity of the *ompA* ribosome-binding site and its flanking sequences contribute to the remarkable longevity of the *ompA* transcript, and together they are sufficient for full activity of the *ompA* mRNA stabilizer. Alone, each of these elements can stabilize mRNA to a lesser extent. Thus, *ompA* transcripts that have both stabilizing elements (wild-type, *ompAΔ65-104*) are more stable than *ompA* messages lacking either the 5′ hairpin or 5′-ss2 (*ompAΔ64*, *ompAΔ104*, *ompAΔ104-114*) which, in turn, are more stable than a message that lacks both of these RNA segments (*ompAΔ115*). Similarly, the half-life of *bla* mRNA, which is increased twofold simply by adding a 5′ stem–loop (*bla202*), increases about fivefold when its 5′ UTR is replaced with the entire *ompA* 5′ UTR (Belasco et al. 1986; Emory and Belasco 1990).

The longevity of *ompAΔ104** mRNA, in which hp1, ss1, and hp2 are replaced by a synthetic stem–loop, rules out any contribution to *ompA* mRNA stability from pos-

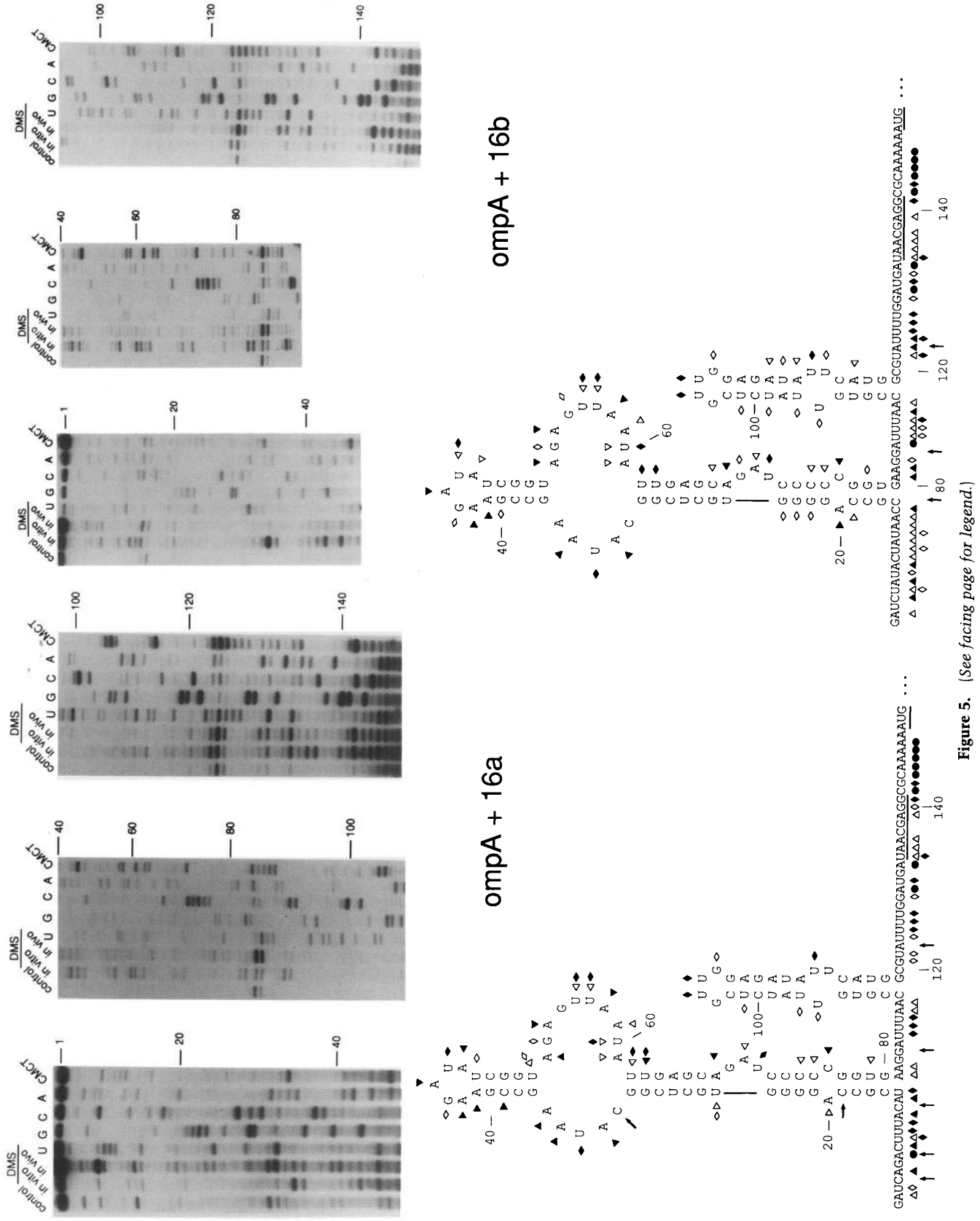


Figure 5. (*See facing page for legend.*)

sible sites of translation initiation upstream of ss2. These sites, which were identified on the basis of sequence analysis (Movva et al. 1980), probably are inaccessible to ribosomes due to occlusion by intramolecular base-pairing (Chen et al. 1991).

Stabilization of mRNA by a 5′ stem–loop

The presence of a 5′-terminal stem–loop can prolong the lifetime of *ompA* mRNA by as much as a factor of 5. The location of this stem–loop at or very near the 5′ terminus is crucial to its stabilizing effect, whereas the sequence of this hairpin and its position relative to the ribosome-binding site appear to be of little consequence to message stability. Up to two unpaired nucleotides upstream of the 5′ hairpin (e.g., *ompA+4*, *ompAΔ29*) are tolerated without any reduction in mRNA stability, but the addition of 10–15 unpaired nucleotides of random sequence to the *ompA* 5′ end (*ompA+12a*, *ompA+16a*, *ompA+16b*) is as destabilizing as deletion of the 5′ hairpin. Moreover, the short lifetime of *ompAΔ73a* mRNA versus *ompAΔ73b* mRNA indicates that 5 unpaired bases at the 5′ end are sufficient to accelerate degradation of *ompA* mRNA, as these two messages are identical in sequence except for the number of nucleotides that precede hp2–ss2. Therefore, the minimum number of 5′-terminal unpaired nucleotides that can destabilize *ompA* mRNA in *E. coli* is no more than five and may be as few as three. The destabilizing effect of unpaired bases at the *ompA* 5′ terminus does not appear to be sequence specific.

These findings explain our previous observation that the 5′ UTRs of the *S. marcescens* and *E. aerogenes ompA* transcripts function as very effective mRNA stabilizers in *E. coli* (Chen et al. 1991). Although these *ompA* 5′ UTRs are quite similar in secondary structure to that of *E. coli* and the sequence of ss1 and ss2 is highly conserved (80%), there is extensive sequence divergence in hp1 and hp2 (Chen et al. 1991). The efficacy of the *Serratia* and *Enterobacter ompA* 5′ UTRs in *E. coli*, despite numerous hp1 and hp2 sequence differences, is now understandable in light of our present finding that virtually any 5′-terminal hairpin followed by ss2 is sufficient to protect *ompA* mRNA from rapid degradation.

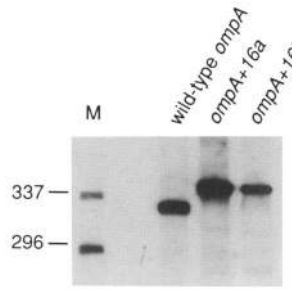


Figure 6. 5′-End mapping of *ompA+16a* and *ompA+16b* mRNA at steady state. Total cellular RNA was isolated from *E. coli* strain C600S or from strain SE600 containing either pOMPA+16a or pOMPA+16b. The wild-type and mutant *ompA* transcripts were detected by S1 analysis with 5′-end-labeled DNA probes complementary to the first 0.32–0.34 kb of each message. Calibration is in nucleotides. A set of molecular size standards (lane *M*) was generated as in Fig. 2.

As the sequence of the 5′ stem–loop is relatively unimportant to message stability and its stabilizing influence is negated by the presence upstream of several unpaired nucleotides, it evidently is the absence of a significant single-stranded RNA segment at the 5′ terminus rather than the presence of *ompA* hp1 that makes messages bearing the *ompA* 5′ UTR resistant to degradation. We note that several other long-lived prokaryotic messages in their mature form (e.g., *E. coli papA* mRNA, T4 gene 32 mRNA, and *Rhodobacter capsulatus pufBA* mRNA) have the potential to form a hairpin structure within 1–4 nucleotides of the 5′ end (Fig. 9), (Youvan et al. 1984; Belasco et al. 1985; Gorski et al. 1985; Baga et al. 1988; McPheeters et al. 1988). Together with our present findings, this observation suggests that a 5′

Figure 5. Methylation of *ompA* transcripts bearing 5′ extensions. (*Top*) Total cellular RNA was isolated from *E. coli* strain SE600 containing pOMPA+16a (*left*) or pOMPA+16b (*right*) after treating each culture with DMS (DMS/in vivo). Alternatively, total cellular RNA was purified from the same cultures without prior DMS treatment and then alkylated in vitro with either DMS (DMS/in vitro) or CMCT. Sites of alkylation within the 5′ UTR of *ompA+16a* and *ompA+16b* mRNA were mapped by primer extension with AMV reverse transcriptase, using complementary oligodeoxynucleotides (AGCGAAACCAGCCAGTGCCACTGC or GATAACACG-GTTAAATCCTTCAC) that annealed to mRNA sequences either 22–45 nucleotides downstream or 50–72 nucleotides upstream of the translation initiation codon of *ompA+16a* and *ompA+16b*. Gel electrophoresis was performed in parallel with the products of primer extension on unmethylated RNA templates in the presence (lanes *U*, *G*, *C*, *A*) or absence (control lane) of dideoxynucleoside triphosphates. The sequencing lanes are labeled to indicate the sequence of the RNA, not the complementary DNA. Blockage of primer extension by an alkylated RNA base results in a complementary DNA fragment that is 1 nucleotide shorter than that arising from incorporation of a dideoxynucleotide opposite the same RNA base. Calibration is in nucleotides from the mRNA 5′ end. (*Bottom*) Summary of alkylation data for the 5′ UTRs of *ompA+16a* (*left*) and *ompA+16b* (*right*) mRNA. Adenosine, cytosine, and uridine residues susceptible to significant methylation by DMS in *E. coli* are indicated. (●) Heavy methylation; (▲) moderate methylation; (△) weak methylation. Also labeled are uridine and guanosine residues that are significantly alkylated by CMCT in vitro. (◆) Heavy-to-moderate alkylation; (◇) weak alkylation. Comparisons of susceptibility to alkylation are meaningful only among nucleotides within the same message and at a similar distance from the annealed primer. Arrows identify the principal sites of termination by reverse transcriptase on an unalkylated template; the structural significance of primer extension products ending at these sites on alkylated RNA is often difficult to assess. Two of these termination sites on unalkylated RNA (at nucleotides 84 and 123) correspond to known RNase K cleavage sites within the *ompA* 5′ UTR (Lundberg et al. 1990); whether or not the others also correspond to RNA 5′ ends is uncertain. No differences were observed between the alkylation patterns of the *ompA+12a* (data not shown) and *ompA+16a* 5′ UTRs.

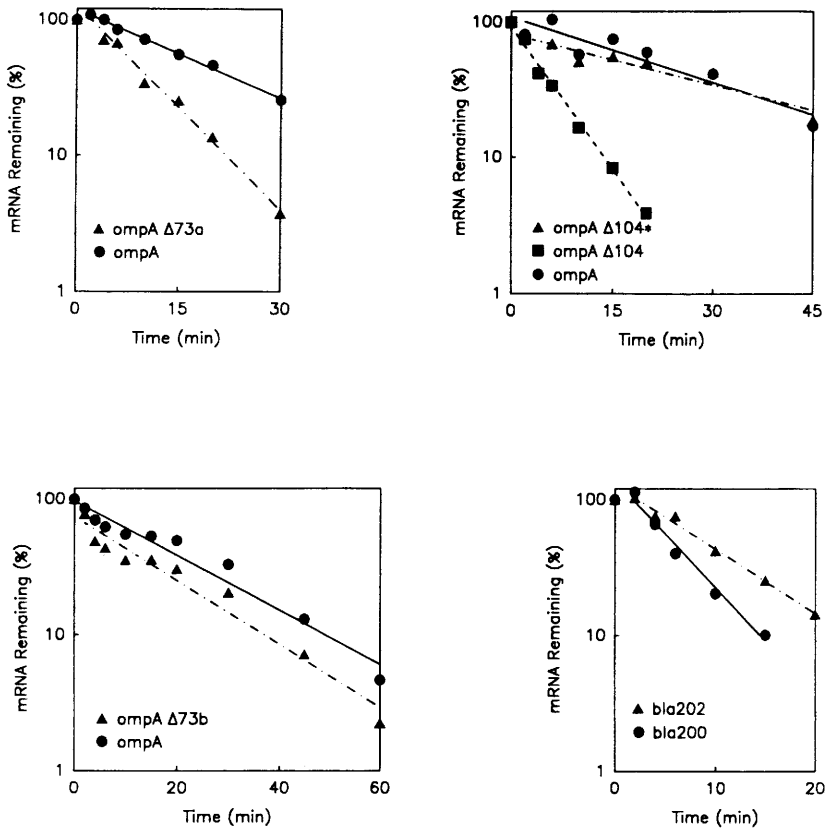


Figure 7. Degradation of a variant *ompA* transcript bearing a minimal 5′ stabilizer preceded by 0, 1, or 5 nucleotides and of *bla* mRNA with an added 5′-terminal stem–loop. After transcription inhibition, total cellular RNA was isolated periodically from *E. coli* strain C600S containing pOMPAΔ*73a (top left)*, pOMPAΔ*73b (bottom left)*, pOMPAΔ*104* (top right)*, pBLA200 (*bottom right*), or pBLA202 (*bottom right).* Transcripts of the *ompA*, *ompAΔ73a*, *ompAΔ73b*, *ompAΔ104**, *bla200*, and *bla202* genes were detected by S1 analysis, and mRNA concentration was plotted semilogarithmically as a function of time. The measured half-lives were 6.0 ± 0.3 min for *ompAΔ73a* mRNA (▲) and 14 ± 1 min for wild-type *ompA* mRNA (●) (*top left*), 13 ± 1 min for *ompAΔ73b* mRNA (▲) and 15 ± 1 min for wild-type *ompA* mRNA (●) (*bottom left*), 24 ± 3 min for *ompAΔ104** mRNA (▲) and 19 ± 3 min for wild-type *ompA* mRNA (●) (*top right*), 3.7 ± 0.3 min for *bla200* mRNA (wild-type) (●) and 18 ± 2 min for wild-type *ompA* mRNA (*bottom right*), and 6.8 ± 0.4 min for *bla202* mRNA (▲) and 17 ± 2 min for wild-type *ompA* mRNA (*bottom right*). For comparison, decay data for *ompAΔ104* mRNA (■) are also shown (*top right*; Table 1).

stem–loop may be of general importance as a means by which prokaryotic organisms can selectively enhance the stability of mRNA.

Mechanism of mRNA degradation in E. coli

The roughly additive contributions to mRNA stability of two distinct structural domains of the *ompA* 5′ UTR suggest that the degradation of messages that can be stabilized by fusion to this 5′ UTR is initiated either by a single ribonuclease whose rate of attack is determined by a combination of 5′ UTR structural features or by a pair of ribonucleases with differing specificities, both of which must be blocked to achieve pronounced mRNA longevity. Undoubtedly there are unstable transcripts whose lability results from attack by a different ribonuclease that is not sensitive to structural features near the mRNA 5′ end; addition of a 5′-terminal hairpin or fusion to the entire *ompA* 5′ UTR is not expected to stabilize such messages. Nevertheless, the lifetimes of many short-lived *E. coli* RNAs (including *bla*, *lacZ*, and *phoA* mRNA) are controlled by the ribonuclease(s) impeded by substituting the *ompA* 5′ UTR or adding a simple 5′-terminal stem–loop (Belasco et al. 1986; M. Hansen and J. Belasco, unpubl.); and, in principle, all long-lived messages must have structures, sequences, or bound factors that shield them from attack by this enzyme.

Our data therefore suggest that *E. coli* contains a ribonuclease that prefers to attack messages beginning with >2–4 unpaired nucleotides. This enzyme is not a 3′ exonuclease, as multiple lines of evidence indicate that *bla* mRNA degradation, which is slowed by addition of a 5′-terminal hairpin, begins upstream of the 3′ end (von Gabain et al. 1983; Belasco et al. 1986). Instead, the sensitivity of this ribonuclease to base-pairing at the mRNA 5′ terminus indicates that it is either a 5′ exonuclease or a 5′-end-dependent endonuclease that cuts internally but interacts, at least initially, with the 5′ end of mRNA. There are a number of reasons to doubt that the ribonuclease obstructed by the *ompA* 5′ UTR is a 5′ exonuclease that removes single nucleotides sequentially from the RNA 5′ end. First, as shown previously for the wild-type *ompA* transcript (von Gabain et al. 1983), rapid degradation of *ompAΔ64*, *ompA+16a*, and *ompA+16b* mRNA appears to begin downstream of the 5′ UTR. In addition, degradation of *ompA* transcripts with either of two single-stranded 5′ extensions does not generate wild-type *ompA* mRNA as a readily detectable decay intermediate; significant accumulation of such an intermediate might be expected if these messages were degraded by a hypothetical 5′ exonuclease that is impeded when it encounters an RNA stem–loop structure. Third, no 5′ exoribonuclease has ever been detected in *E. coli*. Finally, inactivation of the *ams/rne* gene product in *E. coli* stabilizes many RNAs (including *ompA* mRNA) and inhibits their cleavage in vivo by RNase E or RNase K, two *E. coli* endonucleases that appear to be interrelated and may even be identical (Apirion 1978; Ono and Kuwano 1979; Lundberg et al. 1990; Mudd et al. 1990a,b; Babitzke and Kushner 1991; Lin-Chao and Cohen 1991;

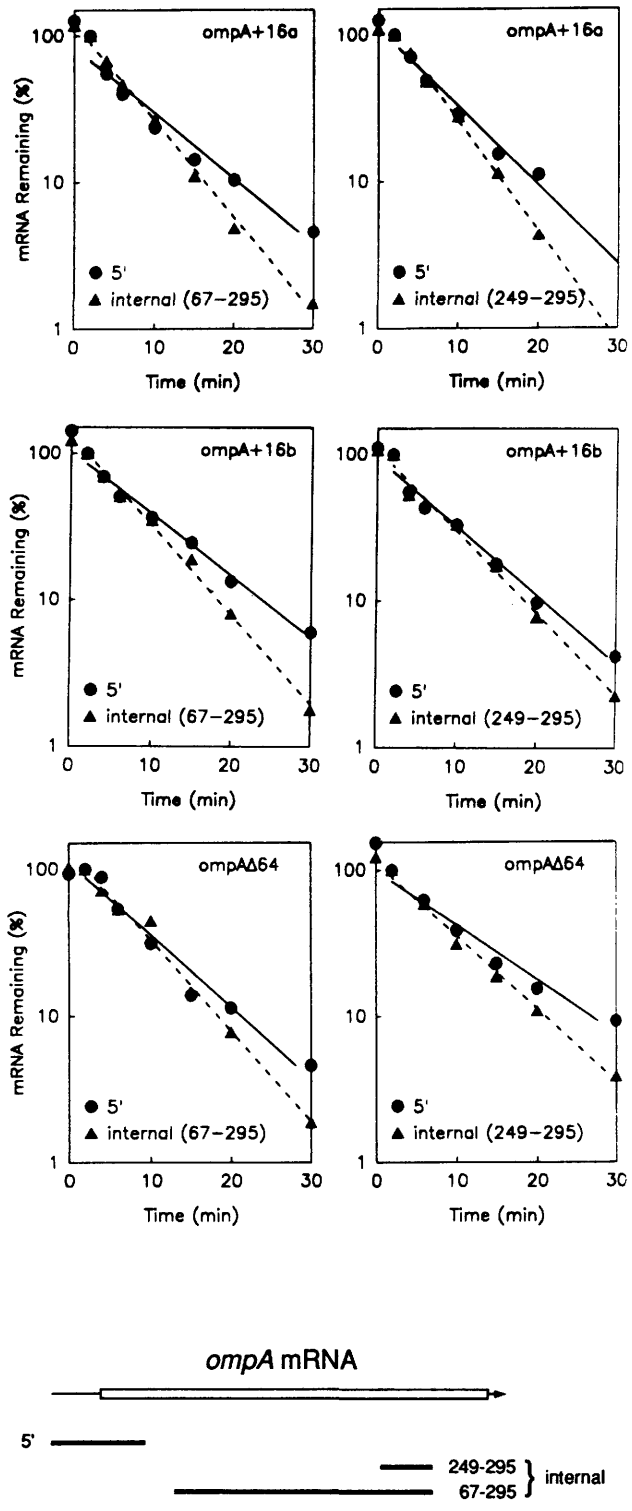


Figure 8. Segmental differences in stability within mutant *ompA* transcripts lacking a 5′-terminal stem–loop. After transcription inhibition, total cellular RNA was isolated periodically from *E. coli* strain SE600 containing pOMPA+16a, pOMPA+16b, or pOMPAΔ64. The degradation of 5′-terminal and internal segments of each mutant *ompA* transcript was monitored simultaneously by S1 analysis with a mixture of two 5′-end-labeled DNA probes, one complementary to the 5′ UTR plus codons 1–37 and the other complementary either to codons 67–295 or to codons 249–295. (*Top*) Semilogarithmic plots of the decay of 5′-terminal (●) and internal (▲) segments of *ompA+16a* (*top*), *ompA+16b* (*middle*), and *ompAΔ64* (*bottom*) mRNA, as determined by S1 analysis with two pairs of segment-specific probes. In every case, decay of the internal segment is faster than decay of the 5′-terminal segment. (*Below*) Map of the 5′ and internal *ompA* mRNA segments detected by S1 analysis with each probe. (Thin lines) *ompA* UTRs; (open rectangle) *ompA* protein-coding region; (arrowhead) *ompA* 3′ end; (thick lines) *ompA* mRNA segments complementary to each of the three probes.

Mackie 1991; Melefors and von Gabain 1991; Nilsson and Uhlin 1991; Taraseviciene et al. 1991); therefore, either or both of these endonucleases are candidates for the key enzyme(s) whose action is impeded by the *ompA* 5′ UTR. It seems likely, therefore, that the *ompA* 5′ UTR protects the *ompA* message from initial, rate-determining endonucleolytic cleavage at one or more sites downstream of the 5′ end. The precise location of these sites is currently under investigation.

If the enzyme that is impeded by the *ompA* 5′ UTR is an endonuclease (i.e., a ribonuclease that cuts internally), then the rate of mRNA cleavage by this endonuclease is determined not only by the presence of RNA sequences that can serve as cleavage sites but also by the presence or absence of structures near the mRNA 5′ end that can control access to these sites. Indeed, the ability of a 5′-terminal stem–loop to shield an entire transcript from degradation suggests that the primary determinant of mRNA half-life may often be the accessibility of potential cleavage sites rather than their number or their intrinsic susceptibility to cleavage. This protective effect of a 5′ stem–loop suggests a degradation mechanism for *E. coli* messages that can be stabilized by fusion to the *ompA* 5′ UTR, whereby a rate-determining, 5′-end-dependent endonuclease first binds in a structure-dependent step to the mRNA 5′ end or to a protein bound there

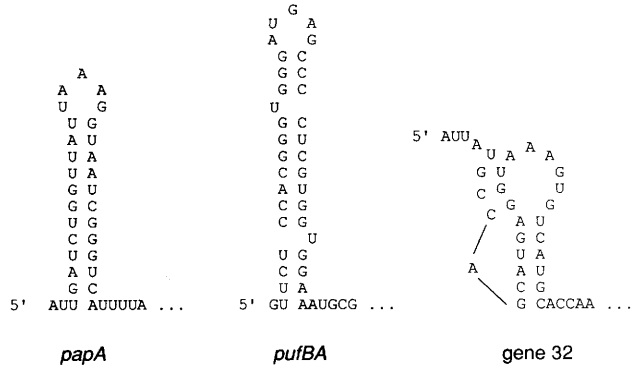


Figure 9. Stem–loop structures near the 5′ end of stable prokaryotic mRNAs. The sequence and likely secondary structure is shown for a 5′-terminal untranslated segment of *E. coli papA* mRNA, *R. capsulatus pufBA* mRNA, and T4 gene 32 mRNA (McPheeters et al. 1988).

and then seeks out cleavage sites downstream. The RNA fragments thereby generated could be rapidly degraded to mononucleotides by further endonuclease cleavage and exonuclease digestion.

The influence of a 5′-terminal structural element on the stability of mRNA bears a striking resemblance to the selective degradation of proteins on the basis of their amino-terminal amino acid residue (Varshavsky et al. 1988). Moreover, the apparent effect of an upstream structural element on mRNA cleavage at downstream sites is reminiscent of cellular regulatory mechanisms that control transcription and translation rates via sequence elements located upstream of initiation sites for RNA or protein synthesis.

Materials and methods

Bacterial strains and plasmids

E. coli K-12 strains C600S and SE600 (Nilsson et al. 1987) are streptomycin-resistant, *supE44*$^-$ variants of strain C600. SE600 is identical to C600S except for a deletion of the chromosomal *ompA* gene (Emory and Belasco 1990).

Plasmid constructions were confirmed by restriction mapping and DNA sequencing. When necessary, protruding DNA ends were made blunt prior to ligation by treatment with T4 DNA polymerase, the Klenow fragment of DNA polymerase I, or mung bean nuclease. Oligonucleotide-directed mutagenesis was performed as described previously (Kunkel 1985; Nakamaye and Eckstein 1986) using purified oligodeoxynucleotides synthesized on an Applied Biosystems 381A instrument.

Plasmid pOMPA+4 is a pBR322 derivative that encodes a pseudo-wild-type *ompA* message transcribed from the *bla* promoter (Emory and Belasco 1990); this plasmid has a *Bcl*I site at the *ompA+4* transcription initiation site. Plasmid pOMPA+4+3 was constructed by oligonucleotide-directed insertion of 3 bp (ATC) into pOMPA+4 to create a second *Bcl*I site at the promoter-distal end of the segment encoding hp1 (GTGAA→GTGATCAA). Deletion of the 0.07-kb *Bcl*I fragment of pOMPA+4+3 generated pOMPAΔ*64*. Plasmids pOMPAΔ29 and pOMPAΔ73a were constructed by deleting from pOMPA+4 a 0.03-kb *Bcl*I (filled-in)–*Eco*RV fragment or a 0.08-kb *Bcl*I (filled-in)–*Hph*I (T4 DNA polymerase) fragment, respectively, thereby reconstituting the *Bcl*I site. Plasmid pOMPAΔ73b was constructed by cleaving pOMPAΔ73a with *Bcl*I, treating the linearized DNA with mung bean nuclease, and religating the resulting blunt ends. The 5′ terminus of *ompAΔ73b* mRNA mapped to 1 nucleotide upstream of *ompA* hp2, and DNA sequencing of pOMPAΔ73b revealed the unexpected loss of one additional base pair upstream of the four that were planned. Plasmid pOMPAΔ74-103 was constructed by oligonucleotide-directed deletion of a 30-bp pOMPA+4 fragment that encodes hp2 and the preceding nucleotide; this mutation created a *Sna*BI at the site of deletion. Plasmid pOMPAΔ104 was created by deleting the 0.08-kb *Bcl*I–*Sna*BI fragment of pOMPAΔ74-103, after first adapting the *Sna*BI end with a *Bcl*I linker (CTGATCAG) and cleaving the linker with *Bcl*I. Insertion of the 0.06-kb *Bcl*I fragment of pOMPA+4+3 in its natural orientation into the *Bcl*I site of pOMPAΔ104 generated pOMPAΔ65-104. Plasmids pOMPAΔ9-52 and pOMPAΔ104-114 were constructed by oligonucleotide-directed deletion from pOMPA+4 of either a 44-bp fragment encoding the top of hp1 or an 11-bp fragment encoding the first 11 nucleotides of ss2, respectively.

Plasmid pOMPA+16a was created by cloning a 1.28-kb *Acc*I (filled-in)–*Pst*I *ompA* fragment of pTU100 (Bremer et al. 1980) between the *Bcl*I (filled-in) and *Pst*I sites of pJB322 (Belasco et al. 1986), thereby reconstituting the *Bcl*I site. Plasmid pOMPA+12a was constructed by cleaving pOMPA+16a with *Bcl*I, removing the protruding ends with mung bean nuclease, and religating the resulting blunt ends. Plasmid pOMPA+21b was constructed by oligonucleotide-directed insertion of 17 bp (GATCTATACTATAACCG) at a site immediately promoter-distal to the *Bcl*I recognition sequence of pOMPA+4, thereby creating a *Bgl*II site adjacent to the *Bcl*I site (TGATCAGATCTATACTATAACCG). Plasmid pOMPA+16b is nearly identical to pOMPA+21b but lacks the 5-bp *Bcl*I–*Bgl*II fragment. Insertion of a symmetrical DNA linker (GCCCACCGGCAGCTGCCGGTGGGC) into the *Bcl*I site (filled-in) of pOMPAΔ64, pOMPA+16a, pOMPA+21b, pOMPAΔ104, and pBLA200 (Emory and Belasco 1990) created pOMPAΔ64*, pOMPA+12a*, pOMPA+16b*, pOMPAΔ104*, and pBLA202, respectively.

Plasmid pOMPAΔ331 was constructed by deleting a 0.33-kb *Bcl*I (filled in)–*Hpa*I fragment from pOMPA+16a, thereby reconstituting the *Bcl*I site. Plasmid pPB101 was created by inserting a 0.39-kb *Bam*HI–*Pst*I fragment of pOMPA+16a between the *Bam*HI and *Pst*I sites of pUC19.

Measurement of mRNA lifetimes

Bacterial culture at 30°C in supplemented LB medium, RNA extraction, S1 analysis, and calculation of mRNA half-lives were performed as described previously (Emory and Belasco 1990). All strains grew rapidly under these conditions, with doubling times between 34 and 44 min. Quantitation of S1-protection data was accomplished either by autoradiography and densitometry (Emory and Belasco 1990) or by gel scanning on a Molecular Dynamics model 400 PhosphorImager. Half-lives were calculated by least-squares analysis of semilogarithmic plots of mRNA concentration versus time. Half-life errors were estimated from the standard deviation of the slope of each plot.

Three different 5′-end-labeled DNA fragments of pOMPA+16a were used as probes to monitor the decay of wild-type *ompA* mRNA and most variants thereof. S1 analysis with any of these probes (a 1.2-kb *Bgl*II–*Eco*RI DNA fragment, a 0.6-kb *Bst*EII–*Eco*RI fragment, or a 0.6-kb *Hin*dIII–*Eco*RI fragment), which have been described previously (Emory and Belasco 1990), gives the same half-life for the wild-type *ompA* transcript because all three probes extend beyond the 3′ boundary of the comparatively stable 5′-terminal segment of *ompA* mRNA. Decay of *ompA+16b* and *ompA+16b** mRNA was monitored by S1 analysis with a 0.6-kb *Hin*dIII–*Eco*RI fragment of a pOMPA+16b* point mutant, which was 5′-labeled at a *Hin*dIII site created by oligonucleotide-directed mutagenesis at a site corresponding to *ompA* codon 94. Differential decay of segments within the *ompA+16a*, *ompA+16b*, and *ompAΔ64* messages was monitored by simultaneous S1 analysis with two segment-specific probes labeled at the 5′ end: a 0.46-kb *Ava*II–*Hin*dIII fragment of pOMPA+16a complementary to the 5′ UTR plus codons 1–37, and either a 0.86-kb *Bgl*II–*Eco*RI fragment of pOMPAΔ331 complementary to codons 67–295 or a 0.16-kb *Bgl*II–*Eco*RI fragment of pPB101 complementary to codons 249–295. Comparative 5′-end-mapping of *ompA*, *ompA+16a*, and *ompA+16b* mRNA was performed by S1 analysis with a 0.5-kb *Bst*EII–*Eco*RI fragment of either pOMPA+16a or pOMPA+16b. Decay of *bla200* and *bla202* mRNA was monitored by S1 analysis with a 5′-labeled 1.03-kb *Hin*fI–*Hin*dIII fragment of pBLA200.

Probing of RNA secondary structure with DMS

Methylation of RNA by DMS in *E. coli* and in vitro, alkylation of RNA with CMCT in vitro, and mapping of reactive nucle-

otides were performed as described previously (Moazed et al. 1986; Chen et al. 1991). Bacteria for DMS and CMCT alkylation (SE600/pOMPA+12a, SE600/pOMPA+16a, SE600/pOMPA+16b, or SE600/pOMPAΔ29) were grown and harvested under the same conditions as were employed for measurements of mRNA stability. Two 5′-end-labeled DNA oligonucleotides (AGCGAAACCAGCCAGTGCCACTGC or GATAACACGGTTAAATCCTTCAC) were used as primers to map methylation sites within the 5′ UTR of *ompA* mRNA variants. These primers annealed to mRNA sequences 22–45 nucleotides downstream or 50–72 nucleotides upstream of the *ompA* translation initiation codon, respectively, and they were extended at 55°C with AMV reverse transcriptase (Boehringer).

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