Ribonuclease V of Escherichia coli: Susceptibility of Heated Ribosomal RNA and Stability of R17 Phage RNA

Abstract. *Native ribosomal RNA and intact phage R17 RNA are insensitive to attack by ribonuclease V, an exonucleolytic activity associated with ribosome movement on the substrate RNA. However, ribosomal RNA becomes a substrate when it is heated under conditions that make it a template for protein synthesis, and R17 RNA is attacked if it is either heated or fragmented. Accessibility of the 5′ terminus of an RNA molecule is probably increased by heating or fragmentation and may determine its susceptibility to ribonuclease V.*

Ribonuclease V is an exonucleolytic activity, associated with ribosomes of *E. coli*, which probably degrades messenger RNA (mRNA) in intact cells (*1*). The activity seems to be attendant on ribosome movement along mRNA, for it requires monosome formation from 30*S* and 50*S* ribosomes, and is inhibited by antibiotics that block ribosome movement and function (*1*).

Here we show first, that ribosomal RNA (rRNA), which is ordinarily not degraded by ribonuclease V (*1*), can be degraded under conditions that permit it to participate in protein synthesis as an mRNA. Second, we show that R17 RNA, which is not usually degraded in vivo (*2*), is also not a substrate for ribonuclease in extracts, but can become one if it is first fragmented or heated.

Ribosomal RNA extracted from ribosomes does not serve as an mRNA (*3*), nor is it degraded by ribonuclease V (*1*). However, McCarthy, Holland, and Buck have shown that rRNA can serve as a template if it is first heated (*4*). We confirmed this result and have shown that heated rRNA also becomes susceptible to ribonuclease V (Fig. 1). The extent of hydrolysis in the conditions employed (30 percent) is comparable to that observed with other substrates of ribonuclease V in similar assays (*1*).

However, the capacity to function as an mRNA probably does not guarantee that an RNA chain will be susceptible to ribonuclease V action. This can be inferred from results when RNA of phage R17 is used. This RNA is resistant to ribonuclease action in intact cells (*2*); the 28*S* RNA purified from this virus (Fig. 2) is highly efficient as an mRNA in vitro (*5*), but is completely resistant to ribonuclease V (Table 1). The stability of R17 RNA in vitro, as in vivo, is consistent with the notion that ribonuclease V degrades mRNA in intact cells.

One way to understand the resistance of R17 RNA to ribonuclease V is to assume that for mRNA degradation, the ribosome involved has to begin at or near the 5′ end of the mRNA molecule. The nucleotide sequences of R17 RNA that bind ribosomes are well within the molecule (*6*), and its 5′ end may therefore be inaccessible to ribonuclease V.

This notion is supported by experi-

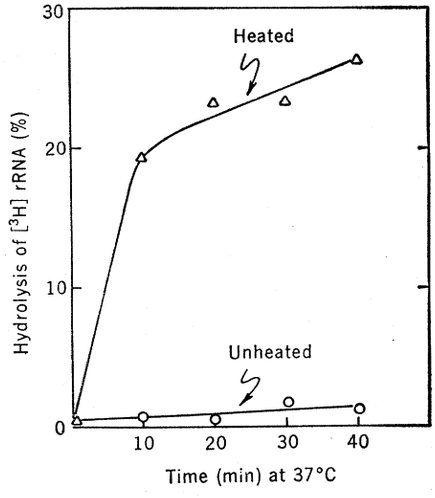

Fig. 1 (right). Sensitivity of heated rRNA to ribonuclease V. The assay mixture contained, per milliliter, guanosine triphosphate, 3×10^{-7} mole; glutathione, 10^{-5} mole; transfer RNA, 200 μg; Mg^{2+}, 2×10^{-5} mole; NH_4Cl, 5×10^{-5} mole; adenosine triphosphate, 2×10^{-5} mole; tris-HCl, *p*H 7.6, 4×10^{-5} mole; 200 μl of cell extract of strain N464 first heated 10 minutes to inactivate ribonuclease II (*11*); and substrate. The substrate, labeled rRNA, was prepared by phenol extraction of ribosomes from cells labeled with [^{3}H]-uracil (15 μc/mmole) for three generations; 360 μg of the rRNA (1050 count min^{-1} μg^{-1}), unheated (lower curve) or heated to 100°C for 5 minutes (upper curve) was used as substrate per milliliter of reaction mixture. (For heating, a thin-walled test tube, containing 5 mg per milliliter of RNA, was placed in boiling water, then transferred to ice water.) Alcohol solubilization of labeled material was measured on 50-μl samples taken after incubation at 36°C for 10, 20, 30, or 40 minutes, as indicated.

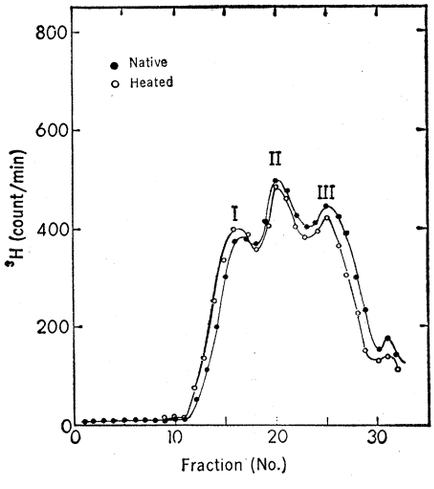

Fig. 2 (left). Separation in sucrose gradients of intact and fragmented R17 RNA. Intact 28*S* R17 [^{3}H]RNA (30 count min^{-1} μg^{-1} peak I) was separated from two size classes of fragments [peak II, about 21*S*, and peak III, about 16*S* (*7*)], by zonal sedimentation in sucrose gradients. Centrifugation was for 23 hours at 4°C in 0.01*M* tris (*p*H 7.5) and 0.1*M* NaCl, at 25,000 rev/min in a Spinco model L2 25.1 rotor. Before centrifugation, one sample tube containing 1 ml of the RNA solution was placed at 90°C for 6 minutes (heated) and then plunged into an ice bath. A 1-ml control sample (unheated) was kept on ice during this time. Each sample was then fractionated in a gradient. The two gradient patterns are superimposed. [For preparation of bacteriophage R17 and its RNA, see (*7*).]

Table 1. Action of ribonuclease on intact and fragmented R17 RNA. Portions of indicated sucrose gradient fractions (from the gradient analysis shown in Fig. 2) were exposed to ribonuclease V action. The conditions were those of Fig. 1; the substrate RNA in each milliliter of reaction mixture was 160 μg of peak I, 240 μg of peak II, or 320 μg of peak III, incubated up to 30 minutes at 37°C. In all cases, degradation of the RNA was approximately linear for 30 minutes. Duplicate assays were reproducible to within ± 2 percent. Consistent results were obtained with samples of fractions across each peak, and from fractionation of two different preparations of R17 RNA. When samples were tested for their capacity to support protein synthesis, the peaks of heated or unheated RNA showed the overall capacity characteristic of intact 28*S* RNA (peak I) and fragmented RNA (peaks II and III; see *7*).

Peak (No.)	Fraction (No.)	Hydrolysis in 30 minutes (%)
	Unheated	
I	15	0
II	19	17
III	25	8
	Heated	
I	15	24
II	19	33
III	25	12

ments in which fragments of R17 RNA are subjected to ribonuclease V action. Large fragments are customarily found in R17 RNA preparations. Such fragments were isolated by sucrose gradient centrifugation of a preparation of total R17 RNA (*7*) (peaks II and III in Fig. 2), and were found susceptible to degradation (Table 1). Furthermore, as had been observed for ribosomal RNA, heating increased the fraction of the RNA that could be rendered soluble in alcohol by ribonuclease V (Table 1).

Even more striking, intact chains of R17 RNA were sensitive to attack by ribonuclease V when they were repurified after heat treatment (Table 1). Fragmentation and heating probably increase the accessibility of the 5′ end to ribosomes. However, this view, if correct, raises an interesting question regarding multicistronic messenger RNA molecules that, like R17 RNA, contain an untranslated portion of informational RNA at the 5′ end. [As another example, the *o* site in the lac operon, which is adjacent to the structural genes of the operon (*8*) is believed to be transcribed (*9*) but not translated (*10*).] Either these initial sequences, when exposed, can somehow be traversed by ribosomes and thus degraded, or they are degraded by a mechanism that does not involve ribosome movement.

Whatever the reason for its resistance to degradation, the stability of intact, unheated R17 RNA suggests that many of the conditions required for protein synthesis are necessary for ribonuclease V action but are not sufficient for it. At least one further requirement seems to be that the mRNA be susceptible to ribonuclease V.

An obvious implication of these results is that individual species of mRNA, even when active in protein synthesis in vivo and in vitro, can probably still escape degradation. Perhaps addition of an appropriate nucleotide or polynucleotide structure at the 5′ end of the molecule can prevent degradation. In phage RNA, in which this might be the case, the resultant stability would help to insure successful infection, and might have offered the selective advantage for the development of the initial, untranslated segment.

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Chloramphenicol: Effect on DNA Synthesis during Phage Development in Escherichia coli

Abstract. *Chloramphenicol added at various times during intracellular development of various bacteriophages causes a reduction in the rate of synthesis of phage DNA. The reduction is faster the smaller the size of the DNA molecule in the mature phage.*

After inhibition of protein synthesis, for example with chloramphenicol (CAP), DNA synthesis in *Escherichia coli* proceeds until completion of the current cycle of duplication and then stops (*1*). When protein synthesis is resumed, DNA replication begins again, after a delay presumably representing the synthesis of the protein or proteins necessary for initiation. In *E. coli* cells infected with T5 or λ bacteriophage, DNA synthesis is sensitive to inhibition by CAP (*2*). Infection with T-even phages seemed to be an exception because inhibition of DNA synthesis was observed only if the drug was added to the infected complexes during the first period after infection (*3, 4*). After the addition of CAP to *E. coli* cells infected with phage λ, T4, T5, or T7, the rate of DNA synthesis declines exponentially and in a manner inversely proportional to the molecular weight of the DNA of the phage.

The phages were wild-type T4, T5, and T7 and *E. coli* B and its photoreactivation deficient mutant *pht*$^-$ and *E. coli* K12 C600 (λ *c*857). All experiments were performed in M9 medium (0.022*M* KH_2PO_4, 0.042*M* Na_2HPO_4, 0.018*M* NH_4Cl, 8.5×10^{-3}*M* NaCl, 0.001*M* $MgSO_4$, and 0.4 percent glucose) supplemented with 1 percent Difco vitamin-free casamino acids decolorized by filtration through activated charcoal.

For phage infection experiments, bacteria concentrated from growing cultures were mixed with phage. After 2 minutes at 37°C the mixture was diluted into warmed medium to a concentration of 5 to 10×10^7 cells per milliliter. Pulse labeling was performed by transferring 1-ml aliquots of the cells to warmed tubes containing 0.2 μc of [^{14}C]thymidine (New England Nuclear; 30 μc/μmole). After 1 minute 1 ml of cold 10 percent trichloroacetic acid (TCA) and 1 percent thymidine were added. Precipitates were collected on Millipore filters, washed twice with 5 ml of 5 percent TCA, and

Table 1. Direct and indirect measurement of total DNA synthesized in T4 infected cells.

DNA measured by	Time	No CAP	Time of addition of CAP (minutes after infection)				
			6	12	14	16	24
Pulse label*	30 min	100†	55	62	69	71	94
	60 min	254		111	120	131	165
Diphenylamine reaction‡	30 min	100†	52	62	69	69	89
	60 min	164§		83	110	121	149

* Measured by integrating the area determined by the curves of [^{14}C]thymidine label plotted on linear scale. † All values are normalized to this control taken as 100. ‡ See (*6*). § In the absence of CAP, partial lysis of bacteria during centrifugation results in the loss of diphenylamine reacting material. No loss of optical density was observed in CAP treated or untreated cultures in the course of the experiment.