Properties of a monovalent and divalent requiring endoribonuclease with novel specificity from calf heart

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ABSTRACT

A new monovalent and divalent requiring endoribonuclease has been identified in extracts of calf heart tissue using a natural substrate molecule. This ribonuclease creates 5' hydroxyl and 3' phosphate groups in its cleavage products. It cleaves predominantly between pyrimidine and A residues though not all such possible sites are attacked in the substrates tested.

INTRODUCTION

Extracts of mammalian cells manifest many ribonuclease activities (1). Relatively few of these have been characterized using natural substrate molecules. Because of the possible involvement of specific endoribonucleases in RNA splicing events, we have searched for such enzymatic activities in a convenient source of mammalian tissue, calf heart, using natural substrate molecules. We have identified a calcium or magnesium activated specific endoribonuclease which cleaves a tRNA precursor molecule, a bacteriophage ϕ 80 encoded small RNA and a transcript from mouse β -globin cloned DNA. This endoribonuclease produces 5' hydroxyl purine and 3' pyrimidine phosphate groups in its cleavage products.

EXPERIMENTAL PROCEDURE**Materials**

DEAE-Sephadex A-50, Sephadex G-100, Sephadex G-200 and Blue Sepharose were purchased from Pharmacia, Uppsala, Sweden. The DEAE-Sephadex was washed and prepared for use according to Robertson et al. (2). Hydroxylapatite, and Bio-beads SM-2 were obtained from Biorad.

Inorganic ^{32}P phosphate and α - ^{32}P -GTP were purchased from New England Nuclear and Amersham Radiochemicals, respectively. S-Adenosylmethionine was obtained from Sigma Chemical Corp.

Methods

Preparation of ^{32}P -labelled substrates: ^{32}P -labelled tRNA^{Tyr} precursor and M3 RNA were extracted from 10% polyacrylamide gels as previously described (3). β -globin mRNA precursor RNA was transcribed from a purified mouse β -globin DNA fragment (BamHI-cleaved MGS-1 DNA) as described (4), and eluted from gel slices by grinding and soaking in 1M NaCl. The eluted β -globin mRNA precursor fragment was further purified (5) and dialyzed versus double distilled water.

Assay of RNase CH²: Unless otherwise noted, all reactions were carried out in a final volume of 30 μ l in silicone-treated test tubes at 37°C for 15 min, under the same conditions as for the RNase P reaction (2). Each reaction mixture contained 30mM Tris-HCl, pH 8.0; 100mM NH₄Cl; 5mM MgCl₂; 0.1mM EDTA; 0.1mM β -mercaptoethanol; substrate containing from 6,000 to 8,000 cpm of ^{32}P ; and enzyme. Reactions were terminated by adding 5 μ l of 0.4M EDTA, pH 9.4, containing 0.1% bromophenol blue. Samples were analyzed on a 10% acrylamide slab gel as described previously (2).

Positions of the bands in the gel were determined by radioautography.

Protein assay - Protein concentration was determined by the method of Bradford (6).

Fingerprint analysis: Fingerprints of RNase T₁ or RNase A digests of substrates and products were carried out on PEI plates according to the method of Platt and Yanofsky (7).

Purification of RNase CH:

All steps of the purification procedures described herein were carried out at 4°C. 6mM β -mercaptoethanol was present in all buffers.

Step 1: Preparation of supernatant fraction (S16) from calf heart - Calf heart (548g), defatted and sliced, was homogenized in 384ml of ice cold buffer TM (20mM Tris-HCl, pH 7.6; 15mM Mg(OAc)₂; 6mM β -mercaptoethanol) containing 0.02M NH₄Cl and 20mM PMSF. The buffer was then made 0.7% in triton X-100 and 0.3% in sodium deoxycholate. The homogenate was left for 30 min on ice and then centrifuged at 16,300xg for 30 min.

Step 2: DEAE-Sephadex A-50 chromatography - The S16 (740ml) was diluted two fold with buffer TM containing 0.02 M NH₄Cl (we call this 0.02M Buffer TM) and loaded onto a DEAE Sephadex A-50 column (bed volume 2.31; 11.5 x 23cm). The column was washed with 2.6l of 0.06M Buffer TM. The enzyme was eluted with 0.15M Buffer TM. (Fig. 1A). After the initial 800 mls of eluate, 20 ml fractions were collected. 3 μ l from each fraction were assayed for protein concentration.

Step 3: Ammonium sulfate fractionation - The pooled, active fraction from Step 2 was passed through a Bio-beads column (1.1 x 10cm) at a flow rate of 100ml/hr to remove the Triton X-100. The resulting active fraction was concentrated using $(\text{NH}_4)_2\text{SO}_4$. The 25-37% (0.25g to 0.37g/ml original volume) $(\text{NH}_4)_2\text{SO}_4$ pellet was resuspended in 0.02M Buffer TM.

Step 4: Sephadex G-100 chromatography - The concentrated fraction (17ml) from Step 3 was applied to a Sephadex G-100 column (bed volume, 1.31; 4.2 x 96cm) equilibrated with 0.02M Buffer TM. (Fig. 1B). 20 ml fractions were collected and 5 μ l of each assayed for protein concentration.

Step 5: Second DEAE-Sephadex A-50 chromatography - The active Sephadex G-100 fraction (fractions 25 and 26, 66mg total protein) was applied to a DEAE-Sephadex A-50 column (1.1 x 23cm) equilibrated with buffer TM as described for Step 1. Elution was carried out with a linear gradient using 60 mls of 0.02 M buffer TM and 60 mls of 0.25 M buffer TM in the reservoirs. 3.2 ml fractions were collected and 3 μ l of each assayed for protein concentration. The enzyme activity eluted from 0.075M to 0.1M NH_4Cl (Fig. 1C).

Step 6: Sephadex G-200 chromatography - The active fraction from Step 5 was concentrated by addition of $(\text{NH}_4)_2\text{SO}_4$ to 37% and subjected to Sephadex G-200 chromatography (Fig. 1D), in 0.2M Buffer TM. Fractions of 1.8 ml were collected. 54% of the enzyme activity could be recovered from Sephadex G-200 (Fig. 1D) and was purified at least 28-fold by this step.

Step 7A: Hydroxylapatite chromatography - An aliquot (2.1ml, 0.46mg protein) of the active fraction from Step 6 was dialyzed first against 20mM Tris-HCl (pH 7.5) for 1.5hr and then against 25mM potassium phosphate buffer (pH 7.0) for 1.5hr and applied to a hydroxylapatite column (0.9 x 0.7cm) (Fig. 1E). Elution was carried out with a linear gradient (8 mls total volume) from 25 mM to 250 mM potassium phosphate (pH 7.0). Fractions 1-4 were 1 ml each and fractions 5-30 0.45 ml each. The enzyme activity separated into two peaks: a flow through fraction (HA I) and a bound fraction (0.15M to 0.2M PO_4^{3-} ; HA II). The ratio of activities of HA I to HA II was 12:1. HA I or HA II were purified at least 40 or 35 times, respectively, from the Sephadex G-200 fraction.

Step 7B: Blue Sepharose chromatography: Active fractions from the G-200 column (0.39 mg) were applied to a small column (0.5 x 3.2 cm) of Blue sepharose (Fig. 1F) equilibrated with 0.1M buffer TM. The column was washed with 50mM Tris HCl (pH 7.5) containing 0.2M NaCl and 10mM Mg

Figure 1: Chromatographic properties of RNase CH. Each panel represents the elution profile on different columns as described in the Methods. The solid horizontal bar represents the peak of enzymatic activity. The left-hand ordinate is the relative protein concentration as determined by the method of Bradford(6) and the right-hand ordinate (when necessary) is the salt concentration. A. first DEAE-Sephadex A-50; B. G-100 Sephadex; C. Second DEAE-Sephadex A-50; D. G-200 Sephadex; E. Hydroxylapatite; F. Blue Sepharose. Arrows indicate application of (a) 0.2 M NaCl; (b) SAM; (c) 0.5 M NaCl. (•) Protein concentration. (—) Salt concentration. For the G-100 Sephadex column shown in panel B and the G-200 Sephadex column shown in panel D, the void volume consisted of the first twenty fractions.

(OAc)₂; the same buffer containing 5mM S-adenosyl methionine (SAM), 50 mM Tris-HCl (pH 7.5) and 0.5M NaCl, and finally with 0.1M Tris HCl (pH 8.9), 10mM Mg (OAc)₂, 0.5M NaCl. The enzyme eluted with the final wash (Fig. 1F) and recovery of activity was more than 50%. SAM does affect enzyme activity (see Results) but had no effect on its elution properties. Fractions 1-15 were 0.35ml each and fractions 16-20 0.7mls each.

RESULTS

Fractionation of RNase CH:

The partial purification of RNase CH from crude cell extracts is illustrated in Figure 1, which represents the various column chromatographic steps used in the fractionation. To optimize yield of the

RNase CH activity, we pooled fractions around the activity peak from each column (as shown by the thick horizontal bars in Figure 1) for use in each successive step. A considerable amount of non-specific nuclease activity can be found contaminating the peak RNase CH fractions until the third column chromatographic step is reached (Fig. 1, C). Because of this problem and the fact that the enzymatic activity is of uncertain stability as it is purified, estimates of the purification factor are difficult to make. RNase CH activity could be estimated with some accuracy only in those fractions free of non-specific nuclease activities (as determined by the recovery of Bands 3 and 4 in the RNase CH assays; see Figure 2). Activity was determined by cutting out gel slices corresponding to substrate and to Bands 1 and 2 resulting from any particular reaction incubation, determining the radioactivity in the gel slices by counting Cerenkov radiation and thereby estimating the extent of the reaction. Purification factors were calculated by comparing specific activities of peak fractions from successive column chromatographic steps. Using these methods, we estimate the purification factor merely from Step 5 through Step 7A (Fig. 1, C and E) is about one thousand-fold (see Methods) and thus the overall purification of RNase CH from the crude extract through Step 7A must be greater than this factor. Purification of the ribonuclease activity can be followed with any of the substrates we describe in the Methods. We found that RNase CH requires either Mg^{2+} or Ca^{2+} and a monovalent cation (see below). However, we routinely carried out assays during purification in the presence of Mg^{2+} in order to follow the separation of RNase CH from other Mg^{2+} requiring nucleases. RNase CH yields a unique pattern of cleavage products from the tRNA^{Tyr} precursor substrate (see Fig. 2). The properties of the enzyme, that is, ion requirements, etc., do not change significantly as the activity is purified. If the purest fractions are dansylated (8), electrophoresed in SDS-acrylamide gels and illuminated with UV light, fewer than ten distinct bands are visible. Thus, our best enzyme preparations are probably between 1-10% pure.

Cleavage Specificity on tRNA^{Tyr} precursor:

There are six distinct products resulting from the action of RNase CH on tRNA^{Tyr} precursor as shown in (Fig. 2). The time-course of digestion experiment shows that Bands 1a, 1b (which are difficult to resolve) and 3a, 3b appear first, and are followed by the appearance of Bands 2 and 4. Subsequently, the amounts of material in Bands 1a, b and 3a, b decrease as

Figure 2: Time course of a RNase CH cleavage of *E. coli* tRNA^{Tyr} precursor. The times, in minutes, when aliquots were taken from the reaction mixture, are shown at the top of the figure. 'P' refers to a control reaction with *E. coli* RNase P. The reaction mixture (180μl) composition is described in the Methods. The enzyme preparation used was the Sephadex G-200 fraction. Samples (30μl) were removed at the indicated times, the reaction stopped, and electrophoresed as described in Methods. At the right-hand side of the figure, 'p', 't' and '5'' indicate, respectively, the positions of intact tRNA^{Tyr} precursor RNA, the RNase P cleavage fragment containing the mature tRNA^{Tyr} sequence, and the RNase P cleavage fragment containing 43 nucleotides proximal to the 5' terminus of the precursor RNA.

the amounts in Bands 2 and 4 increase. Thus, Bands 1a and 1b or 3a and 3b maybe intermediates on a pathway to Band 2 or 4, respectively. [Band 2 migrates in the same position as the 3'-fragment of the tRNA^{Tyr} precursor which results from digestion with RNase P (Fig. 2, Lane P)]. All products shown in Fig. 2 were eluted from the gel and digested further with RNase CA. Bands 1a and 1b yielded a product with the same mobility as Band 2, though Band 1b was more resistant to RNase CH than Band 1a, whereas Bands 3a and 3b gave a product with the mobility of Band 4 as expected. Bands 2

and 4 were resistant to further digestion by RNase CH. We inferred that the Band 2 nucleotide sequence was contained in both the sequences of Band 1a and Band 1b and that the sequence in Band 4 was contained in the sequences of Band 3a and Band 3b. The mode of action of RNase CH was confirmed further by fingerprint analyses of all digestion products³. The results of these analyses are shown in Fig. 3 and the positions of the cleavage sites in the precursor nucleotide sequence are shown in Fig. 4A and B.

The products of a RNase T₁ digestion of Band 1a and 1b are shown in Fig. 3B and 3C, respectively. It can be seen, in comparison with a schematic figure of a RNase T₁ digestion of precursor to tRNA^{Tyr} (Fig. 3A), that both bands contain the mature tRNA sequence (see also Table 1). The 5' terminus of mature tRNA^{Tyr} (pG) is missing in the fingerprints of Bands 1a and 1b, but new Spots X and Y, respectively, appear in these fingerprints. From the results of secondary RNase A analysis of these spots, we infer that Spot X could be AUUACCCG or CAUUACCCG (Table 1). The latter possibility was excluded, because an oligonucleotide, UAAAAAGC, was detected in a RNase A digestion of Band 3b (see below and Fig. 4A). Spot Y was deduced to be ACCCCG (Table 1). Band 2 gave the same pattern of a RNase T₁ digestion as that of mature tRNA^{Tyr} (see Fig. 3A and ref. 2) except that pG was missing. The fingerprints of RNase A digestion of Bands 1a, 1b and 2 were identical. The fingerprint of a RNase A digestion of the 3'-fragment of the RNase P digestion of intact precursor tRNA^{Tyr} has one spot (pGGU) which is absent from the former RNase A fingerprints: they, however, have one new spot, Spot M, (Fig. 3F). Spot M has two moles of G and one mole of U as shown by secondary digestion. We inferred that spot M is GGU and thus we concluded that Band 2 contains the same polynucleotide sequence as the 3'-fragment of the RNase P digestion of tRNA^{Tyr} precursor except for the 5' phosphate group. (We determined separately that our RNase CH preparation had no accompanying phosphatase activity.) We further concluded that Bands 1a and b, within which the sequence of Band 2 is contained, include the 3' terminus of the precursor tRNA molecule.

The products of a RNase T₁ digestion of Band 3a are shown in Fig. 3D. A new Spot Z can be seen and is inferred from secondary digestion to have the sequence CAUU. Band 3b gave the same pattern as Band 3a (data not shown) except for Spot Z. We recovered the polynucleotides (especially pppGU and AAAAAGC) from a RNase A digestion of Band 3a or 3b, indicating

that both bands contain the sequence beginning with the 5' end of the precursor tRNA^{Tyr} substrate. Thus, Bands 1a and 3b, and Bands 1b and 3a respectively are products of single cleavage events at the sites labelled I in Fig. 4A and B. (We did not recover C in a fingerprint of a RNase T₁ digestion of Band 3b because of the slow mobility of this nucleotide in our system.) The yield of products from the cleavages at the sites I in Fig. 4A and B indicate that cleavage by RNase CH occurs at each of those sites with about equal probability.

The products of a RNase T₁ digestion of Band 4 are shown in Fig. 3E. Spot 4 is UAAAAAG as determined by secondary digestion. CCAG is missing and a new Spot N appears. This spot was confirmed to contain the sequence CC by snake venom phosphodiesterase digestion. These data indicate that the sequence contained in Band 4, which includes the 5' terminus of the precursor tRNA molecule, is also a smaller part of the sequence contained in Bands 3a and b. In fact, all the fingerprinting and enzyme cleavage kinetics described above can be summarized as shown in Figure 4A and B: that is, Bands 1a and 3b and Bands 1b and 3a are the result of cleavage events at the sites marked I; Bands 2 and 4 result from the subsequent cleavages at sites marked II in the products generated from the first cleavage at sites I. We did not attempt to recover the decanucleotide and smaller fragments which would result from the cleavages at sites II in site I products.

End groups Produced by RNase CH Cleavage:

The data from which identification of the terminal oligonucleotides in RNase CH products are made also lead to the conclusion that RNase CH generates 5' hydroxyl and 3' phosphate groups in its products. All the oligonucleotides analyzed after RNase T₁ or RNase A digestion had the expected chromatographic mobilities and compositions after secondary

Figure 3: RNase A or RNase T₁ generated fingerprints of products resulting from RNase CH treatment of *E. coli* tRNA^{Tyr} precursor.
 A: Schematic RNase T₁ fingerprint of the precursor to tRNA^{Tyr}. Solid spots represent oligonucleotides from the mature tRNA. Open spots are oligonucleotides found only in the precursor (extra sequences). Half solid-half open spots are oligonucleotides found in both in mature tRNA sequence and in the precursor extra sequences. B, C, D and E: RNase T₁ products of Bands 1a, 1b, 3a and 4 (Fig. 2), respectively. The sequences of critical spots analyzed are summarized in Table 1. F. RNase A products of Band 2. Spot M is new (see Table 1). In panels B and C, spot 18 is the 21-mer containing the 3' end of the tRNA sequence shown in panel A. In panels D and E spot 4 was determined to be AUAAG. Spots 18 and 4 are marked as reference spots.

Table 1: Identification of some important oligonucleotides in the fingerprints (Fig 3) of RNase T₁ or RNase A digestion of RNase CH cleavage products of the tRNA^{Tyr} precursor.

Band	RNase used	Spot	Products of Secondary digestion	Suggested Sequence
1a	T ₁	X	C,U,G,AC,AU	AUUACCCCG
1b	T ₁	Y	C,G,AC	ACCCCG
2	A	M	G,U	GGU
3a	T ₁	Z	C,U,AU	CAUU
	T ₁	13	C,U,G,AAAAAG	CUUCCCG and UAAAAAG
4	T ₁	13	C,U,G	CUUCCCG
		N	C*	CC

Secondary digestion were carried out with RNase A or RNase T₁, respectively, depending on whether RNase T₁ or RNase A was used in the primary digestion.

*Secondary analysis with snake venom phosphodiesterase digestion.

Figure 4: Cleavage sites of RNase CH in E. coli tRNA^{Tyr} precursor (A) and (B), and Ø80-encoded M3 RNA (C). The arrows mark the sites of cleavage. The Roman numbers above the arrows indicate the order of cleavages as determined by time course experiments. Part (B) is a linear schematic diagram of the structure shown in (A). The RNase CH cleavage sites are marked and the resulting fragments labeled to correspond to the bands shown in Figure 2. In part (C), M3 Band 1 extends from site I to the 3' terminus, Band 2 from site II to the 3' terminus, and Band 3 from the 5' terminus to site I.

digestion (in comparison with known standards) of oligonucleotides with 5' hydroxyl and 3' phosphate groups. This is also evident in our analysis of the 5' terminus of Band 2 (Fig. 3F) which lacked the oligonucleotide pGGU after RNase A digestion but which did contain the new spot GGU. Our analysis of the RNase CH digestion products of M3 RNA were also consistent with this conclusion (see below).

Specificity and Mode of RNase CH cleavage of M3 RNA: The deduced cleavage sites of RNase CH in another natural substrate, Ø80 encoded M3 RNA, are shown in Figure 4C. A time course digestion experiment of M3 RNA by RNase CA showed a pattern similar to that seen with tRNA^{Tyr} precursor. That is, one cleavage event occurred first, producing two products, followed by a second cleavage near the base of the long hairpin (see Fig. 4C). The nature of all the products, intermediate and final, was determined by fingerprint analyses (not shown), secondary digestion of key oligonucleotides, and comparison of these products with the published sequence of M3 RNA (9). The result of RNase A and RNase T₁ digestions of M3 RNA and the RNase CH digestion products are summarized in Table 2, and are consistent with the cleavage scheme shown in Fig. 4C, in which the first cleavage occurs at site I and the second at site II.

Ion Requirements of RNase CH: The effect of altered ionic strength on the RNase CH reaction was estimated by visual inspection of autoradiographs (Fig. 5), since extensive quantitation was too cumbersome with the assay we use. The results (summarized in the legend to Fig. 5) show that the enzyme requires both the presence of a divalent and a monovalent cation for maximum activity. The divalent cations Ca²⁺ and Mg²⁺ were effective at similar concentrations. With some enzyme preparations Ca²⁺ appeared somewhat more effective in stimulating RNase CH activity than did Mg²⁺.

The optimum concentration of a monovalent cation was dependent on the concentration of a divalent cation used. Higher concentrations of divalent cation required lower concentrations of monovalent cation for optimum enzyme activity. For instance, 50 to 100mM NH₄Cl was optimum at 5mM Ca²⁺, whereas 100mM NH₄Cl was inhibitory at 10mM Ca²⁺.

Curiously, SAM activated the reaction at concentration of 0.3 mM to 0.6 mM. ATP showed no effect in this reaction. The pH optimum of the reaction is between 7 and 8. The accumulation of the intermediates (for example Bands 1a and 1b rather than Band 2 in Fig. 4B) was enhanced at pH higher than 8.

Other characteristics of RNase CH: E. coli 5S RNA was resistant to attack

Table 2A: Ribonuclease T₁ products obtained from the RNase CH cleavage products of M3 RNA (9).

	Secondary digestion				
<u>RNase T₁ products</u>	<u>products</u> ⁺	<u>M3</u>	<u>Band 1*</u>	<u>Band 2*</u>	<u>Band 3*</u>
pppG	—	+			+
UUACUUG	U, G, AC	+			+
CCG	C, G	+			+
UAACACAG	U, AC, AG, AAC	+			
UG	U, G	+	+		
AUAAG	AU, AAG	+	+		
CUUUG	C, U, G	+	+	+	
AAG	AAG	+(1)	+(1)	+(2)	
UUACCG	C, U, G, AC	+	+	+	
CUUCAAG	C, U, AAG	+	+	+	
CUUUUU(AA) _{OH}	C, U, A(A)	+	+	+	
G	G	+	+	+	
AG	AG		+		
UAACAC	U, AC, AAC				+

⁺The RNase T₁ products were digested further with pancreatic RNase A. Products are listed in the order they occur in the 5' to 3' direction except for the mononucleotide G, and the ones newly appearing in RNase T₁ digestion products. A (+) indicates expected recovery from the digest of the indicated RNA species. The numbers in parentheses on polynucleotide AAG show the molar ratio obtained from counting this spot.

*The locations of Bands 1, 2 and 3 in the M3 RNA sequence are given in the legend to Fig. 4C.

by RNase CH under conditions where tRNA^{Tyr} precursor and M3 RNA were digested completely. A β -globin gene transcript (4) undergoes limited cleavage by RNase CH resulting in the production of seven fragments about 120 nucleotides or larger in size. None of the cleavages occurs precisely at the junction of an intervening sequence (data not shown). The enzyme was inhibited by bulk *E. coli* tRNA^{Tyr} at about 30 μ g/ml and sensitive to inactivation by proteases.

DISCUSSION

Most previous characterizations of mammalian ribonucleases have relied on the use of synthetic substrates (1,10). The properties of RNase CH, although determined with natural substrates, do not appear to be identical to those of enzymes previously characterized with synthetic substrates.

Table 2B: Ribonuclease A products obtained from the RNase CH cleavage products of M3 RNA.

<u>RNase A products</u>	Secondary digestion				
	<u>products*</u>	M3	Band 1	Band 2	Band 3
pppGU		+			+
U (14)	U	+	+	+	+
AC (3)	AC	+	+	+	+
GC (2)	C,G	+	+	+	+
C (4)	C	+	+	+	+
GU (2)	U,G	+	+		+
AAC	AAC	+			+
AGU	AG,U	+	+		
GAU	AU,G	+	+		
AAGGC (2)	C,G,AAG	+	+	+	
GAAGU	U,G,AAG	+	+	+	
(AA) _{OH}		not recovered			

* Secondary digestion was carried out by RNase T₁ digestion of the RNase A products.

Products are listed in the order they occur in the 5' to 3' direction. A (+) indicates the presence of the polynucleotide. Products, AGU and GAU were not resolved in the fingerprint. Number in parenthesis are the molar yields of the designated oligonucleotides.

RNase CH is also separable by virtue of its chromatographic and other properties from the tRNA processing enzyme, RNase P, purified from calf heart and HeLa cells using the same substrate we used for RNase CH (11, 12).

With respect to enzymes characterized with synthetic substrates, RNase CH is different from both an endoribonuclease isolated from calf liver which cleaves its substrates between C and U residues (13, 14) and from RNase L, an interferon-activated enzyme which cleaves synthetic and natural substrates with limited specificity (15, 16). The enzyme is also different from a recently characterized single-stranded endoribonuclease from Ehrlich cell nucleoli which produces 5'-phosphate groups in its cleavage product termini (17). Other endoribonuclease preparations which are capable of cleaving rRNA precursors and which have been prepared from nucleoli have also been described (18-21). These latter nucleases respond differently from RNase CH to various ions (18,19), have different chromatographic behavior (20), or are of too uncertain characterization

Figure 5: Ion requirements of RNase CH activity. The figure shows an autoradiograph of an acrylamide gel analysis of reactions performed with various cation additions as listed below. B1-4 indicate the positions of Bands 1-4 as shown in Figure 1. The reaction mixture contained 1mM β -mercaptoethanol, 0.1mM EDTA, 20mM Tris-HCl (pH 7.5) and 6,000-8,000 cpm precursor with the addition of a divalent cation (5mM) and/or a monovalent cation (100mM). Relative activity was estimated by visual inspection of the autoradiograph. Enzyme fractions (G-200 Sephadex, see Methods) were diluted with 20mM Tris-HCl (pH 7.5) containing 0.05 mg/ml bovine serum albumin prior to use. The data for the last two reactions are not shown in the figure. Similar results were obtained with either G-200 Sephadex or HA I peak fractions (see Methods).

Lane No.	Divalent	Monovalent	Relative Activity
1	----	----	-
2	----	NaCl	-
3	----	KCl	-
4	----	NH_4Cl	-
5	$\text{Mg}(\text{OAc})_2$	----	-
6	"	NaCl	+
7	"	KCl	+
8	"	NH_4Cl	+
9	$\text{Ca}(\text{OAc})_2$	----	-
10	"	NaCl	+
11	"	KCl	+
12	"	NH_4Cl	+
	MnCl_2	----	$\pm$
	"	NH_4Cl	$\pm$

(21) to make a direct comparison possible. There is, however, another endoribonuclease, Endoribonuclease VI, which appears to have an absolute requirement for calcium ions and which has specificity similar though not identical to the activity we report here (22). Endoribonuclease VI has been purified from Artemia larvae and has a specificity for UpN sequences as determined through the use of synthetic substrates (22). RNase CH, while not chemically homogeneous, works optimally with magnesium or calcium ions and has a strong specificity for pyr-A sequences at high pH. We have not used synthetic substrates in our characterization of RNase CH. It is conceivable, though unlikely, that our preparation is a mixture of a mammalian activity similar to Endoribonuclease VI and another activity. In any case, the activity we describe may be of some use in RNA sequencing studies particularly as these studies are always carried out on natural substrates like the ones we have employed to characterize RNase CH.

The cleavage specificity of RNase CH appears to be determined by a combination of sequence and structure. That is, not all possible sites available on the basis of the proposed sequence specificity are cleaved. As determined with all three of the substrates we used, some sites are preferred above others even when there are only two such sites in one substrate molecule. It is apparent, therefore, that structure, secondary and/or tertiary, must play a role in determining the action of this enzyme.

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²Abbreviation: RNase CH - calcium or magnesium activated endoribonuclease from calf heart, PMSF-phenylmethylsulfonylchloride.

³tRNA^{Tyr} precursor RNA has not been isolated in non-radioactive form suitable for end-labeling and ladder sequence analysis. Consequently, analysis of RNase CH cleavage products is carried out on uniformly labeled material.

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