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Chloroplast DNA transcripts are encapsidated by tobacco mosaic virus coat protein

(pseudovirions/subgenomic components)

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ABSTRACT **Preparations of tobacco mosaic virus contain pseudovirions, particles resembling virions but containing host rather than viral RNA. The encapsidated host RNA was found to be composed of discrete-sized species derived from a large portion of the chloroplast genome except that very little, if any, ribosomal RNA is present. Pseudovirions contain the same chloroplast DNA transcripts as those detected in extracts from uninfected leaves, although not always in the same relative amounts. Several strains of tobacco mosaic virus were tested and all were found to contain pseudovirions, with the U2 strain containing more than the others.**

Viruses are assembled from components that are synthesized independently in host cells, and mechanisms usually operate to ensure that only essential components are assembled into virions. Among exceptions to this rule are the occasional instances of host, rather than viral, nucleic acid being packaged in virus capsids. For instance, an efficient process exists to ensure that only viral RNA is incorporated into tobacco mosaic virus (TMV) particles: assembly is initiated by reaction of a capsid protein oligomer with a specific viral RNA encapsidation initiation site (1). Nevertheless, TMV preparations contain a small proportion of pseudovirions, particles that contain host rather than virus RNA (2). The encapsidated host RNA is composed primarily of chloroplast DNA transcript and, thus, questions arise as to the site of pseudovirion assembly within the cell and whether pseudovirions assemble by the same mechanism as that of virions. The present investigation was undertaken to define more clearly the nature of the encapsidated host RNA. We show that it consists of discrete-sized transcripts from a large portion of the chloroplast genome.

MATERIALS AND METHODS

TMV Isolation and Purification of RNA. Strains of TMV (U2, dahlemense, and U1 grown in tobacco and Cc grown in red kidney bean) were multiplied for ≈2 weeks, and rods were purified as follows. Leaf material, macerated in the presence of 0.1 M $Na_2B_4O_7$/0.5 mM EDTA/5 mM 2-mercaptoethanol, pH 7, was filtered through Miracloth (Calbiochem) and centrifuged at 10,000 × *g* for 30 min. NaCl and polyethylene glycol, M_r 8000 (Sigma), were added to the supernatant to 1% and 6%, respectively, and the suspension was stirred for 4–24 hr at 4°C. The virus was pelleted by centrifugation at 10,000 × *g* for 30 min and then resuspended in M/15 phosphate buffer (pH 7) to which CsCl was added to a density of 1.32 g/ml. The solution was centrifuged at 35,000 rpm in the Beckman 50 Al rotor for 24 hr, whereupon the virus formed an opalescent band. This band was collected and the CsCl was removed by dialysis against M/15 phosphate buffer. RNA was extracted from purified rods by the use of phenol/chloroform/$NaDodSO_4$.

Preparation of a Cc LMC Clone. Cc LMC RNA, separated from other Cc RNA components by sedimentation through a sucrose gradient (3), was polyadenylylated (4) and used as a template for synthesis of double-stranded cDNA (5). The cDNA was inserted into the *Sph* I site of pBR322 by G-C tailing. One of the resultant clones, pCcA53, contains a sequence representing ≈550 nucleotides of Cc LMC, including its 3′ terminus, as determined by comparing its restriction map with a published one for this region (6).

Purification of Tobacco Chloroplast DNA. Tobacco chloroplasts were isolated and the DNA was purified as described (7).

Preparation of ^{32}P-Labeled Probes. Random-primed ^{32}P-labeled cDNA (specific activity, 10^9 cpm/μg) was prepared using leaf, root, and TMV RNA templates as described (8). Tobacco chloroplast DNA and plasmid DNA were labeled by nick-translation to a specific activity of 10^8 cpm/μg as described (9).

RNA and DNA Blot Hybridizations. RNA preparations were electrophoresed through methylmercuric hydroxide-containing agarose gels (10) and transferred to diazotized paper (11). Immobilized RNAs were incubated with ^{32}P-labeled probes in the presence of 10% sodium dextran sulfate/50% formamide at 42°C for 16–20 hr with the probe concentration at 1 ng/ml. The filter papers were washed with 2× standard saline citrate (NaCl/Cit; 1× NaCl/Cit is 0.15 M NaCl/0.015 M Na citrate) at 62°C for 30 min and then successively with lower concentrations of NaCl/Cit to a final concentration of 0.1× NaCl/Cit at 62°C. The washed blot was exposed to Fuji XR film with two Quanta III intensifying screens at −70°C. Exposure time ranged from 2 hr to 7 days.

Southern blots were obtained by transferring electrophoretically separated restriction fragments of DNA from a 0.8% agarose gel to nitrocellulose and using the same hybridization and wash conditions as described above for RNA blots.

Plasmid DNA Purification. Chimeric plasmids consisting of petunia chloroplast DNA *Pst* I restriction fragments inserted into pBR322 were kindly provided by J. Palmer (Carnegie Institution of Washington, Stanford, CA). These were replicated in *Escherichia coli* strain HB 101 and isolated as described (12).

RESULTS

Distinguishing Between Encapsidated Virion and Host RNA Species. A number of subgenomic RNA species of different length, 3′ coterminal with virion RNA, are generated during

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Abbreviations: TMV, tobacco mosaic virus; kb, kilobase(s); NaCl/Cit, standard saline citrate.

TMV infection (13, *). Some of these, including the small 700-nucleotide monocistronic messenger for capsid protein, LMC, are messenger RNAs for internal cistrons (14–16). Which species are encapsidated *in vivo* depends on the position of the encapsidation initiation site. In most strains LMC is not encapsidated because the encapsidation initiation site lies 5′ to that portion of genomic RNA represented by LMC. In others, including the cowpea strain Cc, LMC contains the encapsidation initiation site and, therefore, is encapsidated (17). A cDNA clone prepared to the 3′ terminal ≈550 nucleotides of Cc TMV LMC was used as a probe to detect subgenomic TMV RNA species (Fig. 1*A*, lanes A′ and A). The Cc RNA was also probed with cDNA prepared to leaf RNA from uninfected host plants (bean) (lane B). The two probes anneal with different discrete RNA species; the cloned LMC probe hybridizes to 3′-coterminal virion and virion-derived species, whereas the bean leaf RNA probe hybridizes to several encapsidated host species.

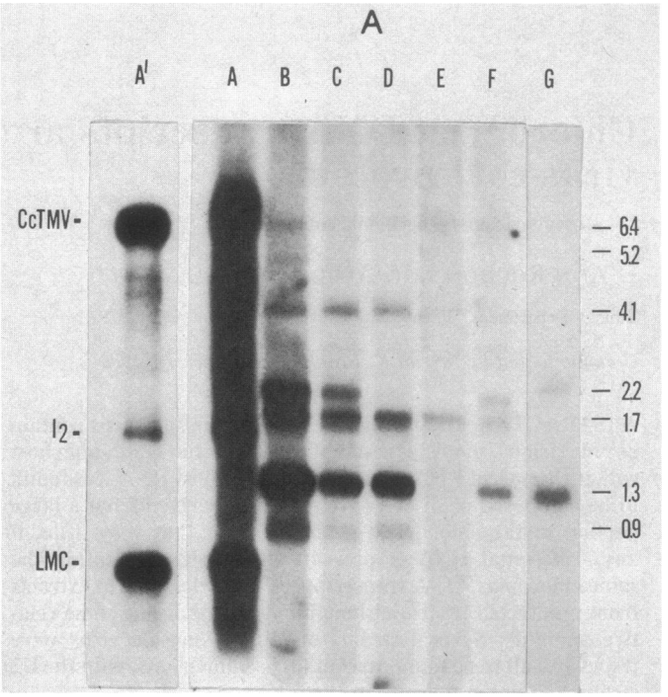
The method adopted for virus purification involves exposure of virions to nuclease-laden plant juice and banding in a CsCl gradient under conditions that would sediment RNA. Thus, it is unlikely that the host RNA species that copurify with the virions are unprotected and not part of virion-like structures. To verify that the host species are not carried into virion preparations as extraneous contaminants, purified virion preparations were incubated with pancreatic ribonuclease (1 μg/ml) for 1 hr at 37°C followed by proteinase K (1 mg/ml) for 30 min at 37°C before virion RNA extraction. This treatment was effective in completely degrading control virion RNA but was ineffective in altering the results shown in Fig. 1. We conclude, therefore, that the host RNA that copurifies with virions is encapsidated in virion-like structures (pseudovirions).

Characteristics of Encapsidated Host RNA. *Discrete species of host RNA are encapsidated.* The cDNAs prepared to healthy leaf RNAs hybridize with distinct species present in virion RNA preparations rather than to randomly sheared RNA. Host species of 6.4, 5.2, 4.4, 4.1, 2.9, 2.2, 1.7, 1.3, and 0.9 kilobases (kb) are detected in RNAs of the Cc and U2 strains (Fig. 1*A*, lanes B–D; Fig. 1*B*, lanes B–D).

Some of the encapsidated species are conserved. As shown in Fig. 1*A*, cDNAs to tobacco and turnip leaf RNAs hybridize to many of the same-sized host species present in Cc strain RNA as does a cDNA to RNA of bean, the host in which Cc TMV was replicated. Not all of the encapsidated RNA species are equally conserved because turnip leaf lacks the material present in bean and tobacco leaves that hybridizes with a prominent 2.2-kb Cc species (Fig. 1*A*, lanes B–D). It is of interest that some of the encapsidated host species show conservation even when the virion RNAs with which they are associated show little homology (18). Thus, when a cDNA prepared to U2 RNA (grown in tobacco) is used to probe immobilized Cc RNA (grown in bean), hybridization is obtained to encapsidated host RNA but not to the virion RNA (Fig. 1*A*, lane G).

The encapsidated RNA contains chloroplast DNA transcripts. The original observation (2) that the encapsidated RNA is mostly chloroplast derived is confirmed here by two types of observation. The first is that cDNA to turnip root RNA hybridizes to TMV host components to an appreciably lesser extent than does cDNA to leaf RNA (Fig. 1*A*, lanes B–E and Fig. 1*B*, lanes B–E). The second is that tobacco chloroplast DNA hybridizes to several of the same components as does cDNA to leaf RNA (Fig. 1 *A* and *B*, lanes F). Additional evidence for the chloroplast origin of encapsidated host RNA is presented in a later section.

*Rochon, D. & Siegel, A., Proceedings of the Fifth International Congress for Virology, Aug. 2–7, 1981, Strasbourg, France, p. 258 (abstr.).



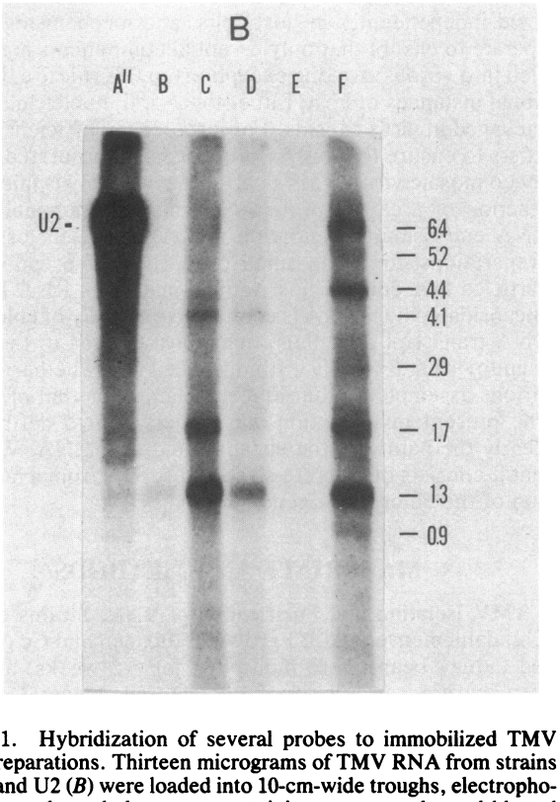


FIG. 1. Hybridization of several probes to immobilized TMV RNA preparations. Thirteen micrograms of TMV RNA from strains Cc (*A*) and U2 (*B*) were loaded into 10-cm-wide troughs, electrophoresed through methylmercury-containing agarose gels, and blotted onto diazotized paper. The paper was cut into 7.5-mm-wide strips, each containing ≈1 μg of RNA, and each strip was incubated with a different ^{32}P-labeled probe, as follows. Lanes: A and A′, cloned Cc LMC (pCcA53) 2×10^6 cpm (2×10^8 cpm/μg); B–D, cDNA to bean, tobacco, and turnip leaf RNA, respectively, each probe 2×10^7 cpm (10^9 cpm/μg); E, cDNA to turnip root RNA, 2×10^7 cpm (10^9 cpm/μg); F, tobacco chloroplast DNA, 2×10^7 cpm (10^8 cpm/μg); G and A″, cDNA to TMV strain U2 RNA, 1.6×10^7 cpm (10^9 cpm/μg). Exposure time is 16 hr except for A′, which is 2 hr. The numbers to the right of each figure indicate the approximate size in kb of the detected host RNA species. Lettering on the left indicates the major genomic and subgenomic virion species.

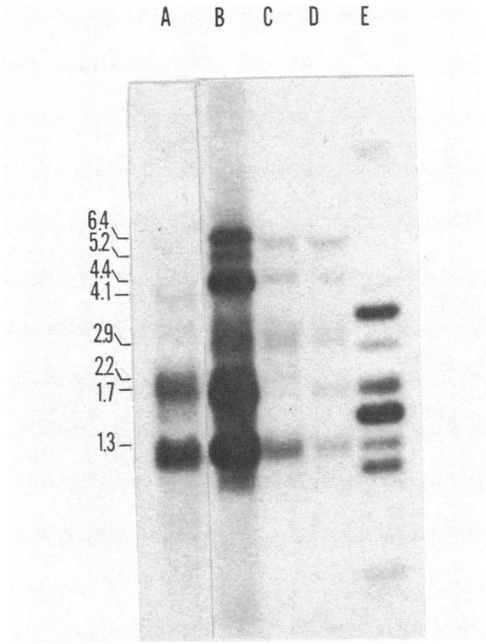


FIG. 2. Hybridization of tobacco chloroplast DNA to the RNAs of several TMV strains. The RNAs were electrophoresed through a methylmercury gel, transferred to diazotized paper, and incubated with ^{32}P-labeled tobacco chloroplast DNA, 2×10^7 cpm (10^8 cpm/μg). Lanes A, C, and D contain 2 μg each of Cc, dahlemense, and U1 TMV RNAs, respectively. Lane B contains 0.5 μg of U2 RNA and lane E contains 0.05 μg of tobacco leaf RNA for use as a size marker. The numbers on the left indicate the size in kb of detected chloroplast transcripts.

Encapsidation of host RNA is general for strains of TMV. Four distantly related strains of TMV—U1, U2, Cc, and dahlemense—all encapsidate host RNA (Fig. 2). Some of the encapsidated species appear to be the same size in all four strains. TMV strains contain different amounts of host RNA, with the U2 strain containing the most. Note that the signal given by this strain is stronger than that given by the others even though there is less of it on the blot than of the others.

Encapsidated host RNA derives from many parts of the chloroplast genome. Fig. 3 illustrates that the encapsidated host RNA is not derived from only a localized region of chloroplast DNA. A cDNA prepared to U2 RNA anneals to almost all of 13 cloned *Pst* I fragments constituting 84% of the petunia chloroplast genome. Petunia DNA was used because it became available and because petunia and tobacco, the U2 host, belong to the same family (Solanaceae) within which chloroplast DNA has diverged very little (5). A stronger signal is obtained with some fragments than with others, not always dependent on fragment size, indicating that more encapsidated RNA is derived from some chloroplast DNA regions than from others. Particularly notable is the paucity of signal given by the 7.6- and 4.6-kb fragments, which lie totally within the inverted repeat sequence (Fig. 4*A*).

The nature of the encapsidated host RNA was explored by incubating the cloned *Pst* I fragments individually with immobilized U2 RNA. Fig. 4 shows that each fragment hybridizes to one or more probe-specific discrete RNA species. It is apparent that the species are not all present in equal amounts. Some of the species are present as several size variants. This can be seen most easily in the signal pattern yielded by the 1.0- and 1.4-kb fragments where in each case several RNA species are present that are greater in size than the fragment. The most intense signal obtained with the 1.0-kb fragment is given by a species with an estimated size of 4.4 kb, but on longer exposure, bands of 5.2, 3.0, and 1.5 kb are also seen. A similar feature is seen with the 1.4-kb probe. However, not all transcripts are present as multiple forms. Only one species (1.7 kb) is made evident by hybridization with the 4.1-kb fragment.

Encapsidated host species are the same as those present in uninfected leaf tissue. To determine whether the host species found in TMV RNA are also present in uninfected leaf RNA extracts, paired samples of U2 and tobacco leaf RNA were incubated with the different cloned *Pst* I petunia chloroplast fragments. The results (Fig. 4) show that for the most part the fragments hybridize to species of the same size in both RNA preparations. For instance, note that the 4.1-kb fragment hybridizes to a single 1.7-kb RNA species present in both U2 and leaf extract. The relative amounts of some species are different in the U2 and leaf preparations. For instance, the short exposure autoradiograph shows that the 9.2-kb fragment gives strong signals with pseudovirion RNA species of ≈4.4 and 1.5 kb and with leaf RNA species of ≈3.2 and 1.3 kb. However, faint bands corresponding to the strong U2 RNA signals are present in the leaf RNA preparation and vice versa. This can be seen more clearly in the long exposure autoradiograph. It occurred to us that the differences observed between the U2 and leaf patterns might result from an electrophoretic artifact, so a number of U2-leaf RNA mixtures were probed with several of the chloroplast DNA fragments. The results (not shown) were what one would expect from a mixture: the strong hybridization bands of both U2 and leaf were present.

Some of the observed transcripts are probably derived from known chloroplast genes. For instance, as indicated on the petunia chloroplast DNA restriction map (Fig. 4*A*), the gene for the large subunit of ribulose bisphosphate carboxylase:oxygenase has been mapped to the border region of the 4.1- and 21-kb *Pst* I fragments (19). Thus, the 1.7-kb RNA species, the only transcript detected by the 4.1-kb fragment

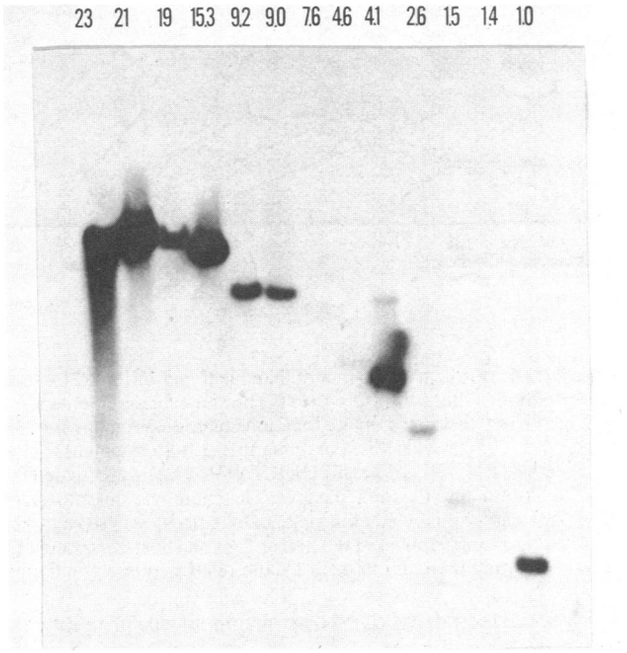


FIG. 3. Hybridization of U2 RNA cDNA to cloned petunia chloroplast DNA fragments. Two hundred nanograms of each chimeric plasmid containing a petunia chloroplast DNA fragment inserted into its *Pst* I site was treated with *Pst* I, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and incubated with a ^{32}P-labeled cDNA to U2 RNA (5×10^8 cpm; 10^9 cpm/μg). The numbers at the top indicate the size in kb of the cloned chloroplast DNA fragment loaded onto each lane (see Fig. 4*A*).

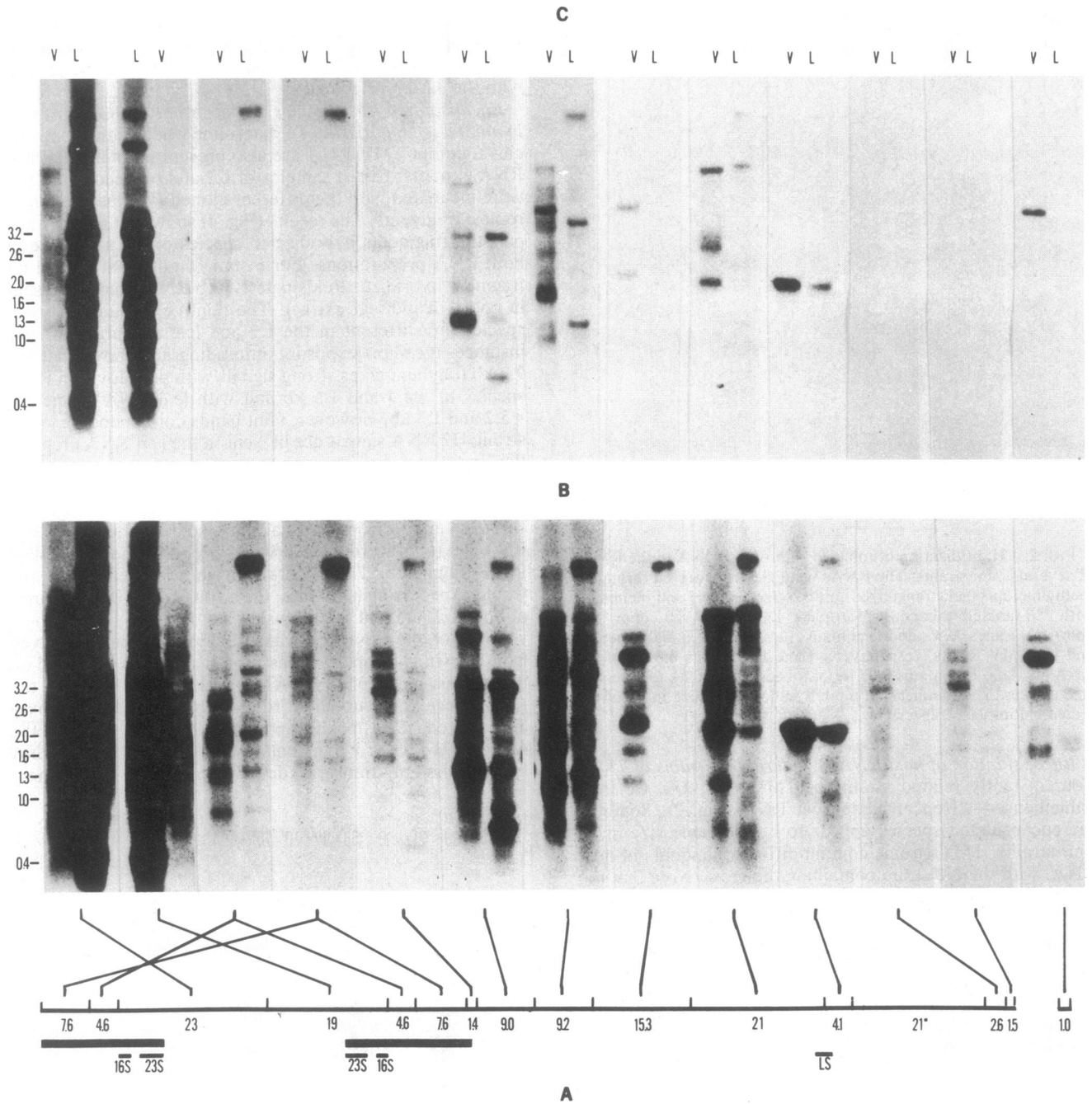


FIG. 4. Hybridization of immobilized leaf and virion RNA to cloned petunia chloroplast DNA fragments. (*A*) A linearized *Pst* I restriction map of petunia chloroplast DNA (19). The size of each fragment is indicated in kb. Thick bars below the map indicate the inverted repeat units. Thin bars show the approximate locations of coding regions for 23S and 16S ribosomal RNAs and for the large subunit of ribulose bisphosphate carboxylase:oxygenase (LS). An unmapped 1.0-kb fragment is at the right. The 21-kb fragment with an asterisk has not been cloned. (*B* and *C*) Tobacco leaf RNA (L, 1 μg) and U2 RNA (V, 1 μg) were loaded in alternate wells, electrophoresed through methylmercury-containing agarose gel, and blotted onto diazotized paper. The paper was cut into strips, each containing a L channel and a V channel, and each strip was incubated with a different ^{32}P-labeled chloroplast DNA fragment (100 ng; $\approx 1 \times 10^8$ cpm/μg) as indicated by the lines between the map and the autoradiographs. *B* is a long exposure (160 hr), and *C* is a shorter exposure (16 hr). The numbers on the left are the size in kb of ribosomal RNA markers. The slowest moving band in each L lane results from a small amount of DNA that copurified with the leaf RNA.

and also detected by the 21-kb fragment, is probably the transcript from this gene. This conclusion is fortified by the observation that the 4.1-kb fragment hybridizes to a 2.2-kb fragment in Cc RNA and to 2.6- and 2.4-kb fragments in bean leaf RNA (the host for the Cc strain) (not shown). This agrees with the report (20) that the large subunit transcript is larger in bean than it is in several other species, including tobacco.

It is interesting to note that the 1.4- and 1.5-kb fragments yield similar patterns. The location of these fragments at the border of the inverted repeat unit and the long unique sequence indicates that the hybridized RNA species are derived from a gene located within the inverted repeat unit but close to its terminus.

The signals yielded by the 23- and 19-kb fragments are much more intense with leaf RNA than with U2 RNA. This phenomenon is illustrated in a short exposure autoradiograph (Fig. 4*C*). As expected, bands in the leaf preparation represent primarily ribosomal RNA. We do not detect species of similar size in U2 RNA, and thus we conclude that

very little, if any, ribosomal RNA is encapsidated. The species hybridizing to the 23- and 19-kb fragments in U2 RNA are probably derived from the short unique region of chloroplast DNA.

DISCUSSION

We report here the general phenomenon that strains of TMV encapsidate discrete transcripts from several regions of the chloroplast genome. This finding may provide an aid to the study of both chloroplast and virus biology.

TMV, in particular the U2 strain, provides the chloroplast biologist with an enriched partially purified source for chloroplast DNA transcripts, some of which may be otherwise difficult to obtain in sufficient quantity for study. An even greater enrichment may be possible by isolating pseudovirions directly from chloroplasts (see below).

Matthews (21), after reviewing the literature, concluded that TMV components are most likely synthesized and assembled in amorphous intracellular cytoplasmic inclusions, called viroplasms, that are not associated with nuclei, mitochondria, or chloroplasts. Thus, a prerequisite for pseudovirion formation is either the presence of chloroplast DNA transcripts in the cytoplasm or virus capsid protein in chloroplasts. The observations of Shalla *et al.* (22) indicate the latter may be the case. They observed that noninfectious rods appear in chloroplasts of TMV infected cells, more so in a U5 [a close U2 relative (23)] than in a U1 infection. These have the same width and serological specificity as TMV but are shorter, with median length one-third that of TMV rods. If these are the same as pseudovirions, then a reasonable hypothesis is that they are assembled in chloroplasts from resident chloroplast transcripts and migrant cytoplasmically synthesized capsid protein. A number of cytoplasmically synthesized proteins are essential chloroplast constituents, and the mechanism by which they enter the chloroplast has been partially elucidated (24). Capsid protein may enter by a similar or different mechanism, but the large amount of pseudovirion in U2 preparations may be a consequence of a greater propensity for U2 than of other capsid proteins to enter chloroplasts.

It seems reasonable to assume that the same principles apply to the encapsidation of host RNA as those that operate in virion assembly; that is, that assembly is initiated by a reaction between a capsid protein oligomer and an encapsidation initiation site, although it is possible that conditions exist within the chloroplast that permit a less specific assembly mechanism. If we assume an encapsidation initiation dependent specific assembly, then those chloroplast transcripts that become encapsidated should have either a common nucleotide sequence or a region of secondary structure that resembles the viral encapsidation initiation site. It is interesting to speculate that such a site may have a normal necessary function that is quite different from that of presenting the opportunity for encapsidation during infection, but what this might be is not immediately obvious. The site may not be present on all chloroplast transcripts; it appears to be absent from the ribosomal RNA transcript, and thus its postulated function may involve messenger RNA maturation or, possibly, ribosomal recognition. The likelihood that viral and host RNAs share such a critical conserved nucleotide sequence may provide insight concerning tobamovirus origin.

There are two infection-associated phenomena—symptomatology and host range—about which little is known. These require an intimate association between virus and host, and we raise the question whether host RNA encapsidation may not have something to do with either one or both of these phenomena. Is it possible that symptoms result in part from the sequestering of particular messenger RNAs into virion-like structures at a critical stage or stages of plant development? In the same vein one may wonder whether host range might not be governed, at least in part, by the ability of capsid protein to react with and encapsidate a specific one or set of host RNAs.

The possibility is presented that pseudovirions might be vehicles for horizontal gene transfer. However, there is no known mechanism by which a plant cell might convert an introduced RNA transcript into DNA and insert it into the genome. Nevertheless, the presence of intronless pseudogenes (25) suggests the existence of such a mechanism.

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