DNA-INHIBITED RNase OF E. COLI

James Eley and Karen Crawford

Department of Biology, Rensselaer Polytechnic Institute
Troy, New York

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SUMMARY: This communication presents evidence which indicates that Escherichia coli contains an RNase which is strongly inhibited by DNA. The enzyme is co-purified with polynucleotide phosphorylase and what appears to be DNA-dependent RNA polymerase. The only product of an exhaustive digest of poly C is 5'-CMP.

To date five different RNases have been found in E. coli. RNase I is an endonuclease which forms nucleoside-3'-monophosphates (1). RNase II, an exonuclease, produces nucleoside-5'-monophosphates and short chain oligonucleotides which are resistant to further attack (2,3). RNase III appears to be an endonuclease which forms 3'-phosphate terminated products and degrades only double-stranded RNA (4). RNase IV, an endonuclease, slowly hydrolyzes high molecular weight RNA to acid-insoluble polynucleotides. It does not hydrolyze poly C under conditions where R17 RNA is hydrolyzed (5). RNase V is an exonuclease which produces nucleoside-5'-monophosphates. It is dependent on many of the factors necessary for the translation process, i.e., GTP, transfer RNA, and the G and T factors, and may be an enzyme specific for the degradation of messenger RNA (6,7).

We report here findings which indicate a sixth RNase may be present in E. coli. The enzyme rapidly hydrolyzes poly C to 5'-CMP and this hydrolysis is strongly inhibited by DNA. This enzyme appears to be different from either RNase II or RNase V, the only other RNases which form nucleoside-5'-monophosphates. RNase II (8,2) is not inhibited by DNA and RNase V (6) requires ribosomes, GTP, and transfer RNA which are not required by the nuclease described here.

Materials and Methods

Assays. Ribonuclease and polynucleotide phosphorylase assays were carried out at 37°C by the filter paper disk technique as described by Bollum (9). Each assay (0.25 ml) contained 10 μmoles Tris-HCl (pH 7.8), 1 μmole $MgCl_2$, and other additions as noted in the Results. All ^{14}C-labeled and unlabeled nucleotides were purchased from Schwarz-Mann. The ^{14}C-CDP used for polynucleotide phosphorylase assays had a sp. act. of 1.0×10^5 cpm/μmole. For all assays 0.2 ml aliquots were pipetted onto 1-inch-square sections of Whatman 3 MM paper and processed as described by Bollum (9). Radioactivity was determined by liquid scintillation using a toluene-Omnifluor scintillator. For time studies the basic assays (0.25 ml) were scaled up six-fold (1.5 ml).

Enzyme Preparation. E. coli Hfr_1, obtained from the laboratory of R. M. Franklin, were grown to early log phase in nutrient broth (10), harvested, and stored at -30°C. Cells were extracted by treatment with lysozyme, freeze-thawing, and sonication. The extract was fractionated on a Bio-Gel A-50 column and the material in the first major absorbing peak (280 mμ) was concentrated with $(NH_4)_2SO_4$ (80% saturation) and then dissolved in buffer (20 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$). This solution was centrifuged at 96,000g for 4 hours. Material in the supernate, after precipitation with $(NH_4)_2SO_4$ (55% saturation), was dissolved in buffer A (20 mM Tris-HCl, 1 mM EDTA, pH 7.8). The absorbance (280 mμ) of this solution should not be greater than 1.5 to minimize self-elution in the following step. The protein was next fractionated on DEAE cellulose using a procedure similar to that of Chamberlain and Berg (11). The column was eluted stepwise, first with buffer A and then with buffer A which was 0.16 M in KCl, 0.23 M in KCl, and finally 0.4 M in KCl. The protein in the 0.23 M and 0.4 M fractions was precipitated with $(NH_4)_2SO_4$ (80% saturation) and dissolved in a minimum volume of buffer (20 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.8). Protein concentrations were usually 8-10 mg/ml. The 0.23 M fraction contained polynucleotide phosphorylase, DNA-inhibited RNase and occasionally some DNA-dependent RNA polymerase. The 0.4 M fraction contained all three enzymes. The

enzymes are stable for as long as 6-8 weeks at 4°C. All assays were done with 0.23 M fractions. Details of the purification will be published at a later date.

Preparation of DNAs. Bacteriophage T4, grown on E. coli B, were purified by differential centrifugation and DNA extracted by the phenol procedure (12). T4 DNA (540 μg/ml) in buffer (100 mM Tris-HCl, 1 mM EDTA, pH 7.8) was heat-denatured by incubation in boiling water for 10 minutes followed by rapid cooling in ice-water. Salmon testes DNA (Worthington) in sterile water (1 mg/ml) was freshly prepared every two weeks. Heat-denaturation of the DNA (1 mg/ml of water) was carried out as described for T4 DNA. Salmon testes DNA was also denatured by incubation in 0.3 M KOH (5 mg DNA/ml) at 37°C for 16-21 hours. It was then neutralized with conc. HCl, precipitated with 2 vols. of 95% ethanol, lyophylized, and dissolved in water (1 mg/ml). This DNA is indicated as KOH-DNA throughout this communication. Salmon testes DNA was treated with KOH to insure that all RNA was removed. KOH-DNA gave results that were identical to those obtained with heat-denatured salmon testes DNA.

Preparation of ^{14}C-Labeled Poly C. Poly C was prepared from ^{14}C-CDP using polynucleotide phosphorylase eluted from DEAE cellulose at 0.4 M KCl. After phenol extraction to remove protein the poly C was thoroughly dialyzed first against buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.8) containing 2 mM pyrophosphate and finally against buffer without pyrophosphate. Poly C prepared in this manner had an average chain length in excess of 150 nucleotides per 3'-terminus.

Other Procedures. Protein was determined by the method of Lowry et al. (13) and diphenylamine reagent was used to determine DNA (14). Descending chromatography was used and appropriate standards were run with each chromatogram.

Results and Discussion

We were led to this work by the observation that polymerization of CDP by polynucleotide phosphorylase was dependent upon the presence of DNA (Fig. 1). Results similar to those shown in Fig. 1 were also obtained for the polymerization of ADP and UDP. DNase appears to be present as a contaminant in the enzyme solution since the stimulatory effect of low quantities of DNA is transitory.

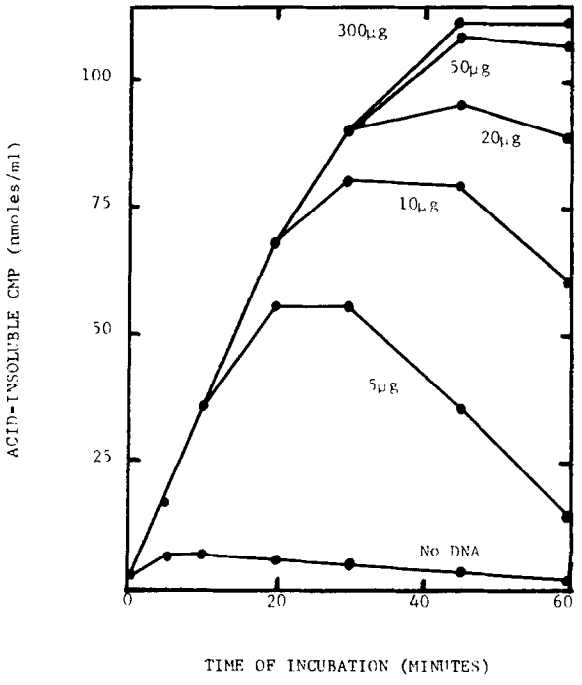

Figure 1. Effect of KOH-DNA on the conversion of ^{14}C-CDP into acid-insoluble polynucleotide as a function of incubation time. Each assay (basic assay scaled up 6-fold) contained 125 μg of protein (0.23 M KCl fraction), 0.48 μmoles ^{14}C-CDP and KOH-DNA (μg) as indicated.

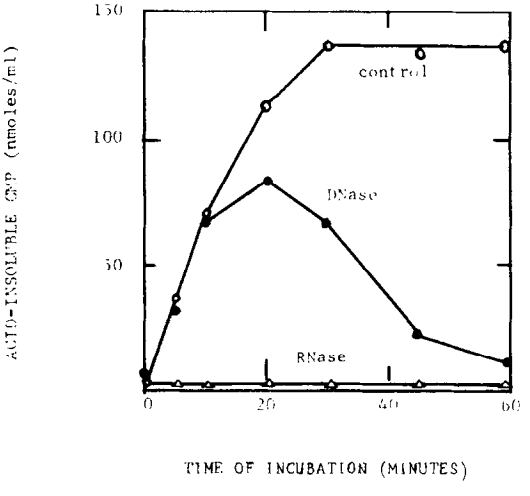

Figure 2. Effects of DNase and RNase on the KOH-DNA dependent conversion of ^{14}C-CDP into acid-insoluble polynucleotide as a function of incubation time. Each assay (basic assay scaled up 6-fold) contained 103 μg of protein (0.23 M KCl fraction), 0.48 μmoles ^{14}C-CDP and 50 μg of KOH-DNA. ○, control; ●, DNase I (50 μg electrophoretically purified, Worthington); △, RNase A (10 μg, Worthington).

Formation of poly C, in the presence of DNA, was inhibited by bovine RNase and the poly C product was made unstable by the addition of DNase I (Fig. 2).

To determine if DNA was stabilizing the poly C product two assays were run, one with DNA and the other without DNA (Fig. 3). Aliquots were removed at times indicated, mixed with EDTA to stop the reaction and chromatogramed to separate CTP, CDP, CMP, cytidine and poly C. When DNA was absent (Fig. 3a) the time-dependent decrease in CDP was paralleled by a simultaneous rise in CMP. At all times less than 1% of the radioactivity applied to the chromatogram was found at the origin. In contrast very little CMP was formed when DNA was present (Fig. 3b) and considerable radioactivity (poly C) remained at the origin. This time-dependent increase of poly C was paralleled by a simultaneous decrease in CDP. By 60 minutes the reactions were complete and the amount of CMP formed (86

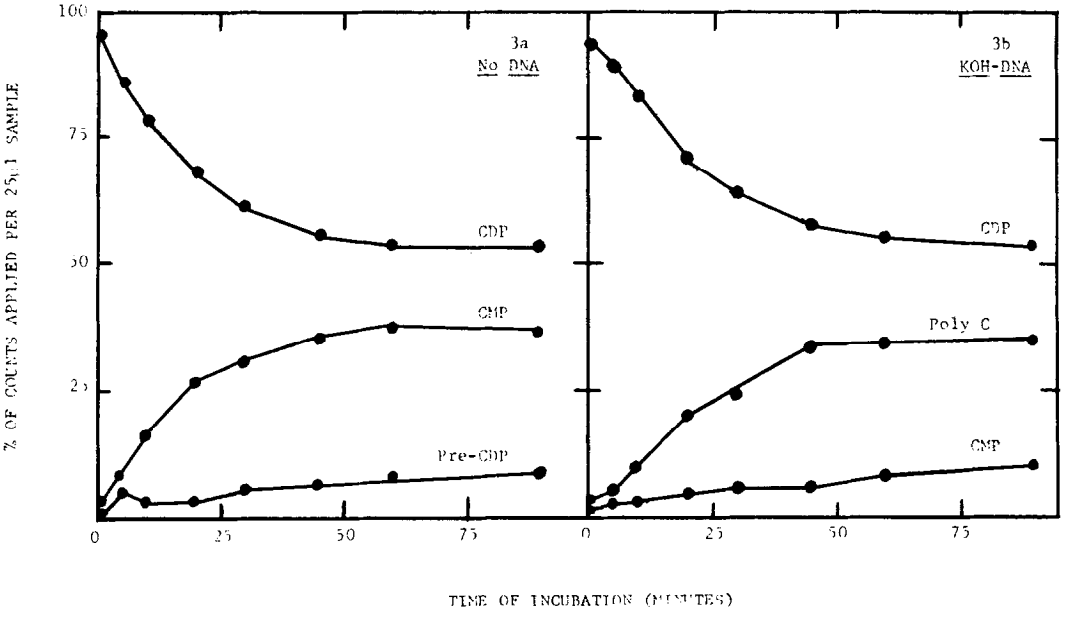

Figure 3. Effect of KOH-DNA on the conversion of ^{14}C-CDP into poly C and CMP as a function of incubation time. The polynucleotide phosphorylase assays (0.25 ml) contained 25 µg of protein (0.23 M KCl fraction) and 80 µmoles of ^{14}C-CDP. One contained no DNA (Fig. 3a) and the other contained 50 µg of KOH-DNA (Fig. 3b). At 0, 5, 10, 20, 30, 45, 60, and 90 minutes 25 µl aliquots were removed from each assay and mixed with 5 µl of EDTA (1 µmole). 25 µl (600 cpm) of the EDTA mixtures were immediately spotted on Whatman 3 MM paper and dried with a stream of cool air. The chromatograms were developed in 1% $(NH_4)_2SO_4$: isopropanol (50/100, v/v) for 72 hours (15). Appropriate regions of the chromatograms were cut into one inch-square sections and counted by liquid scintillation.

nmoles/ml) when DNA was absent is nearly identical to the amount of CMP polymerized (82 nmoles/ml) with DNA present. We interpret these results to indicate that DNA protects the poly C product from breakdown by an RNase. The line in Fig. 3a labeled pre-CDP was the radioactivity found between the origin and the CDP area and may represent oligonucleotides formed by the RNase.

To further establish the inhibitory effect of DNA, ^{14}C-CDP was used as a substrate. These experiments indicated that DNA protected poly C from breakdown and that this protection was dependent upon DNA concentration (Fig. 4). Protection was not complete even at relatively high concentrations of DNA (300 μg DNA/assay). An initial rapid breakdown, completed by 5 minutes, was followed by a relatively slow decrease in acid-insoluble radioactivity.

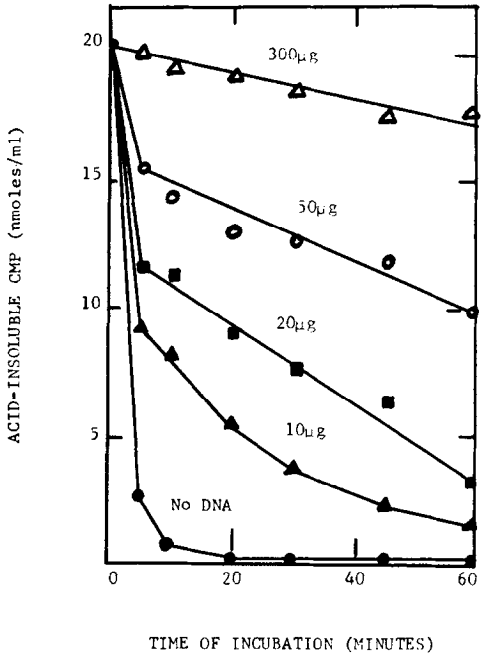

Figure 4. Effect of KOH-DNA on the RNase hydrolysis of ^{14}C-poly C as a function of incubation time. Each assay (basic assay scaled up 6-fold) contained 125 μg of protein (0.23 M KCl fraction), 11 μg of ^{14}C-poly C (34 μmoles CMP, sp. act. 100 cpm/μmole) and KOH-DNA (μg) as indicated.

It appears that large amounts of DNA (300 μg/assay) can completely inhibit the initial rapid breakdown, but the subsequent slow breakdown is largely independent of DNA concentration. We believe that DNA exerts its inhibitory

effect by interaction with the RNase and experiments are presently being conducted to check this point. The initial rapid breakdown of poly C may be a reflection of the time necessary for a DNA-RNase interaction to occur. At present we do not know what causes the subsequent slow breakdown of poly C since we do not know to what extent the enzyme preparations are contaminated by nucleases which are not inhibited by DNA.

DNAs which had been denatured were better inhibitors of the RNase than were the native DNAs (Fig. 5). Native salmon testes DNA inhibited less than did the heat-denatured DNA. Native T4 DNA gave no inhibition whereas denatured T4 DNA did inhibit. Calf Thymus DNA gave results similar to those obtained with salmon testes DNA (data not shown).

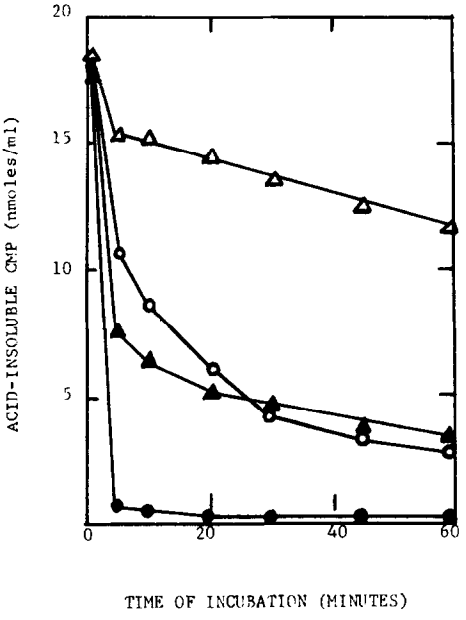

Figure 5. Effect of DNAs on the nuclease hydrolysis of ^{14}C-poly C as a function of incubation time. Each assay (basic assay scaled up 6-fold) contained 125 µg of protein (0.23 M KCl fraction), 50 µg of DNA, and 9.0 µg of ^{14}C-poly C (28 µmoles of CMP, sp. act. 100 cpm/µmole. ●, native T4 DNA; ○, heat-denatured T4 DNA; ▲, native salmon testes DNA; △, heat-denatured salmon testes DNA.

Exhaustive enzyme digests of ^{14}C-poly C were chromatogramed in 95% ethanol, 1 M ammonium acetate (70/40, v/v) saturated with $Na_3B_4O_7$ (7). This solvent separates cytidine, 2'- or 3'-CMP, and 5'-CMP. All the radioactivity in the

digests migrated as 5'-CMP. With one enzyme preparation some cytidine was also detected indicating a phosphatase contaminant.

Preliminary experiments indicate that E. coli ribosomal RNA is also slowly hydrolyzed by the RNase and this hydrolysis is inhibited by DNA.

References

1. Spahr, P. F., and B. R. Hollingworth, J. Biol. Chem., 236, 823 (1961).
2. Singer, M. F., and G. Tolbert, Biochemistry, 4, 1319 (1965).
3. Nossal, N. G., and M. F. Singer, J. Biol. Chem., 243, 913 (1968).
4. Robertson, H. D., R. E. Webster and N. D. Zinder, J. Biol. Chem., 243, 82 (1968).
5. Gesteland, R. F., and P. F. Spahr, in Cold Spring Harbor Symposia on Quantitative Biology, Vol. 34, p. 707 (1969).
6. Kuwano, M., C. Ning Kwan, D. Apirion and D. Schlessinger, Proc. Natl. Acad. Sci. U.S., 64, 693 (1969).
7. Kuwano, M., D. Schlessinger and D. Apirion, J. Mol. Biol., 51, 75 (1970).
8. Spahr, P. F., J. Biol. Chem., 239, 3716 (1964).
9. Bollum, F. J., in Procedures in Nucleic Acid Research, Ed. G. L. Cantoni and D. R. Davies, Harper and Row, p. 296 (1966).
10. Erickson, R. L., M. L. Fenwick and R. M. Franklin, J. Mol. Biol. 10, 519 (1964).
11. Chamberlin, M., and P. Berg, Proc. Natl. Acad. Sci. U.S., 48, 81 (1962).
12. Thomas, Jr., C. A., and J. Abelson, in Procedures in Nucleic Acid Research, Ed. G. L. Cantoni and D. R. Davies, Harper and Row, p. 553, (1966).
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. L. Randall, J. Biol. Chem., 193, 265 (1951).
14. Schneider, W. C., in Methods in Enzymology, Ed. S. P. Colowick and N. O. Kaplan, Academic Press, Vol. III, p. 680 (1957).
15. Hurlbert, R. B., in Methods in Enzymology, Ed. S. P. Colowick and N. O. Kaplan, Academic Press, Vol. III, p. 799 (1957).