

Expression in mammalian cells, purification and characterization of recombinant human pancreatic ribonuclease

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A synthetic cDNA coding for human pancreatic RNase, equipped with a secretion signal sequence, was cloned and stably expressed in Chinese hamster ovary cells. The recombinant RNase, secreted into the culture medium, was purified and characterized. It was found to be indistinguishable, by structural and catalytic parameters, from the enzyme isolated from human pancreas. Furthermore, the glycosylated forms were separated from the non-glycosylated form. Up until now, human RNases have been isolated only in small amounts from autopsied specimens. This has hindered the exploitation of a human RNase for the construction of immunotolerated immunotoxins. On the other hand, the availability of an effective system for the expression of a human RNase may render feasible the transfer, by protein engineering, of the interesting pharmacological actions of non-human RNase [1993 Trends Cell Biol. 3, 106–109] to an immunotolerated, human RNase.

Ribonuclease; Human pancreatic RNase; expression

1. INTRODUCTION

Human pancreas ribonuclease (HP-RNase), already described and partially purified by several investigators [1–4], was isolated and characterized by Weickman et al. [5], and sequenced by Beintema et al. [6]. Compared to bovine pancreatic RNase A [7], the prototype of the pancreatic-like superfamily [8] with whom it shares about 70% of its amino acid residues, HP-RNase has an extension of three residues at the C-terminus, a much higher content in basic residues, and is partially (50%) glycosylated at Asn³⁴ [6,9]. As an enzyme, HP-RNase is less active than RNase A on yeast RNA, but much more active on double-stranded RNA [10,11].

Recently, it has emerged that several ribonucleases (the RISBASES, Ribonucleases with Special Biological Actions) have non-predictable physiological roles or biological actions, in addition to their catalytic activities [12]. The interest in these RISBASES derives also from the potential applications in biotechnology of some of their special actions, such as the antitumor action of bovine seminal RNase [13] and of amphibian onconase [14]. The possibility of using protein engineering to transfer such actions, so far detected only in non-human RISBASES, to an immunotolerated, human RNase, de-

pends on the availability of an effective system for the expression of a cDNA coding for a human RNase. Furthermore, the recently reported construction of specifically targeted immunotoxins with RNases as toxins [15,16] strongly suggests the use of an easily available human moiety in such hybrid molecules.

Here we report the synthesis of a cDNA coding for HP-RNase, its stable expression in cultures of Chinese hamster ovary cells, and the purification and characterization of the recombinant protein.

2. MATERIALS AND METHODS

2.1. Construction of the expression vector

Bacterial strains, and the general procedures for DNA manipulations, and bacterial and Eucaryotic cell growth, as well as the procedure for the construction of the expression vector pMC-E-BS (see below) from plasmid pMThGHSV402 [17], were as previously described [18]. Chinese hamster ovary cells (CHO K1) were obtained from ATCC (Rockville, MD, USA).

Fig. 1 illustrates the strategy employed for the construction of the expression vector for HP-RNase. A cDNA coding for HP-RNase was designed (see Fig. 2) on the basis of the available amino acid sequence of HP-RNase [6], with the aid of a computer program [19]. The cDNA, equipped with the secretion signal sequence of seminal RNase [20], a homologous protein, was constructed from synthetic oligonucleotides (Beckman Analytical), using a novel procedure based on the polymerase chain reaction [21]. The outermost 5'- and 3'-end oligonucleotides contained a *Bam*HI and a *Sal*I restriction site, respectively. The final product of the amplification procedure (491 bp), purified by agarose gel electrophoresis, and trimmed with *Bam*HI and *Sal*I restriction nucleases, was inserted into the plasmid pMC-E-BS, previously freed of the sequence coding for seminal RNase by treatment with the same restriction nucleases. The ligation product was used to transform competent JM101 *Escherichia coli* cells. Sequence analyses of some recombinant clones confirmed that they all harbored the recombinant vector, denominated pMC-E-HP, containing the DNA sequence cod-

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Abbreviations: HP-RNase, human pancreatic ribonuclease; RNase A, bovine pancreatic ribonuclease A; RISBASES, ribonucleases with special biological actions; CHO cells, Chinese hamster ovary cells; poly(A):poly(U), double-stranded polymer made up of poly-AMP and of poly-UMP; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

ing for HP-RNase, with the upstream signal sequence of seminal RNase.

2.2. Other methods

Substrates and RNase A (type XII-A) were purchased from Sigma. Enzymatic assays were performed on yeast RNA after Kunitz [22], and on double-stranded poly(A):poly(U) after Libonati and Floridi [23]. The latter method was modified as suggested by S. Sorrentino (personal communication). Briefly, to 0.1 mM substrate (from its phosphate content), aliquots of enzyme were added to a final volume of 1 ml of 0.1 M MOPS buffer at pH 7.5 containing 0.1 M NaCl. Absorbance at 260 nm was recorded at 25°C as a function of time. One unit of enzymatic activity was defined as the amount of enzyme that produced an increase of A_{260} per min equal to the increase produced by the complete transformation of substrate under the assay conditions.

Polyacrylamide gel electrophoresis (SDS-PAGE), detection on gels of proteins and of RNase activity, and immunoblots were performed as already described [18]. Detection of carbohydrates on gels was obtained with the GlycoTrack Kit from Oxford GlycoSystems following the manufacturer's instructions. Anti-HP-RNase antibodies and HP-RNase from human pancreas [5] were kindly provided by Dohn G. Glitz, University of California, Los Angeles.

3. RESULTS AND DISCUSSION

3.1. Expression of recombinant HP-RNase

Stable transfectants were obtained after co-transfection of CHO cells with both the pMC-E-HP expression vector, containing the cDNA coding for HP-RNase, and the pSV2NEO vector inducing resistance to neomycin [17]. About 100 clones were selected for resistance to G418 (a neomycin analog), individually subcultured, and induced with 40 mM zinc sulphate in the presence of 0.5% fetal calf serum. The medium of each culture was assayed for activity on double-stranded poly(A):poly(U). The clone with the highest secretion level was propagated and found to be stable through 10 population doublings in the absence of the selecting agent G418.

3.2. Purification and characterization of recombinant HP-RNase

Conditioned medium (1 liter) from CHO cells transfected with plasmid pMC-E-HP, and induced as described above, was concentrated 10-fold on an Amicon concentrator equipped with a YM3 membrane. The concentrated material was dialyzed against 50 mM Tris-Cl, pH 7, clarified by centrifugation, and loaded on a column (1.5 × 6 cm) of carboxymethyl-cellulose (CM-32, Whatman) equilibrated in the same buffer. The column was washed with about 200 ml of the equilibration buffer and eluted with 100 ml of a linear NaCl gradient (0–0.4 M) in the same buffer. As shown in Fig. 3, RNase activity distributed among the fraction unadsorbed by the cation exchanger, and two sharp activity peaks, P1 and P2, eluted at about 0.16 M and 0.22 M NaCl, respectively. The P1 and P2 active fractions were de-salted by dialysis against water, and analyzed. The RNase activity unadsorbed by the ion exchange column

was not studied further. Both fractions P1 and P2 were purified, each with a yield of 1.5 mg/l.

After SDS-PAGE and Coomassie blue staining, the RNases in P1 and P2 were found to run as single bands (see Fig. 4, lanes 1–3). The molecular size of P2 was that expected for HP-RNase (about 15 kDa), whereas P1 run with a larger size. Activity staining of a parallel gel (Fig. 4, lanes 4 and 5) confirmed that P2 was a homogeneous protein, whereas P1 was found to be heterogeneous. An identical result was obtained when a parallel gel was blotted onto nitrocellulose and tested with anti-HP-RNase antibodies (Fig. 4, lanes 6, 7). When another identical gel was blotted onto nitrocellulose and treated for carbohydrate detection, only fraction P1 was found to be positive and resolved in at least two components (see Fig. 4, lanes 8 and 9).

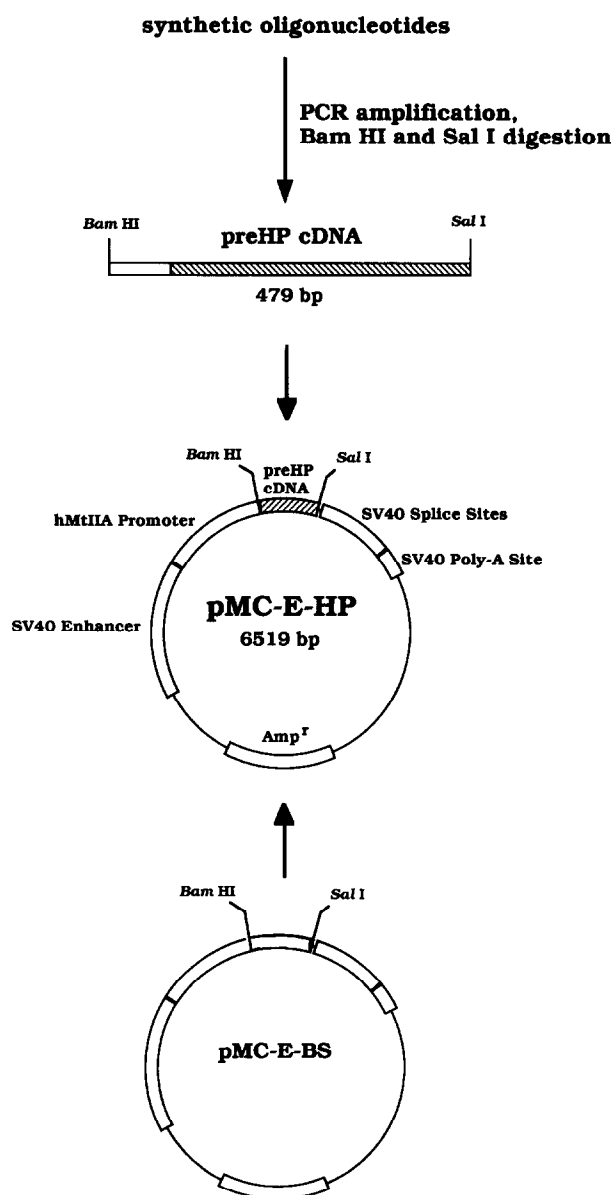


Fig. 1. Strategy for the construction of the plasmid pMC-E-HP for the expression of an HP-RNase cDNA equipped with a signal sequence.

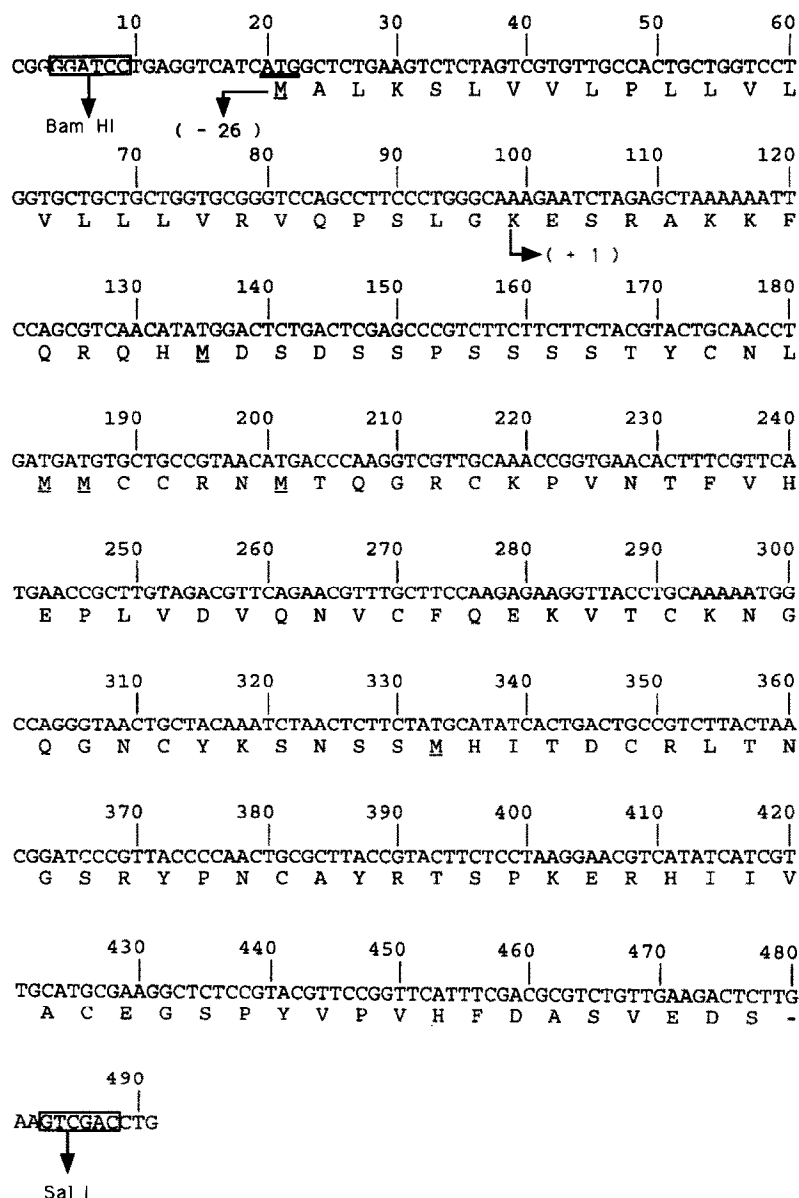


Fig. 2. Sequence of a synthetic cDNA coding for HP-RNase (bases 98–480) with an upstream sequence coding for a secretion signal peptide (bases 20–97). Restriction sites for *Bam*HI (at the 5'-end) and *Sal*I (at the 3'-end) are also shown.

These data indicate that both P1 and P2 fractions can be identified as distinct molecular species of HP-RNase, as they both react with anti-HP-RNase antibodies. They also show that P1 is a mixture of glycosylated forms of HP-RNase, and that P2 does not contain any carbohydrates.

It has been reported [6] that purified HP-RNase consists of two species, one of them glycosylated, in a ratio of about 1:1; furthermore, in that report the authors surmised that the component with the higher mobility separated by SDS-PAGE in these preparations of HP-RNase, was probably the non-glycosylated form of the protein, while the slow-moving components were glycosylated forms. Clearly, the chromatographic system em-

ployed here separated the non-glycosylated form of the enzyme from a composite of glycosylated species.

The amino acid composition of the non-glycosylated form (P2 fraction) was found (not shown) to be identical, within experimental error, with that derived from the sequence of HP-RNase isolated from human pancreas [6]. N-terminal sequence analyses of fractions P1 and P2 of rHP-RNase revealed that for both fractions the sequence of residues 1–19 was identical with that of the protein isolated from human pancreas [6] (data not shown). This indicated a correct processing of the signal sequence.

The two components of rHP-RNase as isolated were also characterized in their main enzymic properties. As

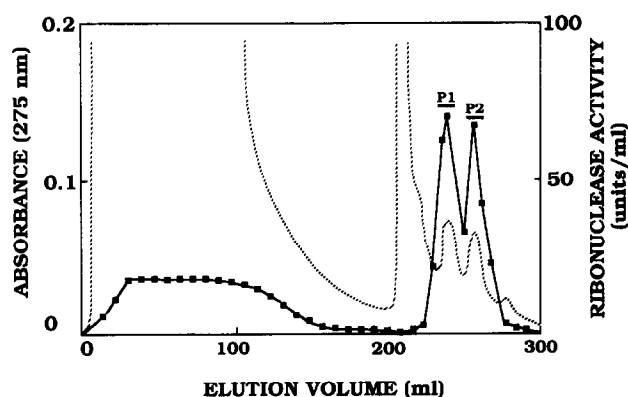


Fig. 3. Carboxymethyl-cellulose chromatography of culture medium of CHO cells transfected with the expression vector pMC-E-HP harboring HP-RNase cDNA. The tracing of absorbance at 275 nm (---) and RNase activity measured on poly(A):poly(U) as a substrate (■-■) are shown.

summarized in Table I, they were found to be virtually undistinguishable one from the other, and both from the enzyme isolated from human pancreas, in their activities on yeast RNA and on a double-stranded poly(A):poly(U) substrate. As previously reported for the enzyme purified from human pancreas [11], rHP-RNase was found to be much more active than RNase A on double-stranded RNA and less active on single-stranded RNA.

3.3. Concluding remarks

HP-RNase was stably produced by CHO cells transfected with a plasmid containing a DNA sequence coding for the protein. Both the non-glycosylated and the glycosylated forms of the protein were obtained and separated.

It should be noted that, to our knowledge, this is the first human RNase, with the exception of angiogenin, an RNase with low catalytic activity on standard RNase

Table I

Comparison among the activities of HP-RNase, rHP-RNase, and RNase A on single- and double-stranded substrates

	Substrate	
	poly(A):poly(U)	yeast RNA
	Specific activity (U/mg)	
HP-RNase	520	33.8
rHP-RNase P1	490	26.1
rHP-RNase P2	502	28.2
RNase A	1.8	104.1

HP-RNase isolated from human pancreas, fractions P1 (glycosylated) and P2 (non-glycosylated) of recombinant rHP-RNase, and RNase A, were tested on double-stranded poly(A):poly(U) and on yeast RNA as described in Section 2.



Fig. 4. SDS-gel electrophoresis of fractions P1 and P2 of recombinant HP-RNase. Lanes 1, 4, 6, and 8: fraction P1 of rHP-RNase. Lanes 2, 5, 7, and 9: fraction P2 of rHP-RNase. Lane 3: a mixture of fraction P1 and P2 of rHP-RNase. Lanes 1-3: staining with Coomassie blue. Lanes 4, 5: RNase zymogram. Lanes 6, 7: immunoblot treated with anti-HP-RNase antibodies. Lanes 8, 9: blot treated for carbohydrate detection.

substrates [24], to be cloned and expressed at satisfactory expression levels (5 mg/l of culture medium).

Recently, the expression in *Saccharomyces cerevisiae* of a synthetic gene for HP-RNase has been reported [25]. The expression product, assayed in the culture medium, was found to be a mixture of glycosylated forms, amounting to less than 1 mg/l of culture medium.

The amount of purified rHP-RNase which can be obtained by the one-step purification procedure reported here from one liter of culture medium is comparable to what can be isolated after a lengthy, several-step procedure, from more than 10 human pancreases.

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REFERENCES

- [1] Delaney, R. (1963) *Biochemistry* 2, 438-444.
- [2] Ukita, T., Takahashi, T., Waku, K. and Hoshino, O. (1964) *J. Biochem. (Tokyo)* 55, 293-302.
- [3] Bardon, A., Sierakowska, H. and Shugar, D. (1976) *Clin. Chimica Acta* 67, 231-243.
- [4] Neuwelt, E.A., Schmukler, M., Niziak, M.S., Jewett, P.B. and Levy, C.C. (1977) *Biochem. J.* 163, 419-426.
- [5] Weickmann, J.L., Elson, M. and Glitz, D.G. (1981) *Biochemistry* 20, 1272-1278.
- [6] Beintema, J.J., Wietzes, P., Weickmann, J.L. and Glitz, D.G. (1984) *Anal. Biochem.* 136, 48-64.
- [7] Blackburn, P. and Moore, S. (1982) *The Enzymes* 15, 417-433.
- [8] Beintema, J.J., Schuller, C., Irie, M. and Carsana, A. (1988) *Progr. Biophys. Mol. Biol.* 1, 165-192.
- [9] Beintema, J.J., Blank, A., Schieven, G.L., Dekker, C.A., Sorrentino, S. and Libonati, M. (1988) *Biochem. J.* 255, 501-505.
- [10] Bardon, A., Sierakowska, H. and Shugar, D. (1976) *Biochim. Biophys. Acta* 438, 461-473.
- [11] Libonati, M. and Sorrentino, S. (1992) *Mol. Cell. Biochem.* 117, 139-151.
- [12] D'Alessio, G. (1993) *Trends Cell Biol.* 3, 106-109.
- [13] D'Alessio, G., Di Donato, A., Parente, A. and Piccoli, R. (1991) *Trends Biochem. Sci.* 16, 104-106.

- [14] Mikulski, S.M., Ardelt, W., Shogen, K., Bernstein, E.H. and Menduke, H. (1990) *J. Nat. Cancer Inst. Lett.* 82, 151–153.
- [15] Rybak, S.M., Saxena, S.K., Ackerman, E.J. and Youle, R.J. (1991) *J. Biol. Chem.* 266, 21202–21207.
- [16] Newton, D.L., Ilercil, O., Laske, D.W., Oldfield, E., Rybak, S.M. and Youle, R.J. (1992) *J. Biol. Chem.* 267, 19572–19578.
- [17] Friedman, J.S., Cofer, C.L., Anderson, C.L., Kushner, J.A., Gray, P.P., Chapman, G.E., Stuart, M.C., Lazarus, L., Shine, J. and Kushner, P.J., (1989) *Bio/Technology* 7, 359–362.
- [18] Russo, N., de Nigris, M., Di Donato, A. and D'Alessio, G. (1993) *FEBS Lett.* 318, 242–244.
- [19] Libertini, G. and Di Donato, A. (1993) *Protein Eng.* 5, 821–825.
- [20] Preuss, K.D., Wagner, S., Freudenstein, J. and Scheit, K.H. (1990) *Nucleic Acids Res.* 18, 1057.
- [21] Di Donato, A., de Nigris, M., Russo, N., Di Biase, S. and D'Alessio, G. (1993) *Anal. Biochem.* 212, 291–293.
- [22] Kunitz, M. (1946) *J. Biol. Chem.* 164, 563–568.
- [23] Libonati, M. and Floridi, A. (1969) *Eur. J. Biochem.* 8, 81–87.
- [24] Shapiro, R., Fox, E.A. and Riordan, J.F. (1989) *Biochemistry* 28, 1726–1732.
- [25] Ribó, M., del Cardayré, S.B., Fernandez, E., De Llorens, R., Raines, R.T. and Cuchillo, C.M. (1993) 3rd International Meeting on Ribonucleases: Chemistry, Biology, Biotechnology, Abstr. P28.