MULTIPLE RIBONUCLEASE H ACTIVITIES FROM BHK-21/C13 CELLS

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1. Introduction

Ribonuclease H, first discovered in calf thymus [1], specifically degrades the RNA component of DNA–RNA hybrid molecules. It has since been isolated from other sources including KB cells [2], *Escherichia coli* [3,4] and purified preparations of RNA-dependent DNA polymerase from avian myeloblastosis virus [4,5]. Two ribonuclease H activities have been isolated from rat liver [6] and from the murine myeloma MOPC-21 [7].

Current evidence [8–12] suggests that DNA synthesis may be initated by RNA, in both prokaryotic and eukaryotic systems. Our interest in ribonuclease H stems from (a) the putative role that the enzyme has in DNA replication, selectively removing initiating RNA strands, and (b) our observation that purified preparations of DNA-dependent DNA polymerases I and II from BHK-21/C13 cells contain ribonuclease H activity [13]. This paper describes multiple forms of ribonuclease H in BHK-21/C13 cells.

2. Materials and methods

2.1. *Materials*

[^{3}H]poly(A)–poly(U) was obtained from Miles Seravac (Pty) Ltd., Maidenhead, Berks., UK; Sephadex G-75, Sephadex G-100 and DEAE-Sephadex A-25 from Pharmacia (G.B.) Ltd., London, UK; phosphocellulose (Whatman) from Reeve Angel Scientific Ltd., London, UK. All reagent chemicals were Analar grade.

2.2. *Extraction and fractionation of ribonuclease H activity*

BHK-21/C13 (baby hamster kidney) cells were grown to midlogarithmic phase in roller bottles [14,15], harvested, washed once in 25 mM Tris–HCl (pH 7.5 at 4°C), 0.14 M NaCl, 0.05 M KCl, 0.2 mM Na_2HPO_4, and allowed to swell in 10 mM Tris–HCl (pH 7.5, 4°C), 5 mM 2-mercaptoethanol for 5 min. The cells were disrupted in a glass homogenizer tube with two strokes of a Teflon pestle (clearance 0.075 mm) using a Tri-R-Stir-R motor at 1100 rpm. The homogenate was centrifuged (800 *g*, 10 min) and the nuclear pellet resuspended in 0.2 M potassium phosphate buffer pH 7.5 and held for 1 hr at 4°C. The nuclear and cytoplasmic fractions were then centrifuged at 100 000 *g*, for 1 hr and the supernatants dialyzed to 0.02 M potassium phosphate, pH 7.5. The supernatants were taken to 80% sutaration at 4°C with $(NH_4)_2SO_4$, the precipitates collected by centrifugation and dissolved in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% (v/v) ethylene glycol. After dialysis, the nuclear and cytoplasmic extracts were centrifuged at 100 000 *g* at 4°C for 1 hr and the supernatants pooled. Subsequent steps in the fractionation are described under Results.

2.3. *Preparation of hybrid substrates*

2.3.1. DNA–[^{3}H]RNA

Heat-denatured BHK-21/C13 cell DNA was transcribed with the homologous DNA-dependent RNA polymerase B using [^{3}H]UTP, ATP, CTP and GTP

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[16]. The product was separated from protein by extracting twice using $CHCl_3$–isoamyl alcohol, the hybrid remaining in the aqueous phase. The hybrid preparation was purified further by passage through a column (24 × 1 cm) of Sephadex G-75 equilibrated with 10 mM Tris–HCl (pH 7.5, 20°C), 0.1 M KCl, 1 mM EDTA. The DNA–[^{3}H]RNA hybrid emerged as a sharp peak (all of the radioactivity was acid-insoluble) cleanly separated from the mononucleotides (all of the radioactivity was acid-soluble) and other small molecules. The fractions containing the hybrid were pooled. Evidence for the hybrid nature of the preparation is presented in section 3.2 (fig. 4) in which it is shown that the hybrid is not degraded by pancreatic ribonuclease unless it is thermally denatured in which case it is susceptible to the action of that enzyme.

2.3.2. [^{3}H]DNA–RNA

This was prepared as for the DNA–[^{3}H]RNA hybrid, [^{3}H]DNA and non-radioactive ribonucleoside 5′-triphosphates being substituted for DNA and [^{3}H]ribonucleoside 5′-triphosphates respectively.

2.3.3. DNA–RNA × [^{3}H]DNA

Non-radioactive DNA–RNA hybrid (see 2.3.1) serves as an excellent primer and template for DNA polymerase I from BHK-21/C13 cells [13]; its primer-template activity is equal to that of 'activated' DNA [13] for DNA polymerase I.

Using this enzyme with the DNA–RNA primer-template, dATP, dCTP, dGTP and [^{3}H]dTTP, stretches of DNA were synthesized, which were presumably attached covalently to the 3′-terminals of the RNA component in the DNA–RNA hybrid. Preliminary evidence using [α-^{32}P]dGTP alone or together with dATP, dCTP and dTTP has indicated that a proportion of the ^{32}P label is transferred to alkali-labile linkage in the product.

The DNA–RNA × [^{3}H]DNA thus produced was extracted and purified by gel filtration through Sephadex G-75 as before (see 2.3.1).

2.4. *Ribonuclease H assay*

The assay (100 μl) contained DNA–[^{3}H]RNA, a divalent metal cation, buffer and enzyme (see Results). After incubation at 37°C, 10 μl of calf thymus DNA (2.5 mg/ml) and 110 μl of 10% (w/v) trichloroacetic acid were added at 0°C. After 20 min, the acid-soluble material was removed by centrifugation at 2000 *g* for 45 min, and 100 μl of the supernatant added to 0.9 ml of water and 10 ml of scintillation fluid [15] for counting in the Tracerlab Corumatic 100 liquid scintillation spectrometer.

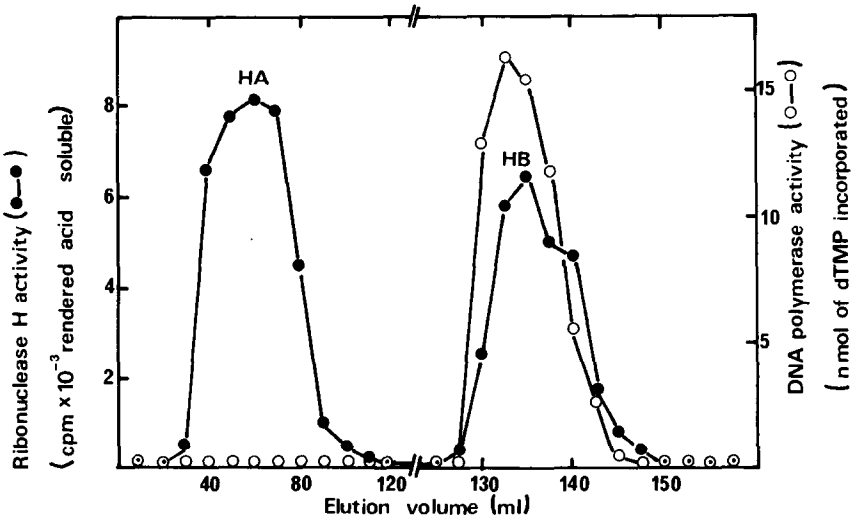

Fig. 1. Chromatography of the cell extract on a column (15 × 1.7 cm) of phosphocellulose. The unbound peak (HA) was collected in 10 ml fractions, and the peak eluted by 400 mM phosphate (HB) in 3 ml fractions. Ribonuclease H assays were incubated for 5 min at 37°C with 5 mM $MgCl_2$ and 50 mM Tris–HCl, pH 8.3.

2.5. *DNA polymerase assay*

DNA polymerases I and II were assayed by standard techniques for the enzymes from BHK-21/C13 cells [13].

3. Results

3.1. *Separation of multiple ribonuclease H activities*

Column chromatography of the cell extract on phosphocellulose separated the ribonuclease H activity into two fractions, one (HA) which did not bind to the column at 100 mM phosphate and another (HB) which was eluted from the column together with DNA polymerase activity, by 400 mM phosphate (fig. 1).

After dialysis against 50 mM Tris–HCl buffer, pH 8.0, 50 mM KCl, 20% (v/v) ethylene glycol, HA was applied to a column of DEAE-Sephadex A-25 and was resolved into two activities (fig. 2). One (Ha1) did not bind to the column under these conditions while the other (HA2) was eluted at 175 mM KCl. HA1 contains a small amount of ribonuclease activity which can be removed by dialysis against 20 mM KCl, 50 mM Tris–HCl, pH 8.0, followed by re-chromatography on DEAE-Sephadex A-25. Under these conditions, HAl binds to the column and is eluted with 125 mM KCl. The enzyme is then free of ribonuclease activity but is highly unstable losing all of its activity after a few days at – 70°C.

The HB activity from the phosphocellulose column (fig. 1) was dialyzed against 100 mM phosphate buffer, pH 7.5, containing 20% (v/v) ethylene glycol and applied to a column of Sephadex G-100 (fig. 3). The ensuing gel filtration resolved the ribonuclease H activity into two fractions. One (HB1) emerged in the void volume and contained also DNA polymerase I (6–8 S) activity [13], while the other (HB2) was retarded on the column and contained DNA polymerase II (3.3 S) activity [13].

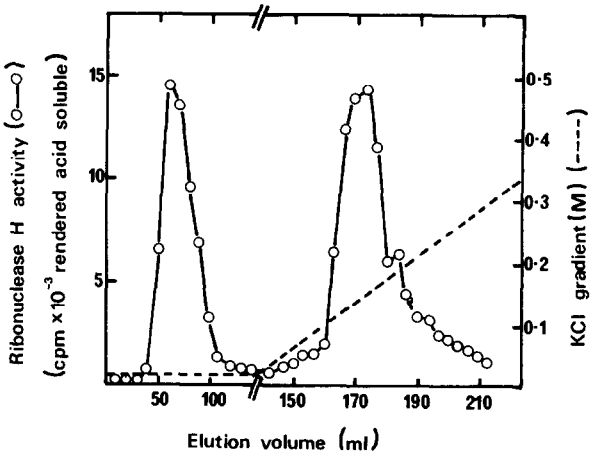

Fig. 2. Chromatography of ribonuclease H fraction HA (see fig. 1) on a column (15 X 1.6 cm) of DEAE-Sephadex A-25. The unbound peak was collected in 10 ml fractions and the peak eluted by the KCl gradient in 3 ml fractions.

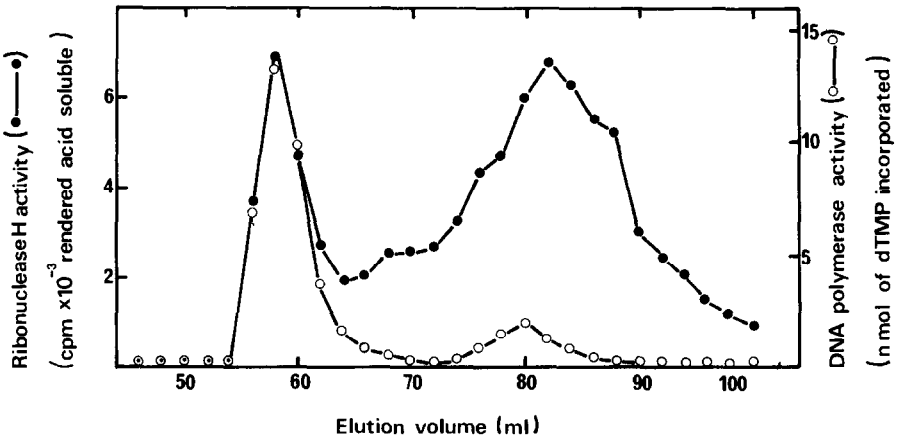

Fig. 3. Gel filtration of ribonuclease H fraction HB (see fig. 1) through a column (68.0 X 1.2 cm) of Sephadex G-100. 2 ml fractions were collected. Ribonuclease H was assayed as described in fig. 1. The void volume of the column is indicated by the arrow.

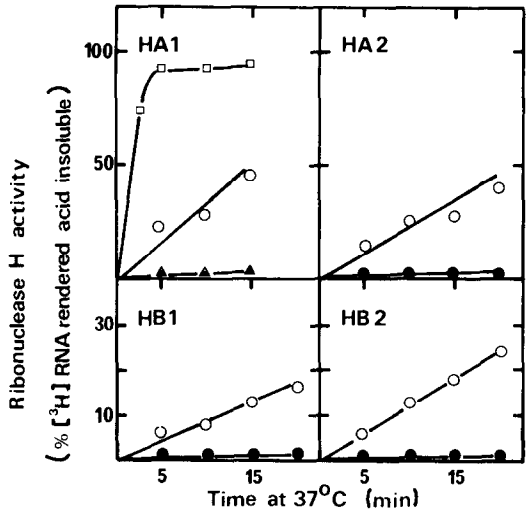

Fig. 4. Ribonuclease H activity with native and heat-denatured DNA–RNA hybrid. HA1 and HA2 were assayed in 5 mM $MgCl_2$, 0.2 M Tris–HCl, pH 8.3, and HB1 and HB2 in 40 mM $MgCl_2$, 0.2 M Tris–HCl, pH 7.6. Hydrolysis of the RNA component of the hybrid using KOH rendered 7163 cpm acid-soluble; the acid-soluble cpm released by enzymic action in the assays are expressed as a precentage of this figure. Pancreatic ribonuclease was used at 4 μg/ml. ○, native DNA–RNA hybrid; ●, denatured DNA–RNA hybrid; △, pancreatic ribonuclease with native DNA–RNA hybrid (no ribonuclease H present); □, pancreatic ribonuclease with denatured DNA–RNA hybrid (no ribonuclease H present).

3.2. *Specificity of the ribonuclease H activities*

All four ribonuclease H activities degrade the RNA of a DNA–[^{3}H]RNA hybrid to acid-soluble material, the reaction being linear up to about 20 min (fig. 4). The susceptibility of the substrate was lost after the hybrid was thermally denatured.

None of the enzyme activities degrades native or denatured BHK-21/C13 cell DNA, poly(A)–poly(U), thermally denatured poly(A)–poly(U), the DNA of a [^{3}H]DNA–RNA hybrid or the DNA of a DNA–RNA X[^{3}H]DNA hybrid.

3.3. *Properties of the ribonucleases H*

The pH optima of the ribonuclease H activities were determined in the presence of 5 mM Mg^{2+} to be 8.3 and 8.6 for HA1 and HA2 respectively, and in the presence of 40 mM Mg^{2+} to be 7.7 and 7.5 for HB1 and HB2 respectively. In the presence of 0.5 mM Mn^{2+} the pH optimum for HA1 had a broad range from 7.4

Table 1
Variation of divalent metal cation optima of the four ribonuclease H activities at pH 7.6 and pH 8.3

Enzyme	pH 7.6					pH 8.3				
	Concentration for optimal activity (mM)		Activity at the optimum (% of control*)		Activity ratio (Mg^{2+}: Mn^{2+})	Concentration for optimal activity (mM)		Activity at the optimum (% of control*)		Activity ratio (Mg^{2+}: Mn^{2+})
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}		Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	
HA1	5	0.5	12.0	10.6	1.13	1	0.5	66	16.1	4.1
HA2	5	0.5	8.5	19.5	0.44	5	0.5	54	28.5	1.9
HB1	40	0.5	37.3	5.3	7.0	60	0.5	15	18.0	0.83
HB2	20	+	43.0	+	–	40	1.0	28	9.1	3.08

HA1 and HA2 were incubated for 5 min, HB1 and HB2 for 20 min, at 37°C, with the DNA–[^{3}H]RNA hybrid, 0.2 M Tris–HCl buffer at the appropriate pH, and either $MgCl_2$ (0–120 mM per assay) or $MnCl_2$ (0–20 mM per assay). Blank samples were incubated and treated in the same manner (see section 2.4) but in the absence of the enzymes; these blanks gave very low values for acid-soluble counts/min.

* The enzyme activity at the optimum concentration of divalent cation is expressed as a percentage of the control which was the counts/min rendered acid-soluble by hydrolysis of the [^{3}H]RNA component of the DNA–[^{3}H]RNA hybrid with KOH.

\+ Optimum concentration for $MnCl_2$ not reached at 20 mM; at the latter concentration the extent of hydrolysis of the hybrid catalysed by HB2 was 7% of the control.

Table 2
The effect of *S*-adenosyl methionine, KCl and *N*-ethylmaleimide on the ribonuclease H activities

Enzyme	Percent of control activities					
	S-adenosyl methionine			KCl (275 mM)		*N*-Ethylmaleimide (1 mM)
	0.5 mM	2 mM	4 mM	Mg^{2+}	Mn^{2+}	
HA1	126	60	10	4	20	30
HA2	137	90	10	3	17	12
HB1	116	45	0	30	49	23
HB2	105	20	6	19	257	21

HA1 and HA2 were incubated for 5 min at 37°C with 5 mM $MgCl_2$ and Tris–HCl buffer, pH 8.3 HB1 and HB2 were incubated for 20 min at 37°C with 40 mM $MgCl_2$ and Tris–HCl buffer, pH 7.6. Where manganese was substituted for magnesium, the assays for all four enzymes contained 0.5 mM $MnCl_2$. Control incubations contained 25 mM KCl; *S*-adenosyl methionine and *N*-ethylmaleimide were omitted.

to 8.8 whilst the optima for HA2, HB1 and HB2 were all 7.6.

In extensions of these experiments in which the Mg^{2+} and Mn^{2+} optima were determined at various pH values for each ribonuclease H, it was clear not only that the divalent cation optima and the relative activities with Mg^{2+} and Mn^{2+} varied with pH, but also that the responses of the ribonucleases H were different from each other (table 1).

The effects of *S*-adenosyl methionine, KCl and *N*-ethylmaleimide on the four ribonuclease H activities were examined (table 2). The enzymes were inhibited differentially by *N*-ethylmaleimide, and by *S*-adenosyl methionine at concentrations of 2 mM or greater. In the presence of $MgCl_2$ and $MnCl_2$ all the enzymes were inhibited by KCl except for HB2 which was stimulated 2.5-fold when Mn^{2+} was used as the divalent cation.

4. Discussion

These observations clearly demonstrate the existence, in BHK-21/C13 cells, of four physically separable enzyme activities capable of degrading the RNA component of DNA–RNA hybrid molecules. Studies with other nucleic acid templates show that all are true ribonuclease H activities and not enzymes analogous to exonuclease III or the 5′-3′ nuclease of DNA polymerase I of *E. coli* [3] which also degrade the RNA of a DNA–RNA hybrid.

Studies on some of the properties of the four enzymes show that they behave differently from one another, but that HA1 and HA2 are rather similar and that HB1 and HB2 resemble each other. The inhibition by *S*-adenosyl methionine is similar to that previously reported for a ribonuclease H from calf thymus tissue [18]. The calf thymus enzyme was also stimulated by KCl in the presence of Mn^{2+} in a similar manner to HB2.

The biological role of these enzymes is unknown but the removal of RNA primers of DNA synthesis is a possibility. For this reason, the apparent association of the HB enzymes with DNA polymerases is at present under investigation.

Acknowledgments

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