The *Rana catesbeiana* rcr Gene Encoding a Cytotoxic Ribonuclease

TISSUE DISTRIBUTION, CLONING, PURIFICATION, CYTOTOXICITY, AND ACTIVE RESIDUES FOR RNase ACTIVITY

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*Rana catesbeiana* ribonuclease (RC-RNase) is a pyrimidine-guanine sequence-specific ribonuclease found in *R. catesbeiana* (bullfrog) oocytes. It possesses both ribonuclease activity and cytotoxicity against tumor cells. We report here for the first time the cloning of RC-RNase cDNA from liver rather than from oocytes where RC-RNase is stored. An internal fragment of cDNA was obtained by reverse transcription-PCR using deduced oligonucleotides as primers. Full-length cDNA was obtained by 5’- and 3’-RACE technique. The cDNA clone, named rcr gene, contained a 5’-untranslated region, a putative signal peptide (22 amino acids), a mature protein (111 amino acids), a 3’-untranslated region, and a polyadenylation site. The cDNA which encoded the mature protein was fused upstream with a modified *pelB* signal peptide DNA and inserted into pET11d for expression in *Escherichia coli* strain BL21(DE3). The secretory RC-RNase in the culture medium was enzymatically active and was purified to homogeneity. The recombinant RC-RNase had the same amino acid sequence, specific activity, substrate specificity, antigenicity, and cytotoxicity as that of native RC-RNase from frog oocytes. Amino acid residues His-10, Lys-35, and His-103 are involved in RC-RNase catalytic activity. Ribonucleolytic activity was involved in and may be essential for RC-RNase cytotoxicity. DNA sequence analysis showed that RC-RNase had approximately 45% identity to that of RNase superfamily genes. This indicates that RC-RNase is a distinct ribonuclease gene in the RNase superfamily.

Ribonucleases are widely found in living organisms and have been proposed to function in RNA metabolism and gene expression (1). Several abundant ribonucleases have been isolated from organs of various animals and have been well characterized. For example, various kinds of ribonuclease have been purified from bovine organs, e.g., pancreas, liver, kidney, brain, and seminal fluids. RNase A, from bovine pancreas, has been extensively characterized and is widely used in molecular biology (2). The occurrence of several homologous ribonucleases in different organs of the same animal suggests the existence of a family of homologous genes regulated in a tissue-specific fashion. Although these ribonucleases are abundant and well characterized biochemically, their biological significance is still not clear (3, 4). Recently, several proteins with known biological functions were found to have intrinsic ribonucleolytic activity. For example, angiogenin from human possesses both angiogenesis and ribonucleolytic activities (5). Eosinophil-derived neurotoxin and eosinophil cationic protein from humans exert both neurotoxicity and ribonucleolytic activity (6, 7). Bovine seminal ribonuclease, a dimer made up of two identical 124 amino acid subunits, exerts both antitumor and ribonucleolytic activity (8). The ribonucleases from frog oocytes exert antitumor activity as well as ribonucleolytic activity, e.g., onconase from *Rana pipiens* (9–11) and RC-RNase from *Rana catesbeiana* (12, 13).

Three distinct properties are found in the frog RNases. First, the substrate specificity of frog RNase is pyrimidine-guanine and that of mammalian RNase is pyrimidine-adenine (14). Second, RNase from the frog is resistant to RNase inhibitor from human placenta, whereas mammalian RNase is susceptible to the inhibitor. Third, the RNases of frogs with antitumor activity are found in oocytes, e.g., bullfrog RC-RNase (14), whereas most of mammalian RNases with cytotoxic activity are found in eosinophils. Cloning and site-directed mutagenesis of the ribonuclease gene and overexpression of the gene product will enable us to elucidate this difference more effectively than traditional chemical modification methods. However, prior to the present study, cloning of a frog RNase gene had not yet been successful, although some efforts were made. For example, a synthetic gene for onconase was made by oligonucleotide synthesis and assembly, and a semisynthetic gene for onconase was made by ligating a genomic DNA fragment with two synthetic DNA fragments. However, the recombinant products of this gene expressed in *Escherichia coli* was not enzymatically active (15, 16). In this report, we present for the first time the successful cloning of ribonuclease cDNA from frog liver rather than from oocytes where RC-RNase is stored. We also describe the expression of active RC-RNase in *E. coli* and purification of recombinant RC-RNase to homogeneity. Site-directed mutagenesis of RC-RNase was made to determine the residues essential for catalytic activity and cytotoxicity.

EXPERIMENTAL PROCEDURES

Frogs—Native bullfrogs (*R. catesbeiana*) were obtained from a local frog farm and housed at 25 °C. The frogs were 300–400 g in body weight. The tadpoles reached the metadactyl stage by day 28 after hatching. After day 28, the tadpoles were fed 50% fresh shrimp and 50% live earthworms daily. Each frog was given about 100 ml of water daily, which included water changes. Each frog was individually housed in a 25-ml container with pond water. Each frog was fed with 100 ml of water daily which included water changes. Each frog was individually housed in a 25-ml container with pond water.

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TABLE I
Oligonucleotides used as primers for PCR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'→3')</th>
</tr>
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<tbody>
<tr>
<td>RC-I</td>
<td>5'TCGCA GAGAG TTACAC ACCTT Y (C/T) CTT3'</td>
</tr>
<tr>
<td>RC-IA</td>
<td>5'ATN (A/C/G/T) CTC D (A/G/T) GCR (A/G) A AAGTC ACTG3'</td>
</tr>
<tr>
<td>RC-5'-RACE</td>
<td>5'CCTAC CAGGCT ATTCT CTAGA GTGAC3'</td>
</tr>
<tr>
<td>RC-3'-RACE</td>
<td>5'CGGGCG CAATG TCCAT GCGT: ACCAG TGGCC ACCAC TTCCAG3'</td>
</tr>
<tr>
<td>5' Nco-MA</td>
<td>5'CAGCC AGATG TCCCC CAATT CCCC</td>
</tr>
<tr>
<td>3' Xba</td>
<td>5'CCATT CCCTT CTTAGA ATTA3'</td>
</tr>
<tr>
<td>3' pelB</td>
<td>5'CCTTA GCCGG GGCGG GGCGG</td>
</tr>
<tr>
<td>3' Bam</td>
<td>5'GCAGG CCTAG TCGAG ATGGT CTTG</td>
</tr>
<tr>
<td>5' H10A</td>
<td>5'GGAAAC TTTCG ACAGCG AGGCC CATTA TAAAC AGACC G3'</td>
</tr>
<tr>
<td>5' K35A</td>
<td>5'CAGGC AGAAT GAAAC CTTCG ATTA3'</td>
</tr>
<tr>
<td>3' H103A</td>
<td>5'TCTG TATTT CCAAG AAAAA GCTAC GGATA TTTG3'</td>
</tr>
</tbody>
</table>

upstream (GGCG ATG GCC ATG G → GCC ATG GCC ATG NNN N; where N represents any nucleotide) by PCR method using a two-point mutated oligonucleotide (3' pelB) as primer without changing its encoded amino acids. The pelB gene and its upstream region were obtained by PCR method ranging from the XhoI site to the modified Neol site. The cDNA of the native RC-RNase was cloned by PCR using oligonucleotides (5' Nco-MA, 3' Bam) containing the Neol and BamHI sites, respectively, as primers. These PCR products containing the modified pelB gene and the RC-RNase gene were digested with Neol, gel-eluted, and ligated by T4 DNA ligase. For amplification of the ligated DNA fragment, PCR was performed using oligonucleotides (5' Xba, 3' Bam) containing the XhoI and BamHI sites, respectively, as primers. The amplified pelB-RC-RNase ligated DNA fragment was introduced into the pET11d expression vector through the XhoI and BamHI sites and named pET-pel-RC.

Expression of RC-RNase in *E. coli*—The pET-pel-RC transformed cells were grown in TB medium containing 100 μg/ml ampicillin to stationary phase. The supernatant and insoluble pellet of cell lysate were collected by centrifugation. The RC-RNase in supernatant and insoluble pellet, and culture medium was analyzed by Coomassie Blue staining, Western blotting, and zymogram assay.

Cloned and Active Residues of a Cytotoxic Ribonuclease Gene
RESULTS

Cloning of the Full-length of RC-RNase cDNA

Six clones with inserts of predicted size were obtained from the reverse transcription-PCR product of liver mRNA using oligonucleotides RC-1 and RC-IA as primers. The sequence of these six clones corresponded to amino acid residues 34–107 of RC-RNase from frog oocytes. To obtain full-length cDNA, oligonucleotides RC-5'-RACE and RC-3'-RACE in combination with adaptor AP-1 were used as primers for PCR amplification of the 5' upstream and 3' downstream regions of the RC-RNase gene. There were eight clones obtained from 5'-RACE and six clones from 3'-RACE. After fusion of the 5'-RACE and 3'-RACE fragments, four clones with correct sequences in the pGEM-T vector were obtained and named pGEM-RC.

Expression of RC-RNase mRNA in the Liver of Female Bullfrog

As shown in Fig. 2A, the RC-RNase mRNA was only expressed in the liver of female bullfrogs, and no signal was found in the liver of male bullfrogs. The RC-RNase mRNA was not found in other tissues or organs of female frogs, i.e. pancreas, kidney, stomach, intestine, ovary, and spleen. The ribosomal RNA content including 28S rRNA and 18S rRNA in each tissue and organ was similar except 28S rRNA of oocyte was partially degraded as detected by ethidium bromide staining (Fig. 2B). This indicates that the rcr gene was specifically transcribed in the liver of female bullfrogs rather than in oocytes where it localized.

Expression and Purification of Recombinant RC-RNase

The RC-RNase gene with pelB signal sequence was subcloned in expression vector and named pET-pel-RC (Fig. 3).
**FIG. 3. Construction of expression vector pET-pel-RC.** The NcoI site of pelB signal peptide from plasmid pET 22b was moved 6 bases upstream by PCR without changing its encoded amino acids. The NcoI-digested, modified pelB DNA fragment and RC-RNase gene were ligated and amplified by PCR using oligonucleotides containing XhoI and BamHI site, respectively, as primers. The XhoI- and BamHI-digested pelB-RC-RNase fragment and vector fragment from pET11d were ligated and named pET-pel-RC.

pET-pel-RC transformed E. coli BL21(DE3) cells were grown at 37 and 34 °C to stationary phase (A_600 of 1.8). IPTG was added to a final concentration of 0.5 mM, and incubation was continued for another 24 h. Equal aliquots of total supernatant, insoluble pellet, and culture media were taken for analysis. The amount of total protein in culture media was quite low compared with that of the supernatant and insoluble pellet of cell lysate (Fig. 4A). Most of the mature RC-RNase protein was equally present in the supernatants and insoluble pellets, but the precursor of RC-RNase, which had slower mobility than RC-RNase on the gel, existed exclusively in the insoluble pellets upon IPTG induction (Fig. 4B). The insoluble pellets, which contained a large amount of RC-RNase, showed no activity, whereas the RC-RNase in the culture medium and supernatant, possibly derived from the periplasm of E. coli, was active (Fig. 4C). The ribonuclease activity in culture medium increased 5–10-fold when the temperature was shifted from 37 to 34 °C. The activity did not increase further when the temperature dropped to 30 °C. There was no apparent effect of IPTG induction on the production of secreted active RC-RNase in the culture medium. The production of secreted RC-RNase in the culture medium reached a plateau after 3 days' incubation as measured by both Western blotting and the acid-soluble method (data not shown).

The RC-RNase in the culture medium was concentrated by PEG absorption and purified by blue dextran-Sepharose CL6B and carboxymethylcellulose column chromatographies. RC-RNase protein seemed visible on the gel with PEG-concentrated crude samples, and it was homogeneous after being purified by two column chromatographies (Fig. 5A). The recombinant RC-RNase had similar antigenicity and identical mobility as that of native RC-RNase from bullfrog as determined by Western blotting analysis (Fig. 5B). Both recombinant and native RC-RNase also showed similar specific activity and identical mobility as observed on the zymogram of RNA-casting SDS-PAGE (Fig. 5C). These results indicated that recombinant RC-RNase had similar properties to that of native RC-RNase. The results of purification after quantitation of ribonuclease activity at each purification step using the acid-soluble method are summarized in Table II. 2.55 mg of RC-RNase was purified from 120 ml of PEG-concentrated medium which came from the original 700 ml of culture medium. The yield of ribonuclease activity was 28.5% from the concentrated medium and 11% from the original culture medium. The specific activity of the recombinant RC-RNase was the same as that of native RC-RNase from bullfrog oocytes as determined by the acid-soluble method (12).

**General Properties of Recombinant RC-RNase**

**Amino Acid Sequence Identity**—The recombinant RC-RNase purified from E. coli culture media has a modified N terminus similar to that of native RC-RNase, and neither yield any signal upon Edman degradation. Therefore, the protein was fragmented with CNBr for internal sequence analysis. One peptide had a sequence of NVLST which was identical to residues 59–63 of native RC-RNase from bullfrog oocytes as determined by the acid-soluble method encoded by the cDNA.

**Substrate Specificity**—Only dinucleotides CpG and UpG were cleaved by the recombinant RC-RNase, whereas the others, i.e., UpA, ApU, CpC, UpU and Cpa, were not cleaved under the same assay conditions. This property was identical to that

**FIG. 4. Expression of RC-RNase in pET-pel-RC transformed E. coli BL21(DE3).** A, total protein analysis of soluble proteins, insoluble debris, and culture media. 40 ml of pET-pel-RC transformed cells were cultivated to stationary phase (A_600 of 1.8) at 34 or 37 °C. IPTG (0.5 mM) was added and incubated for another 24 h. One two-hundredth aliquot of 40-ml culture was taken for analysis. The order of sample alignment was similar to A except RC-RNase in lane 1 of A was absent. RC, RC-RNase, pRC, RC-RNase precursor. C, ribonuclease activity assay on RNA-casting SDS-PAGE. One ten-thousandth aliquot of 40-ml culture was taken for activity assay. The order of sample alignment was identical to that of A.

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FIG. 5. Analysis of RC-RNase purified from E. coli culture media. A, components of proteins at each purification step. Samples were separated by 13.3% SDS-PAGE and stained by Coomassie Blue. Lane 1, PEG concentrated crude media (90 µg); lane 2, blue dextran-Sepharose CL6B column eluate (45 µg); lane 3, CM-cellulose column eluate (3 µg, estimated by SDS-PAGE and Coomassie Blue staining using bovine pancreatic RNase A as standard); lane 4, RC-RNase from bullfrog oocytes (4 µg). B, Western blotting analysis of RC-RNase. One-tenth aliquot of sample as described in A was taken for Western blotting analysis. The order of sample alignment was identical to that of A. C, ribonuclease activity assay on RNA-casting SDS-PAGE. One-fiftieth aliquot of sample as described in A was taken for activity assay. The order of sample alignment was identical to that of A.

TABLE II

Purification of RC-RNase from E. coli culture media

| Step                | Volume (ml) | Protein* (µg) | Total activity b (unit/mg) | Specific activity (unit/mg) | Yield (%)
|---------------------|-------------|---------------|---------------------------|----------------------------|--------
| Concentrated media  | 120         | 1,845,400     | 2,653                      | 35.7                       | 28.5   |
| Blue dextran-Sepharose | 50          | 658,200       | 21,940                     | 35.7                       |        |
| CM-cellulose        | 28          | 2,250         | 525,900                    | 206,130                    | 28.5   |

* Protein concentrations of steps 1 and 2 samples were measured by the Bradford method (21) and that of step 3 sample was estimated on gel stained with Coomassie Brilliant Blue R using bovine pancreatic RNase A as standard.

b One enzyme unit is defined as the amount of enzyme producing one unit of acid-soluble material under standard assay condition as described in the text.

DISCUSSION

The ribonuclease (RNase) gene superfamily combines functionally divergent proteins that share statistically significant sequence and DNA sequence homologies. The amino acid sequence of RC-RNase is homologous to those of frogs’ RNases, i.e., 79.1% identity for Rana japonica oocytic RNase, 65.5% identity for R. catesbeiana liver RNase, and 52.4% identity for R. pipiens oocytic RNase (oncomase) as calculated by the GCG-GAP program (15). It is not highly homologous to human eosinophil-derived neurotoxin (26.7% identity) and eosinophil cationic protein (25.0% identity), which are both cytotoxic to some neurons (6, 7). The homologies of RC-RNase to other mammalian RNase genes are between 27.6 and 34.3% identity.

The DNA sequence homologies among frog RNase genes could not be compared because no DNA sequence data are available except for the RC-RNase gene. The homologies of RC-RNase to the RNase superfamily is between 41.5 and 47.9% which compared with 12 RNase genes by the GCG-GAP program. The relationship of these RNase genes is expressed as a phylogenetic tree created by the GCG-PILEUP program in Fig. 8. Amino acid sequence analysis indicated that RC-RNase is a cognate member in the frog RNase family but it is a distinct class in the RNase superfamily according to both amino acid sequence and DNA sequence.

The amino acid sequence of RC-RNase was toxic to the HeLa Tet-On cell. Typical phenomena of apoptosis were observed in the detached cells after RC-RNase treatment at day 2 and day 3, e.g., nuclear fragmentation, cytoplasmic blebbing, and DNA laddering. The surviving attached cells detached and floated into the medium after further RC-RNase incubation. These results show that the residue His-103 is involved in both RNase activity and cytotoxicity.

Amino Acid Sequence and DNA Sequence Homologies in RNase Superfamily

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![Image](http://www.jbc.org/)

* H. C. Huang and Y. D. Liao, unpublished results.
RC-RNase were found on the RNA-casting gel (data not shown). Therefore, the establishment of a secretory expression system enabled us to obtain a recombinant RC-RNase comparable with native RC-RNase directly from culture media without further treatment.

Antitumor activity of RNase exists only in frog oocytic RNases and bovine seminal dimer RNase but not in other RNases, e.g. RNase A, bovine seminal monomer RNase, and bullfrog liver RNase. Neurotoxicity was found in human EDN, ECP, as well as in frog onconase. Chemical inactivation on RNase activity of onconase, EDN, and ECP by iodoacetate reduced the cytotoxicity/neurotoxicity of these proteins. These results indicate that RNase activity is essential to induce cytotoxic or neurotoxic action (9, 11, 24). However, two single base pair mutations (K35R, H128D) were introduced in human angioatin (9, 11, 24). However, two single base pair mutations (K35R, H128D) were introduced in human angioatin (38), rat pancreatic RNase (39), and chicken bone marrow RNase (40).

Cloning and Active Residues of a Cytotoxic Ribonuclease Gene

Fig. 7. Cytotoxic effects of RC-RNase on HeLa Tet-On cells. HeLa Tet-On cells (1.5 x 10⁶, CLONTECH) were seeded on 60-mm Nunc plates overnight in Dulbecco's modified Eagle's medium containing 10% fetal calf serum before 2 µg RC-RNase treatments. Cell numbers were counted by trypan blue exclusion assay (13). ●, cell without RC-RNase treatment; ○, cells treated by native RC-RNase from frog oocytes; ■, cells treated by recombinant wild type RC-RNase; □, cells treated by H103A RC-RNase. Bars represent standard deviations of three experiments.

Fig. 8. Phylogenetic tree of ribonuclease genes. The DNA sequences of the following RNase genes were aligned by GCG-PILEUP program: bullfrog (R. catesbeiana) oocytic RNase (RC-RNase), bovine pancreatic RNase (19), bovine brain RNase (31), bovine seminal RNase (32), human pancreatic RNase (33), human eosinophil-cationic protein (34), human eosinophil-derived neurotoxin (35), human angioatin (36), mouse pancreatic RNase (37), mouse eosinophil-associated RNases (38), rat pancreatic RNase (39), and chicken bone marrow RNase (40).

amino acid sequence similarity. Known members assigned to this family include secretory and nonsecretory RNases, angioatin, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), antitumor protein (onconase), and RC-RNase from frog oocytes. Most of these genes in the superfamily have been cloned and sequenced and possess DNA sequence homology as shown in Fig. 8. The RNase gene from frogs has not previously been cloned and compared, although they possess several important properties, e.g. substrate specificity for pyrimidine-guanine and cytotoxic activity toward tumor cells. In this report, we succeeded in cloning RC-RNase gene and characterized the active residues for RNase activity and possible sites for cytotoxicity. From DNA sequence analysis, we found that RC-RNase is a distinct class in the RNase superfamily.

Onconase from R. pipoens oocytes combined with tamoxifen is being evaluated in human phase III clinical trials in patients with pancreatic carcinoma, and it is approaching maturity as a new member of the anticancer arsenal of drugs (16). However, a gene from R. pipoens has not been successfully cloned. A synthetic gene was made by oligonucleotide synthesis and assembly, and a semisynthetic gene was obtained by ligating a genomic DNA fragment with two synthetic DNA fragments. Those onconase genes expressed in E. coli and formed inclusion bodies. No RNase activity was observed until it was renatured by extensive steps. The specific activity of renatured onconase is 3–10% of native oonconase from frog oocytes. In addition, an extra methionine is added on onconase's N terminus which is crucial for its RNase activity and cytotoxicity. The N-terminal methionine is removed by CNBr cleavage only if its internal methionine is substituted by other residues (15, 16). In this report, a modified pelB signal peptide was introduced in front of RC-RNase; therefore, the expressed RC-RNase was secreted into the culture medium and was enzymatically active. There were still some RC-RNases with the same size as that of mature form RC-RNase that remained in soluble fractions and insoluble pellets of cell lysates, but they were not enzymatically active until denaturation and renaturation were performed. However, the renatured RC-RNase was not homogeneous because several minor bands with slower mobility than native RC-RNase were found on the RNA-casting gel (data not shown). Therefore, the establishment of a secretory expression system enabled us to obtain a recombinant RC-RNase comparable with native RC-RNase directly from culture media without further treatment.

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Sue Lin-Chao for critical readings and helpful discussions of the manuscript.

Vitellogenin is a major yolk protein used for nutrition during embryogenesis. The vitellogenin gene is transcribed and translated in liver. The protein is secreted into the bloodstream and stored in the yolk granules of oocytes. The RNase activity and cytotoxicity of vitellogenin in the yolk granules is regulated by compartmentation in yolk granules. This is why the vitellogenin gene could be transcribed, translated, and stored in the yolk granules of oocytes, although a putative signal peptide exists in front of the vitellogenin gene.

Ribonucleases, e.g. RNase A. Successful cloning and series mutation of RC-RNase gene followed by RNase activity and cytotoxicity assay in a later study will enable us to find the receptor-binding domain of RC-RNase which may be crucial for understanding the cytotoxicity of RC-RNase.

Vitellogenin is a major yolk protein used for nutrition during embryogenesis. The vitellogenin gene is transcribed and translated in liver. The protein is secreted into the bloodstream and stored in the yolk granules of oocytes in X. laevis (28, 29). In bullfrogs, the RC-RNase gene is transcribed in the liver and the protein is closely associated with vitellogenin in the yolk granules of oocytes (22, 30). No precursor form of RC-RNase is found in the oocyte, although a putative signal peptide exists in front of RC-RNase from DNA sequence analysis of the cloned gene. Therefore, it is suggested that RC-RNase is transcribed, translated, and processed in the liver, secreted into the bloodstream, and then stored in the yolk granules of oocytes. This is why the RC-RNase gene could be cloned from bullfrog liver rather than from oocytes. The possible function of RC-RNase in oocytes and early embryos may be involved in a defense mechanism against predators because of its cytotoxicity. The RNase activity and cytotoxicity of the oocyte itself is regulated by compartmentation in yolk granules and RNase-specific inhibitor binding in the cytosol to prevent self-attack of RNA and susceptible factors (22, 30). Therefore, the absence of receptors on cell surfaces as well as the presence of inhibitors in the cytosol may also protect bullfrogs from self-attack in other tissues.

Acknowledgments—We thank Drs. T. C. Lee, K. King, J. Y. Yeng, and Sue Lin-Chao for critical readings and helpful discussions of the manuscript.

REFERENCES

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