Characterization of double-stranded ribonucleic acid in tobacco leaves

(RNA replication/RNA-dependent RNA polymerase/nucleases)

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Contribution by Heinz Fraenkel-Conrat, April 23, 1979

ABSTRACT Double-stranded RNA was isolated from tobacco leaves and characterized in terms of base composition, density, and nuclease resistance. Although its role in the plant's physiology is not clear, evidence was adduced that it is the product of the RNA-dependent RNA polymerase previously shown to occur in this and other plants. The fact that twice as much fully as partially double-stranded RNA appears to be made favors a regulatory role for the double-stranded RNA rather than a transcriptional intermediate role.

The presence of appreciable amounts of RNA-dependent RNA polymerase activity has been demonstrated in recent years in most plant tissues studied (1-13). The physiological role of these enzymes is, however, unknown. In the search for the function of such enzymes it appears logical to investigate the occurrence of their primary product, double-stranded RNA. There is, to our knowledge, only a single report on the presence of small amounts of double-stranded RNA in plant tissues, as demonstrated solely by induction of interference with rhabdo-viral multiplication (13). We have now isolated double-stranded RNA from 32P-labeled tobacco leaves and collected data on its physical and chemical nature, its location, and its amount in actinomycin D-treated tissue. We conclude from these data that this RNA is produced by the plant's RNA-dependent RNA polymerase.

EXPERIMENTAL PROCEDURE

Materials

[32P]Orthophosphate (carrier free) was purchased from New England Nuclear. Deoxyribonuclease (ribonuclease free, 2465 units/mg) and calf thymus DNA were obtained from Worthington; pancreatic ribonuclease A, Pronase K, and actinomycin D were from Sigma; ribonuclease T1 was from Calbiochem-Behring; yeast transfer RNA was from Calbiochem; cellulose was Whatman CF11 cellulose. Sephadex G-75 was from Pharmacia, and K1 from Mallinckrodt. Cytoplasmic polyhedrosis virus was a gift from Y. Iwashita (Utsunomiya University, Japan), and tobacco mosaic virus RNA was prepared by standard methods from tobacco plants infected with the virus.

Methods

32P Labeling and Actinomycin D Treatment. Tobacco plants (Nicotiana tabacum var Turkish) (weighing about 10 g) with about five fully expanded leaves were cut off and immediately immersed in small beakers containing 1-2 mCi of [32P]orthophosphate in about 0.3 ml of H2O (1 Ci = 3.7 × 1010 becquers). They were placed in a draft and, as this fluid was taken up, more water was added over a 2- to 4-hr period. They were then floated on water, for 16 hr under fluorescent lights, for a total of 24 hr.

Actinomycin D (50 µg per plant) was imbibed in the same manner. The plants in the beakers were held in a moist, closed atmosphere for 24 hr as above and then allowed to take up 32P and the same amount of actinomycin D for another 24-hr period under the same conditions.

Cell Fractionations. 32P-Labeled leaf tissues frozen at −75°C were ground to a fine powder, then allowed to melt in the presence of the following medium: 70 mM Tris-HCl, pH 7.8/0.5 M sucrose/10 mM KCl/40 mM 2-mercaptoethanol (14). The Miracloth-filtered extract was fractionated by repeated sedimentations and washings into a mostly nuclear fraction, which sedimented during centrifugation 2 min at 200 × g, and a mostly chloroplast fraction, which sedimented during centrifugation for 10 min at 2000 × g. The various fractions were at times further purified by discontinuous gradient centrifugation for 1 hr at 53,000 × g. The nuclear fraction was the pellet material from the primary nuclear fraction; the chloroplast fraction was the upper of the two dark green bands from the primary chloroplast fraction. The 2000 × g supernatant was centrifuged for 2 hr at 105,000 × g; the superant was the source material for the cytoplasmic nucleic acid. All these procedures were done at 0-4°C.

Preparation of Double-Stranded RNA. Nucleic acids were usually extracted from 30-40 g of 32P-labeled leaf tissue by being ground in a chilled mortar with 40 ml of buffer (0.1 M Tris-HCl/0.1 M NaCl/10 mM EDTA, pH 7.0), 2% of 10% sodium dodecyl sulfate, and 40 ml of 90% phenol containing 0.1% 8-hydroxyquinoline. The slurry was shaken for 20 min at 25°C. After centrifugation at 3000 × g for 10 min, the buffer phase was extracted three more times by shaking with phenol for 5-10 min. The nucleic acids were precipitated by addition of 3 vol of ethanol and a few drops of 3 M sodium acetate buffer (pH 4.0) and stored overnight at −20°C.

The precipitated nucleic acids were collected by centrifugation, rinsed with ethanol, and dried under reduced pressure. The pellet was dissolved in DNase buffer (50 mM NaCl/2 mM MgCl2/10 mM Tris-HCl, pH 7.4), ribonuclease-free pancreatic deoxyribonuclease I was added to give 20 µg of DNase per ml, and the solution was incubated for 30 min at room temperature. The solution was then brought to 0.25 M NaCl and pancreatic RNase A and RNase T1 were added to give 10 µg/ml each and 25 units/ml respectively. The solution was incubated for 30 min at 37°C. The reaction was terminated by thrice extracting the solution for 10 min with an equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline. The nucleic acid was precipitated from the water phase as above.

The precipitated nucleic acid was dissolved in STE buffer (0.1 M NaCl/1 mM EDTA/50 mM Tris-HCl, pH 6.9) and absolute ethanol was added to give 35% (vol/vol). The sample was applied to a cellulose column (1 × 25 cm) (15), the column was washed with STE buffer/ethanol, 65:35, (vol/vol), and 15

Abbreviation: STE buffer, 0.1 M NaCl/1 mM EDTA/50 mM Tris-HCl, pH 6.9.

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fractions of 3–4 ml were collected. Then the column was eluted with STE buffer/ethanol, 85:15 (vol/vol) (20–25 fractions) and finally with STE buffer alone (20 fractions). The nucleic acid eluting at the final step was concentrated by precipitation with 3 vol of ethanol, with 0.1 mg of yeast transfer RNA added as carrier.

Nucleic acid fractions were further treated with RNase under the standard conditions (see above) at high ionic strength. After phenol extraction and ethanol precipitation, the nucleic acids were dissolved in 0.15 M NaCl/0.015 M sodium citrate, at pH 7.0, and applied to a Sephadex G-75 column (0.8 × 12 cm) equilibrated with the same buffer. The elution front containing the nucleic acid was collected, and the nucleic acid was precipitated with 3 vol of ethanol, with 0.1 mg of yeast transfer RNA added as carrier.

Isopycnic Density-Gradient Centrifugation. The purified RNA preparations were centrifuged to equilibrium in KI (16). The double-stranded RNA was dissolved in 5.1 ml of 6.77 M KI, containing ethidium bromide (20 μg/ml), sodium citrate (15 mM), sodium bisulfite (10 mM), and potassium phosphate (10 mM). The final pH was 7.0. The final density of the solutions was 1.56 g/ml.

Gradients were centrifuged for 48–64 hr at 37,000 rpm (20°C) in an SW50.1 rotor in a Beckman L2-65B ultracentrifuge; 0.2-ml fractions were then collected. The density of the fractions was measured immediately after they were collected with a 0.1-ml pipette calibrated with distilled water. The radioactivity of each fraction was measured in 2 ml of Aquasol 2 scintillant. The fluorescence of marker RNAs was determined after addition of 0.2 ml of 10 mM sodium bisulfite in a Perkin–Elmer model MPF-3-L fluorescence spectrophotometer at 580 nm with 340 nm as the excitation wavelength (16).

Base Composition Analysis. The RNA was digested with 0.3 M KOH at 37°C for 18 hr. Mononucleotides resulting from the alkaline hydrolysis were separated by electrophoresis on Whatman no. 3 MM paper at 2500 V in 0.5% pyridine/5% (vol/vol) glacial acetic acid/1 mM EDTA, at pH 3.5. Carrier nucleotides (50–100 μg of each in 10 μl) were added and co-electrophoresed with radioactive samples. After electrophoresis, the paper was dried and the UV-quenching spots were cut out and their radioactivity was measured.

Isolation of RNA without Nuclease Treatment. Sufficient 10 M LiCl was added to the crude nucleic acid preparation to give a final concentration of 2 M (17, 18). After 16 hr or more at 0°C, the solution was centrifuged for 15 min at 27,000 × g. Three volumes of low-salt buffer were added to the resulting supernatant fraction and several milliliters were added to the pellet. Then the nucleic acids in each fraction were precipitated with ethanol, washed, dissolved in STE buffer, and subjected to two cycles of cellulose chromatography as above.

RESULTS AND DISCUSSION

Purification and characterization of double-stranded RNA

Treatment of crude 32P-labeled nucleic acids with DNase I, followed by pancreatic ribonuclease A and RNase T1 in high-salt buffer, resulted in about 85% digestion, as illustrated by a typical experiment shown in Table 1B. When such nuclease-resistant nucleic acids were applied to a cellulose column, elution at decreasing alcohol concentrations produced a profile illustrated in Fig. 1. The first peak is believed to contain small molecular weight material, including oligonucleotides and any residual DNA. The second peak probably contains relatively nuclease-resistant polyribonucleotides. The third peak, eluting with STE buffer without ethanol and representing only 0.06% of the total radioactivity, is presumed to be double-stranded RNA. This was substantiated by the fact that the double-stranded RNA of cytoplasmic polyhedrosis virus chromatographed only in this region, while the single-stranded RNA of turnip yellows mosaic virus was eluted in the second peak and

![Figure 1](image-url) Cellulose chromatography according to Franklin (15) of the nucleic acids that had been treated with DNase I in DNase buffer and RNases A and T1 in high-salt buffer, extracted with phenol, and then chromatographed on Sephadex G-75. The radioactivity of 1 ml of each fraction was directly measured (without scintillation fluid).
Table 2. Enzyme resistance of purified double-stranded RNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>361</td>
</tr>
<tr>
<td>RNases T1 + A in high salt</td>
<td>354</td>
</tr>
<tr>
<td>RNase T2 in high salt</td>
<td>357</td>
</tr>
<tr>
<td>DNase and RNases T1 + A + T2 in high salt</td>
<td>353</td>
</tr>
<tr>
<td>RNases T1 + A in low salt</td>
<td>37</td>
</tr>
<tr>
<td>Heated (100°C, 3 min) and quick-cooled, then RNases T1 + A in high salt</td>
<td>31</td>
</tr>
</tbody>
</table>

double-stranded DNA and tRNA in the first peak (data not shown).

However, the material in the third peak was not pure double-stranded RNA, since a second RNase treatment (RNase A plus RNase T1) of that nucleic acid in high salt caused degradation of 40–60% (Table 1D). That such double-stranded RNA is a distinct molecular species is supported by the observation that such a third peak was also resolved upon direct cellulose chromatography of plant RNA that had not been treated with enzyme (see below). Cellular double-stranded RNA appears not to have been previously isolated without nuclease treatment.

The identification of this material as double-stranded RNA was confirmed by various methods. When suspended in high salt, this material was highly resistant to digestion by DNase and all RNases, including T2. However, after heat denaturation, it was almost completely digested (Table 2). The possibility that the purified RNase-resistant material contained DNA-RNA hybrid molecules seems to be ruled out because such hybrids are sensitive to DNase (19) and RNase plus DNase (20) and would not, after melting, be 90% digestible by RNase.

Further proof for the double-stranded nature of this RNA was obtained by the following tests. (i) The nuclease-resistant material had a buoyant density of about 1.57 g/ml (Fig. 2). Added tobacco mosaic virus RNA had a buoyant density of 1.62 g/ml and calf thymus DNA a buoyant density of 1.49 g/ml. The density of the RNase-resistant material (1.57 g/ml) is similar to that of double-stranded RNA of cytoplasmic polyhedrosis virus (1.56 g/ml) and reovirus double-stranded RNA (1.57 g/ml) (16). (ii) Base analysis showed 28.3 and 28.1% for A and U, and 21.8 and 21.6% for G and C, respectively.

Fig. 2. Centrifugation of purified double-stranded[^32P]RNA in KI density gradient. Approximately 900 cpm of double-stranded RNA was centrifuged in KI. The radioactivity of 0.2 ml of each fraction was determined in 2 ml of Aquasol 2 scintillant.

Fig. 3. Sedimentation profile of purified double-stranded[^32P]RNA centrifuged in a 15–30% glycerol gradient for 7 hr at 45,000 rpm in the SW50.1 rotor. Radioactivity was measured as in the legend of Fig. 2. BMV, brome mosaic virus; ds RNA, double-stranded RNA. A similar sedimentation rate was observed for double-stranded RNA isolated without nuclease treatment.

To determine the molecular weight of the double-stranded RNA purified by the cellulose column technique, we centrifuged the RNA on glycerol gradients (15–30%) in 10 mM Tris-HCl/0.1 M NaCl/1 mM EDTA, at pH 7.4 for 7 hr at 4°C at 45,000 rpm in an SW50.1 rotor. Brome mosaic virus RNAs and tRNA were used as markers. The double-stranded RNA sedimented at about 6 S (Fig. 3). The double-stranded RNA purified from actinomycin D-treated leaves was also centrifuged and showed the same S value. From the formula of Franklin (molecular weight = 2.42 S^3) (21), a molecular weight of about 25,000 can be calculated for the double-stranded RNA.

To determine whether the double-stranded RNA was completely or only partially double stranded, in the sense of having a single-stranded component, we isolated it without nuclease treatment by LiCl precipitation. Double-stranded RNA is soluble in 2 M LiCl, but partially single-stranded RNA (such as the replicative intermediates of viral nucleic acid replication) is insoluble in 2 M LiCl (17, 18). Total cellular nucleic acids were therefore fractionated with 2 M LiCl. The double-stranded RNA present in both the LiCl-soluble and LiCl-insoluble fraction was then purified by two cycles of cellulose chromatography. Twice as much RNA was found in the soluble fraction.

Table 3. Determination of fully and partially double-stranded RNA in plant tissue

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total[^2P]-labeled nucleic acid</th>
<th>Purified dsRNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 M LiCl soluble</td>
<td>1.0 × 10^6</td>
<td>0.7 × 10^5 (0.26%, fully ds)</td>
</tr>
<tr>
<td>2 M LiCl insoluble</td>
<td>1.7 × 10^6</td>
<td>0.36 × 10^5 (0.13%, partially ds)</td>
</tr>
</tbody>
</table>

* Values are in cpm/g of leaf.

† Purified by cellulose chromatography (see Fig. 1). ds, Double-stranded.
Table 4. Localization of double-stranded RNA in total cell nucleic acid

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total nucleic acid, cpm/g leaf</th>
<th>Double-stranded RNA, cpm</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>0.68 x 10^5</td>
<td>32</td>
<td>0.047</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>0.58 x 10^5</td>
<td>100</td>
<td>0.170</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>16.9 x 10^5</td>
<td>639</td>
<td>0.036</td>
</tr>
</tbody>
</table>

These data are from an experiment in which the nuclear and chloroplast fractions were not purified by discontinuous gradient centrifugation. A similar proportion of replicative form and replicative intermediate RNAs was obtained in preparations so further purified.

Correlation between double-stranded RNA and plant RNA replicase

We have previously reported that most of the RNA-dependent RNA polymerases of healthy, as contrasted to virus-infected, plants is found in the cytoplasm (5, 10); the much smaller amount of particle-bound enzyme was found associated with the chloroplast, rather than the nuclear fraction (unpublished results). We have now determined the distribution of double-stranded RNA in these two particulate fractions and in the cytoplasm and found the same pattern: by far the most double-stranded RNA is in the cytoplasm and very little in the nuclear fraction. In terms of percentage of total labeled RNA, the chloroplast fraction distinctly has the highest proportion of double-stranded RNA, supporting our belief that this enzyme may originate in the chloroplasts (Table 4).

Nuclease-resistant double-stranded RNA has been found in the nuclei of many animal cells (e.g., refs. 18 and 22–24). No convincing evidence for the presence of RNA-dependent RNA polymerase in such cells has been found, and it has been suggested that the double-stranded RNA may result from transcription of both strands of the nuclear DNA (18, 25, 26). Our present results provide evidence against such an origin for the largely cytoplasmic double-stranded RNA of plants. We approached this question by testing the effect of actinomycin D on the synthesis of tobacco double-stranded RNA. The specific activity of the single-stranded [32P]RNA of actinomycin-treated plants was 30% that of the control plants, thus showing that transcription was inhibited. Yet, as shown in Table 1, treatment of plants with actinomycin D did not depress, but rather relatively increased, the synthesis of double-stranded RNA. This may be regarded as strong evidence that this RNA was made in plants not by DNA-dependent RNA polymerase, but by the RNA-dependent RNA polymerase, which is present and not affected by actinomycin D (4, 5, 8–10).

The biological role of this enzyme remains, however, uncertain. Our present finding that considerably more fully than partially double-stranded RNA is present in plants differs from the results obtained in RNA virus-infected tissues when the viral partially double-stranded replicative intermediate usually predominates at early stages of infection (27–29) (see Note Added in Proof). Thus the plant RNA replicase appears not to be engaged in mRNA amplification, as we suggested earlier (30). It now appears more probable that its purpose is the production of double-stranded RNA molecules, possibly for some regulatory function.

Note Added in Proof. When the kinetics of self-annealing of the double-stranded (90% RNase-resistant) RNA of healthy tobacco was compared with that of double-stranded RNA, largely TMV RNA, isolated from infected plants by the same technique, annealing after melting for 2 1/2 min at 100°C in 1.5 mM NaCl/0.15 mM sodium citrate was slightly faster for the former (34 and 85%, as compared to 20 and 82% in 1 and 6 hr, respectively, at 68°C in 0.50 M NaCl/0.030 M sodium citrate). This strongly supports the belief that the double-stranded RNA in healthy plants represents a unique species, its low molecular weight accounting for its slightly faster renaturation. Its uniqueness is also borne out by its base composition and by the fact that its amount exceeds that of partially double-stranded material (2:1; see Table 3), while in 7-day TMV-infected plants the opposite ratio of RF/R1 was obtained (0.44:1). The ratio of cytoplasmic to "bound" double-stranded RNA in the infected plants was 0.9/1, while in the healthy plants it was 3.3/1.

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