RNase III Cleavage of Single-stranded RNA

EFFECT OF IONIC STRENGTH ON THE FIDELITY OF CLEAVAGE

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Ribonuclease III from Escherichia coli has been purified to apparent homogeneity by affinity chromatography on immobilized double-stranded RNA. Polyacrylamide gel electrophoresis of the purified enzyme in the presence of sodium dodecyl sulfate gave one band of protein with a molecular weight of approximately 25,000. Chromatography on Sephadex G-100 is consistent with a molecular weight of 50,000, suggesting that the native enzyme is a dimer.

RNase III cuts some single-stranded RNAs, such as bacteriophage T7 early RNA, at specific sites in vivo. This RNA is cut at these same sites by the purified enzyme under all conditions tested. However, at low ionic strength relatively small increases in enzyme concentration produce cuts at secondary sites. At high ionic strength, the enzyme's preference for the sites cut in vivo is more pronounced and secondary cuts are made only at very high enzyme concentrations.

Secondary cuts are shown to occur at specific sites and are made in a variety of RNAs even from sources other than E. coli. By cutting RNAs at secondary sites it should be possible to generate RNA fragments which would be useful in a number of studies.

RNase III of Escherichia coli cuts the primary transcripts from the ribosomal cistrons of E. coli and the early region of bacteriophage T7 at specific sites and these cuts are an important step in the production of the RNAs normally observed in vivo (1-3). The in vivo pattern of cleavages has been faithfully reproduced by purified RNase III, although additional cuts have been observed at high levels of enzyme (2).

In contrast to its specific cutting of single-stranded RNAs, purified RNase III seems to degrade double-stranded RNA rather nonspecifically (4). Double-stranded RNA is an effective inhibitor of RNase III cleavage of single-stranded RNA, suggesting that the specific cleavage sites in single-stranded RNA may have double-stranded character (2, 5). Specific base sequences might also be required for cleavage (6-8).

Since purified RNase III cuts single-stranded RNA so specifically, this enzyme might be useful as a tool in analyzing RNA molecules from sources other than E. coli. If specific cleavage sites have been preserved in evolution, enzymes similar to RNase III may be present in higher organisms and play important roles in RNA processing (9). If so, RNase III may be useful in studying RNA processing in higher organisms, and in fact is being used for this purpose (9, 10). On the other hand, if no enzymes with cleavage specificity similar to RNase III are present in higher organisms, long RNA molecules might by chance contain cleavage sites, and RNase III could be used to cut such molecules into specific fragments for analysis by other means.

In order for RNase III to be a useful tool for studying a variety of RNAs, it is desirable to know how specific the RNase III cuts are and what factors might influence the specificity of cleavage. In testing the ability of highly purified RNase III to cut RNAs from mammalian sources, it was discovered that the ability to cleave these RNAs depends strongly on the ionic strength of the reaction mixture. This indication that ionic strength influences the specificity of cleavage has been investigated using the well characterized T7 early mRNAs as substrates.

EXPERIMENTAL PROCEDURE

Materials

Nucleic Acids—Polycistronic T7 early RNA, labeled with 32P, was synthesized by transcribing T7 DNA with purified Escherichia coli RNA polymerase in the presence of [α-32P]GTP (specific activity, 1.2 Ci/mmol) (1). The RNA was purified by treatment with DNase (5 μg/ml for 5 min) followed by extraction with phenol, chromatography on cellulose CF 11 (11, 12), and precipitation with ethanol. The precipitated RNA, dissolved in 100 μl of 0.1 M sodium dodecyl sulfate/0.05 M Tris-HCl (pH 6.8)/0.002 M EDTA, was placed in a boiling water bath for 2 min. It was then layered on a 10 to 30% glycerol gradient in 1% sodium dodecyl sulfate/0.05 M Tris-HCl (pH 6.8)/0.002 M EDTA and centrifuged at 40,000 rpm using a Spinco SW 50.1 rotor. Centrifugation was at 20°C for 3½ hours. At the end of the run the gradient was fractionated and the fractions containing the polycistronic RNA were pooled.

RNA from the 0.3 gene of T7 was purified by preparative electrophoresis from T7 early RNAs produced in vivo. E. coli B was grown in low phosphate B2 medium (13) to 4 x 10^6 cells/ml and then irradiated with
ultraviolet light to suppress the synthesis of host RNAs. Irradiated cells were infected with T7 in the presence of 400 μg/ml of chloramphenicol and 150 μg/ml of H₁₅PO₄ (14). RNA was isolated 10 min after infection and electrophoresed on a 4% polyacrylamide slab gel (16). The region of the gel containing the 0.3 mRNA was located by autoradiography, cut out, and transferred to a ground glass homogenizer. The gel was homogenized with 3 ml of 0.1% sodium dodecyl sulfate/0.5 M sodium acetate (pH 6.0)/0.001 μM EDTA, and the RNA was allowed to elute overnight at room temperature. The eluted RNA was extracted repeatedly with an equal volume of water-saturated tert-butanol and then passed through a Millipore filter to remove small particles of polyacrylamide (16).

Purified RNAs were precipitated twice with ethanol in the absence of added carrier RNA and stored frozen in sterile water.

RNase III (Ploy(A-T)) was synthesized by E. coli RNA polymerase using Ploy[d(A-T)] as template in the presence of [³²P]UTP (specific activity, 100 Ci/mole). After DNAse treatment and extraction with phenol, Ploy(A-U) was isolated by chromatography on cellulose CF11.

Double-stranded RNA from a virus associated with the mold Penicillium chrysogenum and single-stranded RNA from bacteriophage 𝜑2 were the kind gifts of H. D. Robertson. Other Materials—[α-³²P]UTP and [³²P]UTP were purchased from New England Nuclear. DNAse (RNAse-free) and lysozyme were obtained from Worthington. Poly[d(A-T)] was purchased from Miles. Agarose-hexane-polyl(poly-C) [AG poly(I)-poly(C)] was from F-L Biochemicals.

Methods

Purification of RNase III—Cell extracts from E. coli A 19 (RNase I⁻) (17) were prepared using minor modifications of the method described by Burgess and Jendrisak for the purification of RNA polymerase (18). Frozen cells (200 g) were resuspended in 600 ml of 0.05 M Tris-HCl (pH 7.9)/0.25 M KCl/0.02 M EDTA/0.01 M 2-mercaptoethanol/0.1 mM dithiothreitol/23 μg/ml of phenylmethylsulfonylfluoride. The resuspended cells were lysed by treatment with 130 μg/ml of lysozyme followed by the addition of sodium deoxycholate to a final concentration of 0.05%. The viscosity of the lysate, which is not further diluted, was reduced by adding MgCl₂ to 0.01 M and then 2 mg of DNase. All subsequent operations were carried out at 0–4°C.

The cell extract was centrifuged at 10,000 rpm for 30 min in a Sorvall G-5 rotor and then at 40,000 rpm for 4 hours in a Spinco 42.1 rotor. The high speed supernatant (600 ml) was fractionated with ammonium sulfate (4) by adding solid ammonium sulfate (22 g/100 ml) slowly with constant stirring. The precipitate that formed was removed by centrifugation. To the supernatant was added an additional 14 g/100 ml of solid ammonium sulfate to attain 33% saturation. The precipitate that formed was removed by centrifugation. The supernatant was added a further 14 g/100 ml of solid ammonium sulfate and centrifugation was repeated. All the supernatant, containing approximately 50% of the activity, was pooled and dialyzed overnight against 2 liters of Buffer A. Dialysis was continued the next day for a few hours against a second 2 liters of Buffer A. The dialyzed ammonium sulfate fraction was clarified by low speed centrifugation and dialyzed with enough Buffer A minus KCl to produce a final concentration equal to that of Buffer A. Usually dilution to a final volume of 100 ml was sufficient.

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The purified RNase III solution was then diluted to 1 mg/ml of crystalline RNase III. The enzyme was dialyzed against 0.01 M Tris-acetate (pH 7.5)/0.1 mM magnesium acetate/2 mM dithiothreitol/0.1 mM EDTA and then stored on ice.

Purified RNase III was applied to a column (4 x 70 cm) of Sephadex G-100 equilibrated and developed with 5% glycerol/0.02 M Tris-HCl (pH 7.9)/0.01 M magnesium acetate/0.2 M NH₄Cl/0.1 mM dithiothreitol/0.1 mM EDTA. This latter step also served to reduce the ion content of the enzyme solution.

RNase III activity against Polyl(A-U)—A simple assay for RNase III measures the release of acid-soluble counts from [³²P]Poly(A-U) (4). Reaction mixtures (100 μl) containing 10 μg/ml of [³²P]Poly(A-U) in 0.02 M Tris-acetate (pH 7.5)/0.1 M KCl/0.01 M MgCl₂/0.1 mM dithiothreitol/0.1 mM EDTA/25 μg/ml of bovine serum albumin were incubated with RNase III for 20 min at 37°C. Reactions were stopped by adding 5% trichloroacetic acid, and the RNA remaining was determined by collecting the precipitates on glass fiber filters and measuring the radioactivity in a toluene-based scintillation fluid.

Polyacrylamide Gel Electrophoresis—RNAs and proteins were analyzed by electrophoresis on slab gels in the presence of sodium dodecyl sulfate essentially according to the procedures described by Studier (14). Two gel systems, 3 to 20% polyacrylamide gradient gels and 2% polyacrylamide gel, were used to degrade DNA-RNA hybrids to low levels of pancreatic RNase (23, 24), the ability of sodium dodecyl sulfate to slowly inactivate pancreatic RNase at room temperature, and the sensitivity of gel electrophoresis for detecting and measuring the size of homogenous RNA fragments. Purified phage particles are used, making the purification of DNA unnecessary. The procedure has been used here to map fragments of the gene 0.3 mRNA of T7.

Briefly, a small amount of radioactively labeled RNA in buffer containing sodium dodecyl sulfate is mixed with purified phage to give approximately 5 μg of DNA in a total volume of 40 μl. The mixture is heated briefly in a boiling water bath to release and denature the DNA, placed at 65°C for 30 min to renature, and then treated with pancreatic RNase for 1 hour at room temperature to degrade any unhybridized RNA. The RNase itself is inactivated by the sodium dodecyl sulfate in the mixture. The protected RNA is then released from the hybrids by brief heating in a boiling water bath and analyzed by gel electrophoresis. The size of the protected fragments, together with the known position of the deletion, determines the location of the RNA fragment in the DNA.

RESULTS

Affinity Chromatography of RNase III—RNase III appears to be the only enzyme in extracts from Escherichia coli that selectively degrades double-stranded RNA (4, 25). Therefore, it

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* F. W. Studier and J. J. Dunn, manuscript in preparation.
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Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions during the purification of RNase III. Portions of the following samples were analyzed by electrophoresis on a 15% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate (14): a, high speed supernatant (10 μg of total protein); b, dialyzed ammonium sulfate fraction (25 μg); c, flow through of DEAE-cellulose column (10 μg); d, flow through of poly(I)-poly(C) column (10 μg); e, 2 M NH₄Cl eluent of poly(I)-poly(C) column (5 μg). Samples a to d were prepared by diluting aliquots with sodium dodecyl sulfate sample buffer (14). Sample e was first dialyzed against water to remove NH₄Cl, lyophilized, and then dissolved in sodium dodecyl sulfate sample buffer. Prior to loading on the gel the samples were placed in a boiling water bath for 2 min.

Fig. 2. Positions of primary RNase III cleavage sites within polycistronic T7 early RNA. *Escherichia coli* RNA polymerase initiates transcription from three sites near the physical left end of the T7 DNA molecule to produce polycistronic T7 early RNA. The arrows, labeled 1 to 5, indicate the positions of the primary RNase III cleavage sites. Cleavage at these sites generates the five individual early mRNAs (0.3, 0.7, 1.1, 1.3) plus the three initiator RNAs (I₀, I₁, and I₂) (1).

under “Experimental Procedure” and summarized in Table I was developed.

As shown in Fig. 1, one major band of protein was observed when the material eluted from the poly(I)-poly(C) column in 2 M NH₄Cl was electrophoresed on a polyacrylamide gel in the presence of sodium dodecyl sulfate. A molecular weight of approximately 25,000 was calculated for RNase III from its migration on the gel relative to bovine serum albumin, pancreatic DNase, lysozyme, and hemoglobin (27), in close agreement with the molecular weight of RNase III reported by Darlix (28). Electrophoresis of larger amounts of the poly(I)-poly(C) eluent indicated the presence of two low molecular weight impurities, which together are estimated to account for less than 5% of the protein in this fraction. These impurities can be removed by gel filtration on Sephadex G-100. The position at which RNase III elutes from G-100 columns (relative to hemoglobin) indicates that in its native form the enzyme probably contains two of the 25,000 subunits.

Effect of Monovalent Salt Concentration on Specificity of Cleavage—Polycistronic T7 early RNA is an ideal system for testing how incubation conditions affect the fidelity of RNase III cleavage in vitro, since the sites at which the enzyme cleaves this RNA in vivo are known (1, 29). Cleavage in vivo occurs at five widely separated sites generating the five individual early mRNAs of T7 and three smaller initiator RNAs (Fig. 2). These RNAs range from approximately 150 nucleotides to 3000 nucleotides in length and produce a characteristic pattern after their electrophoresis on polyacrylamide gels. Any deviations from the normal pattern can be readily detected, thereby providing a sensitive method for analyzing the specificity of cleavage in vitro. Due to the wide range in the size of the products, two different gel systems were used to resolve all eight RNAs. RNAs greater than 600 nucleotides were separated by electrophoresis on 2% polyacrylamide plus 0.5% agarose gels while RNAs of less than 600 nucleotides were separated on 3 to 20% polyacrylamide gradient gels.

Patterns obtained after treatment of polycistronic T7 early RNA with RNase III at different concentrations of NH₄Cl are shown in Fig. 3, and similar patterns have been obtained using NaCl or KCl in place of NH₄Cl. The normal in vivo pattern is obtained at salt concentrations between 0.15 and 0.3 M, but it is evident that little, if any, full length gene 1 mRNA is produced at NH₄Cl concentrations below 0.1 M. At still lower concentrations of salt the gene 0.7 and 1.3 mRNAs also disappear from the patterns. As these larger RNAs disappear from the pattern, many discrete smaller RNAs appear on the gradient gel, indicating that loss of the normal T7 mRNAs is due to endonucleolytic cleavages at specific sites. To facilitate discus-
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FIG. 3. Effect of NH₄Cl concentration on the specificity of cleavage of polycistronic T7 early RNA by RNase III. Polycistronic T7 early RNA was incubated with RNase III for 20 min at 37° in 5% sucrose, 0.02 M Tris-HCl (pH 7.9), 0.005 M MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol, and NH₄Cl as indicated. Each incubation mixture contained, in a final volume of 50 μl, 20,000 cpm of polycistronic T7 early [³²P]RNA (∼10 ng) and 1.5 units of RNase III. Reactions were terminated by adding 50 μl of a solution containing 0.2% sodium dodecyl sulfate, 0.05 M EDTA, and 100 μg/ml of tRNA and then 2.5 volumes of ethanol were added. After 1 hour at -15°, the RNA was collected by centrifugation, dissolved in 50 μl of sodium dodecyl sulfate sample buffer (14) containing 50 μg/ml of tRNA, and then placed in a boiling water bath for 2 min. Equal portions of each sample were then electrophoresed on a 2% polyacrylamide plus 0.5% agarose gel and on a 3 to 20% polyacrylamide gradient gel. The RNA applied to the tracks marked control was from an incubation mixture which received no RNase III. The positions of the early RNAs are indicated to the right of each gel pattern.

sion, the five sites normally cleaved by RNase III in vivo (and at 0.25 M NH₄Cl in Fig. 3) will be referred to as primary sites and the additional sites of cleavage will be referred to as secondary sites.

Patterns identical with those shown in Fig. 3 were obtained if the RNA was heated for 2 min at 100° in 0.01 M Tris-HCl (pH 7.5)/0.001 M EDTA and quenched on ice immediately before incubation with RNase III, suggesting that the secondary cleavages are not caused by intermolecular aggregation of the RNA. A number of lines of evidence suggest that the cutting of secondary as well as primary sites in the T7 RNA is caused by RNase III and not by a contaminating activity. Divalent cations, which RNase III requires for activity (4), are an absolute requirement for cutting at both primary and secondary sites. Furthermore, the new ends produced by cutting at secondary sites have 5'-phosphate termini (see below) and RNase III is known to produce 5'-phosphate end groups when it cuts double-stranded RNA (5, 22) and when it cleaves at the primary sites in polycistronic T7 early mRNA (6, 7). Finally, addition of 20 μg/ml of double-stranded RNA to the reaction mixture inhibits both primary and secondary cleavages (Fig. 4) as is expected if these cuts are made by RNase III. Addition of single-stranded f₂ RNA, which is known to lack primary sites (4), had no effect on cleavage at primary sites in polycistronic T7 early RNA even at concentrations as high as 300 μg/ml (Fig. 4). However, high concentrations of f₂ RNA did seem to inhibit cleavage at secondary sites, probably because f₂ RNA itself contains such sites.

The patterns of Fig. 3 were obtained using a constant amount of RNA and RNase III. Although secondary cuts are
The first partial site most resistant to cutting is less active and partial digestion products are observed. Similar partial products are produced at lower ionic strengths.

Fig. 3. The effect of added single- and double-stranded RNAs on cleavage at primary and secondary sites. Digestion and analysis of samples was essentially as described in the legend to Fig. 3 except that NaCl was used as the monovalent salt: 0.01 M in Samples a to g, 0.15 M in Sample h. Samples a, b, and h received no RNA in addition to the polycistronic T7 early RNA. Single-stranded RNA was added to Samples c to f to give 50, 100, 200, and 300 µg/ml, respectively. Double-stranded *Penicillium chrysogenum* RNA was added to Sample g at a final concentration of 20 µg/ml. These RNAs were added prior to the addition of RNAse III. No RNAse III was added to Sample a, all other samples contained RNAse III at a final concentration of 10 units/ml.

made at low ionic strength at this enzyme concentration (30 units/ml), only primary sites are cut at sufficiently low enzyme concentrations. Polycistronic T7 early RNA was incubated with different concentrations of RNAse III in 0.005, 0.05, and 0.25 M NH4Cl as described in the legend to Fig. 3, except that incubation was for 10 rather than 20 min. At 0.005 and 0.05 M NH4Cl, 3 to 6 units/ml of RNAse III produced complete cutting at the primary sites with little evidence of cutting at secondary sites. However, a 2- to 4-fold increase in enzyme concentration (6 to 24 units/ml) was sufficient to cause the disappearance of all the gene 1 mRNA (the most sensitive index of secondary cuts). At 0.25 M NH4Cl, a slightly higher enzyme concentration (10 units/ml) was required to produce complete cutting at the primary sites, but fifty times as much enzyme (500 units/ml) was required to eliminate the gene 1 mRNA from the patterns. Thus, primary cuts are preferred at all ionic strengths, but the range of enzyme concentration over which only primary cuts are made is rather narrow at low ionic strengths and very broad at high ionic strengths. These results also indicate that the activity of RNAse III in cutting single-stranded RNA increases slightly at low ionic strength, whereas the ability to solubilize poly(A-U) was found to be optimal near 0.3 M NH4Cl (in agreement with the results of Robertson et al. (4)) and decrease by about 2-fold at the lowest concentration of NH4Cl used in Fig. 3.

At the higher concentrations of NH4Cl used in Fig. 3, the RNAse III is less active and partial digestion products are observed. Similar partial products are produced at lower ionic strengths if limiting concentrations of enzyme or short incubation times are used. The first partial most resistant to cutting was required to eliminate the gene 1 mRNA from the patterns. These results indicate that RNAse III is less active and partial digestion products are observed. Similar partial products are produced at lower ionic strengths if limiting concentrations of enzyme or short incubation times are used. The first partial most resistant to cutting by RNAse III migrates behind the gene 1 mRNA. It is produced by cleaving at the third primary cleavage site without cleavage at the fourth and fifth sites, and contains the gene 1, 1.1, and 1.3 mRNAs. At still lower RNAse III concentrations, a set of three RNAs is found between the 0.3 and 1.3 mRNAs of the gel pattern. These RNAs are produced by cleavage at the second primary site without cleavage occurring at the first primary site and consist of the three initiator RNAs (I1, I2, and I3) linked to the 0.3 mRNA. The relative resistance of these specific partial cleavage products indicates that the second and third primary cleavage sites, those on either side of the 0.7 mRNA, are cleaved more efficiently than the remaining three sites.

Two forms of the 1.1 mRNA are observed in Fig. 3. Small amounts of the faster migrating form can also be observed in vivo. The transition is due to an RNAse III cleavage near the 3' end of the 1.1 mRNA, which releases an oligonucleotide 29 nucleotides long. The nucleotide sequence of this fragment has been determined and will be presented elsewhere. This fragment has never been observed in the 1.3 mRNA, indicating that the cleavage which releases it from the 1.1 and 1.3 mRNAs is less efficient than the cleavage that separates the 1.1 and 1.3 mRNAs.

Cleavage at Secondary Sites within 0.3 mRNA—When polycistronic T7 RNA is cleaved by excess RNAse III at low salt concentrations, reproducible patterns of discrete oligonucleotides are obtained. This suggests that the secondary cleavages are specific and that it might be possible to map their location. In order to keep the patterns relatively simple, individual T7 RNAs were isolated by preparative electrophoresis, incubated with RNAse III in 0.05 M NH4Cl, and then analyzed by electrophoresis on 3 to 20% gradient gels. The smallest T7 RNA that was readily cut by RNAse III under these conditions was the gene 0.3 mRNA, each of the initiator RNAs being quite resistant to cleavage. As shown in Fig. 5, the number of fragments produced from the gene 0.3 mRNA was dependent on the monovalent salt concentration; five major fragments were observed at NH4Cl concentrations ≥ 0.05 M. Each of these fragments, labeled 1 to 5 in Fig. 5, was isolated and its location determined with the aid of the mapping procedure described under "Experimental Procedures." Fig. 6 shows the products that were protected against pancreatic RNAse digestion when full length 0.3 mRNA and each of the five fragments was annealed with wild type T7 DNA or with DNA from the deletion phage C5 in which the right-hand one-third of the 0.3 gene is deleted (29). The entire 0.3 mRNA molecule was protected when annealed with wild type DNA but only the portion complementary to the region not deleted was recovered after annealing with the C5 deletion DNA. The entire length of Fragments 3, 4, and 5 were protected when annealed with C5 DNA indicating that they must originate from the portion of the 0.3 gene that is not deleted.  C5 DNA protected only a small portion of Fragment 2, placing this fragment almost entirely within the right-hand one-third of the gene. Two bands were produced from Fragment 1, the upper apparently comes from uncut 0.3 mRNA that contaminated this preparation, the lower coming from Fragment 1 itself. This result shows that Fragment 1 does not extend all the way to the left end of the 0.3 mRNA. The positions deduced for the secondary cleavages in the 0.3 mRNA by this technique are shown in Fig. 7.
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FIG. 6. Mapping of fragments produced by RNase III cleavages at secondary sites within the 0.3 mRNA. Full length 0.3 mRNA and each of the five major fragments produced by cutting at secondary sites within the 0.3 mRNA was annealed with wild type T7 DNA (W) or with DNA from the deletion phage C5 (D). After digestion with pancreatic RNase, the portion of each RNA that remained was determined by electrophoresis on a 3 to 20% polyacrylamide gel. The appearance of double bands, especially when the smaller fragments were annealed, is the result of heterogenous pancreatic RNase digestion at the ends of the hybrids.

FIG. 5. Cutting of secondary sites within 0.3 mRNA. Incubation mixtures, containing the indicated concentrations of NH₄Cl, were prepared as described in the legend to Fig. 3 except that 0.3 mRNA (10,000 cpn) was used in place of polycistronic T7 early RNA. RNase III (20 units/ml) was then added to each sample except the one labeled control which received no RNase III. Addition of RNase III increased the final concentration of NH₄Cl by 4 mM. After 30 min at 37° the samples were precipitated with ethanol and then electrophoresed on a 3 to 20% polyacrylamide gradient gel. The origin of electrophoresis is at the top of the figure. The positions of full length 0.3 mRNA and the five major fragments produced at NH₄Cl concentrations above 30 mM are identified at the right.

FIG. 7. Location of major secondary cleavage sites within 0.3 mRNA. The positions of the secondary cleavage sites within the 0.3 mRNA required to generate the five major fragments were determined from the molecular weights of the individual fragments and their location within the 0.3 mRNA. As shown, these five fragments are produced by cleavage at three sites, Fragments 1 and 3 being partial digestion products.

which generate these five fragments are given in Fig. 7.

Secondary Cuts Produce 5'-Phosphate Termini—RNAs produced by digestion of double-stranded RNA or by cutting at primary sites in T7 early RNA have been shown to have 5'-phosphate termini (5-7, 22). Cutting at secondary sites likewise produces 5'-phosphate ends as shown by the following experiment. A sample of 0.3 mRNA was incubated with 100 units/ml of RNase III in the absence of added NH₄Cl to produce secondary cuts. Polyacrylamide gel analysis indicated that no full length 0.3 mRNA remained in the sample after digestion. The entire sample was then hydrolyzed in alkali and analyzed by two-dimensional electrophoresis (5) to detect nucleoside diphosphates that would be produced from 5'-phosphate ends. All four possible diphosphates were detected. Since the 0.3 mRNA itself ends in 5'-pG (6, 7) this indicates that secondary cuts produce 5'-phosphate ends, and that 5' ends other than G are produced.

Individual fragments produced from 0.3 mRNA by secondary cuts (Fig. 7) were also analyzed for 5'-phosphate ends. As expected, pGp was the only nucleoside diphosphate recovered from full length 0.3 mRNA and from Fragments 3 and 5 (each of which has the original 5' end). The 5' ends of Fragments 1, 2, and 4 are generated by secondary cuts within the 0.3 mRNA and might be expected to give other diphosphates. In fact, Fragment 4 yielded pAp. Fragment 2 gave approximately equal amounts of pGp and pCp, suggesting that its 5' end is probably
heterogenous. Fragment 1 was not analyzed since it appeared to be contaminated with uncut 0.3 mRNA; however, it should have the same 5’ end as Fragment 4.

**DISCUSSION**

Polycistronic T7 early RNA can be cleaved in *vitro* by RNase III at five primary sites and a number of secondary sites. Primary sites are those where cleavage takes place in *vitro* and are the preferred sites of cleavage in *vitro* under all conditions tested. In E. coli, primary sites seem to be cleaved immediately after they are synthesized, and uncut RNAs are produced only in RNase III− strains. In *vitro* transcription also produces uncut RNAs which can then be tested for RNase III processing sites. Knowing the cleavage properties of purified RNase III should aid in determining whether an RNase III cleavage observed in *vitro* would most likely also occur in *vivo*. Presumably the conditions shown here to promote a high fidelity of cleavage of polycistronic T7 early RNA (low enzyme concentration and moderate salt concentration) would also apply for other RNAs. Ideally, the RNA to be tested for the presence of primary sites should be treated with RNase III in parallel with polycistronic T7 early RNA and the T7 digestion pattern used as an index of cleavage at primary sites and the absence of cutting at secondary sites. Studies of this type could provide information concerning the role of RNase III in processing *E. coli* mRNAs if DNAs from specialized transducing phages were used as the templates for RNA synthesis.

Some primary sites in polycistronic T7 early RNA seem to require more enzyme for cleavage than others. The second and third primary sites appear to be cleaved most efficiently. The reason for this apparent difference in susceptibility of primary sites is not known. It might be caused by differences in the nucleotide sequence of the sites or simply reflect the degree to which the conformation of the polycistronic RNA affects the accessibility of the sites to RNase III. However, coupling of transcription with RNase III cleavage should equalize accessibility of all sites to RNase III, and under these conditions the primary sites still seem to be heterogenous.

Evidence that RNase III recognizes particular nucleotide sequences at the primary sites in polycistronic T7 early RNA has been obtained (6, 7). Since RNase III degrades double-stranded RNA and double-stranded RNA inhibits cleavages in single-stranded RNA it seems likely that primary sites also contain regions of base pairing as a common structural element (2, 5). Structures of this type are common in most RNAs that have been sequenced (30) and are thought to be frequent in the individual T7 early RNAs (31). Possibly such regions of localized base pairings are the secondary RNase III cleavage sites. Primary sites might contain in addition to the suggestion sequence element a more perfect or larger helical structure than is found at secondary sites. This might allow primary sites to bind RNase III more efficiently than secondary sites at moderate ionic strength and account for the observation that primary sites are the preferred cleavage sites. Lowering of the monovalent salt concentrations might allow for more efficient binding to secondary sites as well as primary sites resulting in a greater probability that both will be cleaved. This would be consistent with the observation that RNase III cleaves polycistronic T7 early RNA most efficiently at low ionic strength.

It is also possible that the salt concentration effects the enzyme directly causing a change in its conformation or in its monomer-dimer equilibrium with resulting changes in its specificity.

Sites similar to secondary sites in T7 early RNA have also been observed in other RNAs. Paddock et al. (32, 33) have presented evidence that a 140 nucleotide long RNA, synthesized after infection of *E. coli* with bacteriophage T4, T4 species 1 RNA, is cleaved at two sites by RNase III in *vitro*. These sites are apparently secondary sites, since these cleavages are not observed in *vivo* and the optimal salt concentration for cleavage in *vitro* is below 0.03 M (cleavage being strongly inhibited at higher salt concentrations). Both cleavages of T4 species 1 RNA occur at the base of a region of presumed secondary structure containing 17 potential base pairs.

The ability of RNase III to introduce specific breaks at a number of sites should provide information as to how RNase III preferentially recognizes primary sites once the sequence of a number of primary and secondary sites is determined. It also suggests that RNase III might be useful for producing fragments from many RNAs. Both 23 S and 16 S rRNAs from *E. coli* contain several secondary cleavage sites but 5 S RNA apparently has none.2 RNAs from sources unrelated to *E. coli* have also been tried as potential substrates for RNase III. Polio RNA was found to contain no sites equivalent to primary sites but it does contain a number of secondary sites. RNase III cleavage at these sites is currently being used in an attempt to map deletion mutations in the polio genome. Westphal and Crouch (34) have reported that RNase III can cleave adenovirus mRNA, isolated from infected cells, and mammalian 28 S and 18 S rRNAs. In their studies moderately high ionic strength was used and high levels of enzyme were required to effect cleavage, suggesting that these RNAs contain no primary cleavage sites.

Affinity chromatography on double-stranded RNA is an effective way to purify RNase III from *E. coli* and might be useful for purifying similar enzymes from other sources. By analogy with *E. coli* RNase III, enzymes that cut double-stranded RNA might be involved in RNA processing in other organisms.

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