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New sequence-specific human ribonuclease: purification and properties

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ABSTRACT

A new sequence-specific RNase was isolated from human colon carcinoma T84 cells. The enzyme was purified to electrophoretical homogeneity by pH precipitation, HiTrapSP and Superdex 200 FPLC. The molecular weight of the new enzyme, which we have named RNase T84, is 19 kDa. RNase T84 is an endonuclease which generates 5′-phosphate-terminated products. The new RNase selectively cleaved the phosphodiester bonds at AU or GU steps at the 3′ side of A or G and the 5′ side of U. 5′AU3′ or 5′GU3′ is the minimal sequence required for T84 RNase activity, but the rate of cleavage depends on the sequence and/or structure context. Synthetic ribohomopolymers such as poly(A), poly(G), poly(U) and poly(C) were very poorly hydrolysed by T84 enzyme. In contrast, poly(I) and heteroribopolymers poly(A,U) and poly(A,G,U) were good substrates for the new RNase. The activity towards poly(I) was stronger in two colon carcinoma cell lines than in three other epithelial cell lines. Our results show that RNase T84 is a new sequence-specific enzyme whose gene is abundantly expressed in human colon carcinoma cell lines.

INTRODUCTION

Our knowledge of the diversity of cellular ribonucleases (RNases) has expanded immensely in recent years. It is clear now that the small number of non-specific RNases that were identified early on was just a fraction of the great number of highly-specific enzymes that are responsible for RNA metabolism. Most probably, every RNA molecule undergoes some reactions requiring a ribonuclease. These reactions can be classified as (i) reactions of RNA processing, and (ii) reactions in which RNA molecules are degraded. The large number of distinct reactions within the first class probably needs a large number of highly specific RNases in order to ensure accurate processing of the various RNAs. The second class of degradative reactions probably requires only a small number of non-specific RNases.

The *in vivo* function of many of the RNases has not yet been ascertained. Most of the RNases with ascribed function are *Escherichia coli* enzymes, but a variety of other RNases with distinct specificities have also been isolated. For example, the (2′-5′)oligoadenylate-dependent RNase L of mammalian cells plays a significant role in the antiviral state induced by interferon (1). RNase Us is an eosinophile-derived neurotoxin (2), frog onconase is a potent antitumor agent now in phase III human clinical trials against pancreatic and liver cancer (3,4), angiogenin promotes blood vessel formation (5,6), S-RNases from plants are responsible for self-incompatibility (7,8), bovine seminal ribonuclease is a cytotoxic protein (9), and the intracellular ribonucleases modulating RNA metabolism participate in the control of gene expression (10).

It has been suggested that RNases constitute markers for human cancer cells (11), and in the human HT-29 adenocarcinoma cell line two enzymes exhibiting RNase activity have been purified from the cell culture medium. The first, angiogenin (5), catalyzes a limited number of endonucleolytic cleavages of 28S and 18S ribosomal RNA. The second is RNase 4. Although RNase 4 is a secretory enzyme, it shares with the non-secretory RNases the ability to degrade poly(U) faster than poly(C), but its function is not known (11). It should be recalled that two major classes of human ribonucleases, known as the secretory or pancreatic type and the non-secretory or liver type, are specific for pyrimidine nucleotides (12–14). Pancreatic human RNases, found mostly in secretory organs, display optimal activity at a pH of ~8.0. Because the ratio of the rates of cleavage of poly(C) and poly(U) by pancreatic type RNases is >10, they are considered to be highly C preferential enzymes. The liver-type non-secretory RNases are most effective in the pH range of 6.5–7.0 and cleave both poly(C) and poly(U).

We observed, during study of cystic fibrosis transmembrane regulator (CFTR) gene expression in HT-29 and T84 human epithelial cells (15), that these cells produce relatively large amounts of an unknown RNase. Since this RNase T84 does not efficiently degrade poly(C) and poly(U), even a large elevation in this enzyme would not have been detected in previous studies using poly(C) or poly(U) as substrates. The present report therefore deals with the purification and characterization of this new and highly sequence-specific enzyme. After homogenization, pH precipitation, ultrafiltration, ion-exchange and gel permeation

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chromatography (FPLC), the isolated enzyme (RNase T84) was shown to be free of other RNase activities by SDS–PAGE, activity staining and mapping of RNase T84 cleavage positions using tRNAs as substrates.

MATERIALS AND METHODS

Cell lines and cell culture

Human T84 and HT-29 epithelial cells were obtained from the American Type Culture Collection (Rockville, MD) and propagated in DMEM/Ham's F12 medium (1:1) and DMEM medium, respectively, with 10% FCS, L-glutamine and antibiotics. Cultures were grown at 37°C under 5% CO_2/95% air. Both are human colon adenocarcinoma cell lines. Culture conditions for the other epithelial cell lines used—CFPEo– (nasal polyp), CFPAC (pancreatic adenocarcinoma) and HeLa (cervical tumor)—are described elsewhere (16,17,18).

Standard RNase reaction

RNase activity was evaluated by measuring the amount of acid-soluble nucleotides generated by RNase digestion of RNA. The reaction mixture contained 100 µg of polynucleotide and 20 µl of purified RNase T84 solution, in 0.1 M sodium acetate buffer at pH 5.5. This mixture was incubated in the presence or absence of different reagents or divalent cations for 30–90 min at 37°C, and the reaction was stopped by addition of ice-cold, 3% trichloroacetic acid. The non-degraded substrate was removed by centrifugation at 14 000 *g* for 15 min, and absorbance was measured at 260 nm. One unit of RNase activity is defined as the amount of enzyme, which after 1 h incubation with poly(A,G,U), under the standard reaction conditions, led to an increase of 0.1 in A_{260}. Poly(A), poly(C), poly(U), poly(G), poly(I), poly(A,U) and poly(A,G,U) were purchased from Sigma.

Staining for RNase T84

To detect RNase T84 activity, 0.3 mg/ml poly(I) was added to the separating 12% polyacrylamide gel solution along with the customary reagents prior to polymerization. Proteins were separated on SDS–PAGE poly(I)-containing gel as described (19–21). Next, SDS was removed from the gel by soaking it in 25% isopropanol in 0.1 M sodium acetate buffer (pH 5.5), after which it was incubated in 0.1 M sodium acetate buffer at 37°C for 120 min. The gel was then stained with 0.2% toluidine blue in 10 mM sodium acetate buffer (pH 5.5). Proteins with RNase activity appear as white bands. After destaining in 50% methanol/12% acetic acid, the same gel was stained following a silver staining procedure.

Chromatography of proteins

Chromatographic procedures were performed using ion exchanger (HiTrapSP) and gel filtration (Superdex 200) FPLC columns from Pharmacia. The calibrations of Superdex 200 were done with ribonuclease A (13 700), chymotrypsinogen (25 000), ovoalbumin (43 000), albumin (67 000) and Blue Dextran 2000 (Pharmacia). Ultrafiltration was done using Centricon 30 (Amicon).

Preparation of T84 cell extracts and purification of RNase T84

T84 cells were washed five times in buffered saline, and extracts were prepared by homogenization with a glass Dounce homogenizer in buffer containing 10 mM KCl, 1.5 mM magnesium acetate, 20 mM HEPES/KOH, pH 7.4 (22), and protease inhibitor cocktail (Sigma no. P-8340). After lysis, debris was removed by centrifugation (15 min at 16 000 *g*). The crude extract from T84 cells (~30 mg protein) was dialysed overnight against 10 mM sodium acetate buffer, pH 4.5. Precipitated proteins were removed by centrifugation (4000 *g*, 30 min, 4°C) and discarded. The supernatant was applied to a strong cation exchanger (HiTrapSP) column, previously equilibrated with starting buffer (10 mM sodium acetate, pH 4.5). Bound material was eluted with a linear gradient of NaCl up to 1 M at a flow rate of 1 ml/min. One milliliter fractions were collected (Fig. 1B). Active fractions corresponding to RNase T84 were dialysed against 0.2 M sodium acetate buffer, pH 5.8, concentrated by ultrafiltration and loaded on a 125 ml Superdex 200 gel filtration column equilibrated with 0.2 M sodium acetate buffer, pH 5.8. RNase T84 was eluted with the same buffer; 1.5 ml fractions were collected at a flow rate of 1.5 ml/min (Fig. 1C). The purification scheme is shown in Figure 1A.

Protein analysis

Protein concentrations were determined using the BCA kit (Pierce). Protein gels were stained with the silver kit from Bio-Rad.

Determination of terminal groups generated by RNase T84

The unlabeled yeast $tRNA^{Asp}$ (3.2 µg) was incubated with 0.13 or 0.26 U of RNase T84 (or without RNase T84 in a control sample) in 0.25 M sodium citrate, pH 5.0, 7 M urea at 55°C for 10 min. The reaction products after phenol extraction and ethanol precipitation were ligated with [^{32}P]pCp (10 µCi) in the presence of T4 RNA ligase (2 U) in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 30 µg/ml BSA) and analysed on polyacrylamide 12% sequencing gels in parallel to the products of digestion of 5′ [^{32}P]$tRNA^{Asp}$ (1 µg) by RNase T84 (0.03 and 0.06 U).

Identification of terminal groups in poly(I) digests

The termini produced by cleavage of RNA by RNase T84 were analysed according to the method described by Laval and Paoletti (23). Snake venom phosphodiesterase and spleen phosphodiesterase were from Worthington Biochem. Corp. (Freehold, NY).

Mapping of RNase T84 cleavage positions using yeast $tRNA^{Asp}$ and yeast $tRNA^{Phe}$ as substrates

Yeast $tRNA^{Asp}$ and yeast $tRNA^{Phe}$ were purified and kindly provided by Dr Gerard Keith from I.B.M.C., Strasbourg, France. The 5′ ends of tRNAs were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (24,25). Yeast $tRNA^{Phe}$ or $tRNA^{Asp}$ labeled with ^{32}P was incubated for 10 min with the purified RNase T84 either at room temperature in 60 mM sodium acetate buffer, pH 5.5, or at 55°C in 0.25 M sodium citrate, pH 5.0, and 7 M urea. The reaction products were separated on 8 and 12% denaturing polyacrylamide gels. After electrophoresis, the gels

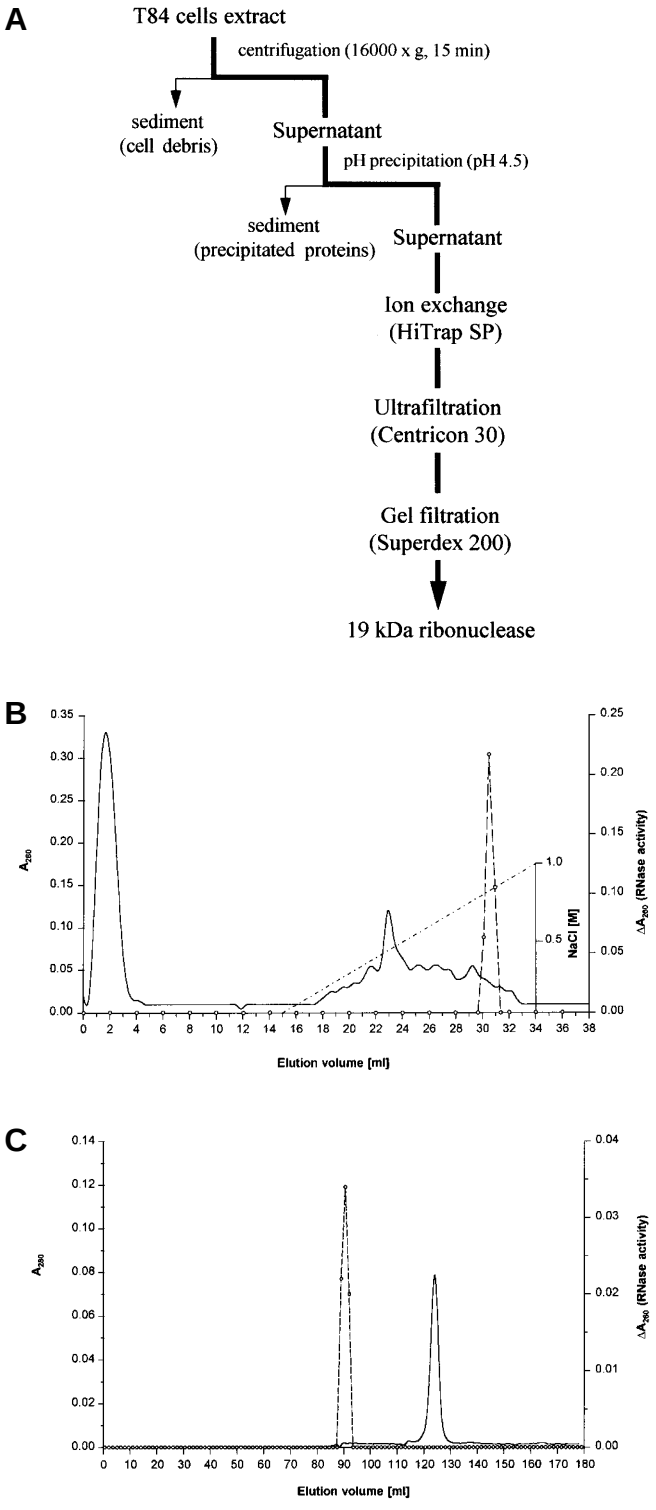


Figure 1. (**A**) Purification of RNase T84 from whole cell extracts. Purification steps are described in the text. (**B** and **C**) Chromatography profiles of RNase-active materials from human T84 cell line: (B) HiTrapSP, (C) Superdex 200. ○, RNase T84 activity at pH 5.5 using poly(I) as a substrate. RNase activity was evaluated by measuring the amount of acid-soluble nucleotides generated by RNase T84 digestion of poly(I). The non-degraded substrate was removed by centrifugation, and absorbance was measured at 260 nm. Chromatographic conditions and RNase activity assay are described in Materials and Methods. The volume of aliquots used for activity assay was 20 µl.

were dried and either autoradiographed or quantified using a PhosphorImager (Molecular Dynamics).

RESULTS

Polymer specificity and optimal conditions for the RNase T84 activity

The preferential cleavage of poly(A), poly(C), poly(G), poly(U), poly(I), poly(A,U) or poly(A,G,U) was investigated. Analysis of the ribohomopolymer degradation kinetics indicated that only poly(I) was appreciably degraded (Fig. 3). Other ribohomopolymers were cleaved with much lower efficiency. Heteroribopolymers poly(A,G,U) and poly(A,U) were also degraded, but to a lesser degree than poly(I). The activity of purified RNase T84 was investigated in different buffers and pH values. The highest activity was observed at pH range 4.5–5.8 depending on the substrate used (Fig. 4).

To verify the specificity of the RNase for RNA, its activity was assayed on native and denatured calf thymus DNA. No nucleolytic activity was observed (not shown).

RNase activity in five cell lines

Enzymatic reactions were done with 10 µg of protein extracted from the following human cell lines: CFPEo–, T84, HT-29, CFPAC and HeLa. RNase activity was tested on poly(I). The highest RNase activity was observed in crude extract from T84 and HT-29 colon adenocarcinoma cells. The extracts from CFPEo–, CFPAC and HeLa epithelial cell lines degraded the substrate to different extents, those from HeLa cells being the least potent (not shown).

Characterization of RNase T84

Three active fractions from the gel filtration column (Fig. 1C) were combined, concentrated by ultrafiltration and loaded on SDS–PAGE gel containing poly(I). After toluidine blue coloration, one activity band corresponding to ~19 kDa was seen (Fig. 2A, lane 3). After decoloration and silver staining, one protein band with the molecular mass of ~19 kDa replaced the activity band seen before (Fig. 2B, lane 3). This result corresponded to the molecular mass found using a calibrated Superdex 200 gel filtration column.

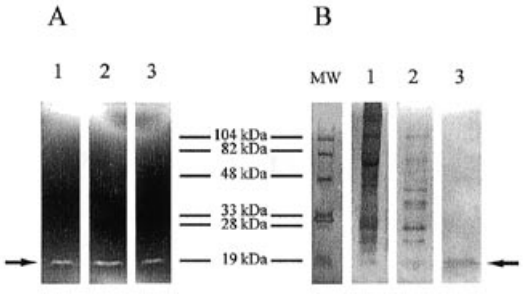


Figure 2. (**A**) Negative staining of RNase T84 activity visualized on SDS–PAGE poly(I)-containing gel: lane 1, crude extract; lane 2, the active fraction eluted from HiTrapSP column; lane 3, the active fractions eluted from Superdex 200 column. MW, molecular weight standard. (**B**) Silver staining of proteins on SDS–PAGE poly(I)-containing gel [lanes 1, 2 and 3 as in (A)]. See text and Figure 1 for the protein purification profiles.

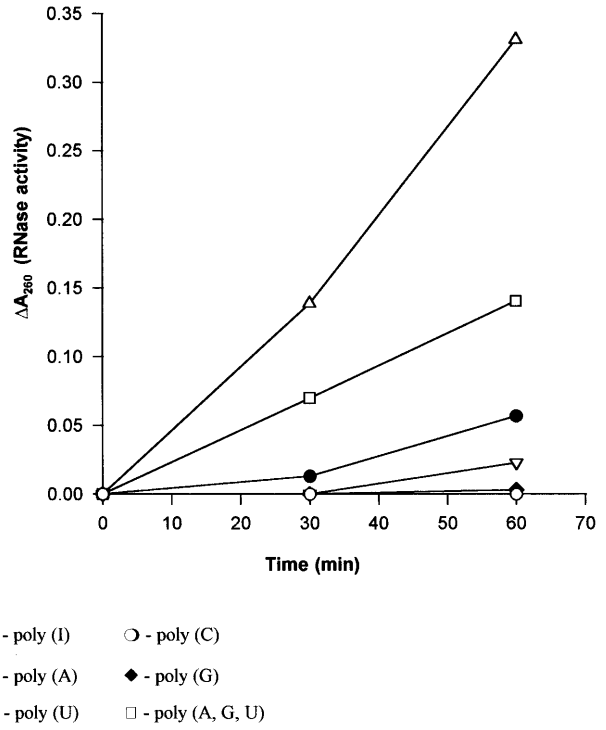


Figure 3. Polymer specificity for the RNase T84 reaction. Purified RNase T84 activity was evaluated by measuring the amount of acid-soluble nucleotides generated by RNase T84 digestion of ribopolymers. A reaction mixture containing 100 µg of polynucleotide and 20 µl of purified RNase T84 in 0.1 M sodium acetate buffer pH 5.5 was incubated for 30 or 60 min at 37°C, and the reaction was stopped by 3% TCA. The non-degraded substrate was removed by centrifugation, and absorbance was measured at 260 nm.

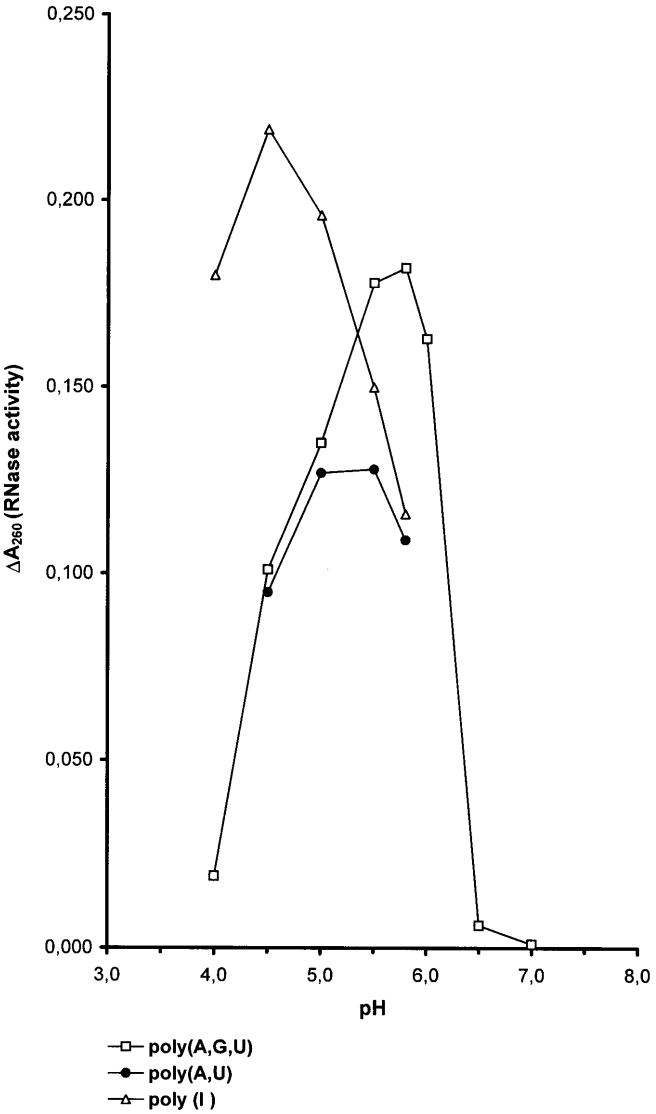


Figure 4. Optimal pH for the RNase T84 activity. Purified RNase T84 activity was evaluated by measuring the amount of acid-soluble nucleotides generated by RNase T84 digestion of ribopolymers: poly(I), poly(A,G,U) and poly(A,U). A reaction mixture containing 100 µg of polynucleotide and 0.6, 1.75 and 2 U of purified RNase T84, respectively, was incubated for 60 min at 37°C. The non-degraded substrate was removed by centrifugation, and absorbance was measured at 260 nm. For details of the reaction conditions see Materials and Methods.

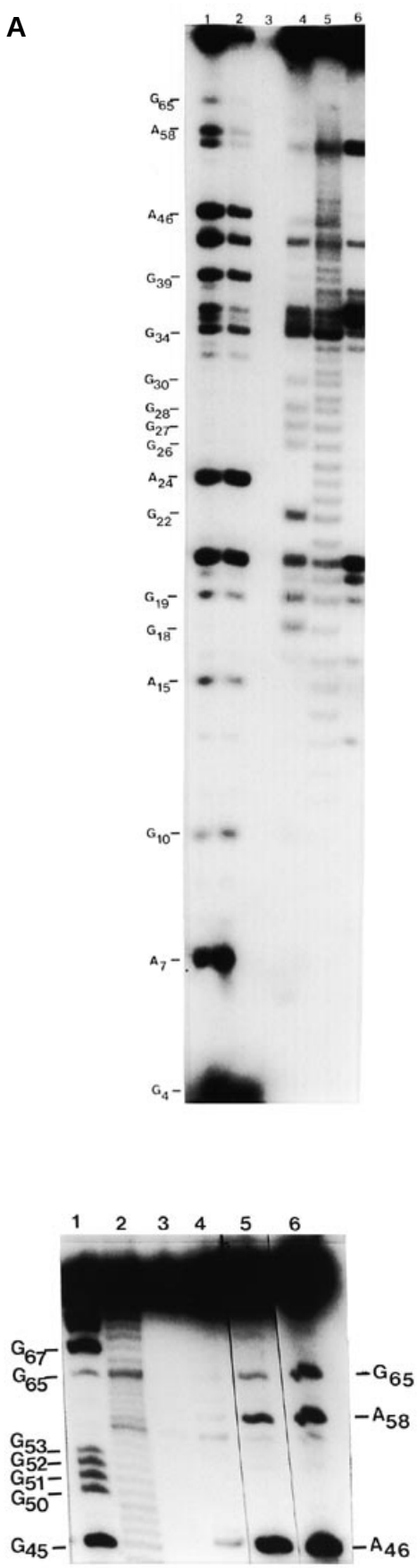
RNase T84 did not require the presence of divalent cations or sulfhydryl compounds to maintain either its stability or activity. This activity was not changed by the presence of 10 mM Mg^{2+} or EDTA. Other divalent cations, including Zn^{2+}, Ca^{2+} and Mn^{2+} inhibited RNase activity. The activity was also inhibited by $H_2PO_4^{2-}$ ions known to inhibit nucleolytic activity (not shown).

The stability of RNase T84 protein was tested at different temperatures. For this purpose RNase T84 was pre-incubated for 15 min at temperatures between 37 and 100°C and then underwent the standard reaction. The RNase remained stable up to 60°C. At higher temperatures, it started to lose its activity (not shown).

RNase T84 specificity

To investigate the preferential cleavage by RNase T84 in a natural polyribonucleotide, we used 5′-^{32}P-end-labeled yeast $tRNA^{Asp}$ (of which primary, secondary and tertiary structures are known) as the substrate. The products of the nucleolytic reaction were analysed on 12 and 8% polyacrylamide sequencing gels to establish precisely the RNase cleavage positions and the nature of the reaction.

In $tRNA^{Asp}$, the cleavages under denaturing conditions (0.25 M sodium citrate buffer, pH 5.0 and 7 M urea, 55°C) occurred between G_4 and U_5, A_7 and U_8, G_{10} and U_{11}, A_{15} and D_{16}, A_{24} and U_{25}, G_{34} and U_{35}, G_{39} and U_{40}, A_{46} and U_{47}, A_{58} and U_{59}, G_{65} and U_{66} (Figs 5 and 6). The other bands seen for example at positions 20:1, 36, 43 or 56 corresponded to non-specific, spontaneous cuts, because they were present in both the control sample and the enzymatic assay. The data obtained show that RNase T84 has a very high specificity for the phosphodiester bonds of the RpU dinucleotide sequences with adenosine or guanosine at the R position. The cleavage occurs at the 3′ side of A or G and the 5′ side of U. In contrast, under the same reaction conditions, RNase T1 specific for G catalysed the hydrolysis of $tRNA^{Asp}$ at all possible G positions regardless of the following nucleotide sequence (Fig. 5A, lane 4 and Fig. 5B, lane 1). RNase U2, which is specific for adenine, recognized all A positions in $tRNA^{Asp}$ (not shown). Although all possible AU and GU



sequences were recognized in denatured $tRNA^{Asp}$ by the T84 enzyme, the relative cleavage rates at different position varies depending most probably on the nucleotide sequence and/or secondary structure of the longer sequence motif (to compare the cleavage preference at different positions the relative cleavage rates were measured as the intensities of the bands corresponding to the products of the initial step of the enzymatic reaction, when only a few percent of the tRNA molecules were cleaved by the enzyme; not shown).

To explore the effect of the nucleotide in the N′ position of the dinucleoside phosphate ApN′ and of the nucleotide in the N position of NpU on the enzymatic activity of RNase T84, we used as substrates for this enzyme two forms of yeast phenylalanine tRNA: $tRNA^{Phe}_M$ (Major) and $tRNA^{Phe}_m$ (minor), which differ by only one base pair: C_6–G_{67} in the minor form instead of U_6–A_{67} present in the Major form (26). A_5pU_6 in $tRNA^{Phe}_M$ changes to A_5pC_6 in $tRNA^{Phe}_m$ and $A_{67}pU_{68}$ changes to $G_{67}pU_{68}$. RNase T84 did not cleave the phosphodiester bond between A_5 and C_6 in $tRNA^{Phe}_m$ (Fig. 7A, line 7—denaturing conditions and B, line 9—semidenaturing conditions; Fig. 8), but cleaved A_5pU_6 in $tRNA^{Phe}_M$ very efficiently either in denaturing conditions (Fig. 7A, lines 1 and 2; B, lines 11 and 12 and Fig. 8) or in 60 mM sodium acetate buffer pH 5.5, 20°C in which secondary and partially tertiary structure of tRNA is maintained (Fig. 7B, lines 5–8; Fig. 8). $G_{67}pU_{68}$ in $tRNA^{Phe}_m$ was also cleaved by this RNase, but with lower efficiency than $A_{67}pU_{68}$ present in the same position in $tRNA^{Phe}_M$, while the cleavage rate observed for two other positions, $G_{15}pD_{16}$ and $G_{51}pU_{52}$, was similar in both $tRNA^{Phe}$ species (Fig. 7B, lines 9 and 7, respectively; Fig. 7C, lines 10 and 7, respectively; Fig. 8). These findings strongly supported our first result (Figs 5 and 6), suggesting that RNase T84 has a high specificity for PupU sequences (the only exception is the cleavage observed at position U_8pA_9). The result shows that ApU was cleaved by the enzyme more efficiently than the dinucleoside phosphate GpU. In Figure 8 we have shown all positions of phosphodiester bonds hydrolysis in $tRNA^{Phe}$ catalysed by RNase T84. The bends seen in both the control and enzymatic samples were the result of non-specific, spontaneous cleavage of tRNA and were not shown on a cloverleaf model. Comparison of the relative cleavage rates of different phosphodiester bonds in yeast $tRNA^{Phe}$ shows that the T84 enzyme cleaves with the highest efficiency within A_5pU_6 either in native or denatured RNA. The final degradation product of complete digestion of $tRNA^{Phe}$ by RNase T84 seen on the sequencing gel is the pentanucleotide with 3′ A_5-OH (Fig. 7B, line 11); other products deprived of 5′ labeled end are not seen on a gel.

Figure 5. The analysis of the reaction products of RNase T84 on 5′-^{32}P-labeled $tRNA^{Asp}$. 5′-^{32}P-end-labeled yeast $tRNA^{Asp}$ was incubated with purified RNase T84 for 10 min at 55°C in 0.25 M sodium citrate buffer, pH 5.0, 7 M urea. The reaction products were separated using (**A**) 12 and (**B**) 8% denaturing polyacrylamide gels. After electrophoresis, the gels were dried and autoradiographed or quantified using PhosphorImager (Molecular Dynamics). (A) Lanes 1 and 2, partial digest of $tRNA^{Asp}$ (0.8 μg) with increasing concentration of RNase T84 (0.03 and 0.06 U, respectively); lanes 3 and 4, T1-RNase ladder of $tRNA^{Asp}$; lane 5, H_2O ladder; lane 6, incubation control. (B) Lane 1, T1-RNase ladder of $tRNA^{Asp}$; lane 2, H_2O ladder; lane 3, incubation control; lanes 4–6, partial digest of $tRNA^{Asp}$ (2 μg) with increasing concentration of RNase T84 (0.005, 0.01 and 0.03 U, respectively). Band intensities correspond to the amount of the enzymatic reaction products.

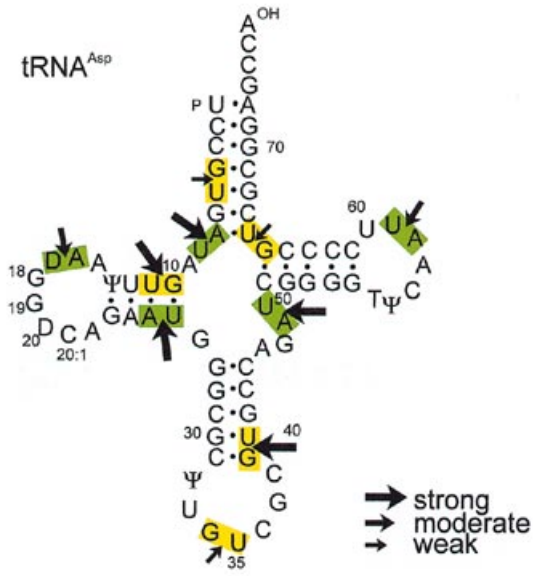


Figure 6. Model of the secondary structure of yeast tRNA^{Asp}. Arrows represent the positions of phosphodiester bonds hydrolysis catalysed by RNase T84. The size of the arrows correspond to the amount of the enzymatic reaction products (band intensities) counted from the initial stage of the reaction, when only a few percent of the tRNA molecules were cleaved by the enzyme and as established by PhosphorImager quantitation of the uncleaved tRNA molecules, the plot of the cleavage rate of tRNA as a function of the concentration of RNase T84 was linear throughout the enzyme concentration range tested (not shown).

In order to determine the influence of RNA secondary structure on RNase T84, we have conducted in parallel the enzymatic hydrolysis of yeast tRNA^{Phe} at 0 or 20°C in 60 mM sodium acetate buffer, pH 5.5, in which secondary structure of RNA is maintained and under denaturing conditions (55°C, 7 M urea, pH 5.0). The RNase T84 did not exhibit absolute specificity for the substrates having secondary structure and cleaved either single or double-stranded RNA (Fig. 7A, B and C). However, when the enzymatic reaction occurred under RNA denaturing conditions, we established that the denatured RNA was a much better substrate for this RNase than the native one.

Determination of terminal groups generated by RNase T84

To determine terminal groups generated by RNase T84, the unlabeled tRNA^{Asp} was incubated with (or without in the control sample) RNase T84 and the reaction products were than ligated with [^{32}P]pCp in the presence of T4 RNA ligase. The ligation products were analysed on polyacrylamide sequencing gels in parallel to the RNase T84 digestion products of 5′[^{32}P]tRNA^{Asp}. The reaction of RNA ligation requires 3′-hydroxyl groups; therefore, we assumed that if RNase T84 generated 3′-OH terminals, 3′-^{32}P-labeled fragments of the length of 1 nt longer than the fragments generated by RNase T84 would be detected among ligation products. Indeed, we detected several bands corresponding to the nucleotides of the expected length: for example fragments of 5, 8, 11, 18, 21 and 25 nt (Fig. 9). Multimeric products were also observed. This result indicates that RNase T84 generates 3′-hydroxyl-terminated products. The comparison of the electrophoretic mobilities of the oligonucleotide fragments generated by RNase T84 and RNases T1 or U2 which are known to generate 3′-phosphoryl-terminated products showed that the fragments produced by RNase T84 migrated further than the corresponding RNase T1 or RNase U2 oligonucleotides, supporting that RNase T84 generates 3′-hydroxyl and 5′-phosphoryl-terminated products.

Identification of terminal groups in poly(I) digests

Treatment of RNase T84 digests of poly(I) with either snake venom phosphodiesterase or spleen phosphodiesterase resulted in more extensive degradation in the case of venom phosphodiesterase treatment (not shown). This also indicated that RNase T84 degrades RNA chains to 3′-OH and 5′-phosphate-terminated fragments.

DISCUSSION

The ribonucleases constitute a superfamily of enzymes with various activities. The large number of distinct RNA processing reactions necessitates a variety of highly specific RNases. The high specificity of these enzymes involves the recognition of specific sequences and/or higher-ordered structures on the RNA substrates. In this study, we described the properties and purification of a novel highly sequence-specific ribonuclease from human colon carcinoma T84 cells, RNase T84. The enzymatic protein was purified to electrophoretic homogeneity and is free of other RNase activities as was shown by SDS–PAGE, activity staining and mapping of RNase T84 cleavage positions. This endonuclease has a molecular weight of 19 kDa and catalyses the cleavage of tRNAs in a dinucleotide-specific manner. RNase T84 recognizes 5′PupU3′ steps and catalyses the hydrolysis of the phosphodiester bonds at the 3′ side of A or G, and the 5′ side of U yielding 3′A(OH) or G(OH) and 5′ pU terminated products. Although the ApU or GpU sequence is sufficient to be recognized by the T84 enzyme, the presence of a longer characteristic sequence is most probably required for its full activity. This apparently new enzyme is not secreted by T84 cells, has no obvious relationship to the pancreatic RNase family enzymes, and seems more similar to other dinucleotide-specific enzymes like RNase L or RNase 4. These enzymes, however, recognize different dinucleotides than RNase T84. For example, human RNase L, the (2′-5′)oligoadenylate-dependent, 80 kDa ribonuclease, catalyzes the cleavage at the 3′ side of the sequences of the forms UpU, UpA, UpG, ApA and ApU (27), while RNase 4 (~16 kDa) catalyzes the hydrolysis of the phosphodiester bonds of dinucleotides UpA and UpG (11). Human liver and spleen RNases also prefer purines at the N′ position (28). All the above enzymes except RNase T84 catalyze the efficient hydrolysis of poly(U).

The observed RNase T84 sequence specificity also differs from the specificity for G or A nucleotides known for RNase T1 from *Aspergillus oryzae* (29) and RNase U2 from *Ustilago sphaerogena* (30), respectively. The latter enzymes recognize all guanosine or adenine nucleotides in the single-stranded RNA regions and do not require the following U. Both RNase T1 and RNase U2 yield 3′ phosphate-terminated products.

Most of the mammalian RNases so far studied are active toward poly(C) and poly(U). RNase T84 displays unusually marked degradation of poly(I), but does not efficiently degrade any of the four common polyribonucleotides [poly(C), poly(U), poly(A) or poly(G)]. It has been suggested that poly(G) could not be used because it forms aggregates in solutions and resists enzymatic action (31). Poly(I) has a hypoxanthine base. Hypoxanthine, with

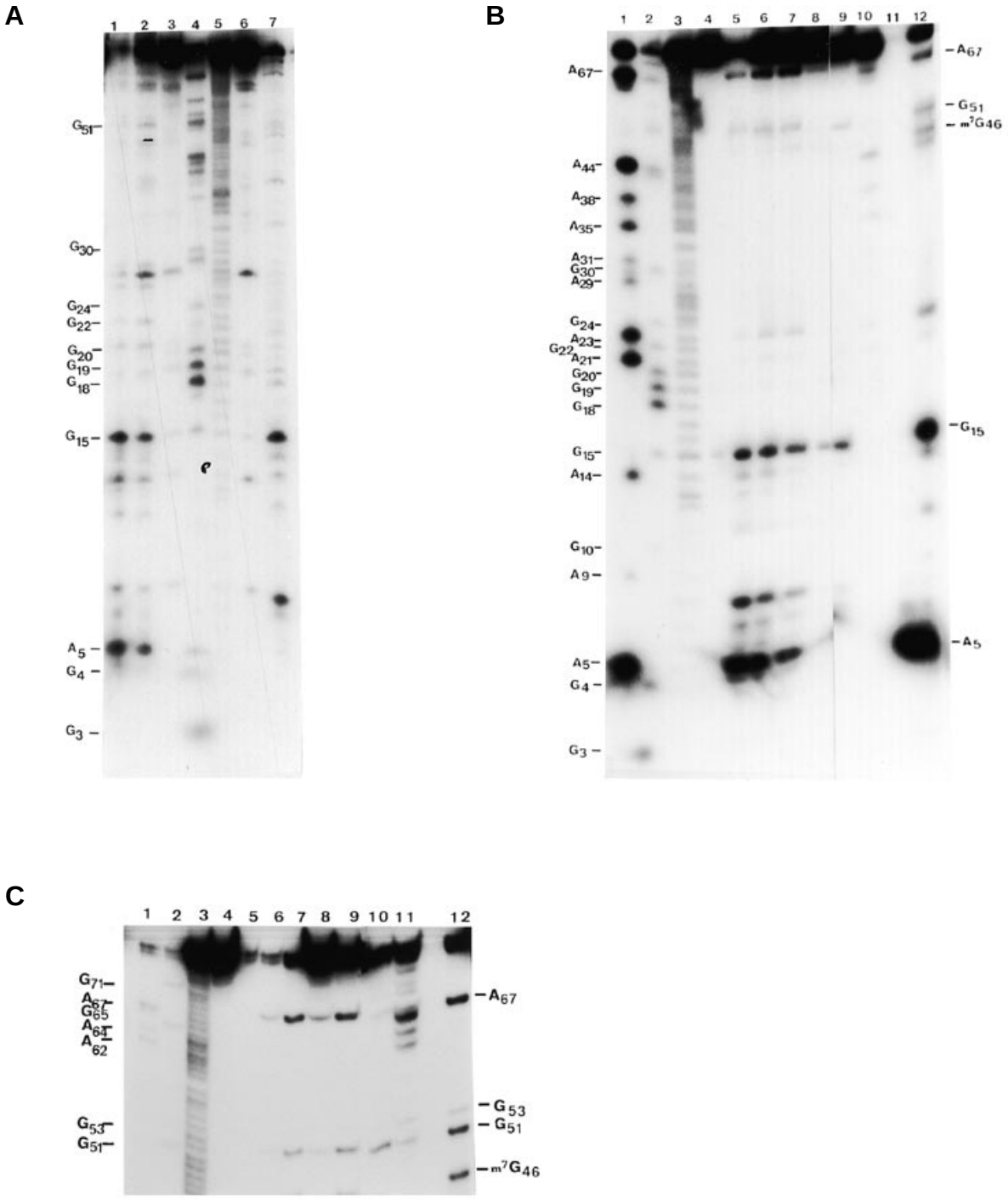


Figure 7. The analysis of the reaction products of purified RNase T84 on 5′-^{32}P-labeled yeast tRNA$^{Phe}_{M}$ and yeast tRNA$^{Phe}_{m}$. (**A**) 12% polyacrylamide gel: lanes 1 and 2, partial digest of tRNA^{Phe} M (0.8 µg) with 0.06 and 0.03 U of RNase T84 respectively, under denaturing conditions; lane 3, U2-RNase ladder of tRNA^{Phe}; lane 4, T1-RNase ladder of tRNA^{Phe}; lane 5, H_2O ladder; lane 6, incubation control; lane 7, partial digest of tRNA$^{Phe}_{m}$ (0.8 µg) with 0.06 U of RNase T84 under denaturing conditions. (**B**) 12% polyacrylamide gel: lane 1, U2-RNase ladder of tRNA$^{Phe}_{M}$; lane 2, T1-RNase ladder; lane 3, H_2O ladder; lane 4, incubation control; lanes 5–8, partial digest of tRNA$^{Phe}_{M}$ (1 µg) with RNase T84 (0.6, 0.24, 0.12 and 0.05 U, respectively) in 0.06 M sodium acetate buffer, pH 5.5, 20°C, 10 min; lane 9, partial digest of tRNA$^{Phe}_{m}$ (1 µg) with RNase T84 (0.24 U) in 0.06 M sodium acetate buffer, pH 5.5, 20°C, 10 min; lane 10, U2-RNase ladder of tRNA$^{Phe}_{m}$; lanes 11 and 12, partial digest of tRNA$^{Phe}_{M}$ (1 µg) with RNase T84 (0.3 and 0.03 U, respectively) in 0.25 M sodium citrate, pH 5.0, 55°C, 10 min. (**C**) 8% polyacrylamide gel: lane 1, U2-RNase ladder of tRNA$^{Phe}_{M}$; lane 2, T1-RNase ladder; lane 3, H_2O ladder; lane 4, incubation control; lanes 5–9, partial digest of tRNA$^{Phe}_{M}$ (1 µg) with RNase T84 (1.2, 0.6, 0.24, 0.05 and 0.12 U, respectively) in 0.06 M sodium acetate buffer, pH 5.5, 20°C, 10 min; lane 10, partial digest of tRNA$^{Phe}_{m}$ (1 µg) with RNase T84 (0.24 U) in 0.06 M sodium acetate buffer, pH 5.5, 20°C, 10 min; lane 11, U2-RNase ladder of tRNA$^{Phe}_{m}$; lane 12, partial digest of tRNA$^{Phe}_{M}$ (1 µg) with RNase T84 (0.03 U) in 0.25 M sodium citrate, pH 5.0, 55°C, 10 min.

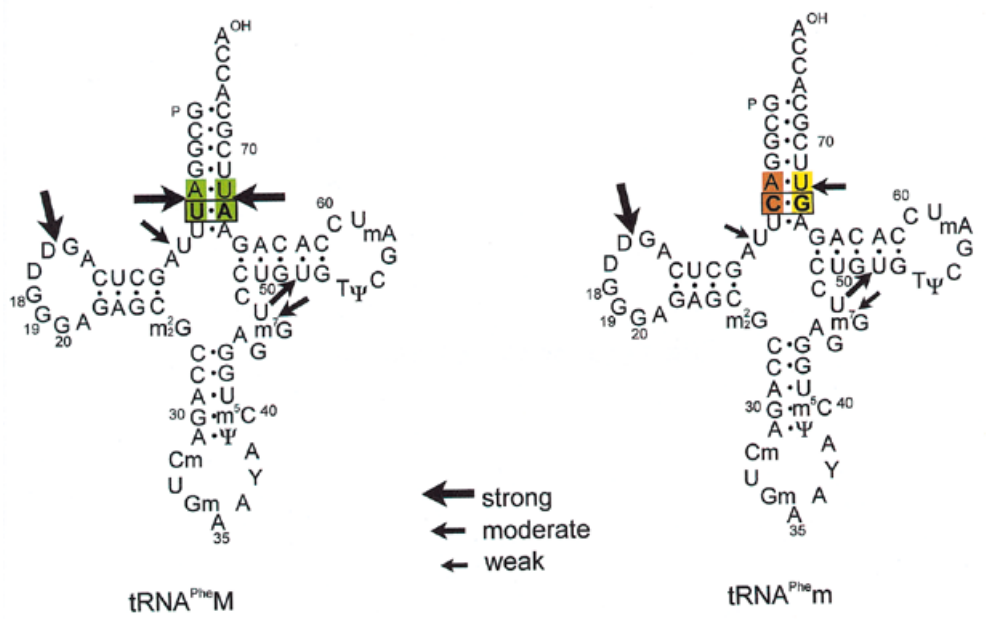


Figure 8. The cleavage positions of yeast tRNA$^{Phe}_{M}$ and yeast tRNA$^{Phe}_{m}$ hydrolyzed by RNase T84.

its structural resemblance to guanine, may be recognized by the enzyme as a nearly legitimate base. This is the case for RNase T1 (11 kDa) of *A.oryzae* (29).

RNase T84 differs also from I-RNase (partially purified from a pig brain extract), which is Mg^{2+} dependent and specifically degrades single-stranded I-RNA. The site of cleavage by I-RNase is non-specific; I-RNase acts as a exonuclease generating 5′ NMPs as products. I-RNase is able to degrade RNAs that previously have been modified by the RED-1 double-stranded RNA adenosine deaminase (dsRAD). dsRADs destabilize dsRNA by converting adenosine to inosine, and some of these enzymes are interferon inducible (32).

The optimal conditions for RNase T84 activity are different from those of most of the human RNases so far described. The pH optimum defined, together with the inhibitory effects of Zn^{2+} ions, suggests that RNase T84 might be active in hydrolyzing RNAs under certain very specific cellular conditions. The fact that neither Mg^{2+} nor low concentrations of Ca^{2+} inhibited RNase T84 activity, combined with the inhibitory effects of $H_2PO_4^{2-}$, suggest that these ions, which are known to regulate many different cell functions, including transmembrane active and passive ion transport, do not participate in the regulation of the activity of RNase T84.

The yeast tRNA^{Asp} and yeast tRNA^{Phe} substrates used in our experiments to establish the sequence specificity of RNase T84 have clearly established secondary and tertiary structure, which therefore enabled us to study the structural preference of RNase T84. RNase T84 hydrolyzed both single- and double-stranded regions of tRNA, but its activity was facilitated by denaturation of tRNA, in contrast to RNase L, which cleaves in single-stranded RNA regions only (27).

Using two naturally occurring yeast tRNA^{Phe} species differing by a single base pair substitution allowed us to show that ApU or GpU dyad sequences are absolutely required for the enzymatic activity of RNase T84 and that the ApU dinucleotide is much better recognized by the enzyme than GpU at the same longer sequence context. The change of ApU present in tRNA$^{Phe}_{M}$ into ApC present in tRNA$^{Phe}_{m}$ resulted in complete inaccessibility of this position to the RNase, while the change of ApU to GpU resulted in a >10-fold lower cleavage rate. Although the ApU or GpU sequence is sufficient to be recognized by T84 enzyme, the presence of a longer characteristic sequence is most probably required for its full activity. We have established that in denatured or native yeast tRNA^{Phe} the T84 enzyme cleaves at the highest rate the phosphodiester bond of the A_5pU_6 dinucleotide.

The observation showing that RNase T84 activity is higher in T84 cells, which are highly differentiated epithelial cells (33), than in HT-29 cells, which under normal culture conditions display an undifferentiated phenotype (34), suggests that RNase T84 activity is especially strong in differentiated human colon adenocarcinoma epithelial cells. This is supported by the low RNase activity found here in pancreatic adenocarcinoma (CFPAC), nasal polyp (CFPEo–) and cervical tumor (HeLa) epithelial cells.

The finding of a new RNase T84 in human tumor cells is of considerable interest in relation to the large body of research exploring the effect of cancer on RNase content (35). The question therefore arises of whether the new tumor-derived RNase T84 could serve as a useful marker for the detection, diagnosis or monitoring of carcinoma of the colon, or cancer in general. Investigations in normal and cancerous human tissues will be necessary to answer this question.

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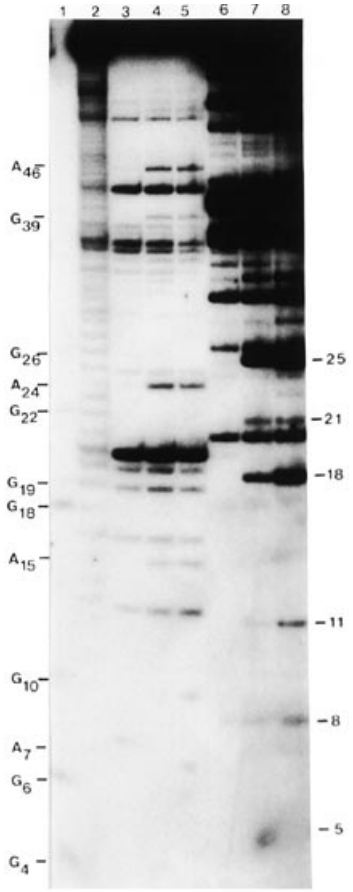

Figure 9. Determination of terminal groups generated by RNase T84. The unlabeled yeast $tRNA^{Asp}$ was incubated with purified RNase T84 and the reaction products were ligated with [^{32}P]pCp as described in Materials and Methods. The final products were analysed on 12% sequencing gels in parallel to the products of digestion of 5′[^{32}P]$tRNA^{Asp}$ by RNase T84. Lane 1, T1 RNase ladder of 5′[^{32}P]$tRNA^{Asp}$; lane 2, H_2O ladder; lane 3, incubation control; lanes 4 and 5, partial digest of 5′[^{32}P]$tRNA^{Asp}$ (1 µg) with increasing concentration of purified RNase T84 (0.03 and 0.06 U, respectively)——the reaction was carried out under denaturing conditions (0.25 M sodium citrate, pH 5.0, 7 M urea, 55°C, 10 min); lane 6, control-$tRNA^{Asp}$ ligated with [^{32}P]pCp; lanes 7 and 8, $tRNA^{Asp}$ (3.2 µg) partially digested with increasing concentration of purified RNase T84 (0.13 and 0.26 U, respectively) under denaturing conditions and than ligated with [^{32}P]pCp.

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REFERENCES

1 Lengyel,P. (1982) *Annu. Rev. Biochem.*, **51**, 251–282.
2 Gleich,G.J., Loegering,D.A., Bell,M.P., Checkel,J.L., Ackerman,S.J. and McKean,D.J. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 3146–3150.
3 Ardelt,W., Mikulski,S.M. and Shogen,K. (1991) *J. Biol. Chem.*, **266**, 245–251.
4 Mikulski,S.M., Chun,H.G., Mittelman,A., Panella,T., Puccio,C.A., Shogen,K. and Constanzi,J.J. (1995) *Int. J. Oncol.*, **6**, 889–897.
5 Fett,J.W., Strydom,D.J., Lobb,R.R., Alderman,E.M., Bethune,J.L., Riordan,J.F. and Vallee,B.L. (1985) *Biochemistry*, **24**, 5480–5486.
6 Raines,R.T., Toscano,M.P., Nierengarten,D.M., Ha,J.H. and Auerbach,R. (1995) *J. Biol. Chem.*, **270**, 17180–17184.
7 McCluree,B.A., Harting,V., Ebert,P.R., Anderson,M.A., Simpson,R.J., Sakiyama,F. and Clarke,A.E. (1989) *Nature*, **342**, 955–957.
8 Taylor,C.B., Bariola,P.A., delCardayrt,S.B., Raines,R.T. and Green,P.J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 5118–5122.
9 Kim,J.-S., Soucek,J., Matousek,J. and Raines,R. (1995) *J. Biol. Chem.*, **270**, 31097–31102.
10 Deutscher,M.P. (1988) *Trends Biochem. Sci.*, **13**, 136–139.
11 Shapiro,R., Fett,J.W., Strydom,D.J. and Vallee,B.L. (1986) *Biochemistry*, **25**, 7255–7264.
12 Sierakowska,H. and Shugar,D. (1977) *Prog. Nucleic Acid Res. Mol. Biol.*, **20**, 59–130.
13 Beintema,J.J., Hofsteenge,J., Iwama,M., Morita,T., Ohgi,K., Irie,M., Sugiyama,R.H., Schieven,G.L., Dekker,C.A. and Glitz,D.G. (1988) *Biochemistry*, **27**, 4530–4538.
14 Sorrentino,S. and Libonati,M. (1997) *FEBS Lett.*, **404**, 1–5.
15 Besancon,F., Przewlocki,G., Baro,I., Hongre,A.-S., Escande,D. and Edelman,A. (1994) *Am. J. Physiol.*, **267**, C1398–C1404.
16 Cozens,A.L., Yezzi,M.J., Chin,L., Simon,E.M., Finkbeiner,W.E., Wagner,J.A. and Gruenert,D.C. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 5171–5175.
17 Schoumacher,R.A., Ram,J., Iannuzzi,M.C., Bradbury,N.A., Wallace,R.W, Tom Hon,C., Kelly,D.R., Schmid,S.M., Gelder,F.B., Rado,T.A. and Frizzell,R.A. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4012–4016.
18 Weber,L.A., Feman,E.R. and Baglioni,C. (1975) *Biochemistry*, **14**, 5315–5321.
19 Morita,T., Niwata,Y., Ohgi,K., Ogawa,M. and Irie,M. (1986) *J. Biochem.*, **99**, 17–25.
20 Blank,A., Sugiyama,R.H. and Dekker,C.A. (1982) *Anal. Biochem.*, **120**, 267–275.
21 Bravo,J., Fernandez,E., Ribo,M., de Llorens,R. and Cuchillo,M. (1994) *Anal. Biochem.*, **219**, 82–86.
22 Minks,M.A., Benvin,S., Moroney,P.A. and Baglioni,C. (1979) *J. Biol. Chem.*, **254**, 5058–5064.
23 Laval,J. and Paoletti,C. (1972) *Biochemistry*, **11**, 3604–3610.
24 Berkner,K.L. and Folk,W.R. (1977) *J. Biol. Chem.*, **252**, 3176–3184.
25 Keith,G., Pixa,G., Fix,C. and Dirheimer,G. (1983) *Biochimie*, **65**, 661–672.
26 Keith,G. and Dirheimer,G. (1987) *Biochem. Biophys. Res. Commun.*, **142**, 183–187.
27 Floyd-Smith,G., Slattery,E. and Lengyel,P. (1981) *Science*, **212**, 1030–1032.
28 Neuwelt,E.A., Frank,J.J. and Levy,C.C. (1976) *J. Biol. Chem.*, **251**, 5752–5758.
29 Irie,M. (1965) *J. Biochem.*, **58**, 599–603.
30 Uchida,T., Arima,T. and Egami,F. (1970) *J. Biochem.*, **67**, 91–102.
31 Hashiguchi,M., Iizuka,M. and Yoshida,H. (1994) *Bull. Shimane Med. Univ.*, **17**, 67–70.
32 Scadden,A.D.J. and Smith,C.W.J. (1997) *EMBO J.*, **16**, 2140–2149.
33 Adams,R.B., Planchon,S.M. and Roche,J.K. (1993) *J. Immunol.*, **150**, 2356–2363.
34 Van Belzen,N., Diesveld,M.P.G., Van Der Made,A.C.J., Nozawa,Y., Dinjens,W.N.M., Vlietstra,R., Trapman,J. and Bosman,F. (1995) *Eur. J. Biochem.*, **234**, 843–848.
35 Maor,D. and Mardiney,M.R.,Jr (1979) *CRC Crit. Rev. Clin. Lab. Sci.*, **10**, 89–111.