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Purification and characterization of human ribonuclease HII

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ABSTRACT

A ribonuclease H activity from human placenta has been separated by ion exchange chromatography from the major RNase HI enzyme. Additional chromatographic steps allowed further purification, more than 3,000 fold compared to the crude extract in which it represents about 15% of the total RNase H activity. The enzyme requires Mg^{2+} ions for its activity, is strongly inhibited by the addition of Mn^{2+} ions or other divalent transition metal ions, and exhibits a pH optimum between 8.5 and 9. It shows a strong sensitivity to the SH-blocking agent *N*-ethylmaleimide. It has a strict specificity for double-stranded RNA–DNA duplexes and exhibits neither single-stranded nor double-stranded RNase (or DNase) activities. Therefore, this enzyme displays the characteristics of class II RNase H and is now termed RNase HII. Renaturation gel assays and gel filtration experiments proved a monomeric structure for the active enzyme with a native molecular weight of about 33 kDa. The human RNase HII acts as an endonuclease and releases oligoribonucleotides with 3′-OH and 5′-phosphate ends. It is therefore a candidate for the RNase H-mediated effect of antisense oligodeoxynucleotides.

INTRODUCTION

Ribonucleases H (RNases H) are enzymes which degrade specifically the RNA moiety of DNA–RNA hybrids. A protein with this specificity was first discovered by Hausen and Stein in calf thymus (1, 2). Later on, RNases H have been found in procaryotes, other eucaryotes and retroviruses, there as a part of the reverse transcriptase (for reviews see 3–5).

Despite their ubiquitous occurrence, detailed knowledge of their biological function is restricted to the retroviral and procaryotic enzymes. The major *E.coli* enzyme is probably involved in the removal of RNA primers for lagging strand DNA synthesis and generates RNA primers for replication of the ColE1 plasmid. The retroviral RNase H cleaves the viral RNA strand and yields primers for second strand DNA synthesis, prior to integration of the retroviral genome.

In higher eucaryotes, RNases H can be classified into two classes according to their molecular weight, chromatographic behavior on ion exchangers, activation by divalent cations and sensitivity to SH-blocking agents like *N*-ethylmaleimide (NEM). In addition, they are immunologically distinct proteins. The class I enzymes display higher native molecular weights (68–90 kDa) than the class II enzymes (30–45 kDa). Class I RNases H are activated by either Mn^{2+} or Mg^{2+} and are insensitive to NEM. They are likely involved in DNA replication, as their activity rises in parallel with DNA synthesis after mitogen stimulation of resting lymph node cells (6).

In contrast, class II RNases H (formerly named RNases HIIb) are active only in the presence of Mg^{2+}, are inhibited by additional Mn^{2+} and are highly sensitive to NEM. They seem to be linked to RNA transcription, as it was shown that the bovine RNase HII activity increased with RNA synthesis (7). In addition, partially purified human class II enzyme has been discussed in the context of RNA transcription (8). The two classes also show serological distinctness: the polyclonal antibody raised against the bovine RNase HI was reported to recognize only the class I enzymes and to neutralize their activity, including that of human origin (9–11).

Much information, including crystallographic structure, is available for RNases H of *E.coli* and of retroviral origin but very little is known about the structure and properties of these enzymes in higher eucaryotes. Both bovine RNases HI and HII have been purified and characterized, but only the human class I enzyme has been prepared from cultured cells and studied up to now. We clearly need to improve our knowledge about these enzymes which certainly play a key role in DNA replication and transcription. It is also of interest to define more precisely the substrate requirement of these enzymes, as they have been shown to be involved in antisense oligonucleotide-mediated effects (12, 13).

In this report we describe the purification and characterization of a human RNase H to near homogeneity from placenta. This enzyme was termed RNase HII according to its properties (molecular weight and cation requirements). We showed that this enzyme can act on substrates where short DNA oligonucleotides are hybridized to the inner part of complementary RNAs, and

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therefore can be classified as an endonuclease. The characterization of the human RNase HII underlined its similarity with the corresponding bovine enzyme (14).

MATERIALS AND METHODS

Chemicals, enzymes and isotopes

Ribonucleoside triphoshates were obtained from Boehringer (Mannheim, Germany). DEAE-cellulose (DE52) and CM-cellulose (CM52) were purchased from Whatman (Maidstone, UK), phenyl-Sepharose, Sephadex HR 200 and native DNA-cellulose from Pharmacia and heparin-agarose from Pierce. DE52 and CM52 celluloses were pre-conditionned as recommended by the supplier. Isotopes were from Amersham ([γ-^{32}P]ATP, 110 TBq/mmole; [^{32}P]pCp 110 TBq/mmole) or NEN ([α-^{3}H]ATP, 1.7 TBq/mmole). T4 polynucleotide kinase, T4 RNA ligase and phosphodiesterase I from *Crotalus durissus* were from Boehringer. Molecular weight standards have been purchased from Pharmacia, protease inhibitors from Sigma and Fluka and pre-stained proteins from BioRad.

Substrates and oligonucleotide synthesis

The standard [^{3}H]RNA-DNA hybrid was synthesized by transcription of heat-denatured calf thymus DNA with *E.coli* RNA polymerase as previously described (15). Incubation conditions were adjusted to give a specific activity in the labeled duplex of 66 dpm/pmol. [^{32}P]Poly(rA)·poly(dT) was prepared by transcription of poly(dT) by *E.coli* RNA polymerase according to Sarnghadaran *et al.* (16) and purified on a cellulose column, in the presence of ethanol. Oligodeoxynucleotides for RNase H specificity assays were synthesized on a Milligen 7500 DNA synthesizer using phosphoramidite chemistry. DNA-RNA hybrids of defined sequence were prepared by hybridization of chemically synthesized oligodeoxynucleotides with the complementary oligoribonucleotide obtained by *in vitro* transcription with T7 RNA polymerase (17).

The oligoribonucleotide was either 5'-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, after dephosphorylation with calf intestinal phophatase, or 3'-end-labeled using T4 RNA ligase and [^{32}P]pCp according to standard procedures (18). Both DNA and RNA oligonucleotides were purified by electrophoresis on denaturating 7 M urea gels.

Ribonuclease H assay and analysis of RNase H cutting sites

Assays were carried out according to Büsen and Hausen (7) with minor modifications. [^{3}H]-Labeled RNA (60 pmol) in double-stranded DNA-RNA hybrids was incubated with the enzyme in 0.5 ml of assay mixture containing 30 mM Tris-HCl, pH 7.8, 0.01% 2-mercaptoethanol, 0.05 M $(NH_4)_2SO_4$ and 0.02 M $MgCl_2$. After incubation at 37°C for the appropriate time, the acid-insoluble material was precipitated by trichloroacetic acid.

For analysis of cleavage sites, labeled RNA (0.1 μM final concentration) and complementary oligonucleotides (1 μM) were mixed with 0.3 units of the human RNase HII preparation in a total volume of 20 μl containing 30 mM Tris-HCl, pH 7.8, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$ and 0.01% 2-mercaptoethanol. The samples were incubated for 30 min at 37°C and the reaction was stopped by adding 20 μl of electrophoresis loading mix (50 mM EDTA, 7 M urea, 0.1% bromophenol blue, 0.1% xylene cyanol). Analysis was carried out by electrophoresis on denaturing 20% polyacrylamide gels. To determine the nature of 3' termini, labeled RNA fragments were incubated for 30 min at 37°C in the presence of (9×10^{-3} units) of snake venom phosphodiesterase I.

Determination of kinetic parameters

Fraction 3 (phenyl-Sepharose pool) of the human RNase HII preparation was used for steady-state kinetic experiments. 10 μl of this fraction (12.5 μg/ml) were added to 490 μl of 30 mM Tris-HCl, pH 8.5, buffer containing 0.01% 2-mercaptoethanol, 75 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$ and the labeled hybrid at the chosen concentration. At defined times of incubation at 37°C, 50 μl aliquots were withdrawn, spotted onto Whatmann 3 MM filters and immediately immersed in 5% trichloroacetic acid (T-CA), 1% Na pyrophosphate. The amount of hybrid hydrolysed was measured and the Michaelis-Menten parameters (K_M, V_{max}) were calculated (19).

Purification of RNase HII

After removal of connective tissue, fresh human placenta was washed in 0.9% NaCl, chopped into small pieces and stored in 85 g portions at −80°C. For enzyme preparation all operations were carried out at 4°C. A portion (85 g) of fresh or frozen placenta was homogenized with a Waring Blendor (1 min at low and 3 min at high speed), in a total volume of 500 ml of buffer A (10 mM Tris-HCl, pH 7.8, 2 mM EDTA, 0.1% 2-mercaptoethanol, 0.5 mM PMSF, 10 mM benzamidine, 1 μg/ml aprotinin and 1 μg/ml leupeptin). $(NH_4)_2SO_4$ was added to a final concentration of 100 mM during low speed homogenization to precipitate chromatin, which has a low solubility at this concentration. The resulting precipitate containing mainly deoxyribonucleoprotein (1) was removed by centrifugation at 8,000 *g* for 45 min in a Kontron A6.14 rotor. Glycerol was added to the supernatant up to a final concentration of 10%. This crude extract (fraction 0) was diluted 5-fold with buffer B (buffer A containing 10% glycerol) and stirred gently with 500 g diethylaminoethyl (DEAE)-cellulose (Whatman DE-52 preconditioned after the supplier's manual) for 2 h. The suspension was filtered on a Buchner funnel and washed with 1 l of buffer B to recover unbound material. This fraction (fraction 1) was gently stirred overnight with 500 g carboxymethyl (CM)-cellulose (Whatman CM-52). The suspension was then extensively washed with 3 l of buffer B on a Buchner funnel and poured into a column (4.6×40 cm). The column was then washed at 1 ml/min with 900 ml of buffer B and eluted, at the same rate, with a linear 0-600 mM KCl gradient in buffer B. Protein concentration was determined by the method of Bradford (12) with bovine serum albumin as a standard. RNase H activity was assayed with [^{3}H]RNA-DNA hybrid as described above.

Fractions of the RNase H activity peak from the CM-cellulose column were pooled (fraction 2). $(NH_4)_2SO_4$ was added to a final concentration of 1 M and the sample (150 ml) was loaded onto a phenyl-Sepharose column (30 ml bed volume), equilibrated in buffer B containing 1 M $(NH_4)_2SO_4$. The column was then washed with 100 ml of the same solution and bound proteins were eluted with a linearly decreasing 1-0 M $(NH_4)_2SO_4$ gradient in buffer B, at a flow rate of 0.5 ml/min. Activity-containing fractions were pooled (fraction 3) and dialysed overnight against 2 l of buffer B (with one change after 3 h). The solution was then loaded on a native DNA-cellulose column (bed volume 5 ml), washed with 100 ml buffer B. A 0-500 mM KCl linear gradient in buffer B was applied (volume 60 ml, flow rate 0.16 ml/min). RNase H activity-containing fractions were pooled (fraction 4) and stored at −80°C.

Alternatively, we used a modified purification procedure starting with fraction 3, dialysed against buffer C (30 mM Tris−HCl, pH 7.8, 20% glycerol, 2 mM EDTA, 0.1% 2-mercaptoethanol) and then adsorbed to 5 ml heparin−agarose. Proteins were eluted with a 0−600 mM KCl linear gradient in buffer C (50 ml). Fractions containing RNase H activity were pooled (fraction 4b), concentrated 20-fold using a Filtron Macrosep™ centrifugal concentrator (exclusion size 10 kDa) and analysed by preparative SDS−PAGE, as described below. Enzyme activity-containing fractions were determined by renaturation gel assay (see below) and the corresponding proteins detected by silver staining (20, 21).

Analytical and preparative SDS−polyacrylamide gel electrophoresis

Analytical gel electrophoresis of proteins was carried out on 1 mm thick SDS−polyacrylamide gels according to Laemmli (22). After electrophoresis, the gels were stained with Coomassie Brilliant Blue or processed for silver staining (20, 21). Molecular weights of the corresponding proteins were determined, using the following markers: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.1 kDa). Western blot analysis was performed as previously described (15).

Preparative SDS−polyacrylamide gel electrophoresis was carried out on 12% vertical cylindrical sieving gels, using a BioRad Model 491 Prep Cell, which allowed the continuous elution and fractionation of proteins. The small prep cell column (28 mm inner diameter) was used with a separation gel height of 6 cm and a stacking gel height of 1 cm. Two prestained proteins covalently linked to dyes, soybean trypsin inhibitor (27.5 kDa) and carbonic anhydrase (32.5 kDa), were used as markers to adjust the running conditions.

Renaturation gel assay

[^{32}P]-Labeled poly(rA)·poly(dT) hybrid gels were performed by running classical 13% SDS−polyacrylamide gels (18×15×0.1 cm) according to Laemmli except that [^{32}P]-labeled hybrid (about 450,000 cpm, corresponding to 1.7 nmol of hybrid of a fresh preparation) was included in the resolving gel before polymerization (23). An aliquot of each sample was mixed with loading buffer containing SDS and 2-mercaptoethanol, heated for 2 min at 95°C, and loaded. At the end of the electrophoresis the part of the gel on which molecular weight markers were run was cut out and stained with Coomassie Brilliant Blue R-250. The other part of the gel was processed according to the basic procedure described by Rong and Carl (24) with the exception that $MgCl_2$ was replaced by $MnCl_2$ to promote renaturation, as described previously (23). The gel was then dried and autoradiographed overnight.

RESULTS

Purification of human ribonuclease HII

Analysis of RNase H activity from human placenta under different ionic conditions revealed that this tissue contained two classes of RNase H, the predominant class I enzyme and the less prominent class II RNase H. To separate these two classes of enzyme we took advantage of the fact that RNase HII does not bind to anion exchangers but to cation exchangers, whereas the reverse is true for RNase HI. This procedure was successfully used for the bovine enzymes (9). At the initial step, the placenta crude extract (fraction 0) was mixed with DEAE−cellulose to adsorb RNase HI and recover an unbound fraction, containing the class II activity. This chromatography was designed as a batch procedure to allow handling of large quantities in a short time.

The DEAE−cellulose unbound fraction (fraction 1) was then exposed to a batch of CM−cellulose and, after washing and pouring a column, the proteins were eluted by a linear KCl gradient: a single RNase H activity peak, eluting at around 110 mM KCl was obtained (fraction 2; data not shown). Binding of human placenta crude extract to anion and cation exchangers revealed that only about 15% of total RNase H activity is due to a class II enzyme. As shown in Table 1, after the application of 4.5 g of total protein to DEAE−cellulose, the class II enzyme was eluted with about 6 mg of protein from the CM−cellulose column, corresponding to more than a 600-fold increase in specific activity.

The next purification step was hydrophobic chromatography on phenyl−Sepharose. Under the loading conditions used (see Materials and Methods) no RNase H activity was found in the flow-through; as shown in Figure 1 a linear decreasing gradient of ammonium sulfate yielded a single RNase H activity peak close to 200 mM (fraction 3). The yield from this step was only 26%, indicating the high sensitivity of the enzyme to denaturation in preparations with low protein concentration.

Fraction 3 was then loaded on a native DNA−cellulose column. The activity profile of this affinity chromatography is displayed in Figure 2a. After an extensive wash with buffer B (see Materials and Methods), the enzyme eluted at around 400 mM KCl within a sharp peak (fraction 4). As shown by

Table 1. Purification procedure of human RNase HII

Fraction	Volume (ml)	Protein (mg)	Total activity (units[a])	Specific activity (units/mg)	Enrichment (fold)	Yield (%)
0 Crude extract	450	4500	20800 (3300[b])	4.6 (0.73[b])		
1 DEAE−cellulose	3300	2300	3300	1.4	1.9[b]	100[b]
2 CM−cellulose	120	6.1	2660	440	600	80
3 Phenyl−sepharose	40	0.4	680	1700	2300	21
4 Native DNA−cellulose	9	0.02	46	2300	3150	1.4

Crude extract was prepared from 85 g of human placenta.
[a]One unit was the amount of RNase H required to produce 100 pmol of acid-soluble RNA from [^{3}H]poly (rA)·poly (dT) in 10 min at 37°C.
[b]The activity of the class II enzyme was not measured in fraction 0 due to the presence of class I RNase H. The estimate was based on the total activity in the DEAE flow-through assuming a 100% yield at the first step.

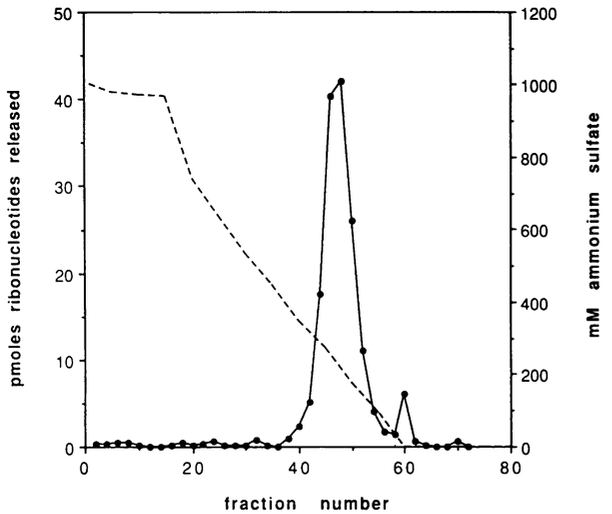


Figure 1. Hydrophobic chromatography of class II RNase H from human placenta. Fraction 2 (150 ml) obtained from CM−cellulose chromatography was loaded on a 30 ml phenyl−Sepharose column eluted by a 1−0 M $(NH_4)_2SO_4$ decreasing gradient (···) in buffer B (gradient volume 200 ml). The RNase H activity (●) of 2.5 ml fractions was determined in the presence of magnesium as described in Materials and Methods.

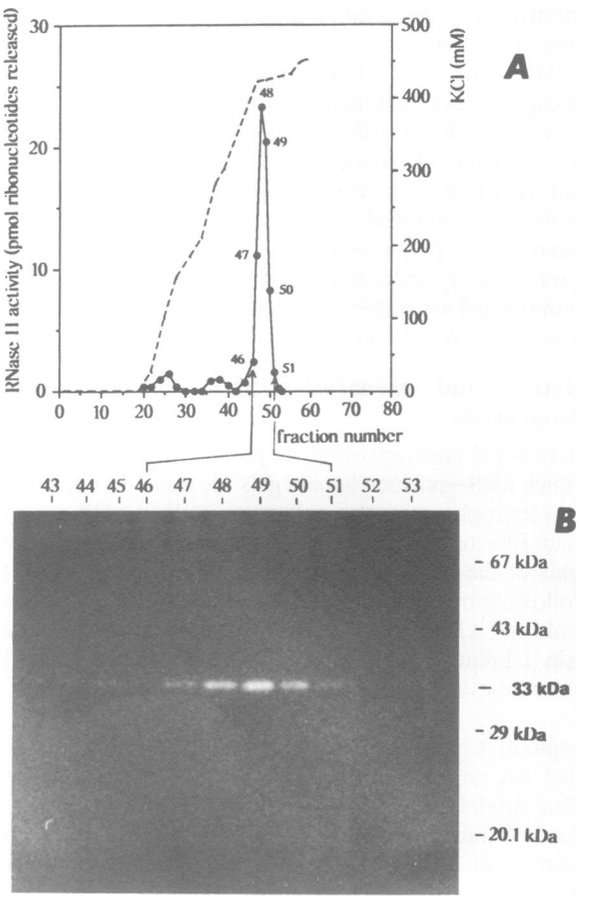


Figure 2. Affinity chromatography of human RNase HII. (**a**) Following dialysis against buffer B, fraction 3 obtained from phenyl−Sepharose chromatography was applied to a native DNA−cellulose column (5 ml bed volume) eluted by a 60 ml linear KCl gradient (···) increasing from 0 to 500 mM. The RNase H activity of 1 ml fractions (●) was determined with [^{3}H]RNA−DNA hybrids as described in Materials and Methods. (**b**) Fractions eluted from the phenyl−Sepharose column were assayed for RNase H activity on a 13% polyacrylamide−SDS gel in which [^{32}P]RNA−DNA hybrid was embedded. RNase H digestion was allowed to proceed after renaturation of the protein as indicated in Materials and Methods. Size markers are given to the right. RNase H activity was detected at a position corresponding to 33 kDa.

renaturation gel assay (Figure 2b), a [^{32}P]poly(rA)·poly(dT)-degrading activity was detected in fractions corresponding to the RNase HII peak on DNA−cellulose and was associated with protein(s) of about 33 kDa molecular weight under denaturing conditions. Fraction 4 (DNA−cellulose pool) displayed a specific activity of 2300 U/mg. Table 1 summarizes the purification procedure. The purification factor starting from placenta extract was calculated to be close to 3,000, the most efficient step being the cation exchange chromatography. The overall yield of purification up to the DNA−cellulose column, based on our estimate of the RNase HII activity in the crude extract, as indicated in the legend of Table 1, was about 1.4%, assuming a 100% yield for the first step.

The fraction purified on DNA−cellulose was used for further characterization (except where indicated). However, the yield from this step was very low (about 7%), mainly due to the 'bleeding out' of DNA and enzyme from the column. Moreover, after this purification procedure no protein bands on a silver stained SDS−polyacrylamide gel could be seen in association with the fractions containing renaturable hybrid-degrading activity, underlining the high sensitivity of the hybrid gel analysis but preventing the evaluation of purity.

We therefore used an alternative procedure aimed at improving the purification yield. We took advantage of the fact that the class II RNase H binds to heparin−agarose and modified the purification procedure correspondingly. Starting with the phenyl−Sepharose fraction (fraction 3), the enzyme eluted at around 250 mM KCl (fraction 4b; data not shown) from the heparin−agarose (5 ml bed volume). However, this did not significantly improve the specific activity of the enzyme. This fraction was further purified by the use of preparative SDS−PAGE (see Materials and Methods). The peak fraction of the RNase H activity obtained from preparative electrophoresis (fraction 5b) was finally analysed by silver staining and renaturation gel assay. Three distinct protein bands were observed, the upper one (33 kDa) corresponded to the hybrid-degrading band (Figure 3). This 33 kDa band is therefore the human RNase HII protein. The other two bands either correspond to contaminating proteins or to proteolytic fragments. The peak fraction of the preparative SDS−PAGE can be considered to contain roughly 30% pure active enzyme.

Physical and enzymatic properties of human RNase HII

The native molecular weight of the human RNase HII was determined by gel filtration on Sephacryl S 200 HR from comparison with globular proteins used as standards for the enzyme. RNase H activity was found in fractions 175−205 with a maximum in fraction 190 (Figure 4). The elution position of standard proteins was determined using the Bradford method allowing estimation of a native molecular weight of about 30 kDa for the human RNase HII, i.e close to that of carbonic anhydrase used in the standard mixture, assuming a globular shape for all

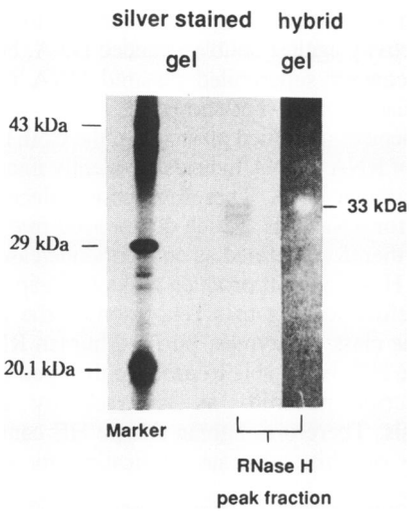


Figure 3. Identification of the human RNase HII. Electrophoretic analysis of fractions obtained by preparative SDS–PAGE (see text) was performed either by silver staining of a 13% polyacrylamide–SDS gel (left) or by renaturation assay on a hybrid gel (right) as described in Materials and Methods. Size markers are given to the left. The RNase H activity detected on the hybrid gel corresponded to a 33 kDa protein.

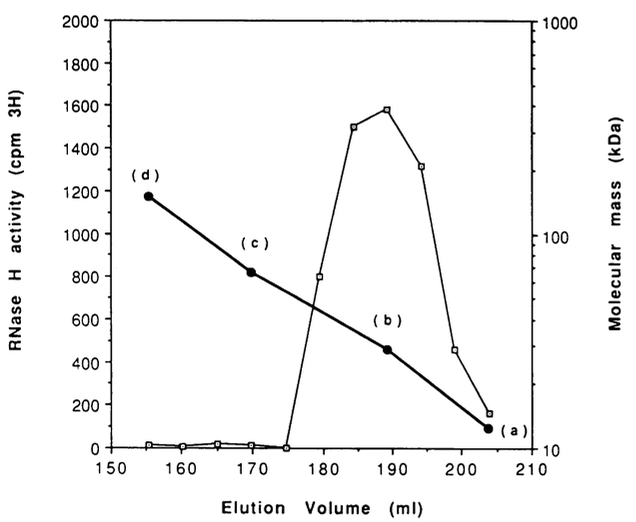


Figure 4. Determination of the native molecular weight of human RNase HII. A sample of concentrated fraction 3 (human RNase HII, phenyl–Sepharose pool; 170 units; 0.2 mg protein) was loaded on a Sephacryl S 200 HR gel filtration column (100 cm) together with the molecular weight markers (5 mg each): (a) cytochrome c (12.4 kDa), (b) carbonic anhydrase (29 kDa), (c) bovine serum albumine (66 kDa) and (d) alcohol dehydrogenase (150 kDa). The chromatography was performed in buffer B including 500 mM KCl to avoid aggregation of proteins (flow rate 26 ml/h). The position of the RNase H activity peak is indicated (□). The elution volumes of the marker proteins (♦) were determined by the Bradford method.

proteins. This result agrees with the value (33 kDa) obtained by renaturation gel assays. As gel filtration chromatography was performed under native conditions, whereas renaturation assays followed electrophoresis on a denaturing polyacrylamide gel, this implies that the active human RNase HII is a monomeric enzyme of about 33 kDa.

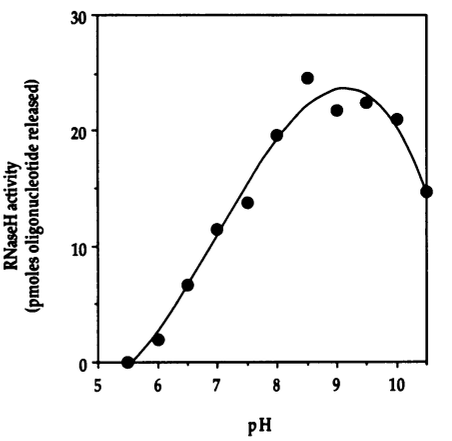


Figure 5. pH dependence of human RNase H II activity. The activity was measured as described in Materials and Methods in the presence of Mg^{2+} ions. The DNA–cellulose pool (fraction 4) was used for this analysis.

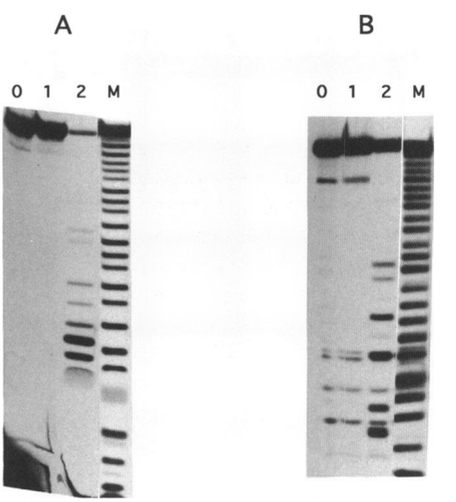


Figure 6. Cleavage of RNA–DNA hybrids by RNase H. RNA24 (**a**) or RNA23 (**b**) (see Scheme 1 for sequences) were incubated with RNase HII in the absence (lanes 1) or in the presence (lanes 2) of complementary oligonucleotides DNA24 or DNA23, respectively, under the conditions indicated in Materials and Methods. Lanes 0 correspond to the samples incubated in the absence of RNase H and lanes M to size markers obtained by alkaline hydrolysis of [^{32}P]-labeled RNA.

RNase HII was assayed under different pH and ionic conditions, varying both the nature and the concentration of divalent cations, according to the general procedure described in Materials and Methods. The pH optimum was observed between pH 8.5 and 9.0, as measured in imidazole–HCl, Tris–HCl and glycine–NaOH buffers. However, at pH 7.4 the enzyme still possessed about 60% of the activity measured at pH 8.5 (Figure 5).

The human RNase HII was totally dependent on the presence of a divalent cation. It showed a broad optimum around 20 mM $MgCl_2$ and was only slightly active with $MnCl_2$. In the presence of 20 mM $MgCl_2$, the activity was strongly inhibited by additional divalent cations introduced as chloride salts. The

inhibitory efficiency decreased in the order: Fe^{2+} (5 μM), Mn^{2+}(50 μM), Cu^{2+} (50 μM), Co^{2+} (50 μM), Zn^{2+} (50 μM), Ni^{2+} (200 μM) and Ca^{2+} (1000 μM) (the cation concentration inducing 50% inhibition is indicated in parentheses). In addition, we observed that the enzyme acted optimally in the presence of 75 mM ammonium sulfate and was strongly inhibited by the SH-blocking agent *N*-ethylmaleimide, 50% inhibition being achieved at 2 mM (data not shown).

The kinetic parameters have been determined for the human RNase HII with tritiated hybrid, obtained by transcription of calf thymus DNA (see Materials and Methods). Derived from a Lineweaver–Burk plot (not shown), we found the following values: K_M = 1.4 μM and V_{max} = 0.043 μM/min. For comparison, the kinetic parameters obtained with poly (rA)·poly(dT) were K_M = 1.6 μM and V_{max} = 0.038 μM/min.

As expected, the human RNase HII is highly specific for RNA–DNA hybrids and did not exhibit any detectable activity either on double-stranded (not shown) or on single-stranded RNA (Figure 6). In addition, the enzyme was shown to be free of any nucleolytic activity against double-stranded DNA, because it was not able to convert supercoiled plasmid DNA into the open circular or linear forms (not shown).

Chloramphenicol-amplified plasmids of the ColE1 type contain short pieces of RNA–DNA hybrids covalently integrated in this supercoiled circular DNA. Therefore these molecules can serve as substrates for RNases H, which do not need free RNA 3′-OH ends and are therefore defined as endo-ribonucleases. An endo-ribonuclease H action will produce nicks and gaps in the RNA moeity, therefore leading to a relaxation of the molecule. In contrast to the class I enzymes, purified human RNase HII (as bovine RNase HII) is not able to produce the open circular form of a Bluescript plasmid, as analysed by agarose gel electrophoresis. Therefore, human RNase HII cannot act as an endonuclease on this substrate, indicating its sensitivity to supercoiling.

We then examined the mode of cleavage of RNase HII using short synthetic RNA–DNA duplexes as substrates. As shown on Figure 7a, incubation of a 15 base pair heteroduplex (RNA21–DNA15) resulted in the quantitative cleavage of the RNA strand: less than 10% intact RNA was present after 1 h incubation in the presence of 0.3 units of human RNase HII. Multiple breakdown products were detected following analysis on polyacrylamide denaturing gel. All of them seem to be due to initial cleavage events and not from further processing of short RNA fragments, as indicated by the kinetic analysis of individual fragments (Figure 7b). As the RNA 21mer and DNA 15mer were bound in such a way that RNA was protruding at both the 3′ and 5′ ends (Figure 6), this demonstrated that human RNase HII is an endonuclease, although we cannot exclude that in addition it could have an exonuclease activity.

The pattern of cleavage sites introduced in three different RNA–DNA hybrids by RNase HII indicates that the enzyme did not show any simple sequence specificity (Scheme 1). But

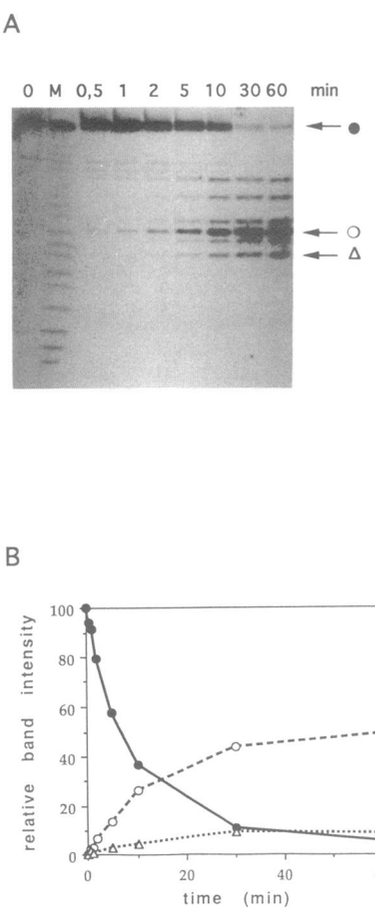
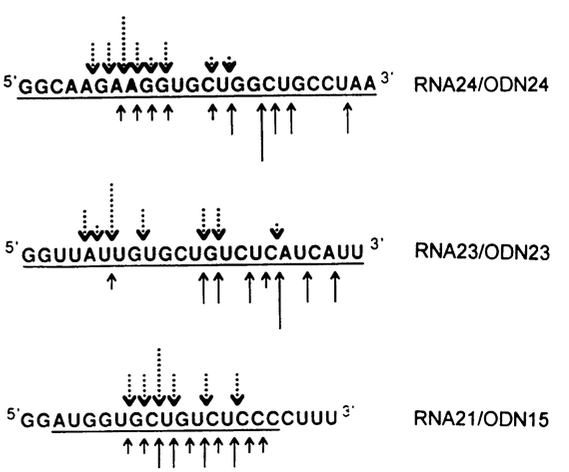


Figure 7. Cleavage products of RNA–DNA hybrids by human RNase HII. (**a**) [^{32}P] 5′-end-labeled RNA21 was incubated alone for 30 min (lane 0) or with RNase HII in the presence of the complementary DNA sequence, DNA15, as described in Materials and Methods for the time indicated at the top of the lanes (see Scheme 1 for sequences). Lane M corresponds to the alkaline hydrolysis of [^{32}P]-labeled RNA. (**b**) Kinetic analysis of the formation of RNA fragments by RNase HII. Bonds indicated by an arrow (a, right) were cut out and counted. Their intensity was calculated with respect to the total amount of radioactivity in each lane and plotted versus time (○, ●). A similar estimate was performed for full length RNA (▲).



Scheme 1. Location of cleavage sites induced by human RNase HII on three different RNA–DNA hybrids. Only the RNA sequence is shown; the underlined nucleotides correspond to the region paired with the complementary DNA sequence. The arrows indicate both the location and the relative intensity of the cleavage sites deduced from densitometric analysis of autoradiographs similar to those shown in Figures 7 and 8: long, medium and short tailed arrows correspond to 25–50%, 5–25% and 0–5% cleavage, respectively. Solid and dotted arrows refer to 3′- and 5′-end-labeled RNA, respectively.

different products were detected on either 5′- or 3′-end-labeled RNA. In addition, no cleavage took place at a distance shorter than 4 nucleotides from the RNA 5′ end, whereas cleavage sites were seen close to the 3′ end, indicating a non-symmetrical position of the catalytic site with respect to the binding domain.

In order to analyse the cleavage products, the oligoribonucleotides produced by RNase HII were subsequently incubated with snake venom phosphodiesterase I. They were degraded (not shown), indicating that the human RNase HII gives 3′-OH and 5′-phosphate ends, as phosphodiesterase I requires 3′-hydroxyl groups.

DISCUSSION

Two main distinct RNases H (HI and HII) are found in higher eucaryotes, displaying different biochemical, physical, physiological and serological properties (7, 9, 10). Class II RNases H have low native molecular weights (30−40 kDa) and are active exclusively in the presence of Mg^{2+} ions. In addition, they are sensitive to *N*-ethylmaleimide. There are few reports dealing with eucaryotic class II enzymes; activities were described and characterized in rat liver (25, 26), chick embryo (27), Krebs II ascites cells (28), HeLa cells (8) and calf thymus (14). They all share the common aforementioned properties.

In this paper, we described the purification of the human RNase HII from placenta. Human placenta is not very rich in total RNase H activity compared to calf thymus (only about 5%) from which bovine RNases H have been extracted. In addition, class II RNase H represents a minor activity in eucaryotic cells, compared to the class I enzyme. We had to face problems about the yield, mainly during the last purification steps, as the human RNase HII was very sensitive to inactivation in dilute solutions. Moreover, it did not support repeated freezing and thawing.

According to biochemical and physical criteria, the RNase H that we purified was clearly a class II enzyme: (i) it was optimally active under 20 mM Mg^{2+} and was inhibited by other divalent cations; (ii) this enzyme was very sensitive to *N*-ethylmaleimide; and (iii) the molecular weight of the native protein determined by gel filtration (around 30 kDa) and that found by gel renaturation assay (33 kDa) allowed one to assign a monomeric structure to the human RNase HII.

We have shown that this enzyme acts as an endonuclease, at least with hybrid substrates in which a short DNA oligonucleotide is hybridized to the central part of a longer complementary RNA leading to 3′-OH and 5′-phosphate ends, as do other characterized RNases H (3). However, the human RNase HII is unable to relax plasmids containing RNA−DNA hybrids, like its bovine counterpart.

The determination of the Michaelis−Menten parameters of the human RNase HII allows comparison with values determined for other RNases H. With poly(rA)·poly(dT) as substrate the K_M was found equal to 1.6 μM, to be compared with the K_M of 1.9 μM for the bovine class I enzyme (29) or the K_M of $\approx$1.4 μM for the *E.coli* enzyme (30). These values are higher than the K_M of RNases H from Krebs cells, estimated to be lower than 0.5 μM (28), but lower than the K_M of $\approx$4.2 μM determined for the *E.coli* enzyme when using an oligo(rA)·oligo(dT) (31) as a well-defined substrate compared to poly(rA)·poly(dT) used in most studies, including this one.

The cleavage pattern of three different RNA−DNA heteroduplexes brings interesting insights on the enzyme−hybrid complex and on the mode of action of the human RNase HII (Scheme 1). The fragments obtained from 3′- or 5′-end-labeled RNA are different for two hybrids (RNA24−DNA24 and RNA23−DNA23). As only the shortest fragments with the labeled end can be detected, this corresponds to the processed duplexes until the remaining fragments are not efficiently recognized as a substrate by the enzyme. The kinetic analysis (Figure 7b) shows that the cleavage products formed initially accumulate in the time scale of the experiment (>30 s). This suggests that the enzyme binds to the hybrid and degrades processively the RNA strand leading to a mixture in which the major product is about 6−9 base pairs long. It is striking that with a shorter hybrid (RNA21−DNA15) the two patterns obtained with 3′- or 5′-end-labeled RNA strands are more or less in register. This is in agreement with the previous explanation: on a short heteroduplex once bound to the duplex the enzyme has a restricted freedom to move for cleavage.

It is also clear that the cleavage pattern is non-symmetrical. Cuts were detected close to the 3′ end of the hybrid: the first or the second ribonucleotide of the heteroduplex can be cleaved. In contrast, on the 5′ side, the shortest RNA fragments are 4−5 nucleotides. The enzyme needs a few base pairs on the 5′ side of the RNA strand to bind, leading to the location of its catalytic site 4−5 nucleotides away, inside the duplex. This may be related to the fact that the *E.coli* enzyme is active on a 4 base pair heteroduplex (32). It has also been reported that this enzyme has a preference to cut between the sixth and the seventh nucleotides on the RNA strand of hybrid duplexes (33).

The human RNase HII has an endonucleolytic character. This is of interest for its potential contribution to antisense oligonucleotide-mediated effects. The involvement of RNase H in sequence-selective inhibition of gene expression by complementary oligonucleotides has been demonstrated in cell-free extracts (34). In wheat germ extracts the RNase H activity responsible for the inactivation of targeted mRNAs is a class II enzyme (15). In eucaryotic cells both RNase HI and RNase HII are located mainly in the nucleus (9, 10; W.Büsen, unpublished data), nevertheless a minor cytoplasmic ribonuclease H activity must exist and has been shown clearly to be implicated in mediating antisense effects in *Xenopus* oocytes (35, 36; Cazenave *et al.*, unpublished results). Indirect evidence strongly suggests that this might also be the case in other types of eukaryotic cells (37, 38).

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