The Structure of Tobacco Rattle Virus Ribonucleic Acids: Comparison of Large Oligonucleotides Derived from the 3' Ends

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SUMMARY

The RNA species of the CAM isolate of tobacco rattle virus have been labelled at their 3' termini and partially digested with T1 ribonuclease. The two RNAs give an identical pattern of labelled fragments following acrylamide gel electrophoresis, showing considerable structural similarity between the 3' ends of these molecules. RNA fragments derived from the 3' ends of short particle RNA and long particle RNA labelled in vitro with 125I were tested for their ability to anneal to complementary long particle RNA. Fragments derived from short particle RNA failed to hybridize, showing that despite their structural similarity, the 3' sequences of these RNAs are not homologous and that the homologous sequence known to be shared by TRV RNAs are not at the 3' ends of the molecules.

INTRODUCTION

Tobacco rattle virus (TRV) is unusual among multicomponent viruses in that the interdependence of the two virus components is not complete. The long particle of each strain is infectious (Harrison & Nixon, 1959) and yields long particle RNA which fails to become encapsidated in the absence of short particle RNA (Lister, 1966; Frost, Harrison & Woods, 1967). The short particle RNA contains the coat protein gene (Sänger, 1968, 1969; Ball, Minson & Shih, 1973). Since the short particle is not infectious it seems that the long particle contains the gene for an RNA replicase responsible for the replication of both long and short particle RNA, and it follows that the RNA of both particles contains a recognition site for such an enzyme. RNA–RNA hybridization experiments with the CAM strain of TRV have shown that about 25 % of the nucleotide sequences of the short RNA are also present in the long RNA (Darby & Minson, 1973) and since the short RNA of this strain has a mol. wt. of 0.7 x 10⁶ (Cooper & Mayo, 1972) this represents homologous sequences of about 500 nucleotides in total. The location of these homologous sequences is unknown but it is reasonable to suppose that the replicase binding sites would be at the 3' ends of the molecules. The RNAs of brome mosaic virus (BMV) have been shown to contain long, nearly identical, sequences at their 3' ends (Bastin et al. 1976) and we have shown the presence of the short sequence GCCCO₂ at the 3' ends of both TRV RNAs (Minson & Darby, 1973a). The object of the work described here was to examine long sequences derived from the 3' ends of TRV RNA molecules to find whether such sequences showed structural similarity and whether they were homologous.

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METHODS

Biological materials. The CAM isolate of TRV was used throughout and was a gift from Dr B. D. Harrison. A ‘defective isolate’ of this strain that produces only infectious long particle RNA has been described previously (Darby & Minson, 1973).

Virus RNA. Short and long particles of the virus were separated by two cycles of sucrose gradient centrifugation and the RNA extracted and further purified on sucrose gradients (Darby & Minson, 1972). Double stranded RNA was extracted from infected White Burley tobacco plants and used in RNA – RNA hybridization experiments as described before (Minson & Darby, 1973b).

Periodate oxidation and borohydride reduction. This was performed by the method of Weber & Weissmann (1970). After reduction with $^3$H-borohydride the RNA was precipitated with ethanol and purified by electrophoresis in $24\%$ acrylamide, $05\%$ agarose gels. The RNA was detected by staining briefly with toluidine blue and the stained bands were extracted from the gel by grinding with equal volumes of phenol and $005\text{ M-tris}$, $01\text{ M-NaCl}$, $5\text{ mm-EDTA at pH 75}$. The RNA was precipitated from the aqueous phase with ethanol and precipitated again from aqueous solution with cetyltrimethylammonium bromide. The precipitate was then washed three times with $70\%$ ethanol.

Iodination of RNA. TRV RNA species were radiolabelled by an adaptation of the method of Commerford (1971). The reaction was performed in $02\text{ ml }01\text{ M-sodium acetate at pH 48}$, $25\times10^{-5}\text{ M-KI}$, $5\times10^{-4}\text{ M-TICl}_3$ containing $200\mu\text{ g RNA and }5\text{ mCi Na}^{125}\text{I}$. After 5 min at $60^\circ\text{C}$ the reaction was stopped by transfer to ice and addition of $2\mu\text{l }2\text{-mercaptoethanol}$, and the RNA was precipitated with 2 vol. of ethanol. The precipitate was dissolved in $025\text{ ml }02\text{ M-ammonium acetate at pH 89}$, containing $1\%$ SDS, and the solution was heated at $45^\circ\text{C}$ for 10 min to remove unstable derivatives. The solution was then transferred to ice, made $05\text{ M}$ with respect to NaCl, and the RNA precipitated with ethanol. The RNA was finally dissolved in $005\text{ M-tris}$, $01\text{ M-NaCl}$, pH 75, precipitated with cetyltrimethylammonium bromide, and the precipitate washed twice with $70\%$ ethanol.

Digestion of RNA. Partial RNase digestion was achieved by digesting $40\mu\text{ g RNA}$ with $15$ or $40$ units $\text{T}_{1}\text{ RNase}$ for $1\text{ h at }0^\circ\text{C}$ in $50\mu\text{l }005\text{ M-tris}$, $02\text{ M-NaCl, }001\text{ M-MgCl}_2$ at pH 75. The reaction was stopped by addition of an equal volume of $2\%$ SDS, $8\text{ m-urea}$ and the mixture immediately heated to $80^\circ\text{C}$ for $10\text{ min}$. Complete $\text{T}_{1}\text{ digestion}$ was performed in the same buffer but using $100\text{ units }\text{T}_{1}\text{ RNase}$ for $1\text{ h at }37^\circ\text{C}$.

Total enzymic hydrolysis of RNA was achieved by digestion of $40\mu\text{ g RNA}$ with $50$ units $\text{T}_{1}\text{ RNase}$, $10\text{ units }\text{T}_{2}\text{ RNase}$ and $20\mu\text{ g pancreatic RNase}$ in $05\text{ ml }005\text{ M-sodium acetate, }001\text{ M-MgCl}_2$, pH 55, for $1\text{ h at }37^\circ\text{C}$. Total alkaline hydrolysis was for $15\text{ min at }100^\circ\text{C in }0\text{ M-NaOH}$.

Separation of digestion products. Partially digested RNA was fractionated in $12\%$ acrylamide gels in $01\text{ M-tris}$, $015\text{ M-boric acid, }25\text{ mm-EDTA, pH 83}$, containing $6\text{ m-urea}$ and $01\%$ SDS. Electrophoresis was usually for $14\text{ h at }10\text{ V/cm}$. When required, RNA fragments were extracted from the gel using phenol in the presence of unlabelled carrier RNA. Fragments were sometimes purified by a second electrophoretic separation in the absence of urea.

Total $\text{T}_{1}\text{ digests}$ were fractionated in two dimensions by electrophoresis according to Sanger, Brownlee & Barrell (1965).

Total hydrolysis products were separated by electrophoresis on Whatman paper 2 at pH 35.
3'-terminal structure of TRV RNAs

Fig. 1. Total hydrolysis products of 3H-borohydride reduced TRV short RNA. RNA was completely digested with nucleases and electrophoresed for 20 min at 80 V/cm with 100 μg cytosine as a marker. The same result was obtained with long RNA and with RNA hydrolysed with alkali. Electrophoresis was also done for 5 min at 50 V/cm to ensure that no negatively charged species had migrated into the anode buffer. The arrow marks the position of the cytosine marker.

RESULTS

Purity of the RNA species

The interpretation of the results which follow depends heavily on the purity of the RNA species. After separation of the two types of virus particle by gradient centrifugation, examination of the particles with the electron microscope revealed less than 1% contamination of short particles by long particles and 10% contamination of long particles by short particles. After extraction, the RNAs were further purified by gradient centrifugation, and RNA radiolabelled with 3H-borohydride was purified by acrylamide gel electrophoresis. The molar contamination of long particle RNA by short particle RNA must therefore be much less than 10% (2.5% by weight).

Terminally labelled RNA

TRV RNAs oxidized with periodate and reduced with 3H-borohydride were found to be labelled only at their 3'-hydroxyl terminus. Following total hydrolysis of the RNA either with nucleases or alkali, the products were separated by electrophoresis and all the isotope migrated with the cytosine marker (Fig. 1). Abou Haidar & Hirth (1977) have reported that the short particle RNA of TRV is blocked at its 5' terminus by a 5'-linked 7-methyl guanosine, and since this group contains a cis-hydroxyl pair, periodate oxidation and borohydride reduction should yield a hydroxymethylmethyleneglycol derivative. In two experiments done with different RNA preparations, we failed to find such derivatives associated with either long particle or short particle TRV RNA. We are unable to explain these results, which are inconsistent with the findings of Abou Haidar & Hirth (1977).

Partial digestion

TRV RNAs labelled at their 3' termini with 3H-borohydride were partially digested with T1 ribonuclease and the products electrophoresed in parallel on 12% acrylamide gels. Equal weights of the two RNAs were used throughout. E. coli 3H-RNA was used as a
Fig. 2. Electrophoresis of RNA fragments derived from the 3' ends of TRV RNAs. RNA reduced with $^3$H-borohydride was partially digested with T1 RNase and electrophoresed in 12% acrylamide gels with 2 µg $^{3H}$-tRNA. (a) 20 µg RNA digested with 8 units T1 RNase for 1 h at 0 °C and electrophoresed for 13 h at 10 V/cm. (b) as (a) but digested with 20 units and electrophoresed for 8 h. The arrow marks the position of t-RNA. Note that since equal weights of RNA were used the molar concentration of short RNA is four times that of long RNA. Migration is from left to right.
Fig. 3. Electrophoresis of iodinated RNA. Ten µg of TRV short or long particle RNA was mixed with 10^5 ct/min of iodinated RNA of the same type. After electrophoresis in 2-4% acrylamide gels for 3 h at 8 V/cm the gels were stained, dried on to filter paper and autoradiographed for 15 h. Gels (a) and (c) are stained gels containing long and short RNA respectively; (b), (d) are autoradiographs of (a) and (c). Migration is from top to bottom.

gel marker and its position determined by staining before the gels were cut into 1 mm slices, dissolved in 'NCS Tissue Solubilizer' (Amersham-Searle), and the isotope content determined by scintillation counting. The results of two experiments are shown in Fig. 2, and both experiments show a striking similarity between the digestion products of the two TRV RNAs. The patterns are virtually identical, and major products are seen which migrate at rates of 0.7, 0.87, 2.0 and 2.5 relative to t-RNA. The results indicate considerable structural similarity between the 3' ends of the two RNA molecules.

Iodinated RNA

To find whether the structural similarity at the 3' ends of TRV RNAs was accompanied by sequence homology in this region the RNAs were labelled internally with ^125I. After iodination 100 to 150 µg RNA was recovered with a specific activity of 5 × 10^6 d/min/µg. Under these conditions about 1% of the cytosines are iodinated and as shown in Fig. 3 the RNA is undegraded. Digestion of this RNA with pancreatic ribonuclease yields from the 3' end ^3C_{out} which is readily identified by electrophoresis at pH 3.5 since it has less negative charge than all other iodinated products. Similarly, digestion with Tt ribonuclease yields ^1(CCC_{out}), which can be identified in a two dimensional fingerprint (Fig. 4a). Both these methods could therefore be used to identify large fragments derived from the 3' ends of these molecules. Note that a minor spot co-migrates with ^1(CCC_{out}) in the first dimension. This spot yields only ^1Cp following pancreatic ribonuclease digestion and is present only in digests containing ^1(CCC_{out}). We conclude that this is either ^4CCC_{out} or C^0CC_{out}, the major spot comprising a mixture of the two remaining isomers.

Forty µg of iodinated long particle and short particle RNA were digested with 5 units Tt
Fig. 4. Tt oligonucleotide fingerprint of iodinated RNA. RNA was digested with Tt-RNase as described in the text and electrophoresed in two dimensions as described by Sanger et al. (1965). The arrowed spot is \(^{1}(\text{CCC}_{\text{olh}})\). This spot yields only \(^2\text{Cp}\) and \(^{1}\text{Coh}\). (a) Tt-fingerprint of short particle RNA. (b) Tt-fingerprint of fragment VII derived from short RNA.

Table 1. Percentage of \(^{125}\text{I}\) recovered as \(^{1}\text{Coh}\) from RNA fragments following pancreatic ribonuclease digestion

<table>
<thead>
<tr>
<th>Fragment number</th>
<th>Derived from</th>
<th>Mobility relative to (^{30}\text{t-RNA})</th>
<th>(^{1}\text{Coh}) (%)</th>
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<tr>
<td>I</td>
<td>short particle</td>
<td>0.45</td>
<td>0.5</td>
</tr>
<tr>
<td>II</td>
<td>RNA</td>
<td>0.60</td>
<td>0.7</td>
</tr>
<tr>
<td>III</td>
<td>RNA</td>
<td>0.70</td>
<td>0.5</td>
</tr>
<tr>
<td>IV</td>
<td>RNA</td>
<td>0.72</td>
<td>0.1</td>
</tr>
<tr>
<td>V</td>
<td>RNA</td>
<td>0.93</td>
<td>0.1</td>
</tr>
<tr>
<td>VI</td>
<td>RNA</td>
<td>1.75</td>
<td>0.15</td>
</tr>
<tr>
<td>VII</td>
<td>RNA</td>
<td>2.00</td>
<td>0.90</td>
</tr>
<tr>
<td>I</td>
<td>long particle</td>
<td>0.44</td>
<td>0.2</td>
</tr>
<tr>
<td>II</td>
<td>RNA</td>
<td>0.70</td>
<td>0.3</td>
</tr>
<tr>
<td>III</td>
<td>RNA</td>
<td>0.75</td>
<td>0.3</td>
</tr>
<tr>
<td>IV</td>
<td>RNA</td>
<td>2.00</td>
<td>1.5</td>
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ribonuclease as before and the samples electrophoresed in parallel in a 12 % slab gel. Autoradiography of the gel gave very poor resolution but after staining with toluidine blue the pattern of bands was readily discerned, though this was more difficult for the long particle RNA digest because of the greater complexity and lower molar concentration of this RNA. A number of major fragments were extracted from the gel, electrophoresed again and extracted, after detection by autoradiography. To find whether any of these fragments were derived from the 3' ends of the molecules they were digested with pancreatic ribonuclease and the fraction of \(^{125}\text{I}\) recovered as \(^{3}\text{Coh}\) was determined. Table 1 shows the results of these determinations together with the mobility of each fragment relative to \(^{30}\text{t-RNA}\), and it is apparent that fragments III and VII from the short particle RNA digest are derived from the 3' end of the molecule. This was confirmed by two dimensional fingerprinting of Tt digests. Fig. 4(b) shows a fingerprint of a Tt digest of fragment VII and shows a very considerable enrichment for \(^{1}(\text{CCC}_{\text{olh}})\). The fingerprint was done using material obtained after a single gel separation because insufficient isotope was available following the second separation. Fragments III and VII have mobilities of 0.7 and 2.0 respectively relative to \(^{30}\text{t-RNA}\) and presumably correspond with products of these mobilities found in digests of terminally labelled RNA (Fig. 2). Fragments 2 and 4 from the long RNA
3’-terminal structure of TRV RNAs

Table 2. Hybridization of fragments from long RNA and short RNA to complementary long RNA*

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Derived from</th>
<th>Hybridized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>long RNA</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>III</td>
<td>short RNA</td>
<td>6·5</td>
</tr>
<tr>
<td>VII</td>
<td></td>
<td>1·7</td>
</tr>
</tbody>
</table>

* The source of complementary long RNA was double stranded RNA from infected plants. This was denatured immediately before hybridization. Since this RNA contains equal contributions of (+) and (−) strands very high levels of hybridization are obtained only when the concentration of denatured double stranded RNA is high relative to the concentration of isotope labelled (+) strand – a condition which has proved difficult to achieve with this virus (see Darby & Minson, 1973).

digest also have mobilities of 0·7 and 2·0, and have a high ¹⁵N content, but the evidence here is less convincing, and it is apparent that these fragments are much less pure than those derived from short RNA.

To find out whether these 3’ terminal fragments were homologous as judged by nucleic acid hybridization, 10⁶ ct/min of each fragment was annealed to a preparation of denatured, double stranded, long particle RNA extracted from plants infected with a ‘defective isolate’ of the virus (Darby & Minson, 1973). The purity of the fragments derived from long particle RNA is not important in this experiment because they are used merely as positive controls; they are homologous oligonucleotides of approximately the same size as the fragments derived from the 3’ ends of short particle RNA. The results in Table 2 show that, as expected, long particle RNA fragments hybridize efficiently to their homologous complementary strand. Fragments III and VII from short particle RNA, however, hybridize very poorly. We have no direct evidence for the purity of fragments III and VII so the interpretation of these data is not straightforward. The high ¹⁵N content suggests that a 3’ terminal fragment must be the major component of bands III and VII and it follows from the hybridization results that the 3’ end of the short particle RNA is not homologous with sequences present in long particle RNA. This is confirmed by a comparison of the hybridization behaviour of fragment VII before and after purification by a second electrophoretic separation. After the first separation fragment VII contained 6 % ¹⁵N and gave 5 % hybridization to complementary long RNA, whereas after a second electrophoretic separation it contained 9 % ¹⁵N and hybridized to 1·7 %. Thus enrichment for the 3’ terminal component reduces the level of heterologous hybridization.

DISCUSSION

The results presented here show that the 3’ ends of the RNAs of TRV have considerable structural similarity. Partial nuclease digestion yields a nearly identical pattern of 3’-derived fragments as judged by polyacrylamide electrophoresis, and this presumably reflects an identity of folding and base-pairing such that the available sites for hydrolysis are in the same positions in both molecules. We suppose that these structural similarities represent recognition sites for a shared replicase. Our results are similar to those obtained by Bastin et al. (1976) with the RNAs of BMV. Despite the structural similarities between the 3’ ends of TRV RNAs, oligonucleotides derived from this region of the molecules do not cross hybridize, and the primary sequences must therefore contain many differences. Our results in this respect are different from those obtained by Bastin et al. (1976) for although
hybridization studies have not been done with BMV RNAs it is apparent that differences between the 3' sequences of the RNAs of this virus are limited to only a few base changes. It is not clear why the constraints imposed on these sequences in BMV RNA should be greater than those imposed on TRV RNAs.

The finding that the 3' sequences of TRV RNAs are structurally similar but not homologous leaves unanswered the question of the position of the homologous sequences which we have previously shown to be present in these RNAs (Darby & Minson, 1973). Abou Haidar & Hirth (1977) have shown that the encapsidation of TRV RNA is polar and consider that initiation is at the 5' ends of the molecules. It is tempting therefore to speculate that this is the position of the homologous sequences and that these are responsible for coat protein binding.

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REFERENCES


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