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The *ompA* 5′ Untranslated RNA Segment Functions in *Escherichia coli* as a Growth-Rate-Regulated mRNA Stabilizer Whose Activity Is Unrelated to Translational Efficiency

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The 5′ untranslated region (UTR) of the long-lived *Escherichia coli ompA* message can function in vivo as an mRNA stabilizer. Substitution of this *ompA* mRNA segment for the corresponding segment of the labile *bla* gene transcripts prolongs their lifetime by a factor of 6. We show here that the function of this *ompA* mRNA stabilizer requires the presence of a 115-nucleotide *ompA* RNA segment that lies upstream of the ribosome-binding site. Although deletion of this segment reduced the half-life of the *ompA* transcript by a factor of 5, its absence had almost no effect on the translational efficiency of *ompA* mRNA. Like the *ompA* transcript, but unlike *bla* mRNA, hybrid *ompA-bla* messages containing the complete *ompA* 5′ UTR were significantly less stable under conditions of slow bacterial growth. We conclude that the stabilizing activity of the *ompA* 5′ UTR is growth rate regulated and that the mechanism of mRNA stabilization by this RNA segment is not related to the spacing between translating ribosomes.

In addition to its protein-coding capacity, mRNA contains untranslated segments that serve functions crucial to gene regulation. For example, structural elements in the 3′ untranslated regions (UTRs) of bacterial messages direct transcription termination (44). In a number of instances, gene expression is known to be modulated through changes in the efficiency of transcription termination at similar elements located upstream of protein-coding regions (27). Signals that control rates of translation initiation also reside in untranslated regions of mRNA (20).

Recently, it has become clear that untranslated segments of procaryotic messages also can influence gene expression through their impact on mRNA stability. There is now considerable evidence that RNA stem-loop structures found at the 3′ ends of most bacterial messages and at intercistronic sites of many operon transcripts play an important role in protecting mRNA from digestion by bacterial 3′ exoribonucleases (11, 24, 36, 38). On the other hand, intercistronic and 3′ UTRs of mRNA can have a destabilizing effect when they contain RNA elements that are targets of cellular endoribonucleases (2, 45, 49).

The 5′ UTR of mRNA also can control mRNA stability (3, 4, 6, 12, 14, 21, 48). For example, in *Escherichia coli* the 5′ leader segments of certain long-lived transcripts can prolong the lifetimes of otherwise labile messages to which they are fused (6, 21). Because 5′ UTRs contain signals for ribosome binding, it is possible that their influence on mRNA stability is simply an indirect consequence of differences in translation initiation frequency. According to this model (10, 14, 25, 26), translating ribosomes sterically protect mRNA from otherwise rapid RNase attack, and 5′ UTR substitutions that decrease the spacing between translating ribosomes could prolong message lifetimes by reducing the fractional length of unshielded RNA accessible to cellular RNases. Alternatively, the sequences or structures of some 5′ UTRs might make them poor targets for a bacterial RNase that initially associates at or near the 5′ end of mRNA, and their stabilizing influence might therefore be an immediate consequence of their ability, either directly or in concert with an associated protein, to impede binding by such an RNase to mRNA.

One *E. coli* message whose long lifetime apparently is determined by its 5′ UTR is the transcript of the *ompA* gene. The *ompA* gene encodes a major outer membrane protein of *E. coli*. The abundance of the OmpA protein is a consequence of both the strength of the *ompA* promoter and the unusual longevity of the *ompA* transcript, whose half-life of 15 to 20 min in rapidly growing cells makes it one of the most stable bacterial messages known (55). OmpA protein synthesis is regulated by the rate of cell growth and declines as the cell doubling time increases; this regulation is achieved primarily through modulation of the stability of *ompA* mRNA, whose half-life can fall by as much as a factor of 4 in slowly growing cells (29, 39).

Two lines of evidence indicate that the remarkable stability of the *ompA* message is attributable to its long (133-nucleotide [nt]) 5′ UTR (Fig. 1). Substitution of the 5′ UTR and first few codons of *ompA* mRNA for the corresponding portion of the labile *bla* gene transcripts increases the half-life of *bla* mRNA in rapidly growing cells from 3 min up to 17 min (6). In addition, the relative concentrations of *ompA* mRNA fragments resulting from endonucleolytic cleavage in the 5′ UTR increases with decreasing message stability at slow bacterial growth rates (32).

We show here that deletion of the 115-nt segment of the *ompA* 5′ UTR that precedes the Shine-Dalgarno element reduces the stability of the *ompA* transcript to that of an ordinary *E. coli* message. Stabilization of *ompA* mRNA by the presence of this segment is not related to the frequency of translation initiation; instead, the *ompA* 5′ UTR appears to act in a direct manner to ward off RNase attack. We also present evidence that, in addition to its ability in *cis* to confer stability on *bla* mRNA in rapidly growing cells, the *ompA* 5′ UTR mediates growth rate regulation of mRNA stability.

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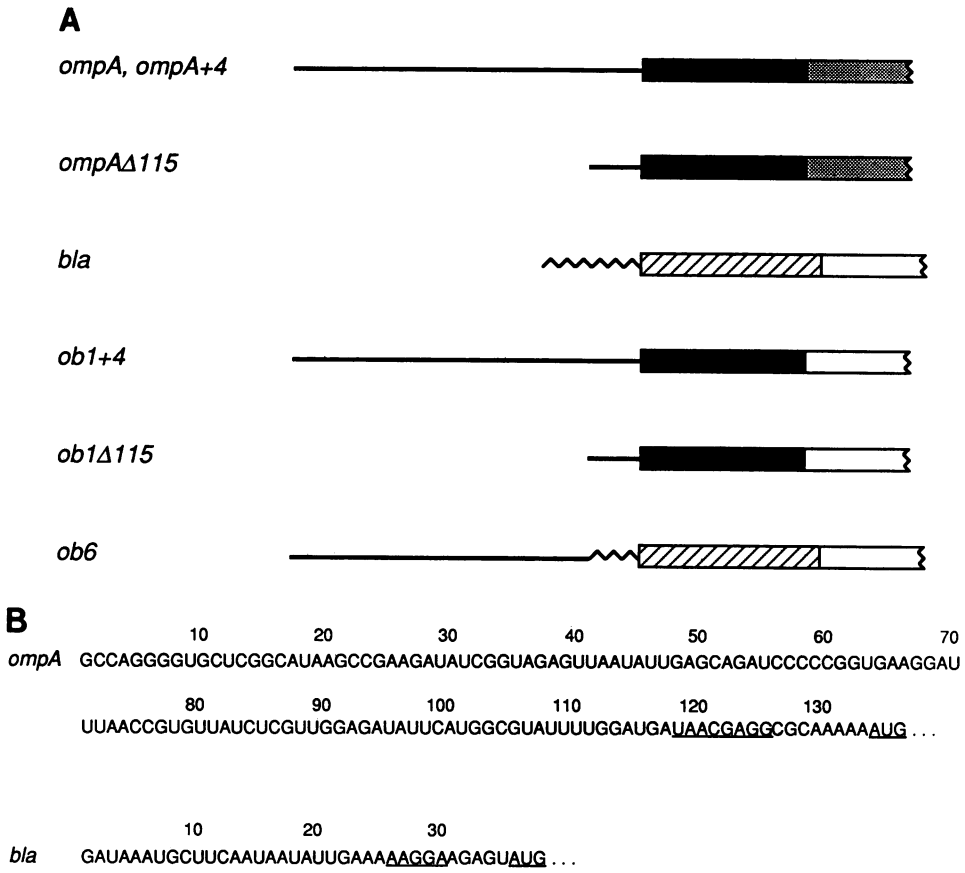

FIG. 1. The 5′ segments of *E. coli* messages examined in this study. (A) Maps of the 5′-terminal segments of *ompA* and *bla* mRNA and variants thereof. ——, Full-length or truncated *ompA* 5′ UTR; ∿∿∿, full-length or truncated *bla* 5′ UTR. Translated mRNA segments encoding the pre-OmpA signal peptide (■) and pre-β-lactamase signal peptide (▨) are indicated, as are mRNA segments encoding the amino termini of the mature OmpA protein (▩) and of mature β-lactamase (□). The central and 3′-terminal segments of these messages are not shown. (B) Sequences of the 5′ UTRs of wild-type *E. coli ompA* and *bla* mRNAs (7, 22, 54). The 5′ terminus of each wild-type transcript is at position 1 (8, 15; von Gabain, personal communication). In each case, the Shine-Dalgarno element and translation initiation codon are underlined. The mRNA sequence downstream of this AUG codon is not shown. The 5′ UTR of *ompA+4* and *ob1+4* mRNAs differs from the wild-type *ompA* 5′ UTR in that four nucleotides (GAUC) have been added at the 5′ end and the G at position 1 of the wild-type sequence has been changed to A. In *ompAΔ115* and *ob1Δ115* mRNAs, nt 1 to 115 of the wild-type *ompA* 5′ UTR have been deleted and replaced with the sequence GAUCAG. In *bla200* mRNA, nucleotide 4 of the wild-type *bla* sequence has been changed to C; this mutation does not affect the half-life of *bla* mRNA (6). In *ob6* mRNA, nucleotides 1 to 18 of *bla* mRNA have been replaced with the first 118 nt of the *ompA+4* 5′ UTR (nt 118 of the *ompA+4* transcript corresponds to nt 114 of wild-type *ompA* mRNA).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strains used as hosts for transcript half-life measurements were C600S, PM191, and SE600, all of which are derivatives of C600. C600S is a streptomycin-resistant, *supE44* variant of C600 (40); PM191 is a *recA* mutant (31).

SE600 is a derivative of C600S with a chromosomal deletion in the *ompA* gene that prevents *ompA* mRNA synthesis. It was constructed by two successive P1 transductions into C600S (33). C600S was first transduced with a P1 lysate of CC111 (*ompA901*::Tn*5 pyrD* [30]). Kanamycin-resistant transductants were screened for a *pyrD* phenotype and for resistance to infection by the OmpA-specific bacteriophage K3h1. SE600 was then generated by transduction to a *pyrD*$^+$, kanamycin-sensitive phenotype, using a P1 lysate grown on the *ompA* deletion mutant BRE50 (6). SE600 is K3h1 resistant and contains no *ompA* mRNA, as judged by gel blot analysis.

For RNA or protein isolation under rapid growth conditions, *E. coli* cultures were maintained for 5 to 7 generations of steady exponential growth at 30°C in LB medium (33) supplemented with 0.4% glucose and 0.5% Casamino Acids (Difco Laboratories). To achieve slower growth rates, cells were grown at 30°C in 2× MOPS medium (37) supplemented with 2 mM $FeSO_4$ and either 0.8% sodium acetate or 0.8% sodium succinate as a carbon source. Cultures were continuously monitored to ensure a stable growth rate and harvested at an optical density of 0.30 ± 0.02 at 650 nm.

Plasmids. To construct pOMPA+4, a *Bcl*I site was created at the transcription initiation site of the *E. coli ompA* gene by oligonucleotide-directed mutagenesis (ACATCG → TGATCA). The *ompA* transcription unit was then inserted as a 1.2-kilobase-pair (kb) *Bcl*I-*Pst*I fragment between the *Bcl*I and *Pst*I sites of pJB322 (6). This brought *ompA+4* transcription under the control of the *bla* promoter, which directs transcription initiation at a site (8) just 4 nt upstream of the wild-type *ompA* 5′ end.

Plasmid pOMPAΔ115 was constructed from pOMPA+4 by deleting a 0.12-kb *Bcl*I-*Mnl*I fragment after adapting the *Mnl*I end with an 8-base-pair (bp) *Bcl*I linker (CTGATCAG). Sequence analysis of pOMPAΔ115 showed that an additional base pair had unexpectedly been lost from the *Mnl*I end of

the deletion. Therefore, in *ompAΔ115* mRNA, the first 115 nt of the wild-type *ompA* message have been deleted and replaced with the hexanucleotide GAUCAG.

Plasmid pOB6 was constructed from pBLA200, which differs from pBR322 in two ways. First, like pJB322 (6), pBLA200 has a unique *Bcl*I site at the *bla* transcription initiation site, thanks to an A→C mutation engineered at position 4187 of the pBR322 sequence (42, 54). Second, introduction of the fd phage transcription terminator as a 0.35-kb insert at the *Hin*dIII site (6, 19) blocked transcription of the *bla* gene from promoters upstream of its natural promoter but allowed transcription of the *tet* gene from an fd phage promoter also present on the cloned fragment. To create pOB6, an 18-bp *Bcl*I-*Ssp*I fragment from the promoter-proximal end of the *bla* transcription unit of pBLA200 was replaced with a 118-bp *Bcl*I-*Mnl*I fragment from the promoter-proximal end of the *ompA* transcription unit of pOMPA+4.

Plasmids pOB1+4 and pOB1Δ115 were derivatives of pBB322, a variant of pBLA200 with a unique *Bst*EII site created by a C→G mutation in *bla* codon 23, which encodes the last amino acid of the pre-β-lactamase signal peptide. In pOB1Δ115, a 0.10-kb *Bcl*I-*Bst*EII (filled-in) fragment of pBB322 encoding the *bla* 5′ UTR and the pre-β-lactamase signal peptide was replaced with a 0.08-kb *Bcl*I-*Hae*III fragment of pOMPAΔ115 encoding the *ompAΔ115* 5′ UTR and the pre-OmpA signal peptide. Plasmid pOB1+4 is identical to pOB1Δ115 except that it instead contains the corresponding 0.20-kb *Bcl*I-*Hae*III fragment of pOMPA+4. Both *ob1+4* and *ob1Δ115* were in-frame gene fusions that encoded a chimeric preprotein consisting of the pre-OmpA signal peptide joined to mature β-lactamase.

RNA isolation. *E. coli* culture samples (12 to 25 ml) were rapidly cooled to 0°C by addition to crushed ice. The cells were pelleted and suspended in ice-cold 0.3 M sucrose–0.01 M sodium acetate (pH 4.5) (0.125 ml). After addition of 2% sodium dodecyl sulfate (SDS)–0.01 M sodium acetate (pH 4.5) (0.125 ml), the cell suspension was heated for 3 min at 70°C and extracted three times for 3 min at 70°C with hot phenol (0.25 ml) that had been preequilibrated with unbuffered water. RNA was ethanol precipitated and treated for 30 min at room temperature with RNase-free DNase I (30 U; Bethesda Research Laboratories, Inc.) in 20 mM sodium acetate (pH 4.5)–10 mM magnesium chloride–10 mM sodium chloride. EDTA was then added (to 25 mM), and the RNA was phenol extracted, ethanol precipitated, and stored at −20°C in 20 mM sodium phosphate (pH 6.5)-1 mM EDTA.

Probes for S1 analysis of mRNA. Three different 5′-end-labeled DNA fragments were used as probes of *ompA* mRNA; all gave the same half-life for the wild-type *ompA* transcript, which served as an internal standard. One probe, a 1.2-kb *Bgl*II-*Eco*RI fragment of pOMPA+4, was labeled at a *Bgl*II site corresponding to codon 293 of *ompA* mRNA. A second probe, a 0.6-kb *Bst*EII-*Eco*RI fragment of pOMPA+4, was labeled at a *Bst*EII site corresponding to *ompA* codon 105. A third probe, a 0.6-kb *Hin*dIII-*Eco*RI fragment of a pOMPA+4 point mutant, was labeled at a *Hin*dIII site that was created by oligonucleotide-directed mutagenesis at a site corresponding to *ompA* codon 94.

Hybrid *ompA-bla* transcripts *ob1+4* and *ob1Δ115* were detected with a 1.0-kb *Hin*fI-*Eco*RI probe from pBLA200 or pBB322; these probes were 5′ end labeled at a *Hin*fI site corresponding to *bla* codon 264. The transcript of the *ob6* gene was probed with a 1.1-kb *Hin*fI-*Eco*RI fragment of pOB6; this probe was 5′ end labeled at a *Hin*fI site corresponding to *ob6* codon 264.

In some experiments, an additional DNA probe was included to detect *lpp* mRNA. This probe was derived from a 1.0-kb *Xba*I-*Hpa*I fragment of pKEN113 (28), 3′ end labeled at an *Xba*I site located between the *lpp* transcription and translation initiation sites.

Measurement of mRNA half-life. To measure mRNA decay rates, total cellular RNA was extracted at time intervals after transcription inhibition with rifampin (0.2 mg/ml), and equal amounts (2 μg) of each RNA sample were subjected to S1 analysis with one or more end-labeled DNA probes (30 ng). Hybridization was carried out for 4 h at 45°C and was followed by treatment with S1 nuclease (100 U) for 90 min and electrophoresis beside a set of molecular size standards (not shown in Fig. 2 to 6) on a 4 or 6% polyacrylamide gel containing 40% urea. Data from autoradiograms were quantitated by scanning laser densitometry on an LKB Ultroscan XL instrument, using LKB GelScan software. Correction for the slightly nonlinear response of the X-ray film was made possible by including on each gel a time-zero sample diluted two-, four-, and eightfold (not shown in Fig. 2 to 6). Half-lives were calculated by least-squares analysis of a semilogarithmic plot of mRNA concentration as a function of time, using only measurements from RNA samples collected at least 2 min after rifampin addition to allow time for completion of nascent transcripts. Half-life errors were estimated from the standard deviation of the slope of each plot.

Translational efficiency. Relative translational efficiencies were measured by quantitating relative steady-state concentrations of mRNA and either OmpA protein or β-lactamase activity in samples taken simultaneously from a single culture and quenched rapidly on ice.

Relative steady-state concentrations of *bla*, *ob1+4*, and *ob1Δ115* mRNA were determined in parallel by S1 analysis of equal amounts of total cellular RNA, using the same mixture of two probes: one complementary to the *bla* segment of these messages and the other complementary to the chromosomal *lpp* transcript, which served as an internal standard. Relative β-lactamase production was determined by measuring β-lactamase enzyme activity per microgram of total cellular protein. Cells were lysed by treatment for 30 min at 0°C with lysozyme (2 mg/ml) in 20 mM Tris hydrochloride–10 mM EDTA (pH 7.3), followed by three freeze-thaw cycles. After centrifugation for 30 min at 4°C, β-lactamase activity in the lysate supernatant was determined from the rate of benzylpenicillin hydrolysis, which was monitored at 240 nm (46, 47). Total cellular protein in the same lysate supernatant was measured by using the Pierce Coomassie protein assay reagent as instructed by the manufacturer.

Relative steady-state concentrations of *ompA*, *ompA+4*, and *ompAΔ115* mRNAs were measured in parallel by S1 analysis of equal amounts of total cellular RNA, using the same mixture of two probes: one complementary to the *ompA* message and the other complementary to the *lpp* transcript, which served as an internal standard. Relative OmpA protein production was determined from the ratio in *E. coli* outer membranes of OmpA to OmpC, another outer membrane protein that served as an internal protein standard. *E. coli* outer membrane proteins were isolated as described previously (23). Outer membrane protein samples were heated to 100°C for 5 min in loading buffer (10 mM Tris hydrochloride, 4 mM sodium phosphate [pH 7.8], 0.5% SDS, 0.5% 2-mercaptoethanol, 52% glycerol, 0.25% bromophenol blue) and fractionated by electrophoresis on a polyacrylamide-urea gel. The running gel consisted of 15% polyacryl-

amide (14.6% acrylamide plus 0.4% bisacrylamide), 8 M urea, 0.1% SDS, and 0.375 M Tris hydrochloride (pH 8.9). The stacking gel consisted of 5% polyacrylamide, 0.1% SDS, 62.5 mM Tris hydrochloride, and 56 mM sodium phosphate (pH 7.8). The gel was stained with Coomassie blue (17), and the concentration of OmpA protein relative to OmpC was quantified by densitometry.

RESULTS

Mutations in the *ompA* 5′ UTR can reduce mRNA stability. The half-life of *bla* mRNA in *E. coli* increases from 3 min up to 17 min when a *bla* message segment comprising the 5′ UTR and the first 22 codons is replaced with the 5′ UTR and the first 4 codons of the *E. coli ompA* transcript (6). To define more precisely the portion of this *ompA* segment that is sufficient to stabilize *bla* mRNA, the decay of transcripts from three additional *ompA-bla* fusions was examined.

In the prototype fusion, *ob1+4* (Fig. 1), a *bla* gene segment encoding the 5′ UTR and signal peptide was replaced with the corresponding segment of the *ompA* gene. The chimeric messages produced from this plasmid-borne in-frame gene fusion were transcribed from the *bla* promoter beginning at a site just 4 nt upstream of the wild-type *ompA* 5′ end; β-lactamase produced from these messages was exported to the periplasm with the aid of the OmpA signal peptide. The decay rate of *ob1+4* mRNA was measured in *E. coli* C600S carrying plasmid pOB1+4 and growing with a 42-min doubling time by S1 analysis of RNA samples collected at time intervals after transcription inhibition with rifampin (Fig. 2). Simultaneous monitoring of the degradation of the chromosomal *ompA* transcript provided an internal standard. The *ob1+4* transcripts decayed slowly and uniformly with a half-life of 17.8 ± 0.5 min, a value virtually indistinguishable from the half-life of the *ompA* transcript itself (15.3 ± 1.4 min) and six times longer than the half-life of wild-type *bla* mRNA (55). This finding is consistent with the long lifetime previously observed for an *ompA-bla* hybrid message (*ob2*) that contained the entire *ompA* 5′ UTR but lacked nearly all of the RNA segment encoding the OmpA signal peptide (6), except that the biphasic decay reported for *ob2* mRNA was not evident in these experiments. The longevity of *ob1+4* mRNA confirms that the *ompA* 5′ UTR can function in *cis* as a stabilizer of heterologous mRNA and shows that the stabilizing activity of this untranslated RNA segment is independent of the promoter from which it is transcribed.

The 133-nt *ompA* 5′ UTR (Fig. 1) can be subdivided into two domains: an 18-nt ribosome-binding site and a 5′-terminal 115-nt segment. Each of these domains was tested separately for its ability to prolong the lifetime of *bla* mRNA. To determine whether *bla* mRNA can be stabilized simply by replacing the *bla* ribosome-binding site with that of *ompA*, we deleted from the *ob1+4* 5′ UTR all but the last 18 nt, leaving only the *ompA* ribosome-binding site intact (Fig. 1). The half-life of the resulting *ob1Δ115* transcripts was only 6.9 ± 0.3 min (Fig. 3A). This finding shows that substitution of the *ompA* ribosome-binding site for the *bla* ribosome-binding site enhances the stability of *bla* mRNA only twofold and is not sufficient to explain the 18-min half-life of *ob1+4* mRNA.

To ascertain whether the *ompA* 5′ UTR segment that precedes the Shine-Dalgarno element is alone sufficient to stabilize *bla* mRNA, we replaced the first 18 nt of *bla* mRNA with the first 115 nt of the *ompA* transcript (Fig. 1). The resulting fusion (*ob6*) placed this untranslated *ompA* mRNA segment 7 nt upstream of the *bla* Shine-Dalgarno element (AAGGA), a location nearly equivalent to its position in the *ompA* transcript. The half-life of *ob6* mRNA was 4.1 ± 0.3 min (Fig. 3B), a lifetime barely distinguishable from that of wild-type *bla* mRNA (3 min). Thus, although the *ompA* mRNA segment preceding the ribosome-binding site is necessary for full stability of *ob1+4* mRNA, this segment alone is not able to stabilize *bla* mRNA. Either this RNA segment and the segment encompassing the *ompA* ribosome-binding site function synergistically, or they both overlap a single element that must remain intact for full message stability.

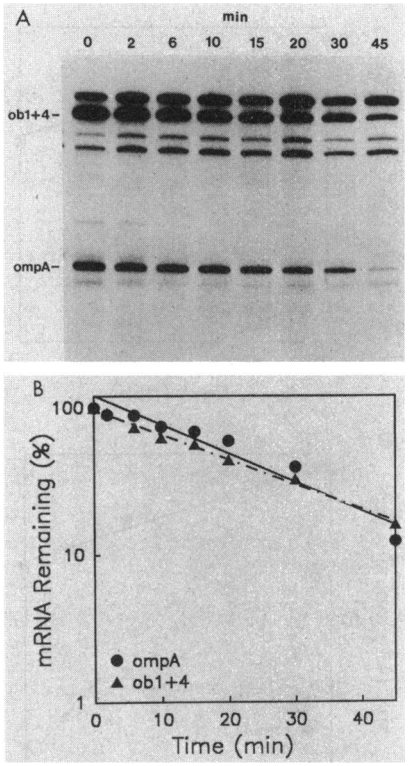

FIG. 2. Decay of *ob1+4* mRNA in rapidly growing *E. coli* cells. (A) S1 analysis. At time intervals after transcription inhibition, total cellular RNA was isolated from *E. coli* C600S containing pOB1+4 and growing with a doubling time of 42 min. The *ob1+4* and endogenous wild-type *ompA* transcripts were detected by S1 analysis of RNA samples (2 μg) with a mixture of transcript-specific DNA probes. Bands that represent *ob1+4* and *ompA* mRNA are indicated (protected probe fragment lengths of 0.83 and 0.45 kb, respectively). The remaining three major bands in each lane correspond to reannealed probe DNA. (B) Semilogarithmic plot of mRNA concentration as a function of time. The measured half-lives were 17.8 ± 0.5 min for *ob1+4* mRNA and 15.3 ± 1.4 min for wild-type *ompA* mRNA.

To examine further the components of the *ompA* 5′ UTR that are important for its function as an mRNA stabilizer, we next determined the contribution of the first 115 nt of this untranslated RNA segment to the stability of the *ompA* transcript itself. A complete *ompA* transcription unit was first fused to the *bla* promoter of pJB322 (a plasmid derivative of pBR322 [6]) so as to allow synthesis in *E. coli* of an *ompA* transcript (*ompA+4*) that was identical to the wild-type transcript except for four additional nucleotides (GAUC) at the 5′ end and a G→A mutation at position 1 of the wild-type *ompA* mRNA sequence (Fig. 1). Substitution of the inefficient *bla* promoter for the strong *ompA* promoter balanced the effect of increasing the *ompA* gene copy

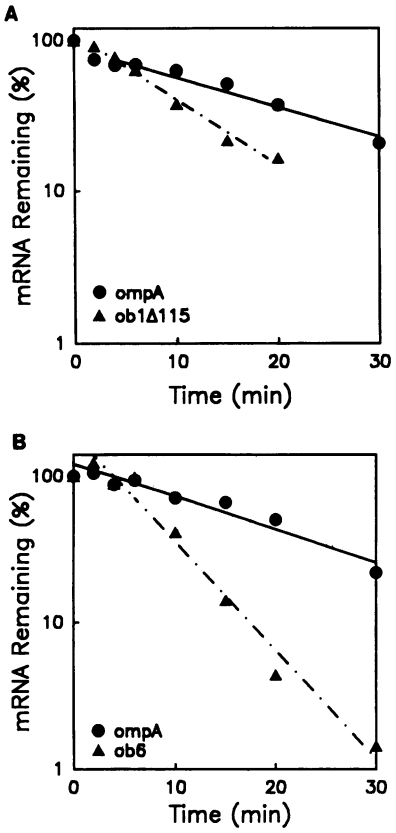

FIG. 3. Decay of *ob1Δ115* and *ob6* mRNAs in rapidly growing *E. coli* cells. At time intervals after transcription inhibition, total cellular RNA was isolated either from *E. coli* PM191 containing pOB1Δ115 and growing with a doubling time of 60 min (A) or from *E. coli* C600S containing pOB6 and growing with a doubling time of 48 min (B). The *ob1Δ115*, *ob6*, and endogenous wild-type *ompA* transcripts were detected by S1 analysis of RNA samples (2 μg) with a mixture of transcript-specific DNA probes. Data from autoradiograms were quantitated by densitometry and analyzed as semilogarithmic plots of mRNA concentration versus time. The measured half-lives were 6.9 ± 0.3 min for *ob1Δ115* mRNA and 15.3 ± 1.1 min for wild-type *ompA* mRNA (A) and 4.1 ± 0.3 min for *ob6* mRNA and 13.4 ± 1.4 min for wild-type *ompA* mRNA (B).

number, so that introduction of plasmid pOMPA+4 into *E. coli* resulted in only a modest (fourfold) increase in the cellular concentration of *ompA* mRNA and OmpA protein (Table 1).

The decay rate of the *ompA+4* transcript was measured in *E. coli* SE600, an isogenic derivative of C600S with a chromosomal *ompA* gene deletion. Two independent experiments gave half-lives of 21.5 ± 1.7 and 17.1 ± 3.4 min (Fig. 4A and C), for an average *ompA+4* half-life of 19.3 min, essentially the same as that of wild-type *ompA* mRNA. The equivalent half-lives of *ompA+4* and wild-type *ompA* mRNA show that in addition to being promoter independent, the stability of *ompA* mRNA is not affected by whether it is chromosome or plasmid encoded or by small changes in the cellular concentration of *ompA* mRNA or OmpA protein. In the second measurement (Fig. 4A and C), degradation of another long-lived *E. coli* transcript, *lpp*, was monitored simultaneously to provide an internal control. Under the cell growth conditions used in this experiment, the *lpp* transcript, which encodes the major *E. coli* outer membrane lipoprotein, decayed very slowly with a half-life of 52 ± 3 min.

Deletion of 115 promoter-proximal bp of the *ompA+4* transcription unit (the same segment deleted in *ob1Δ115*) generated *ompAΔ115* (Fig. 1). Degradation of the resulting 5′-truncated transcript and of the wild-type *ompA* message was monitored simultaneously in *E. coli* C600S containing plasmid pOMPAΔ115. In two independent experiments, the *ompAΔ115* message decayed with half-lives of 3.1 ± 0.1 and 4.1 ± 0.2 min (Fig. 4B and D). This average half-life of 3.6 min was about five times shorter than the half-life of the wild-type *ompA* transcript and of the pseudo-wild-type *ompA+4* message. Clearly, the *ompA* 5′ UTR makes a crucial contribution to the unusual longevity of the *ompA* transcript. Without the 5′-terminal 115-nt segment that precedes the ribosome-binding site, the *ompA* transcript would be no more stable than a typical *E. coli* message.

mRNA stabilization by the *ompA* 5′ UTR is not related to translational efficiency. In principle, the stabilizing influence of the *ompA* 5′ UTR might be the direct result of the ability of this RNA segment to protect the *ompA* transcript from cleavage by a hypothetical cellular RNase that is sensitive to structural features near the 5′ end of mRNA. Alternatively, if deletion of 115 nt from the 5′ end of *ompA+4* and *ob1+4* mRNAs interferes with translation initiation, instability might result indirectly from a proportionate increase in the spacing between translating ribosomes, which might leave these transcripts sterically more accessible to endoribonuclease attack.

To distinguish between these possibilities, we determined the effect on translational efficiency of deleting 115 nt from the 5′ end of *ompA+4* and *ob1+4* mRNAs and of substituting the *ompA* 5′ UTR for the *bla* 5′ UTR. The relative

TABLE 1. Relative translational efficiency of *ompA*-related transcripts versus *ompA* mRNA[a]

mRNA	Strain (doubling time)	OmpA protein[b]	mRNA concn[c]	Relative translational efficiency[d]
	SE600 (45 min)	0	0	
ompA	C600S (37 min)	1.00	1.00	1.00
ompA+4	SE600(pOMPA+4)[e] (40 min)	3.92; 3.29	2.97; 2.53	1.21 ± 0.07 (mean)
ompAΔ115	SE600(pOMPAΔ115)[e] (40 min)	1.00; 0.98	1.28; 1.02	0.81 ± 0.12 (mean)

[a] All data have been normalized to the values obtained for *ompA* mRNA in C600S.
[b] Relative moles of OmpA protein per mole of OmpC protein in the *E. coli* outer membrane.
[c] Relative moles of *ompA*-related mRNA per mole of *lpp* mRNA.
[d] Calculated by determining the ratio of the relative OmpA protein concentration to the relative *ompA*-related mRNA concentration and dividing this value by the relative cell doubling time. Compensation for small differences in cell growth rate is necessary in order to relate steady-state protein concentration to the protein synthesis rate. OmpA is a stable protein in the *E. coli* outer membrane (18).
[e] Data from two independent experiments are reported.

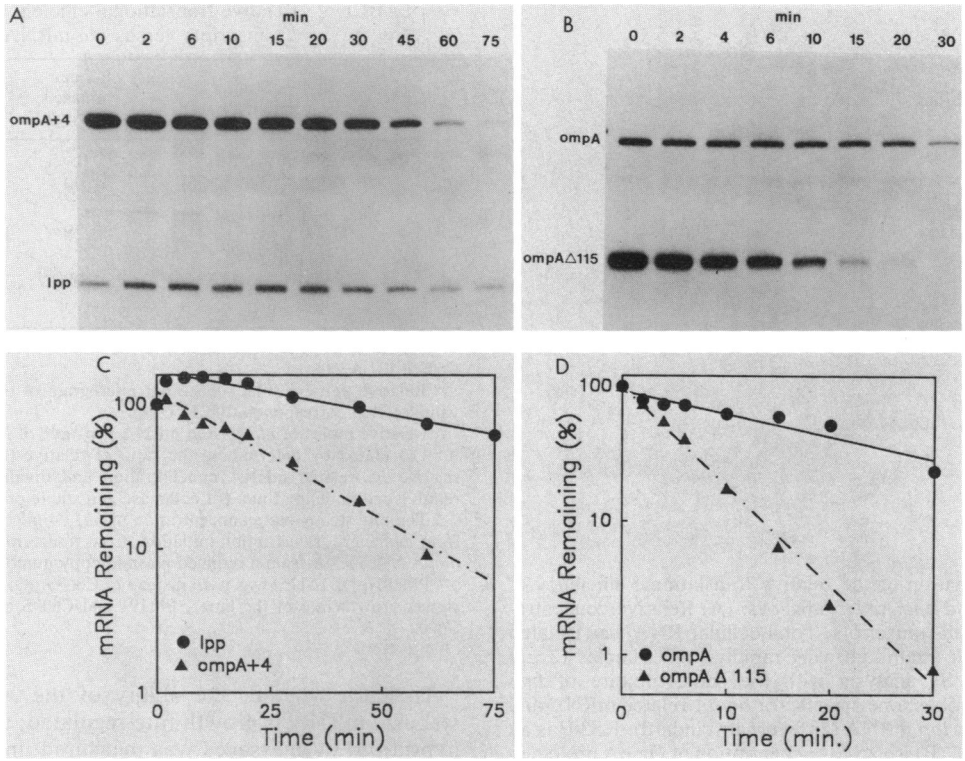

FIG. 4. Decay of *ompA+4* and *ompAΔ115* mRNAs in rapidly growing *E. coli* cells. (A) Analysis of *ompA+4* mRNA. At time intervals after transcription inhibition, total cellular RNA was isolated from *E. coli* SE600 containing pOMPA+4 and growing with a doubling time of 41 min. The *ompA+4* and endogenous *lpp* transcripts were detected by S1 analysis of RNA samples (2 μg) with a mixture of two transcript-specific DNA probes (protected probe fragment lengths of 0.45 and 0.30 kb, respectively). In this experiment, the measured half-lives were 17.1 ± 3.4 min for *ompA+4* mRNA and 52 ± 3 min for *lpp* mRNA. (B) Analysis of *ompAΔ115* mRNA. At time intervals after transcription inhibition, total cellular RNA was isolated from *E. coli* C600S containing pOMPAΔ115 and growing with a doubling time of 39 min. The *ompAΔ115* and endogenous wild-type *ompA* transcripts were detected by S1 analysis of RNA samples (2 μg) with a single DNA probe (protected probe fragment lengths of 0.34 and 0.45 kb, respectively). In this experiment, the measured half-lives were 4.1 ± 0.2 min for *ompAΔ115* mRNA and 17.8 ± 2.5 min for wild-type *ompA* mRNA. (C and D) Semilogarithmic plots of mRNA concentration as a function of time.

translational efficiencies of *ompA*, *ompA+4*, and *ompAΔ115* mRNAs were measured by comparing OmpA protein production with the cellular concentration of the relevant transcript. *E. coli* C600S, or strain SE600 containing either pOMPA+4 or pOMPAΔ115, was grown in rich medium to log phase and harvested for simultaneous extraction of outer membrane proteins and total cellular RNA. The relative steady-state concentration of *ompA*, *ompA+4*, or *ompAΔ115* mRNA in each strain was measured in parallel by S1 analysis, using *lpp* mRNA as an internal standard (Fig. 5A). In addition, the OmpA protein content of each outer membrane sample was determined by gel electrophoresis and staining (Fig. 5B); for these measurements, outer membrane protein OmpC served as an internal standard. (At normal levels of *ompA* gene expression, essentially all of the OmpA protein in *E. coli* is stably associated with the outer membrane [18].) The relative translational efficiency of each transcript was then calculated from the ratio of OmpA protein production to mRNA concentration, with a minor correction for small differences in cell growth rates (Table 1). Because the protein-coding sequences of these messages are identical, their respective polypeptide chain elongation rates should not differ significantly; therefore, for this set of transcripts, relative translational efficiency should reflect the average spacing of translating ribosomes along each message.

The threefold-greater abundance in vivo of the pseudo-wild-type *ompA+4* transcript compared with wild-type *ompA* mRNA led to a proportionate three- to fourfold increase in the relative concentration of OmpA protein in the outer membrane of SE600(pOMPA+4) versus C600S. This finding suggests that despite possible cross-talk in the production of *E. coli* outer membrane proteins (13), in this concentration range the OmpA-to-OmpC protein ratio reflects the relative rate of OmpA protein synthesis. In addition, the cellular concentration of the short-lived *ompAΔ115* transcript in SE600(pOMPAΔ115) was nearly identical to that of wild-type *ompA* mRNA in C600S, and translation of each of these messages generated equivalent amounts of OmpA protein. Thus, deletion of the 5′ RNA segment lying upstream of the *ompA* ribosome-binding site had hardly any effect on the translational efficiency of *ompA* mRNA. Apparently, the fivefold contribution of this 115-nt segment to *ompA* mRNA stability is not an indirect consequence of a change in ribosome spacing.

To corroborate this conclusion, we measured the effect on translational efficiency of deleting the first 115 nt of the *ompA* 5′ UTR in the context of *ob1+4* mRNA. *E. coli* containing pBLA200, pOB1+4, or pOB1Δ115 was grown in rich medium and harvested in log phase for simultaneous extraction of both total cellular protein and total cellular RNA. The relative steady-state concentration of *bla*, *ob1+4*, or *ob1Δ115* mRNA in each strain was measured in parallel by S1 analysis, again using *lpp* mRNA as an internal standard. In addition, relative β-lactamase production from each of these mRNA templates was determined by assaying

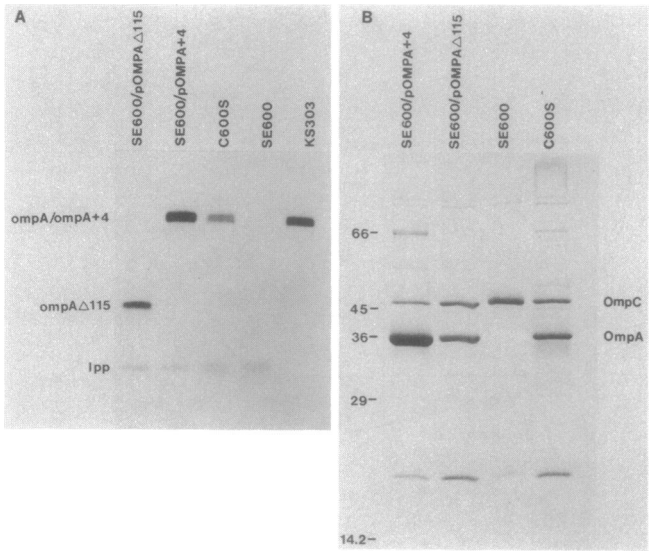

FIG. 5. Determination of the relative translational efficiency of *ompA*, *ompA+4*, and *ompAΔ115* mRNAs. (A) Relative concentrations of *ompA*-related transcripts. Total cellular RNA was isolated from various *E. coli* strains growing rapidly, and samples (2 μg) were subjected to S1 analysis with the same mixture of two end-labeled DNA probes: one specific for *ompA*-related mRNA and the other specific for *lpp* mRNA (an internal standard). KS303 is an *lpp E. coli* strain (53). (B) Relative concentration of OmpA protein in the *E. coli* outer membrane. Outer membrane protein samples (unequal amounts) from various *E. coli* strains growing rapidly were fractionated by denaturing gel electrophoresis and stained. Bands corresponding to proteins OmpA and OmpC (an internal standard) are indicated. Calibration is in kilodaltons.

β-lactamase activity per microgram of total cellular protein. In this manner, *bla* was used as a reporter to compare translation initiation from full-length and truncated versions of the *ompA* 5′ UTR.

As in the case of the *ompA*, *ompA+4*, and *ompAΔ115* transcripts, the translational efficiency of *ob1Δ115* mRNA was nearly the same as that of *ob1+4* mRNA (Table 2). This finding confirmed that removal of the first 115 nt of the *ompA* 5′ UTR hardly affects the frequency of translation initiation at the *ompA* ribosome-binding site. Moreover, the translational efficiency of *ob1+4* mRNA, which contains the *ompA* ribosome-binding site, was somewhat lower than that of *bla* mRNA (Table 2); therefore, the sixfold stabilization of *bla* transcripts that results from substitution of the *ompA* 5′ UTR is not due to an increase in ribosome loading frequency. This latter finding is consistent with a previous report that the translational efficiencies of *ompA* and *bla* mRNAs are quite similar in rapidly growing *E. coli* cells (29). Together, our data indicate that the stabilizing effect of the *ompA* 5′ UTR is not related to ribosome spacing and must instead be a direct consequence of the ability of this RNA segment to impede RNase attack.

mRNA stabilization by the *ompA* 5′ UTR is growth rate regulated. The stability of *ompA* mRNA is regulated by the growth rate of *E. coli* (39). Under conditions of slow growth, the half-life of this transcript can fall by as much as a factor of 4. In contrast, the stability of other *E. coli* transcripts, such as *bla* and *lpp*, is growth rate independent (39). Recent evidence suggests that growth rate regulation of *ompA* mRNA stability might be explained by an acceleration in the rate of endonucleolytic cleavage at specific sites in the *ompA* 5′ UTR (32).

TABLE 2. Relative translational efficiency of *bla*-related transcripts versus *bla* mRNA[a]

mRNA	Strain (doubling time)	β-Lactamase activity[b]	mRNA concn[c]	Relative translational efficiency[d]
	C600S(40 min)	0	0	
bla	C600S(pBLA200) (48 min)	1.00	1.00	1.00
ob1+4	C600S(pOB1+4) (41 min)	0.28	0.66[e]	0.50
ob1Δ115	PM191(pOB1Δ115)[f] (60 min)	1.08	2.70	0.32

[a] All data have been normalized to the values obtained for *bla* mRNA in C600S(pBLA200).

[b] Relative units of β-lactamase per microgram of total cellular protein; a value of 1.00 corresponds to 0.15 U/μg.

[c] Relative moles of *bla*-related mRNA per mole of *lpp* mRNA.

[d] Calculated by determining the ratio of relative β-lactamase activity to relative *bla*-related mRNA concentration and dividing this value by the relative cell doubling time. β-Lactamase is a stable protein in *E. coli* (52).

[e] The low steady-state concentration of *ob1+4* mRNA presumably results from inefficient transcription initiation, from transcription attenuation in the *ompA* 5′ UTR, or from a reduced plasmid copy number.

[f] PM191(pOB1Δ115) was used instead of C600S(pOB1Δ115) because of the slower growth rate of the latter. PM191 and C600S are both derivatives of C600.

To learn whether the ability of the *ompA* 5′ UTR to stabilize mRNA is growth rate regulated, the half-life of the hybrid *ob1+4* messages was measured under conditions of slow bacterial growth. In *E. coli* growing exponentially in MOPS-acetate medium (37) with a doubling time of 265 min, *ob1+4* mRNA decayed with a half-life of 3.0 ± 0.2 min (Fig. 6), just one-sixth of its half-life in cells doubling every 42 min. The half-life of the chromosomal *ompA* transcript in the same slowly growing cells fell to 5.3 ± 0.5 min. In *E. coli* growing in MOPS-succinate medium with a doubling time of 145 min, the decay rate of *ob1+4* mRNA was intermediate between that observed in cells growing with doubling times of 42 and 265 min. Because the stability of *bla* mRNA is growth rate independent, these data indicate that all of the structural information necessary for growth rate regulation of *ompA* message stability resides in the first 194 nt of the *ompA* transcript and that the activity of the *ompA* 5′ UTR as an mRNA stabilizer is regulated by the rate of cell growth.

DISCUSSION

In *E. coli*, the lifetimes of individual messages can differ by as much as 2 orders of magnitude, with half-lives ranging from seconds up to nearly an hour. The structural and mechanistic basis for these marked stability differences is not well understood. Although 3′-terminal stem-loop structures serve an important function as barriers to 3′-exonuclease digestion of mRNA, most bacterial messages end in an RNA hairpin, and there is hardly any evidence that mRNA half-life differences arise from differential susceptibility of 3′ stem-loop structures to exonuclease penetration (11). Instead, given the apparent absence of 5′-exoribonuclease activity in *E. coli* (16), most mRNA degradation seems likely to begin with endonucleolytic cleavage (1, 10, 11).

Two models have been advanced to explain why some messages are attacked by endonucleases more readily than others. According to one, the endonucleases that initiate mRNA degradation in bacteria are specific for the presence or absence of certain features of mRNA sequence or structure, and the lifetime of a given message reflects the degree

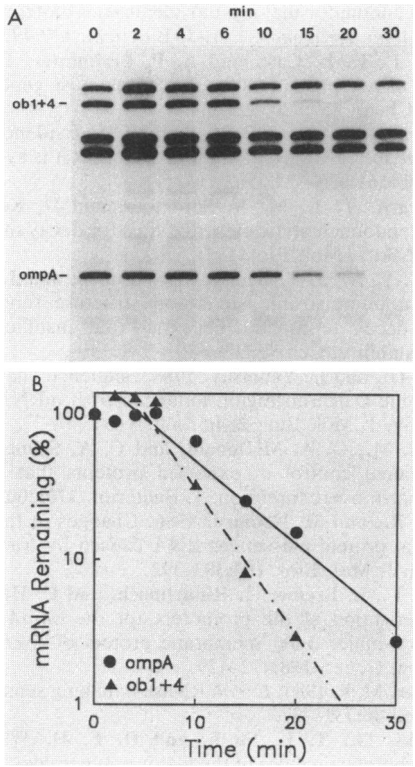

FIG. 6. Decay of *ob1+4* mRNA in slowly growing *E. coli* cells. (A) S1 analysis. At time intervals after transcription inhibition, total cellular RNA was isolated from *E. coli* C600S containing pOB1+4 and growing in MOPS-acetate medium with a doubling time of 265 min. The *ob1+4* and endogenous wild-type *ompA* transcripts were detected by S1 analysis of RNA samples (2 μg) with a mixture of transcript-specific DNA probes. Bands that represent *ob1+4* and *ompA* mRNAs are indicated. The remaining three bands in each lane correspond to reannealed probe DNA. (B) Semilogarithmic plot of mRNA concentration as a function of time. The measured half-lives were 3.0 ± 0.2 min for *ob1+4* mRNA and 5.3 ± 0.5 min for wild-type *ompA* mRNA.

to which it fits the substrate specificity of these RNases (5). Advocates of the other model propose that the collective substrate specificity of bacterial endoribonucleases is so low that they would cleave any message in a matter of seconds were it not for the steric protection afforded mRNA by translating ribosomes (10, 26); accordingly, mRNA stability differences are attributed largely to differences in ribosome spacing resulting from disparate frequencies of translation initiation. These two models are not mutually exclusive, and there is some experimental evidence in support of each.

Any successful theory of bacterial mRNA degradation must account for the ability of the 5′ segments of certain stable transcripts (e.g., *ompA*) to prolong the lifetime of segments of otherwise labile messages fused downstream. Because signals controlling the frequency of translation initiation generally reside in mRNA 5′ UTRs, this phenomenon would seem to be readily explained by a theory that relates mRNA stability to ribosome spacing. However, our data show that a large 5′ UTR deletion that markedly destabilizes *ompA* mRNA does not significantly alter its frequency of translation initiation. This finding indicates that the mechanism by which the *ompA* 5′ UTR stabilizes mRNA is not a secondary consequence of a change in the average spacing of ribosomes along the message. Indeed, our data show that replacement of the *bla* 5′ UTR with that of *ompA* increases the stability of *bla* mRNA by a factor of 6 despite a slight reduction in translational efficiency.

How then might a small 5′ segment of the *ompA* message stabilize a much longer 3′ segment of *bla* mRNA to which it is fused? A reasonable explanation is that *bla* mRNA is vulnerable to cleavage by an *E. coli* RNase that associates, at least initially, at or near the 5′ end of the message, and that attack by this RNase is directly impeded by some feature(s) of the sequence or structure of the *ompA* 5′ UTR. The ability to block this hypothetical RNase might be an intrinsic property of the *ompA* 5′ UTR, or it might be mediated by a cellular protein that wards off RNase attack by binding specifically to this *ompA* RNA segment.

What then of the model for bacterial mRNA degradation that emphasizes the importance of translational efficiency to mRNA stability? Although ribosome spacing may affect mRNA stability in many cases, it is important not to overstate the inherent vulnerability of untranslated segments of transcripts. Despite being untranslated, the 5′ UTR of the *ompA* transcript is among the most stable segments of that message in rapidly growing cells (55). Moreover, although inhibitors of translation initiation and certain nonsense mutations can destabilize mRNA (14, 40, 50), an amber mutation in codon 56 of the *bla* gene, which leaves more than 80% of each *bla* transcript inaccessible to ribosomes, has little effect on the half-life of *bla* mRNA (40), nor is the *Bacillus subtilis ermC* message destabilized by a Shine-Dalgarno element mutation that blocks translation initiation (4). Modest differences in ribosome spacing also may have little impact on mRNA stability. For example, analysis of *lacZ* mutations that cause up to a sixfold change in translational efficiency has shown no correlation of *lacZ* mRNA half-life with ribosome spacing (43, 51). The ambiguous role of translating ribosomes in protecting mRNA from degradation may reflect the variety of messages that have been examined and the substrate specificity of *E. coli* endoribonucleases.

The stability of the *ompA* transcript is growth rate regulated (39) and falls by as much as a factor of 4 in cells growing slowly under conditions that do not impair the translational efficiency of *ompA* mRNA (29). Our data show that stabilization of *bla* mRNA by fusion of the *ompA* 5′ UTR also is regulated by the rate of cell growth. Since the half-life of *bla* mRNA itself is independent of growth rate (39), the growth rate dependence of *ob1+4* mRNA stability indicates that the same segment of the *ompA* transcript responsible for its unusual longevity also mediates growth rate regulation of *ompA* message stability. Consistent with this finding is evidence that the rate of endonucleolytic cleavage by RNase K at specific sites in the *ompA* 5′ UTR increases at slow bacterial growth rates (32, 41; A. von Gabain, personal communication). This 5′ *ompA* mRNA segment survives no longer than other parts of the *ompA* transcript under conditions of slow growth (39), whereas in rapidly growing cells the *ompA* 5′ UTR is so resistant to cleavage that it is among the last segments of the *ompA* message to be degraded (55). Taken together, these findings suggest that as the cell growth rate slows, the *ompA* 5′ UTR grows increasingly vulnerable to RNase attack until cleavage there becomes the dominant mechanism for triggering degradation of the entire *ompA* message.

The relative concentration of the RNA fragments produced in vivo by RNase K cleavage in the *ompA* 5′ UTR is very low, apparently because these fragments are subsequently degraded extremely rapidly, with half-lives of less than 1 min (32; von Gabain, personal communication). Undoubtedly, the hyperlability of the 5′ cleavage products,

which have been severed from the *ompA* 3′ stem-loop, is due to rapid 3′-exonuclease digestion. The basis for the extreme instability of the 3′ cleavage products, which have simply lost a terminal portion of the *ompA* 5′ UTR, is less readily apparent. Nearly all of these 3′ cleavage products contain an intact ribosome-binding site, and since deletion of all but the last 18 nt of the *ompA* 5′ UTR has almost no effect on translational efficiency, it is unlikely that translation of these degradation intermediates is significantly impaired. Moreover, although deletion of the entire 115-nt segment preceding the Shine-Dalgarno element reduces the half-life of *ompA* mRNA by a factor of 5, this effect is far from sufficient to account for the fleeting existence of the *ompA* 3′ cleavage products, all but one of which retain more of the *ompA* 5′ UTR than does *ompAΔ115* mRNA. We have examined the stability of transcripts of additional *ompA* deletion mutants with 5′ truncations smaller than 115 nt; these messages closely resemble some of the naturally occurring *ompA* mRNA degradation intermediates, but none decays any faster than *ompAΔ115* mRNA, and all are efficiently translated (S. Emory, unpublished observations).

Why should an endonucleolytic cleavage product be so much more labile than a similar RNA molecule produced as a direct product of transcription? We propose that the marked lability of these 3′ cleavage products may result from processive digestion of *ompA* mRNA by the same endonuclease (RNase K) responsible for the initial cleavage events that generate these intermediates. We hypothesize that the rate-limiting step in message degradation by this enzyme is its association with the 5′ UTR of mRNA. Subsequently, it migrates swiftly along the message from 5′ to 3′, periodically cleaving it at vulnerable sites. The RNA fragments thereby generated would then be rapidly degraded to mononucleotides by 3′ exoribonucleases. Alternatively, the 3′ fragments generated when RNase K cleaves mRNA might be rapidly digested by a hypothetical 5′-exonuclease activity that acts exclusively on RNA 5′ ends created by RNase K cleavage. Either of these two models for mRNA degradation by RNase K would help to explain the 5′-to-3′ directionality in decay reported for some *E. coli* transcripts (9, 34, 35), as well as the ability of the *ompA* 5′ UTR to control the stability of a long segment of *bla* mRNA fused downstream. Definitive testing of these models will require characterization of this endoribonuclease in vitro (41).

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