Location of Unique Sequences in Tobacco Mosaic Virus Ribonucleic Acid*

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SUMMARY

Tobacco mosaic virus has been partially stripped of its protein coat by sodium dodecyl sulfate. The nucleic acid exposed was digested with T1 ribonuclease and the products were examined for the presence of three oligonucleotides of unique sequence. The results indicate the location of two of the unique oligomers to be near the 5'-OH-linked end and the third to be near the middle of tobacco mosaic virus RNA. They also provide evidence that the cistron for tobacco mosaic virus coat protein is not situated at the 5'-OH-linked terminus.

The mapping of bacterial or viral genomes is carried out by taking advantage of the phenomenon of genetic recombination. No such phenomenon has been observed during the multiplication of viruses containing ribonucleic acid and, as a result, the feasibility of mapping RNA has received little attention. Two notable exceptions are the work of May and Knight (1) who showed that tobacco mosaic virus is stripped sequentially of its protein coat by sodium dodecyl sulfate in a polar fashion, and Kado and Knight (2) who used this property to locate a local lesion gene on tobacco mosaic virus ribonucleic acid. Bruening1 has suggested that other unique sequences of TMV RNA may be similarly located. In this regard, Mundry (3) has reported the isolation of a polyribonucleotide oligomer from a T1 ribonuclease digest of TMV RNA that appears to be unique. Independently, we have isolated three oligomers that appear to be unique in that each occurs only once in the TMV RNA molecule. For convenience, they are termed “unique-mers.” This report describes the initial experiments to locate the position of these three unique-mers in the TMV RNA chain.

Preparation of TMV—Tobacco mosaic virus was prepared from infected Nicotiana tabacca leaves by a modification of the methods of Steere (4) and Boedtker and Simmons (5) as described in detail elsewhere (6). These preparations were of a high degree of purity as evidenced by the sharp boundaries obtained during velocity sedimentation and the fact that successful base sequence determinations have been made at the 5'-hydroxy-linked end of the nucleic acid core (7).

Polar Stripping—The protein coat of TMV was removed in a stepwise fashion essentially as described by May and Knight (1). The stripping solutions were made as follows. A suspension of 0.5 g of TMV in 0.02 M cacodylate and 0.002 M EDTA, pH 7.5, was diluted to 27.5 ml with the same buffer. To this was added 1.0 ml of a suspension containing about 10 mg of bentonite (8) and 1.5 ml of 20% SDS. This suspension was stirred at 26° and, at various intervals, the optical density was read at 400 mp to provide a rough measure of the extent of stripping. The reaction was stopped by diluting the suspension to 300 ml with cold 0.02 M cacodylate-0.002 M EDTA buffer. The diluted suspension was then centrifuged at 15° in a No. 30 Spinco rotor at 27,000 rpm for 16 hours. At the conclusion of the centrifugation, the rotor was allowed to come to rest without the aid of a brake. The clear supernatant liquids were removed carefully and the optical absorption spectrum was measured. The absorption at 282 mp was used to determine the amount of protein (1 mg per ml yields 1.27 optical density units at 282 mp). The pellets were suspended in small amounts of cacodylate-EDTA buffer containing 20 μg of bentonite per ml. The suspensions were combined, diluted to 60 ml, and centrifuged at 37,000 rpm in a No. 40 Spinco rotor for 16 hours. The absorption of the supernatant liquid from this centrifugation was measured at 282 mp and the combined protein content was used to estimate the degree of stripping. The protein content of the second supernatant was usually less than 5% of the first supernatant.

Hydrolysis of Exposed Nucleic Acid—The pellets of partially stripped virus were suspended in a total of 10 ml of 0.01 M Tris, pH 7.5, containing 20 μg of bentonite per ml. The bentonite was removed by centrifugation at 10,000 rpm for 30 min and the supernatant of bentonite-free partially stripped virus was incubated at 40° with 1,000 units of T1 ribonuclease (Calbiochem) for 6 hours. At the end of this time, 1 ml of bentonite, contain-

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1 Dr. George Bruening, Department of Biochemistry and Biophysics, University of California, Davis, California, personal communication.

The abbreviations used are: TMV, tobacco mosaic virus; SDS, sodium dodecyl sulfate.
Fig. 1. Chromatographic pattern from chain length separation of oligomers from a T1 RNase hydrolysis of TMV RNA. TMV RNA digest, 10 mg, was chromatographed on a DEAE-Sephadex A-25 column, 0.5 × 27 cm. The gradient was linear from 7 M urea, 0.1 M NaCl-7 M urea, 0.5 M NaCl. The total gradient volume was 1000 ml; 6-ml fractions were collected.

Fig. 2. Upper, rechromatography of 30 optical density cm² units of a-mer after digestion with T1 RNase. Conditions were the same as in Fig. 1, except that 10-ml fractions were collected. Lower, chromatography of 28 optical density cm² units of µ-mer on a DEAE-Sephadex A-25 column, 0.5 × 27 cm, at pH 3. The gradient (400 ml) was linear from 7 M urea, pH 3-7 M urea, pH 3-0.5 M NaCl. Fractions, 5 ml, were collected.

Fig. 3. Upper, rechromatography of 30 optical density cm² units of %-mer after digestion with T1 RNase. Conditions were the same as in Fig. 2. Lower chromatography of 28 optical density cm² units of µ-mer at pH 3. Conditions were the same as those in Fig. 2 (lower).

RESULTS

Isolation of Unique-mers—When oligomers from a T1 RNase digest of TMV RNA were chromatographically separated according to chain length, the pattern shown in Fig. 1 was obtained. It can be seen that two small peaks, designated Ψ and Ω, were separated from the main pattern of oligomers. No other ultraviolet-absorbing material was eluted beyond the Ω peak even when the concentration of the NaCl in the solvent was increased to 1 M. In a separate experiment, 30 optical density cm² units of material in the Ψ peak were isolated and treated with 100 units of T1 RNase at 40°C for 3 hours. The pattern obtained from chromatography of this material according to chain length is shown in Fig. 2a. It can be seen that essentially all of the Ψ peak was resistant to further digestion with T1 RNase. When the material in the Ω peak was rechromatographed on DEAE-Sephadex, 7 M urea, NaCl gradient, pH 3, only one symmetrical peak was obtained, as shown in Fig. 2b. Digestion of 30 optical density cm² units of the material in the Ψ peak with T1 RNase resulted in the elution of a single, slightly asymmetrical peak when separated according to chain length (Fig. 3a). However, chromatography of the Ψ peak on DEAE-Sephadex, 7 M urea, NaCl gradient, pH 3, yielded two peaks; one component was eluted early (Ψ₁-mer), and the other was eluted late (Ψ₂-mer). This is shown in Fig. 3b. Preliminary experiments indicate that the Ψ-mer contains approximately 70 bases and that 1 eq of Ψ-mer is produced per eq of TMV RNA digested with T1 RNase. Briefly, the evidence is as follows. Equilibrium sedimentation analysis indicates a molecular weight of 21,500 ± 800. Analysis of oligomers from a pancreatic RNase digest of the Ψ-mer yields A₃₅C₄₁U₁₉G as the empirical formula. Labeling of the free 5'-
OH group at the 3'-linked end with $^{32}\text{P}$ establishes U, exclusively, as the end base. Finally, approximately $1.1\%$ of the ultraviolet-absorbing material present in the original TMV RNA (6400 bases) is recovered from the $\Omega$-peak, indicating that the $\Omega$-mer (70 bases) probably occurs only once.

The total amount of material in the $\Psi$ peak was slightly greater than the amount in the $\Omega$ peak. From this it may be deduced that the two components in the $\Psi$ peak, $\Psi_1$-mer and $\Psi_2$-mer, contain approximately 30 bases each, and that each occurs only once in a molecule of TMV RNA.

These unique-mers were chosen as markers for mapping be-

<table>
<thead>
<tr>
<th>Stripping</th>
<th>$\Psi$-mer</th>
<th>$\Omega$-mer</th>
<th>Ratio ($\Psi$:$\Omega$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>57.5</td>
<td>52.5</td>
<td>1.10</td>
</tr>
<tr>
<td>3%</td>
<td>0.67</td>
<td>3.61</td>
<td>0.186</td>
</tr>
<tr>
<td>8%</td>
<td>2.1</td>
<td>4.7</td>
<td>0.448</td>
</tr>
<tr>
<td>12%</td>
<td>1.12</td>
<td>2.4</td>
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</tr>
<tr>
<td>22%</td>
<td>0.83</td>
<td>1.71</td>
<td>0.485</td>
</tr>
<tr>
<td>33%</td>
<td>5.94</td>
<td>9.16</td>
<td>0.590</td>
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<tr>
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<td>6.24</td>
<td>7.21</td>
<td>0.805</td>
</tr>
<tr>
<td>64%</td>
<td>1.87</td>
<td>1.80</td>
<td>1.04</td>
</tr>
</tbody>
</table>

$^a$ $\Psi$-mer and $\Omega$-mer content of 101 mg of TMV RNA.
$^b$ Data obtained from 1 g of TMV.

cause they could be isolated and determined easily after the chain length separation procedure.

Location of Unique-mers—TMV was stripped of its protein by treatment with SDS at $26^\circ$ for various periods of time. The average degree of stripping as measured by the release of TMV protein into solution is shown in Table I. Supernatant protein from the samples stripped as much as $30\%$ gave absorption spectra indistinguishable from those of known TMV protein. After removal of the solubilized protein and SDS, the exposed nucleic acid was cleaved with T$_1$ RNase and the oligonucleotides obtained were separated according to chain length. Fig. 4, a, b, c, and d, shows the separation of $\Psi$-mers and $\Omega$-mers from 100, 3, 22, and 64% stripped virus. It can be seen in Fig. 4b that the $\Psi$-mer peak appeared in substantial quantity when as little as $3\%$ of the total protein coat was removed. In this sample, the peak with the $\Psi_1$-mers contained less than half as much ribonucleotide as was present in the $\Omega$ peak. In Fig. 4c, the amount of $\Psi$-mer has increased to about one-half of the $\Omega$-mer. The $\Psi$-mer obtained from TMV RNA exposed up to $22\%$ was diluted with 4 volumes of 7 M urea and loaded on a Sephadex A-25 column. The column was washed with 7 M urea, pH 3, NaCl gradient. A single peak was eluted early, indicating that the first $\Psi$-mer exposed corresponds to $\Psi_1$-mer. As the degree of stripping was increased, the quantity of $\Psi$-mer eventually increased until it approximately equalled the $\Omega$-mer (Fig. 4d).

The relative amounts of oligonucleotides obtained from T$_1$ RNase digests of the remaining portions of the partially stripped virus are shown in Fig. 4, b, c, and d. Owing to the fact that recovery of the RNA was not quantitative, only the relative amounts of unique-mers could be compared. The patterns show
a complementary relationship between the RNA exposed by stripping and the RNA remaining.

The ratio of Ψ-mer to Ω-mer in the last column of Table I is plotted in Fig. 5. For completely stripped TMV, the value of this ratio is 1.1. It appears that most of the Ω-mer was exposed after 3% stripping and that the ratio in Ψ-mer is very low. This implies that Ψ-mer is located closer to the 5'-OH-linked end of TMV RNA than the Ψ-mers. In addition, it indicates that the Ω-mer is probably within 180 bases of that end. As more of the TMV RNA is exposed, the ratio of Ψ-mers to Ω-mer increases so that, at 12% stripped, the ratio is 0.6 of the value obtained when stripping is complete. This indicates that one of the Ψ-mers is exposed at this point and that it is probably located at about 6% of the distance, or 380 bases, from the 5'-OH-linked end. This ratio remains constant from 12% stripped to about 35% stripped, after which it again rises and eventually equals the ratio for completely stripped TMV. This second rise in Ψ-mer:Ω-mer ratio is evidence that the second Ψ-mer has been exposed. From the graph, it may be inferred that the position of Ψ-mer is about 44% of the distance in from the 5'-OH-linked end. These results may be summarized as shown in Fig. 6.

**DISCUSSION**

One of the main difficulties in determining the base sequence of viral ribonucleic acid, such as TMV RNA, stems from the large number of bases in the nucleic acid chain (6400 for TMV RNA). Stepwise degradation (10) and end-labeling methods (7) have established the position of only a relatively small number of bases near the 5'-hydroxy-linked terminus of TMV RNA. Similar results have been reported with f-2 (11), MS-2 (12), and ribosomal RNA (13). Obviously, some additional procedures are necessary for continuation of progress in base sequence analysis. One such approach is the location of unique sequences in TMV RNA by biochemical mapping, which depends on two characteristics of the virus and its RNA:

1. The protein molecules of TMV are removed by SDS sequentially, starting from the 3'-hydroxy-linked end of the RNA and progressing toward the 5'-hydroxy-linked end (1).
2. TMV RNA contains a number of unique sequences that can be visualized as single, long chain oligomers in T1 ribonuclease digests (3, 7).

The precision with which the unique-mer can be located depends largely on the distribution of chain lengths obtained for any degree of stripping. Symington and Commeron (14), in experiments with low concentrations of SDS and TMV, have found that the distribution of chain lengths widens as more nucleic acid is exposed. The location of the Ω-mer and the Ψ-mer in our experiment appears to be fairly precise. This is most probably due to the fact that they are located very close to the point where stripping begins and where the distribution of chain lengths exposed is fairly narrow. The second Ψ-mer probably cannot be located closer than ±10% of the 44% of stripping as indicated in our experiments. For this reason, Bruening has suggested that the products of the stripping reaction be fractionated into narrow size classes and each examined for the presence of unique-mer. In this case, the narrower the distribution, the more precise the location. Experiments of this kind are currently underway.

The use of this method for mapping unique-mer is not necessarily limited to the three described here. It is likely that other oligomers of unique sequence can be isolated from T1 RNase digests and assigned positions in the TMV RNA molecule. From theoretical considerations, Thomas (15) has proposed that in DNA molecules that recombine the sequences of all oligomers above the chain length of 12 may be unique. This principle may be extended in message and virus RNA. In a T1 RNase digest of TMV RNA, a substantial fraction of the total oligomers is longer than 12. Furthermore, a different set of unique-mer may be obtainable by endonucleolytic cleavage specific for cytosine rather than guanine (16).

With regard to the TMV coat protein, our results show that the Ω-mer sequence cannot be placed wholly within any model of the coat cistrons. The Ω-mer consists of a sequence of 69 bases without guanine. This codes for a peptide of 23 adjacent amino acids from which, by virtue of the amino acid code (17, 18), the following must be excluded: valine, alanine, aspartic acid, glutamic acid, glycine, tryptophan, and arginine. Wild type TMV protein (19) contains no sequence of more than 10 amino acids from which, by virtue of the amino acid code (17, 18), the following must be excluded: valine, alanine, aspartic acid, glutamic acid, glycine, tryptophan, and arginine. Wild type TMV protein (10) contains no sequence of more than 10 amino acids from which any of those listed above is missing. This means that the coat cistron may not contain a sequence of more than 33 bases without a guanine, and therefore cannot include the entire Ω-mer.

Because the Ω-mer is situated within 180 bases of the 5'-OH-linked terminus, it may be deduced that the TMV coat protein cistron is no closer than 180 bases to that terminus, and possibly as far as a whole cistron away.

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