RIBONUCLEASE V OF ESCHERICHIA COLI, I. DEPENDENCE ON RIBOSOMES AND TRANSLOCATION*  

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Communicated by Herman N. Eisen, August 14, 1969  

Abstract.—A new RNase activity, tentatively named RNase V, was found in cell-free extracts of *E. coli*. This activity requires ribosomes, G and T factors, tRNA, K+ or NH4+, Mg2+, GTP, and a sulfhydryl compound to degrade poly U, poly A, T4 phage mRNA, or *E. coli* mRNA. RNase V is specific for mRNA; it does not attack ribosomal RNA. It is inhibited by antibiotics that decrease breakdown of mRNA in *vivo*, such as chloramphenicol and streptomycin, and by such agents as 5'-β, γ-methylene-guanosine triphosphate, and fusidic acid, which inhibit ribosome-dependent GTPase and translocation of ribosomes along mRNA. The evidence suggests that RNase V is either an integral part of the ribosome or is tightly associated with it, and that it selectively degrades mRNA in intact cells.  

The instability of messenger RNA is central to many questions in biology. What determines how long a molecule of mRNA persists, and how does it break down? The mechanisms have remained unknown, though recent work has suggested that at least in some cases, both functional inactivation and degradation of mRNA proceed from the 5' to the 3' end of the molecule.  

A clue to the mechanism and regulation of mRNA breakdown has been obtained from studies of mRNA metabolism in antibiotic-treated cultures of *E. coli*. When protein synthesis is blocked by chloramphenicol or streptomycin, mRNA synthesis continues, but mRNA breakdown is inhibited. This implies that mRNA breakdown is linked to some feature of protein synthesis.  

Therefore, we have looked for an RNase activity linked to the protein synthetic machinery. Here we report the finding of such an activity, tentatively designated RNase V, and describe some of its properties. It is specific for mRNA and requires ribosomes, tRNA, T factor, and G factor. RNase V is inhibited by chloramphenicol and streptomycin, which block mRNA breakdown in *vivo*, and by fusidic acid and 5'-β,γ-methylene-guanosine triphosphate which block translocation. These properties suggest that RNase V is an mRNAse in the cell.  

Materials and Methods.—Strains: The strains used were all derived from *E. coli* K12- N11 (met- his- ura- RNase I) was isolated from strain D10 (met- RNase I). N2022 was one of a number of mutants of N11 selected to have RNase II that is temperature sensitive. N46 was derived from strain 1113 B; it grows in minimal medium containing methionine and tyrosine, and retains the characteristic nucleases of strain 1113B. Strain 1113B lacks RNase I, contains a temperature-sensitive RNase II, and has a lowered level of polynucleotide phosphorylase.  

G and T factors: The isolation of G and T polymerization factors, and the preparation of ribosomes free of G, was greatly facilitated by the use of strain G1, a strain with temperature-sensitive G factor (isolated by Tocchini-Valentini and Mattoccia). Their
modifications\textsuperscript{22, 23} of published techniques\textsuperscript{6-8} have been followed to prepare S-30 cell extracts, S-100, G, and T.

Ribosomes and S-100: Ribosomes of G1 were obtained from S-30 extracts and freed of T by two centrifugal washes in 0.01 M MgSO\textsubscript{4}, 0.01 M Tris-HCl (pH 7.4), followed by one centrifugation in the same buffer containing 15\% sucrose. Their remaining content of G was inactivated by incubation in the Tris-Mg\textsuperscript{2+} buffer at a ribosome concentration of 1 mg/ml (17 A\textsubscript{260} units/ml) for 10 min at 45\°C just before use.

Ribosomes of other strains (N464, for example) were freed of endogenous G and T factors by five washes with buffers containing NH\textsubscript{4}Cl, according to Lucas-Lenard and Lipmann.\textsuperscript{7} This procedure also removed RNase II.\textsuperscript{27} When residual G and T levels were not critical, ribosomes isolated from S-30 by two cycles of differential centrifugation in Tris-Mg\textsuperscript{2+} buffers\textsuperscript{24} could be used. Ribosomes prepared by any of these procedures functioned to an equivalent extent (±15\%) in assays for polyphenylalanine synthesis\textsuperscript{26} or poly U breakdown (RNase V).

S-100 was either used as such or was first heated for 10 min at 50\°C, a treatment sufficient to inactivate RNase II in strains N464\textsuperscript{20} or N2022.\textsuperscript{19}

Radioactive compounds: The substrates used to test for RNase V were either \textsuperscript{3}H-poly U or \textsuperscript{14}C-poly A (Miles Laboratories: 2.74 \mu g/\text{mg} and 0.15 \mu g/\text{mg}, respectively); purified T\textsubscript{4} phage-specific \textsuperscript{3}H mRNA (the gift of Dr. D. E. Kennell of this department); or \textsuperscript{3}H-pulse-labeled E. coli mRNA extracted from polyribosomes.\textsuperscript{26} \textsuperscript{32}P-ribosomal RNA was prepared according to the method of Yankofsky and Spiegelman.\textsuperscript{77} Radioactive samples were counted in a three-channel liquid scintillation spectrometer (Packard, model 3314), with efficiencies of 15, 65, and 95\% for \textsuperscript{3}H, \textsuperscript{14}C, and \textsuperscript{32}P, respectively.

Results.—(a) Detection and requirements of RNase V: An RNase activity dependent on protein synthesis would presumably require the presence of K\textsuperscript{+} or NH\textsubscript{4}\textsuperscript{+} ions in incubation media, since K\textsuperscript{+} is required for protein synthesis.\textsuperscript{28, 29} The activity could thus easily be confused with, or masked by, the K\textsuperscript{+}-dependent RNase II of E. coli.\textsuperscript{16} We therefore undertook the search for a new RNase in strains selected to contain temperature-sensitive RNase II. In extracts of such strains (N464, N2022; see Materials and Methods) heated to inactivate RNase II, the new activity, RNase V, could be demonstrated. RNase V activity (measured by formation of alcohol-soluble radioactive material from \textsuperscript{3}H-polyU) was assayed in presence of ribosomes, S-100, tRNA, K\textsuperscript{+} or NH\textsubscript{4}+, Mg\textsuperscript{2+}, glutathione, and GTP. (All the experiments presented here, unless otherwise specified, were carried out with fractions from strain N464.) Figure 1 shows the dependence of the reaction on each of the reactants, and illustrates some of the specificities encountered: Na\textsuperscript{+} did not substitute for K\textsuperscript{+} or NH\textsubscript{4}+, nor could GTP be replaced by a non-hydrolysable analog, 5'-\textbeta,\textgamma-methylene-guanosine triphosphate,\textsuperscript{14} or by ATP. In absence of glutathione, \textsuperscript{3}H-poly U breakdown decreased from 30 to 4–8\% in various experiments.

(b) G and T factors are the required components of S-100: Column chromatography on DEAE-Sephadex A50 permitted the demonstration that factors G and T, required for the elongation of polypeptide chains,\textsuperscript{6-8} are also necessary to support RNase V activity. In Figure 2, the positions of G and T factors, assayed by previously described techniques,\textsuperscript{22, 23} are indicated on the column elution profile. If instead of S-100, T factor (from its column peak) was added to reaction mixtures, no activity was observed unless a sample from a column fraction containing G factor was also included (this is shown in Fig. 2 for strain N464); a corresponding requirement for T in the presence of G was also observed.
Fig. 1.—Requirements for poly U degradation by RNase V. Each panel shows the response to one additive in presence of all the others. The complete assay mixture contained: GTP, $5 \times 10^{-4}$ M; GSH, $10^{-4}$ M; Mg$^{2+}$, $2 \times 10^{-3}$ M; NH$_4$Cl, $5 \times 10^{-2}$ M; Tris-HCl, $4 \times 10^{-2}$ M, (pH 7.6); as well as *E. coli* tRNA, 100 µg; $^3$H-poly U, 40 mµc (14.6 µg); ribosomes, 2 mg; and S-100 200 µl; per ml. Assays were carried out at 36°C for 30 min, an interval during which the reaction is essentially linear. Samples of 50 µl were processed and counted according to Spahr; alternatively, the $^3$H-poly U that remained alcohol-insoluble after incubation was collected and counted on filters.

The complete mixture rendered 30 to 40% of the added $^3$H-poly U alcohol-soluble in various experiments, with a precision in duplicate samples of ±3%. Background samples (time zero) showed about 1 to 4% soluble cpm, which has not been subtracted.

(a) tRNA, µg/ml; (b) monovalent cation, M; (c) At levels of GTP indicated, inhibition by added 5'-β, γ-methylene-GTP (GMP-PCP); (d) Mg$^{2+}$, M; (e) Ribosomes, µg; (f) S-100, µl (S-100 contains 15 mg protein/ml).

(this is shown in Fig. 2 for strain G1). When column fractions were tested individually for their capacity to support RNase V activity, a peak was found which includes fractions containing both T and G activity (compare Fig. 2, top panel, with Fig. 1 in ref. 6).

The fractions containing G and T were free of detectable amounts of several known nucleases, including RNase II.
(c) **RNase V requires translocation of mRNA on ribosomes:** Five lines of evidence strongly support the notion that translocation of mRNA on ribosomes is a precondition for RNase V activity: 1) G factor, which catalyzes the ribosome-dependent GTPase (thought to provide the energy for ribosome movement), and T factor, which catalyzes specific tRNA binding to ribosomes, are both required (Fig. 2). 2) GTP, tRNA, K⁺, sulfhydryl compounds—requirements for translocation—are all required for RNase V function. 3) Fusidic acid, a specific inhibitor of G-factor GTPase and translocation, inhibits RNase V (Table 1). 4) Heated ribosomes from a strain with a temperature-sensitive G factor (see Materials and Methods) fail to show RNase V activity when supplemented with heated S-100 of the same strain, but were fully competent in RNase V activity when supplemented with heated wild-type S-100 (Table 2). 5) RNase V activity was competitively inhibited by 5′-β,γ-methylene-guanosine triphosphate (Table 1 and Fig. 1). This GTP analog binds to ribosomes in the absence of any added column fractions showed no RNase V activity. **Top panel:** Fractionation of S-150 (ref. 22) from N464. G-factor activity, ---; RNase V assayed with 0.01 ml of each column fraction (no additions from tubes 50 (T) or 70 (G)), ---; RNase V activity in presence of relative excess of T-factor from tube 50, ---. **Bottom panel:** fractionation of S-150 from strain G1, T-factor, ---; RNase V in presence of a relative excess of G-factor from tube 70 of the N464 fractionation, ---; G-factor, ---; RNase V in presence of a relative excess of T-factor, ---.

**Fig. 2.—Requirements for G and T amino acid polymerization factors for RNase V activity.** Elution profile of a DEAE-Sephadex A50 fractionation, with a linear gradient of 0.15 to 0.35 M KCl of soluble protein from strains N464 (top) and G1 (bottom). G factor was located by its capacity to supplement an inactive extract of strain G1 in polyphenylalanine synthesis. T was located by its capacity to catalyze polyphenylalanine synthesis from charged tRNA in presence of washed ribosomes and 0.01-ml aliquots of the peak tube (70) of G (strain N464). (0.01 ml volumes of each tube were tested.) The peak tubes (50 and 70) contained an amount of T or G equivalent to about 50 to 80% of that in an equal volume of S-100. 0.01-ml volumes from each tube were also tested for RNase V activity in the absence of any added S-100. Each factor was then tested for its capacity to support RNase V activity in the presence of the other factor (under conditions similar to those in Fig. 1). In place of S-100, 0.01 ml of one factor (a relative excess) from column tube 50 (factor T) or tube 70 (factor G) from strain N464, were added with 0.01 ml of each fraction to be tested. Washed
TABLE 1. Inhibition of RNase V by blockage of ribosome function.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Poly U</th>
<th>% Inhibition</th>
<th>T4 mRNA</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td>None</td>
<td>1410</td>
<td>...</td>
<td>2010</td>
<td>...</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>510</td>
<td>43</td>
<td>1190</td>
<td>41</td>
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<tr>
<td>Fusidic acid</td>
<td>680</td>
<td>52</td>
<td>460</td>
<td>77</td>
</tr>
<tr>
<td>5'-G,γ-methylene GTP</td>
<td>240</td>
<td>83</td>
<td>300</td>
<td>85</td>
</tr>
<tr>
<td>Rifampycin</td>
<td>1405</td>
<td>&lt;1</td>
<td>1980</td>
<td>1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>480</td>
<td>66</td>
<td>300</td>
<td>85</td>
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</table>

RNase V was assayed as in the Legend to Fig. 1, except with 4700 counts/10 min of 3H-poly U or 6440 counts/10 min of T4 phage-specific mRNA as substrate; incubations were for 30 min at 36°C. Comparable inhibitions were observed in points taken at 2, 5, 10, 15, 20, and 30 min; dose response curves for the various inhibitors will be published elsewhere. The antibiotics were used at final concentrations of 200 μg chloramphenicol, 250 μg fusidic acid, 100 μg rifampycin, and 100 μg streptomycin/ml; and 5 × 10⁻⁴ M 5'-methylene GTP. No corrections were made for zero-time values, or values in controls without added ribosomes (1-4%).


<table>
<thead>
<tr>
<th>S-100</th>
<th>Ribosomes</th>
<th>% RNA solubilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>N464</td>
<td>N464</td>
<td>35</td>
</tr>
<tr>
<td>G1</td>
<td>G1</td>
<td>2</td>
</tr>
<tr>
<td>N464</td>
<td>G1</td>
<td>29</td>
</tr>
<tr>
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<tr>
<td>G1</td>
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<td>1</td>
</tr>
<tr>
<td>...</td>
<td>G1</td>
<td>3</td>
</tr>
</tbody>
</table>

Assay for RNase V as in Legend to Fig. 1, except with washed ribosomes and S-100 from strains N464 or G1, as indicated. S-100 and ribosomes were heated 10 min at 50°C. This completely inactivated RNase II in N464 fractions; 5% breakdown of poly U due to residual RNase II activity in G1 fractions has been subtracted.

ribosomes but cannot be hydrolyzed. Thus, the hydrolysis of GTP is necessary for RNase V function.

(d) **RNase V is specific for mRNA**: RNase V can use as substrates 3H-poly U, 14C-poly A, purified T4 phage-specific 3H-mRNA, and 3H pulse-labeled E. coli mRNA (Table 3). In contrast, stable RNA (23P-ribosomal RNA) was not attacked at all (Table 3). Thus, RNase V appears to be specific for mRNA.

(e) **Inhibitors of mRNA breakdown in vivo inhibit RNase V in vitro**: The antibiotics chloramphenicol and streptomycin, which had been shown to prevent mRNA breakdown in whole cells, inhibit RNase V activity at levels inhibitory for in vitro protein synthesis (Table 1). As a control, rifampycin, which only affects synthesis of RNA does not inhibit degradation by RNase V (Table 1). The inhibitors used here do not affect RNase II (results to be published).

**Discussion.**—mRNase, as presently visualized, degrades mRNA in the 5' to 3' direction. However, RNase II and polynucleotide phosphorylase, two well-characterized ribonucleases, both degrade RNA in the opposite direction (3' to 5'). Therefore, mRNA is broken down in growing cells either by these enzymes in some complex scheme, or by a different enzyme(s). RNase V is an attractive candidate for such an activity, since its properties are those expected of mRNase.
The uniqueness of RNase V will be further detailed in subsequent reports but its specificity and requirements already make it distinct from other reported nucleases. Here we have emphasized its tight association with ribosomes and its dependence upon all factors, including T and G, associated with ribosome movement along mRNA; these components are not required by either RNase II or polynucleotide phosphorylase. Also, the antibiotics that inhibit mRNA breakdown in vivo inhibit RNase V—but not, for example, RNase II.

G and T are probably the only S-100 proteins required for RNase V function, and it is therefore likely that RNase V activity is either tightly associated with the ribosome, or is an integral part of it. (The peptidyl transferase activity is one that has been assigned to the ribosome itself.) A number of alternatives are not excluded, but seem improbable, e.g., that a soluble RNase cochromatographs with G and T (Fig. 2), or that G or T is itself RNase V.

Since ribosome movement proceeds in the 5' to 3' direction along mRNA, it is probable that RNase V also acts 5' to 3', as expected for an mRNase in the cell. In forthcoming publications we present evidence that RNase V requires both 30S and 50S ribosomal subunits, and that it is an exonuclease that degrades mRNA to 5'-mononucleotides in the 5' to 3' direction.

We propose as a working model that each time a ribosome adds to a 5'-sequence of mRNA to begin translation, there is a certain potential that mRNase associated with it will begin to degrade the mRNA chain. Such an event should not occur every time a ribosome adds to mRNA, since in the cell each chain of mRNA tends to function many times in a polyribosome. Supporting this notion that mRNase and ribosome movement can be uncoupled is the discovery that in whole cells chloramphenicol inhibits mRNA breakdown without significantly affecting translocation. Consistent are the findings in extracts that chloramphenicol inhibits RNase V (Table 1) without affecting ribosome-dependent G-factor GTPase.

This proposed mechanism for mRNase action would have evident advantages for the cell. It would ensure that unless ribosomes are actually moving along mRNA and actively engaged in protein synthesis, mRNA would tend to be preserved.

The overall features of such a model are consistent with data about mRNA breakdown in whole cells. Random jeopardy at the beginning of mRNA would generate the exponential decay kinetics often observed for mRNA, while in some cases a fixed number of ribosomes might function on each molecule of mRNA before degradation begins, which results in linear decay of that mRNA species.
It is not clear what determines whether a ribosome beginning to translate a molecule of mRNA will also begin to degrade it. Four factors may be involved to varying extents: (1) A critical protein might be present only in some ribosomes. (2) Some small molecule may be necessary and may be limiting the initiation of the reaction. (3) Some specific feature of mRNA, such as the 5’-terminus, might require critical modification to expose it to RNase V action. (4) The ribosome might have to be in a rare configuration to permit initiation of degradation. The inhibition of RNase V by antibiotics that bind to the ribosome and inhibit protein synthesis, but permit translocation, suggests that the configuration of the ribosome can be critical.

* Supported by NIH grants GM-10447 and HD-01956, Training Grant AI-257, and American Cancer Society grant P-477. D. S. holds Research Career Development Award GM-11710. We are grateful to Drs. C. Gurgo, L. Gorelic, and D. E. Kennell for preparations of labeled RNA, and to Ritz Mazzola and Dan Longo for valuable assistance in some of the experiments.

8 Conway, T. W., and F. Lipmann, these *Proceedings*, 52, 1462 (1964).
19 Isolated in the laboratory of W. Gilbert.
24 Tissieres, A., D. Schlessinger, and F. Gros, these Proceedings, 46, 1450 (1968).
29 Conway, T. W., these Proceedings, 51, 1216 (1964).
32 Gordon, G., these Proceedings, 59, 179 (1968).
33 Egel, R., N. Brot, B. Redfield, J. E. Allender, and H. Weissbach, these Proceedings, 59, 861 (1968).
37 Morse, D. E., and C. Yanofsky, personal communication.


Singer, M. F., and G. Tolbert, Biochemistry, 4, 1319 (1965).


Spahr, P. F., and R. F. Gesteland, these PROCEEDINGS, 59, 876 (1967).


Levinthal, C., A. Keynan, and A. Higa, these PROCEEDINGS, 48, 1631 (1962).