Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 653–657, February 1983
Biochemistry

Decay of mRNA in *Escherichia coli*: Investigation of the fate of specific segments of transcripts

(*bla*/*ompA*/RNA half-life/transcriptional termination/S1 nuclease)

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Contributed by Stanley N. Cohen, September 23, 1982

ABSTRACT **An assay was developed to investigate the fate of specific segments of β-lactamase (*bla*) and *ompA* gene transcripts in *Escherichia coli*. DNA probes cloned in bacteriophage M13 were treated with an endonuclease capable of cleaving single-stranded DNA, the fragments produced were annealed with total cellular RNA, and the resulting RNA·DNA hybrids were subjected to S1 nuclease treatment and gel fractionation. By using this assay, direct evidence was obtained for 3′-to-5′ directionality in the decay of the long-lived mRNA encoded by the *ompA* gene, and no preferential stability was observed for translated versus untranslated mRNA segments. In the case of *bla* mRNA, initial cleavage of the full-length transcript was rate limiting, and no decay intermediates were detected. No difference in degradation rate was seen for *bla* transcripts having variant 3′ or 5′ termini.**

The level of expression of a gene is determined primarily by three elements: the rate of transcription, the stability of the RNA transcript, and the efficiency of translation. In *Escherichia coli*, the factors influencing the efficiencies of transcription and translation have been the subject of considerable study, and the enzymes and nucleic acid sequences involved in these processes have been well characterized. It has been shown that the strengths of promoters span at least two orders of magnitude (1) and that translation rates can differ by as much as 10-fold (2).

By contrast, the factors that determine mRNA stability are not well understood. Almost nothing is known about the initiation sites of mRNA degradation; although a number of RNases have been identified in *E. coli* (3), it is still unclear which are primarily responsible for determining the rate of mRNA decay *in vivo*.

A major barrier to the detailed study of mRNA degradation has been the lack of an adequate assay system. Typically, one of three methods has been used to monitor the rate of decay of individual mRNA species. One assay, which yields a so-called "functional half-life" of a transcript, involves the pulse-labeling of proteins synthesized after the inhibition of transcription (4). Other methods are based on RNA blotting techniques or hybridization of pulse-labeled RNA to DNA fragments (5).

In order to investigate directly the factors that influence the decay of mRNA, we have developed an assay based in part on the method of Berk and Sharp (6). A uniformly radioactively labeled single-stranded DNA probe complementary to an entire transcript is digested with one of several endonucleases that accomplish site-specific cleavage of single-stranded as well as double-stranded DNA (7). After being annealed with total cellular RNA, the fragmented probe is treated with S1 nuclease, and those DNA fragments protected from digestion by hybrid formation with complementary RNA sequences are then fractionated by gel electrophoresis. This procedure has made it possible to determine the relative decay rates of different regions of the transcript and thus to investigate possible directionality and segmental specificity of the decay process.

We describe here experiments designed to elucidate the pattern and kinetics of decay of two different mRNA species in *E. coli*: (*i*) the RNA for the β-lactamase (*bla*) enzyme encoded by a Tn*3* transposon-derived gene and (*ii*) the RNA for the abundant *E. coli* outer membrane protein II* (*ompA*). These RNA transcripts are both monocistronic, have roughly the same size, and encode proteins secreted through the bacterial cell inner membrane.

MATERIALS AND METHODS

Enzymes. S1 nuclease was obtained from Boehringer Mannheim; activity units are as defined by the supplier. DNase I free of RNase activity was obtained from Worthington (DPRF grade). *Hae* III endonuclease was purchased from New England BioLabs.

Phage and Plasmid Constructions. Single-stranded probes for mRNA were prepared by cloning of the complementary DNA strand in the single-stranded bacteriophage vector M13G*ori*1 (8), kindly provided by J. Kaguni. DNA fragments cloned in this phage are stably maintained over many generations (J. Kaguni, personal communication). The plasmid pTU102, a pSC101 derivative that contains an amber mutation in the *ompA* gene of *E. coli* (9), was the generous gift of U. Henning. M13*ompA* was prepared by a multistep procedure. First, an M13 construct containing the complement of the 3′-terminal 340 nucleotides (nt) of the *ompA* gene was made by insertion at the *Pvu* II site of M13G*ori*1 of a 1.83-kilobase (kb) *Bam*HI fragment from pTU102. Subsequently, a 1.78-kb *Bam*HI fragment from pTU102 encoding the first 880 nt of the mutant *ompA* transcript was subcloned at the *Bam*HI site of pBR322 (9, 10), excised as part of an *Eco*RI/*Hin*fI fragment, and then inserted between the *Eco*RI and *Pvu* II sites of the aforementioned *ompA* construct in M13. This procedure resulted in a noncontiguous arrangement of the two subcloned portions of the *ompA* gene in a single phage genome. M13*bla* was constructed by inserting at the *Pvu* II site of M13G*ori*1 a 1.5-kb fragment of pBR322 containing the β-lactamase gene and bounded at one end by the plasmid's unique *Cla* I site. The plasmid pJLS2010 was a dele-

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Abbreviations: kb, kilobase(s); nt, nucleotide(s).

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tion mutant of pBR322 lacking the 650-base pair *Eco*RI/*Hin*cII fragment that contains the *tet* promoter region.

RNA Isolation. Rifampicin (0.2 mg/ml) was added to a culture of *E. coli* strain C600 growing exponentially at 30°C, thereby blocking any further initiation of transcription by RNA polymerase. At selected time intervals, samples were withdrawn from the culture and rapidly chilled on ice. The bacteria were pelleted by centrifugation and lysed at 65°C in 0.15 M sucrose/0.01 M sodium acetate, pH 4.5/1% sodium dodecyl sulfate. Total cellular RNA was recovered by extraction with hot phenol (11). After treatment with RNase-free DNase I (100 units/mg of RNA) for 30 min at room temperature, the RNA was extracted with phenol/chloroform and precipitated with ethanol. Samples were stored at −20°C in 20 mM sodium phosphate, pH 6.5/1 mM EDTA.

Preparation of Radioactive Single-Stranded Probe DNA. Single-stranded phage DNA was uniformly labeled *in vivo* with [^{32}P]orthophosphate and extracted from the phage with phenol. Its specific radioactivity was 1×10^6 dpm/μg. Restriction digestions of single-stranded DNA (7) were performed overnight at 37°C in 6 mM Tris·HCl, pH 7.6/6 mM $MgCl_2$/0.5 mM dithiothreitol/0.1 mg of bovine serum albumin per ml, containing *Hae* III (≈4 units/μg of DNA).

Determination of mRNA Half-Life. Autoradiograms were analyzed by densitometry. Rates of decay were calculated by least-squares determination of the slope of a plot of −ln(band intensity) versus time. Errors were estimated from the standard deviation of the slope.

RESULTS

Analysis of *bla* mRNA Decay by RNA Blotting. An exponentially growing culture of *E. coli* carrying the plasmid pJLS2010 was treated with rifampicin to block further initiation of transcription, and total cellular RNA was isolated at 2-min intervals. [pJLS2010 is a deletion mutant of pBR322 lacking the *tet* promoter region, so that all β-lactamase transcripts are initiated from the promoter indigenous to the *bla* gene (12, 13).] RNA blot analysis using a probe for β-lactamase mRNA suggested the existence of multiple, unresolved transcripts decaying uniformly with a half-life of about 3 min (Fig. 1 *Left*).

Analysis of *bla* mRNA Decay by Protection from S1 Nuclease. In order better to quantitate mRNA amounts and to resolve multiple transcripts, the decay of the β-lactamase messages was examined by a modification of the method of Berk and Sharp (6). Total cellular RNA isolated at time intervals after rifampicin treatment was hybridized with uniformly radioactively labeled single-stranded DNA complementary to the entire *bla* transcript (M13*bla*). After thorough digestion with S1 nuclease, the resistant *bla* RNA·DNA hybrids were heated to 100°C and then electrophoresed on a denaturing polyacrylamide gel. Autoradiography revealed three β-lactamase transcripts about 1,000, 1,100, and 1,150 nt in length and of roughly equal abundance (Fig. 1 *Right*). All three species decayed with approximately the same half-life as that observed in the simple RNA blot. In this experiment as well as those described below, the RNA·DNA hybridization was performed under conditions of DNA excess and was allowed to proceed to completion, as demonstrated by the fact that the band intensities were unaffected by tripling the DNA input. These conditions are necessary to ensure that the observed bands accurately reflect the populations of the various mRNA species present.

Analysis of *bla* mRNA Decay by Protection of *Hae* III Fragments from S1 Nuclease. In order to map the three β-lactamase transcripts and, more importantly, to assess whether different segments of these transcripts display differing stabilities *in vivo*, a novel method of analysis was devised. This method was similar

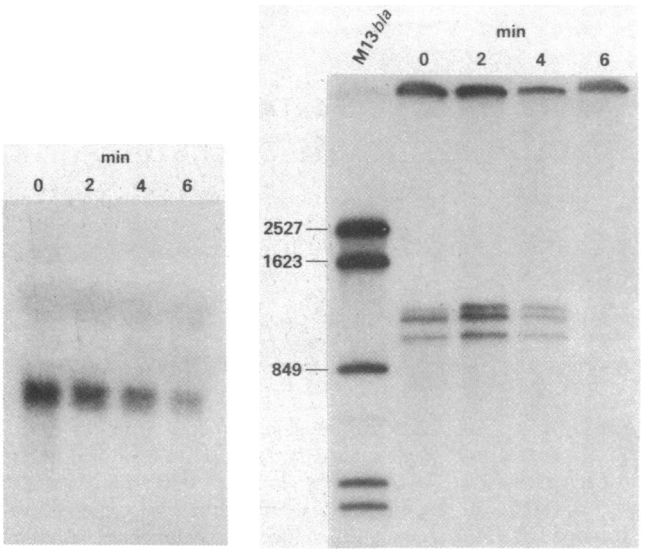

FIG. 1. Decay of full-length *bla* mRNA. (*Left*) RNA blotting analysis. Total cellular RNA (25 μg) isolated 0, 2, 4, and 6 min after rifampicin treatment of *E. coli* carrying the plasmid pJLS2010 was treated with glyoxal (14) and fractionated on a 1.5% agarose gel. The RNA was blotted onto diazophenyl-thioether-paper (15) and probed with nick-translated *bla* gene DNA. (*Right*) S1 nuclease analysis. Total cellular RNA (6 μg) isolated as for *Left* was allowed to hybridize with a 3- to 5-fold molar excess of uniformly radiolabeled intact M13*bla* DNA in 30 μl of 0.04 M sodium phosphate, pH 6.5/0.4 M NaCl/0.4 mM EDTA/80% (vol/vol) formamide (42°C, 3 hr). The hybridized sample was treated with S1 nuclease (2×10^4 units) for 2 hr at 37°C in 0.05 M sodium acetate, pH 4.5/0.28 M NaCl/2.5 mM $ZnSO_4$. The reaction was quenched by the addition of EDTA (4 mM) and extraction with phenol/chloroform. After three precipitations with ethanol, the sample was denatured at 100°C in 0.5× TBE buffer/80% formamide and fractionated by electrophoresis on a denaturing polyacrylamide gel (4% acrylamide/40% urea/1× TBE) beside a *Hae* III digest of M13*bla* DNA, which served as a molecular size standard (calibration is in nt). TBE buffer is 89 mM Tris borate, pH 8.0/2 mM EDTA.

to the S1 protection procedure described above, except that the complementary single-stranded DNA was digested with *Hae* III endonuclease (7) prior to hybridization. In this manner we hoped to examine the decay of specific mRNA segments.

The results of this analysis are shown in Fig. 2 *Left*. Six bands were observed. Two of these (267 and 80 nt long) correspond to *Hae* III-generated fragments mapping within the *bla* gene (Fig. 3 *Upper*). A third strong band (432 nt) is attributable to the common 5′ end of the three β-lactamase transcripts, beginning at the initiation site for transcription (12, 13) and ending at the first downstream *Hae* III site. The three remaining weak bands (≈200, 300, and 360 nt) reflect three distinct 3′ ends of the β-lactamase messages, all extending from the last *Hae* III site but differing with respect to the site of termination of transcription. The length of each transcript is consistent with termination at a site corresponding to a sequence (16) commonly observed at sites of transcriptional termination in *E. coli* (i.e., a stem and loop structure followed by a brief run of U residues). The structure of the DNA region encoding these transcripts is shown in Fig. 3 *Upper*.

Comparison of the bands for DNA fragments protected by RNA isolated at the moment of rifampicin addition versus 2 min later shows that most bands hardly diminished in intensity. Presumably, this brief delay in the onset of net decay of β-lactamase mRNA is due to the completion of nascent transcripts during the first minute or so (19) after the initiation of transcription was blocked. From 2 to 6 min after rifampicin addition, all bands diminished in intensity at the rate observed earlier for

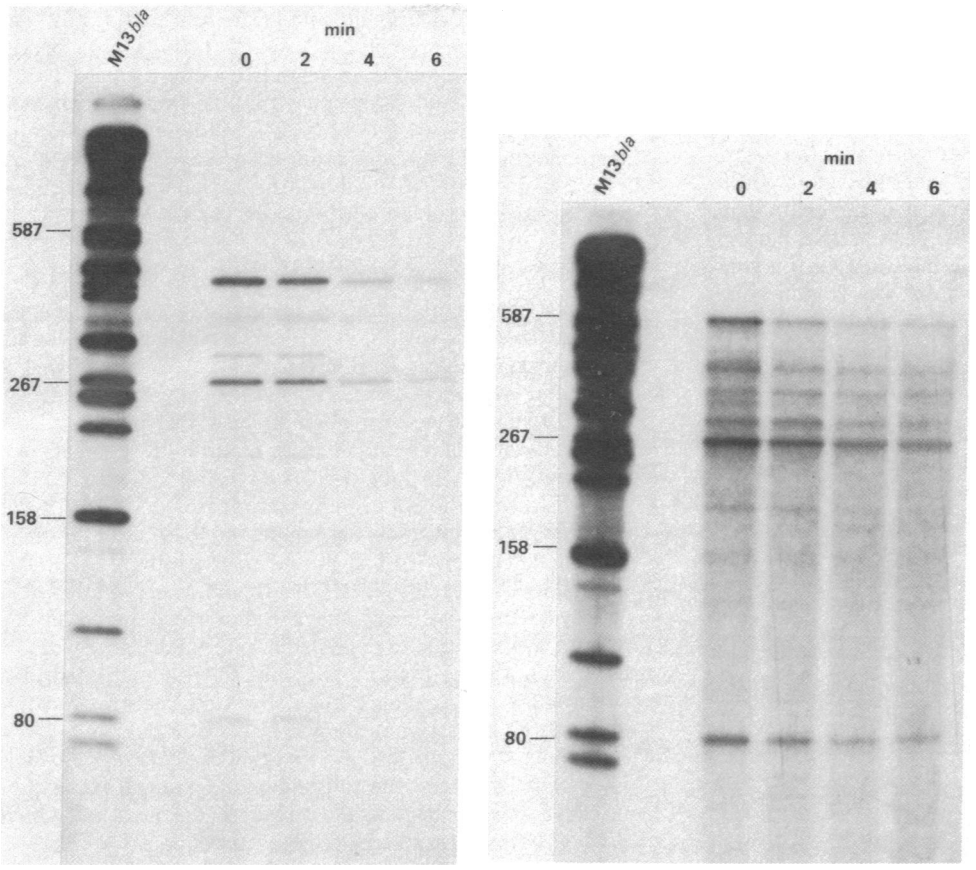

FIG. 2. Decay of *bla* mRNA segments. (*Left*) Transcripts from pJLS2010. (*Right*) Transcripts from pBR322. S1 nuclease analysis and molecular size markers were as in Fig. 1 except that the M13*bla* DNA probe was digested thoroughly with *Hae* III endonuclease prior to hybridization with total cellular RNA. In addition, a second S1 nuclease treatment was performed after ethanol precipitation of the products of the first treatment. The segment half-lives in min were 3.3 ± 0.4 (587 nt), 2.9 ± 0.1 (432 nt), 3.5 ± 0.2 (360 nt), 2.4 ± 0.2 (300 nt), 3.1 ± 0.1 (267 nt), 2.9 ± 0.6 (200 nt), and 2.8 ± 0.9 (80 nt).

the full-length mRNA. Such a decay pattern indicates that the rate constant for degradation of *bla* mRNA decay intermediates is at least as great as that for their formation, so that only segments of full-length messages were observed experimentally. (It should be noted that the cleavage of a single phosphodiester linkage within a particular segment of a mRNA molecule is scored by this assay as the degradation of that segment; unless cleavage occurs near the end of the segment, the size of the DNA fragment protected by the cleaved RNA is noticeably reduced.)

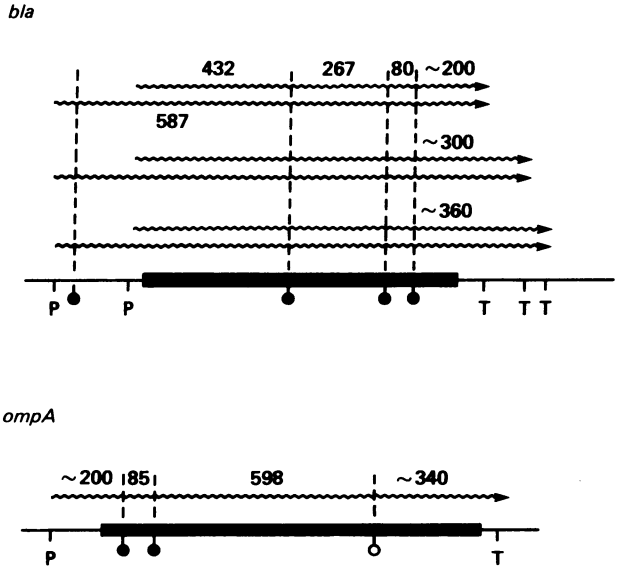

FIG. 3. Genetic maps. (*Upper*) *bla* gene transcriptional unit (12, 13, 16). (*Lower*) *ompA* gene transcriptional unit (17, 18). Numbers refer to the lengths of mRNA in nt. Solid bars define the translated portions of these genes. P, promoter; T, terminator; ●, *Hae* III endonuclease cleavage site; ○, *Bam*HI cleavage site. Wavy lines represent transcripts, and arrows indicate the 3′ termini.

Despite differences in their 3′ ends, all three β-lactamase transcripts of pJLS2010 were found to decay at the same rate. To determine whether altering the site of initiation of *bla* mRNA would affect its half-life or pattern of decay, the experiments were repeated with RNA from *E. coli* carrying the plasmid pBR322. In this plasmid, transcription of the β-lactamase gene is initiated at two sites, the natural *bla* gene promoter and a second promoter mapping near the *Hin*dIII site and reading away from the *tet* gene (12, 13). Consequently, six different β-lactamase transcripts were observed by virtue of the two promoters and three terminators that define the *bla* transcriptional unit in pBR322 (Fig. 3 *Upper*).

On the basis of both RNA blotting and S1 nuclease analysis using a full-length complementary probe, the half-life of β-lactamase messages initiated in the *tet* promoter region was identical to that of *bla* mRNA initiated at the natural *bla* gene promoter (3 min). When the *bla* transcripts from pBR322 were subjected to S1 nuclease analysis with a complementary single-stranded probe that had been cleaved with *Hae* III (Fig. 2 *Right*), an additional band (587 nt) was observed, corresponding to a *Hae* III fragment encoding all but the first 54 nt of the 5′ end of transcripts initiated in the *tet* promoter region. This segment, which includes an extensive untranslated portion of 191 nt, decayed with the same 3-min half-life as the 5′-terminal fragment of mRNA beginning at the *bla* promoter. Thus, there was no significant difference in the *in vivo* stability or manner of decay of β-lactamase messages with different 5′ ends. [The 5′ terminal segment containing the first 54 nt of *bla* transcripts initiated in the *tet* promoter region was not detected. Presumably, the 44-nt fragment of M13*bla* DNA protected by this segment was too small and too low in radioactivity to be visible

under these experimental conditions. The origin of a faint band (≈340 nt), seen only for *bla* mRNA from pBR322 isolated at the moment of rifampicin addition (0 min), has yet to be identified.]

Analysis of *ompA* mRNA Decay. The transcripts of the β-lactamase gene are representative of the class of *E. coli* mRNA having a moderate half-life. With the rationale that the degradation intermediates of a long-lived *E. coli* message might be sufficiently stable to be detected, the decay of the *ompA* gene transcript (17, 18) was examined. On the basis of RNA blotting (Fig. 4 *Left*), a half-life of 15 min was established for this transcript, 5 times longer than that for *bla* mRNA. To determine whether the decay kinetics of this mRNA molecule would allow an assessment of segmental specificity in its degradation, S1 nuclease analysis with a fragmented single-stranded probe was performed.

The DNA sequence complementary to the *ompA* message was cloned in M13G*ori*1 as two separate *Bam*HI fragments. One of these fragments contained the first ≈880 nucleotides of the *ompA* transcript and included the only two *Hae* III sites within the *ompA* gene; the other encoded the last 340 nt of the message (Fig. 3 *Lower*). Total cellular RNA isolated from *E. coli* at time intervals after rifampicin treatment was hybridized with M13*ompA* DNA that had been radiolabeled and digested with *Hae* III. After treatment with S1 nuclease and gel electrophoresis, four bands were detected by autoradiography (Fig. 4 *Right*). Two of these, 85 and 598 nt in length, correspond to a *Hae* III fragment and a *Hae* III/*Bam*HI fragment mapping within the *ompA* gene (Fig. 3 *Lower*). The second largest fragment (≈340 nt) represents the 3′ end of the transcript, beginning at the internal *Bam*HI site and ending in a short tract of U residues following a stem and loop structure (17). The remaining fragment (≈200 nt) corresponds to the 5′ end of the *ompA* message, beginning at the initiation site for transcription (18) and ending at the first *Hae* III site.

Three conclusions may be drawn from the data in Fig. 4 *Right*. First, there is a single *ompA* gene transcript in *E. coli* defined by a unique promoter and a unique terminator. Second, a heterogeneous population of degradation intermediates is observed to accumulate, as evidenced by the fact that two segments (200 and 85 nt) exhibit longer half-lives than the full-length message. Finally, the order of segment stability (200 nt > 85 nt > 598 nt ≈ 340 nt) demonstrates that the 5′ end of the *ompA* message is the last portion to decay.

DISCUSSION

Both degradation from mRNA termini and endonucleolytic cleavage have been suggested as possible mechanisms accounting for message decay in *E. coli* (5, 20–22). With the aim of elucidating the role of specific nucleotide sequences in mRNA degradation, we have developed an assay that permits direct analysis of the relative rate of decay of different segments of a transcript.

In the case of the *bla* gene transcripts encoded by pBR322, all segments of a given message appeared to decay at the same rate, a rate identical to that for the degradation of the entire transcript. This result suggests that the decay of *bla* mRNA occurs by an "all-or-nothing" mechanism in which there is no significant accumulation of degradation intermediates. Evidently the decay of these transcripts is limited by some initial rate-determining step, and once this step occurs, rapid degradation of the entire transcript ensues.

In contrast, despite the low rate of initial cleavage of the long-lived *ompA* message, the subsequent steps in its degradation occurred slowly enough to allow a heterogeneous population of decay intermediates to accumulate. Consequently, differences in the rates of segmental decay could be detected. The two 5′-proximal segments of this transcript (200 and 85 nt) were nearly twice as stable as the central (598 nt) and 3′ (340 nt) segments. This observation is most simply explained by a decay mechanism involving a 3′-to-5′ exoribonuclease (e.g., RNase II) that

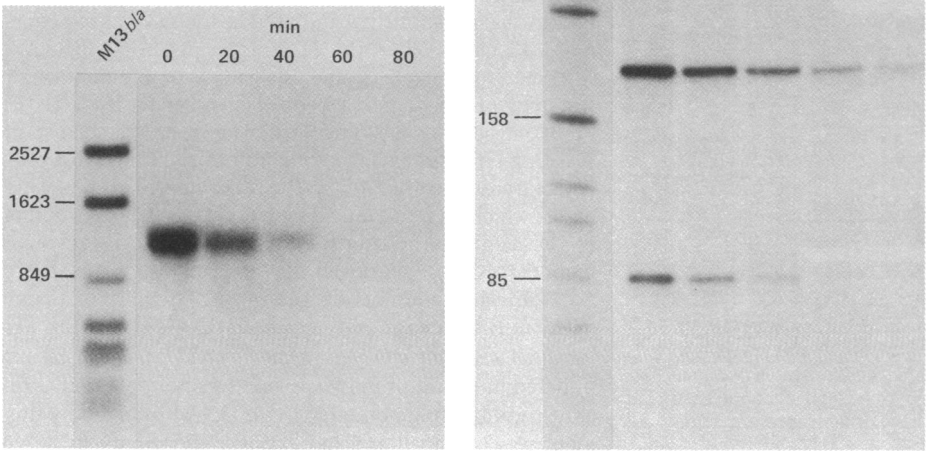

FIG. 4. Decay of *ompA* mRNA. (*Left*) RNA blotting analysis. Total cellular RNA (10 μg) isolated 0, 20, 40, 60, or 80 min after rifampicin treatment was electrophoresed and blotted as in Fig. 1 *Left* and then probed with nick-translated *ompA* DNA. A *Hae* III digest of M13*bla* was used as a molecular size standard. (*Right*) S1 nuclease analysis with a fragmented probe. Total cellular RNA isolated as in *Left* was hybridized with radioactively labeled M13*ompA* DNA that had been cleaved with *Hae* III endonuclease. Otherwise, conditions were as for Fig. 2. A *Hae* III digest of M13*ompA* DNA served as a molecular size standard and was electrophoresed beside the S1 nuclease-treated samples. The segment half-lives in min were determined to be 13 ± 3 (598 nt), 15 ± 2 (340 nt), 25 ± 2 (200 nt), and 21 ± 4 (85 nt).

initiates either at the 3′ terminus of the intact transcript or at an internal site generated endonucleolytically near the 3′ end of the message. The molecular basis for the 5-fold greater stability of the *ompA* gene transcript compared to *bla* mRNA remains to be elucidated.

The multiple promoters and terminators for the *bla* gene in the pBR322 plasmid provide a convenient set of related transcripts differing only in their termini. Interestingly, all six transcripts encoding the β-lactamase structural sequence had similar half-lives; thus, message stability was not affected by altering the site of initiation or termination of the transcript. If mRNA stability is determined by structures located at the 5′ or 3′ end of the transcript, there must be little sequence specificity in this process.

Significantly, no difference in the stabilities of translated versus untranslated segments was observed with either the *bla* or *ompA* gene. Thus, the absence of bound ribosomes at the untranslated 5′ and 3′ termini appears to render these segments no less stable than the translated portion of these transcripts. Indeed, the most long-lived portion of the *ompA* message is the 200-nt 5′ segment, of which only 30% is translated (Fig. 3 *Lower*). In contrast, studies of nonsense mutants of the *trp* operon have indicated that premature release of ribosomes within a protein-coding mRNA segment destabilizes the transcript distal to the site of mutation (23).

Previously, it was established that the *bla* gene in the pBR322 plasmid is transcribed from two promoters, one indigenous to the gene and one acquired in the construction of the plasmid (12, 13). The present work demonstrates that termination of β-lactamase mRNA encoded by pBR322 also occurs at multiple sites, only the first of which is indigenous to the transposon Tn3, from which the *bla* gene was originally derived. Consistent with this interpretation is the presence of DNA sequences that can form characteristic hairpin-loop RNA termination signals at the locations postulated, on the basis of the length of the transcripts, to contain the putative termination sites. It is unlikely that the observed variation in length of the 3′ ends of the *bla* transcripts results from RNA processing. In such a case, one would expect degradation of the longest 3′ terminal segment to precede that of the two shorter 3′ termini. Instead, roughly the same half-life was observed for all three of these 3′ segments as well as for the full-length transcripts from which they derived. We conclude from these data that the indigenous *bla* gene transcriptional terminator must be leaky, because more than half of the β-lactamase transcripts extend beyond this site. This conclusion is consistent with observations indicating that insertion of Tn3 can turn on expression of genes adjacent to the *bla* gene end of the transposon (24).

The steady-state concentration of a messenger RNA species is determined by the ratio of its rates of synthesis and decay. In the case of the *bla* gene, our analysis has permitted the dissection of these two components in a single experiment. The degradation rates of the transcripts initiated at each of the two *bla* promoters in pBR322 are equal. Consequently, the relative strengths of these promoters can be assessed directly from the steady-state amounts of these two classes of mRNA. By this means we have determined that transcription from each of these promoters *in vivo* is initiated at approximately the same rate, a result that is in accord with previous *in vitro* studies (12).

The assay used in these studies should be broadly applicable to the investigation of many aspects of RNA metabolism, including transcription and processing. It has proven to be both reliable and convenient. The use of a single-stranded DNA probe avoids both the problem of competition between DNA·DNA and RNA·DNA hybridization and the complication of transcription from the opposite strand of the DNA template. Fragmentation of the probe by endonuclease digestion allows direct comparison of the fate of different transcript segments. For purposes of quantitation, fluid hybridization and direct autoradiography of the gel appear to be superior to filter blotting techniques for analysis of RNA. Furthermore, because of the logarithmic nature of the decay process (4), the precision of RNA half-life determination is not compromised even by moderate variability of gel band intensities.

Although in these studies we have relied on the availability of suitably located *Hae* III endonuclease cleavage sites, alternative approaches can be employed. For example, other restriction endonucleases have been shown to cleave single-stranded DNA (7) and RNA·DNA hybrids (25). Alternatively, genes can be fragmented by endonuclease digestion, and the gene segments can be subcloned in phage M13 (as was here done with the *ompA* gene), thereby allowing any restriction site to serve as the terminus of a probe for a specific mRNA segment. The same end can be achieved if foreign DNA fragments showing no complementarity to the cellular RNA under study are inserted at intragenic endonuclease cleavage sites of full-length probes already cloned in M13.

We thank Dr. C. Yanofsky for his helpful comments on the manuscript. These studies were supported by Grants GM27241 and GM26355 from the National Institutes of Health and in part by American Cancer Society Grant MV-44E. J.B. was supported by Fellowship DRG-525 of the Damon Runyon–Walter Winchell Cancer Fund.

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