Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4890–4894, July 1987
Biochemistry

Effect of premature termination of translation on mRNA stability depends on the site of ribosome release

(*bla*/*ompA*/ribosome coverage/stop codons/RNA half-life)

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Contributed by Stanley N. Cohen, April 15, 1987

ABSTRACT **Translational stop codons were introduced at various locations in the protein-coding regions of the monocistronic *bla* and *ompA* gene transcripts of *Escherichia coli*, and the decay characteristics of the upstream and downstream mRNA segments were analyzed. Premature termination of translation at codon position 26 reduced the stability of both the translated and ribosome-free segments of *bla* mRNA, whereas release of ribosomes just 30 codons further downstream resulted in normal stability for both segments. Normal stability of an untranslated *bla* gene mRNA segment required its linkage to a ribosome-bound segment of *bla* gene mRNA. These findings indicate that depriving an mRNA segment of ribosomes does not necessarily render it more susceptible to degradation. However, premature termination of translation at a location that allows ribosomes to traverse only a short segment of *bla* mRNA can lead to destabilization of the entire transcript.**

Increasingly, evidence indicates that mRNA stability is an important factor controlling gene expression. Different mRNA species are known to decay at different rates within the same cell: in bacteria the half-lives of transcripts range from seconds to as long as 20 min (1–3), and the differential stability of the component segments of polycistronic transcripts can accomplish the differential expression of genes within the same operon (4–6). In addition, changes in the rate of bacterial growth can alter the stability of certain messages by as much as 5-fold (3). In eukaryotic cells, the rate of degradation of individual messages can change in response to hormonal signals (7, 8).

The interaction of transcripts with ribosomes is also known to influence transcript stability. Studies that have used drugs or translation stop codons to examine the effects of prematurely terminated protein synthesis on mRNA half-life have suggested that stripping an mRNA segment of its ribosomes accelerates its degradation (9, 10). However, in other investigations, the 5′-untranslated segment of a transcript has been found to be at least as stable as the downstream protein-coding region (2).

To study further the relationship of ribosome coverage to transcript stability, we introduced stop codons at different locations in the protein-coding regions of two monocistronic mRNA species and examined the decay characteristics of the upstream and downstream mRNA regions using a procedure that enables analysis of the degradation of specific segments of transcripts (2). These studies of *Escherichia coli* β-lactamase (*bla*) and outer membrane protein A (*ompA*) gene transcripts, which are similar in size but are known to show marked differences in stability and characteristics of degradation (2, 3), confirm that ribosome coverage of mRNA can affect transcript stability. However, premature termination of translation beyond a site located early in the protein-coding region of bla mRNA has no effect on the half-life of either the translated upstream mRNA segment or the unprotected mRNA segment located downstream from the site of translation termination.

MATERIALS AND METHODS

The suppressor-minus (*supE44*$^-$) strain, *E. coli* C600S, was derived from C600 by selection for streptomycin resistance. Its suppressor genotype was verified by comparing β-lactamase production (3) in C600S and C600 cells containing the plasmid pBLA103 (*bla*^{amb}). The plasmid pTU102 (11) was kindly provided by U. Henning (Max-Planck-Institut für Biologie, Tübingen, F.R.G.); the pBR322 derivatives, pBR322ocu8 (ref. 12; herein renamed pBLA4) and pTG1 (ref. 13; herein renamed pBLA26), were gifts from D. Botstein (Massachusetts Institute of Technology) and J. Knowles (Harvard University), respectively. The pBR322 derivatives pBLA56 and pBLA103 were constructed *in vitro* by oligonucleotide-directed mutagenesis (14). Amber mutations were verified by comparison of β-lactamase expression in the isogenic strains XA100 (suppressor$^+$) and XA106 (suppressor$^-$) (15). Plasmid pBLA192 was obtained by trimming with T4 DNA polymerase the 3′-protruding ends of linearized pBR322 DNA that had been cut by *Pst* I and subsequently religating the resulting blunt DNA termini; the removal of 4 base pairs at the *Pst* I site introduced a frame shift into the *bla* gene that resulted in translation termination at codon 192 (16). A truncated peptide of the predicted size was produced upon addition of pBLA192 to an *in vitro* coupled protein-synthesizing system (Amersham). Plasmid pBLAΔ63 was derived from pJB322 (17) by deleting the plasmid DNA segment between the *Bcl* I site (filled-in) at the point of transcription initiation and the *Xmn* I site at codon 63 within the *bla* gene.

C600S*ompA29* was made by homologous recombination of the C600S chromosome with a mutant *ompA* gene carried by a hybrid of the temperature-sensitive replication plasmid pPM103 (18) and plasmid pTU102 (11). Recombinants were selected by growth at 42°C in tetracycline and were grown further at the nonpermissive temperature for several generations to eliminate any plasmids formed by recombinational excision from the chromosome. Double recombinants containing only the *ompA29* allele in the chromosome were then selected for their ability to survive infection by bacteriophage K3 (19). Replacement of the wild-type *ompA* allele by one containing the *ompA29* amber mutation was verified by bacterial susceptibility to K3 when a suppressor gene was introduced (15, 20).

Extraction of cellular RNA from *E. coli* growing at 30°C and analysis of mRNA decay have been described (2).

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Abbreviation: nt, nucleotide(s).

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RESULTS

Introduction of Stop Codons Within the *bla* and *ompA* Genes. Translation stop codons were introduced at five different positions within the monocistronic *bla* gene of plasmid pBR322 and at one position near the promoter-proximal end of the monocistronic, chromosomally located *ompA* gene. The creation of stop codons corresponding to amino acid positions 4, 26, 56, 103, and 192 of the protein encoded by the *bla* gene yielded alleles designated *bla4*, *bla26*, *bla56*, *bla103*, and *bla192* (Fig. 1) and respective plasmids named pBLA4, pBLA26, etc. The stop codons of *bla56* and *bla103* were amber mutations, whereas *bla4*, *bla26*, and *bla192* contained ochre mutations. Introduction of an amber mutation at codon 29 of the *ompA* gene yielded an allele designated *ompA29*, which was substituted for the wild-type *ompA* locus in the chromosome of *E. coli* strain C600S.

Effect of Premature Termination of Translation on *bla* mRNA Stability. The stability of *bla* transcript segments upstream and downstream from the point of translation termination was analyzed in C600S cells containing pBLA4, pBLA26, pBLA56, pBLA103, or pBLA192. Radiolabeled *Hae* III-generated single-stranded DNA fragments that collectively extended the length of the *bla* gene transcript were hybridized as described (2) to cellular RNA samples isolated at various time intervals after rifampicin blockage of further transcription initiation, treated with S1 nuclease, and analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 2). The observed 587-nucleotide (nt) and 432-nt fragments represent the 5′-terminal segments of *bla* transcripts initiated at the two promoters reported previously, the 267-nt and 80-nt DNA fragments correspond to internal segments of *bla* mRNA, and the 360-nt, 300-nt, and 200-nt DNA fragments represent the 3′ segments of transcripts that terminate at the three *bla* mRNA terminators located on the pBR322 plasmid (2). The 267-nt DNA fragment protected from S1 digestion by mRNA encoded by *bla4*, *bla26*, and the wild-type *bla* gene was partially replaced by two other protected fragments, 175-nt and 88-nt long, when the *bla192* transcripts were analyzed. The deletion of four nucleotides in the *bla* gene of the pBLA192 construct and the consequent failure of the mutant transcript to base pair with the wild-type probe at this location (see Fig. 1) provided a cleavage site for S1 nuclease and resulted in an altered pattern of protected DNA fragments.

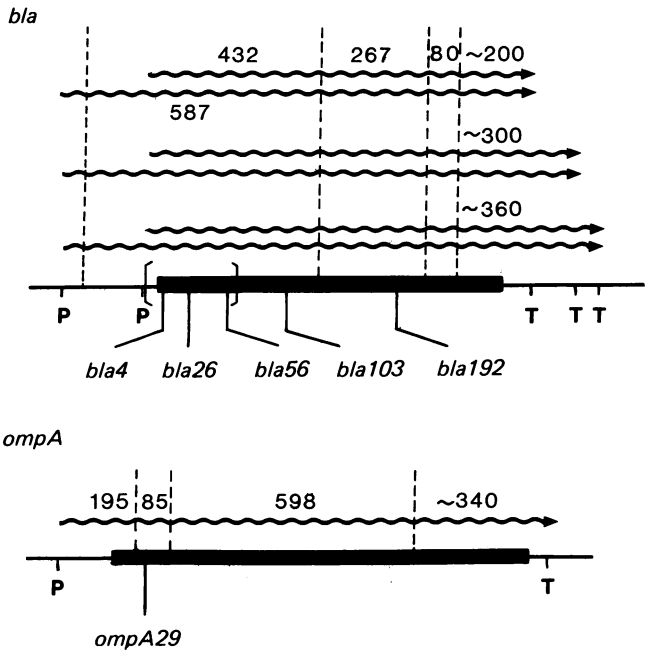

FIG. 1. Genetic maps of the *bla* and *ompA* genes and mutants thereof. (*Upper*) The *bla* gene transcriptional unit; (*Lower*) the *ompA* gene transcriptional unit. Solid bars define the protein-coding region of these genes. Wavy lines represent transcripts, with arrowheads at their 3′ termini. Vertical dashed lines mark the boundaries of mRNA segments defined by the fragmented DNA probes used for S1 analysis; numbers between these arrows indicate lengths of these segments in nt. P, promoter; T, terminator. The position of the stop codon for each *bla* allele is marked. The brackets indicate the termini of the deletion in pBLAΔ63.

The *bla56*, *bla103*, and *bla192* gene transcripts had a half-life (2–3 min) approximately the same as that determined for transcripts encoded by the wild-type *bla* gene (2), whereas mRNA derived from the *bla4* and *bla26* genes was less stable, with half-lives ≈0.6 and 0.8 min, respectively (Figs. 2 and 3). No significant difference was observed between the rate of decay of the translated and untranslated segments for any of these mRNA species. However, the dramatic change observed for the half-life of *bla* mRNA when a translation stop signal was introduced at codon 56 versus codon 26 suggests the existence of a biochemical "boundary" with regard to the effects of ribosomes on mRNA decay: termination of translation at codon 26 of the *bla* gene transcripts led to rapid degradation of both the translated and untranslated segments of these messages, whereas termination of translation just 30 codons downstream (at codon 56) resulted in a normal decay rate for the resulting messages, all segments of which had roughly the same half-life as *bla* transcripts encoded by the wild-type gene (cf. Fig. 2 and ref. 2). Thus, provided that ribosomes can traverse the *bla* mRNA segment preceding codon 56, the site of their subsequent dissociation from the message does not affect the stability of even the lengthy unprotected segment of the transcript downstream from the site of translation termination.

The *blaΔ63* gene transcripts [which are not nonsense mutants, but which instead lack the entire 5′ *bla* mRNA segment up to codon 63, including the ribosome binding site (see Fig. 1)] had a half-life of ≈0.8 min (see Fig. 2). For both the *bla56* and *blaΔ63* messages, a structurally similar lengthy 3′ segment lacks ribosomes; however, the stability of this naked segment is normal for *bla56* mRNA but is reduced for *blaΔ63* mRNA. This finding suggests that the stability observed for the untranslated 3′ segment of *bla56* mRNA requires the initial assembly of ribosomes on the 5′ segment of that transcript.

The Effect of Premature Termination of Translation on *ompA* mRNA Stability. Messenger RNA encoded by the *ompA* gene has a much longer half-life (15 min) than *bla* gene mRNA and most other *E. coli* transcripts (2–3 min) (2). Introduction of a translation stop signal at codon 29 of the *ompA* protein-coding sequence and replacement of the wild-type chromosomal gene with the mutant allele resulted in a new strain called C600S*ompA29*. Segmental analysis of the decay of *ompA29* gene transcripts showed four mRNA-protected *Hae* III-generated DNA fragments (Fig. 2): two of these (195 nt and 340 nt) corresponded respectively to the 5′ and 3′ segments of the transcript, whereas two others (598 nt and 85 nt long) corresponded to internal segments (2). Densitometric analysis of the autoradiogram shown in Fig. 2 revealed half-lives between 1.2 and 1.8 min for the various segments of the *ompA29* transcripts. The enhanced stability previously observed for the 5′-terminal 195-nt segment of the wild-type *ompA* transcript (2) was not observed for that segment of the *ompA29* transcript.

DISCUSSION

Our studies indicate that depriving an mRNA segment of its normal complement of ribosomes does not necessarily render

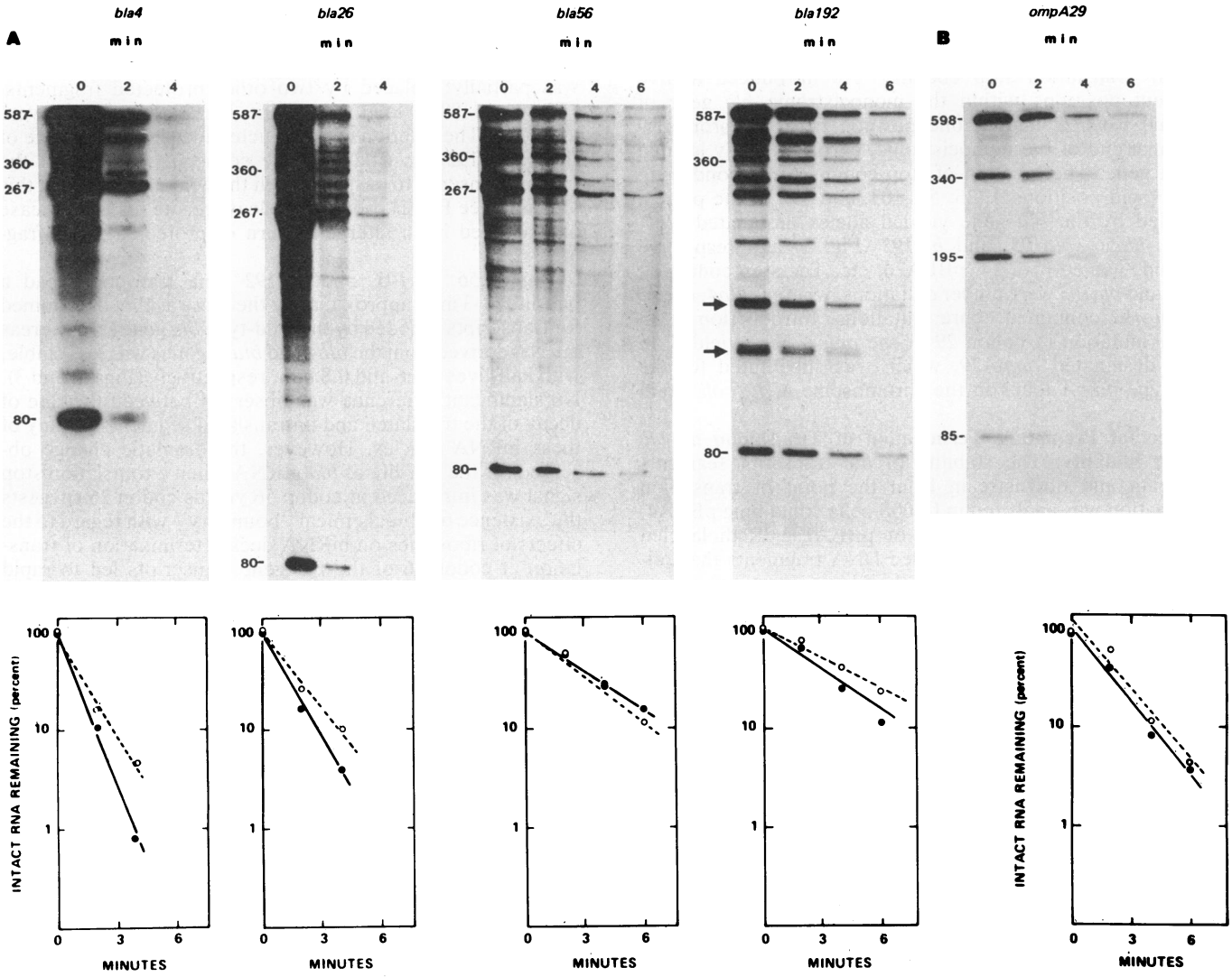

FIG. 2. S1 analysis of the decay of mutant *bla* and *ompA* gene transcripts in *E. coli*. (*A*) The decay of *bla4*, *bla26*, *bla56*, and *bla192* mRNA; (*B*) the decay of *ompA29* mRNA. (*Upper*) S1 analysis with fragmented probes was performed as described (2) with RNA samples isolated at time intervals after rifampicin addition. Arrows indicate the two new bands that result from a mismatch between the probe and the *bla192* transcript due to a 4 base-pair deletion in pBLA192. Calibration is in nt. (*Lower*) Beneath each autoradiogram is a semilogarithmic plot of the decay of the 587-nt 5′ segment (solid line) and 360-nt 3′ segment (dashed line) of the corresponding *bla* transcripts or of the 195-nt (solid line) and 340-nt (dashed line) segments of the *ompA29* transcript. The segment half-lives in minutes were as follows: 0.6 ± 0.1 (587 nt), 0.9 ± 0.1 (267 nt), 0.9 ± 0.1 (360 nt) for *bla4*; 0.9 ± 0.1 (587 nt), 0.8 ± 0.1 (267 nt), 1.2 ± 0.1 (360 nt) for *bla26*; 2.3 ± 0.1 (587 nt), 2.1 ± 0.2 (267 nt), 2.0 ± 0.1 (360 nt) for *bla56*; 2.0 ± 0.2 (587 nt), 1.7 ± 0.1 (267 nt), 2.2 ± 0.2 (360 nt) for *bla103* (not shown); 2.2 ± 0.2 (587 nt), 3.0 ± 0.2 (177 nt), 3.0 ± 0.1 (360 nt) for *bla192*; and 1.2 ± 0.1 (195 nt), 1.8 ± 0.2 (85 nt), 1.7 ± 0.2 (598 nt), 1.3 ± 0.2 (340 nt) for *ompA29*. The half-life for *blaΔ63* mRNA was 0.8 ± 0.1 min (data not shown).

the segment more susceptible to ribonuclease cleavage. Termination of translation at codon position 56 in *bla56* gene mRNA, which is devoid of ribosomes for more than four-fifths of its length, resulted in normal stability for both the upstream (ribosome-covered) and downstream (ribosome-free) segments. However, introduction of a translation stop signal just 30 codons further upstream (i.e., at codon 26) reduced dramatically the stability of both the translated and untranslated segments of the transcript. The untranslated *blaΔ63* messages were labile, despite the structural similarity of *blaΔ63* mRNA to the relatively stable, untranslated segment of *bla56* mRNA; therefore, the normal stability observed for the untranslated segment of the *bla56* transcripts appears to require the assembly of ribosomes at the 5′ end of the message.

Earlier investigations of wild-type *ompA* and *bla* gene mRNA stability have shown that 5′- and 3′-noncoding regions, which normally lack ribosomes, are not preferentially degraded (2). Such findings, along with the present demonstration of the stability of long segments of *bla* mRNA that have been deprived by mutation of their normal complement of ribosomes, contrast with results obtained for the *trp* operon transcript (10). The disparity between our findings and reports of *trp* mRNA decay (10) could arise from the fact that entirely different messages with potentially different decay characteristics were examined; some *E. coli* transcripts may be destabilized by the absence of ribosomes over much of their length. However, the significance of our finding is its demonstration that large mRNA segments devoid of ribosomal coverage are not necessarily more vulnerable to attack by cellular ribonucleases.

Analysis of the decay of mRNA encoded by the *ompA29* allele indicated that introduction of a translation stop signal at codon position 29 resulted in a 10-fold increase in the rate of mRNA degradation, as compared with the wild-type transcript. Nevertheless, the half-life observed for both the

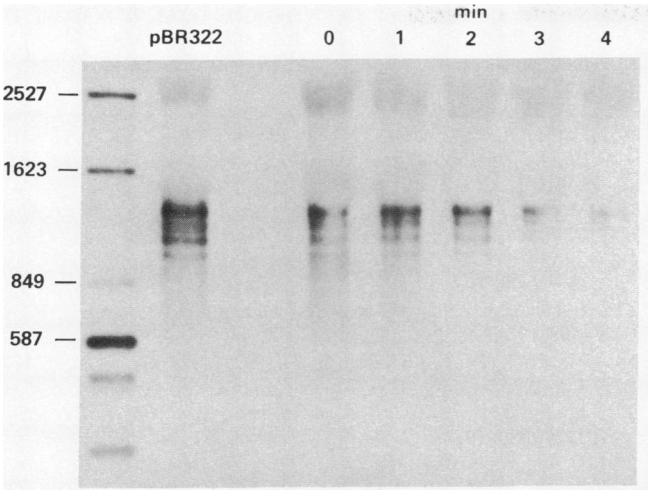

FIG. 3. RNA blot analysis of *bla26* gene mRNA decay in *E. coli*. RNA samples (20 μg) from a logarithmic-phase culture of C600S containing plasmid pBLA26 were isolated at time intervals after rifampicin addition and fractionated on a 1.5% agarose/formaldehyde gel beside an RNA sample (10 μg) from a logarithmic-phase culture of C600S containing pBR322. Blotting and hybridization with radiolabeled *bla* DNA were performed as described (3). Multiple bands resulted from multiple sites of *bla* gene transcription initiation and termination (2). A *Hae* III digest of uniformly labeled M13*bla* DNA (2) (left lane) served as a molecular size standard. Calibration is in nt. The half-life of *bla26* mRNA following cessation of transcription was 0.8 ± 0.1 min. The faint, nondecaying broad band present in every RNA lane near the top of the gel often appears in *E. coli* RNA blots and represents nonspecific hybridization of the probe to 23S rRNA.

translated and untranslated segments of the *ompA29* gene transcript still exceeded that found for the *bla4* and *bla26* messages (which also lack ribosomes for most of their length).

In the S1 analysis used in these experiments to assay mRNA decay, the cleavage of a single phosphodiester linkage within a particular mRNA segment is scored as the degradation of that segment (2). The advantage of this assay compared to an assay involving the simple hybridization of labeled RNA to DNA immobilized on a filter (9, 10) is that intact message segments are distinguished from short, functionally inactive (but nevertheless hybridizable) RNA fragments (except in the highly improbable circumstance that an RNA cleavage site coincides precisely with a *Hae* III restriction site in the DNA probe). Our studies have shown (2) that the initial cleavage of full-length wild-type *bla* mRNA (half-life ≈3 min) is rate-limiting for turnover of the *bla* message and that once this initial cleavage occurs, rapid degradation of the entire transcript ensues. These decay kinetics explain the absence of detectable *bla* mRNA decay intermediates.

Two lines of evidence indicate that the decay rate of only full-length messages was measured in the present studies. The first is the roughly equimolar representation of all segments of any given transcript at time zero in the S1 analyses and the absence of unexpected bands or smears. Second, the half-life of full-length *bla26* mRNA as determined by RNA blot analysis (Fig. 3) was about the same as that determined by segmental S1 analysis. Thus, if premature ρ-dependent transcription termination occurs to some extent for any of the nonsense mutants under investigation, the products that would result from such premature termination must, because of their low rate of production or their rapid decay, constitute an insignificant fraction of the total message population.

Collectively, our findings are compatible with either of two models. In one, the assembly of ribosomes on mRNA and their transit past certain transcript loci are required to maintain normal stability. According to this model, if ribosomes traverse a putative stability-determining region located between codons 26 and 56 of *bla* mRNA, then their subsequent premature dissociation will not affect the rate of degradation of either the translated or untranslated segments of the message. The rapid degradation observed for *bla4* and *bla26* mRNA cannot result from ρ-dependent premature termination of transcription due to translation termination at codons 4 and 26 since only the half-lives of full-length transcripts were measured. Furthermore, the role of ribosomes cannot be simply to translate and thereby protect from cleavage a specific ribonuclease-sensitive site between codons 26 and 56 because the absence in *blaΔ63* mRNA of the segment between these codons did not prevent rapid degradation of *blaΔ63* transcripts. Moreover, an in-frame deletion that includes the segment between codons 26 and 56 but does not otherwise interrupt translation does not significantly affect the rate of degradation of *bla* mRNA (17).

A second possible model is that the short half-life observed for *bla4* and *bla26* gene mRNA results from the complete absence of ribosomes on a substantial fraction of these mutant transcripts. This model is based in part on calculations indicating that the transcripts encoded by these mutated genes allow space for no more than one or two ribosomes between the start and stop codons (21). Because the rate of translation initiation by ribosomes is slow compared to the rate of peptide elongation (22), the average number of bound ribosomes will be less than one per transcript for the *bla4* and *bla26* mutants if the average ribosome spacing for *bla* transcripts is >78 nt. Thus, the mutant *bla4* and *bla26* gene transcripts may alternate between translated (one ribosome bound) and untranslated (no ribosomes bound) states; during the time that a given transcript molecule is awaiting binding and initiation of translation by the next ribosome, the transcript would (like *blaΔ63* mRNA) be naked and, therefore, highly vulnerable to ribonuclease cleavage, resulting in the short half-life observed. According to this model, association of even one ribosome with a *bla* transcript is sufficient to protect both translated and untranslated mRNA segments from accelerated degradation. Based on the average rate of translation of individual messages in *E. coli* (≈17 codons per sec at 37°C, ref. 23) the average transit time for a ribosome translating *bla56* mRNA at 30°C should be <5 sec. If continual association with at least one ribosome is in fact the basis for the relatively normal (2 min) half-life of *bla56* mRNA, then the average interval between consecutive translation initiation events at the *bla* mRNA ribosome binding site would also have to be <5 sec. By this reasoning, the rate of equilibration of *bla4* or *bla26* messages between the translated and untranslated states would be rapid compared to their rate of decay; consequently, only a single weighted-average half-life would be measured for either of these messages, and the observed monophasic decay plots would be anticipated.

The notion that events associated with ribosome binding are important in determining the stability of downstream mRNA segments is consistent with evidence that fusion of the 5′-terminal segment of a stable transcript on the 5′ end of a less stable mRNA segment can substantially increase the half-life of the downstream segment (17, 24). However, the molecular mechanism by which degradation of an mRNA segment that lacks ribosomes is affected by ribosome coverage of an upstream segment of that transcript is presently unclear, given the absence of known 5′ exoribonucleases in *E. coli* (25). What is certain is that deprivation of an mRNA segment of its usual complement of ribosomes need not result in increased susceptibility to ribonucleolytic cleavage.

We are grateful to Charles Yanofsky for his helpful comments on this manuscript. These studies were supported by Grant GM27241 from the National Institutes of Health (to S.N.C.), by Grant GM35769 from the National Institutes of Health (to J.G.B.), and by a grant from the Riksföreningen mot Cancer of Sweden (to A.v.G.). Much of this work was carried out at the Institute of Applied Cell and Molecular Biology, University of Umeå, Umeå, Sweden.

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