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The Detection and Characterization of Viral-Related Double-Stranded RNAs in Tobacco Mosaic Virus-Infected Plants

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The 2 *M* LiCl-soluble RNA fraction extracted from tobacco mosaic virus (TMV)-infected tobacco plants contains, in addition to the viral replicative form of 4×10^6 MW, three smaller double-stranded (ds) RNA species with apparent molecular weights (estimated by polyacrylamide gel electrophoresis, using ds RNAs as markers) of 2.25, 1.1, and 0.23 $\times 10^6$. The synthesis of all four ds RNAs is insensitive to actinomycin D. They are completely RNase insensitive at high salt concentrations and are found both in directly inoculated and in apical tissues. In tissues incubated in the presence of ^{3}H-uridine and actinomycin D, the three small ds RNAs accounted for 6 to 11.5% of the total radioactivity incorporated into viral ds RNA. On a molar basis, however, in apical leaves the smallest ds RNA was synthesized to almost the same level as the replicative form. By molecular hybridization, the three small ds RNAs have been shown to be of viral origin, and each contains sequences represented in the 5′ end of complementary (negative strand) TMV RNA. Based on molecular weight data, none of the ds RNAs can be considered to be a ds form of the subgenomic TMV coat protein mRNA (the LMC), suggesting that it is not replicated independently. None of the small ds RNAs was found to be an endogenous product of the bound TMV RNA replicase.

INTRODUCTION

Characteristic of the replication of RNA plant viruses is the presence of double-stranded (ds) nucleic acids in diseased tissues. In TMV infections, structures with the properties of replicative form (RF; fully double stranded) and replicative intermediate (RI; double stranded with single-stranded "tails") have been observed (Nilsson-Tillgren, 1970; Jackson *et al.*, 1971, 1972; Aoki and Takebe, 1975), although their exact role in the replication of TMV has not been fully elucidated. There is, however, reasonable evidence to indicate their involvement (Jackson *et al.*, 1972; Kielland-Brandt and Nilsson-Tillgren, 1973; Aoki and Takebe, 1975).

As a part of the TMV replication process, subgenomic mRNAs are formed (Hunter *et al.*, 1976), at least one of which is encapsidated and is found in the population of rods comprising TMV preparations (Beachy and Zaitlin, 1977). It was the purpose of the current study to determine if there were ds RNA molecules in TMV-infected plants which corresponded in size to twice the MWs of the subgenomic single-stranded (ss) TMV mRNAs. Rationalization for this approach can be found in some studies with multicomponent plant viruses where ds counterparts of the virion ss RNAs have been observed (Bol *et al.*, 1975; Bastin and Kaesberg, 1976; Kaper and Diaz-Ruiz, 1977; Diaz-Ruiz and Kaper, 1978; Henriques and Morris, 1979). We did find at least three new species of ds RNA in TMV-infected plants, but based on their MWs, only one of them could conceivably

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be the ds counterpart of a subgenomic TMV mRNA.

MATERIALS AND METHODS

Plants. Fully expanded leaves of tobacco (*Nicotiana tabacum* cv. Samsun) were inoculated on both sides with TMV, U_1 strain (0.5 mg/ml), in 0.05 *M*, pH 7.0, phosphate buffer and with Celite as an abrasive. Following inoculation, plants were kept in a growth chamber at 28.5° with a 16-hr photoperiod (mixed incandescent and fluorescent illumination, 12,000 lux). Directly inoculated leaves or young apical leaves showing vein clearing were harvested usually 3 to 4 days after inoculation, the midribs were removed, and the leaf blades were kept frozen until processed.

Extraction of RNA. Frozen leaf tissue (usually 50–200 *g*) was ground in a chilled mortar in the presence of liquid nitrogen with 1 vol of GPS buffer (0.2 *M* glycine, 0.1 *M* Na_2HPO_4, 0.6 *M* NaCl, pH 9.6), 0.2 vol of 10% SDS, 1% mercaptoethanol, 1 vol of water-saturated phenol containing 0.1% 8-hydroxyquinoline, and 1 vol of chloroform–butanol (25:1). The resulting slurry was allowed to melt and was then stirred at room temperature for 30 min. The homogenate was centrifuged at 12,000 *g* for 10 min to separate the phases, and the aqueous phase was removed and precipitated for several hours with 2 vol of ethanol at −20°. The resulting precipitate was collected by centrifugation (12,000 *g*, 20 min), resuspended in 50 m*M* Tris-HCl buffer, pH 7.5, and dialyzed overnight against 2 liters of the same buffer at 4°. On the next day, the nucleic acid solution was clarified by centrifugation, made 10 m*M* in $MgCl_2$, and incubated with 50 μg/ml deoxyribonuclease I (Worthington, DPFF) for 40 min at room temperature. The material was subsequently precipitated with 2 vol of ethanol and 100 m*M* sodium acetate, pH 4.8, for several hours at −20°.

The precipitate was collected by centrifugation (12,000 *g*, 20 min), thoroughly dried under vacuum, and resuspended directly in 5 to 10 ml 2 *M* LiCl. After vigorous vortexing for 2 min, the slurry was centrifuged (12,000 *g*, 20 min) and the salt-soluble supernatant fraction was removed and precipitated with 3 vol of ethanol at −85° for 40 min.

Isolation of ds RNA. The ds RNA fraction was isolated by CF-11 cellulose chromatography with a method somewhat modified from the original procedure of Franklin (1966). These modifications were developed by Dr. Elizabeth Dickson of Rockefeller University. The salt-soluble RNA alcohol precipitate (collected by centrifugation) was resuspended in STE buffer (100 m*M* NaCl, 50 m*M* Tris-HCl, 1 m*M* EDTA, pH 7) and made to 50% ethanol. CF-11 cellulose (Whatman, Catalog 1113) had previously been suspended in 0.1 *M* NaOH for 30 min at room temperature. It was washed repeatedly with distilled water to lower the pH and was stored in 1 m*M*, pH 7, EDTA at 4°. To avoid overloading, it is important to have sufficient CF-11 and to treat the sample with DNase before application. Generally a minimum of 1 ml of column volume of CF-11 is required for 200 μg of RNA. All solutions applied to the column were degassed. Columns of CF-11, made in syringe barrels with glass wool plugs, were first eluted with water to allow the fine CF-11 to pass through, then with STE, and finally with a 1:1 mixture of STE and 100% ethanol (termed 50% ethanol-STE). Columns were loaded with the RNA solution in 50% ethanol-STE and were eluted stepwise with several aliquots of degassed solutions of 50% ethanol-STE, 17.5% ethanol-STE, which elutes the ss RNA, and finally with water, which elutes the ds RNA. The optical densities of the different fractions were monitored with a spectrophotometer at 260 nm and appropriate fractions were pooled and precipitated with 2 vol of ethanol and 100 m*M* sodium acetate, pH 4.8. Yeast RNA at 5 μg/ml was used as a precipitation carrier when needed. One cycle of CF-11 chromatography was usually enough to fractionate ds RNA efficiently. If needed, a second cycle was applied under the same conditions. When ^{3}H-RNA was fractionated on CF-11 columns, the elution profile was determined as follows: 50–100 μl of each fraction was spotted on a Whatman

GF/A or 3MM filter; filters were air dried and incubated at 60° for 30 min with 0.4 ml of NCS solubilizer-H_2O (9:1). A toluene-based scintillation mixture was added and radioactivity was estimated with a Beckman liquid scintillation counter.

Gel electrophoresis of RNAs. Double-stranded RNA (and in one instance, ss RNA) was electrophoresed in 3% polyacrylamide slab gels, containing 6 *M* urea (Schuerch *et al.*, 1975). Gels were normally 2.2 mm thick, 15 cm wide, and 12 cm long. The electrode chambers as well as the gels contained 40 m*M* Tris, 20 m*M* Na-acetate, 1 m*M* EDTA, pH 7.2. Samples were electrophoresed at 30 to 35 V for 16 hr. Gels were soaked in water for 0.5 to 1 hr, stained with 20 μg/ml ethidium bromide, and rinsed in water, and RNA species were visualized and photographed using transmitted uv illumination and Polaroid Type 55 negative-positive film.

In vivo labeling of RNA. Leaf tissue (2 g) from healthy or infected plants was cut into 1-mm strips and infiltrated several times under vacuum with 3 ml of a solution containing 50 to 100 μCi/ml ^{3}H-uridine, 250 μg/ml Carbenicillin, in 10 m*M* KH_2PO_4 and, in some experiments, with 50 μg/ml actinomycin D.

Strips were incubated in the light (1900 lux) for 6 hr at 28.5°, rinsed, and frozen at −85° until processed. The extraction of nucleic acids, isolation of ds RNA, and electrophoresis were performed as described for nonradioactive samples.

Radioactive RNA in gels were detected by fluorography, using either the original protocol (Bonner and Laskey, 1974) or a commercially available preparation (Enhance, New England Nuclear Corp.). The relative radioactivity in different RNA species was estimated by scanning the fluorogram films in an Ortec Model 4310 densitometer, photocopying the tracings, and cutting out and weighing individual peaks.

Replicase assay. Bound TMV RNA replicase from 3 day-infected directly inoculated leaves was prepared after Zaitlin *et al.* (1973). The ^{3}H-labeled product was phenol extracted and subjected to chromatography on CF-11 cellulose as described, retaining and analyzing the fraction eluted from the column with water.

Protoplast isolation. Protoplasts were isolated from inoculated tobacco leaves as previously described (Zelcer and Galun, 1976), using a mixture of 0.5% Cellulysin (Calbiochem), 0.2% Drieselase (Plenum Scientific, Hackensack, N. J.), and 0.1% Macerozyme (Kinki Yakult, Tokyo) in a solution containing 0.55 *M* mannitol and 250 μg/ml Carbenicillin.

Preparation of iodinated full-length TMV RNA and of the one-sixth of the RNA containing its 3′ end. TMV virions were extracted as described (Bruening *et al.*, 1976) in a manner which reduces end-to-end aggregation. Full-length enriched particles were collected after sucrose gradient fractionation [7.5 to 30% (w/v) sucrose in 1 m*M* EDTA, pH 7.0, Beckman SW 27 rotor, 27,000 rpm, 2.75 hr, 4° (Beachy and Zaitlin, 1977)]. Fractions containing full-length virions were pooled and RNA was extracted from them by phenol–SDS treatment (Bruening *et al.*, 1976). The resuspended RNA was further treated with 40 μg/ml proteinase K (E. Merck, Darmstadt) in 50 m*M* Tris-HCl, pH 7.8, containing 0.05% SDS. After incubation for 1.5 hr at room temperature, the RNA was phenol extracted two additional times, alcohol precipitated, and resuspended in water.

Alkaline digestion of TMV virions yields an alkali-resistant core (PSV 6) of ca. 48 nm which has been shown to represent that end of the rod which contains the 3′ end of the RNA (Perham and Wilson, 1976, 1978). This fraction was prepared by dialysis of TMV for 48 hr in 10 m*M* Na_2CO_3, pH 10.25, at 4°. The dialysate was treated with micrococcal nuclease at room temperature for 30 min (Perham and Wilson, 1976). The RNA was released from the resultant small rods by heating for 2 min at 60° in disruption buffer [using 1/8 vol of a solution containing 500 mg/ml sucrose, 25 mg/ml SDS, 20 m*M* EDTA, pH 7, and 0.25 mg/ml bromphenol blue (Bruening *et al.*, 1976)] and loading directly on a 2.4% polyacrylamide gel. Samples were run for

4 hr at 50 V in the presence of 40 m*M* Tris, 20 m*M* Na-acetate, 1 m*M* EDTA, pH 7.2. PSV 6 RNA (which also had some PSV 5 RNA as a contaminant) was visualized by ethidium bromide staining; bands were excised and RNA was eluted by homogenization of the gel pieces in 10 vol of STE buffer containing 1% SDS and 10 vol of phenol saturated in 1 m*M* EDTA. Following the separation of phases by centrifugation, the RNA solution was sequentially freed from contaminating acrylamide by chromatography on hydroxylapatite and CF-11 columns (Dickson *et al.*, 1978).

Full-length TMV RNA and PSV 6 RNA were iodinated by Dr. Elizabeth Dickson as described (Dickson *et al.*, 1979).

Double-stranded RNA molecular weight markers. Reovirus was kindly provided by Dr. W. K. Joklik. Virions were phenol extracted and RNA was precipitated by conventional procedures. *Penicillium chrysogenum* virus dsRNA and CARNA 5 ds RNA were kindly provided by Dr. H. A. Wood and Dr. J. M. Kaper, respectively.

Hybridization of denatured ds RNAs on nitrocellulose paper. The procedure followed was modified from that described by Thomas (1980). Horizontal 1.1% agarose (Sigma Type 1) gels containing 10 m*M* Na-phosphate buffer, pH 6.7, and 0.5 μg/ml ethidium bromide, 14 cm long and 3 mm thick, were run for 2.5 to 3 hr at 90 V and at room temperature. The running buffer was 10 m*M*, pH 6.7, Na-phosphate buffer containing 0.5 μg/ml ethidium bromide. Samples consisted of approximately 10 μg of CF-11-purified undenatured ds RNA from 4 day-infected leaves, plus low molecular weight yeast RNA as a carrier. A solution containing 50% sucrose and 0.05% bromphenol blue was added at the level of 20–50% of the sample volume immediately before application to the gel.

After electrophoresis, the gel was photographed with transmitted ultraviolet light and Polaroid Type 55 film and was then treated with 200 ml of 50 m*M* NaOH for 40 min at room temperature to fragment and denature the RNA (Alwine *et al.*, 1977), followed by four washes of 5 min each of 125 ml 50 m*M* Na-borate, pH 8.0. As described for the blotting technique of Southern (1975), the denatured RNAs were transferred with 20× SSC (1× SSC is 0.15 *M* NaCl, 0.015 *M* trisodium citrate, pH 7.0) overnight at room temperature to BA85 nitrocellulose paper (Schleicher and Schuell, Inc., Keene, N. H.) which had been soaked in 20× SSC. The nitrocellulose paper was then placed between two sheets of Whatman 3MM filter paper and was baked for 2 hr at 80° in a vacuum oven.

Hybridization and prehybridization were carried out in sealed plastic bags at 44°. The nitrocellulose papers were prehybridized for 7 hr in 5 ml of a solution containing 50% formamide (which had been deionized with Biorad AG 501-X8 mixed-bed resin), 5× SSC, 50 m*M* Na-phosphate, pH 8, containing 0.02% bovine serum albumin, 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrrolidone, and 250 μg/ml of phenol-extracted low molecular weight yeast RNA (Schwarz/Mann).

The hybridization mixture itself consisted of 0.4 ml of the above solution (except that the Na-phosphate was pH 6.7) and 0.1 ml of 50% potassium dextran sulfate (Meito Sangyo, Tokyo) plus ^{125}I-labeled TMV RNA or ^{125}I-labeled TMV PSV 6 RNA as indicated in the legend to Fig. 3. When the hybridization reaction was completed, the nitrocellulose paper was washed with SSC/SDS as described by Thomas (1980) and was exposed to Kodak XR-5 X-Ray film at −80° using a Kodak X-Omatic regular intensifying screen and preflashed film (Laskey and Mills, 1977).

RESULTS

Isolation and characterization of ds RNAs. The CF-11 chromatography fractionation yielded a ds RNA preparation which, judging from its nuclease resistance (see below), was virtually free of ss RNA contaminants, provided that the columns were not overloaded in excess of the binding capacity cited in the Materials and Methods and provided that DNA in the sample was removed by enzymatic digestion.

When such a ds RNA preparation from TMV-infected tissue was analyzed by gel electrophoresis, a main component with

a molecular weight consistent with the TMV replicative form (RF) (Jackson *et al.*, 1971) was predominant. In addition, at least three minor discrete bands were revealed, migrating faster than the RF (Fig. 1). The migration of the three additional ds RNA species, named for the sake of convenience, ds-1, ds-2, and ds-3, was compared with the migration of ds RNA species of known molecular weight. Figure 1 also includes a profile or reovirus ds RNAs. [Not all of the 10 known ds RNA species (Shatkin *et al.*, 1968) were resolved in the particular gel system used in these experiments.] Additional molecular weight markers included the ds RNA from *Penicillium chrysogenum* virus (Castanho *et al.*, 1978) and CARNA-5 ds RNA (Diaz-Ruiz and Kaper, 1978. ds-1, ds-2, and ds-3 had apparent molecular weights of 2.25 $\times 10^6$, 1.1 $\times 10^6$, and 0.23 $\times 10^6$, respectively

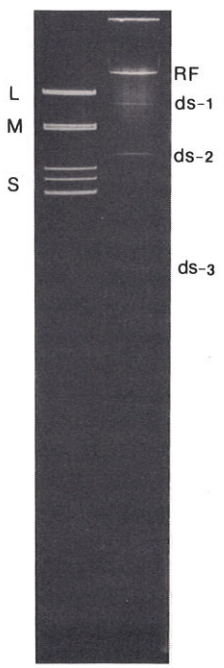

FIG. 1. Electrophoretic profile of the ds RNA fraction extracted from TMV-infected plants run in a 3% polyacrylamide:6 *M* urea gel. Gel was stained with ethidium bromide and photographed with transmitted UV light. Left: reovirus ds RNAs. The large (L), middle (M), and small (S) ds RNAs are only partially resolved in this gel system. Right: TMV ds RNA fraction from apical leaves, 5 days postinoculation.

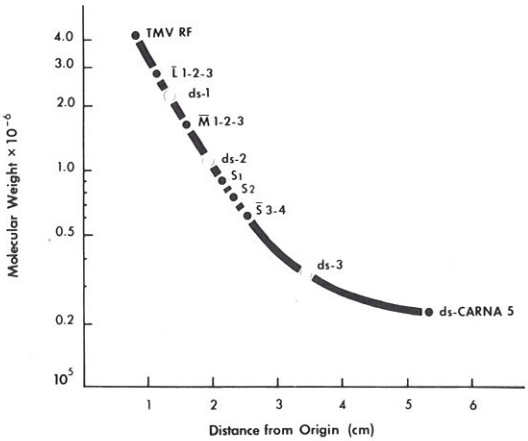

FIG. 2. Apparent molecular weights of TMV ds RNAs as interpolated from the electrophoetic migration of ds RNA markers. Various ds RNAs and the ds RNA fraction from TMV infected tissue were analyzed on a 3% acrylamide:6 *M* urea gel in adjacent lanes. L, M, and S: large, middle, and small species of reovirus ds RNA. Where the several components in the size class were not resolved, the average molecular weight was plotted and designated $\bar{L}$, $\bar{M}$, or $\bar{S}$.

(Fig. 2). The molecular weights of ds-1 and ds-2 were also confirmed with the 7.5% acrylamide:0.2% bis:6 *M* urea system of Schuerch and Joklik (1973) using reovirus ds RNAs as markers. In this system, the 10 reovirus ds RNAs are resolved. Gels were run at room temperature with recirculated buffer for 48 hr at 50 V. (Data not shown).

All four ds RNA species were detected both in directly inoculated and in systemically infected leaves in the apex of the plant. Our preliminary observations suggested that apical leaves yield higher extractable amounts of ds RNA on a tissue weight basis.

No discrete ds RNA bands were detected when mock inoculated leaves were subjected to identical extraction procedures, although trace amounts of RNA were eluted from the CF-11 columns in the ds RNA fraction (water elution). Possibly this RNA is equivalent to the low molecular weight healthy leaf ds RNA seen by Ikegami and Fraenkel-Conrat (1979), but it is too small and too polydisperse to appear on our gels as discrete bands.

TABLE 1

RNase Sensitivity of ds RNA and 2 *M* LiCl-Insoluble RNA[a]

	CF-11 ds RNA	2 *M* LiCl-insoluble RNA
Total acid-precipitable cpm in aliquot	3163	7420
After RNase in 2× SSC[b]	2851	657
% RNase resistance	90.1	8.9
After RNase in 0.1× SSC[b]	21	151
% RNase resistance	0.6	2.0

[a] RNA fractions were prepared from 2 g of 3 day-infected tissue which was incubated for 16 hr in the presence of 4 ml of 10 m*M* KH_2PO_4 containing 250 μg/ml Carbenicillin and 62.5 μCi/ml ^{3}H-uridine. RNA was processed on CF-11 cellulose as described in Materials and Methods, except that the ds RNA preparation was collected after two cycles of CF-11 chromatography, rather than one.

[b] RNase treatment (0.5 μg/ml RNase A plus 0.01 μg/ml RNase T_1 for 30 min at 37°. The reaction was stopped with an equal volume of ice cold 10% trichloroacetic acid (TCA) and the precipitates were filtered onto Schleicher and Schuell No. 30 glass-fiber filters, washed three times in 5% TCA, and counted (Bruening *et al.* 1976).

We also considered whether the smaller ds RNAs in TMV-infected leaves could be artifacts of the isolation process, representing breakdown products of the RF. Such a possibility might be suggested from the studies of Dawson *et al.* (1976), who found that when homogenization was used to isolate RF, or when isolated RF was homogenized, smaller ds RNs appeared. Although our isolation procedures do not include homogenization, we wished to consider this possibility and to extract ds RNAs by the gentlest procedure we could devise. Accordingly, protoplasts were prepared from directly inoculated leaves infected for 3 days. The preparation was divided into two portions: In one the protoplasts were lysed in SDS-containing buffer without homogenization, followed by phenol extraction and recovery of the ds RNAs from CF-11 as described (no vortexing was employed); the other portion was similary treated, but the lysed protoplast solution was homogenized for several minutes at high speed with a Virtis homogenizer before phenol extraction. The results (not shown) revealed that all four ds RNA species were present in both preparations, with a suggestion of a lower yield in the homogenized extracts. These results indicate that ds RNAs 1, 2, and 3 are most probably not generated by the isolation procedures.

Nuclease resistance of RF and ds RNAs 1, 2, and 3. Double-stranded RNA was extracted from ^{3}H-labeled TMV-infected tissues as described in Table 1. The RNase sensitivity of the preparation was tested at high (2× SSC) and low (0.1× SSC) salt concentrations. As seen in Table 1, the ds RNA preparation was very insensitive to RNase at high salt and very susceptible at low salt, confirming its double-stranded nature. Control RNA consisting of 2 *M* LiCl-insoluble RNA extracted from the same tissue was much more susceptible to RNase at high salt, as would be expected for molecules with more single strandedness.

To see if each of the four species of ds RNA was resistant to ribonuclease in high salt, aliquots of the RNA preparations were treated with 0.05 μg/ml RNase A + 0.002 μg/ml RNase T_1, incubated for 30 min at 37°, and analyzed on 3% acrylamide:6 *M* urea gels. To destroy the nuclease before electrophoresis, the samples were incubated for a further 2 h at room temperature with 4 μg/ml proteinase K in 2× SSC and 0.05% SDS. Examination of the gels showed that all of the four principal species of ds RNA were completely unaffected by the nuclease treatment, while the 2 *M* LiCl pellet (mostly ss RNA) used as a control was digested and migrated off of the gel (data not shown).

Hybridization of ds RNAs with TMV RNA and a fragment of TMV RNA representing its 3′ end. Agarose gels were run using a ds RNA preparation. With this gel system, the RF and the three smaller ds RNAs were well resolved (Fig. 3, Lanes 1 and 4). Transfer of these RNAs, after denaturation, was made to nitrocellulose paper as described in Materials and Methods. For hybridization, two types of RNA probes were used: One was iodinated TMV

RNA which was full-length at the time it was iodinated (2 months prior to these experiments), but which had degraded to much smaller pieces at the time it was used. The second probe was ^{125}I-PSV 6 TMV RNA, representing approximately the one-sixth of the RNA from the 3′ end.

Both probes hybridized with the RF and all of the three ds RNAs (Lanes 3 and 5, Fig. 3). The PSV 6 probe and the TMV RNA probe gave bands of equal intensity in the RF region, but the PSV 6 probe was more effective in hybridizing to each of the other ds RNAs, showing that each of them contains the 5′ end of the complementary TMV RNA strand. The fuzziness of some of the bands was not explained; it is possible that there are small amounts of other ds RNAs which are not resolved as bands in the ethidium bromide-stained gels, but which are sufficiently concentrated to hybridize with the probes. A control representing transfer of ss RNAs from TMV infected tobacco showed no hybridization with this procedure (not shown).

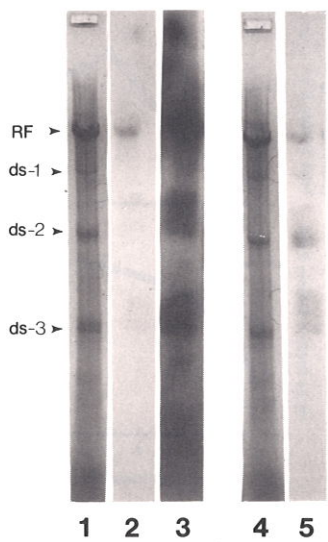

FIG. 3. Hybridization of ^{125}I-TMV RNA to ds RNAs which had been transferred to nitrocellulose paper. Lanes 1 and 4 are photographs of ethidium bromide-stained agarose gels showing the positions of the RF, ds-1, ds-2, and ds-3 extracted from TMV-infected plants. Lanes 2 and 3 represent autoradiographs of two exposures of the nitrocellulose transfer hybridization of the RNAs shown in Lane 1 using 10 ng ^{25}I-TMV RNA (2.4×10^5 dpm/ng). Lane 5 is an autoradiograph of the nitrocellulose transfer hybridization of the RNAs shown in Lane 4, using 40 ng ^{125}I-PSV 6 of TMV RNA (1.6×10^5 dpm/ng).

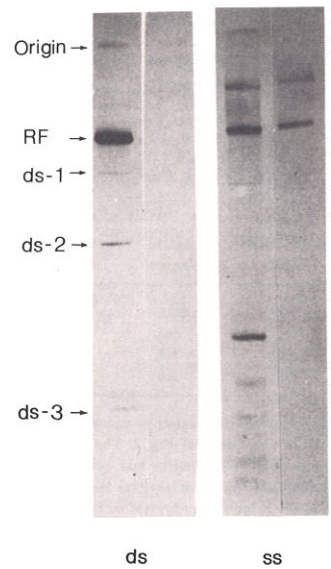

FIG. 4. Electrophoretic profile of 2 *M* LiCl-soluble ds and ss RNAs synthesized in TMV-infected and uninfected tobacco leaves. Directly inoculated leaf tissue (3 days postinoculation) and comparable healthy tissue was allowed to incorporate ^{3}H-uridine for 6 hr (no actinomycin D). The 2 *M* LiCl-soluble ss and ds RNA fractions were analyzed on 3% acrylamide:6 *M* urea gels and processed by fluorography. The left-hand track in each case was from infected, and the right-hand track from uninfected, tissue.

Relative rates of synthesis of TMV ds RNAs. To estimate the rate of synthesis of the different ds RNA species over a 6-hr period, infected leaves were incubated with ^{3}H-labeled uridine, and the radioactivity incorporated into each ds RNA species was determined by scanning the generated fluorograph. Figure 4 shows the presence of labelled RF and the three addition ds RNAs in 2 *M* LiCl-soluble RNA, as well as the efficient fractionation between ss and ds RNA achieved by CF-11 chromatography of 2 *M* LiCl-soluble RNA.

Similar experiments were conducted with both inoculated and apical leaves and the relative incorporation of label into each individual ds RNA species was expressed as a molar equivalent (Table 2).

TABLE 2

RELATIVE SYNTHESIS OF DS RNA SPECIES IN TMV-INFECTED PLANTS[a]

ds RNA	Percentage of radioactivity in given ds RNA		Relative number of molecules[b]	
	Directly inoculated leaves	Apical leaves	Directly inoculated leaves	Apical leaves
RF	93.9	88.4	=1.0	=1.0
ds-1	1.0	0.4	0.02	0.01
ds-2	3.9	5.4	0.15	0.22
ds-3	1.2	5.8	0.16	0.84

[a] ds RNA from directly inoculated or apical leaves was labeled *in vivo* as described in the legend of Fig. 4. The incorporation of radioactivity in individual ds RNA species was determined from scans of fluorograms as described in Materials and Methods.

[b] Calculation of the relative numbers of molecules utilized molecular weights values calculated in Fig. 2.

The three newly described ds RNAs represent a minor proportion of the total radioactivity incorporated into total ds-RNA: 6.1% for inoculated and 11.6% for apical leaves. The comparison of the molar abundance for the different species revealed, however, that the synthesis of ds-3 was substantial in apical leaves, reaching a level almost equal to the RF.

The relative synthesis of the different ds RNA species during different stages of the infection process in the directly inoculated leaves was estimated by labeling leaves at different times after the inoculation. The labeling was done in the presence of actinomycin D, an efficient inhibitor of translation, although it does not affect substantially the ongoing replication of TMV (Semal, 1967). In several experiments, actinomycin D inhibited the incorporation of ^{3}H-uridine into cellular RNA by as much as 85%. Nevertheless, it did not seem to affect the synthesis of RF and the three smaller ds RNAs. Figure 5 shows the increase in incorporation of label into 2 *M* LiCl-soluble RNA during the course of replication in inoculated leaves and the relative proportion of labeled ds RNA for every infection stage.

Subsequently, the ds RNA fraction from each of the different sampling days was analyzed by electrophoresis and visualized by fluorography. Our preliminary results suggested that no substantial qualitative changes occur in the electrophoretic profile of the ds RNAs in the different infection stages studied in spite of the marked stimulation of the total ds RNA fraction synthesis (not shown).

Double-stranded RNA product of the TMV RNA replicase. A membranous fraction from TMV-infected leaves has been shown to be capable of synthesizing a double-stranded, RNase-resistant product which comigrates with RF (Bradley and Zaitlin, 1971). This enzyme is template independent, i.e., for activity it does not require, nor does it respond significantly to, added RNA [0–50% stimulation (Zaitlin *et al.*, 1973)]. To determine if the minor species of ds RNA were also synthesized by this enzyme, replicase product (^{3}H-la-

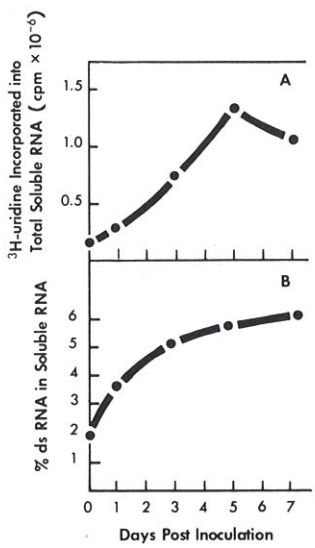

FIG. 5. Incorporation of ^{3}H-uridine into 2 *M* LiCl-soluble RNA and into ds RNA as a function of time after inoculation. For each time point, 2 g of leaves was labeled for 6 hr with ^{3}H-uridine in the presence of 50 μg/ml actinomycin D. Nucleic acid was extracted, treated with DNase, and fractionated with 2 *M* LiCl, and part of the extract was processed on CF-11 cellulose as described in Materials and Methods. Radioactivity in aliquots of the extracts was determined after drying on filter disks and solubilization with 90% NCS reagent (Amersham). (A) Incorporation into total 2 *M* LiCl-soluble RNA; (B) percentage of that incorporation isolated as ds RNA from CF-11 columns.

beled), first treated with CF-11 to enrich for ds molecules, was examined by electrophoresis. A ^{3}H-labeled ds RNA preparation from a TMV-infected plant was coelectrophoresed to identify the positions of all of the ds RNAs. In two experiments only TMV RF was observed as the replicase product, with no suggestion of the other ds RNAs even with prolonged exposure of the fluorogram (data not shown).

DISCUSSION

In this paper we report the finding of three new species of ds RNA in TMV-infected plants, in addition to the known RF. although not specifically emphasized in their reports, small ds TMV RNAs may be seen on the gels shown in some other studies. For example, in Fig. 3D and 4A of Aoki and Takebe (1975), at least two small ds RNAs are evident in gels of labeled ds RNAs from TMV-infected protoplasts, and there are at least two in Fig. 5B of Diaz-Ruiz and Kaper (1978). Moreover, Derrick (1978), who used a serologically specific electron microscope procedure to identify TMV ds RNA, observed molecules of the size of RF and what could be interpreted as several size classes of small ds RNAs. On the other hand, no small bands may be seen on the TMV ds RNA gel presented by Morris and Dodds (1979). Nevertheless, it is apparent that small ds TMV RNAs are not artifacts of our laboratory or of any given preparation procedure—a finding substantiated by our own isolation of them from lysed protoplasts. Furthermore, our hybridization results (Fig. 3) indicated that each individual species contains the 5′ end of the (−) strand, suggesting that they are not randomly cleaved fragments of the RF.

What role small ds RNAs play in either replication or pathogenesis (if any) is mysterious. Double-stranded RNAs are enigmatic in many biological systems (Burke, 1977). Our original rationale was to see if they represented ds counterparts of subgenomic mRNAs. During TMV replication, two major classes of subgenomic mRNAs are formed: One, the I_2 RNA of ~680,000 MW (Beachy and Zaitlin, 1977), is encapsidated into rods and can serve as a mRNA for a 30,000 MW polypeptide; the other, the LMC (Jackson *et al.*, 1972), codes for the viral coat protein, but is not encapsidated in the common strain (Hunter *et al.*, 1976; Siegel *et al.*, 1976). By sequence analysis it is known to consist of nearly 700 nucleotides (Guilley *et al.*, 1979), which would assign it a MW of ~235,000. There are also several other distinct size classes of RNA isolated from TMV virion populations, but their possible mRNA function is not known (Beachy and Zaitlin, 1977).

None of the ds RNAs, 1, 2, and 3, of molecular weights 2.25, 1.1, and 0.23×10^6 seems to be the ds RNA from the I_2 RNA or LMC, which would be expected to have weights of 1.36×10^6 or 0.47×10^6, respectively. With the reasonable uncertainty usually ascribed to MW determinations, and to ds RNA MW determinations in particular (Bozarth and Harley, 1976), ds-2 could possibly be equivalent to a ds I_2 RNA. Moreover, ds-1 could be the ds counterpart of the 1.1×10^6 MW RNA seen by Siegel *et al.* (1976) as a labeled species in actinomycin D-treated tissue. Certainly though, none of the ds RNAs appears to be equivalent to a ds LMC. This is surprising because of the significant synthesis of viral coat protein in TMV biosynthesis, probably requiring concomitant generation of its mRNA, the LMC. Thus, if the subgenomic LMC were replicated independently of the genomic RNA, a ds RNA equivalent to it would probably have been observed. These results suggest that it is not replicated. Based on other sorts of evidence, a similar conclusion was drawn by Dasgupta *et al.* (1980) concerning the subgenomic RNA 4 of brome mosaic virus. Speculation on the mode of synthesis of subgenomic mRNAs is beyond the scope of this paper.

One interesting possibility for a source of LMC derived from a ds RNA could be inferred from the work of Czarniecki and Sreevalsan (1980) in studies with Sindbis virus. The replication of this alphavirus encompasses some of the features seen in TMV, in that infected cells contain one subgenomic mRNA and several classes of ds RNA. The Sindbis virus genome is a 42

S single-stranded RNA of 4.0×10^6 MW. In addition, infected cells also contain a 26 S subgenomic RNA of 1.8×10^6 MW, a subset of the 42 S RNA, which is equivalent to ca. one-third of the molecule containing its 3′ end; it functions as the mRNA for the structural proteins of the virus (e.g., Clegg and Kennedy, 1975). Czarniecki and Sreevalsan found three species of ds RNA in virus-infected cells. One (RF I) corresponded in MW to that expected for the sum of the (+) and (−) strands of the complete genome. Of the other two, RF III of 3.1×10^6 MW yielded 26 S subgenomic RNA upon denaturation. They further demonstrated that there was a second form of RF I, which had an intact (−) strand but a nicked (+) strand. After nuclease treatment, these RF I molecules were cleaved to yield RF III and a 5.6 $\times 10^6$ MW RF II. By this analogy, the LMC could be a constituent of one of our small ds RNAs which might have a nicked (+) strand. To test for such a possibility it would be necessary to denature the various ds RNAs to see if a small LMC-sized RNA with coat protein mRNA activity could be generated.

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