Vol. 103, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1981 Pages 543-551

RIBONUCLEASE F, A PUTATIVE PROCESSING ENDORIBONUCLEASE FROM ESCHERICHIA COLI

Ned Watson and David Apirion

Department of Microbiology and Immunology
Washington University School of Medicine
Box 8093, St. Louis, Missouri 63110

Received October 9,1981

Summary: A new endoribonuclease activity, RNase F, was partially purified from Escherichia coli cells. This activity can cleave a precursor RNA molecule (of Species 1), isolated from T4 infected cells, in a specific site. This activity is different from the other three known processing endoribonucleases of E. coli RNase III, RNase E and RNase P.

In all organisms maturation of many RNA molecules results from a complex set of reactions, whereby, precursor RNAs are being processed by a series of specific ribonucleases (1-3). Three endoribonucleases RNase III, E and P have been shown to participate in the processing of rRNA and tRNA transcripts of E. coli. A fourth processing endoribonuclease, which cleaves near the 3' end of tRNAs, has been implicated to participate in the maturation of tRNA (1-3). Therefore, we set out to isolate and characterize such an activity. We report here the isolation and partial characterization of an activity from E. coli which can introduce a specific cleavage near the 3' end of a tRNA-like structure contained in a precursor RNA derived from the tRNA coding region of bacteriophage T4.

MATERIALS AND METHODS: Strains. E. coli strain N2069 (4) is an RNase III$^-$ (rnc) derivative of strain N7060 (5). Strains N3431 RNase E$^-$ (rne) and N3433 (rne$^+$) were described previously (6). Phage T4Δ27 (7, 8) was a generous gift from Dr. W. McClain.

Growth of Cells and Phage. See Refs. 9 and 10 respectively.

^{32}P-Labeled RNA Substrates. These were prepared as described in Ref. 10.

Assay for RNAase F Activity. The assay mixture contained (per 20 μl) 1.0 μg phenol extracted yeast RNA, 2 mM $MnCl_2$, 170 mM NH_4Cl, 0.1 mM Na_2EDTA, pH 7.0, 25 mM Tris/maleate buffer, pH 7.0, 1,000 to 3,000 cpm [^{32}P]substrate (10.1S or p2Sp1) and enzyme. Any modifications of the assay are noted in the figure legends or the text.

0006-291X/81/220543-09$01.00/0

Protein Determination. See Refs. 11 and 12.

RNase E and RNAse P Assays. See Refs. 13 and 14 respectively.

Quantitation of Gels. See Ref. 15.

Enzyme Purification. Preparation of S30, S200, ammonium sulfate fractionation, DEAE-sephadex, DEAE-cellulose and gel filtration (Sephadex G-150) were all done according to published procedures (9,13).

Affinity Chromatography. A 25.8 x 0.7 cm Affi-Gel Blue was packed by gravity at 6 ml/hr and washed overnight at the same flow rate with 10 volumes of Buffer C [0.02 M Tris/HCl, pH 8.0; 0.1 mM dithioeritritol; 10% glycerol (v/v); 0.01 M $MgCl_2$.] The equilibrated column was loaded with 15 ml of the concentrated, dialyzed G-150 material (55 mg protein) and eluted with 15 ml Buffer C at a flow rate of 6 ml/hr. When all the unbound protein came through the column it was eluted with a 0 to 1.0 M NH_4Cl gradient in the same buffer at the same flow rate. 2 ml fractions were collected and from these 0.1 ml samples were taken, dialyzed overnight in Buffer C and assayed for RNase F activity. The fractions showing RNase F activity were combined, concentrated by dialysis against Buffer C containing 15% polyethylene-glycol 6000, and dialyzed versus Buffer C overnight. This material (1.5 ml at 0.44 mg protein/ml) was frozen at -70°C and is referred to as RNase F.

RESULTS: Partial Purification of RNAase F. When an E. coli rne mutant is infected with T4Δ27, a deletion strain missing seven of the ten genes in the T4 tRNA cluster (7,8), two novel RNAs are accumulated: 10.1S and p2Spl (Fig. 1). 10.1S RNA contains $tRNA^{Gln}$ and $tRNA^{Leu}$, the first two tRNAs in the cluster, as well as Spl RNA, the last RNA in the cluster. The 3' end of 10.1S RNA is produced by termination and extends about 75 nucleotides beyond the 3' end of Spl. p2Spl contains Spl and the 3' end tail (10,16). The first of these RNAs, 10.1S, was used as a substrate for the purification of an endonuclease and later, 10.1S and p2Spl were used to characterize the isolated enzymatic activity, which we call RNase F. The availability of these RNAs which accumulate only in an rne mutant and contain nucleotides distal to a tRNA-like structure enabled for the first time a systematic search for an enzyme which can cleave at the 3'side of RNAs.

In early trials we found that E. coli extracts devoid of RNase III, RNase E, and RNase P could cleave rather efficiently and specifically 10.1S RNA to discrete fragments. We opened a large amount of cells [1.5 kg from strain N2069 (rnc)] spun out the cell debris, and prepared S200 supernatant. RNase F activity was precipitated at 40% saturation with ammonium sulfate. It did not

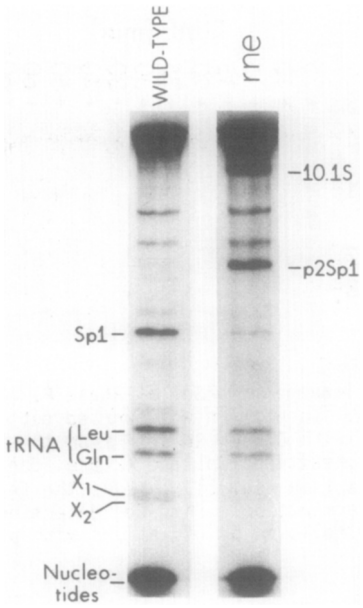

Fig. 1. Appearance of 10.1S and p2Sp1 in vivo. Cultures of a wild type strain (N3433, rne$^+$) and an rne mutant (N3431) were grown in TB medium (15) containing 0.2% glucose and 0.6% peptone at 30°C with shaking to an A_{560} of about 0.3 (~3 x 10^8 cells/ml), shifted to 43°C for 30 min, and infected with T4Δ27 and labeled with $^{32}P_i$. The wild type strain was labeled for 15 min and the rne mutant for 30 min. Labeling was stopped, cells lysed, and samples were applied to a 5%/10% tandem polyacrylamide gel containing 7 M urea. The gel was soaked in a 25% methanol, 5% glycerol solution to remove the urea, dried, and autoradiography performed. The picture shows only the 10% portion of the gel; this practice was also followed in Figs. 2 and 3.

bind efficiently to either CM-cellulose or DEAE-cellulose. Material which was enriched for RNase F activity by using these two resins was concentrated, dialysed, centrifuged, and the supernatant was applied to a Sephadex G-150 column. The elution profile showed a broad protein peak. RNase F activity was detected primarily in the descending portion of the peak.

The active fractions from the descending portion of the peak (about 20% of the protein applied to the G-150 column) were concentrated, dialyzed, and applied to an Affi-Gel Blue affinity chromotography column. Most of the protein, about 65%, did not bind to the column and had only slight RNase F activity. The column was eluted with a linear NH_4Cl gradient (0 to 1 M). Two major protein peaks appeared between 0.02 and 0.2 M NH_4Cl and they did not contain appreciable RNase F activity. Several minor protein peaks

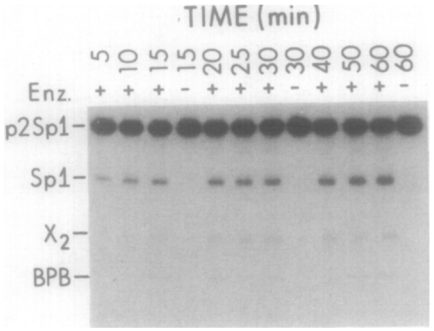

Fig. 2. Kinetics of cleavage of p2Sp1 by RNase F. The experiment was carried out with p2Sp1 RNA. Incubation was carried out at 37°C for the indicated lengths of times with 0.5 μg RNase F from the last purification step. The products of the reactions Sp1 and X2 are identical to RNAs which appear in T4 infected cells (see Fig. 1). [X2 is the fragment from the 3' end of Sp1 till the point of termination. This was determined by comigration in gels and T1 fingerprinting followed by digestions with pancreatic and T2 RNase and P1 nuclease (23).]

appeared later. One of these (between 0.30 to 0.34 M NH_4Cl) had significant RNase F activity and contained less non-specific nuclease activity against 10.1S RNA than the starting material. This fraction (~3% of the recovered protein) was used to further chracterize RNase F activity. This activity cleaved 10.1S RNA to specific products and it also cleaved p2Sp1 to two specific fragments (see below). Because of the simplicity of the reaction with p2Sp1 (Fig. 2) we continued the characterization of RNase F using this RNA. During all the purification, no separation of the activities against 10.1S and p2Sp1 was observed.

Since the cell extracts, as well as the various fractions isolated, contain various nucleases which degrade the substrate p2Sp1 as well as the products, Sp1 and X2 (see Fig. 2), it is not very practical to calculate the level of enrichment achieved in each step.

Properties of RNAase F: Cations. Tests for monovalent cations were carried out in the presence of 2 mM $MnCl_2$ in Tris/maleate buffer, pH 7.0. The monovalent cations Na^+, K^+, and NH_4^+ (only these were tested) were found to stimulate the cleavage of p2Sp1 with the optimum concentration being between 150 to 200 mM. Tests for divalent cations were carried out in the

presence of 170 mM NH_4Cl in Tris/maleate buffer, pH 7.0. The divalent cations Mg^{2+} and Mn^{2+} were unnecessary for the cleavage of p2Spl. We chose to use 2 mM Mn^{2+} in subsequent assays because slightly less degradation products accumulated when this cation was used. (For an example of an RNase F reaction, see Fig. 2.)

pH Optimum. The pH range of the activity was tested using a variety of buffers in a pH range from 5 to 9. The enzyme was active throughout this range, maximum activity was found between pH 6.8 and 7.6.

Activity at Different Temperatures. The enzyme is active up to 60°C. Above this temperature the substrate begins to breakdown spontaneously and it is impossible to assess enzymatic activity.

The Activity Contains a Protein Moiety. Activity was completely lost after 20 min digestion with proteinase K. Proteinase K alone did not digest the p2Spl substrate and when proteinase K was added simultaneously with RNase F the reaction was only slightly inhibited. The last experiment indicated that proteinase K does not interfere with the RNAase F reaction in some unexpected fashion.

Uniqueness of RNAase F. Since we isolated RNase F from an RNase III$^-$ mutant we knew that the purified activity was not RNase III. In order to demonstrate that the RNase F activity was different from the other known E. coli RNA processing enzymes, RNase P and RNase E, we tested RNase F against substrates for RNase E (9S RNA) and RNase P (K band RNA; it contains $tRNA^{Gln}$ and $tRNA^{Leu}$). As can be seen in Fig. 3, purified RNase E and RNase P can cleave their respective substrates, while RNase F, which cleaves 10.1S RNA, and p2Spl (see Fig. 2) cannot act on 9S RNA or the K band. The substrate specificity and its other characteristics clearly distinguish RNase F from all the other endonucleolytic RNases known in E. coli.

Kinetics. To further investigate the mode of production of Spl and X2 by RNase F we carried out the reaction for various lengths of time. Some of the results are shown in Figs. 2 and 4. The reaction appeared to proceed most

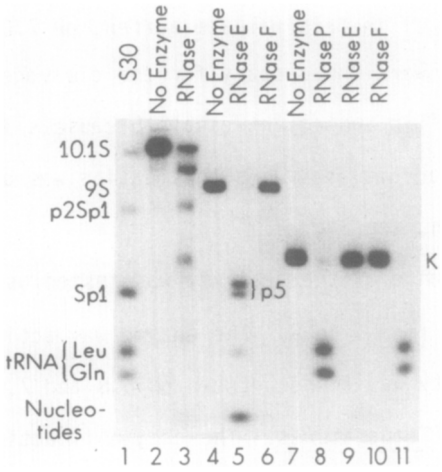

Fig. 3. Comparison of RNase F, RNase E, and RNase P using 10.1S, 9S, and K band RNAs as substrates. Lane 1: 10.1S RNA incubated with S30 (N2069) for 60 min at 37°C using RNase E assay conditions to provide markers. Lanes 2 and 3: 10.1S RNA incubated for 60 min at 37°C using RNase F conditions. Lanes 4-6: 9S RNA incubated for 20 min at 30°C using RNase E assay conditions. Lanes 7-11: K band incubated for 60 min at 37°C using RNase P assay conditions. Amount of protein per assay: 0.2 μg RNase P; 1 μg RNase E; 2 μg RNase F; 5 μg S30. Lane 11 contained S30.

rapidly in the first 5 min. From 5 min to 60 min the rate of the reaction is decreasing and eventually it comes to a halt.

DISCUSSION: The experiments presented here describe an endonucleolytic RNase activity, RNase F, which can introduce a specific cleavage in a precursor RNA at the 3' end of a stable tRNA-like molecule (17) contained in the precursor.

Several factors hindered the purification of the enzyme. For example, the existance of other nucleases in the extract that degrade the substrates 10.1S and p2Sp1 RNA made it difficult to evaluate early fractionation steps. Also, the enzyme appeared either to have affinity for other proteins or to aggregate, since during gel filtration and affinity chromotography we found RNase F-like activity in some, but not all, fractions other than the RNase F peaks. Another possibility is that the cell contains more than a single RNase F-like activity.

The RNase F activity seems to be unique. Its ionic requirements are very different from those reported for other known RNA processing enzymes. Moreover, RNase E or RNase P cannot cleave the RNase F substrates p2Sp1 or

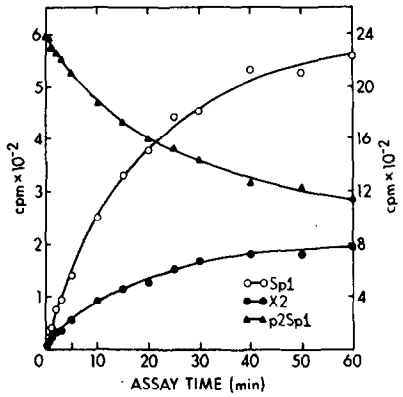

Fig. 4. Quantitative analysis of an RNAse F reaction. An experiment similar to the one shown in Fig. 2 was quantitated. The left scale is for Sp1 and X2 and the right scale is for p2Sp1. Notice that while p2Sp1 disappears Sp1 and X2 appear. About 40% of Sp1 is degraded rather than matured. The relative degradation of X2 is larger as compared to Sp1. The size ratio of Sp1 and X2 is about 2 to 1 (140 to 74 nucleotides).

10.1S RNAs, while the preparation of RNase F cannot cleave 9S or K band RNAs, which are unique substrates for RNase E and RNase P, respectively. RNase F is also different from RNase III; RNase III, for instance, requires a divalent cation (18). Moreover, RNase III is completely missing from strains carrying the rnc-105 mutation (19).

Other workers found indications for an endonucleolytic activity from E. coli which can cleave at the 3' side of a tRNA contained in a precursor molecule from E. coli (20). This activity, however, was not characterized and therefore it cannot be compared with RNase F. Another activity which could be similar to RNAse F is the one reported by Goldfarb and Daniel (21). However, there is not suffieicnt information to compare these two activities.

It is interesting to note that T4 10.1S RNA, which contains a tRNA dimer at its 5' end as well as Sp1 and X2 sequences, is cleaved into 5 products by RNase F, one of which is about the size of the K band (K band contains the tRNA dimer $tRNA^{Gln-Leu}$), while two others are the size of Sp1 and X2. The other two products correspond in size to the other two expected molecules, one being a trimer containing $tRNA^{Gln}$, $tRNA^{Leu}$ and Sp1 RNA, and the other corresponds to p2Sp1 RNA. This is what would be expected if RNase F makes a

cleavage at a site to the 3' end of the dimer, as well as between Spl and X2 RNA. If this is the case, then the 5 products would be the result of non-sequential cleavage of one, the other, or both RNase F cleavage sites. Detailed analysis of the RNase F cleavage products of 10.1S RNA should determine if this is really the case. (In Fig. 3, lane 3, one can see the three larger products, while Spl and X2 are not seen. They can be observed after longer incubation times. Some degradation, especially of X2, does take place, see also Figs. 2 and 4.)

We would like to suggest that RNase F could be the RNA processing enzyme which introduces endonucleolytic cleavages in RNA precursors at or near the 3' ends of tRNA molecules. Such an activity(ies) was implied by various workers studying RNA processing in E. coli and T4 (20,22). None of the known three endonucleolytic RNA processing enzymes, RNase III, RNase E or RNase P posseses such an activity.

While Spl is not a tRNA molecule, it is clear that it resembles sufficiently a tRNA molecule to be a substrate for a variety of enzymes which recognize tRNA molecules (17). Therefore, an enzyme like RNase F, which can introduce a specific cleavage near the 3' end of Spl RNA, might be able to introduce similar cleavages in tRNA precursors.

Further purification of RNase F, determination of the precise positions of cleavages in 10.1S and p2Spl, investigation of possible cleavages of other tRNA precursors, and isolation of mutants defective in this enzyme should help to determine whether or not RNase F plays a role in the cell in the processing of RNA moleucles.

Acknowledgments: We are most grateful to Dr. Sidney Altman for a generous gift of RNAse P, and to Dr. Monoj Roy for a gift of RNase E. Our colleagues Drs. P. Gegenheimer, B.K. Ray and particularly, B. Pragai were a continuous source of advise and inspiration. We also wish to thank Stephanie Hedstrom for technical assistance. This investigation was supported by National Institute of Health Grants GM 25890 and GM19821 and National Cancer Institute Grant CA24727.

References

1. Abelson, J. (1979) Ann. Rev. Biochem. 48, 1035-1069.
2. Altman, S. (1981) Cell 23, 3-4.

3. Apirion, D., and Gegenheimer, P. (1981) FEBS Lett. 125, 1-9.
4. Apirion, D., and Watson, N. (1975) J. Bacteriol. 124, 317-324.
5. Weatherford, S.C., Rosen, L., Gorelic, L., and Apirion, D. (1972) J. Biol. Chem. 247, 5404-5408.
6. Goldblum, K., and Apirion, D. (1981) J. Bacteriol. 146, 128-132.
7. Wilson, J.H., and Abelson, J.N. (1972) J. Mol. Biol. 69, 57-73.
8. Wilson, J.H., Kim, J.S., and Abelson, J.N. (1972) J. Mol. Biol. 71, 547-556.
9. Misra, T.K., Rhee, S., and Apirion, D. (1976) J. Biol. Chem. 251, 7669-7674.
10. Pragai, B., and Apirion, D. (1982) J. Mol. Biol., in press.
11. Ehresmann, B., Imbault, P., and Weil, J.H. (1973) Anal. Biochem. 54, 454-463.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Misra, T.K., and Apirion, D. (1979) J. Biol. Chem. 254, 11154-11159.
14. Lund, E., and Dahlberg, J.E. (1977) Cell 11, 247-262.
15. Gegenheimer, P., Watson, N., and Apirion, D. (1977) J. Biol. Chem. 252, 3064-3073.
16. Pragai, B., and Apirion, D. Manuscript in preparation.
17. Paddock, G.V., and Abelson, J. (1975) J. Biol. Chem. 250, 4185-4206.
18. Robertson, H.D., Webster, R.E., and Zinder, N.D. (1968) J. Biol. Chem. 243, 82-91.
19. Gegenheimer, P., and Apirion, D. (1980) Nucleic Acids Res. 8, 1873-1891.
20. Sekiya, T., Contreras, R., Takeya, T., and Khorana, H.G. (1979) J. Biol. Chem. 254, 5802-5816.
21. Goldfarb, A., and Daniel, V. (1980) Nucleic Acids Res. 8, 4501-4516.
22. Fukada, K., and Abelson, J. (1980) J. Mol. Biol. 139, 377-391.
23. Gurevitz, M., Watson, N., and Apirion, D. Manuscript in preparation.