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Cleavage reaction of a synthetic oligoribonucleotide corresponding to the autocleavage site of a precursor RNA from bacteriophage T4

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A fragment (GUUUCGUACAAAC) having a consensus sequence for the self-cleavage domain in a precursor of an RNA molecule from T4-infected *Escherichia coli* cells (p2Sp1; precursor of species 1) was chemically synthesized and found to be cleaved either between CA (139–140) or between UA (137–138) in the presence of monovalent cations and a non-ionic detergent. The cleaved products had 5′-hydroxyl and 3′-phosphate groups, of which some were in the form 2′,3′-cyclic phosphates.

New catalytic RNA; Synthetic oligoribonucleotide; Autocleavage activity; Hairpin structure; Bacteriophage T4 RNA precursor

1. INTRODUCTION

One viroid, several virusoids and the RNA transcripts of satellite 2 DNA of the newt have been shown to undergo a site-specific self-cleavage reaction in the presence of Mg^{2+} at neutral pH [1–4]. Comparison of several self-cleavage RNA sequences led to the identification of a consensus 3-stemmed secondary structure, termed the 'hammerhead' containing 11 conserved nucleotides at the junction of 3 helices that are precisely positioned with respect to the self-cleavage sites [5–12]. On the other hand, satellite RNA of tobacco ringspot virus, (–)sTRSV, and the genomic RNA of hepatitis δ virus (HDV) are new catalytic RNA motifs that differ from the hammerhead model proposed for other satellite and viroid RNAs [13–15]. Watson et al. [16] have also reported that a precursor of an RNA molecule from T4-infected *E. coli* cells (p2Sp1 RNA) (Fig. 1) has the capability to cleave itself in a specific position (UA, 137–138). This self-cleavage reaction requires at least a monovalent cation and is enhanced by non-ionic detergents.

To confirm that the self-cleavage reaction occurs autolytically, we studied the selective hydrolysis of RNA using a chemically synthesized 13mer (GUUUCGUACAAAC) having a sequence corresponding to G131–C143 of the p2Sp1 RNA. We found that cleavage occurred at phosphodiester bonds of UpA (137–138) and CpA (139–140) in the presence of NH_4Cl or $MgCl_2$ and a non-ionic detergent (Brij 58).

Abbreviations: (pGUUUCGUAC>p), (pGUUUCGU>p), (pppGUUUCGUAC>p), pppGUUUCGU>p)=5′-phosphoryl-oligoribonucleotide-2′,3′-cyclic phosphate; pC>p, pU>p = 5′-phosphoryl-nucleoside-2′,3′-cyclic phosphate

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2. MATERIALS AND METHODS

The oligoribonucleotide was synthesized by the phosphoramidite approach using an Applied Biosystem Model 381A synthesizer as described previously [17]. The phosphoramidite units were prepared from the reaction of 5′-*O*-dimethoxytrityl-*N*-protected-2′-*O*-1-(2-chloroethoxy)ethyl-*N*-protected nucleosides [17] or 5′-*O*-dimethoxytrityl-*N*-protected-2′-*O*-methylnucleosides [18] and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite [19]. The oligoribonucleotide was purified by reverse-phase and ion-exchange HPLC. Oligoribonucleotides were labeled by kination of the 5′-terminal OH with T4 polynucleotide kinase and [γ-^{32}P]ATP, and separated by 20% polyacrylamide/7 M urea gel electrophoresis. The RNA was eluted from the gel, and the labeled RNA was recovered from the eluate by ethanol precipitation.

The assay mixture contained [^{32}P]RNA (R1,3,4), 170 mM NH_4Cl or 25 mM G $MgCl_2$ and 1 mM Na_2EDTA (pH 7.0). The non-ionic detergent (Brij 58) in water was added to a final concentration of 0.5% (w/v). All assay reagents were autoclaved before use. Analytical reactions were usually carried out in 20 μl volumes. The reaction was started by adding the Brij 58 solution to the cleavage reaction mixture (on ice), vortexing and transferring to a 37°C water bath. The control experiment was performed without Brij 58, NH_4Cl, and $MgCl_2$. After 2 h, the reaction was stopped by addition of 5 μl of the stop solution [20] and the reaction was analyzed by electrophoresis on 20% polyacrylamide in 7 M urea. Markers were obtained by hydrolysis of 5′-[^{32}P]-labeled fragments (R1,3,4) with tRNA (2.5 μg), 1 mM Na_2EDTA (pH 7.0) and 50 mM Na_2CO_3 (pH 9.0) at 90°C for 15 min. The 2′,3′-cyclic phosphates in the oligonucleotides were characterized as described by Uhlenbeck [5].

3. RESULTS AND DISCUSSION

Fig. 2 shows the requirements for the self-cleavage

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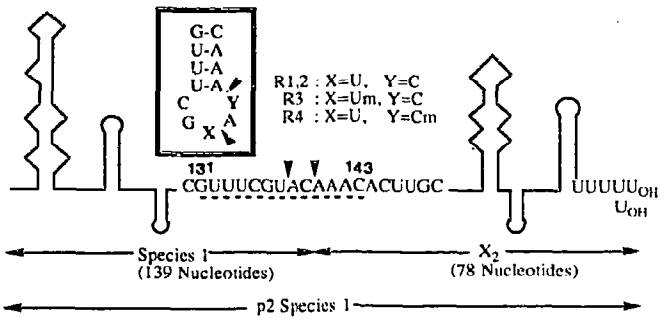

Fig. 1. Structure of p2Sp1 RNA. The nucleotides (R1–4) shown in the inset correspond to the region indicated with a broken underline in the sequence, where X=U or Um and Y=C or Cm. The cleavage site is indicated by an arrow.

reaction of the synthetic RNA 13mer (R1). The complete reaction mixture included the ^{32}P-labeled RNA (R1, X=U, Y=C), 170 mM NH_4Cl or 25 mM $MgCl_2$, 1 mM Na_2EDTA (pH 7.0) and 0.5% Brij 58. Lane 6 (NH_4Cl) and lane 7 ($MgCl_2$) show R1 cleavage (UA (137–138); CA (139–140)) after 2 h at 37°C in the complete reaction mixture. No reaction occurred when only NH_4Cl (lane 3), $MgCl_2$ (lane 4) or Brij 58 (lane 5) was incubated with the R1. This shows that the non-ionic detergent and NH_4Cl or $MgCl_2$ are necessary for the self-cleavage of p2Sp1 RNA. However, this self-cleavage in the presence of Mg^{2+} was much less than in the presence of NH_4^+. In addition, this RNA was cleaved into 4 smaller fragments that were identified to be 9mer (pGUUUCGUAC> p) (P1) and 4mer (AAAC), and 7mer (pGUUUCGU> p) (P2) and 6mer (ACAAAC).

To further characterize the products of RNA autocleavage, [α-^{32}P]ATP-labeled RNA 13mer (R2) was

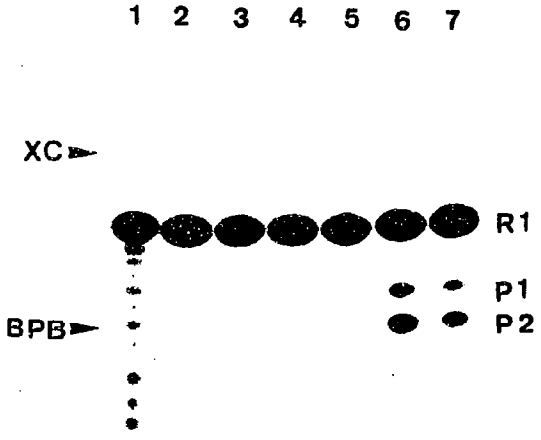

Fig. 2. 20% Polyacrylamide gel electrophoresis of the cleaved products. R1, P1 and P2 show 5′-labeled 13mer RNA (X=U, Y=C) and cleaved products (P1=9mer and P2=7mer). (Lane 1) marker oligonucleotide; (lane 2) control; (lane 3) without 170 mM NH_4Cl; (lane 4) without 25 mM $MgCl_2$; (lane 5) without Brij 58; (lane 6) with Brij 58 and 170 mM NH_4Cl; (lane 7) with Brij 58 and $MgCl_2$.

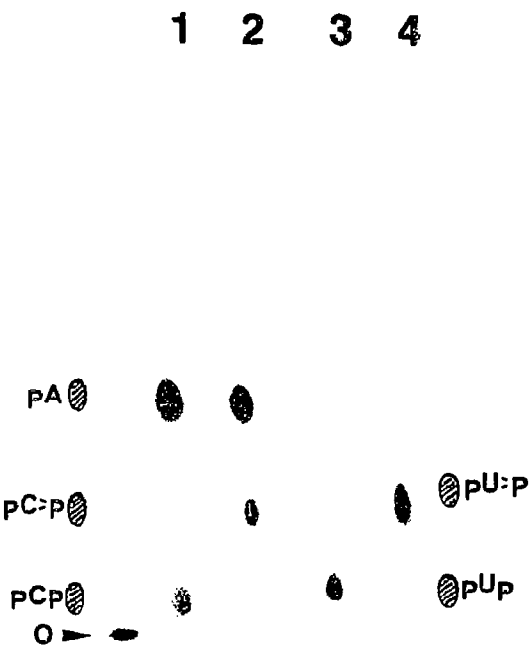

Fig. 3. Thin-layer chromatography after digestion of the cleaved products P1 (pppGUUUCGUAC>p) and P2 (pppGUUUCGU>p) labeled with [α-^{32}P]ATP by nuclease P1. (Lanes 2 and 4) nuclease P1 digestion of P1 and P2; (lanes 1 and 3) acid hydrolysis of 2′,3′-cyclic phosphates. Positions of nonradioactive markers are indicated on each side of the autoradiogram.

made with [α-^{32}P]ATP using T7 RNA polymerase [5]. The RNA cleavage products were digested with nuclease P1 and separated by thin-layer chromatography with 1 M ammonium acetate and subjected to autoradiography (Fig. 3). The labeled phosphate, originally attached to the 5′-terminal adenosine residues of 4mer (AAAC) and 6mer (ACAAAC), was transferred to 9mer (pppGUUUCGUAC> p) (P1) and 7mer (pppGUUUCGU> p) (P2), respectively, as the terminal 2′,3′-cyclic phosphate. This finding was confirmed by the analysis of nuclease P1 digestion products of each of the labeled 7mer and 9mer molecules. Nuclease P1 cleaved RNA to give nucleoside 5′-monophosphates and did not hydrolyze 2′,3′-cyclic phosphates. Only the [α-^{32}P]ATP-labeled P1 and P2 molecules showed the expected pU> p and pC> p (Fig. 3). Additional evidence for 2′,3′-cyclic phosphate includes the observation of the expected product 2′- or 3′-phosphate after acid treatment.

Finally, we chemically synthesized oligoribonucleotides for modified fragments containing 2′-*O*-methylcytidine (Cm) instead of C, or 2′-*O*-methyluridine (Um) instead of U. The modified 13mer RNAs (R3, X=Um, Y=C; R4, X=U, Y=Cm) were designed to confirm participation of the 2′-hydroxyl group in the self-cleavage reaction. No cleavage was observed, as expected (Fig. 4). This does not conflict with the above analysis of the 3′-end of cleaved products.

It is notable that the p2Sp1 RNA cleavage requires

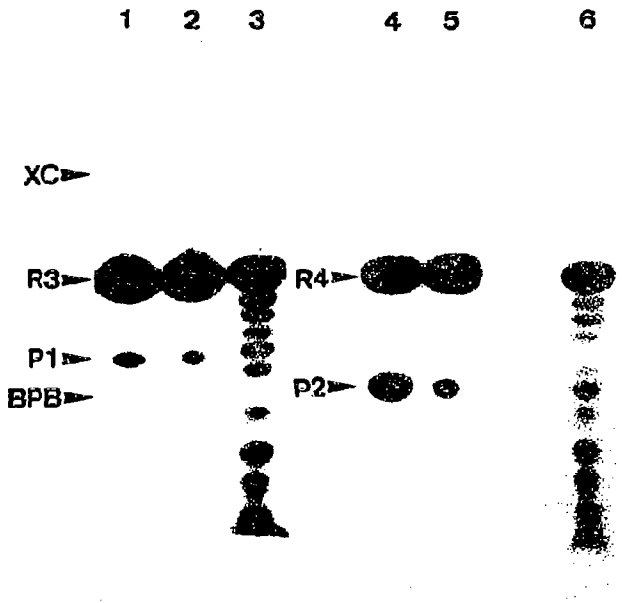

Fig. 4. 20% Polyacrylamide gel electrophoresis of the cleaved products of modified 13mer RNAs. R3, R4, P1 and P2 show 5′-labeled 13mer RNAs (R3: X=Um, Y=C; R4: X=U, Y=Cm) and cleaved products (P1=9mer and P2=7mer). R3 and R4 were incubated in 170 mM NH_4Cl, 0.5% Brij 58, and 1 mM Na_2EDTA (pH 7.0) for 2 h at 37°C. (Lanes 3 and 6) marker oligoribonucleotides; (lanes 1 and 3) with NH_4Cl and Brij 58; (lanes 2 and 5) with $MgCl_2$ and Brij 58.

at least NH_4^+ or Mg^{2+} and occurs with RNA fragments as small as 13 nucleotides. This cleavage occurs at the phosphodiester bond at nucleotides CA (139–140) and UA (137–138), generating the 5′-fragment with the terminal 2′,3′-cyclic phosphate group and the 3′-fragment with 5′-hydroxyl group. We have further shown that this sequence does not contain the hammerhead structure or consensus sequence proposed for the viroid RNA self-cleavage.

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