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Cleavage of T4 species I ribonucleic acid by Escherichia coli ribonuclease III

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ABSTRACT

T4 Species I RNA, a molecule 140 nucleotides in length with some structural features very much like a tRNA, is specifically cleaved by an enzymatic activity in Escherichia coli extracts to give three segments with 19, 48 and 73 nucleotides. We report the purification and characterization of the E. coli RNase which cleaves two 3' phosphodiester bonds of T4 Species I RNA. This reaction has many properties in common with those catalyzed by E. coli RNase III, although the optimal salt conditions for T4 Species I RNA cleavage differ significantly from those for other RNase III-catalyzed reactions. The reaction is not catalyzed by extracts from an E. coli strain lacking RNase III activity. Furthermore, T4 Species I RNA is cleaved by highly purified E. coli RNase III to yield the same three specific fragments. We conclude that this specific cleavage is due to the action of RNase III, and that the requirement for lower ionic strength may reveal further important properties about this RNA processing enzyme.

INTRODUCTION

In previous papers,[2,3] we reported the nucleotide sequences of Species I RNA coded for by T2, T4 and T6 bacteriophages. When Species I RNA is incubated with a crude S30 extract of Escherichia coli, it is cleaved specifically to give segments of 19, 47 and 73 nucleotides with nucleotide 67 missing from the fragments. These digestion products are fairly stable in the crude extract and can be separated by polyacrylamide gel electrophoresis.

Using electrophoretic separation of the fragments as an assay, we have purified the enzyme from E. coli which cleaves Species I RNA specifically. The partially purified enzyme makes two chain scissions producing three polynucleotide chains with 5' phosphates and 3' hydroxyls. Stoichiometric amounts of the three cleavage products containing 19, 48 and 73 nucleotides are obtained. Optimal conditions for cleavage of T4 Species I RNA are described. In addition, studies with a bacterial strain lacking RNase III activity, as well as reactions carried out on T4 Species I RNA with highly purified E. coli RNase III, lead to the conclusion that this reaction is catalyzed by RNase III.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. E. coli strain W3110, obtained from M. Green, was used as the host strain for preparing ^{32}P-labeled RNA. E. coli strain Q13 (rna)4 obtained from M. Hayashi, was used for making the enzyme extracts. E. coli strains A19 (rna-19) and AB301-105 (rna-19, rnc-105), a derivative of A19[5] were the gift of D. Schlessinger. (Hereafter, we shall abreviate AB301-105 as AB105 in this paper.) Bacteriophage strains were obtained as previously described.[2,3]

Enzymes. The ribonucleases used were obtained as described previously.[14] Bovine serum albumin (BSA, Pentex crystallized) and ovalbumin (Pentex 5X crystallized chicken egg albumin) were obtained from Miles Laboratories, Inc. Myoglobin (whale skeletal muscle) was A grade from Calbiochem. Chymotrypsinogen A (Grade CG) and Deoxyribonuclease I were obtained from Worthington Biochemical Corp. RNase III fractions VI and VII were prepared as before.[6]

Chemicals. All routine chemicals were reagent grade. Ammonium sulfate was the ultra-pure special enzyme grade manufactured by Mann Research Laboratories. Bromphenol Blue standard solution was obtained from Van Waters and Rogers, Inc.

Buffers. Buffer A was 0.05 M Tris-HCl, pH 7.5, 0.06 M NH_4Cl. 0.01 M $MgCl_2$, 0.006 M 2-mercaptoethanol. The NH_4Cl concentration was varied for ribosomal washes as described in the text.

Buffer B was 0.02 M Tris-HCl, pH 7.6, 0.02 M NH_4Cl, 0.015 M $MgCl_2$ and 0.006 M 2-mercaptoethanol. Glycerol was added in various concentrations as described in the text. The NH_4Cl concentration was increased for elution from various chromatographic media as described in the text.

Buffer C was 0.01 M potassium phosphate, pH 6.8, 0.015 M $MgCl_2$, 0.006 M 2-mercaptoethanol, and 40% glycerol. The phosphate concentration was increased for elution from hydroxyapatite as described in the text.

Chromatographic media. Sephadex G100 was obtained from Pharmacia Fine Chemicals, Inc., and prepared for use according to the manufacturer's recommendations. DEAE-cellulose was Whatman DE32 which was prepared by the method of Peterson and Sober.[7] The columns were made using 0.02 M Tris, pH 7.6, followed by equilibration with Buffer B with 40% glycerol. Carboxymethylcellulose (standard grade) was purchased from Brown Co., Berlin, N.H. It was soaked in 0.2 N NaOH and 0.5 M NaCl for 48 hours, then filtered and extensively washed with water. The carboxymethylcellulose was resuspended in 0.02 M Tris and titrated to pH 7.6 with 6N HCl. It was then poured into a column and washed extensively with 0.02 M Tris-HCl, pH 7.6, followed by equilibration with Buffer B containing 40% glycerol.

Hydroxyapatite, prepared by the method of Siegelman et al.[8] as modified by Kennel[9], was the kind gift of Dr. Robert Bartsch. Columns were poured from a thick slurry of the hydroxyapatite, enough to give a final column volume of 1 ml, into a 1 ml volume of 0.01 M phosphate, pH 6.8. The crystals were allowed to settle before starting the column flow. The column was extensively washed with 0.01 M phosphate, pH 6.8, followed by equilibration with Buffer C.

Collection of column fractions. Column flow rates were controlled with a peristaltic pump (LKB Type 4192A) and fractions were collected in an LKB Ultrorac fraction collector for all chromatography except for elution from hydroxyapatite in which fractions were collected manually by gravity flow.

[^{32}P]RNA. ^{32}P-labeled RNA was obtained as previously described from E. coli infected with one of the T-even bacteriophages.[2,3]

Standard assay conditions for enzymatic cleavage of Species I RNA. All reactions were carried out in a final volume of 0.1 ml in dichlorodimethylsilane treated tubes at 37° in 0.01 Tris-HCl, pH 8.0; 0.005 M $MgCl_2$, 10^{-4} M EDTA, 10^{-4} M 2-mercaptoethanol for 30 min. ("low salt condition") unless otherwise noted. Substrate was T4[^{32}P]Species I RNA or other ^{32}P-labeled RNA as specified at 5,000-20,000 cpm of ^{32}P radioactivity. Total RNA in the reactions varied from 0.01 µg to 0.1 µg. When it was desired to obtain T1 or pancreatic fingerprints of the cleavage products, 5 to 10 x 10^6 cpm with 5 to 70 µg total RNA was used. Reactions were terminated, and the digestion products were electrophoresed in a 10% polyacrylamide gel as before.[2] Extent of cleavage was determined and reaction rates estimated as described by Robertson et al.[10]

Preparation of subcellular fractions. Subcellular fractions were prepared in a manner modified from that of Robertson et al.[10] E. coli Q13 was grown to late-log phase in L-broth and harvested by centrifugation. The cells (5 to 20 g) were washed and resuspended in Buffer A using 2 ml buffer per gram of cells. The cells were disrupted in a French press at 15,000 lb/in^2, and 10 µg per ml of Deoxyribonuclease I was added to the slurry for reaction at 4° for 30 minutes. The mixture was centrifuged at 13,000 rpm in an SS34 rotor for 20 minutes. The supernatant was then centrifuged for 30 minutes at 16,000 rpm as above and the upper three-fourths volume was collected. This 30,000 x g supernatant called S30 was then used as the source extract for further purification steps. Further fractionation of the S30 and salt washing of ribosomes were carried out as described previously.[10,11]

Protein determinations. Protein concentrations were determined by the procedure of Lowry et al.[12]

RESULTS

Partial Purification of Cleavage Activity

Since in the past it proved beneficial to purify RNase activities by gentle washing of ribosomes with increasing salt concentrations, this approach was adopted here. All steps were carried out at 4°. Fig. 1 shows the effect of the various subcellular fractions upon [^{32}P] species I RNA both with and without added carrier tRNA: Fig. 1A shows that without added carrier, non-specific cleavage can occur to such an extent that some or all of the specific

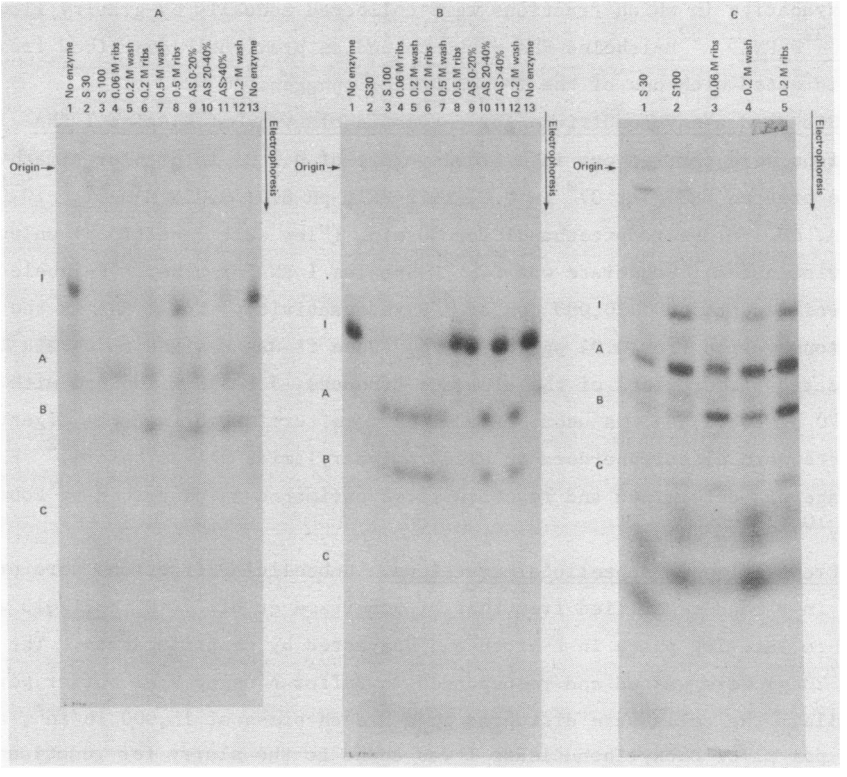

Fig. 1. Subcellular and ammonium sulfate fractionation of T4 Species I RNA cleavage activity. (A) and (B): Ribosomes were prepared from S30 obtained from E. coli Q13 and washed with the indicated NH_4Cl concentrations as described in Materials and Methods. Ammonium sulfate (AS) fractions were prepared from the 0.2 M NH_4Cl ribosomal wash and dialyzed against Buffer B as described in the text. The ammonium sulfate percentages in the figure indicate the number of grams of ammonium sulfate added per 100 ml of original enzyme solution. Volumes of enzyme used per assay of each subcellular fraction were normalized to the S30 starting volume. Thus approximately 175 µg protein in a 5 µg volume was used in the S30 reaction and 8 µg protein in 3 µl volume was used in the 0.2 M ammonium chloride wash fraction. The ammonium sulfate fractions were assayed using 1 µl each for the 0-20% and 20-40% fractions and 5 µl for the >40% fraction. Each reaction contained 1.8 x 10^4 cpm [^{32}P]Species I RNA and approximately 0.9 µg RNA except that reaction mixtures in (B) contained an additional 20 µg carrier tRNA. Assays were incubated and analyzed as described in Materials and Methods. The amount of enzyme used for the 0.2 M wash assays in lanes 12 was 1/3 that used for the assays in lanes 5. (C) Incubations were carried out in the same manner as in (B) except that reaction mixtures contained twice the amount of enzyme, 1.6 x 10^5 cpm of [^{32}P]Species I RNA and 20 µg carrier tRNA. Cleavage fragment B has approximately the same electrophoretic mobility as bromphenol blue dye marker.

cleavage products are missing in the autoradiograph. The nonspecific cleavages may be blocked somewhat by adding carrier tRNA as shown in Fig. 1B and 1C, although as is readily apparent in Fig. 1C, products which are the result of nonspecific degradation still occur to a large extent when enzyme from these subcellular fractions is used.

a. Ribosomal wash

A large proportion of the cleavage activity can be washed off the ribosomes with 0.2 M NH_4Cl buffer as shown in Fig. 1C, column 4, although there are significant amounts in the S100 fraction and in the 0.2 M NH_4Cl washed ribosomes which can be released by washing with 0.5 M NH_4Cl buffer. This behavior is identical to that observed both with RNase P and RNase III.[10,11]

b. Ammonium sulfate precipitation

Crude purification by ammonium sulfate fractionation was then carried out on the enzyme from the 0.2 M NH_4Cl ribosomal wash according to Robertson et al.[10] and the results are shown in Fig. 1. The series of experiments in Fig. 1A without added RNA show that the cleavage activity is in both the 0 to 20% and greater-than-40% fractions. In the 20-40% fraction Species I RNA is completely and nonspecifically degraded. The experiment with added RNA displayed in Fig. 1B, however, shows all of the cleavage activity to be in the 20-40% fraction.

From this it is evident that the bulk of the cleavage activity as well as the nonspecific degradation activity precipitate when ammonium sulfate concentrations between 20 and 40 grams per 100 ml of original solution (20 to 40%) are achieved. The data suggest that the Species I RNA cleavage enzyme is competitively inhibited by tRNA, and that a nonspecific degrading enzyme which may be the "obliterase" described by Robertson et al.[10] can effectively have its activity screened out by appropriate use of additional bulk tRNA. The nonspecific RNase described by Robertson et al.[10] does not wash off ribosomes until 0.5 M NH_4Cl is used. Thus the nonspecific RNase found in the 0.2 M wash by the Species I RNA cleavage assays could be a different RNase activity or it could be the same activity possibly needed in only small amounts if Species I RNA is a more susceptible substrate than the tyrosine tRNA precursor. The cleavage activity in this fraction and in all previous pruification stages is stable for months when stored frozen at -20° in buffer B + 10% glycerol.

c. DEAE cellulose chromatography

The 20-40% ammonium sulfate fraction was then fractionated stepwise on a DEAE-cellulose column (1 x 10 cm) equilibrated with Buffer B containing 40% glycerol (Fig. 2A).

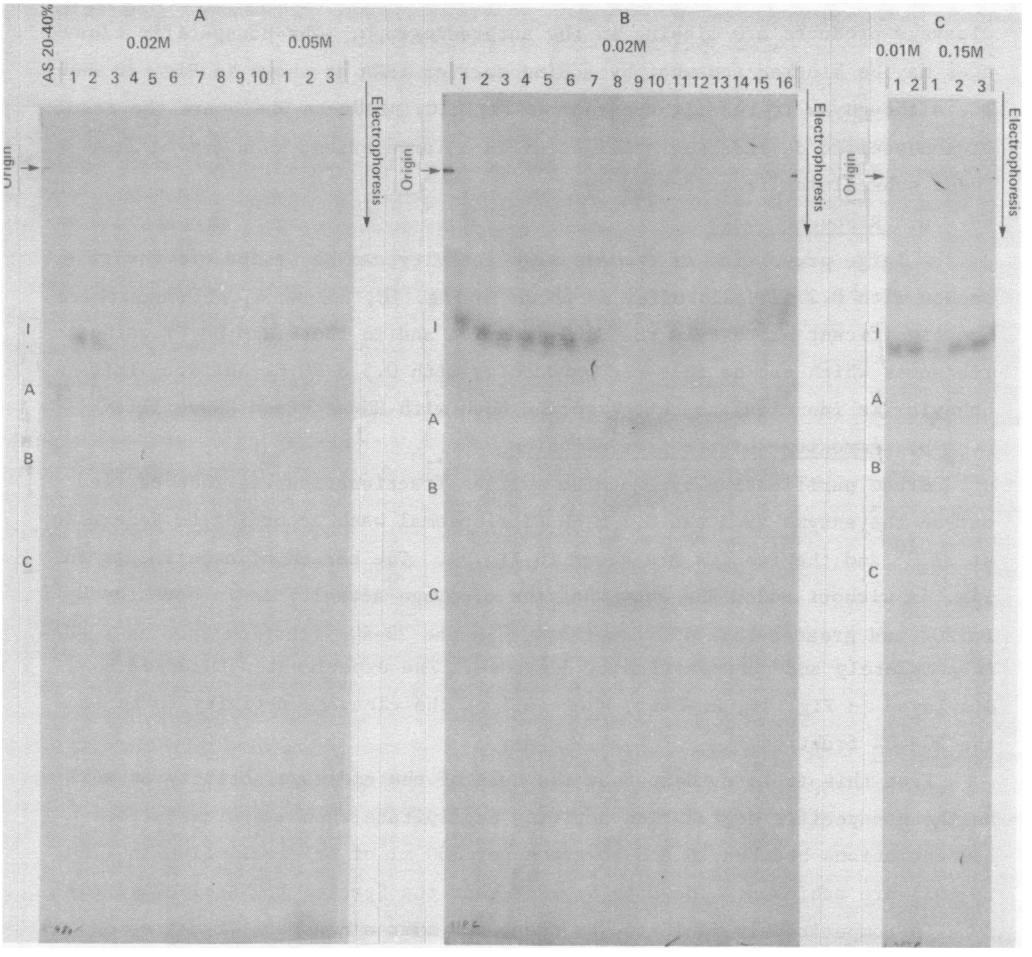

Fig. 2. DEAE-cellulose, carboxymethylcellulose, and hydroxyapatite fractionation of the cleavage activity. (A) DEAE-cellulose fractionation of 20 to 40% ammonium sulfate fraction. A DEAE-cellulose column (1 x 10 cm) was poured and equilibrated with Buffer B + 40% glycerol as described in the text, and 0.7 ml of the dialyzed 20 to 40% ammonium sulfate fraction was loaded into the column. Elution was with ten 3-ml fractions each of Buffer B with 40% glycerol containing 0.02 M NH_4Cl, 0.05 M NH_4Cl, and 0.10 M NH_4Cl, respectively, with a flow rate of 3 ml every 13 min. 5 µl of each fraction assayed as described in Materials and Methods. Each assay contained 5 x 10^3 cpm of [^{32}P]Species I RNA and approximately 0.1 µg RNA. Only the active and neighboring fractions are shown in (A). The assay for the ninth 0.02 M NH_4Cl fraction was anomalous. Repetition of the assay showed that fraction to have reactivity intermediate between the eight and tenth 0.02 M NH_4Cl fractions. Thus it was not included in the pool of active fractions. The assay for the 20-40% ammonium sulfate fraction contained 1 µl enzyme and 60 µg added carrier tRNA. (B) CMC fractionation of DEAE-cellulose pool of active fractions. A CMC column (1 x 10cpm) was poured and equilibrated with Buffer B + 40% glycerol as described in the text, and 6 ml of a pool of the active factions (0.02 M NH_4Cl fractions 3-8 from Fig. 2A) purified through the DEAE-cellulose step were loaded into the CMC column. Elution was with 20 2-ml fractions each of Buffer B with 40% glycerol containing 0.02 M NH_4Cl, 0.05 M NH_4Cl, and 0.2 m NH_4Cl, respectively, with a flow rate of 2 ml every 5 minutes. 10 µl of each fraction was assayed as described in Materials and Methods. Each assay contained 2 x 10^4 cmp of [^{32}P]Species I RNA and approximately 0.1 µg RNA. Only the active and neighboring fractions are shown in (B). (C) Hydroxyapatite fractionation of DEAE-cellulose pool of active fractions. A hydroxyapatite column (1 x 1 cm) was prepared and equilibrated with Buffer C as described in Materials and Methods. 1.5 ml of the DEAE-cellulose pool of active fractions shown in Fig. 2A was loaded onto the hydroxyapatite column followed by additional Buffer C and collected in two 4-ml fractions. Three 4-ml fractions of material eluted with Buffer C containing 0.15 M phosphate were then collected. Other experiments have shown that the size of column used would have been sufficient for purification of the entire DEAE-cellulose pool of active fractions. 10 µl of each fraction was assayed as described in Materials and Methods. Each assay contained 5000 cpm of [^{32}P]Species I RNA and approximately 0.1 µg RNA.

Most of the activity is eluted in the 0.02 M NH_4Cl wash although some activity does elute in the later fractions. Furthermore, the enzyme in these fractions appears to be completely free of other RNases that are able to degrade Species I RNA. The cleavage reaction assay can be carried out with no additional protective RNA added and still yield stoichiometric amounts of the cleavage products with no visible yields of other degradation products. In a series of assays using increasing amounts of enzyme, an assay using 50 times the amount of enzyme necessary to give 63% cleavage showed no non-specific degradation. In another experiment in which the same size DEAE-cellulose column was used, the activity eluted with a 200 ml 0.02 M-0.6 NH_4Cl gradient. Nonspecific RNase activity eluted in fractions containing 0.15 M to 0.2M salt.

At this stage of purification it is possible to conclude that this activity is not identical with RNase P, which binds more tightly to DEAE columns.[10] However, the behavior of this activity on DEAE columns is identical to that previously observed for RNase III.[11,15]

d. Hydroxyapatite chromatography

If the enzyme was to be purified with hydroxyapatite, the pool of active fractions eluted from the DEAE-cellulose column was dialyzed against three 1-liter changes of Buffer C. The dialyzed pool was loaded onto the hydroxyapatite column and washed with an additional 6 ml of Buffer C. No cleavage activity was found in these 0.01 M phosphate elutions. The phosphate concentration was then increased to 0.15 M and all of the cleavage activity could be eluted in 4 ml as shown in Fig. 2C. This step can, therefore, be used to concentrate the enzyme into a smaller volume as well as for purification. The large increase in phosphate molarity was used not only to elute the enzyme sharply into a smaller volume, but because an earlier experiment indicated that while the enzyme binds tightly to hydorxyapatite in 0.01 M phosphate, when 0.03 M increments with increasing phosphate concentrations are used, the enzyme is eluted about equally in every fraction from 0.03 M through 0.15 M. The RNase at this stage of purification is stable for months when stored unfrozen in Buffer C at -10°.

e. Carboxymethylcellulose chromatography

If the enzyme was to be purified using a carboxymethylcellulose (CMC) column, the pooled active fractions eluted from the DEAE-cellulose column were loaded directly on a CMC column (1 x 10 cm) previously equilibrated with Buffer B containing 40% glycerol. As shown by the experiments in Fig. 2B, most of the cleavage activity elutes in the 0.02 M NH_4Cl wash buffer, although

small amounts do come off in later fractions. In this respect the behavior of this activity differs from that reported for RNase III.[13] However, both the amount of glycerol and the nature of the monovalent cation differ markedly in the two cases. The enzyme purified through CMC is fairly stable at -20° in the elution buffer.

Protein determinations showed a decrease in protein concentration during this and previous steps. However, as with RNase P,[10] there is no accurate way to estimate the recovery of cleavage activity.

Estimate of Molecular Weight

An estimation of size for the cleavage enzyme was obtained by passage of the enzyme through a G-100 Sephadex column. The cleavage activity elutes just after ovalbumin, but well before chymotrypsinogen A, indicating a molecular weight of approximately 40,000 daltons.[14] This estimate does not exclude RNase III as a candidate for this activity.[15]

Properties of the Species I RNA Cleavage Enzyme

The endonuclease was tested for activity at various pH and salt conditions which are summarized in Table I. The assays were performed as

Table I. Activity Requirements of Purified Species I RNA Cleavage Enzyme

Constituent	Concentration	Effect on Activity[a]
1. $MgCl_2$	5×10^{-3} M	●
2. 2-mercaptoethanol	less than 10^{-2} M	●
3. EDTA	less than 10^{-3} M	+
4. EDTA	greater than 10^{-2} M	-
5. $CaCl_2$	less than 10^{-3} M	0
6. $MnCl_2$	greater than 10^{-5} M	-
7. NH_4Cl	0.03 M and greater	-
8. NaCl	" " "	-
9. KCl	" " "	-
10. KBr	" " "	-
11. KI	" " "	-
12. $CuCl_2$	greater than 10^{-5} M	-
13. $FeCl_3$	" " "	-
14. $HgCl_2$	" " "	-
15. $CoCl_2$	greater than 10^{-4} M	-
16. $CaCl_2$	5×10^{-3} and greater	-

The cleavage enzyme was assayed under various conditions as described in the text. Constituents 1-4 were varied in concentration from standard conditions, 5 and 6 were tested in assays both by addition and substitution for $MgCl_2$ at various concentrations in otherwise standard conditions, and 7-16 were added at various concentrations to the standard buffer.

[a]Symbol in this column describes the effect on enzyme activity. ●, essential; +, positive effect as regards reliability but does not increase reactivity; 0, no effect; -, inhibitory effect.

described in Materials and Methods, in all cases with enzyme purified through the hydroxyapatite step, although many of the experiments were repeated with enzyme purified through the carboxymethylcellulose step. The standard reaction buffer was 5 x 10^{-3} M $MgCl_2$, 10^{-4} M 2-mercaptoethanol, 10^{-4} M EDTA and 0.02 M Tris, pH 8.0. Conditions were changed by varying the pH or concentrations of the standard ingredients or by addition or substitution of other materials. Enzyme was diluted to the proper strength into suitable buffer before addition to the reaction mixture such that carryover from storage buffers B or C never caused significant effect on the concentration of reaction buffer constituents. Relative reactivities were determined by comparing reaction completion percentages under various conditions. The Species I RNA cleavage enzyme has the following set of properties: The enzyme requires magnesium and other divalent cations cannot be substituted for it. The optimal magnesium concentration is that of the standard reaction buffer. When concentrations of 10^{-3} molar and lower or 10^{-2} M and higher were tried, there was little or no reactivity. Divalent cations Ca^{++} or Mn^{++} could not be substituted for Mg^{++}, and in fact, Mn^{++} showed inhibitory effects causing 50% inhibition at 10^{-4} M and total inhibition at 10^{-3} M when added to the standard buffer. A reducing agent such as 2-mercaptoethanol is also required. While not tested on the more purified enzyme, dithiothreitol worked equally well for cleavages performed with S30 extract. Tests indicated a broad range of useful 2-mercaptoethanol concentrations from as low as 3 x 10^{-5} M to 10^{-2} M; however, 0.1 M completely inhibited the reaction. Little or no reaction was observed when buffers at pH 7.5 or lower were used, but buffers with pH 8 to pH 10 allowed relatively equivalent activity. In this regard, 0.02 M Tris pH 8 instead of 0.01 molar is used in the standard buffer to prevent lowering of pH when hydroxyapatite fractions are assayed. Increasing the Tris concentration to 0.05 M, however, cut the activity by half. Salt (NaCl, KCl, NH_4Cl, KBr, and KI) at concentrations of 0.03 M decreased activity by approximately 50% and total inhibition was observed at concentrations of 0.06M. Enzyme in the S30 extract seemed a little more tolerant of salt with complete inhibition not achieved until 0.09 M salt was added to the buffer solution. Salts of heavy metals were particularly lethal to enzyme activity. $CuCl_2$ and $FeCl_3$ caused complete inhibition and $HgCl_2$ 50% inhibition at 10^{-5} M. Both $HgCl_2$ and $CoCl_2$ resulted in complete inhibition at 10^{-4} M. EDTA could be tolerated with no loss of activity up to 10^{-3} M but 10^{-2} M caused complete inhibition of cleavage reaction. The enzyme was found to have approximately equal activity when the reaction mixture was incubated

at 30°, 37°, and 45°, but no reaction was observed for incubations done at 0° or 17.5°. A rather interesting result was obtained when the enzyme was heated for a short time at 100°, quickly cooled in ice and then allowed to digest Species I RNA under the standard reaction conditions at 37°C for 15 minutes. Heating for 1 minute resulted in an enzyme that nonspecifically cleaved and degraded Species I RNA. Heating for two minutes caused the enzyme to loose all activity.

Charcaterization of the cleavage products

The previous papers[2,3] have described how crude E. coli extracts cleave Species I RNA at two points to yield three segments of 19, 47, and 73 nucleotides, with nucleotide A, at position 67 in the sequence missing from the products as indicated by Fig. 3. Fig. 4 shows fingerprints of T1 ribonuclease digests of the three S30 cleavage products from T4 Species I RNA, and Fig. 5 shows an autoradiograph of the acrylamide gel on which they were

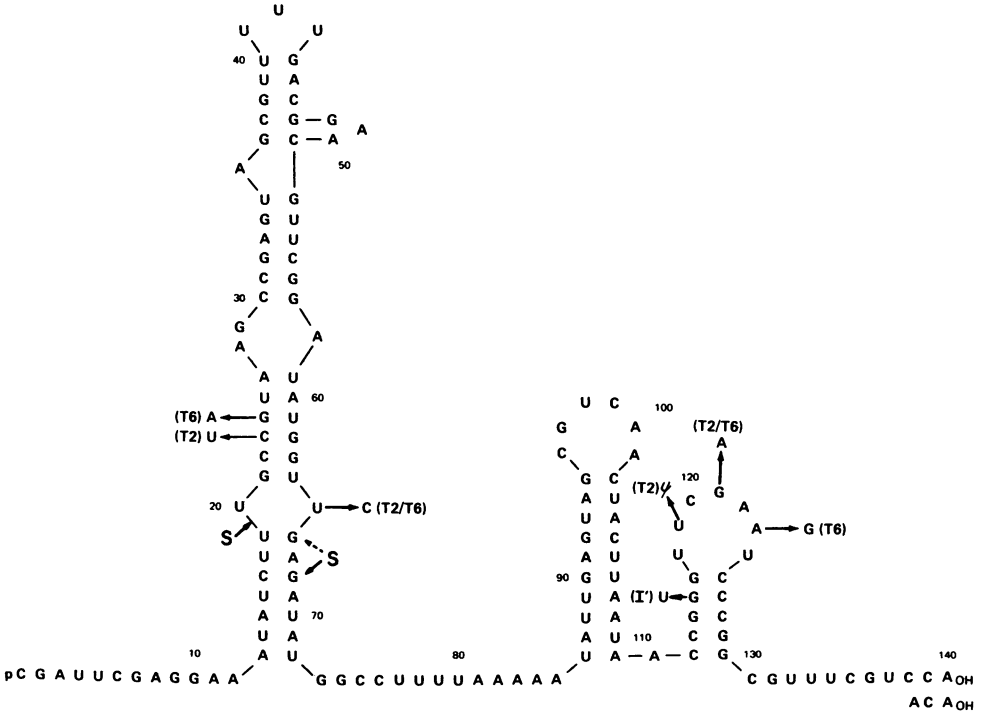

Fig. 3. Possible arrangement for Species I RNA secondary structure. The T4 Species I RNA nucleotide sequence was arranged to give the most stable configuration[2] according to the rules cited in Tinoco et al.[22] Newer rules stated in Tinoco et al.[23] suggest that the base pair between nucleotides 40 and 44 would not be formed and that the first hairpin loop would thus contain five nucleotides.[2] The differences in the sequences for T2 and T6 Species I RNA and the I' mutation in Species I RNA from T4-HA2 are also shown. The endonuclease cleavage points are indicated by S➤. The dotted arrow indicates that nucleotide 67 is missing from the cleavage products when Species I RNA is cleaved by crude extract from E. coli. As discussed in the text, it is present when the cleavage is performed by the purified enzyme. The molecule may be folded to pair the 5' and 3' ends as previously explained.[2]

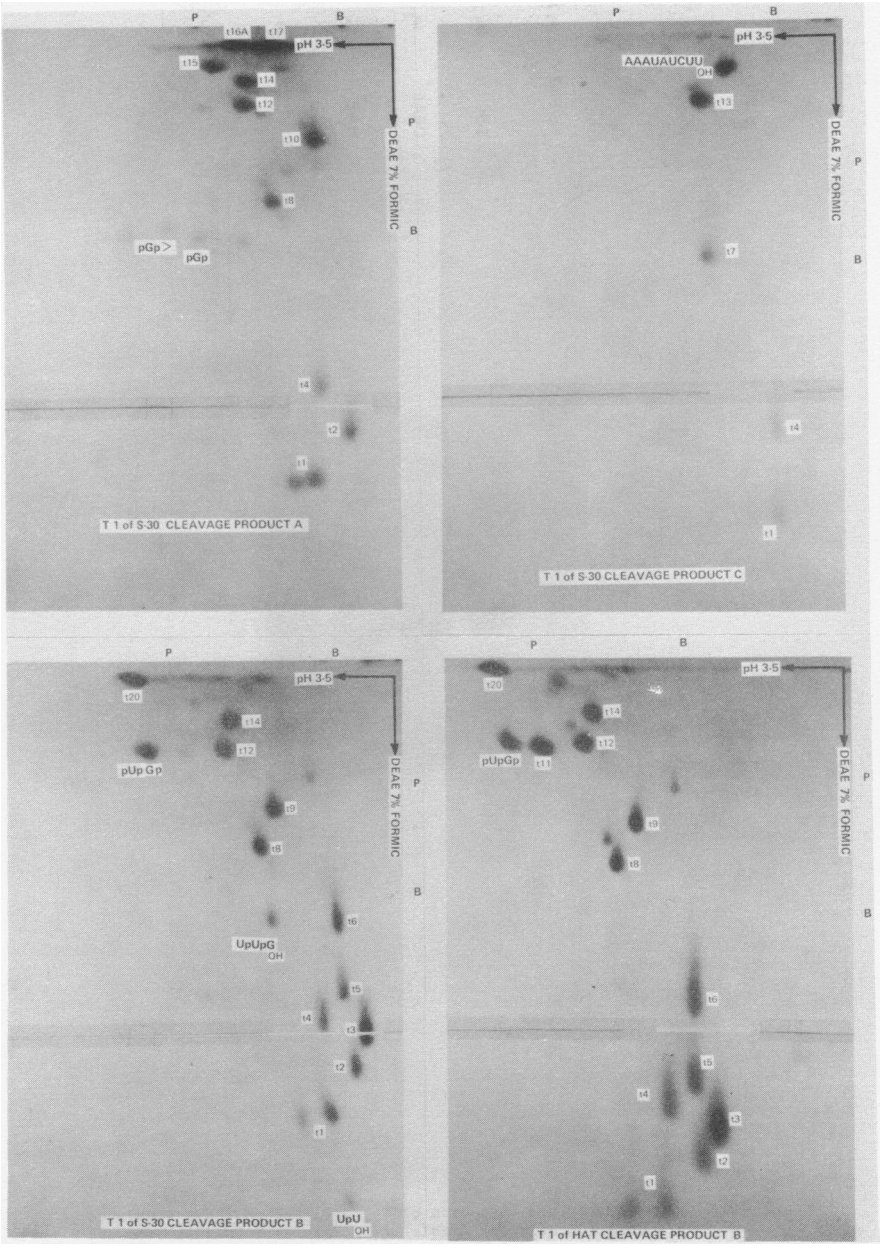

Fig. 4. T1 ribonuclease digestion products obtained from the cleavage products of T4 [^{32}P]Species I RNA. T1 ribonuclease digestions were performed on segments A (upper left), B (lower left), and C (upper right) obtained through cleavage of [^{32}P]Species I RNA by S30 and on segment B (lower right) obtained through cleavage of [^{32}P]Species I RNA by enzyme purified through the hydroxyapatite step. The numbered spots are the same as from a T1 fingerprint of the entire molecule.[2] Nucleotide, t11, is UpUpGp. Separation was by electrophoresis in two dimensions as described in Materials and Methods.

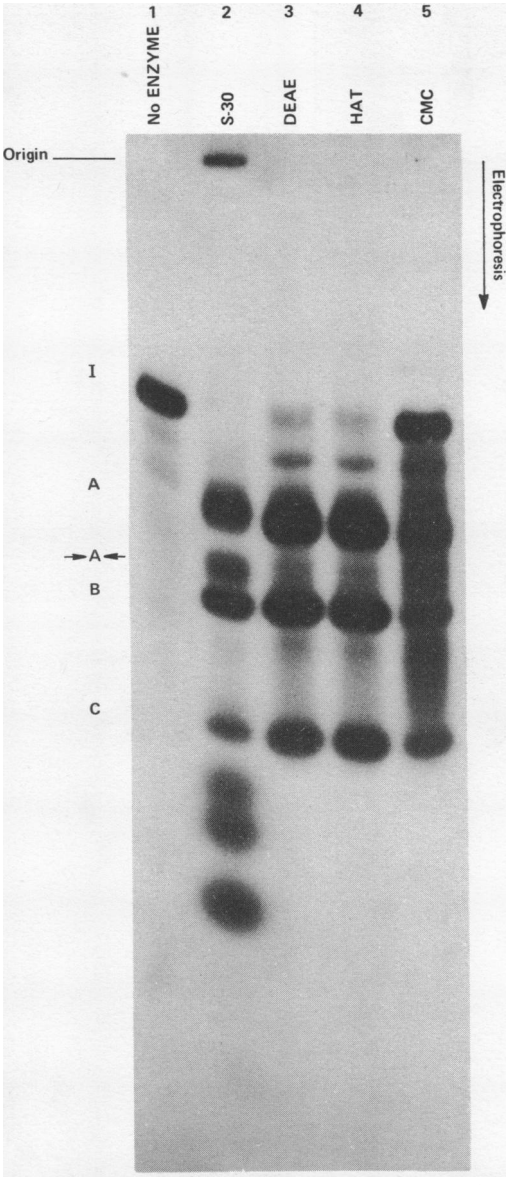

Fig. 5. Cleavage products from digestion of T4 [^{32}P]Species I RNA separated by electrophoresis on a 10% acrylamide gel. [^{32}P]Species I RNA was digested with 5 µl S30, 10µl enzyme purified through the DEAE-cellulose step, and 20 µl of enzyme purified through either the hydroxyapatite step (HAT) or the carboxymethylcellulose (CMC) step. Incubations were as described in Materials and Methods with reaction mixtures containing approximately 5 x 10^6 cpm and 6 µg carrier tRNA except that the digestion with S30 used 66 µg carrier tRNA in the mixture. S30 cleavage product, →A←, is segment A degraded approximately 10 nucleotides from both the 5' and 3' ends. The digestion with the CMC purified enzyme was performed with enzyme that apparently had lost much activity and some specificity subsequent to the pilot experiments.

separated. Segment C from the 5' end of T4 Species I RNA ends with T1 product ApApApUpApUpCpUpU-OH. Segment B from the middle of T4 Species I RNA begins with T1 product pUpGp and terminates primarily with T1 product UpUpG-OH although some UpU-OH is also seen in the fingerprint. Segment A starts with T1 product pGp at position 68 in the sequence. When the cleavage is done with enzyme purified through the hydroxyapatite step, segments C and A remain the same, but segment B must now terminate with UUGA-OH since T1 product t11, UpUpGp, appears in the fingerprint of segment B with UpUpG-OH and UpU-OH completely absent as shown in Fig. 4. The expected pancreatic digest product, GpA-OH, from segment B was also found and identified through alkaline and snake venom phosphodiesterase digestions. Other experiments have shown that nucleotide A, at position 67 is no longer removed when the cleavage is carried out with enzyme purified through the DEAE-cellulose step. Also, when the cleavage is done with the more purified enzyme, T1 products UpApCpA-OH and UpCpCpA-OH from the 3' end of Species I RNA are found in their usual yields in fingerprints of segment A, whereas when the cleavage is with S30, these products are hardly visible on the autoradiograph. In fact, when the cleavage is done with S30, as shown in Fig. 5, another band with mobility intermediate between that of segments A and B often appears in the gel autoradiograph. Analysis of the RNA contained in this band by digestion with T1 and pancreatic ribonucleases shows it to be segment A that has been degraded approximately ten nucleotides from both the 5' and 3' ends. The purification procedure used thus seems to have rid the cleavage enzyme of any exonucleases that degrade either Species I RNA or its cleavage products.

Identity of Endonuclease Activity

The properties of the activity which specifically cleaves Species I RNA were sufficiently similar to those of RNAase III that further experiments were performed to test the possibility that the two are identical.

Cleavage Activity in RNase III$^-$ Strain

T4 Species I RNA cleavage activity in the extract (S30) of AB105 (rna-19, rnc-105) was compared with that of Q13 (rna) (Fig. 6). No cleavage activity could be seen in the extract (S30) of AB105. In another experiment, even with 10 µl of AB105 extract (27.5 mg/ml) no cleavage activity could be seen by gel electrophoresis(data not shown). Therefore, the extract from AB105 had less than 1% of the activity found in the extract from Q13. The possibility that the absence of the activity could be due to some inhibitors in the AB105 was tested. When a mixture of

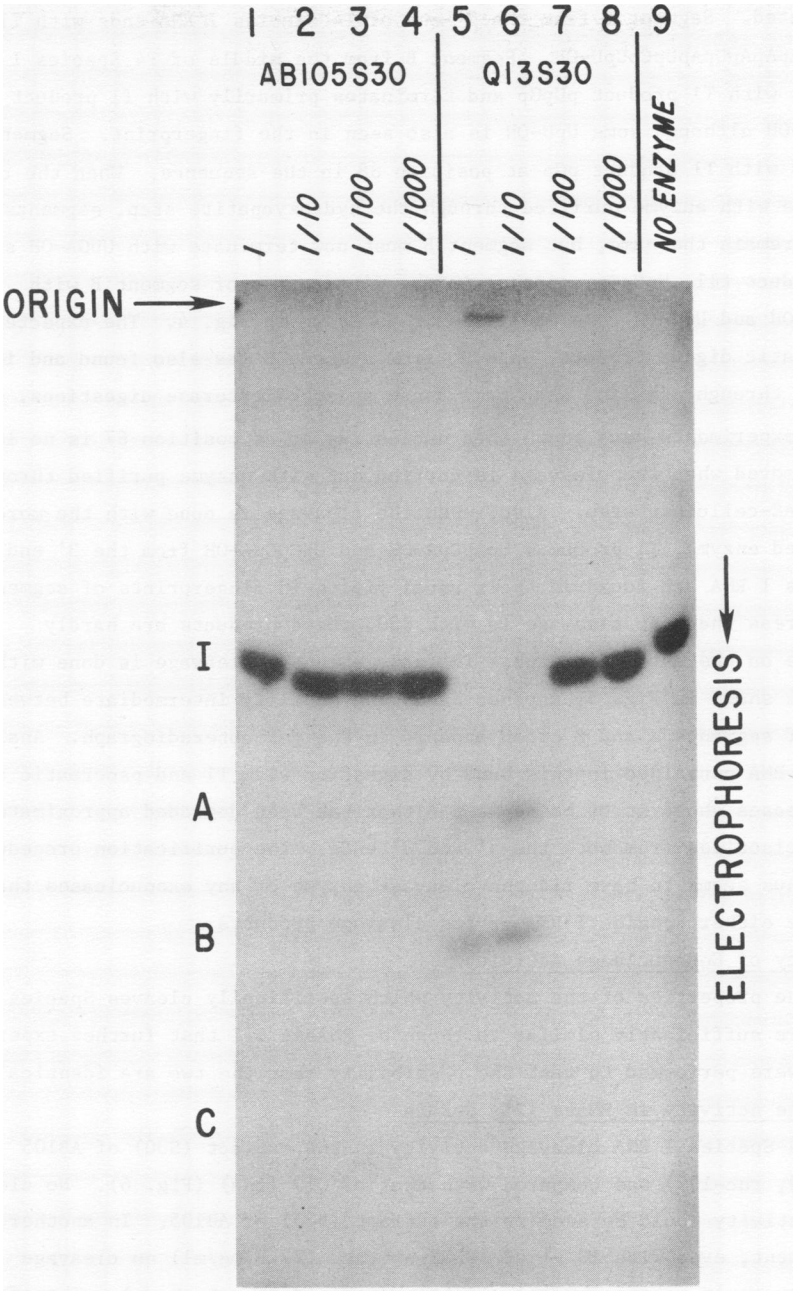

Fig. 6. Comparison of Species I RNA cleavage activity in various E. coli strains. [^{32}P]Species I RNA at approximately 2 x 10^4 cpm was incubated with each of 5 µl of AB105 S30 and Q13 S30 diluted to 1, 1/10, 1/100 and 1/1000 under the "low salt condition" as described in Materials and Methods. Protein amounts of AB105 S30 and Q13 S30 are approximately 27.5 mg/ml and 26 mg/ml, respectively.

A19 S30 (rna-19 , parent strain of AB105) and AB105 S30 was incubated with Species I RNA, no cleavage was seen. However, in another experiment, A19 S30 alone, AB105 S30 alone, and A19 S30 plus AB105 S30 were preincubated for various lenghts of time at 37° in the assay buffer prior to incubation with Species I RNA. If the inhibitory effect of AB105 S30 on Species I RNA cleavage activity is due to the in vivo accumulation of RNAse III substrate, then the Species I RNA cleavage activity should appear after a certain length of preincubation. The result is shown in Fig. 7A. Species I RNA cleavage activity increased, in proportion to the length of preincubation time, until at 120 minutes almost no inhibitory effect of AB105 S30 could be seen. A similar experiment using cleavage activity further purified through the CM cellu-

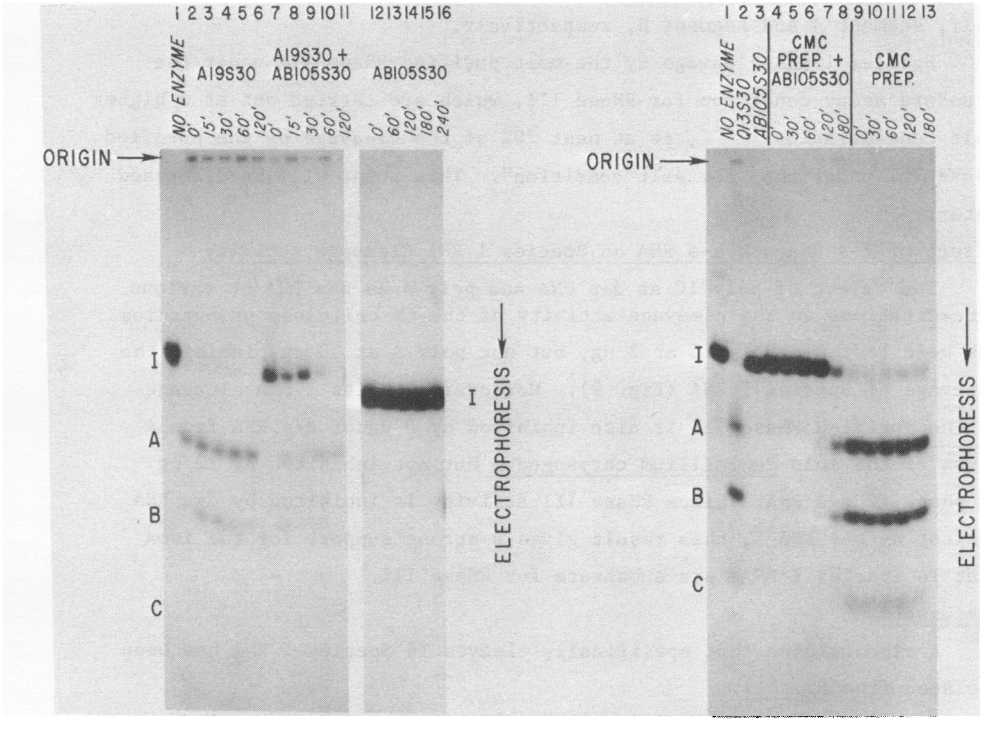

Fig. 7. Analysis of inhibitory effect of crude extract (S30) of AB105 on Species I RNA cleavage activity. (A) (left) 5 µl of A19 S30 alone and 5 µl of A19 S30 plus 5 µl of AB105 S30 were preincubated for 15 min., 30 min., 60 min., and 120 min. at 37° in the assay buffer while 5 µl of AB105 S30 alone was preincubated for 60 min., 120 min., 180 min., and 240 min. After the preincubation T4 Species I RNA at 2 x 10^4 cpm and 30 µg carrier tRNA were added to each tube and incubated for 30 min. more at 37°. (B) (right) 20 µl of CM cellulose preparation alone and that preparation plus 5 µl of AB105 S30 were preincubated in the same manner as in (A). T4 Species I RNA at 2 x 10^4 cpm was added and incubated as in (A).

lose step instead of A19 S30 gave the same result in Fig. 7B. This eliminated the possibility that some inhibitor in AB105 S30 except RNase III substrate was degraded by Q13 S30. Species I RNA cleavage activity is, therefore, missing in the strain AB105. This result suggests that Species I RNA cleavage activity is really RNase III, although there is some possibility that the cleavage activity and RNase III are distinct enzymes and yet both are missing in AB105.

Cleavage of T4 Species I RNA by RNase III

Using a very purified preparation of RNase III[6], T4 Species I RNA was cleaved into the three characteristic fragments under the "low salt condition." (Fig. 8). The cleavage products have been proved, by fingerprint analysis to be exactly the same as segment A, segment B and segment C of T4 Species I RNA. The 5' end group analysis of cleavage products has shown that 5' end groups are pCp, pGp and pUp for Species I RNA itself, segment A and segment B, respectively.

Species I RNA cleavage by the most purified RNase III under the standard assay condition for RNase III, which are carried out at a higher salt concentration [11,15], is at best 20% of the cleavage by the purified RNase III under the "low salt condition". This point will be discussed later.

Effect of d-s RNA and s-s RNA on Species I RNA Cleavage Activity

The effect of poly IC as d-s RNA and poly A as s-s RNA at various concentrations on the cleavage activity of the CM cellulose preparation has been tested. Poly IC at 2 µg, but not poly A at 50 µg, inhibit the cleavage of Species I RNA (Fig. 9). Moreover, Species I RNA cleavage by the purified RNase III is also inhibited by 3 µg of d-s RNA from a virus of the mold Penicillium chrysogenum but not inhibited by 10 µg of phage f2 s-s RNA. Since RNase III activity is inhibited by d-s RNA but not by s-s RNA[15], this result gives a strong support for the idea that T4 Species I RNA is a substrate for RNase III.

Discussion

A ribonuclease that specifically cleaves T4 Species I RNA has been isolated from E. coli.

We have offered several lines of evidence that the enzymatic activity in E. coli that cleaves Species I RNA is RNase III:

1) The activity behaves the same as RNase III in most purification steps.
2) The activity is absent in AB105, an RNase III mutant.
3) Authentic RNase III cleaves Species I RNA in the same specific

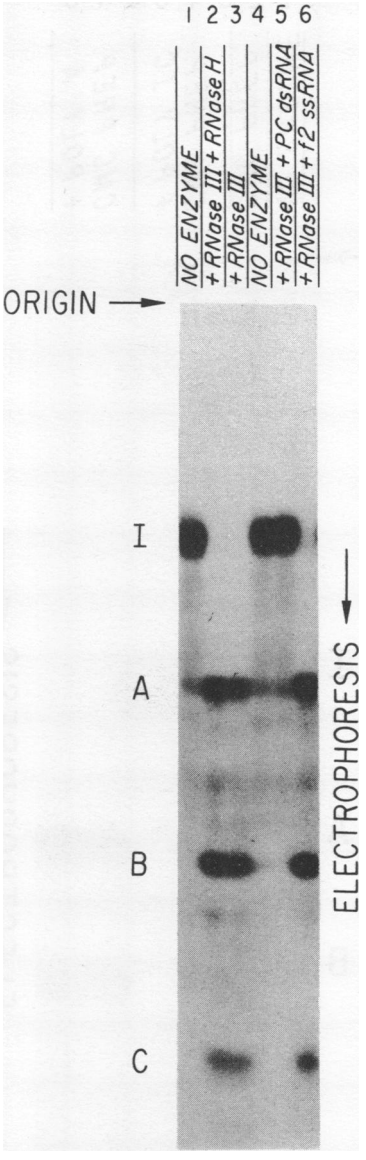

Fig. 8. Cleavage of T4 Species I RNA with highly purified RNase III. RNase III was prepared as described by Robertson and Dunn[6] through the Fraction VI stage (which still contains RNase H activity) and the Fraction VII stage (free of RNase H). Reactions were carried out as before[6] in the presence of 10^5 cpm ^{32}P-labeled T4 Species I RNA and 1 unit of RNase III activity. (1) No enzyme; (2) 1 unit of Fraction VI RNase III; (3) 1 unit of Fraction VII RNase III; (4) No enzyme; (5) Reaction identical to that carried out in (3), but with the added presence of 1 microgram of the competitive inhibitor, double-stranded RNA from a virus of Penicillium chrysogenum; (6) Reaction identical to that carried out in (3), but with the added presence of 10 micrograms of bacteriophage f2 single-stranded RNA.

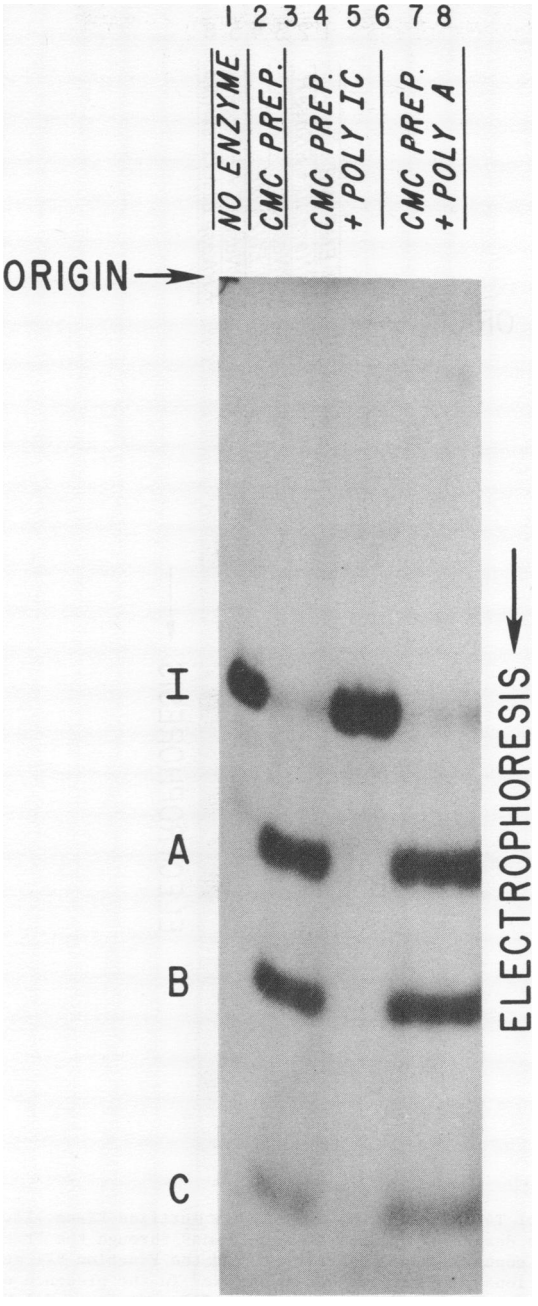

Fig. 9. Effect of d-s RNA and s-s RNA on Species I RNA cleavage activity. Digestion of T4 Species I RNA was carried out in the presence of poly IC (3-5) or poly A (6-8). (1) No enzyme; (2) 20 µl of CM cellulose preparation alone; (3) plus 0.2 µg poly IC; (4) plus 2 µg poly IC; (5) plus 20 µg poly IC; (6) plus 1 µg poly A; (7) plus 10 µg poly A; (8) plus 50 µg poly A.

manner as the activity purified here.

4) The activity is inhibited by double-stranded RNA but not by single-stranded RNA, a property also seen in the processing of T7 mRNAs or ribosomal RNA[16]. It is of interest to compare what is known about the various molecules processed by RNase III.

Kramer et al.[17] and Rosenberg et al.[18] have determined that the nucleotide sequence at the 5' ends of T7 mRNA are pGpApUp. These ends are created by RNase III processing. Inspection of Fig. 3 shows that a pGpApUp sequence is also created in the RNase III cleavage of Species I RNA.

Another fragment that is released in the cleavage of T4 Species I RNA has the 5' end pUpGp. Ginsburg and Steitz have determined that the sequence of the 5' end of the 16S precursor RNA produced in RNase III cleavage of the 30S ribosomal RNA precursor[19] is pUpGp.

In addition to T4 Species I RNA other RNA substrates have been tested for cleavage by enzyme purified through the hydroxyapatite step. Among these are Species IA RNA from T2-infected cells, T4 Species I' RNA, and T6 Species I RNA[2,3]. These RNAs are related in sequence and structure to T4 Species I RNA[2,3].

As described in the previous paper[3], T6 Species I RNA is cleaved normally, but T2 Species IA RNA is cleaved somewhat abnormally causing a buildup of RNA containing nucleotides 1-67. Fig. 3 shows two T2 base substitutions near potential cleavage sites in Species I RNA. Fig. 3 shows that T6 also has two such substitutions, one of which is identical to one of those observed in T2[3]. It is possible, therefore, to propose that the C to U exchange at position 23 affects the rate of cleavage by RNase III between the bases at positions 19 and 20 of Species I RNA. The fragment comprising bases 1-67 can itself be cleaved after isolation from polyacrylamide gels by an additional RNase III treatment. However, an excessive amount of enzyme must be used to accomplish this cleavage.

Cleavage of T4 Species I' RNA[2] occurs at the correct sites, but not always at both sites. Analysis of T1 and pancreatic fingerprints shows the presence of fragments containing nucleotides 1-67 and 20-140 in addition to the normal fragments. Thus the mutation at position 116 must hinder the reaction to some extent.

This is the first instance in which it has been shown that RNase III can produce a single cleavage in a substrate molecule.[20] The action of RNase III in cleaving double-stranded RNA might have led one to expect that obligatory double-strand scissions would be a feature of the mechanism of

action of RNase III. Further studies of mutationally altered RNA substrates may lead to a detailed understanding of the structural requirements for specific RNase III cleavage.

At this stage we can make two points concerning the specificity of RNase III. The enzyme must be recognizing the double-stranded nature of the substrate because double-stranded RNA is a potent inhibitor (and, of course, a substrate) of RNase III reactions. In this connection it is interesting to note that the limit digest products of RNase III acting on double-stranded RNA have an average length of 15 base pairs.[6] There are 17 potential base pairs from the top of the hair pin loop (Fig. 3) to the cleavage site in T4 Species I RNA. The comparison of the ends produced by RNase III cleavage of T4 Species I RNA, T7 mRNA and ribosomal RNA leads us to the conclusion that RNase III may also have sequence specificity in the processing of natural RNAs.

RNase III acting in vivo must have a precise and genetically determined set of cleavage sites. Apparently T4 Species I RNA is not an in vivo substrate because it is quite stable and the products of RNase III digestion of Species I RNA are not found (our unpublished results). We believe that the monovalent cation concentration can strongly affect the specificity of RNase III cleavage. In this study we have found that monovalent cation concentrations below 0.03M are required for optimum cleavage of T4 Species I RNA. In marked contrast monovalent cation concentrations between 0.1 and 0.2 M are required for optimal processing of T7 mRNA or 30S ribosomal RNA presursor[21], as well as digestion of double-stranded RNA[11,15].

Lowering the salt concentration probably perturbs each of these potential substrates for RNase III in a particular way. It may be that sequence recognition is facilitated in lower salt where double-stranded structures are destablilized. The fact that the RNase III reaction is so sensitive to changes in the environment should open the possibility of studying its mode of action in an expanded set of substrates, thus leading to further understanding of its substrate specificity.

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