ADDRESSED FRAGMENTATION OF RNA MOLECULES

Determination of a specific cleavage site on MS2 bacteriophage RNA

V. G. METELEV, N. P. RODIONOVA, N. V. CHICHKOVA, I. G. ATABEKOV, A. A. BOGDANOV, Z. A. SHABAROVA, V. BERZIN+, I. JANSONE+ and E. J. GREN+

Laboratory of Bioorganic Chemistry and Department of Virology, Moscow State University, Moscow 117234 and +*Institute of Organic Synthesis, Latvian Academy of Sciences, Riga 226006, USSR*

Received 18 August 1980

1. Introduction

In [1–3], an experimental approach to specific cleavage (addressed fragmentation) of polycistronic RNAs in a certain site of polynucleotide chain has been developed. Ribonuclease H from *Escherichia coli* is capable of cleaving RNA of bacteriophages MS2 and R17 only in the presence of synthetic oligodeoxyribonucleotides complementary to a certain RNA sequence. Determination of the molecular weights of RNA fragments produced by RNase H allowed us to conclude that specific cleavage of bacteriophage RNA takes place, i.e., fragmentation occurs only in the regions of complementary oligodeoxyribonucleotide binding. The site of cleavage of bacteriophage RNA was located more precisely using a short 59-nucleotide fragment isolated from MS2 RNA as a target for RNase H attack. This fragment contains an intercistronic nucleotide sequence between coat protein and replicase genes (nucleotides 1708–1766) and is designated as MS2 R(-53→6) (see fig.1).

This work describes the primary structure of RNase H cleavage products of the heteroduplex formed by MS2 R(-53→6) with complementary synthetic oligodeoxyribonucleotide d(CTCATGTT) (see fig.1). Our results show that RNase H splits the molecule of RNA in the position corresponding to the 3′-end of the heteroduplex.

2. Materials and methods

Ribonuclease T_1 (Calbiochem) and pancreatic RNase A (Worthington) were used throughout. RNase H was isolated from *E. coli* as in [3]. DEAE paper (DE-81), DEAE cellulose (DE 32) and 3 MM paper were purchased from Whatman. Cellulose acetate strips (3 × 55 cm) from Schleicher and Schüll, thin-layer plates (20 × 20 cm) with PEI-cellulose from Merck, acrylamide and *N′,N*-methylenebisacrylamide from Reanal were used. ^{32}P-Labeled polynucleotide MS2 R(-53→6) was obtained as in [4]. Oligonucleotides were synthesized in our laboratory by N. F. Sergeeva and M. B. Gottich by the method developed in [5,6].

2.1. *Limited RNase H digestion of heteroduplex*

Hybridization and cleavage of heteroduplex by RNase H were done using a modified method of [1–3]. The reaction mixture (20 μl) contained 0.6 μg ^{32}P-labeled MS2 R(-53→6) (26.8 nmol, 39 300 cpm) and

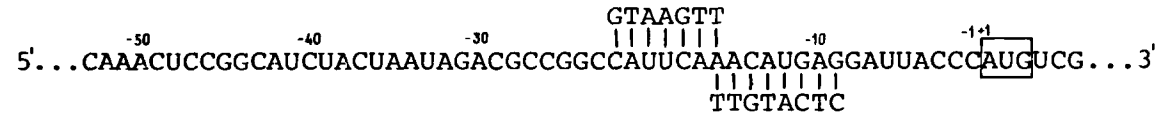

Fig.1. Heteroduplexes of MS2 R(-53→6) with complementary octanucleotide d(CTCATGTT) and heptanucleotide d(TTGAATG). The untranslated region between the coat protein and replicase cistrons of MS2 phage RNA is localized in the sequence U_{-36}–C_{-1}; the initiatory AUG of replicase cistron is boxed.

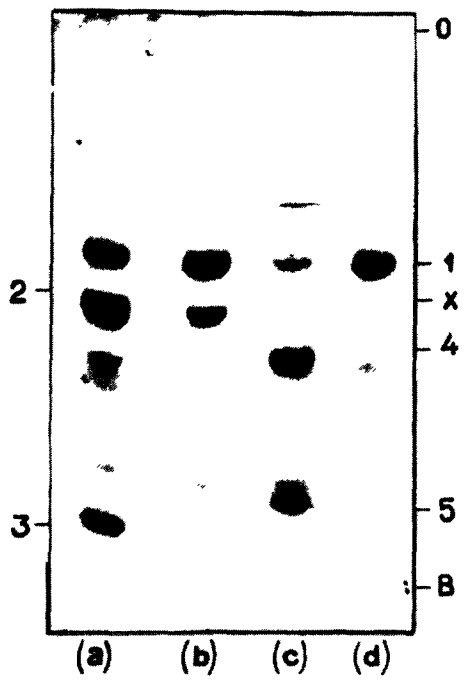

Fig.2. 16% polyacrylamide–6 M urea gel electrophoresis of fragments obtained by digestion of MS2 R(-53→6)–oligomer heteroduplexes with *E. coli* RNase H: MS2 R(-53→6)–d(CTCATGTT) complex; incubation at 2°C with RNase H for 6 h (a) and 3 h (b); MS2 R(-53→6)–d(TTGAATG) complex (c) and MS2 R(-53→6) (d) both after incubation with RNase H for 6 h at 2°C. O, origin; X, xylene cyanol FF; B, bromphenol blue dye marker.

a 40-fold molar excess of oligomers in 0.01 M Tris–HCl, (pH 7.8), 0.15 M NaCl, 0.001 M $MgCl_2$ and 0.0001 M dithiothreitol. The mixture was preincubated for 30 min at 2°C, then 0.1 unit of *E. coli* RNase H was added and the incubation was continued for 6 h. The mixture was supplemented with a solution of neutralized EDTA to 0.005 M final conc. and 0.25 vol. 50% sucrose containing 9 M urea and 0.02% dyes (xylene cyanol FF and bromphenol blue). The solution was loaded onto 1 cm indentations of a 30 × 20 × 0.2 cm slab of 6 M urea-containing 16% polyacrylamide gel (pH 8.0) prepared as in [7]. After 18 h electrophoresis at 400 V the gel was radioautographed.

To obtain preparative amounts of specific fragments of MS2 R(-53→6), the volume of the reaction mixture and the amounts of components were increased 4-fold and, after radioautography, bands '2' and '3' (fig.2a) were eluted as in [4].

2.3. *Sequence analysis of ^{32}P-labeled MS2 R(-53→6) fragments*

For complete hydrolysis, polynucleotides were dissolved in the reaction mixture (10 μl) containing 0.1 μmol Tris–HCl (pH 7.3), 0.01 μmol EDTA and RNase T_1 (enzyme–substrate ratio, 1:40 (w/w)) then incubated for 30 min at 37°C. Unlabeled MS2 RNA was added as a carrier to 4 mg/ml).

The digests were fractionated by a two-dimensional separation method involving high-voltage electrophoresis on cellulose acetate (pyridine–acetate buffer in 7 M urea (pH 3.5)) in the first direction and homochromatography with mixture 'β' on thin-layer PEI-cellulose plates at 60°C [8] in the second direction. The spots of ^{32}P-oligonucleotides were located by autoradiography and eluted with 30% triethylammonium bicarbonate. The sequence of oligonucleotides was determined by further enzymatic digestion and separation of the products by electrophoresis on DE 81 or 3 MM paper at pH 3.5 [8,9].

3. Results and discussion

To provide evidence of regioselective cleavage of RNA at heteroduplex sites and to obtain specific fragments, hybridization of complementary octanucleotide d(CTCATGTT) or heptanucleotide d(TTGAATG) with ^{32}P-labeled MS2 R(-53→6) was performed (fig.1). The resulting complex was treated with RNase H from *E. coli* and the products of hydrolysis were separated electrophoretically in 16% polyacrylamide gel containing 6 M urea. The amounts of the fragments formed and their mobility in gel (fig.2), compared with the mobility of standard fragments obtained after MS2 R(-53→6) hydrolysis by nuclease S_1 and RNase T_1 [10,11], allowed us to conclude that MS2 R(-53→6) fragmentation with RNase H takes place at the sites of polynucleotide complementary to oligomers. Hydrolysis of MS2 R(-53→6)–d(CTCATGTT) complex with RNase H results in only two fragments, '2' and '3', of an expected length of about 44 and 15 nucleotides (fig.2a,b) the yield of which depends on the time of incubation. A similar picture is observed during the cleavage of MS2 R(-53→6)–d(TTGAATG) complex (fig.2c). In this case, too, the formation of two new fragments, '4' and '5' (about 36 and 23 nucleotide residues, respectively) provides evidence of polynucleotide hydrolysis at the site of hybridization with heptanucleotide.

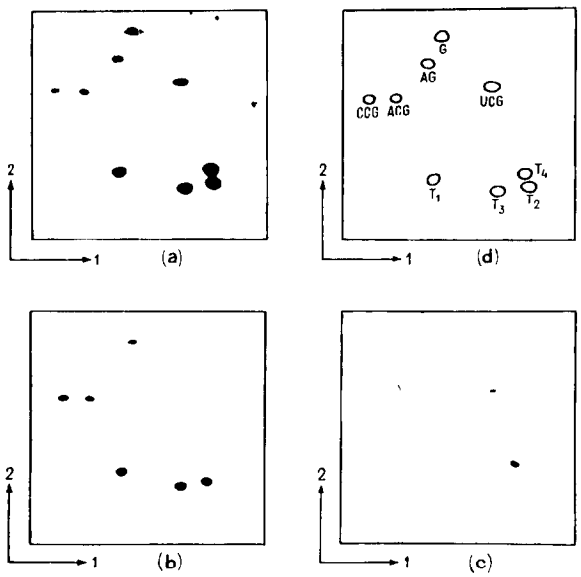

Fig.3. Autoradiograms of RNase T_1 mini-fingerprints of ^{32}P-labeled MS2 R(-53→6) fragments (see fig.2): (a) MS2 R(-53→6); (b) fragment '2'; (c) fragment '3'; (d) tracing of the autoradiogram (a) with identification of spots (cf. table 1). The 3′-terminal phosphate groups of nucleotides are not designated in (d). Electrophoresis in the first dimension is on cellulose acetate at pH 3.5, development of the PEI-cellulose thin-layer plate in the second direction is with homomixture 'β'.

It should be noted that hydrolysis of heteroduplexes with RNase H is strictly specific since incubation of MS2 R(-53→6) with the enzyme in the absence of complementary oligodeoxyribonucleotides can hardly cause marked fragmentation of polynucleotide '1' (fig.2d).

The next series of experiments was performed to locate the site of RNase H attack in the ^{32}P-labeled MS2 R(-53→6)–d(CTCATGTT) heteroduplex. To this end, an analysis of the primary structure of the specific fragments was done. The material from bands '2' and '3' (fig.2a) was isolated from gel by electroelution, purified chromatographically on DEAE cellulose and subjected to complete hydrolysis with RNase T_1. The oligonucleotides obtained were resolved by two-dimensional fractionation. After autoradiography, mini-fingerprints of fragments '2' and '3' (fig.3b,c) were compared with the map of initial MS2 R(-53→6) (fig.3a) which is explained in fig.3d and table 1. As can be seen the RNase T_1 digest of fragment '2' contains characteristic oligonucleotides T1,T2,T3, trinucleotides C–C–Gp and A–C–Gp and mononucleotide Gp, which, when added up, provide the sequence of polynucleotide like MS2 R(-53→-10). The mini-fingerprint of RNase T_1 hydrolyzate of fragment '3' (fig.3c; table 1) revealed the presence of oligonucleotide T4, trinucleotide U–C–Gp and traces of Gp and pGp (not visible in fig.3c) which correspond to the 3′-terminal part of the initial MS2 R(-53→6), most likely polynucleotide MS2 R(-9→6). Since we failed to reveal in the fingerprints such a typical dinucleotide as $A–G_{OH}$ the action of RNase H on MS2 R(-53→6) under the conditions of limited hydrolysis is characterized by main cleavage of phosphodiester bond $A_{-10}–G_{-9}$ localized in the 3′-end of the heteroduplex.

Localization in the 3′-end of the heteroduplex the RNA site attacked by RNase H from *E. coli* is in agreement with [12]. The method of addressed fragmentation of RNA in [1–3] provides RNA splitting of a definite site.

Table 1
Oligonucleotides obtained by RNase T_1 digestion of ^{32}P-labeled MS2 R(-53→6) and its specific fragments

Oligonucleotide	Sequence, molar yield in MS2 R(-53→6)	Fragment '2' MS2 R(-53→-10)	Fragment '3' MS2 R(-9→6)
	Gp (3)	+(2)	+
	pGp (0)	–	+
	A-Gp (1)	–	–
	A–C–Gp (1)	+	–
	C–C–Gp (1)	+	–
	U–C–Gp (1)	–	+
T1	C–A–A–A–C–U–C–C–Gp (1)	+	–
T2	C–A–U–C–U–A–C–U–A–A–U–A–Gp (1)	+	–
T3	C–C–A–U–U–C–A–A–A–C–A–U–Gp (1)	+	–
T4	A–U–U–A–C–C–C–A–U–Gp (1)	–	+

References

[1] Metelev, V. G., Stepanova, O. B., Chichkova, N. V., Rodionova, N. P., Smirnov, V. D., Bogdanova, S. L., Sergeeva, N. F., Ratmanova, K. I., Bogdanov, A. A., Shabarova, Z. A. and Atabekov, I. G. (1978) Biol. Nauk. 8, 27–30.

[2] Stepanova, O. B., Metelev, V. G., Chichkova, N. V., Smirnov, V. D., Rodionova, N. P., Atabekov, I. G., Bogdanov, A. A. and Shabarova, Z. A. (1979) FEBS Lett. 103, 197–199.

[3] Metelev, V. G., Stepanova, O. B., Chichkova, N. B., Smirnov, V. D., Rodionova, N. P., Berzin, V. M., Jansone, I. V., Gren, E. J., Bogdanov, A. A., Shabarova, Z. A. and Atabekov, I. G. (1980 Molekul. Biol. 14, 200–211.

[4] Berzin, V., Borisova, G. P., Cielens, I., Gribanov, V. A., Jansone, I., Rosenthal, G. and Gren, E. J. (1978) J. Mol. Biol. 119, 101–131.

[5] Smirnov, V. D., Ratmanova, K. L., Shabarova, Z. A. and Prokofiev, M. A. (1975) Dokl. Akad. Nauk. SSSR 223, 1153–1155.

[6] Potapov, V. K., Veiko, V. P., Koroleva, O. N. and Shabarova, Z. A. (1979) Nucleic Acids Res. 6, 2041–2056.

[7] De Wachter, R. and Fiers, W. (1972) Anal. Biochem. 49, 184–197.

[8] Volckaert, G., Min Jou W. and Fiers, W. (1976) Anal. Biochem. 72, 433–446.

[9] Brownlee, G. G. (1972) in: Determination of Sequences in RNA (Work, T. S. and Work, E. eds) vol. 3, pp. 67–99, Elsevier/North-Holland, Amsterdam, New York.

[10] Jansone, I., Berzin, V., Gribanov, V. and Gren, E. (1979) Nucleic Acids Res. 6, 1747–1760.

[11] Berzin, V. M., Jansone, I. V., Gribanov, V. A., Tsimanis, A. Yu. and Gren, E. J. (1978) Molekul. Biol. 12, 1288–1298.

[12] Donis-Keller, H. (1979) Nucleic Acids Res. 7, 179–192.