Preface

Nucleases, enzymes that restructure or degrade nucleic acid polymers, are vital to the control of every area of metabolism. They range from "housekeeping" enzymes with broad substrate ranges to extremely specific tools ***(1)***. Many types of nucleases are used in lab protocols, and their commercial and clinical uses are expanding. The purpose of *Nuclease Methods and Protocols* is to introduce the reader to some well-characterized protein nucleases, and the methods used to determine their activity, structure, interaction with other molecules, and physiological role. Each chapter begins with a mini-review on a specific nuclease or a nuclease-related theme. Although many chapters cover several topics, they were arbitrarily divided into five parts:

Part I, "Characterizing Nuclease Activity," includes protocols and assays to determine general (processive, distributive) or specific mechanisms. Methods to assay nuclease products, identify cloned nucleases, and determine their physiological role are also included here.

Part II, "Inhibitors and Activators of Nucleases," summarizes assays for measuring the effects of other proteins and small molecules. Many of these inhibitors have clinical relevance.

Part III, "Relating Nuclease Structure and Function," provides an overview of methods to determine or model the 3-D structure of nucleases and their complexes with substrates and inhibitors. A 3-D structure can greatly aid the rational design of nucleases and inhibitors for specific purposes.

Part IV, "Nucleases in the Clinic," summarizes assays and protocols suitable for use with tissues and for nuclease based therapeutics.

Part V, "Nucleases in the Lab," includes protocols for the use of nucleases in cloning and in determining the activity of other proteins.

The experienced reader will immediately recognize several of the nucleases used as examples throughout this book, especially RNase A and restriction enzymes. However, new nucleases with novel specificity, often performing unexpected functions, are constantly being discovered. For example, a regulator of the unfolded protein response, identified initially as a kinase, is also a sequence-specific ribonuclease (Chapter 3). A human analog of a plant ribonuclease was discovered in the search for a tumor suppressor protein (Chapter 7), whereas angiogenin (Chapter 25) was cloned as a factor stimulating blood vessel formation. RNase L is one of the mediators of Interferon activity (Chapter 12).

Researchers who unmask a nuclease disguised as a cytokine, mating factor, toxin, and so forth should find the methods for characterizing their protein described in the first section of this book particularly useful. These chapters suggest questions to ask about the nuclease's activity or primary structure. Is the amino acid sequence novel or similar to one of the major families of nucleases (Chapters 7 and 18)? Is the cleavage processive or distributive, i.e., does the nuclease scan the nucleic acid polymer

and cleave repeatedly before separating, as has been shown for restriction endonucleases and glycosylases (Chapter 1) or, does it, in the fashion of RNase A (Chapter 2), cleave and simultaneously release the polymer, generating products that are at the same time novel substrates? Does the back (synthesis) reaction affect the kinetics of the cleavage process? Is the nuclease activity essential for metabolic activity, as McClure and coworkers (Chapter 5) have elegantly demonstrated for the stylar RNases? Finally, is the activity sensitive to known inhibitors or activators (Chapters 8, 9, and 12)?

These questions all pave the way for characterization of the 3-D structures of nucleases and their complexes with substrates and activity modulators. The chapters at the center of this book were selected to introduce the reader to methods that can be used to define the tertiary structure of nucleases. Of course, a complete tertiary structure determination by X-ray crystallography (Chapters 13, 14, 17, 19, and 20) or NMR (Chapter 16) requires a good deal of time and specialized techniques too complicated to be summarized here. However, if the sequence has significant identity to a protein for which a structure has been determined, tools now available on the Internet allow one to model the probable 3-D structure (Chapter 18). The methods described aid in the design of nucleases with new properties (Chapters 15 and 20) and improved inhibitors (Chapters 13 and 14).

Nuclease-based therapies and diagnostics are slowly coming into the clinic. DNase I therapy (Chapters 20 and 21) has improved the lives of thousands of cystic fibrosis victims. Nucleases with demonstrated antitumor activity (Chapter 24) stimulated clinical trials of other members of the RNase A family and modified forms (Chapters 23 and 26). Better understanding of nucleases that repair damaged DNA (Chapters 1 and 18), mediate retroviral integration and replication (Chapters 10 and 22), or play a role in cytokine and growth factor mechanisms (Chapters 9, 12, and 25) is important both in understanding disease progression and developing better therapeutic modalities. Antisense therapies, for example, depend on directing the activity of intracellular RNase H (Chapter 11).

As more medical professionals become aware of the importance of nucleases in metabolism and the improved assays for their activity, there will almost certainly be an increase in their use in diagnostics. A recent report, for example ***(2)***, correlated increases in the levels of eosinophil cationic protein in asthmatics allergic to grass pollen with the risk of onset of prolonged asthmatic symptoms.

Finally, nucleases are a major research tool in molecular biology. The exquisite specificity of restriction endonucleases (Chapters 19, 27, and 28) is routinely used in gene cloning. Exploiting the special qualities of a subclass, hapaxoterminers, can make subcloning and gene modification easier (Chapter 29). There are many uses for nonspecific nucleases as well. DNase I can be used to locate the binding sites of proteins on DNA (Chapter 30) and S1-nuclease (Chapter 31) or ribonuclease (Chapter 32) mapping to quantitate specific mRNAs. Degradation of nucleic acid polymers with nonspecific nucleases, including DNase I, RNase A, and the endonuclease from *Serratia* (Chapter 17), can be used to clarify lysates and ease protein purification.

All the chapters describe why and when to use the assays, and the thinking that went into the development of the protocols. These comments, and the Notes on the method, provide guidance and insight when things go wrong (i.e., not as planned),

and for how to go about correcting them. Protocols change in their details constantly. A Northern blot to check the expression in a tissue from various organs of mRNA for a specific protein, which 10 years ago required weeks to prepare, can be done today in a few hours starting with a commercially prepared membrane. The reader is welcome to simplify these protocols further as new developments allow.

We can anticipate that the model proteins used to develop new biophysical methods and clinical therapies, which have changed little in the past 30 years, will show more variety in the future. Most scientists will claim that they use RNase A since the protein is small, soluble, and easy to assay and to refold from a completely denatured state (Chapter 15). However, ready availability and force of habit contribute to the attention paid this "ideal" protein. RNase A was first crystallized ***(3,4)***, Bill Wyckoff has suggested only partially in jest, because a meat packing company made it available free of charge in a highly purified form. The easy purification of pancreatic RNase from the cadavers of zoo animals made this family a Rosetta stone for comparative biology as well. However, many new nucleases are commercially available, and the genome projects are revealing copious sequence information about nuclease families that may be more important metabolically. The examples in *Nuclease Methods and Protocols*, as varied as they are, are only starting points for exploration in the wide world of nucleases.

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2

Analysis by HPLC of Distributive Activities and the Synthetic (Back) Reaction of Pancreatic-Type Ribonucleases

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1. Introduction

1.1. Distributive vs Processive Cleavage of Polymeric Substrates

Nucleic-acid cleavage can be processive, with the enzyme moving from one site to the next in the polymer before dissociating from the substrate, or distributive, with partially cleaved substrates released to the medium after the initial reaction. Kinetics indicate that either mechanism can be seen in reactions with polymerases, helicases, or nucleases ***(1)***. However, many factors affect these kinetics, including the strength of the enzyme–substrate binding, which can be altered by the salt concentration of the buffer, and intermediates in the formation of the complex or the release of the products that may be poorly characterized. The distinction between processivity and distributivity is based primarily on the ratio of rate constants for cleavage and dissociation. Unlike the distinction between endo- and exo-nucleases (i.e., those cleaving within or from one end of the nucleic acid), which will not change with reaction conditions, although in some cases enzymes show only a preference from one or the other activity, assay conditions can blur the distinction between processivity and distributivity.

An example of the analysis of a processive reaction, that of DNA repair by bacterial glycosylases at low salt concentrations, is presented in Chapter 1. Here we present HPLC-based methods to analyze the distributive cleavage of RNA by bovine pancreatic ribonuclease (RNase A), and other enzymes of this family.

1.2. The Enzymatic Mechanism of Pancreatic Ribonucleases

Bovine pancreatic ribonuclease A (RNase A) and related enzymes catalyze the breakdown of their natural substrate, RNA, in two steps. The first step in the cleavage of the 3'-5' phosphodiester bonds of RNA is a transphosphorylation reaction from the 5' position of one nucleotide to the 2' position of an adjacent pyrimidine nucleotide to form two polynucleotides, one ending in a 2',3'-cyclic phosphate and another with a free 5'-OH end (**Fig. 1A**). These RNases have some degree of base specificity, cleaving polyadenylic acid (poly(A)) poorly in comparison to polycytidylic acid (poly(C)) or

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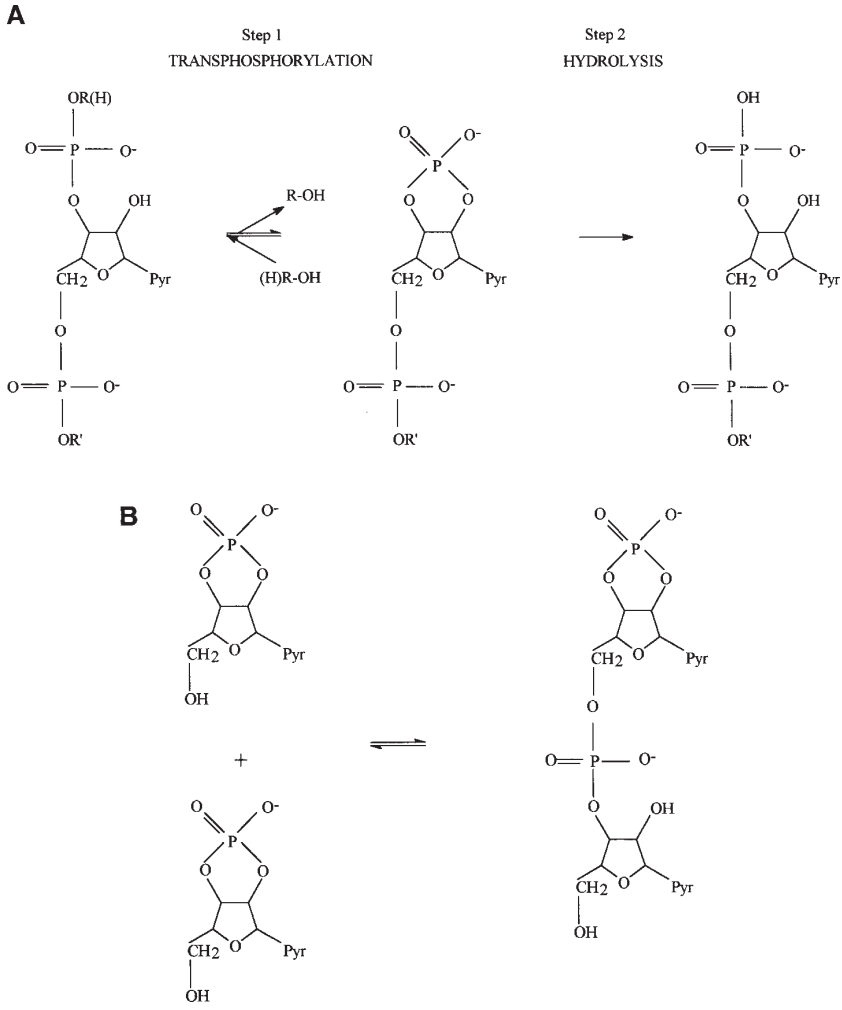

Fig. 1. **(A)** The reaction catalyzed by RNase A. The reaction for the complete breakdown of RNA takes place in two steps: transphosphorylation and hydrolysis. The pyrimidine 2',3'-cyclic phosphate intermediates are released to the medium as true products of the reaction. The hydrolysis of these cyclic compounds only begins when the transphosphorylation reaction is nearly finished. **(B)** The RNase A synthetic (back) reaction from cytidine 2',3'-cyclic phosphate (C>p) to cytidylyl 3',5'-cytidine 2',3'-cyclic phosphate (CpC>p).

polyuridylic acid (poly(U)) ***(2)***. Thus the RNA cleavage will yield purine-core oligonucleotides ending with a pyrimidine 2',3'-cyclic phosphate nucleotide ($(Pur)_nPyr>p$). In the second step of the enzyme reaction, the terminal 2',3'-cyclic phosphodiester of the core oligonucleotides is hydrolyzed to a 3'-nucleotide ***(3)*** (**Fig. 1A**). The transphosphorylation reaction needs a dinucleoside monophosphate as the minimum size for the substrate, but the hydrolysis reaction can take place with a 2',3'-cyclic pyrimidine mononucleotide.

1.3. Cleavage of Polymeric Substrates

Characterizing the RNA breakdown kinetically is complicated by the structural complexity of the RNA molecule, the difficulty of monitoring a very fast reaction, and the fact that most products of the reaction are also substrates, although with different specificity constants *(4)*. In **Subheadings 3.1.** and **3.2.** we describe assays to study different aspects of the processes catalyzed by RNase A *(4–6)* and the eosinophil cationic protein (ECP) *(7)*, using methods based on the separation of the reaction products by HPLC. The pattern of product formation from poly(C) or poly(U) to oligonucleotides of different size, $(Cp)_nC>p$ or $(Up)_nU>p$ respectively, indicates the endo- or exonuclease preference of these "nonspecific" enzymes. The pattern of poly(C) cleavage, for example, indicates that RNase A does not act in a random fashion. Rather, it preferentially binds longer-substrate molecules and cleaves phosphodiester bonds 6–7 residues from the end of the chain *(5)*. In contrast, ECP cleaves predominantly in an exonuclease-like manner *(7)*.

1.4. Characterizing the Synthetic (Back) Reaction

At sufficiently high concentrations of cytidine 2',3'-cyclic phosphate (C>p) as substrate, RNase A generates the hydrolysis product 3'-CMP, or the synthesis product cytidylyl 3',5'-cytidine 2',3'-cyclic phosphate (CpC >p) (*see* **Fig. 1B**). The hydrolysis reaction must be considered formally as a special case of the transphosphorylation back reaction in which the R group of the R-OH substrate is H (*see* **Fig. 1A**). We show in **Subheading 3.3.** how the products of the RNase A reaction of C>p at concentrations above 10 m*M* (pH 5.5), 3'-CMP and CpC>p, can be separated and quantified by means of anion-exchange HPLC. This method was used to characterize the kinetics of both reactions that take place in the same assay mixture *(4,8,9)*.

The methods described here, developed for RNase A, can be applied to different RNases by adjusting the enzyme and substrate concentrations and the assay conditions (pH, ionic strength, temperature, and reaction time).

2. Materials

2.1. Equipment

1. Apparatus: An HPLC system with two pumps (or one pump with a gradient mixer system), a liquid chromatography injector, a UV absorbance detector, and a computer for both control of the system and data processing. We have used the following systems: Amersham Pharmacia Biotech, Waters Corp. (Mildford, MA), and Varian Associates (Sunnyvale, CA).
2. Chromatography columns: reversed-phase HPLC column, Nova Pak C_{18}, 4 μm, 3.9 × 150 mm (Waters Corp.), anion-exchange column, Nucleosil 10 SB, 300 mm × 4 mm I.D., and Vydac-310 SB precolumn stationary phase (Macherey, Nagel and Co., Düren, Germany).
3. Use distilled water treated with a MilliQ water purification system (Millipore Corp.) for the preparation of all solutions.
4. HPLC solvents: acetonitrile 240/farUV HPLC grade (Scharlau Chemie [Barcelona, Spain] or Carlo Erba [Milano, Italy]), ammonium acetate GR (Merck) and acetic acid (analytical grade) (Carlo Erba).
5. Substrates and enzymes: poly(C), C>p (ε_{254} = 7214 M^{-1}/cm), 3'-CMP, 2'-CMP, pepsin from porcine stomach mucosa and bovine pancreatic RNase A (Type XII-A) ($\varepsilon_{277.5}$ = 9800 M^{-1}/cm *[10]*) from Sigma. All other reagents are of analytical grade.

2.2. Solutions

1. Poly(C) solution: 5 mg/mL in 10 m*M* HEPES, pH 7.5, with KOH (or 10 m*M* Tris-HCl, pH 7.5).
2. C>p solution: between 10 and 40 m*M* in 0.2 *M* sodium acetate, pH 5.5.
3. Dissolve RNase A (or the enzyme that is analyzed) in the same buffer as the substrate, and adjust the enzyme concentration according to its activity.
4. Pepsin solution: 3.6 mg/mL in 0.2 *M* H_3PO_4 in 0.1 *M* HCl.
5. Solvent A: 10% ammonium acetate (w/v) in water, degassed before use, to which 1% acetonitrile (v/v) is added.
6. Solvent B: 10% ammonium acetate (w/v) in water, degassed before use, to which 11% acetonitrile (v/v) is added.
7. 0.1 *M* ammonium acetate, pH 5.5, and 0.6 *M* ammonium acetate, pH 5.5, solutions are degassed before use.

3. Methods

3.1. Analysis of the Digestion Products of Poly(C) by Ribonuclease A (Fig. 2)

1. Combine 50 µL of poly(C) solution with 10 µL of 30 n*M* RNase A at 25°C. Stop the reaction by applying samples at different time intervals depending on the enzyme activity (between 0 and 45 min in this case) directly to the HPLC.
2. HPLC procedure:
   a. Nova Pak C_{18} column conditions: flow-rate: 1 mL/min, pressure: 1000–2000 psi (68–136 atm). Fix the maximum pressure limit to 6000 psi (408 atm) to avoid damaging the column. Store the column in 100% acetonitrile, and wash for 20 min with 1% acetonitrile in HPLC-grade water before use.
   b. Separation procedure: Wash the column for 15 min with solvent A. Inject 20 µL of the reaction mixture onto the column. Wash for 10 min, and then elute with a 50-min linear gradient from 100% solvent A to 10% solvent A plus 90% solvent B.
   c. After each run, wash the system for 5 min with water containing 1% acetonitrile and for 10 min with 100% acetonitrile.
   d. Before reequilibrating with solvent A, wash 5 min with water containing 1% acetonitrile. Slight differences in the retention times of oligonucleotides can be produced, depending on the equilibration time of the column. The intermediate washes with water are important to avoid contact between a highly concentrated saline solution and a concentrated organic solvent, which can produce some precipitates with the subsequent clogging of the column and a sharp increase in the pressure of the system.
   e. Monitor the absorbance at 260 nm of the eluate to detect and quantify product. Previously, obtain the number of integration counts per absorbance unit using a standard solution of nucleotide.
   f. Identification of the products: at the initial conditions (**Fig. 2**, $t = 0$), although poly(C) is not electrophoretically homogeneous (according to the information provided by Sigma), all high-molecular-mass components elute as a single peak, and no oligonucleotides or other small-molecular-mass contaminants are present in the sample. The elution position of the small oligonucleotides (**Fig. 3**) is deduced from the pattern of the poly(C) digestion by RNase A after a long incubation time (100 min) when no high molecular mass poly(C) is left, the pattern found by McFarland and Borer ***(11)*** for the chemical hydrolysis of polynucleotides, and the MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass-spectrometry analysis of the individual peaks, except for the two mononucleotide species (3'-CMP and C>p) which yield

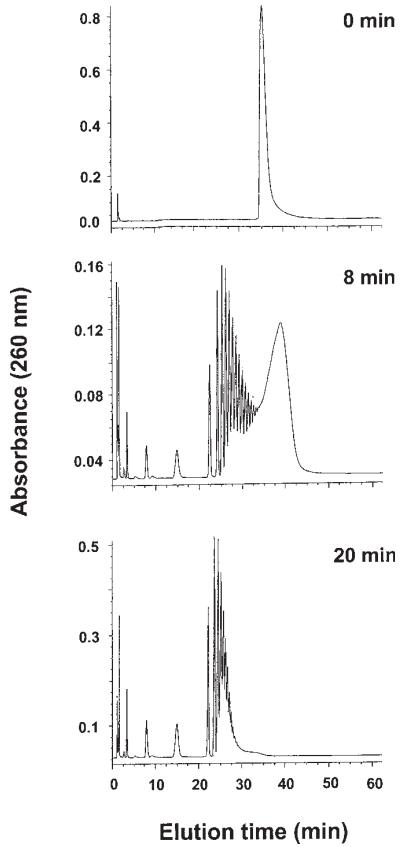

Fig. 2. Analysis by reversed-phase HPLC column (Nova-Pak C_{18} column) of products obtained from poly(C) digestion by RNase A at different time intervals. In each case, 50 µL of a 5 mg/mL poly(C) solution in 10 m*M* HEPES-KOH, pH 7.5, is digested with 10 µL of 30 n*M* RNase A at 25°C. At the initial conditions (t = 0 min) poly(C) elute as a single peak and small molecular mass contaminants are not observed. Poly(C) fraction diminished with the increase of the reaction time (t = 8 and 20 min) with the subsequent formation of oligonucleotides.

unclear spectra. The two mononucleotide products 3'-CMP and C>p elute sequentially, but very close in the first peak, and oligomers of increasing size elute with increasing retention times (**Fig. 3**). Oligonucleotides up to nine residues can be separated with a good resolution.

3. Quantification of results:
   a. Determine the relative formation of mononucleotides and low-mol-mass oligonucleotides (up to nine nucleotides) by integration of the 260-nm peak area and, to normalize the relation between the peak area and the quantity of oligonucleotides, divide the area of each peak by the number of nucleotide residues. As the system cannot resolve nucleotides >10 bases, for the calculation of the relative distribution of each oligonucleotide, the area corresponding to all oligonucleotides with $n > 9$ can be considered as a high-mol-mass product together with the undigested substrate fraction ***(9)***.

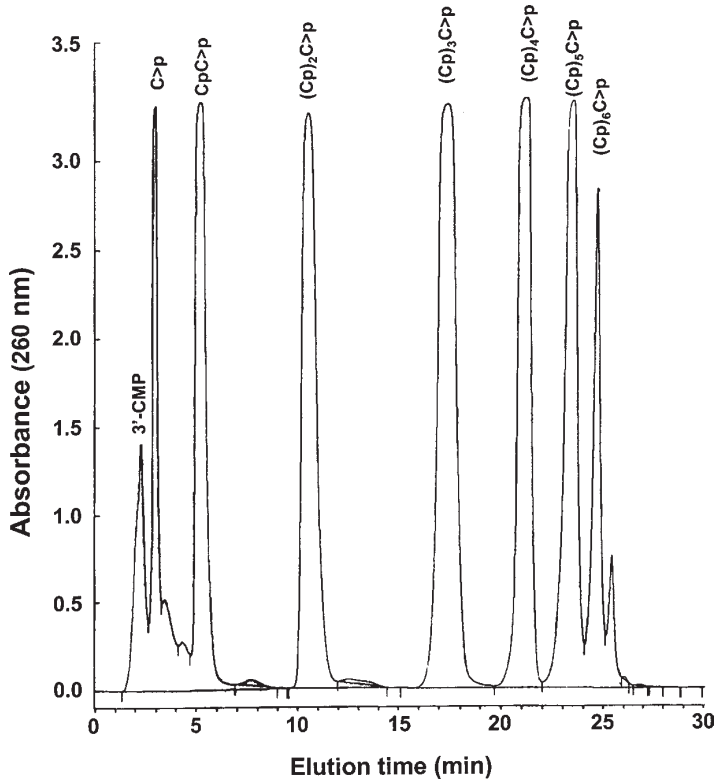

Fig. 3. Elution profile on a reversed-phase HPLC column (Nova-Pak C_{18} column) of oligocytidylic acids $(Cp)_nC>p$ ($n = 0–6$) from poly(C) digestion. 200 µL of 10 mg/mL poly(C), in 10 m*M* HEPES-KOH, pH 7.5, are digested with 15 µL of 0.5 µ*M* RNase A at 25°C for 16 min.

b. To quantify the relative amount of oligonucleotides up to seven residues, divide the 260-nm peak area by the extinction coefficient at 260 nm (ε_{260}): 15,175 M^{-1}/cm for CpC>p, 20,745 M^{-1}/cm for $(Cp)_2C>p$, 24,282 M^{-1}/cm for $(Cp)_3C>p$, 28,683 M^{-1}/cm for $(Cp)_4C>p$, 37,711 M^{-1}/cm for $(Cp)_5C>p$, and 42,428 M^{-1}/cm for $(Cp)_6C>p$.

3.2. Analysis of Product Formation from Oligocytidylic Acids $(Cp)_nC>p$ by Ribonuclease A (Fig. 4)

The method described in **Subheading 3.1.** can also be used for the large-scale preparation of oligonucleotides of the general structure $(Cp)_nC>p$ or $(Up)_nU>p$, in which *n* ranges from 0–6. These oligonucleotides are good substrates for assessing the role of the noncatalytic binding subsites, adjacent to the active site, in the catalytic process.

1. Preparation of oligocytidylic acids: Digest 500 µL of poly(C) solution with 50 µL of 7 µ*M* RNase A at 25°C for 5 min. Separate oligonucleotides of different length according to the method described in **Subheading 3.1.** Collect the fractions that correspond to tetra, penta, hexa, and heptacytidylic acids, pool with the corresponding fractions of several chromatographic runs, freeze-dry, and keep at –20°C until use.

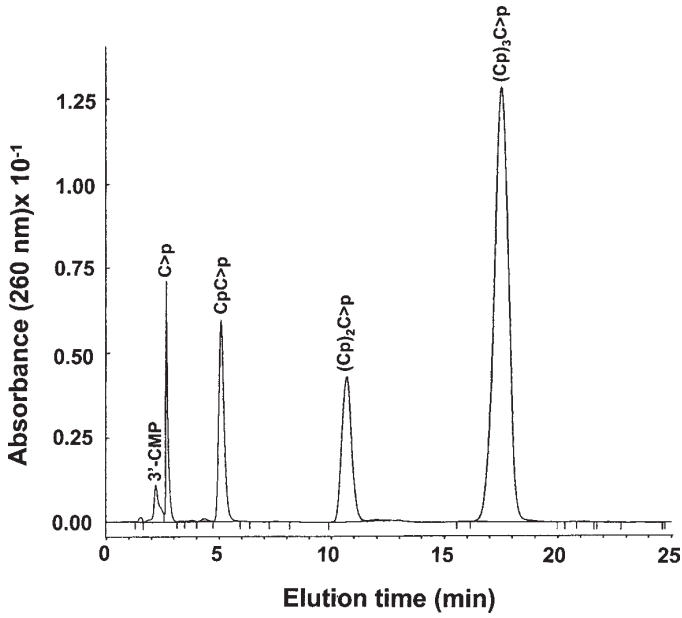

Fig. 4. HPLC separation by reversed-phase HPLC (Nova-Pak C_{18} column) of the products obtained from $(Cp)_3C>p$ digestion by RNase A. 100 µL of $(Cp)_3C>p$ solution (0.3 U of *A* at 260 nm) in 10 m*M* HEPES-KOH, pH 7.5, are digested with 10 µL of 0.1 n*M* RNase A at 25°C for 15 min.

2. Digestion of oligonucleotides: Use the general reaction conditions described for poly(C) digestion. Specific characteristics are related to the substrate and enzyme concentrations. Use substrate solutions with an approximate A_{260} of 0.3; the enzyme concentration depends both on the substrate and the enzyme species used. **Figure 4** is an example, which shows the specific conditions applied to the digestion of $(Cp)_3C>p$ by RNase A.
3. Separate the reaction products by the HPLC procedure described in **Subheading 3.1.**
4. Calculate the amount of each oligonucleotide product by integration of the area of the peaks corresponding to the absorbance at 260 nm divided by the corresponding extinction coefficient (ε_{260}). The number of integration counts per *A*U, using a standard solution of nucleotide, should be obtained beforehand.

3.3. Analysis of the Hydrolysis (3'-CMP Formation) and Synthesis (CpC>p Formation) Reactions by RNase A Using C>p as Substrate (Fig. 5)

The synthetic reaction is only measurable at substrate concentrations above 10 m*M*. At lower substrate concentrations, only the hydrolysis reaction is observed.

1. In a typical assay the reaction mixture contains 30 µL substrate solution and 5 µL RNase A.
2. Quenching procedure: Incubate the assay mixture (35 µL) at 25°C for the desired time. Quench the reaction by the addition of 25 µL 0.2 *M* H_3PO_4 in 0.1 *M* HCl; the final pH of the reaction is 2.0. Immediately add 5 µL of the pepsin solution and incubate at 25°C for 15 min. Pepsin cleaves the Phe120–Asp121 bond in RNase A, and irreversible inactivation takes place. The mixture can be analyzed immediately or stored at –20°C. Storage

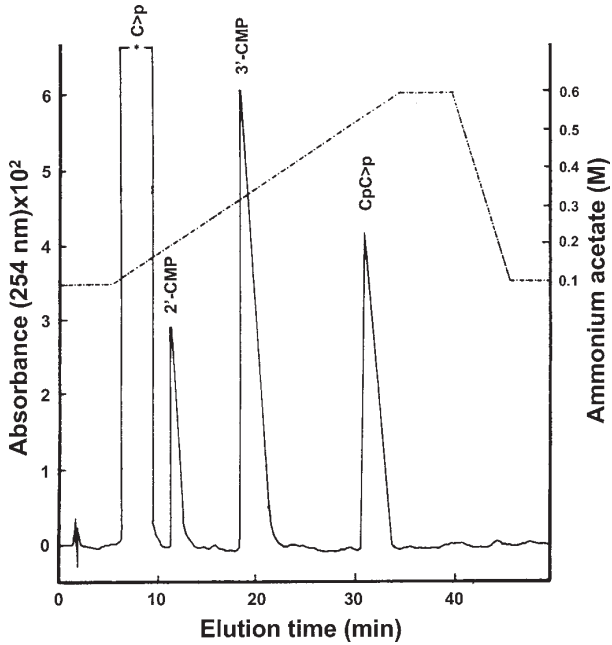

Fig. 5. HPLC separation by anion-exchange chromatography (Nucleosil 10 SB) of the products of the simultaneous hydrolysis and synthesis activities of RNase A. In this case, a reaction mixture containing 40 m*M* C>p and 0.6 μ*M* RNase A in 0.2 *M* sodium acetate, pH 5.5, is incubated at 25°C for 5 min, and the reaction is stopped by the quenching procedure described in the text. Product separation is carried out with a gradient from 0.1–0.6 *M* ammonium acetate, pH 5.5.

of the samples for several months at –20°C does not affect the reproducibility of the results if the correct controls are included.

3. Increase the pH of the sample to 5.5 by the addition of 4 μL 2 *M* NaOH before injecting the sample onto the HPLC column.
4. HPLC procedure:
   a. Nucleosil 10 SB anion-exchange column and Vydac-310 SB precolumn stationary phase conditions: flow-rate: 1 mL/min, pressure: 500–600 psi (34–40 atm). Fix the maximum pressure limit to 3000 psi (204 atm) to avoid problems in the event of collapse of the column. The precolumn prevents the clogging of the HPLC column with protein (pepsin or digested RNase). Change the precolumn after approx 20 runs. Store the column in 20% methanol and wash the column for 20 min with MilliQ-water before use.
   b. Separation procedure: wash the column with 0.1 *M* ammonium acetate, pH 5.5 for 30 min. Inject 20 μL of the sample onto the column. Elute with a linear-salt gradient from 0.1 to 0.6 ammonium acetate, pH 5.5.
   c. Monitor and quantify product elution from the absorbance at 254 nm.
   d. Identification of the products: **Fig. 5** shows a typical chromatogram. Although the hydrolytic action of RNase A on C>p produces only 3'-CMP, a small amount of 2'-CMP appeared in the chromatogram. Both nucleotide monophosphates are produced by direct hydrolysis of C>p in the acid quench. The peaks corresponding to C>p, 2'-CMP, and 3'-CMP can be confirmed by the use of the respective standards. C>p, the sub-

strate of the reaction, elutes with a retention time of 10 min, the 2'-CMP a product of the acid treatment elutes at 12 min; the 3'-CMP (the product of the hydrolysis reaction) at 16 min, and CpCp (the product of the synthesis reaction) at 34 min. The effect of the acid in the formation of 2'-CMP and 3'-CMP is evaluated by a blank containing all the components of the assay except RNase A. It must be considered that the chemical hydrolysis yields equal amounts of 2'-CMP and 3'-CMP; thus, in order to quantify the amount of 3'-CMP formed by RNase A, an amount of 3'-CMP equal to the amount of 2'-CMP is subtracted from the total amount of 3'-CMP.

e. Quantification of the results: Determine the amount of each nucleotide from the integration of the peak areas divided by the corresponding extinction coefficient, ε_{254}, which are 7214 M^{-1}/cm for C>p, 6686 M^{-1}/cm for 3'-CMP, and 14,400 M^{-1}/cm for Cp >p. The number of integration counts per *A*U from standard solutions of C>p or 3'-CMP should be determined beforehand.

4. Notes

1. The method described for poly(C) digestion analysis (**Subheading 3.1.**) has been applied to poly(U) digestion with similar results, although the digestion pattern depends on the specificity of the enzyme *(7)*.
2. In the digestion of oligocytidylic acids $(Cp)_nC>p$ by RNase A (**Subheading 3.2.**) the enzyme concentrations must be extremely low (in the nanomolar range). To avoid denaturation, prepare a concentrated solution of the enzyme and dilute further immediately before the assay. It is also advisable to distribute the substrate in Eppendorf tubes, each one with the exact volume for an individual assay, and keep them frozen. They are thawed immediately before use, equilibrated to 25°C, and then the enzyme solution is added.
3. In the quenching procedure described in **Subheading 3.3.** the final pH (2.0) is critical. Below this pH, too much acid hydrolysis to 2'-CMP and 3'-CMP occurs. At higher pH levels, residual RNase activity can falsify the results.

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