SPECIFICITY OF CLEAVAGE BY RIBONUCLEASE III

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SUMMARY: The specificity of RNase III for various synthetic homopolymeric double-stranded RNA substrates have been examined. Although RNase III appears to cleave all homopolymeric RNA duplex structures, with Poly (U)·Poly (A) as the substrate, the enzyme cleaves the Poly (U) strand much faster than it cleaves the Poly (A) strand. Under conditions where the Poly (U) strand is quantitatively cleaved into acid-soluble fragments ranging in size between 5-8 nucleotides in length, the poly (A) strand is cleaved into large fragments 40-60 nucleotides in length. These results indicate that RNase III recognizes duplex RNA structures for binding, and makes single-stranded scissions and suggests that the enzyme has a preference for cleaving adjacent to UMP residues over AMP residues in polynucleotide chains.

INTRODUCTION: RNase III has been shown to specifically cleave a variety of synthetic and native double-stranded RNAs to yield oligonucleotides possessing 5'-phosphoryl and 3'-hydroxyl termini (1-3). Recently the enzyme has received considerable attention because of its role in the maturation of *E. coli* rRNAs (4-6) and of high molecular weight T7 early mRNAs (2,7).

In the present communication we have studied the specificity of RNase III for various synthetic homopolymeric double-stranded RNA substrates. It has been observed that with a duplex homopolymer Poly (A)·Poly (U) as substrate, the enzyme cleaves the Poly (U) strand into acid-soluble nucleotides much faster than it cleaves the Poly (A) strand indicating that the enzyme specifically discriminates between different nucleotide bases in polynucleotide chains. Furthermore the size of the products formed from the Poly (A) strand are much larger (40-60 nucleotides in length) than those produced from the Poly (U) strand (5-8 nucleotides in length). These results suggest that RNase III recognizes duplex RNA structures for binding, and makes single-stranded scissions.

MATERIALS AND METHODS: RNase III was prepared by a modification of the procedure described by Robertson et al. (1,2). The final enzyme preparation had a specific activity of 68,000 units/mg protein under standard assay conditions described below. The enzyme preparation was free of endo- and exoribonuclease activities towards f2 RNA. [^{3}H]Poly(I)·Poly(C) was prepared as follows: RNA·RNA hybridization was carried out at room temperature for 2 hrs in reaction mixtures (1 ml) containing 10 mM sodium phosphate buffer, pH 7.7, 0.1 M NaCl, 18 A_{260} units each of Poly (C) and Poly (I), one of which was labeled with ^{3}H (specific activity 12,000 cpm/nmole). Poly(A)·Poly(U) was similarly prepared as were synthetic DNA·RNA hybrids with equimolar amounts of polyribonucleotides and polydeoxynucleotides. [^{3}H]RNA·fd DNA hybrids were prepared with E. coli RNA polymerase as described by Berkower et al. (8).

Assay of RNase III activity - The assay for RNase III activity was carried out as follows: reaction mixtures (0.1 ml) containing 50 mM Tris·HCl, pH 7.8, 10 mM magnesium acetate, 0.1 M KCl, 10 μg of dialyzed bovine serum albumin, 500 pmoles (expressed as residues of ribonucleotides) of [^{3}H]Poly(I)·Poly(C) (12,000 cpm per nmole), and varying amounts of RNase III (usually between 0.05 to 0.1 unit) were incubated for 15 min at 37°. The reaction was stopped by the addition of 0.1 ml of bovine serum albumin (10 mg per ml) and 2 ml of ice-cold 5% CCl_3·COOH. After standing in ice for 5 min, the acid-insoluble material was recovered by centrifugation for 5 min at 8,000 x g in an International refrigerated centrifuge. The pellet was dissolved in 0.5 ml of 0.2 M NH_4OH and the solution thus obtained was counted in 10 ml of scintillation fluid in a scintillation counter. One unit of RNAse III activity was the amount of enzyme which resulted in a decrease of 1 nmole of acid precipitable radioactivity in 15 min at 37°.

Table I

Substrate Specificity of RNase III

Additions	Activity
	(pmoles/15 min)
[^{3}H]Poly(I)·Poly(C)	110
[^{3}H]Poly(C)·Poly(I)	209
[^{3}H]Poly(U)·Poly(A)	355
[^{3}H]Poly(A)·Poly(U)	9
[^{3}H]Poly(C)·Poly(dI)	< 3
[^{3}H]Poly(U)·Poly(dA)	< 3
[^{3}H]RNA·ØX174 DNA	< 3

Reaction mixtures (0.1 ml) with 0.15 unit of RNase III were prepared as described in "Methods" except that 500 pmoles of various double-stranded synthetic polyribonucleotides (specific activity 10,000-20,000 cpm/nmole) were added as indicated. Incubation was at 30° for 15 min and the decrease in acid-precipitable radioactivity was determined as described in Methods.

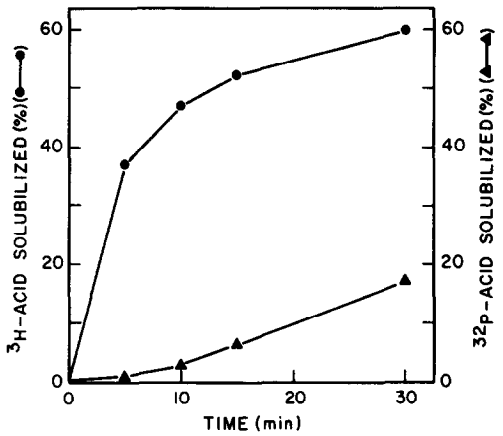

Fig. 1. Degradation of [^{3}H]PolyU)·[^{32}P]Poly(A) by RNase III. Reaction mixture (0.5 ml) was prepared as described under Methods except that [^{3}H]Poly(U)·[^{32}P]Poly(A) (8.6 nmoles containing 48,000 cpm of ^{3}H and 23,000 cpm of ^{32}P) was used as the substrate with 2.7 units of RNase III. Incubation was at 30°; at different intervals, aliquots of 0.1 ml were removed and 5% CCl_3·COOH-insoluble radioactivity was determined as described under Methods.

RESULTS

Substrate specificity of RNase III - The specificity of RNase III for various synthetic homopolymeric double-stranded RNA substrates was examined (Table I). Each ribohomopolymer was cleaved by RNase III after it was annealed to its complementary ribopolymer. Little activity was detected with polyribonucleotides alone (data not shown) or with polymers annealed to their complementary deoxyribopolymers.

The rate of degradation of different homopolymeric RNA·RNA duplexes by RNase III varied as much as 40-fold (Table I). When [^{3}H]Poly(U)·[^{32}P]Poly(A) was used as the substrate for RNase III, the rate of cleavage of the Poly (U) strand into acid-soluble fragments was nearly 6-10 times faster than that of the Poly (A) strand (Fig. 1).

The extent of degradation of each strand as a function of RNase III concentration was also measured (Fig. 2). At a concentration of RNase III where essentially all of the Poly(U) strand was degraded to acid-soluble oligonucleotides, only 15-20% acid-solubilization of the Poly (A) strand was detected. In the presence of 3-4-fold excess of RNase III, only 23% of Poly (A) was degraded

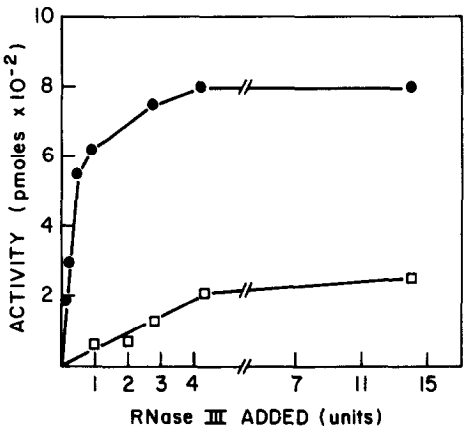

Fig. 2. Dependency of RNase III activity on enzyme concentration. RNase III activity was measured in two separate reaction mixtures as described under Methods in the presence of increasing amounts of enzyme except that one reaction mixture contained 870 pmoles of [^{3}H]Poly(U)·Poly(A) while the other contained 870 pmoles of [^{3}H]Poly(A)·Poly(U) as substrate. The reaction mixtures were incubated for 15 min at 30°. ●——●, [^{3}H]Poly(U)·Poly(A); □——□, [^{3}H]Poly(A)·Poly(U).

into acid soluble fragments (Fig. 2). Increasing the time of incubation to 1 hr with 14 units of RNase III did not lead to further acid-solubilization of Poly (A) (data not shown).

The extent of degradation of [^{3}H]Poly(A)·Poly(U) was further studied (data not shown). In the presence of a 6-fold excess of RNase III (14 units), only 20% of Poly (A) was degraded to an acid-soluble form in 15 min. The addition of more enzyme at 15 min had no further effect. Since under the conditions of the assay the Poly (U) originally present in the duplex structures was quantitatively cleaved into an acid-soluble form, the absence of further acid-solubilization of Poly (A) could be attributed to the lack of Poly(A)·Poly(U) duplex substrates. However, the addition of an equal amount of Poly (U) after 15 min exposure of [^{3}H]Poly(A)·Poly(U) substrate to the enzyme resulted in no further degradation of Poly (A). In contrast, the addition of an equal amount of [^{32}P]Poly(A)·[^{3}H]Poly(U) followed by incubation for an additional 30 min resulted in quantitative cleavage of [^{3}H] Poly (U) (within 5 min) while [^{32}P]Poly(A) was only solubilized to the extent of 18% (data not shown).

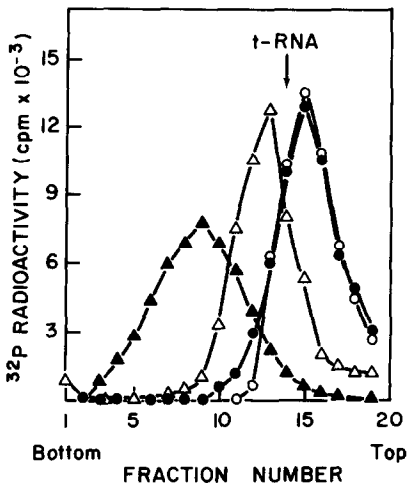

Fig. 3. Cleavage of [^{32}P]Poly(A)·Poly(U) by RNase III. Reaction mixtures (0.1 ml) were the same as described under Methods except that [^{3}H]Poly(I)·Poly(C) was replaced by [^{32}P]Poly(A)·Poly(U) (7.5 nmoles containing 60,000 cpm). Incubation was for 1 hr at 30° with various levels of RNase III as indicated below. The reaction was terminated by adding EDTA to 50 mM and sodium dodecyl sulfate to 0.5%. After incubation at 37° for 2 min, the reaction mixture was chilled in ice and insoluble material removed by centrifugation. Formaldehyde treatment to destroy RNA secondary structure was carried out by adding sodium phosphate buffer, pH 7.7 to 0.1 M and formaldehyde to 3% (v/v), and then heating the mixture at 65° for 15 min followed by cooling to 20° as described by Boedtker (10). Aliquots were layered onto 5 ml of a 5 to 25% linear sucrose gradient. Each gradient contained 0.1 M sodium phosphate buffer, pH 7.7, 3% HCHO (v/v), and 1 mM EDTA. Tubes were centrifuged in an SW 50.1 rotor for 3.5 hrs at 49,000 rpm at 20°. Following centrifugation, fractions of 0.25 ml were collected from each tube and their radioactivity content determined. ▲——▲, no RNase III added. Δ——Δ, treated with 0.5 unit of RNase III; 0——0, treated with 2.5 units of RNase III; ●——●, treated with 5 units of RNase III.

Nature of Products formed by RNase III action on Poly(U)·Poly(A) - The nature of the acid-insoluble Poly (A) product remaining after exhaustive RNase III treatment of [^{32}P]Poly(A)·Poly(U) was investigated by HCHO-sucrose gradient analysis (Fig. 3). It is evident that all the high molecular weight Poly (A) was endonucleolytically cleaved to yield Poly (A) products which sedimented slightly slower than 4S. This value suggests that the chain length of the Poly (A) product is between 40 and 60 nucleotides. These results show that RNase III cleaves Poly (A), and that the resulting product of cleavage is largely acid-insoluble and is much larger in size than those obtained from the cleavage of Poly (U).

The products formed after exhaustive digestion of [^{3}H]Poly(U)·Poly(A) by

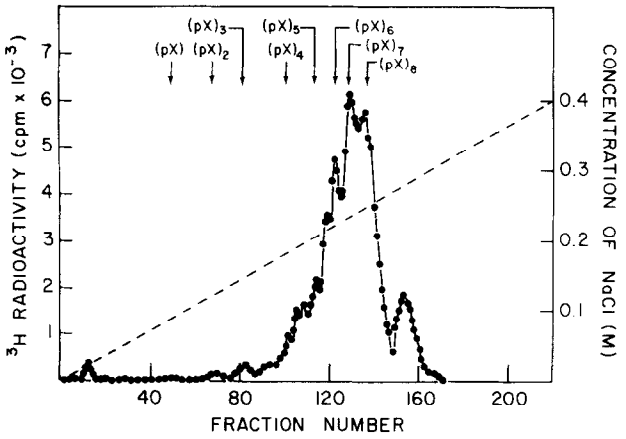

Fig. 4. Size distribution of complete digests of [^{3}H]Poly(U)·Poly(A) by RNase III. Reaction mixture (1.0 ml) was prepared as described in Methods except that various additions were scaled up 10-fold and 87 nmoles of [^{3}H]Poly(U)·Poly(A) (containing 410,000 cpm) was used as the substrate. Incubation was at 30° for 1 hr with 80 units of RNase III. Under these conditions all [^{3}H]Poly(U) present in duplex structures were quantitatively cleaved into 5% CCl_3COOH-soluble form. (The amount of RNase III used in this experiment was actually 3-fold more and the time of incubation was 6 times longer than necessary to obtain complete acid solubility of [^{3}H] Poly(U) strand.) The reaction was terminated by diluting the reaction mixture to 5 ml with water and adding solid urea to a final concentration of 7M. The mixture was applied to a column of DEAE-cellulose (0.7 x 30 cm) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0 containing 7 M urea. After washing the column with 5 ml of the above column-equilibrating buffer, a linear gradient of 400 ml total volume from 20 mM Tris-HCl buffer, pH 8.0 containing 7 M urea to 20 mM Tris-HCl buffer, pH 8.0 containing 7 M urea + 0.4 M NaCl was then applied. Fractions of 1.8 ml were collected and their radioactivity content were determined by counting 0.5 ml aliquots with 10 ml of "Aquasol" (New England Nuclear Corp.). The elution positions of oligonucleotides of varying chain length in this column was ascertained by determining the elution profile of products formed by exhaustive digestion of 5 mg of total E. coli t-RNA with 0.5 mg of pancreatic RNase in 10 mM Tris-HCl buffer, pH 7.4, in a total volume of 1 ml. Under the above reaction conditions, incubation at 37° for 7 hrs gave limit digest of tRNA which consist of nucleoside monophosphates and oligonucleotides of chain lengths varying from 2 to 8 nucleotides in length.

RNase III were also analyzed by chromatography on DEAE-cellulose in 7 M urea (pH 8.0) (Fig. 4). Under the conditions of the analysis, most of the untreated [^{3}H]Poly(U)·Poly(A) substrate remained absorbed in the column and less than 5% of the radioactivity eluted in 0.3 M KCl as a single peak (data not shown). In contrast, when the products formed after complete digestion of [^{3}H]Poly(U)·Poly(A) with RNase III were analyzed, radioactivity at the position of untreated substrate was not detected (Fig. 4). However, there was a marked increase in the label eluting from the column corresponding to penta-, hexa-, hepta- and octa-

nucleotides. This experiment shows that Poly(U)·Poly(A) duplex structures are endonucleolytically cleaved by RNase III, and the main products of degradation of the Poly (U) strand are 5-8 nucleotides in length.

DISCUSSION: Results presented in this communication clearly demonstrate that when Poly(U)·Poly(A) is used as substrate for RNase III, the enzyme shows a marked preference for the cleavage of the Poly(U) strand over the Poly(A) strand. Under conditions where Poly(U) is quantitatively cleaved to acid-soluble fragments ranging in size between 5-8 nucleotides in length, the Poly (A) strand is cleaved to fragments, 40-60 nucleotides in length which are largely acid-precipitable. These results indicate that RNase III recognizes duplex RNA structures for binding, and makes single-stranded scissions and that the enzyme presumably has a preference for cleaving UMP residues over AMP residues in polynucleotide chains.

In vivo, RNase III is involved in introducing cleavages at a limited number of specific sites during maturation of RNA precursors (2,4-7). It is now generally accepted that a combination of both the double-stranded structure and the nucleotide sequence may be required for the processing event to occur. It is conceivable that precursor RNA molecules containing double-stranded regions of homopolymeric Poly(A)·Poly(U) sequences of about 40 nucleotides or less in length can be recognized by RNase III. If this were the case, in such regions the enzyme would cleave the UMP residues without attacking Poly (A) residues. Thus, RNase III could exhibit base specificity in its action on duplex polynucleotide chains. It is noteworthy that Rosenberg et al. (9) have determined the 5'- and 3'-terminal oligonucleotide sequences of several T7 RNA species produced by the action of purified RNase III on a precursor molecule transcribed in vitro by E. coli RNA polymerase. They observed the presence of a unique 3'-terminal sequence, $C_{2-3}U_3A\text{-}U_{OH}$ in all RNA products formed after RNase III action which suggests that sequence recognition is probably involved in RNase III catalyzed hydrolysis. It would be of considerable interest to sequence the 5'- and 3'-terminal nucleotides of products formed after RNase III action on several natural double-stranded RNA substrates in order

to determine whether sequence recognition is indeed involved in the action of RNase III.

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