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Difference Between Functional and Structural Integrity of Messenger RNA

(S13 phage/*Escherichia coli*/long-lived mRNA/rifampicin/gel electrophoresis)

ALVARO PUGA*, MARÍA-TERESA BORRÁS†, ETHEL S. TESSMAN‡, AND IRWIN TESSMAN‡

Department of Molecular Biology and Biochemistry, University of California, Irvine, Calif. 92664

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ABSTRACT **Messenger RNA molecules that are structurally stable, as measured by their ability to hybridize to DNA, may nevertheless be considerably less stable in retaining their ability to function in protein synthesis. The structure of the majority of the mRNA of phage S13 decays with a half-life of 10.6 ± 0.5 min. In contrast, much of the function of the mRNA that is involved in synthesis of a capsid protein (product of the F gene) decays rapidly with a half-life of 1.4 ± 0.8 min; a residual amount of function decays with a half-life of 14.0 ± 4.0 min. The measurements were made in the presence of rifampicin, which was used to prevent the formation of new mRNA. A proposed model for the functional decay is based on the polycistronic nature of the mRNA. Degradation of the mRNA would proceed in two steps: the first step would be a fast attack at a region near the 5′-terminus of each molecule that would eliminate the function of the proximal message; the second step would be a slow attack on the remaining messenger molecule precipitating a subsequent rapid degradation of the physical structure.**

Studies on degradation of messenger RNA of bacteriophages φX174 (1, 2), M13 (3), and T7 (4, 5) have shown that their mRNA is relatively resistant to decay. At least in T7 infection only the viral mRNA is stabilized, not the mRNA of the infected host, thus ruling out a viral-directed general interference with mRNA degradation.

For the above three viruses, only decay of the ability of the mRNA to hybridize to DNA was measured, and no information was obtained on decay of function of the mRNA. But the functional half-life is critical in protein synthesis. Alteration or removal of perhaps just one nucleotide could rapidly eliminate the function of mRNA without significantly reducing the amount of mRNA detected by hybridization. As a result, a structurally long-lived messenger could have a quite short functional life.

Since it is likely that degradation of mRNA plays an important role in regulation of protein synthesis, we have studied the decay of both biological function and structural integrity in the S13-φX174 phage system. Functional decay was studied by examination of the rate of protein synthesis during inhibition of new RNA synthesis by rifampicin. The rate of protein synthesis was measured by the uptake of a labeled amino acid during short pulses into the major phage capsid protein, the F protein. Host-protein synthesis was eliminated by pulsing later than 20 min after infection, by which time host-protein synthesis is shut off (Linney and Hayashi, personal communication; Borrás and E. S. Tessman, unpublished observation). We shall show that there is a remarkable difference between the functional and structural decay curves.

* Present address: Department of Experimental Pathology, Scripps Clinic and Research Foundation, 476 Prospect Street, La Jolla, Calif. 92037.
† Present address: Department of Biology, Muir College, University of California, San Diego, Calif. 92037.
‡ Present address: Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907.

DECAY OF STRUCTURAL INTEGRITY

Structural degradation of mRNA was measured by the decay of hybridizing ability. A relatively stable mRNA is made at late times after infection with the related φX174 (1, 2) and with the small filamentous phage M13 (3). We examined early and late S13 mRNA to see if both have the same decay rate, particularly to examine the possibility that after infection some phage-specified protein may be synthesized that selectively protects phage mRNA against degradation; if this were true, the early messenger that would have to code for such a protein could not be protected, and would be degraded as fast as the host mRNA. We also examined the effect of messenger concentration on its degradation by using a mutant in gene A, blocked in DNA replication, that produces four-times less mRNA than the wild type (6). Thus, we could test the possibility that stability of phage mRNA depends on the degree of saturation of the degradative machinery.

The rate of degradation of phage mRNA was the same whether the pulse was given at early (2.5–3.5 min), intermediate (6.5–7.5 min), or late (10–11 min) times after infection with either wild-type S13 (Fig. 1*A–C*) or a nonsense mutant in gene A, *am*A100 (Fig. 1*D–F*). The half-life of the phage-specific messenger was 10.6 ± 0.5 min and that of the bulk of the host mRNA, 1.9 ± 0.1 min. The similarity of the half-lives of early and late mRNAs argues against the existence of a phage-coded factor that might stabilize the messenger. Since host mRNA is not stabilized after phage infection the stability of the phage message must be an inherent property of the phage message. The results do not rule out the possibility that the cell may have more than one mechanism for degradation of messages, e.g., a major mechanism that would degrade the bulk of the host messenger rapidly and a minor mechanism that would be used for certain foreign mRNAs and perhaps for some of the cellular messages too, but the choice of mechanism would depend on the sequence and structure of the mRNA itself.

The concentration of phage mRNA varied over a 10-fold range from early times after infection with *am*A100 to late times after infection with the wild type, yet the decay rate was the same. That finding excludes the possibility that the

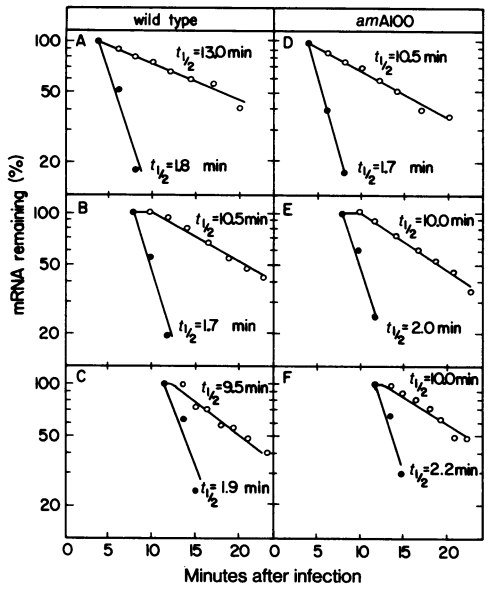

FIG. 1. The decay of S13 (○) and host (●) mRNA in infected cells at 37.0 ± 0.5°. *Escherichia coli* C, a nonpermissive host for amber mutants of S13, was prelabeled in its stable RNA components (ribosomal and transfer RNA) with 0.7 μCi/ml of [^{14}C]uridine (53 Ci/mol) while grown to 1 to 2 × 10^8 cells per ml. The [^{14}C]uridine was then removed by centrifugation and the cells were grown for one more generation. Either wild-type S13 (*A*, *B*, *C*) or a gene-A mutant, *am*A100 (*D*, *E*, *F*) was added to give a multiplicity of five phage per cell. After 2.5 min (*A*, *D*), 6.5 min (*B*, *E*), and 10 min (*C*, *F*) the infected cultures were pulsed for 1 min with 10 μCi of [^{3}H]uridine (27 Ci/mmol) per ml. The pulse was terminated by the addition of a 1000-fold excess of cold uridine and rifampicin (Rifadin, The Dow Chemical Co.) to a concentration of 800 μg/ml to inhibit further mRNA synthesis. Total mRNA and S13-specific mRNA were assayed at different times after infection by removal of 0.5-ml samples of the infected cultures. Each aliquot was pipetted into 6 times the volume of ice-cold 0.03 M Tris·HCl (pH 8.1)–0.03 M NaN_3–400 μg of chloramphenicol per ml. RNA was extracted with phenol–chloroform in the presence of sodium dodecyl sulfate and precipitated with 2.5 volumes of 100% ethyl alcohol. Recovery of pulse-labeled RNA was calculated from the fraction of material prelabeled with ^{14}C that was recovered. S13-specific mRNA was determined by hybridization to replicative form DNA bound to filters (6). Host mRNA was determined by first subtracting the amount of phage mRNA from the amount of total acid-precipitable RNA as a function of time after rifampicin was added. The resulting curve for the decay of bulk host RNA leveled off at about 55% of the initial value and represents the stable RNA. The unstable 45% component is taken to represent the host mRNA (7). The stable level was calculated from the mean of the last four points in each experiment. The mean values and the standard deviations of the individual points were as follows: *A*, 53.7 ± 0.8; *B*, 54.0 ± 1.2; *C*, 58.2 ± 1.1; *D*, 52.3 ± 0.8; *E*, 55.1 ± 0.8; *F*, 57.0 ± 3.0. Control experiments showed that synthesis of phage-specific mRNA was completely inhibited by rifampicin. By 3 min after addition of the drug, synthesis of mRNA was reduced to 1.5% of the untreated control, and by 6 min to 0.2%. A residual 15% level of ^{3}H counts incorporated into host nucleic acid was observed even after longer periods of preincubation with the drug, probably the combined effect of residual RNA synthesis and of [^{3}H]uridine incorporation into DNA after addition of rifampicin (7). This residual level of incorporated counts was eliminated when excess unlabeled uridine was added with the drug.

stability of the phage messengers was the result of an oversaturation of the degradative machinery, which would allow a large fraction of mRNA molecules to escape degradation.

DECAY OF FUNCTION

The functional life of phage mRNA was determined by measurement of the amount of a complete phage-specified protein made at different times after inhibition of mRNA synthesis with 800 μg of rifampicin per ml. Aliquots of infected cells were given a 2-min pulse of [^{3}H]leucine, each at a different time. The amount of phage proteins made during a pulse was examined by gel electrophoresis. Several phage proteins are easily identified (Fig. 2), but only the gene-F product had the high rate of incorporation needed for accurate measurements of half-lives.

The measurement of the decay of phage mRNA function might be distorted to reflect the decay of host function if peak F also contained an appreciable amount of host protein made after infection. However, this is not the case, for when the phage peak was displaced by an amber mutation in gene F there was no significant amount of host protein (Fig. 2).

To provide a standard for calibration of the protein peaks in the gels, at the end of each pulse there was added to each ^{3}H-pulsed aliquot a constant amount of a culture that had been infected in the absence of rifampicin and labeled with [^{14}C]-leucine. Therefore variations in the absolute recovery of proteins from extract to extract had no effect, for it was the ratio ^{3}H/^{14}C in a protein peak that measured the amount of that

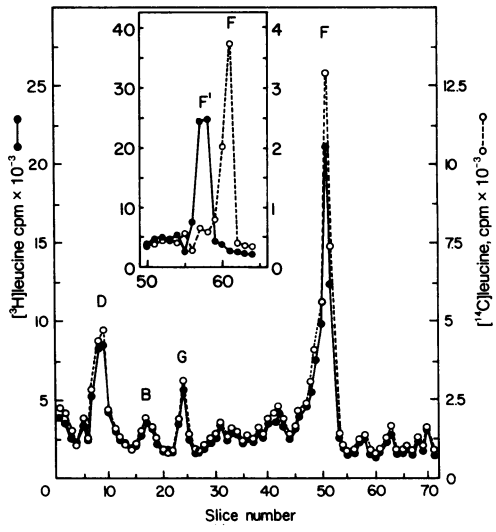

FIG. 2. Polyacrylamide-gel electrophoresis patterns of S13 proteins synthesized during a short pulse with labeled leucine. *E coli* C was grown to 2 × 10^8 cells per ml, infected with *am*En15, a mutant in the lysis gene (E), at a multiplicity of 15 phage per cell. 20 Min after infection two aliquots were removed; one was pulsed for 2 min with 20 μCi/ml of [^{3}H]leucine (40 Ci/mmol diluted with unlabeled leucine to 1 μg/ml) and the other was pulsed for 5 min with 2 μCi/ml of [^{14}C]leucine (315 Ci/mol). The aliquots were pooled, lysed, and electrophoresed as described (8). Electrophoretic movement is from right to left. The *letters* correspond to the genes coding for the respective polypeptides. Both ^{3}H-labeled and ^{14}C-labeled leucine give identical patterns. The *insert* contains the patterns of a normal and a shifted F peak showing the absence of host protein at the normal peak position. The normal peak (*F*) was obtained with wild-type phage and the shifted peak (*F'*) with the amber mutant *am*Fh61.

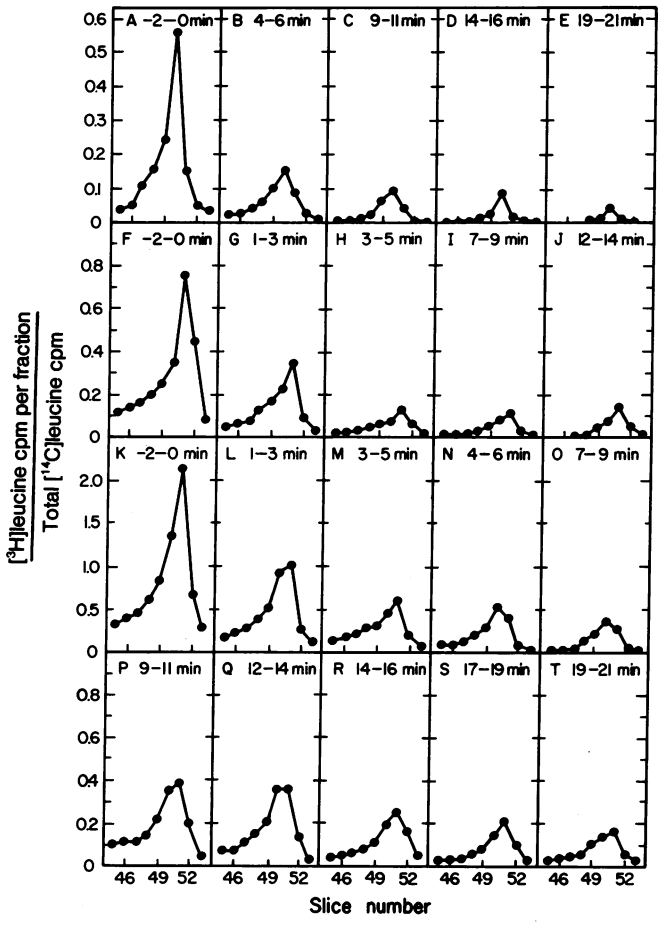

FIG. 3. Polyacrylamide gel electrophoresis patterns in three independent experiments (*A–E; F–J; K–T*) showing the amount of protein F made at different times after addition of rifampicin. *E. coli* C was grown to 2×10^8 cells per ml, infected with *am*En15, a mutant in the lysis gene, at a multiplicity of 15. 20 Min after infection two aliquots were removed; one was pulsed for 2 min with 18 μCi/ml of [^{3}H]leucine and the other, used for standardization of the gel, was pulsed for 5 min with [^{14}C]leucine (315 Ci/mol) as described (8). The three experiments differed in the specific activity of [^{3}H]leucine, the concentration of [^{14}C]-leucine, and the times at which the rifampicin-treated cultures were pulsed. *A–E:* [^{3}H]leucine, 1.9 Ci/mmol; [^{14}C]leucine, 0.2 μCi/ml. *F–J:* [^{3}H]leucine, 3.4 Ci/mmol; [^{14}C]leucine, 0.2 μCi/ml. *K–T:* [^{3}H]leucine, 2.8 Ci/mmol; [^{14}C]leucine, 0.1 μCi/ml. The pulse intervals are given in each panel. The ordinate gives the ^{3}H counts relative to the total number of ^{14}C counts in the entire F peak.

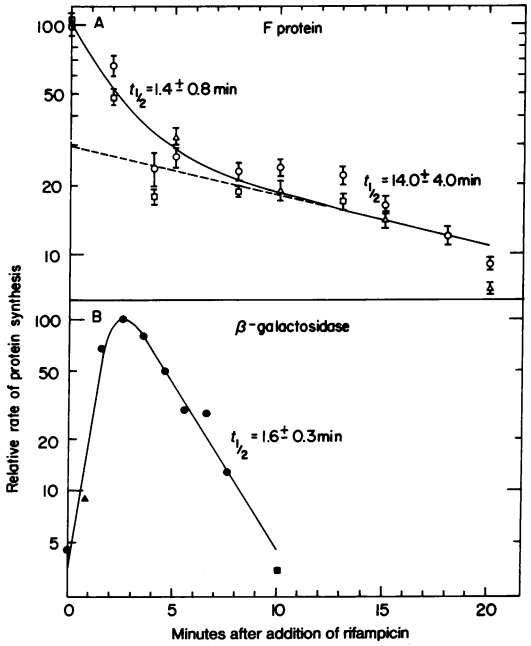

FIG. 4. Functonal decay of mRNA for (*A*) the S13 F protein and (*B*) the bacterial β-galactosidase. For the F protein the ^{3}H counts in each panel of Fig. 3 were integrated and plotted relative to the value at zero time. From Fig. 3*A–E*, △——△; from Fig. 3*F–J*, □——□; from Fig. 3*K–T*, ○——○. The *error bars* represent 2σ derived from counting errors. The relative rate of protein synthesis, R, at time t has been fitted by the two-component decay curve $R = 0.70 \exp(-0.50\,t) + 0.30 \exp(-0.050\,t)$. For measurement of β-galactosidase activity, the inducer isopropyl-β-D-thiogalactopyranoside was added 1.5 min before addition of rifampicin at time 0. Enzyme activity was determined by the method of Revel, Luria, and Rotman (9) with the following modification: the samples were taken into two volumes of 0.1 M phosphate buffer (pH 7.0) containing 500 μg of chloramphenicol per ml, and the cells were pelleted and washed with the same buffer. This was done in order to remove the rifampicin, whose red color interferes with the colorimetric assay of the enzyme. Measurements were made at intervals ranging from 0.5 to 4 min. The rate of β-galactosidase synthesis was calculated from the increments in enzyme activities over the intervals used: 0.5 min (▲); 1 min (●); 4 min (■). Each point was plotted at the time corresponding to the middle of the corresponding interval.

protein synthesized during the pulse. The decrease in ^{3}H counts relative to the ^{14}C counts determines the decay of functional mRNA for a particular protein, in this case, the F protein.

Three independent experiments were performed (Fig. 3*A–E; F–J; K–T*). These three experiments differed in the times at which the pulses were given and in the specific activity of the [^{3}H]leucine added. Rifampicin was added at zero time, which was 20 min after infection. The rate of protein synthesis at $t = 0$ was measured by a 2-min pulse given just before addition of rifampicin.

A control experiment showed that in the absence of rifampicin there was no loss of protein-synthesizing ability. An aliquot of cells was left untreated with rifampicin and pulsed with [^{3}H]leucine at the same time as the 19- to 21-min pulse given to the treated culture, i.e., 39–41 min after infection. This aliquot, which could continue to synthesize new mRNA, in fact showed a 30% increase in amount of counts in each protein species relative to the counts in the time-zero pulse.

The decay curve (Fig. 4*A*) of functional mRNA was obtained by plotting the integrated values for each F-protein peak in Fig. 3. (The entire gel pattern was obtained for every pulsed sample, but only the F peaks of each gel are shown in Fig. 4.) Corrections for the background level of counts in each panel of Fig. 4 had a negligible effect on the slopes of the decay curve. In contrast to the structural stability of the phage mRNA, the functional ability to synthesize the F protein initially decays very rapidly ($t_{1/2}$ = 1.4 ± 0.8 min), accounting for about 70% of the F-protein-synthesizing ability; the remaining 30% of the synthesizing ability decays

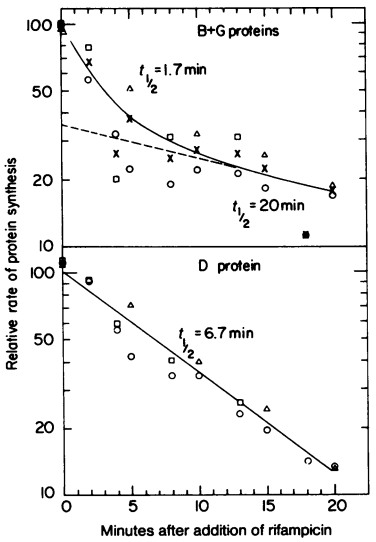

FIG. 5. Functional decay of mRNA for the combined B + G proteins and the D protein of S13. The results from three separate S13 experiments are represented by the symbols △, □, and ○. (The symbols correspond to those used in Fig. 4.) The relative error in each point in terms of 2σ derived from counting errors is 10–15%. Because of the wide scatter in the data for the B + G proteins, the average values were also plotted (×), and these were fitted by a two-component decay curve $R = 0.65 \exp(-0.40\ t) + 0.35 \exp(-0.035\ t)$.

at a rate ($t_{1/2}$ = 14.0 ± 4.0 min) that is like the rate of decay of the structural integrity.

A decay curve of functional cell mRNA that codes for β-galactosidase (Fig. 4*B*) was determined by measurement of the accumulated activity of β-galactosidase as a function of time; the rate of change was then calculated from the experimentally observed increments. Uninfected cells had to be used because synthesis of β-galactosidase is shut off between 10 and 20 min after S13 infection (Borrás and E. S. Tessman, unpublished data). After an initial 3-min lag, the ability to synthesize β-galactosidase decays with a half-life of 1.6 ± 0.3 min in good agreement with other determinations (10–12). The functional decay of β-galactosidase mRNA occurs at nearly the same rate as the structural decay of bulk cell mRNA (1.9 ± 0.1 min). Thus, the behavior of the viral mRNA is quite distinctive.

Decay curves were also determined for protein peaks D, B, and G (Fig. 5), but these curves are less accurate than the one obtained for the F peak because these peaks are smaller. The curves are also less reliable because of uncertainties in the true background level for these peaks in each gel. Since B and G were not always well separated, the data for the two peaks were combined. The rate of synthesis of B + G as a function of time is consistent with a two-component decay curve similar to that found for F; about 65% of the synthesizing capacity decays with $t_{1/2} \approx 1.7$ min and 35% with $t_{1/2} \approx 20$ min. The results for D have been fitted by a single-component curve with $t_{1/2} \approx 6.7$ min; a two-component curve could conceivably fit.

DIFFERENCE BETWEEN STRUCTURAL AND FUNCTIONAL STABILITY

By the criterion of hybridizability, the viral mRNA is relatively stable with a half-life of 10.6 min compared to 1.9 min for bulk bacterial mRNA. Hybridization can occur for fragments of mRNA, so stability of hybridizable material does not prove that the mRNA remains intact. At the least, however, the mechanism of degradation must be different for the viral mRNA because the cell's mRNA is rapidly degraded to acid-soluble lengths. Furthermore, the amount of high-molecular-weight viral mRNA decreases slowly (2), so it is unlikely that the mRNA is degraded rapidly to small hybridizable fragments.

The ability of the mRNA to function in the synthesis of phage F protein shows two components. The major component decays rapidly, like bacterial mRNA; a large minor component is relatively stable functionally.

A trivial explanation for the decay of protein-synthesizing ability could be that rifampicin affects the pool size of amino acids and lowers the intake of the [^{3}H]leucine present in the medium, resulting in an apparent functional decay of the messenger. However, the pool size as determined by measurement of trichloroacetic acid-soluble counts after a pulse with [^{3}H]leucine is the same whether the cells are treated with the drug or not (unpublished data).

The stability that we observe for the physical structure of S13 mRNA in the presence of rifampicin is in good agreement with previous results on mRNA of the related phage φX174 (1, 2) and the similar phage M13 (3), which were obtained in the absence of the drug, suggesting that rifampicin does not affect the rate of decay of the physical structure. It is conceivable that the more rapid functional decay arises from some unknown effect of rifampicin on the rate of mRNA translation. However, experiments (11) in which the synthesis of β-galactosidase after dilution of the inducer was compared with synthesis after addition of rifampicin have shown that the amount of protein translated from previously initiated mRNA chains is not modified by the drug. This result suggests that translation itself is not impaired by the presence of rifampicin. However, we have not ruled out the possibility that inhibition of transcription somehow prevents initiation of translation of preexisting, structurally long-lived phage mRNA.

We conclude that for the F protein [which is coded for by approximately one-fourth of the genome (13, 14)] most of the functional ability of the viral mRNA decays rapidly and, therefore, the observed structural stability is probably largely illusory, for since much of the hybridizable mRNA cannot function, it must have been structurally modified.

It is likely that the structural modification is caused by a rapid but limited attack at sites proximal to the 5′-terminus of the viral mRNA, thereby accounting for conservation of the high molecular weight of the mRNA (2). The attack could prevent ribosome binding or some other aspect of the translation process.

An explanation for the two-component nature of the decay curve is suggested by the existence of long viral mRNA molecules (2). We have found that they range in size from 0.1- to 2-times the length of the genome, with 50% of the molecules having molecular weights of 0.1 to 0.7 × 10^6 and 50% having molecular weights of 1.5 to 3.3 × 10^6 (6). From a study of polar mutants it has been inferred that there is a promoter before gene A (15) and one before gene F (2), the two genes being about 180° apart on the circular chromosome (15) (other promoter sites are possible). Therefore, a viral gene, such as F, will be located in some mRNA molecules

proximal to the 5′-terminus and in some molecules distal; in the larger molecules a gene could be repeated in its cyclic order and thereby occur in both proximal and distal locations. Ribosomal binding sites must exist before genes A and F when the genes are proximal to the 5′-terminus and, therefore, most likely when they are distal. Although an F gene proximal to the 5′-terminus of the mRNA would be inactivated rapidly, a distal gene could continue to function, producing the long-lived second component of the decay curve. The half-life of the second component suggests that a distal gene loses its function when the structural integrity of the mRNA is completely lost.

The difference between the structural stability of phage and bacterial mRNA might reside in a greater resistance of the phage messenger to endonucleolytic attack, for several endonucleases have been implicated in bacterial mRNA decay (4, 16–20). It can be inferred (Fig. 3 of ref. 2) that the viral mRNA is apparently degraded by an all-or-nothing process. At any time, relatively few molecules are being attacked, so there is a slow overall rate of degradation, but those that are being attacked degrade rapidly. It would then be expected that all genes distal to the 5′-end of a mRNA molecule would be inactivated functionally with about the same long half-life.

The observed functional instability of S13 mRNA justifies our reservations about the use of hybridization to measure messenger stability. It is clear that other cases of "stable" mRNA require reexamination by methods that can detect the effects of subtle changes in messenger structure.

NOTE ADDED IN PROOF

Recent studies have shown that there are differences between the functional and structural decay rates for mRNA of the *lac* operon (21). Also, for the two messenger species studied, β-galactosidase and transacetylase, each had a different functional decay rate, which is in accord with the idea that the functional decay rates of the mRNA species in a polycistronic message may depend on their positions within the message.

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