Specificity of acid RNase from HeLa cell lysosomes

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ABSTRACT

The specificity of the lysosomal acid RNase [Saha, B.K., Graham, M.Y. and Schlessinger, D. (1979). J. Biol. Chem. 5951-5957] has been determined by 5'-end labeling and sequencing gel electrophoresis of rRNA. The enzyme has a predilection for the dinucleotide sequence ...NU..., which is cleaved into ...Np and HOU.... Sequences AU and GU are far more susceptible than CU and UU. The susceptibility of UU and CU sequences is enhanced by the presence of adjacent purine residues. The specificity remains unaltered after partial denaturation of the RNA.

INTRODUCTION

We have purified and characterized an acid ribonuclease from HeLa cell lysosomes (1). The enzyme degraded natural RNAs and polyuridylic acid, but other homopolymers like polycytidylic acid and polyadenylic acid were quite resistant. In this report, I have used 5'-end labeling and gel sequencing techniques to define rigorously the specificity of the acid RNase. This enzyme will be a useful addition to the other specific RNases for RNA sequence analysis.

MATERIALS AND METHODS

Preparation of E. coli rRNA. RNA was isolated from *E. coli* MRE600 ribosomes by the SDS-phenol-chloroform method and precipitated with 2 M LiCl. The 16S rRNA was prepared from the 30S subunit by the above procedure.

End-labeling of RNA. The 16S RNA was first treated with *E. coli* alkaline phosphatase to remove the 5'-p (2), then end-labeled (3) using γ -[³²P]-ATP (New England Nuclear) and polynucleotide kinase (P-L Biochemicals). The labelled RNA was passed through a column of Sephadex G-100, concentrated by alcohol precipitation in presence of carrier tRNA and electrophoresed through a 3% acrylamide-8.3 M urea gel. The band corresponding to the 16S RNA was excised, electrophoretically eluted (ref. 4 and procedure to be published) and

alcohol precipitated with carrier 16S RNA. In 5'-terminal analysis (see below), RNA fragments had 5'-OH ends and were end-labeled directly.

Purification of the acid RNase. Acid RNase was purified from HeLa cells by the published procedure (1) with an additional step of phosphocellulose (P-11) chromatography. The enzyme was at least 80% homogeneous as judged by SDS-polyacrylamide gel electrophoresis and staining, and stable for over 2 yr at -20°C. One unit is defined as that amount of the acid RNase which will render 1 µg of rRNA trichloroacetic acid-soluble in 1 min at 37°C.

Analysis of 5'-termini. Two micrograms (2 µg) of *E. coli* rRNA were treated with 0.03 unit of the acid RNase in 0.1 ml of 0.1 M Tris-acetate (pH 5.2), 10 mM EDTA, 0.02% Triton X-100 and 5% glycerol. After 30 min at 37°C, the reaction was terminated by the addition of 0.1% SDS on ice. RNA fragments were isolated by phenol-chloroform extraction and alcohol precipitation in presence of another 0.2 M Tris-acetate. More than 90% of the RNA was recovered in the pellet as determined in other experiments using [³H]RNA. After end-labeling, the fragments were precipitated three times with alcohol in presence of 10 µg of 16S RNA and 50 mM Na-pyrophosphate to remove unincorporated γ-[³²P]-ATP. The precipitate was dissolved in 0.3 M KOH and hydrolysed for 16-20 h at 37°C. The hydrolysate was neutralized with CO₂, an aliquot was spotted on a cellulose thin-layer plate along with 5 µg of each of the pNps, Nps and ATP. After two-dimensional chromatography (5), the thin-layer plate was exposed to an X-ray film when radioactivity was found to be confined only to a few pNps (see results) and no extraneous radioactivity was present. The area corresponding to each of the pNps, Nps and ATP (as visualized under the UV light) was excised and counted in a liquid scintillation system.

Polyacrylamide gel analysis. The [5'-³²P]16S rRNA (2 x 10⁵ cpm) was mixed with cold 16S RNA (50 µg) and precipitated with alcohol. After drying, the pellet was dissolved in 25 µl of 0.1 M Na-acetate (pH 5.6), 2 mM EDTA and 5% glycerol. The mixture was aliquoted and treated with different levels of the acid RNase as described in the Figure legend. All the tubes were incubated at 37°C for 20 min and then chilled on ice. Five microliters (5 µl) of deionized formamide containing bromphenol blue and xylene cyanol (0.05% each) were added to each tube, and 3 µl from each tube was electrophoresed through 20% acrylamide sequencing gels (40 cm long). Under these conditions, bromphenol blue and xylene cyanol migrated with fragments 7 ± 1 and 23 ± 2 nucleotides in length, respectively (6).

RESULTS

The acid RNase degrades single-stranded RNAs yielding oligonucleotides bearing 3'-p and 5'-OH termini, and little or no mononucleotides (Fig. 8, ref. 1). Also, amongst the homopolymers, only polyuridylic acid is degraded by the acid RNase (Fig. 6, ref. 1). These two observations taken together imply that a uridine residue is involved at the cleavage site of the acid RNase and that oligonucleotides produced by the RNase would bear uridine residues at the 3'- or the 5'-termini. I first analyzed the 5'-terminal bases of the products arguing that these would either be all uridine or all non-uridine residues.

Ribosomal RNA from *E. coli* was digested extensively with the acid RNase and the 5'-OH ends of the oligonucleotides produced were labeled with γ -[^{32}P]-ATP and polynucleotide kinase. Subsequently, the 5'-[^{32}P]-labeled oligonucleotides were hydrolyzed with T_2 RNase or alkali, and the ^{32}pNp residues were separated by two-dimensional chromatography on cellulose thin-layer plates. An analysis of the ^{32}pNp residues is shown in Table 1. It is clear that uridine was present at the 5'-OH ends (77%) of most of the oligonucleotides produced by the acid RNase. This establishes that the acid RNase predominantly cleaves the $\cdots\text{NpU}\cdots$ sites producing $\cdots\text{Nps}$ and $\text{HOUs}\cdots$. A significant amount of ^{32}pGp (15%) was also produced indicating that cleavage occurred at $\cdots\text{NpG}\cdots$ sites to a minor extent. This could arise from contamination by another nuclease in the acid RNase preparation, or

Table 1. Analysis of the 5'-termini of fragments generated after the acid RNase treatment of *E. coli* ribosomal RNA. Counts were about 3,000 cpm for ATP and under 1,500 for each of the Nps, in both the control (without enzyme) and the treated samples.

^{32}pNp	Control cpm $\times 10^{-3}$	Treated cpm $\times 10^{-3}$	Net cpm $\times 10^{-3}$	% Net
^{32}pCp	5.5	8.9	3.4	1.2
^{32}pUp	6.1	225.7	219.6	77.4
^{32}pGp	7.0	48.4	41.4	14.6
^{32}pAp	16.6	35.8	19.2	6.8

could imply a secondary specificity of the acid RNase itself (see below).

In order to establish if there is a specificity at the 3'-side of the phosphodiester bond as well and to see if the enzyme could be useful in the elucidation of the primary structure of RNA, I undertook rapid gel sequencing analysis with 5'-[³²P]-16S rRNA (Fig. 1). Since the primary sequence of *E. coli* 16S RNA has already been elucidated (7-9), one can determine the specificity of the acid RNase directly from the gel pattern. In the first one hundred nucleotides of the 16S RNA, strong cleavages occurred only within the first 70 nucleotides. Analysis on 12% gels confirmed these results and indicated that the next strong cleavage site is at G₁₀₂-U₁₀₃ (results not shown; but see Fig. 1, right panel).

An analysis of the cleavage sites in the first one hundred nucleotides from the 5'-end of the 16S RNA is shown in Table 2. There are 23 NU sequences, of which 5 are AU, 3 GU, 7 CU and 8 UU. Of these, all the AU and GU sequences were cleaved strongly. Only one of the CU (C₃₆-U₃₇) and two of the UU (U₄-U₅ and U₂₉-U₃₀) were cleaved appreciably (Fig. 1). Analysis of the neighboring bases reveals that in each of the latter cases, the cleavage site is flanked by two purines, although C₉₅-U₉₆ was not cleaved appreciably in this experiment (Fig. 1, right panel). Presence of only one purine before or after CU and UU may not be sufficient for a cleavage. Although many of these PyrU sequences are located away from the 5'-end of the RNA, their resistance is not due to involvement in secondary structures as predicted by various models (see Table 2 and refs. 7 - 9). In addition, attempts to denature the RNA by including 50% formamide or 5 M urea in the reaction mixture did not render these sites more susceptible (results not shown). However, the acid RNase was more active (~5 fold) under these conditions (requiring proportionately less enzyme to digest the RNA), perhaps because the secondary structure of the RNA had been disrupted, rendering the nucleotides more accessible to the enzyme.

There are additional weaker cleavage sites in the RNA (Fig. 1). Most noticeably, these are cleavages at UG sites (e.g., U₅-G₆; U₂₀-G₂₁; U₃₀-G₃₁ and U₃₇-G₃₈). In each of these cases UG is preceded by a strong cleavage site. Also, all these cleavages occur near the 5'-end of the RNA, which in general is more susceptible to ribonucleases (10). It is not clear whether these weak cleavages are caused by the acid RNase itself or a contaminating enzyme in the preparation. However, the gel pattern does agree with the chromatographic data indicating presence of a fewer but significant number of HOG residues at the 5'-termini of the oligonucleotides produced by

Fig. 1. Acrylamide (20%)-urea (8.3M) gel electrophoresis of [5'- 32 P]-labeled 16S rRNA after treatment with the acid RNase. Electrophoresis was conducted at 2 KV until xylene cyanol reached ~16 cm and bromphenol blue ~30 cm (left panel); or until xylene cyanol just ran out of the gel (right panel). L, formamide ladder (11) and C, without the acid RNase. Lanes a, b, and c indicate successive two-fold dilution of the acid RNase ($a > b > c$). Tubes a, b, and c contained 8, 5 and 5 μ l of the reaction mixture as described in the Methods. Two microliter of the acid RNase (0.05 unit) was added to tube a, mixed, and 5 μ l was transferred serially to b, c and an empty tube. Incubations and electrophoresis conditions are in the Methods.

the acid RNase (Table 1). Another minor cleavage at $A_{50}-A_{51}$ was absent in other experiments.

The results are summarized in Figure 2. In the first one-hundred

Table 2. Cleavage of NU sequences in the first one hundred nucleotides of *E. coli* 16S rRNA. The arabic numerals represent locations of the nucleotides from the 5'-end of the RNA. A strong cleavage is indicated by (+), a weak cleavage by (±) and no cleavage by (-).

AU	Location	3-4	16-17	19-20	28-29	a,b,c	55-56								
	Cleavage	+	+	+	+		+								
GU	Location	11-12	c	61-62	c	69-70									
	Cleavage	+		+		+									
CU	Location	23-24	c	36-37	a,b,c	48-49	c	87-88	a,b,c	90-91	a,b,c	95-96	b,c		
	Cleavage	-		+		±	-	-		-		±			
UU	Location	4-5	c	12-13	13-14	29-30	a,b,c	84-85	c	88-89	a,b,c	91-92	a,b,c	92-93	b,c
	Cleavage	+		±	-	+		-	-		-		-		-

a,b,c nucleotides involved in double-stranded hair-pin structures according to refs. 7,8 and 9, respectively.

nucleotides of the 16S rRNA, there are eleven strong and eight weak cleavages. The latter are observed only when a higher level of the acid RNase is used (compare Lanes a, b and c in Fig. 1). If one considers only the strong bands, they are due entirely to PurU and PurPyrUPur sequences.

DISCUSSION

RNA is commonly sequenced by enzymatic procedures (6,11,12). The chemical method for the sequencing of RNA (13) works only with 3'-end labeled RNA,

Fig. 2. Cleavage sites at the first one hundred nucleotides from the 5'-end of the *E. coli* 16S rRNA (7,8,9). Solid arrows indicate strong cleavage sites and broken arrows, weak cleavage sites (observed with a higher level of the acid RNase).

whereas the enzymatic procedure works both for the 3'- and the 5'-end labeled RNA. The latter is the only choice if one wants to sequence the RNA from the 5'-end.

Only a few specific ribonucleases have been described which are useful for sequencing. These include T_1 RNase, U_2 RNase, pancreatic RNase, Phyl 1 RNase (14) and the recently described CL_3 RNase (10). Although these enzymes are fairly base-specific, often they will cleave at other nucleotides. Thus T_1 (which is specific for guanine residues) will sometimes cleave at adenine residues and U_2 (which is more specific for adenine residues) will frequently cleave at guanine residues. Pancreatic RNase (which is specific for pyrimidines) is often sequence specific (6) and does not produce limit-digests of pyrimidine stretches (10). Phyl 1 and CL_3 RNases have base specificities in the order $U \gg G \sim A > C$ and $C \gg A > U$, respectively. Clearly there is a need for more of the base specific RNases in order to facilitate direct sequencing of RNA.

The acid RNase analyzed here is specific for a uridine residue at the C5'-oxygen of the phosphodiester bond. The catalytic activity also depends on the nature of the base attached to the C3'-oxygen. Thus cleavage of PurU is much stronger than PyrU. This is in accordance with the observation that rRNA or tRNA is a much better substrate than polyuridylic acid, the only homoribopolymer that is degraded by the acid RNase (1). The PyrU cleavage can be stimulated by the presence of surrounding purines (Figs. 1 and 2).

Assuming that about 1/8 of the purines and 1/8 of the uridines in an RNA are present as PurU sequences, one will be able to detect all these purines and uridines (a total of 25% of the bases) in the RNA with acid RNase. Thus in a sequencing gel, these purines will result in strong bands often followed by empty spaces, indicating the presence of adjacent uridines. Two successive bands would imply a purine and an adjacent uridine from PurUUPur sequences. Few other bands would arise from cytidines present in PurCUPur sequences. The application of the acid RNase in sequencing is demonstrated here with 16S rRNA.

The acid RNase can be obtained from a variety of sources including other cell types and organs like the hog spleen (unpublished results). The ease of its purification, unusual stability during storage and its activity under denaturing conditions, make it a useful tool for sequencing RNA. Two other RNases have been described that could have a similar specificity. One of them was isolated from Citrobacter species (15) and the other from Rhizopus nivosus (16). Like the acid RNase, they both cleave at uridine residues in RNA and hydrolyse polyuridylic acid, the only homoribopolymer degraded to 3'-UMP.

However, no sequencing studies of products have been done to permit any valid comparison of these enzymes with the acid RNase.

Another RNase relevant mentioning here is the (2'-5') oligoadenylate-dependent endoribonuclease, RNase L (17,18). Like the acid RNase, RNase L degrades only polyuridylic acid amongst the homoribopolymers (18). RNase L cleaves primarily after UN sequences (N = A, G or U). In comparison, the acid RNase cleaves within the NU sequences (N = A or G, predominantly). Thus, uridine could be involved in the recognition site for either of these enzymes. The physiological role of both these RNases remains speculative.

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