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Site-specific enzymatic cleavage of TMV RNA directed by deoxyribo- and chimeric (deoxyribo-ribo)oligonucleotides

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The TMV RNA molecule can be cleaved at a single site by RNase H directed by chimeric oligo(deoxyribo-ribo)nucleotide with an internucleotide pyrophosphate bond

Site specificity; RNA cleavage; RNase H; Oligo(deoxyribo-ribo)nucleotide

1. INTRODUCTION

The method of site-specific cleavage of large RNAs with *Escherichia coli* RNase H in the presence of oligodeoxyribonucleotides complementary to a given site of an RNA molecule has been proposed by our group [1–4]. This method was applied to the site-specific cleavage of RNA of satellite tobacco necrosis virus [5], RNA of barley stripe mosaic virus [6,7], RNAs of bacteriophages MS2 and R17 [3], tobacco mosaic virus (TMV) [4], brome mosaic virus [8] and potato virus X (Miroshnichenko et al., in preparation). RNase H was suggested to cleave the RNA at the 3′-end of the RNA-DNA heteroduplex [3,5]. However, cleavage of the selected RNA site by RNase H can occur ambiguously in more than one position within the RNA-DNA hybrid and in the case of homopolymeric sequences can even lead to the complete excision of homopolymeric RNA regions of RNA-DNA hybrids (e.g. see [9]). Recently, synthetic chimeric oligo(deoxyribo-ribo)nucleotides were used to direct site-specific attack of RNase H [10–12]. RNase H cleaved RNA (90-mer) at a single site or two positions in the presence of chimeric oligonucleotides containing 2′-*O*-methylribonucleotides and contiguous 3–5-deoxyribonucleotides [11]. Here, we describe experiments on TMV RNA cleavage at a position 3′-proximal to the first AUG initiation codon. RNase H was directed by oligodeoxyribonucleotides (7–10-mer) as well as by chimeric oligonucleotides containing pentaribonucleotide linked to seven contiguous deoxynucleotides at the 5′-end by either a phosphodiester or pyrophosphate bond (fig.1). In the latter case, TMV RNA within the heteroduplex was cleaved at a single site, two residues from the 3′-end of the RNA-DNA region.

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2. MATERIALS AND METHODS

TMV strain U1 was isolated from *Nicotiana tabacum* var. Samsun EN. RNA was isolated using the phenol procedure from the purified virus [13]. Oligodeoxyribonucleotides and the modified chimeric oligo(deoxyribo-ribo)nucleotides were synthesized as in [12]. RNase H was isolated from cells of *E. coli* MRE-600 as described by Darlix [14]. RNase H cleavage reactions were carried out in buffer containing 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM DTT; 0.2 A_{260} units of oligonucleotide were added to 10 μg TMV RNA, incubated for 3 min at 65°C, and cooled slowly at room temperature. The buffer and 0.01 U RNase H were then added and the reaction mixture was incubated for 1 h at 4°C. The reaction was stopped by addition of 2.5 vols ethanol. The

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products of TMV RNA cleavage by RNase H were analysed by electrophoresis in 12% polyacrylamide gel after 3′-terminal labeling with 5′-^{32}P-labeled cytidine 5′,3′-biphosphate by T_4 RNA ligase [9]. Each of the TMV RNA fragments formed upon RNase H cleavage reaction directed by each of the oligonucleotides was isolated from the gel and sequenced via a direct chemical method [15]. The 3′-terminally ^{32}P-labeled nucleotides were determined in each individual RNA fragment derived from TMV RNA by RNase H (complete digestion with nuclease T_2 followed by two-dimensional chromatography in thin layers of cellulose).

3. RESULTS AND DISCUSSION

Different oligonucleotides (I–IV in fig.1) were used complementary to the region of TMV RNA, 3′-proximal to the 5′-terminal 70-base non-translated leader sequence and the initiation AUG codon of the 126/183 kDa gene [16].

Analysis of the 3′-labeled TMV RNA fragments produced by RNase H directed by different oligonucleotides is presented in fig.2. The number of TMV RNA fragments revealed depended on the type of oligonucleotide by which RNase H was directed: from one to four RNA fragments could be produced (1–4 in fig.2) differing in length by one nucleotide. In the presence of chimeric oligo-(deoxyribo-ribo)nucleotides the target of RNase H attack is narrowed down to two (oligonucleotide

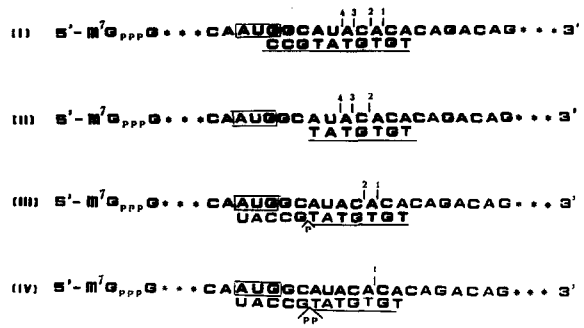

Fig.1. Heteroduplexes, presumably formed by the TMV RNA region 3′-proximal to the leader sequence and the first AUG codon (70–72nd residues from the 5′-end, boxed) with complementary oligonucleotides. Roman numerals correspond to different types of oligonucleotides used to direct the RNase H reaction: oligodeoxynucleotides (I,II), and chimeric oligonucleotides with a phosphodiester (III) or pyrophosphate (IV) bond between the 3′-terminal ribo- and 5′-terminal deoxyribo- (underlined) segments. Arabic numerals above bars indicate the sites at which the RNase H cleaved TMV RNA, producing the RNA fragments designated in fig.2 by the corresponding Arabic numerals 1–4.

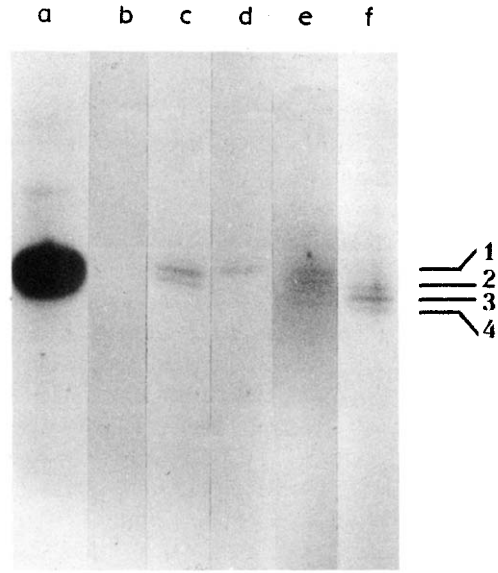

Fig.2. TMV RNA cleavage fragments produced by RNase H in the presence of the oligonucleotides indicated by the Roman numerals in fig.1: (a) $tRNA^{Phe}$ taken as a 76-base marker; (b) TMV RNA + RNase H without oligonucleotides (control); lanes c,d,e and f show TMV RNA fragments produced in the presence of oligonucleotides III,IV,I and II, respectively. Electrophoresis in 12% polyacrylamide gel.

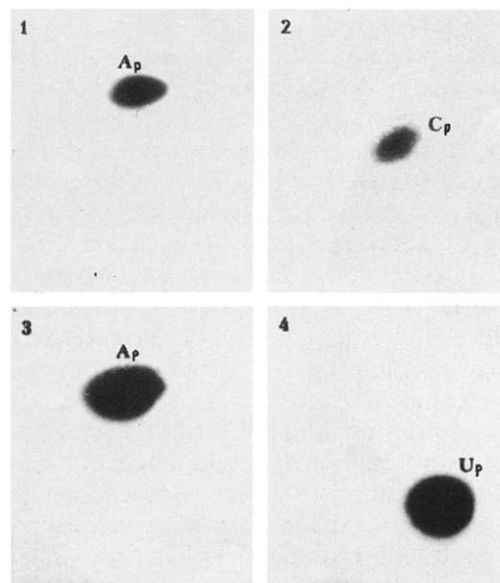

Fig.3. Determination of the 3′-terminal nucleotides in individual RNA fragments cleaved from TMV RNA by RNase H directed by oligonucleotides I–IV. Arabic numbers correspond to those of the TMV RNA fragments in fig.2. Two-dimensional chromatography in thin layers of cellulose (Eastman Kodak) of the 3′-labeled terminal nucleotides (radioautography).

III) or even one (oligonucleotide IV) cleavage site in contrast to oligodeoxynucleotides I and II (four and three cleavage sites, respectively).

Each of the cleavage products of TMV RNA was isolated and sequenced individually. All the products (bands 1–4 in fig.2) were found to correspond to the 5′-terminal fragments of TMV RNA in nucleotide sequence (not shown), i.e. in all cases site-specific oligonucleotide-directed cleavage of TMV RNA took place. However, exact determination of the 3′-terminal residues upon sequencing was complicated by the compression of bands due to the effect of the salts in the sample. Therefore, the 3′-terminally labeled nucleotides were determined in individual TMV RNA fragments derived from TMV RNA by RNase H in the presence of oligonucleotides I–IV (fig.3).

These data show that four TMV RNA fragments, designated 1–4 in fig.2, have 3′-terminal A, C, A and U, respectively. Since these fragments differ in length by one nucleotide, the tetranucleotide target sequence can be deduced.

Taken together, the results of direct sequencing and 3′-terminal nucleotide determination in individual RNA fragments allowed us to reveal the exact site(s) of TMV RNA cleavage by RNase H summarized in fig.1.

In the presence of deca- and hepta-oligodeoxynucleotides RNase H cleaved TMV RNA at four and three sites, respectively (fig.1). Formation of multiple cleavage products either can be due to the ability of RNase H to nick RNA at different sites (1–4 in fig.1) within the heteroduplex or can result from successive excision of nucleotides in the 3′–5′-direction of RNA after its cleavage at a single site (1 in fig.1). It should be noted that the number of cleavage sites can be decreased to only a single position (fig.1) if RNase H is directed by chimeric oligonucleotides.

Our expriments with TMV RNA cleavage are in general conformity with recent model experiments on site-specific cleavage of synthetic polyribonucleotides [10,11] and short oligoribonucleotides [12] by RNase H directed by chimeric polyribonucleotides. It should be mentioned that RNase H directed by chimeric oligonucleotides with an internucleotide pyrophosphate bond cleaved at a single site the native TMV RNA molecule (fig.1) as well as the dodecaribonucleotide pAAUGGCAUACAC [12]. However, the exact site of cleavage by RNase H may be rather different in the cases of native TMV RNA and short oligo- [12] and polyribonucleotides [10,11].

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