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Preferred Sites of Digestion of a Ribonuclease from *Enterobacter sp.* in the Sequence Analysis of *Bacillus stearothermophilus* 5S Ribonucleic Acid†

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ABSTRACT: The preferred sites of digestion of a ribonuclease from *Enterobacter sp.* have been characterized by analysis of the oligonucleotides produced after digestion of 5S [^{32}P]-RNA from *Bacillus stearothermophilus* with this enzyme. The major degradation products obtained contain cytidylic acid at the 3′-phosphorylated end and adenylic acid at the 5′-hydroxylated end. Less commonly, cleavage occurred between cytidylic and guanylic or between uridylic and adenylic acid phosphodiester bonds. There was no cleavage between two purines or between two pyrimidines and the phosphodiester bonds between uridylic and guanylic acid residues remained intact. The ribonuclease has proved valuable in determining the primary structure of *B. stearothermophilus* 5S RNA and is useful in general for nucleotide sequence analysis of RNA.

Recently progress has been made in determining the nucleotide sequences of a wide variety of RNA molecules including such large molecules as the genomes of Qβ (Billeter *et al.*, 1969) and R17 (Nichols, 1970; Jeppesen *et al.*, 1970), RNA phages (Adams and Cory, 1970), and the 16S rRNA of *Escherichia coli* (Fellner, 1969) as well as discrete RNA species transcribed from DNA *in vitro* (Lebowitz *et al.*, 1971). Although continuing advances are being made in the preparation and isolation of discrete RNA fragments which contain genetic information of substantial biological interest, a number of serious technical problems remain in the sequence analysis of even moderately long RNA chains. Among the common problems in nucleotide sequence determination are the analysis of long stretches of pyrimidines and purines, the analysis of very large oligonucleotides produced by T_1 ribonuclease digestion, and the ordering within an RNA chain of fragments produced by specific nucleases.

Because of these problems we have continued to examine a wide variety of nucleases which may prove to be of use in this area. We report here that an enzyme ("C" ribonuclease)[1] isolated originally by Levy and Goldman (1970) from *Enterobacter sp.* grown on poly(cytidylic acid), has particular utility since it produces clean, discrete, and relatively large oligonucleotides from a wide variety of RNAs. The products obtained are useful in arranging in order oligonucleotides produced by T_1 ribonuclease digestion. Furthermore this nuclease has potential utility in the sequence analysis of large oligonucleotides lacking internal guanylic acids. The 5S RNAs are convenient to prepare in reasonably pure forms and are complex enough to provide a range of sequences with various amounts of secondary structures and yet simple enough so that all the products of a nuclease digest can be analyzed. We have used 5S [^{32}P]RNA from *Bacillus stearothermophilus* to determine the preferred sites of digestion of this nuclease.

Materials and Methods

B. stearothermophilus 1430R (kindly provided by Dr. P. Lengyel) was grown at 60° with vigorous shaking initially in a rich medium containing 1% yeast extract, 1% Casamino acids, 1% glucose, $FeCl_3 \cdot 5H_2O$, 0.016 g; NH_4Cl, 2.0 g; Na_2HPO_4, 6.0 g; $NaH_2PO_4 \cdot H_2O$, 3.0 g; Na_2SO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; in a total volume of 1 l.; the pH was adjusted to 7.2. Cells from a log-phase culture were harvested by centrifugation and resuspended in a low phosphate medium containing 0.5% glucose; Tris-HCl, 14.5 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; 0.1 g of aspartic and glutamic acids; 0.005 g of methionine, phenylalanine, and glutamine. The pH of the medium was adjusted to 7.5 and the final volume was 1 l.

The generation time in the low phosphate medium was 70

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[1] "C" ribonuclease refers to the *Enterobacter sp.* ribonuclease.

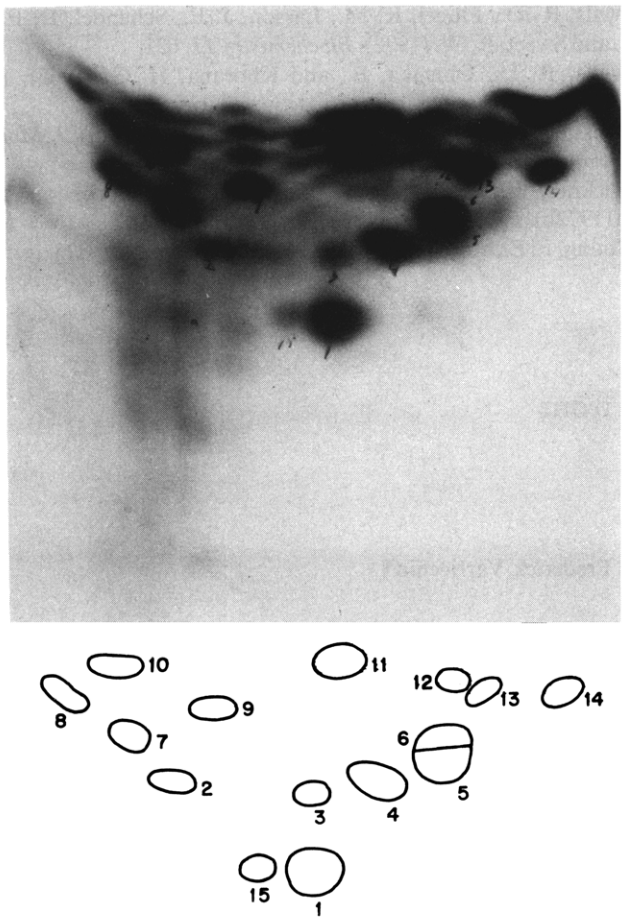

FIGURE 1. Two-dimensional fractionation of products of digestion of *B. stearothermophilus* 5S RNA with the "C" enzyme. Digestion of 40 μg of RNA containing 2×10^6 dpm of ^{32}P-labeled 5S RNA, was with 2 μl of "C" enzyme for 30 min at 37°. First dimension: on Cellogel in pyridine–acetate buffer containing 7 M urea (DuBuy and Weissman, 1971). Second dimension: chromatography from below upward with the "homo A" mixture of Brownlee and Sanger (1969) on a 40 × 20 cm. Tlc plate coated with a 9:1 mixture of cellulose to DEAE-cellulose: (a, top) photograph of autoradiograph; (b, bottom) schematic sketch of autoradiograph.

min. An actively growing culture was split into two parts and to one part was added 10–20 mCi of carrier-free [^{32}P]P_i (New England Nuclear Corp.) which had previously been neutralized with NaOH. Cell growth was monitored in the second culture at A_{600}. When cell growth diminished, the ^{32}P-containing culture was made 0.3% in Casamino acids and after 15 min longer the cells were harvested by centrifugation.

The pellet was resuspended in 2 ml of 0.01 M Tris (pH 7.5 at 4°) and 0.02 ml of Na_2EDTA (0.2 M) and 20 μl of deoxyribonuclease (2 mg/ml) was added; the suspension was incubated for 5 min at 25° and then sodium dodecyl sulfate was added to a final concentration of 0.2%. The suspension was extracted with one-half volume of water-saturated phenol by shaking for 75 min at room temperature; the phenol phase was extracted with one-half volume water and the combined washings were again extracted with an equal volume of phenol. The RNA was precipitated with ethanol and the 5S RNA component was purified by electrophoresis on 10% polyacrylamide gels containing 7 M urea by previously described methods (Varricchio, 1971).

The "C" ribonuclease was isolated from *Enterobacter sp.* (laboratory strain 3CA) grown in a defined medium containing poly(cytidylic acid). The purification procedure, general properties, and definition of units of activity of this nuclease were described previously (Levy and Goldman, 1970). The enzyme was dissolved in water and stored frozen. To digest 5S RNA with "C" ribonuclease, a total of 10–40 μg of RNA was dissolved in 4 μl of a buffer containing 0.1 M Tris-HCl (pH 7.5) and 1 mM $CoCl_2$. In a typical reaction, 0.4 unit of "C" ribonuclease was added in 4 μl of water and the reaction mixture was incubated for 40 min at 37°. The sample was then immediately applied to Cellogel strips. Fractionation of the digests was carried out by electrophoresis on Cellogel in a pyridine–acetate buffer at pH 3.5 in the presence of 7 M urea in the first dimension (Sanger *et al.*, 1965), and in the second dimension on DEAE-cellulose thin-layer plates by the "homochromatography" method (Brownlee and Sanger, 1969).

Autoradiographs were prepared and the oligonucleotides were further characterized by digestion with T_1 ribonuclease or pancreatic ribonuclease and electrophoresis of the resulting fragments on DEAE-cellulose paper at pH 1.7. The procedure for elution of "C" ribonuclease products from chromatography plates and for their identification with T_1 and pancreaticribonucleases were the same as the methods described previously for characterization of large oligonucleotides derived from limited digestion of RNA with T_1 ribonuclease (Brownlee *et al.*, 1968; Brownlee and Sanger, 1969).

Results

5S RNA Cleavage Products Produced by "C" Ribonuclease. To study the preferred sites of digestion and the utility of the "C" ribonuclease, 5S RNA was digested with this enzyme as described above and the products were separated by two-dimensional electrophoresis and chromatography in either the "homo A" (Figure 1) or "homo B" system of Brownlee and Sanger (1969). In each case, a discrete set of oligonucleotides was obtained with a low background of fragments produced by alternative cleavage patterns, even when the digestion was carried out for 90 min with 0.8 unit of enzyme/15 μg of RNA.

The oligonucleotides were analyzed initially by digestion with T_1 ribonuclease and electrophoresis of the products on DEAE paper at pH 1.7 as described in the legend to Table I.

Because of the knowledge already available about the sequence of the *B. stearothermophilus* 5S RNA and its complete T_1 RNase digestion products (C. A. Marotta, S. M. Weissman, and F. Varricchio, in preparation), it was possible to deduce the sequence of each of the "C" enzyme digestion products illustrated in Table I and the positions of these oligonucleotides within the 5S RNA sequence (Figure 2).

Discussion

As with other 5S RNA species which have been completely analyzed (Brownlee *et al.*, 1968; Forget and Weissman, 1969; DuBuy and Weissman, 1971; Hindley and Page, 1972), *B. stearothermophilus* 5S RNA possesses a well-defined sequence. We did not detect variation at any position. The sequence permits extensive base pairing, for example, between the 5′ and 3′ ends. The derivation of the complete sequence and a discussion of possible models of base pairing will be presented elsewhere.

The "C" ribonucelase produced no product that arose from cleavage between two purines. On the other hand, the oligonucleotides produced contained a purine at the 5′-hydroxyl end. In the majority of products, cleavage occurred between a cytidylic acid and adenylic acid. Several products were ob-

TABLE I: Oligonucleotides Produced by Digestion of *Bacillus stearothermophilus* 5S RNA with "C" Ribonuclease.[a]

Oligonucleotides[b]	Products Obtained from Extensive T_1 RNase Digestion[c]	Sequence Deduced[d]
1	A-Gp, Gp, C-Gp, A-A-A-Cp	A-G-C-G(G,(A)-G, A-G, A-G)A-A-A-Cp
2	A-Gp, Gp, U-U-Gp, U-C-Gp, C-U-A-Gp	A-G-G-U-U-G-U-C-G-C-U-A-G-G-C
3	A-Gp, Gp, A-A-A-Cp	G(G, (A)-G, A-G, A-G)A-A-A-Cp
4	A-A-Gp, C-U-C-U-C-C-A-Gp, Cp	A-A-G-C-U-C-U-C-C-C-A-G-Cp
5	C-C-C-C-U-Gp, A-Gp, C-Gp, Cp	A-G-C-G-C-C-C-U-G-Cp
7	U-U-Gp, A-Gp, Gp, C-Cp	A-G-U-U-G-G-G-G-C-Cp
8	U-U-Gp, U-C-Gp, A-Gp, Gp (C, U)[e]	A-G-G-U-U-G-U-C-G-C-Up
9	A-C-Gp, Gp, A-A-Gp, U-Up	A-C-G(G, A-A-G)U-Up
10	Gp,A-A-Gp, U-Up	G(A-A-G, G)U-Up
11	A-Gp, U-Gp, A-Cp, A-A-Gp, A-Gp, Up	A-G-U-G-A-Cp, A-A-G-A-G-Up
12	A-A-Gp, (U_2, C_4)[e]	A-A-G-C-U-C-U-C-C-Cp
13	(A-Cp, Cp, Cp)Gp,[e] (U_2, C_4)[e]	A-C-C-C-G-U-C-U-C-Cp
14	(A-U, C_3)[e]Gp, A-A-Cp	A-U-C-C-C-G-A-A-Cp
15	pC(C, U)[e]	pC-C-Up
16	Gp, C-C-Gp, A-U-Gp, Up	G-C-C-G-A-U-G-G-Up
17	Gp, A-U-Gp, Up	G-A-U-G-G-Up
18	(A-C, C_2)Gp[e], A-A-Cp, U-C-U-C-C-A-U-C-C-C-Gp	A-C-C-C-G-U-C-U-C-C-A-U-C-C-C-G-A-A-Cp
19	U-U-Gp, C-C-C-C-U-Gp, C-C-A-Gp, A-Gp, C-Gp, Cp, Gp	A-G-U-U-G-G-G-G-C-C-A-G-C-G-C-C-C-C-U-G-Cp
20	p-C-C-U-A-Gp, U-Gp, (A-A-U, A-C)[e]	pC-C-U-A-G-U-G-A-C-A-A-Up
21	Gp, Up	G-G-Up
22	A-Gp, Cp	A-G-Cp
23	AGp, Gp, A-A-A-C-A-C-C-C-Gp, Up, Cp	A-G-C-G-(A-G, (A)-G, G, A-G)A-A-A-C-A-C-C-C-G-U-C-U-C-Cp
24	AGp, Gp, A-A-A-C-A-C-C-C-Gp, U-C-U-C-C-A-U-C-C-C-Gp,[e] A-A-Cp	A-G-C-G-(A-G, (A)-G, G, A-G)A-A-A-C-A-C-C-C-G-U-C-U-C-C-A-U-C-C-C-G-A-A-Cp

[a] Abbreviations used are: C, U, A, G, cytidylic, uridylic, adenylic, and guanylic acids, respectively. A hyphen between two nucleotides indicates an intact phosphodiester linkage. [b] The numbers 1–13 refer to oligonucleotides shown in the "homo A" plate of Figure 1. The other numbers refer to oligonucleotides found in other digests and fractionated by either the "homo A" or "homo B" chromatographic systems (Brownlee and Sanger, 1969). [c] Each oligonucleotide produced by digestion with the "C" RNase was isolated from the DEAE-cellulose plates and redigested with 15 μl of T_1 RNase, 2500 IU/ml, and incubated at 37° for 60 min; digests were applied to DEAE-cellulose paper and fractionated by electrophoresis at pH 1.7. Further identification was obtained by alkaline hydrolysis, 15 μl of 0.5 M NaOH, 37°, 24 hr; the resulting mononucleotides were separated at pH 3.5 on Whatman No.1 paper; or, by digestion with pancreatic RNase, 15 μl, 1 mg/ml, 37°, 60 min, and separation of resulting products on DEAE-cellulose paper at pH 3.5. Lines under a product indicate it was present in more than 1 mol compared to other products. [d] These are the only sequences consistent with the data obtained from analysis of the pancreatic and complete and partial T_1 RNase products of *B. stearothermophilus* 5S RNA (C. A. Marotta, S. M. Weissman, F. Varricchio, in preparation). [e] The internal order of these oligonucleotides was not determined directly but was deduced from knowledge of the internal sequence of the products of T_1 RNase digestion of *B. stearothermophilus* 5S RNA. [f] This product was a mixture of the oligonucleotides whose sequences are shown.

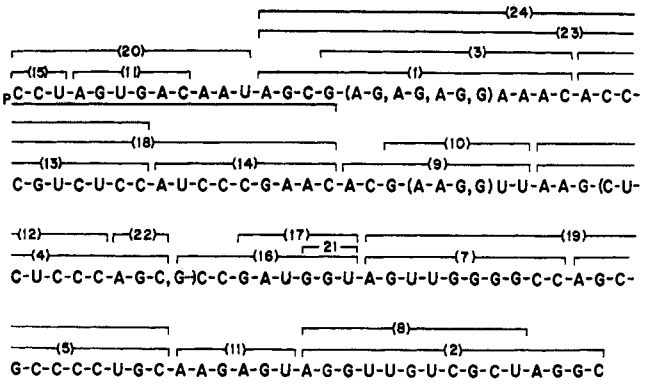

FIGURE 2: Origin of oligonucleotides produced by "C" RNase digestion of *B. stearothermophilus* 5S RNA. The sequence of the RNA is shown with brackets indicating the "C" RNase fragments described in Table I.

served in which cleavage had occurred either between cytidylic and guanylic acids or between uridylic and adenylic acids. In this group of analyses no product was found in which the phosphodiester link between uridylic and guanylic acids had been hydrolyzed even after 90-min incubations. Bonds between pyrimidines were not hydrolyzed. Levy and Goldman (1970) noted hydrolysis of poly(C) by this enzyme after incubation for 4 hr with enzyme to substrate ratios of 1–10 units/400 μg of RNA. Under our conditions cleavage between pyrimidines was not noted but, in view of the results of Levy and Goldman, it would seem probable that with more extensive digestion one might get CpC cleavage with 5S RNA. In practice there is a considerable range of conditions over which this does not occur. It is to be noted that degradation products of 5S RNA included material (oligonucleotides 2 and 20 of Figure 2) that arose by cleavage of the presumptively base-paired regions at the end of the 5S RNA molecule, so that secondary structure of the RNA is not the predominant factor in limiting cleavage of pyrimidine-linked phosphodiester bonds.

Results on larger RNA molecules, such as portions of the RNA transcribed from SV40 DNA (B. S. Zain, R. Dhar, S. M. Weissman, and A. M. Lewis, in preparation) have confirmed the mode of action of the "C" ribonuclease. Although this latter RNA lacks sufficient secondary structure to give reproducible, simple, partial T_1 ribonuclease digestion products, it has been possible to obtain a variety of discrete digestion products with the "C" enzyme which overlap T_1 oligonucleotides and which expedite very considerably the ordering of these oligonucleotides during the sequence analysis. When the time of digestion with "C" ribonuclease was decreased to 15 min at half the usual enzyme concentration, the large oligonucleotides obtained contained two or more of the products of a more extensive digestion. The products obtained were discrete and the maps showed a lower background than those prepared by partial T_1 ribonuclease digestion.

The "C" enzyme may be useful in determining the internal sequence of large oligonucleotides produced by T_1 ribonuclease digestion. Thus, if there are several internal adenylic acids in such oligonucleotides, U2 ribonuclease (Arima *et al.*, 1968) would cleave at positions between the 3′-phosphoryl of the pyrimidine and the 5′-hydroxyl of adenylic acid, while with both enzymes pyrimidine stretches remain intact. Therefore, "C" RNase digests give information about the products linked to the terminal 3′-phosphate of the U2 RNase product. We are currently using this approach with success to analyze T_1 oligonucleotides of chain length 20 or longer in digests of SV40 virus RNA.

While the current work was underway, Contreras and Fiers described the use of carboxymethylated pancreatic ribonuclease at a mildly acid pH to produce a limited digest of RNA (Contreras and Fiers, 1971). This modified pancreatic ribonuclease has specificity in part resembling that of the "C" enzyme, since it tends to cleave between C–A or U–A bonds. However, the modified ribonuclease will still occasionally cleave between pyrimidines. Because it is employed at an acid pH one may further see different effects of secondary structure on the production of partial digestion products of the RNA, than with "C" ribonuclease digests. When intact *B. stearothermophilus* 5S RNA is digested with carboxymethylated pancreatic ribonuclease several of the resulting oligonucleotides are different from the principal products of digestion with the "C" enzyme (C. A. Marotta, S. M. Weissman, and F. Varricchio, in preparation).

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