ESCHERICHIA COLI RNASE III CLEAVES HELA CELL NUCLEAR RNA

by

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SUMMARY: Highly purified RNase III, which is specific for double-stranded RNA gives limited cleavage of nucleolar 45S RNA and nuclear heterogeneous RNA from HeLa cells. Major cleavage products of 45S RNA are comparable in mobility to the *in vivo* products. 45S RNA which had incorporated toyocamycin or which was treated with ethidium bromide was not cleaved by ribonuclease III. The results suggest that there are specific signals at double-stranded regions in the nuclear RNA, which are recognized by an enzyme during RNA maturation.

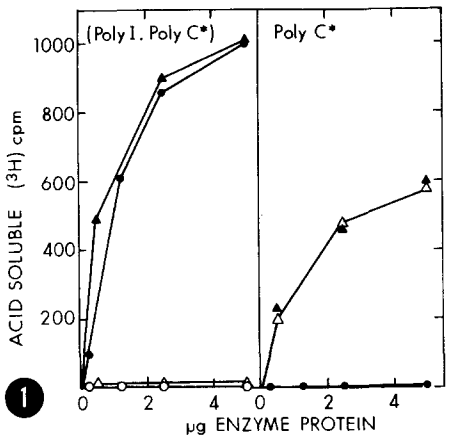
INTRODUCTION

RNase III of *E. coli* has been implicated in maturation of *E. coli* pre-ribosomal RNA (1-3) and T7 messenger RNA (2-4). The enzyme acts specifically at double-stranded regions (5). Such regions may also be important sites in the processing of nuclear RNA of mammalian cells, for intercalating agents or base analogues that weaken base-pairing (6) inhibit nuclear processing of both rRNA (7) and heterogeneous nuclear RNA (8).

We have treated HeLa 45S RNA and heterogeneous nuclear RNA with *E. coli* RNase III. Both types of RNA are cleaved to a limited extent. The products have sizes consistent with the possibility that RNase III may, with some specificity, select processing sites.

MATERIALS AND METHODS

[^{3}H] poly (C), 8.2 mCi/mmole nucleotide phosphate; [^{3}H] uridine (23 Ci/mmole); [^{3}H] adenosine (15 Ci/mmole); and [^{14}C] uridine (50 mCi/mmole) were from Schwarz/Mann. Unlabeled poly I was from Miles. DEAE-cellulose (Whatman), CM cellulose (Serva), and Sephadex G-200 (Pharmacia) were used. Ethidium bromide and RNase A were Sigma products.



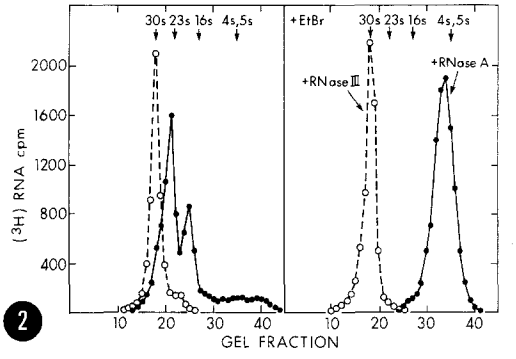


Fig. 1. Specificity of purified RNase III, and inhibition by ethidium bromide. Assays for acid-soluble products were performed in 50 μl, using the conditions recommended for RNase III (1,2,5) with 30 min incubations at 37°C. Each sample contained buffer; 2000 cpm [^{3}H] poly(C) or 1200 cpm [^{3}H] poly(C)·poly(I); the indicated amount of protein; and in some samples, 1.5 mM ethidium bromide. Pure RNase III (see Methods) assayed in the absence (—●—) or in presence (--o--) of ethidium bromide; less pure RNase III in the absence (—▲—) or in presence (--Δ--) of ethidium bromide.

Fig. 2. Gel electrophoretic analysis of RNase III cleavage of E. coli 30S pre-ribosomal RNA, and inhibition by ethidium bromide. Reaction and electrophoresis as in fig. 1, using 1 μg enzyme and 0.1 μg RNA (5000 cpm) in a 30 min incubation. Where indicated, 5 μg pancreatic RNase was used. [^{14}C] labeled E. coli 16S and 23S rRNA added as markers for electrophoresis. At left, 30S pre-rRNA incubated with (—●—) or without (--o--) RNase III. At right, incubation in the presence of ethidium bromide, with RNase III (--o--) or with RNase A (—●—).

RNase III was purified from E. coli A19 according to Robertson et al. (5), except that before the DEAE and CM-cellulose columns, the ammonium sulfate fraction, instead of being dialyzed, was run through a 2 x 30 cm Sephadex G-200 column equilibrated with 0.01 M Tris·HCl, pH 7.5, containing 0.01 M $MgCl_2$; 0.02 M NH_4Cl; 0.005 M β-mercaptoethanol, and 10% glycerol. To remove detectable activity against [^{3}H] poly C (right panel of fig. 1), the three chromatographic steps were repeated using columns half the length, and with a direct elution from CM-cellulose by 0.2 M NH_4Cl, instead of the linear salt gradient.

[^{3}H] poly (C)·poly (I); E. coli [^{14}C] 16S and 23S RNA; and [^{3}H] 30S pre-rRNA were prepared according to ref. 1. [^{3}H] 45S RNA was prepared from 2 x 10^6 S_3 HeLa cells/ml labeled for 15 min with [^{3}H] uridine (25 μC/ml, 23 Ci/mmole).

Toyocamycin containing 45S RNA was prepared from HeLa cells which were preincubated with 0.1 µg/ml toyocamycin for 10 min and labeled 3 hr. Nuclei and nucleoli were made essentially according to Penman (9). The 45S RNA was extracted from nucleoli using 0.5% sodium dodecyl sulfate-phenol at 60°, and the RNA was purified by sucrose density gradient centrifugation (10).

[^{3}H] HnRNA was prepared from 3 x 10^6/ml HeLa cells labeled 20 min with [^{3}H] adenosine (50 µC/ml; 15 Ci/mmole). Nuclei were prepared (9) and nucleic acid extracted with 0.5% sodium dodecyl sulfate-phenol and sedimented through two successive 5 to 20% sucrose gradients in 10 mM sodium acetate, pH 6; 1 mM EDTA; 100 mM NaCl; 0.2% sodium dodecyl sulfate. From each gradient, the RNA larger than 45S was pooled, precipitated with ethanol, and stored at -20°C until just before use.

Each sample prepared as in fig. legends was applied to a 2.95% acrylamide gel and electrophoresed 6 hr at 5 mamp/gel (11). The gels were then sliced and counted (1,12).

RESULTS

Purity of RNase III. RNase III can be separated from single-stranded endonuclease activity (fig. 1: see Methods). More highly purified RNase III (fig. 1: right panel) degraded only the double-stranded substrate. Also, (fig. 1), the action of RNase III on double-stranded RNA was inhibited by the intercalating agent ethidium bromide, while in less purified fractions (fig. 1) the action of a contaminant enzyme against [^{3}H] poly(C) was not inhibited.

Additional control experiments were carried out with 30S pre-ribosomal RNA from E. coli (1-3) as a substrate. Highly purified enzyme cleaved essentially all the 30S pre-rRNA to 25S, 17.5S and smaller RNA fragments within 30 min (fig. 2, left). In contrast to earlier trials with less purified enzyme (1), there was no requirement for added unlabeled mature rRNA to compete away non-specific attack on the primary cleavage products. Intercalation of ethidium bromide

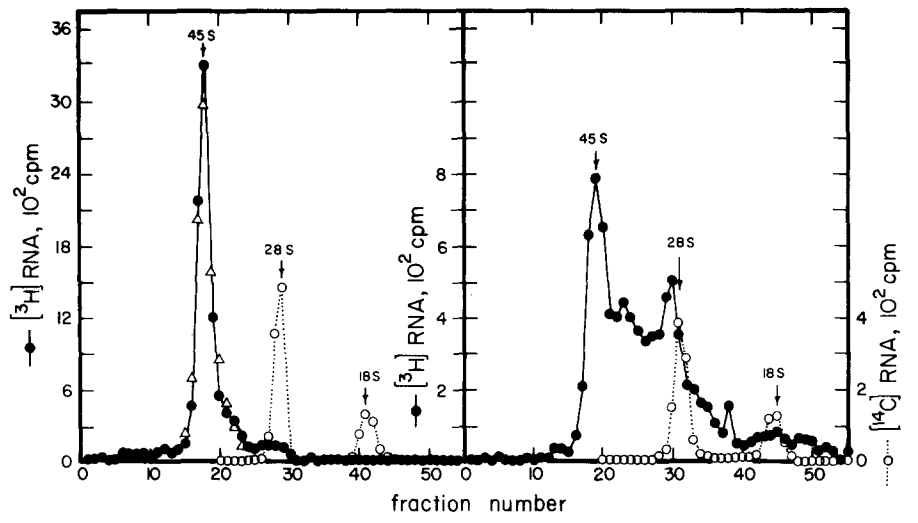


Fig. 3. Gel electrophoretic analysis of cleavage of HeLa cell 45S RNA, and of the effect of ethidium bromide. 45S RNA 0.1 µg; (8000 cpm) was incubated in 120 µl with repurified RNase III (about 1 µg protein) at 37°C for 60 min in buffer (5). In one sample (—Δ—), 0.5 mM ethidium bromide was added. After the reaction, 40 µl of a solution containing 40% sucrose and 2% sodium dodecyl sulfate was added to each sample, and tubes were incubated 5 min at 55°C. A small amount of [^{14}C] labeled 28S and 18 S cytoplasmic rRNA, purified separately, was added to each sample to provide internal markers for electrophoresis. At left, 45S RNA incubated in absence of RNase III (—●—), or in presence of RNase III and 0.5 mM ethidium bromide (—Δ—). At right, analysis of the RNA preparation after incubation with enzyme.

again completely blocked cleavage by RNase III (fig. 2, right). As expected from the trials with single-stranded [^{3}H] poly(C) (fig. 1), ethidium bromide did not prevent degradation of 30S pre-rRNA by RNase A (fig. 2, right).

Cleavage of HeLa nuclear RNA. Purified RNase III cleaved 45S RNA to major fragments with electrophoretic mobility corresponding to 41S and 29S RNA, along with lower levels of smaller fragments (fig. 3). The large products are comparable in size to 41S and 29S cleavage products of 45S RNA *in vivo* (13), though no major peak corresponding to the *in vivo* 32S RNA (13) is seen. In trials with six preparations of 45S RNA and two preparations of enzyme, about 50 to 75% of the RNA was cleaved in 30 to 60 min, after which little if any further cleavage was observed in an additional 60 min incubation. As with the artificial double-stranded RNA (fig. 1) and *E. coli* 30S RNA, cleavage was inhibited by ethidium bromide (fig. 3, left).

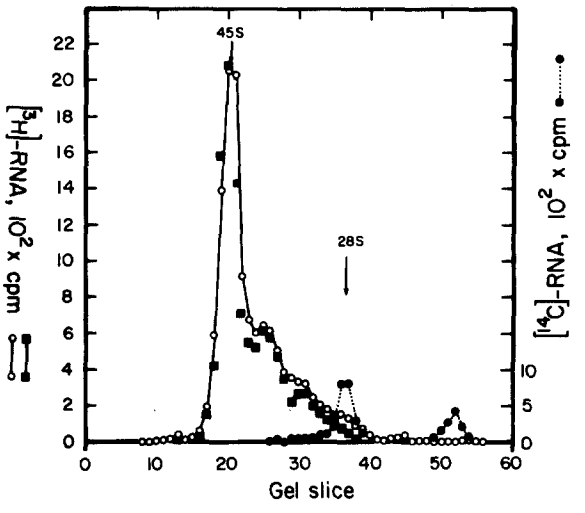


Fig. 4. Gel electrophoresis analysis of cleavage of toyocamycin containing 45S RNA by RNase III. Toyocamycin containing 45S RNA was incubated with RNase III as in Fig. 3. Toyocamycin-containing 45S RNA incubated with (—■—) or without (—o—) RNase III. (--•--), marker RNA.

Cells treated with the adenosine analogue toyocamycin are blocked in rRNA processing (6,14,15). Toyocamycin-containing 45S RNA was purified and found to be resistant to RNase III cleavage (fig. 4).

Limited fragmentation of purified heterogeneous nuclear RNA from growing HeLa cells was also observed (fig. 5). The fraction used was purified by size (> 45S), and at least 60% of it contained 3'-terminal poly(A) sequences characteristic of mRNA, as judged by its capacity to bind to poly dT-columns ([16]; data not shown).

DISCUSSION

These results demonstrate that HeLa cell HnRNA and 45S RNA contain double-stranded regions recognized by an enzyme from an organism at the evolutionary extreme. Double-stranded fragments of nuclear RNA have been prepared by several groups of workers (17-18); probably they exist at a number of points along the RNA, as demonstrated for 45S RNA by Wellauer and Dawid (19). However, the electron micrographs show a large number of such double-stranded segments (19), whereas only a small number of major cleavages by RNase III are observed. By

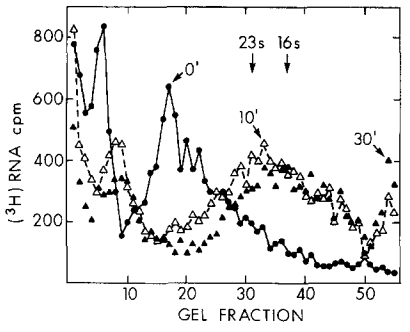


Fig. 5. Gel electrophoresis of RNase III cleavage products of heterogeneous nuclear RNA. 3.5 µg RNA (4.8 x 10^5 cpm) was incubated in 450 µl with RNase III, with or without 12.5 µg enzyme protein, in buffer as in fig. 2. At the indicated times, 50 µl samples were extracted with 0.5% sodium dodecyl sulfate-phenol for 1 min at 60°C, then washed with an equal volume of chloroform. Markers were added and the samples applied to gels for electrophoresis.

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the criterion of gel electrophoresis, the fragments formed are comparable in mobility to the products *in vivo*.

Ethidium bromide, which binds to double-stranded sequences in RNA, inhibits cleavage of 45S RNA (7) and HnRNA (8) *in vivo*, and cleavages by RNase III *in vitro* (fig. 1,2,3). In controls, several RNases that attack single-stranded regions were not inhibited by ethidium bromide (figs. 1,2). Also, RNA containing toyocamycin is resistant to cleavage *in vivo* (15) and resists cleavage by RNase III *in vitro* (fig. 4). This result is especially suggestive because analogues of this class are very similar to adenine, and analogue-substituted ribopolymers differ little from normal ones in many respects, including coding functions (20), but can adopt the syn conformation which would specifically weaken double-helical base-pairing (6).

These results suggest that a specific type or size of double-stranded cleavage signal for the formation of ribosomal and messenger RNA may have been preserved throughout evolution. A possible analogue of RNase III, an RNase active against double-stranded RNA has now been purified from HeLa cell nuclei (21).

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