REGIOSPECIFIC CLEAVAGE OF RNA BY RNASE H FROM *E. coli* IN THE PRESENCE OF A COMPLEMENTARY OLIGONUCLEOTIDE WITH INSERTED ALTERNATING 2'-O-METHYLCYTIDINE RESIDUES

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Received November 15, 1989
Accepted April 3, 1990

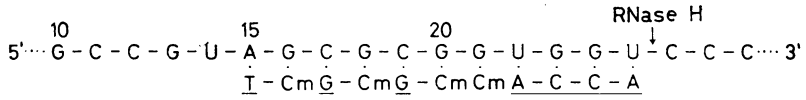
Undecanucleotide dA-dC-dC-dA-Cm-Cm-dG-Cm-dG-Cm-dT (Cm = 2'-O-methylcytidine) was prepared and used as a probe for splitting of 5S rRNA fragment by RNase H (*E. coli*). It was found that substitution of 2'-deoxycytidine of d(ACCACCGCGCT) by 2'-O-methylcytidine in positions 5, 6, 8 and 10 provided regiospecific splitting of RNA fragment by RNase H in the presence of modified probe between U_{25} and C_{26}.

It was shown[1,2] that RNase H (*E. coli*) specifically cleaves RNA hybridized to oligodeoxyribonucleotides and that the RNA is cut within regions complementary to the DNA-oligomers, or in positions immediately adjacent to them. In the comprehensive paper of Shibahara et al.[3], who investigated the cleavage of RNA in the presence of chimeric oligonucleotides containing contiguous oligo(2'-O-methyl)ribonucleotides and oligodeoxyribonucleotide clusters at their 5'- or 3'-ends, no cleavage was observed at regions complementary to oligo(2'-O-methyl)ribonucleotide sequences. Four contiguous deoxynucleotides within these chimeric chains were found to be highly significant for site-specific cleavage by RNase H (*E. coli*). The cutting site preference is supposed to depend on strand conformation, sugar puckering, and sequence.

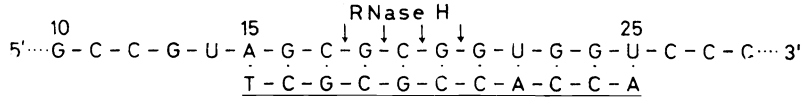
In continuation of our efforts in this line[4,5] aimed at further development in the probe design for regiospecific RNA cleavage by RNase H, we constructed a chimeric undecanucleotide containing alternating 2'-O-methylcytidine (*I*) and compared the products of cleavage of 1-41 5S rRNA fragment in the presence of *I* by means of RNase H (*E. coli*) with the products of cleavage of the same RNA fragment in the presence of unmodified undecanucleotide (*II*) of the same sequence (Figs 1 and 3). We found that the presence of the inserted alternating 2'-O-methylcytidine residues is sufficient to inhibit the cleavage of RNA in duplex in positions of the highest

sensitivity to splitting which were revealed in a separate experiment with unmodified *II*. In the presence of *II*, a multi-site cleavage of RNA fragment took place between C_{17} and G_{18}, G_{18} and C_{19}, C_{19} and C_{20}, G_{20} and G_{21}. In the presence of *I* a slow (Fig. 2) regiospecific splitting proceeded whereby the cleavage took place, in accordance with the earlier observations[3], in position immediately adjacent to the unmodified DNA-tetramer region.

The hydrolysis efficiency was determined by comparing the amount of undegraded RNA to that of all the hydrolytic cleavage products (by measuring the radioactivity of the corresponding bands). These data are shown in Fig. 2. As it can be seen, under the conditions tested the increase in specificity of the RNA hydrolysis is accompanied by the decrease, to some extent, in the efficiency of hydrolysis.



I



II

FIG. 1

Heteroduplexes and their hydrolysis by RNase H from *E. coli*. Deoxynucleotides are underlined; Cm = 2′-O-methylcytidine

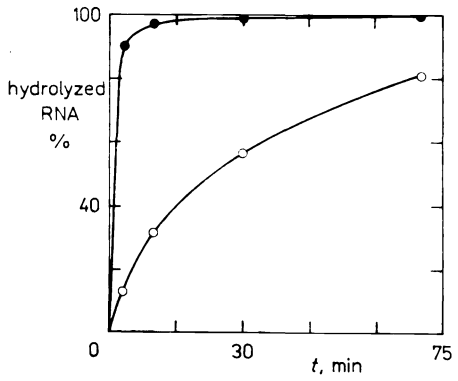


FIG. 2

The hydrolysis of 1—41 fragment of 5S rRNA by RNase H in the presence of **ACCACCG. CGCT** (●) and **ACCACmCmGCmGCmT** (○). Deoxyribonucleosides are semi-boldfaced

The result opens the way to the construction of easily accessible chimeric oligomers containing only one 2'-O-methyl-nucleoside instead of all four[3]. The position of the modification may follow from cleavage studies with unmodified oligodeoxyribonucleotides.

The synthesis of the undecadeoxyribonucleotide *II* was carried out by phosphoramidite solid phase method[7]. Modified *I* was synthesized by H-phosphonate solid phase approach[8,9] according to the protocol shown in Table I. The 3'-H-phosphonates as building units were prepared by PCl_3/imidazole method[8] from the corresponding 5'-O-dimethoxytritylated, N-protected (N^4-benzoyl for dC, N^6-dimethylaminoethylidene for dA (ref.[10]), N^2-dibutylaminomethylene for dG (ref.[10])) deoxyribonucleosides. 2'-O-Methylcytidine was prepared as reported in the literature[6], benzoylated at N-4 by benzoic anhydride in ethanol[11], converted in the 5'-O-dimethoxytrityl derivative, and treated with PCl_3/imidazole reagent[8] to give the appropriate 3'-H-phosphonate for the automated synthesis. The oligomers were purified by reversed phase HPLC and their structure was confirmed by Gilbert–Maxam sequencing technique (data not shown).

EXPERIMENTAL

The phosphoramidite synthesis was performed[7] on Victoriya-4M automated synthesizer (U.S.S.R.). For H-phosphonate synthesis, Syngen 1 automated synthesizer (Czechoslovakia) was used. Reversed-phase HPLC purification was performed using ZORBAX C8 column (0·46 × 25 cm; DuPont). Buffers used: A 0·1M ammonium acetate; B 0·1M ammonium acetate in 40% acetonitrile. Separation conditions: A 3 min, then linear gradient from A to B over 40 min; flow rate 1 ml min^{-1}, temperature 45°C. The product corresponding to the main peak

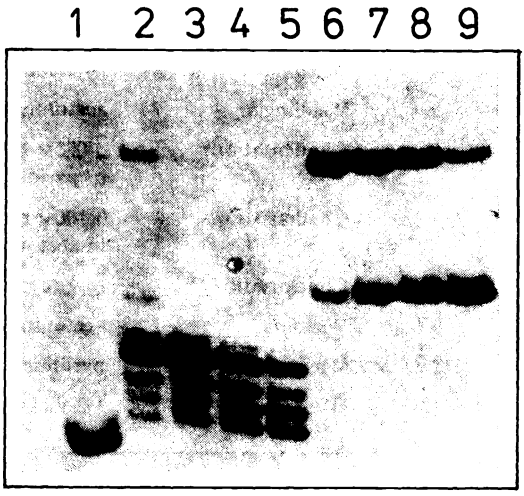


FIG. 3

Autoradiography of cleavage products of 5'-end labelled 1—41 fragment of 5S rRNA after incubation with RNase H for 3, 10, 30 and 70 min, respectively. Lane 1 marker 5'-[^{32}P]-GGGAGCGCGGUGGUCC; lane 2, 3, 4, 5 RNA + ACCACCGCGCT; lane 6, 7, 8, 9 RNA + ACCACmCmGCmGCmT. Deoxyribonucleosides are semi-boldfaced

was repeatedly evaporated with 50% ethanol at 50°C. The homogeneity of isolated dA-dC-dC--dA-Cm-Cm-dG-Cm-dG-Cm-dT was confirmed by electrophoresis in 20% PAAG after 5′-[^{32}P]--labelling by γ-[^{32}P]-ATP and T4 polynucleotide kinase.

E. coli RNase H was purchased from Biolar (Olaine, U.S.S.R.). The 5S rRNA fragment (1—41) was obtained by partial enzymatic hydrolysis of the 5S rRNA from *E. coli* with guanine--specific nuclease C_2 (Olaine, U.S.S.R.) and isolated by electrophoresis in 12% PAAG.

Hydrolysis of the 1—41 5S rRNA fragment by RNase H was performed as follows: 12 u.a. of RNase H from *E. coli* were added to the reaction mixture containing (total volume 10 μl): 0·2M Tris-HCl, pH 7·9; 0·3M NaCl; 0·1M $MgCl_2$; 0·5 mM DTT, 1 mM EDTA; 0·05 A_{260} of oligonucleotide and 1 . 10^5 c.p.m. of the 5′-[^{32}P]-labelled 1—41 5S rRNA fragment (0·001 A_{260}). The obtained mixture was thoroughly mixed and incubated at 25°C (preliminary annealing had no influence on hybridization of oligonucleotides with RNA). Aliquots (2 μl) were taken at 3, 10, 30 and 70 minutes after addition of the enzyme. The reaction was terminated by adding 2·5 μl of formamide. The hydrolysis products were analyzed by 20% PAAG electrophoresis (Fig. 3) followed by the autoradiography. The positions of hydrolytic cleavage were revealed by comparing the electrophoretic mobility of the reaction products to that of the 5′-[^{32}P]-

TABLE I

Protocol for automated oligonucleotide synthesis. CPG-500A (40 mg) with aliphatic arm[12] loaded with 0·6 μmol of nucleoside was used

Step	Operation	Solvent/Reagent	Time s	Flow rate ml min^{-1}
1	Washing	1,2-dichloroethane	60	4
2	Detritylation	3% dichloroacetic acid in 1,2-dichloroethane	110	4
3	Washing	1,2-dichloroethane	20	4
4	Washing	pyridine	5	4
5	Washing	acetonitrile	10	4
6	Condensation	0·025M H-phosphonate in pyridine	2	4
7	Condensation	0·100M pivaloylchloride in acetonitrile	2	4
8	Condensation	repeat steps 6, 7 seven times		
9	Washing	acetonitrile	80	2
10	Washing	pyridine	40	4
11[a]	Washing	1,2-dichloroethane	10	4

[a] After step 11 of the last elongation cycle, steps 1—3 were repeated. Then a two-step oxidation[9] was performed (15 min each step).

-GGGAGCGCGGUGGUCC (marker) and of the partial basic hydrolysis products of 1—41 5S rRNA fragment (not shown).

^{31}P NMR spectra were measured on a Varian XL-200 NMR spectrometer (80·96 MHz for ^{31}P) in deuteriochloroform with 85% H_3PO_4 as external standard. Chemical shifts are given in ppm (δ-scale), coupling constants (J) in Hz.

N^4-Benzoyl-5′-O-dimethoxytriphenylmethyl-2′-O-methylcytidine 3′-Hydrogenphosphonate

2′-O-Methylcytidine[6] (0·50 g, 1·95 mmol) was benzoylated[11] with benzoic anhydride (6 × 0·50 g, the portions being added in 1 h intervals) in absolute ethanol under reflux. After the last addition the mixture was refluxed for 1 h, the solvent was evaporated and the residue dissolved in ether (3 ml). Petroleum ether was then added gradually under stirring and checking by TLC (chloroform–methanol, 9 : 1) to afford a precipitate of the product while leaving most of the unreacted reagent and benzoic acid in solution. The solid was collected and purified by chromatography on silica gel (63—200 μm, 25 g; Fluka). Elution started with chloroform (300 ml), then a gradient of methanol in chloroform (1 to 5%, 100 ml each addition) was applied. Yield, 0·380 g of the benzoyl derivative (R_F 0·25 in chloroform–methanol, 9 : 1) (ref.[13]). The product (0·316 g, 0·875 mmol) was evaporated with toluene (3 × 20 ml) and anhydrous pyridine (2 × 20 ml), dissolved in anhydrous pyridine (20 ml), 4,4′-dimethoxytriphenylmethyl chloride (0·326 g, 0·963 mmol; 1·1 eq.) was added and the whole was left to stand overnight at room temperature. The reaction was quenched by methanol (0·5 ml). After 10 min the mixture was evaporated to one-third volume, the residue partitioned between saturated aqueous solution of sodium hydrogen carbonate (40 ml) and chloroform (3 × 40 ml), the pooled chloroform extracts were dried (sodium sulphate) and evaporated to dryness. After evaporation with toluene (2 × 30 ml) the product was chromatographed on a silica gel column (50 g) in chloroform–triethylamine (99·5 : 0·5, v/v) to afford 0·51 g of a foamy product (73%, based on the benzoyl derivative) which was then phosphitylated using the PCl_3/imidazole reagent[8] to give the title compound (0·51 g, 80%). ^{31}P NMR spectrum: 3·14 dd ($^3J(^{31}P, H) = 8·7$; $^1J(^{31}P, H) = 633·8$).

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Translated by the author (Z.T.).