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Posttranscriptional Regulation of Ribosomal Protein S20 and Stability of the S20 mRNA Species

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I have tested whether selective degradation of mRNA for ribosomal protein S20 of *Escherichia coli* occurs under conditions for which the expression of S20 is regulated posttranscriptionally. Blot hybridization of total RNA extracted from cultures at different times after addition of rifampin has permitted the estimation of relative levels of the two S20 mRNA species and their half-lives. In a strain harboring a plasmid containing the complete gene for S20, including the transcriptional terminator, moderate posttranscriptional repression of S20 synthesis is accompanied by a substantial increase in the half-lives of both S20 mRNAs relative to those in the haploid parental strain. In an otherwise identical strain in which the transcriptional terminator is deleted from the plasmid-borne S20 genes, the half-life of total S20 mRNA declines more than twofold relative to that in the haploid parent. Thus accelerated decay of the mRNAs for ribosomal protein S20 appears to be an artifact of deletion of the transcriptional terminator, rather than a physiologically significant consequence of translational repression.

Posttranscriptional regulation appears to be a major mechanism of control of the synthesis of ribosomal protein in *Escherichia coli* (7, 12, 19). When the capacity of a cell to synthesize one or more ribosomal proteins exceeds the availability of the nascent rRNA to sequester them, then the translational efficiency of the mRNA(s) encoding the proteins in question decreases. This decline is postulated to be a consequence of the binding of one or more free ribosomal proteins to a site on the mRNA(s) in a manner which reduces the efficiency of translational initiation or, in one instance, leads to premature termination of transcription (28). For ribosomal protein S20, gene dosage experiments (6, 22), the ability of S20 to inhibit its own synthesis in vitro (26, 27), and homologies between the leader region of S20 mRNA (deduced from the DNA sequence of its gene) and the 5′ third of 16S rRNA (13) are consistent with the basic model for autogenous translational control outlined above.

The stability of the mRNAs encoding ribosomal proteins, particularly under conditions of autogenous repression, is a potentially important factor in this control process. Indeed, selective inactivation of the excess mRNA in multicopy strains has been proposed to explain the compensation observed in some ribosomal protein operons for increased gene dosage (4, 24). Olsson and Gausing have pointed out, however, that an apparently accelerated rate of decay of an mRNA could be a consequence of its repression rather than the root cause (20). We previously observed that the apparent half-life of S20 mRNA in a strain carrying 58 copies of the gene decreased to 60 s from 150 s in the haploid parental strain (22). The construction used in that experiment deleted a putative rho-independent terminator normally located immediately distal to the gene for S20 (14). The secondary structure found at the 3′ end of transcripts generated at rho-independent sites can contribute to the stability of mRNAs so terminated (9, 18, 21, 23). In this work, therefore, I investigated the stability of the native S20 mRNA as a function of growth rate and gene number, factors which should influence translational efficiency.

MATERIALS AND METHODS

Strains. The bacterial strains and plasmids used in this work have been described previously (14) and are based on C600 *galK* (17) or pKO-1 (17). Strain GM286 is C600 *galK* (pGM51) in which the plasmid contains residues 1 to 691 of the S20 sequence (14) in pKO-1. Strain GM291 is C600 *galK* (pGM46) in which pGM46 contains residues 1 to 551 of the S20 sequence in the same vector.

Media. Bacterial cultures were grown routinely in M9 medium supplemented with glucose or glycerol as carbon sources. Strains derived from C600 *galK* were further supplemented with either 0.25% Casamino Acids (Difco Laboratories) (or 50 μg of each amino acid per ml) or threonine and leucine to 50 mg/liter. When needed, ampicillin was included at 25 to 40 mg/liter.

Analysis of S20 mRNAs. RNA was extracted from exponential-phase cultures, which had been harvested by centrifugation, by lysis of the cell pellet in boiling sodium dodecyl sulfate, followed by extraction with phenol as described previously (3). Crude RNA was dissolved in 4 M guanidinium thiocyanate and freed of DNA by being pelleted through a cushion of CsCl (2). For the analysis of total S20 mRNA by slot-blotting, samples of total cellular RNA were denatured and diluted serially. Portions of 5.0, 1.25, and 0.32 μg in 1.5 M NaCl–0.15 M sodium citrate (pH 7) were applied to a sheet of nylon (Biodyne A; Pall, Inc.) by gentle suction with a commercial slot-blot manifold (Schleicher & Schuell, Inc.). RNA was fixed to the nylon by baking in vacuo. For the resolution of individual species of S20 mRNA by Northern blotting, samples of total RNA, typically 4.0 μg, were separated by electrophoresis on a 1.5% agarose gel and transferred to a sheet of nylon (Biodyne A). In both cases, S20 mRNAs were visualized by annealing, as described previously, with a single-stranded RNA probe complementary to residues 232 to 551 in the S20 mRNA (14). Blots were quantified by densitometry, taking care to ensure that exposures were in the linear range of the film.

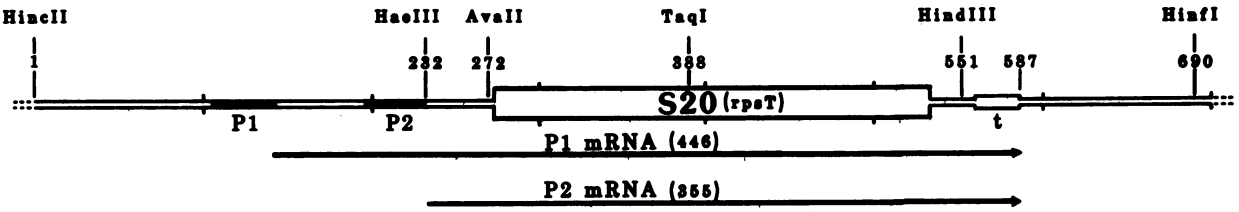

FIG. 1. Structure of the gene for ribosomal protein S20 (*rpsT*). Symbols: ▭, coding region for S20; = noncoding sequences are denoted by the thin double line; ▬, positions of the two S20 promoters (15). The numbers above the line give the coordinates (in base pairs) for cleavage by selected restriction enzymes (13, 14), while the small open box labeled t indicates the position of a rho-independent terminator (14). The two arrows below the line show the extents of the two S20 mRNA species and their sizes (in residues) (14). The single-stranded RNA probe used to detect the S20 mRNAs extends from residue 232 to residue 551 (14).

Measurement of the rate of synthesis of S20. Aliquots (0.5 ml) of exponential cultures were labeled for 2.0 min with 15 to 20 μCi of [^{35}S]methionine, chased for 30 s with excess (final concentration, 1 mM) unlabeled methionine, and then mixed with an equal volume of M9 salts containing 350 mg of chloramphenicol per liter and 20 mM NaN_3. Cells were harvested by centrifugation, suspended in twofold-concentrated sample buffer (10), and boiled for 5 min. Aliquots containing equal amounts of radioactivity were diluted into a buffer containing 10 mM sodium phosphate, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 400 μg of bovine serum albumin per ml, so that the final concentration of sodium dodecyl sulfate would not exceed 0.1%. Labeled S20 was precipitated from this mixture with antibody to S20 (kindly supplied by L. Kahan, University of Wisconsin) and killed *Staphylococcus aureus* cells. After extensive washing, immunoprecipitates were further resolved by electrophoresis on polyacrylamide gels, visualized by fluorography, and quantified by densitometry.

RESULTS

Properties of strains which overproduce S20 mRNAs. The gene for ribosomal protein S20 (*rpsT*) is transcribed from two promoters and terminates at a rho-independent terminator, leading to the steady-state accumulation of two monocistronic mRNA species of approximately 350 and 450 residues (14, 15). This is illustrated schematically in Fig. 1. Plasmids pGM46 and pGM51 contain both promoters and the complete coding sequence for S20 (13). A fragment of 140 base pairs encompassing the complete rho-independent terminator normally found distal to *rpsT* is present in pGM51 but is missing from pGM46. In both plasmids, the coding sequences for S20 would be transcribed in the same sense as that for galactokinase present in the parental vector, pKO-1. The absence of the transcriptional terminator in pGM46 therefore creates an operon fusion between *rpsT* and *galK*.

The data in Table 1 summarize some basic properties of strains GM286 and GM291 which contain, respectively, pGM51 (terminator positive) and pGM46 (terminator negative). Both strains display a marked increase in the steady-state level of S20 mRNA relative to that of the haploid parental strain as determined by slot-blot hybridization or RNA (Northern) blotting. In strains GM286 (terminator positive) and GM291 (terminator negative), growing at 1.4 to 1.5 doublings per h, the levels of S20 mRNA are 5.7- and 23-fold higher, respectively, than in the parental haploid strain (Table 1). These levels appear to reflect, in part, the copy number of the relevant plasmids; the yield of pGM51 from strain GM286 growing at this rate is very low, while that of pGM46 is severalfold higher. Since the steady-state S20 mRNA in GM291 is a mixture of species in various stages of synthesis and decay (14), the relative level given in Table 1 probably overstates the portion which is intact and translationally competent. The rate of synthesis of S20 in these and in control strains was measured by immunoprecipitation of S20 from extracts of pulse-labeled cultures. A typical experiment is illustrated in Fig. 2. Both strains GM286 and GM291 exhibit modest increases in the rate of synthesis of S20 (2.8-fold and 3.6-fold at 1.4 to 1.5 doublings per h, respectively; data summarized in Table 1). Nonetheless, the relative translational efficiency of the S20 mRNA(s) decreases significantly in both strains at both growth rates tested (Table 1, last column). Thus, in agreement with previous work (6, 22), these strains exhibit posttranscriptional control of synthesis of S20. The reduction in translational efficiency is more pronounced in strain GM291. This may be a reflection of an overestimate of the functional S20 mRNA in this strain, as noted above.

Stability of S20 mRNAs during steady-state growth. To determine how dosage compensation affects the stability of S20 mRNAs, RNA was extracted from various strains after exponential cultures had been treated with rifampin to a final concentration of 200 μg/ml to block further accumulation of RNA. A control experiment was performed to assess the effectiveness of the treatment with rifampin. A culture of C600 *galK* was radioactively pulse-labeled with uridine for periods of 30 s before and after the addition of rifampin. Incorporation of [^{3}H]uridine into acid-precipitable material fell by 40 and 95% in 30-s pulses beginning 15 s and 5 min,

TABLE 1. Translational repression in derivatives of C600 *galK*

Strain	Plasmid	Terminator	Relative rate of S20 synthesis[a,b]	Relative abundance of S20 mRNA(s)[b,c]	Relative translational efficiency of S20 mRNA(s)[b,d]
C600 *galK*		NA[e]	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)
GM286	pGM51	+	2.3 (3.4)	5.7 (10)	0.40 (0.34)
GM291	pGM46	−	3.6 (1.6)	23 (7.9)	0.16 (0.20)

[a] Cultures were pulse-labeled and the incorporation into S20 was determined by immunoprecipitation and densitometry as described in the text. The data represent the average of duplicate experiments.

[b] Numbers outside parentheses are for cultures growing at 1.4 to 1.5 doublings per h, while numbers inside parentheses are for 0.6 to 0.7 doublings per h.

[c] Total RNA was extracted from exponential cultures of each strain and the relative level of total S20 mRNA was determined by slot-blot hybridization as described in the text. Values are the average of duplicate experiments.

[d] Relative translational efficiency is the ratio of the rate of synthesis of S20 to the steady-state level of S20 mRNA and is independent of plasmid copy number.

[e] NA, Not applicable. The chromosomal copy of *rpsT* does, of course, contain an intact terminator.

respectively, after the addition of the drug. Since growth of S20 mRNA should be completed within 10 s after its initiation, it is reasonable to assume that the relative stability of S20 mRNA can be measured by its abundance in cells treated with rifampin. If inhibition of transcription were not instantaneous, then the apparent half-lives of the S20 mRNAs might be inflated and there would be a lag in the approach to steady-state exponential decay. Such a lag is not obvious in the data (see Fig. 3).

We previously reported a half-life of 60 s for total S20 mRNA in the multicopy strain GP2 (22). This strain contains a plasmid, pGP2, in which residues 1 to 551 of S20 are cloned into pBR322, deleting the rho-independent terminator in the process. Attempts to include the terminator in the same host-vector system failed. A 690-base-pair fragment encompassing the entire S20 gene could, however, be maintained stably in the vector pKO-1 in C600 *galK* (17). The construction analogous to pGP2 in pKO-1 is pGM46 (in strain GM291). Individual species of S20-galactokinase fusion mRNA from GM291 cannot be resolved cleanly by Northern blotting (cf. Fig. 4c in reference 14); therefore, I used slot-blot hybridization to measure the rate of decay of total S20 mRNA from this strain. In cultures growing at 1.4 to 1.5 doublings per h, the total S20 mRNA decayed with monophasic kinetics (data not shown) and an apparent half-life of 66 s (Table 2). For comparison, the initial rates of decay of total S20 mRNA from strains C600 *galK* and GM286 were also measured by slot-blot analysis at 126 s and 145 s, respectively (Table 2). The former number may be a small overestimate owing to a low level of annealing of the probe to rRNA (visible in Northern blots), which has the effect of inflating the apparent amount of S20 mRNA at longer times after addition of rifampin. In any event, it is clear that the half-life measured here for S20 mRNA lacking its natural 3′ terminus in GM291 (66 s) is very close to the value obtained previously for strain GP2 by using filter hybridization (60 s) and that in both cases, the stability of the S20 fusion mRNA is reduced by a factor of about 2 compared with that of the S20 mRNAs which retain the normal transcriptional terminator.

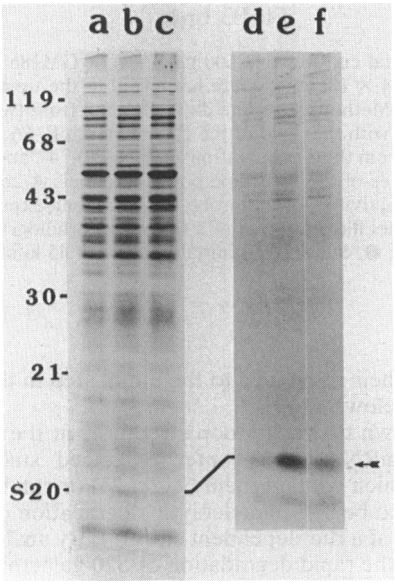

FIG. 2. Analysis of S20 in crude cell lysates. Extracts of cultures (growing in this case at 0.6 doublings per h) which had been pulse-labeled for 2.0 min with [^{35}S]methionine were prepared, and aliquots containing equal amounts of acid-precipitable radioactivity were separated on a 12.5% polyacrylamide containing sodium dodecyl sulfate (10) (lanes a to c). In lanes d to f, portions of each extract were fractionated by immunoprecipitation with an anti-S20 serum (or a control serum not shown in the figure) before being subjected to electrophoretic analysis. Extracts were prepared from strains C600 *galK* (lanes a and d), GM286 (lanes b and e), and GM291 (lanes c and f). The position of authentic S20 resolved on the same gel is indicated in the left-hand margin and by an arrow on the right; the numbers in the left-hand margin give the molecular weights (in thousands) of standard proteins.

TABLE 2. Half-lives of S20 mRNAs in haploid and multicopy strains

Strain	Plasmid	Terminator	Half-life (s)[a,b]		
			Total S20 mRNA[c]	P1 mRNA[d]	P2 mRNA[d]
C600 *galK*		NA[e]	125	73 (41)	116 (108)
GM286	pGM51	+	145	108 (250)	180 (690)
GM291	pGM46	−	66	ND[f]	ND

[a] Total RNA was extracted from exponential cultures at various times after addition of rifampin to 200 mg/liter and purified as described in Materials and Methods.

[b] Values outside parentheses are for cultures growing at 1.4 to 1.5 doublings per h, while values inside parentheses are for 0.6 to 0.7 doublings per h.

[c] The total S20 mRNA species remaining at various times after addition of rifampin were estimated by slot-blot hybridization as described in the text.

[d] Individual S20 mRNAs were resolved from total RNA by electrophoresis and Northern blotting, followed by hybridization to an S20-specific probe (see the text and Fig. 3).

[e] NA, Not applicable

[f] ND, Not determined. Individual species of chromosomally encoded S20 mRNAs in strain GM291 could not be resolved from the heterogeneous S20-*galK* fusion mRNAs (see the text and reference 14).

With Northern blotting, it is possible to separate both species of S20 mRNA and measure their relative abundances (14), whereas previously we used filter hybridization, which would not permit this distinction (22). Figure 3 illustrates decay curves for S20 mRNAs extracted from cultures growing at 1.4 to 1.5 doublings per h. In C600 *galK*, both species exhibit monophasic exponential decay (Fig. 3A). The species initiated at P1 (450 residues; hereafter designated the P1 mRNA) decays more rapidly than the species initiated at P2 (350 residues; the P2 mRNA). The half-lives of these mRNAs are 73 and 116 s, respectively (summarized in Table 2). At a lower growth rate (0.67 doublings per h), the half-life of the P1 mRNA decreases significantly to 41 s, whereas that of the P2 mRNA is almost unchanged at 108 s (Table 2). Increasing the copy number of the gene for S20 to about 5 in strain GM286 not only increases the half-lives of both S20 mRNA species relative to those in the haploid parent, but also alters the shape of the decay curve. In Fig. 3B, it is clear that both S20 mRNA species display biphasic decay curves. The apparent initial rates of decay, determined with three independent RNA preparations, are 108 and 180 s for P1 and P2 mRNAs, respectively. The slower decay phase for both mRNA species begins 12 min after the addition of rifampin and has a half-life of over 480 s in both cases. The reason for the slower component of decay is not clear. If the decay curves in Fig. 3B are treated as the sums of fast and slow components, then the half-lives of the fast (initial) components are calculated as 80 and 109 s for P1 and P2 mRNAs, respectively. At lower growth rates, both mRNA species display monophasic decay curves (data not shown), with apparent half-lives of 690 and 250 s for P2 and P1 mRNA, respectively (Table 2).

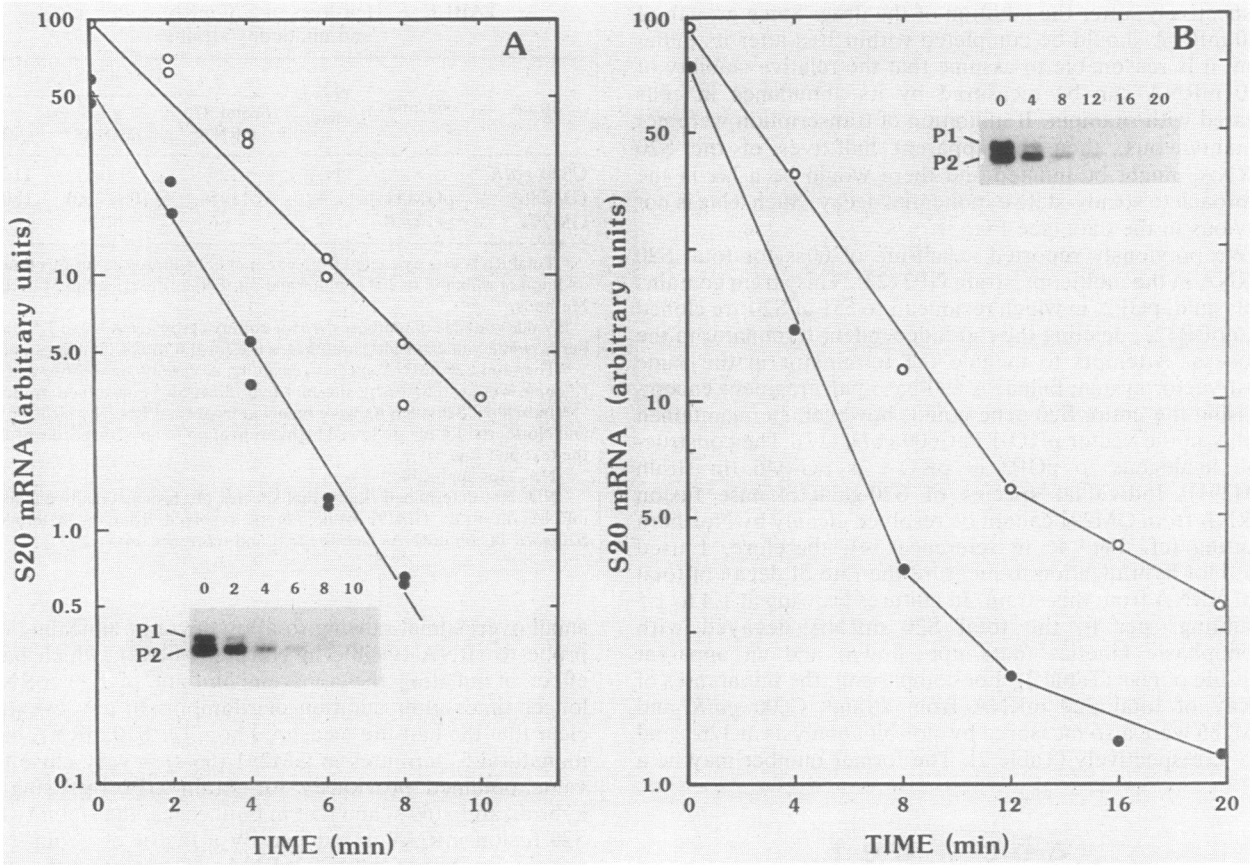

FIG. 3. Decay of S20 mRNA species in haploid and multicopy strains. Exponential cultures of C600 *galK* (A) or GM286 (B) growing at 1.5 doublings per h were treated with rifampin to 200 μg/ml, and portions (12.5 ml; 4×10^9 cells) were harvested at the times indicated on the ordinate. Extraction of the RNA and its analysis are described in Materials and Methods. (A) The data obtained from two independent preparations of RNA, probed once or twice each (on independent blots), are plotted with the yield of the P2-initiated mRNA at zero time in each preparation set as 100. (B) The datum points represent average values obtained from three preparations of RNA (0-, 4-, and 8-min points) or two preparations (12 to 20 min) probed once or twice each. The absolute intensities of the zero time points in panels A and B cannot be compared directly as presented, since the exposures, loading of RNA, and specific activities of the probe varied among experiments. Note that both axes in panels A and B are drawn to different scales. The insets in each panel illustrate part of a typical autoradiogram from which the data were obtained. Longer exposures were used for later time points. Symbols: ●, S20 mRNA initiated at P1 (0.45 kilobases); ○, S20 mRNA initiated at P2 (0.35 kilobases).

DISCUSSION

The most important ramification of the work reported here is that translational repression of the synthesis of ribosomal protein S20 is not obligatorily accompanied by an accelerated decay of its mRNA. Indeed, in strain GM286 (terminator positive), in which moderate repression occurs, both species of S20 mRNA are at least as stable as in the corresponding haploid strain at 1.4 to 1.5 doublings per h and are considerably more stable at lower growth rates. Other data (G. D. Parsons, B. C. Donly, and G. A. Mackie, manuscript in preparation) confirm this and show that both chromosomally encoded S20 mRNAs can be stabilized over twofold when S20 is overexpressed from a plasmid containing a derepressing mutation in the S20 leader. Since translational repression probably results from the exclusion of ribosomes from initiating translation on an mRNA, the apparent stablization afforded by S20 must differ from that known to occur when elongation of translation is blocked by chloramphenicol (11, 16). Instead, binding of S20 to its mRNAs may permit them to fold into a configuration which enhances their resistance to the initial step in their degradation (see below).

It is known that stem-loop structures at the 3′ termini of bacterial mRNAs can confer enhanced stability to the mRNAs which contain them (9, 21, 23) or can block what is presumed to be 3′ exonucleolytic degradation of *trp* mRNA terminated at a rho-dependent site (18). By analogy, it seems likely that the rapid degradation of S20-galactokinase fusion mRNA in strain GM291 (terminator negative) (Table 2) is a consequence of the deletion of the normal S20 transcriptional terminator in pGM46 rather than of translational repression. The same argument will explain our previous observation that S20 mRNA appears to decay 2.6-fold faster in a multicopy strain than in the haploid parent (22). In the latter strain, the plasmid used to increase the copy number of S20 (pGP2) also deleted the sequences containing the rho-independent terminator. In other words, the accelerated decay of S20 mRNA measured previously (22) is an artifact of the deletion of the rho-independent terminator distal to the S20 coding sequences. In the alpha ribosomal operon, translational repression is accompanied by decreased stabil-

ity of the corresponding mRNA (24). Why the S20 and alpha mRNAs should differ in their response to translational repression is not immediately clear, but may reflect features of the mRNAs themselves which determine the rate-limiting steps in their decay.

Several additional features of the process by which S20 mRNAs are degraded also emerge from this work. The synthesis of the two S20 mRNA species would require 8.1 and 6.4 s, assuming an RNA growth rate of 55 nucleotides per s (5). The lifetimes of the two S20 mRNAs are long by comparison (Table 2). Thus virtually all S20 mRNAs are completed before the onset of degradation. Attempts to visualize degradative intermediates of the S20 mRNAs in haploid or multicopy strains have failed. Reconstruction experiments have shown that the techniques used here would have detected mRNA fragments one-third the size of the complete mRNA in 5 to 10% relative abundance (data not shown). Both S20 mRNAs therefore decay in an all-or-none mode (1, 25), consistent with the hypothesis that the initial step in the decay is rate limiting. It is surprising, however, that the two mRNA species decay with significantly different lifetimes (Table 2; additional data not shown). Since the two S20 mRNAs differ only in the 90 additional residues present in the leader of the P1 mRNA, at least one determinant of stability lies in the 5′ untranslated portion of the S20 mRNAs (8). It is noteworthy that fusion of the 5′ portion of the *ompA* mRNA to the coding sequences for β-lactamase can confer a threefold increase in stability to the latter mRNA (1). Attempts to identify portions of the untranslated part of the two S20 mRNAs which contribute to translational efficiency, translational repression, and mRNA stability are in progress and may yield explanations for the differential stabilities of the S20 mRNAs.

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