Infectious in vitro transcripts from cloned cDNA of a potyvirus, tobacco vein mottling virus

(cDNA cloning/T3 RNA polymerase/T7 RNA polymerase/in vitro mutagenesis/electroporation)

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ABSTRACT Full-length cDNA copies of tobacco vein mottling virus (TVMV) RNA were constructed downstream from bacteriophage T7 or T3 RNA polymerase promoters. The plasmids were designed to produce in vitro transcripts containing, respectively, one or two guanosine residues at the 5' terminus not derived from the TVMV sequence and a single cytidine residue at the 3' terminus following the poly(A) tail. Introduction of transcripts from either plasmid into tobacco mesophyll protoplasts resulted in the accumulation of TVMV coat protein and RNA. Neither coat protein nor viral RNA accumulated in protoplasts inoculated with linearized cDNA or with in vitro transcripts synthesized in the absence of 7-methylguanosine(5')triphospho(5')guanosine (m7GpppG). Tobacco seedlings inoculated with native TVMV RNA developed symptoms a few days before those inoculated with in vitro transcripts; however, 3 weeks after inoculation, the symptoms produced by the two inocula were indistinguishable.

The potyviruses are considered to have the most serious economic impact on crop plants and to be the largest of all the recognized families and groups of plant viruses (1). Potyvirus particles are flexible rods of length 700–800 nm. Their monopartite genome consists of a single-stranded, (+)-sense RNA molecule of 9.5 kilobases that is 3'-polyadenylated and contains a 5'-terminal genome-linked protein (VPg).

The complete nucleotide sequences of the RNAs of two potyviruses, tobacco vein mottling virus (TVMV) and tobacco etch virus, have been reported (2, 3). In both cases, the sequence contains a single open reading frame capable of encoding a polypeptide of ~340 kDa. Upon proteolytic processing, this polypeptide yields the viral coat protein (CP) and four nonstructural proteins: helper component protein (HC, an aphid transmission factor), cylindrical inclusion protein (CI), and two nuclear inclusion proteins (4). In addition, at least two other gene products for which proteins have yet been identified are predicted by the nucleotide sequences.

The production of infectious RNA transcripts from full-length cloned cDNAs (5–8) has significantly facilitated investigations of the functions of some plant viral proteins. For example, transcripts of cDNA mutated in vitro have been used to study the promoter involved in the synthesis of brome mosaic virus (BMV) subgenomic RNA 4, which encodes the viral CP (9, 10). The role of the tobacco mosaic virus (TMV) CP in movement and symptomatology was studied by altering the cDNA of the CP cistron and observing the effects of the mutant transcripts in plants (11). In vitro transcripts have been used to map a nucleotide sequence in TMV RNA involved in the hypersensitive response in host plants (12), to study the TMV 30-kDa protein gene and its role in cell-to-cell movement (13), and to analyze the properties of the putative replicase gene of TMV (14, 15).

As a first step in the development of a genetic system for the study of potyviruses, we have constructed full-length cDNA copies of TVMV RNA and have used them to produce infectious transcripts in vitro. The ability to produce biologically active viral RNA in vitro provides a major tool for the investigation of gene expression, replication, and pathogenicity of a large and very important group of viral plant pathogens.

MATERIALS AND METHODS

Construction of a Full-Length cDNA Copy of TVMV RNA with a T7 Promoter. TVMV RNA was isolated from purified virus by sucrose gradient centrifugation as described (16). A full-length cDNA copy of TVMV RNA was synthesized by the method of Ahlquist (17) with the following modifications. Two oligonucleotides were designed to specifically prime first- and second-strand cDNA synthesis from TVMV RNA. The 3' oligonucleotide primer (5'-GGCGGAGCTCTTTTTTTTTTTTTTTTTTTT-3') contained an oligo(dT) tract to hybridize to the TVMV poly(A) tail and an Sst I recognition site. The 5' oligonucleotide primer (5'-GGGCCGAGCTAGATACTGACCCCAGTACTATAGAAAAATACAAAATACAACAACC-3') contained an Xho I recognition site, a bacteriophage T7 RNA polymerase promoter, and the first 23 nucleotides (nt) of the TVMV sequence. After first-strand cDNA synthesis and digestion with RNase A, the cDNA was purified by binding to "glass milk" (Bio101, Inc.) according to the manufacturer's recommendations. The cDNA was resuspended in 20 μL of 50 mM Hepes, pH 7.3/20 mM MgCl2/5 mM dithiothreitol and annealed to the 5' primer. Deoxyribonucleotides (200 μM final concentration) and 5 units of phage T4 DNA polymerase (New England Biolabs) were added. The mixture was incubated for 1 hr at 37°C and the reaction was terminated by heating at 65°C for 15 min. The cDNA was then digested with either Xho I and BamHI, BamHI alone, or BamHI and Sst I and ligated into similarly digested pUC20H (Fig. 1A; ref. 19).

Abbreviations: TVMV, tobacco vein mottling virus; BMV, brome mosaic virus; TMV, tobacco mosaic virus; CP, coat protein; CI, cylindrical inclusion protein; HC, helper component protein; VPg, viral genome-linked protein; nt, nucleotide(s).

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The original nucleotide sequence of the 5' terminal region of TVMV RNA was obtained from direct RNA sequencing and indicated the presence of a guanosine at position 5 (2). Subsequently isolated clones of the region have contained an adenosine at this position. More recent RNA sequencing has indicated that the 5' terminus of the viral RNA is heterogeneous, having either three or four terminal adenosine residues (18). Hence, the oligonucleotides used in this report were designed to reflect the change at position 5 and to produce in vitro transcripts with four 5'-terminal adenosine residues.

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and the constructions were used to transform *Escherichia coli* JM109.

Clones containing the three *BamHI* fragments of TVMV cDNA were selected by insertional inactivation of the β-galactosidase gene of pIC20H. Plasmids were isolated (20) and analyzed by restriction enzyme digestion. Those which agreed with the restriction map predicted from the nucleotide sequence of TVMV RNA (2) were analyzed further. Clones from the 5' terminus were assayed for the presence of an active T7 promoter in *in vitro* transcription reactions (see below). One 5' cDNA clone, pXB2, was selected for further constructions. The sequence of the junction between the T7 promoter and the 5' terminus of the viral RNA was confirmed by DNA sequence analysis (21). pTV-Bam7 was selected as representative of the internal *BamHI* fragment. Clones from the 3' terminus were sequenced to determine the length of the poly(A) tail. One plasmid, pBS6, contained a 96-nt poly(A) tail and was selected for the construction of the full-length clone.

The insert of pXB2 was removed by digestion with *Xho I* and *BamHI* and ligated into pUC119 (22) that had been cleaved with *Sal I* and *BamHI* (Fig. 1A). The resulting plasmid was named pUCXB2. The insert of pBS6 was liberated by *Sst I* and *BamHI* digestion and ligated into similarly digested pUCXB2 to produce pXS2-6. The insert of pTV-Bam7 was removed by *BamHI* digestion and ligated into pXS2-6 that had been cleaved with *BamHI* and treated with alkaline phosphatase. Plasmids were screened by restriction enzyme digestion to identify those containing the *BamHI* insert in the proper orientation. The resulting plasmid was named pXBS7 and represented a complete DNA copy of the TVMV genome downstream from a T7 promoter.

**Construction of a Full-Length cDNA Copy of TVMV RNA with a T3 Promoter.** The insert in mTV-H8 (18), which contains a cDNA copy of nt 1–1177 of TVMV RNA in M13mp19, was excised by *Sal I* and *Sst I* digestion and ligated to similarly cleaved pBluescript KS(−) (Stratagene) to produce pBSH8 (Fig. 2A). The predicted sequences between the T3 promoter and the 5' terminus of TVMV cDNA were removed by *in vitro* mutagenesis (24) using the *E. coli* strain BD2399 (*dut* −*/ung* −) and helper virus M13K07 (22) to produce single-stranded DNA for the mutagenesis reaction. The oligonucleotide used in the reaction, 5'-AACCTCTACTAAAGGAAAATAAAAAAATCAACA-3',6 contained nt −12 through +3 of the T3 promoter followed by the first 19 nt of TVMV RNA. The resulting plasmids were sequenced to verify the deletion and tested in *in vitro* for an active T3 promoter. The selected plasmid, pBSH88, was digested with *Bgl II* and *Sst I* and ligated to an 8785-base-pair *Bgl II*–*Sst I* fragment liberated from pXBS7. The resulting plasmid, pBS1220, contained the complete TVMV sequence downstream from a T3 promoter and was used for *in vitro* transcription experiments.

**In Vitro Transcription.** Transcription reaction mixtures contained 100 ng of linearized plasmid DNA per μl; 4–7 units of T7 or T3 RNA polymerase (Pharmacia) per μl; 40 mM Tris-HCl (pH 7.9); 6 mM MgCl2; 10 mM NaCl; 10 mM dithiothreitol; 2 mM spermidine; 250 μM each ATP, CTP, and GTP; 50 μM TTP; 500 μM m7GpppG (New England Biolabs); and 1 unit of RNase Block (Stratagene) per μl.

![Fig. 1.](image1.jpg) **Fig. 1.** (A) Construction of a full-length cDNA copy of TVMV RNA downstream from a T7 promoter. Cistron map of TVMV is shown at top as described by Hellmann *et al.* (23). Restriction fragments of TVMV cDNA were cloned into the vectors shown. Thick arrows indicate T7 promoter sequences. The final plasmid was named pXBS7 and represents a complete DNA copy of the TVMV genome. (B) Predicted site of initiation and direction of transcription from pXBS7 (arrow). The junction of the T7 promoter and TVMV sequence is indicated.

![Fig. 2.](image2.jpg) **Fig. 2.** (A) Construction of a full-length cDNA copy of TVMV RNA downstream from a T3 promoter. Thick arrows indicate T3 and T7 promoter sequences as designated. The final plasmid was named pBS1220 and represents a complete DNA copy of the TVMV genome. (B) Predicted site of initiation and direction of transcription from pBS1220 (arrow). The junction of the T3 promoter and TVMV sequence is indicated.
Transcription mixtures were incubated for 20 min at 37°C, GTP was added to 250 μM, and the incubation was continued for 1 hr. In vitro transcripts were used without further treatment in infectivity assays.

**Inoculation of Protoplasts and Leaves with in Vitro Transcripts.** *Nicotiana tabacum* L. cv. Xanthi mesophyll protoplasts were prepared and inoculated by electroporation as described by Luciano et al. (25), using a Bio-Rad Gene-Pulser with either 5 μg of TVMV RNA or 100 μl of in vitro transcription reaction mixture. After incubation of the protoplasts for 48–60 hr, extracts were assayed for TVMV proteins by immunoblotting using rabbit antiserum prepared against TVMV CP.

Inoculum applied to *N. tabacum* L. cv. Ky14 seedlings was prepared by mixing 100 μl of in vitro transcription reaction mixture with 100 μl of 6 mM sodium phosphate buffer (pH 9.0) containing 1 mg of bentonite and 1 mg of Celite per ml (6). Each plant was mechanically inoculated with 20 μl of this mixture. Control seedlings were mock-inoculated or inoculated with 0.5 μg of TVMV RNA in 20 μl of phosphate buffer/bentonite/Celite per plant. All plants were rinsed with water 5 min after inoculation. Those which developed symptoms were analyzed for accumulation of TVMV-encoded proteins by immunoblotting.

**Immunoblotting.** Leaf tissue was homogenized (1 g/ml) in disruption buffer [10% (vol/vol) glycerol/2.3% (wt/vol) SDS/60 mM Tris-HCl, pH 8.0/5% (vol/vol) 2-mercaptoethanol]. The extracts were boiled for 5 min and centrifuged for 5 min in an Eppendorf microcentrifuge. Protoplast extracts were collected by centrifugation and resuspended at a concentration of 4 × 10^6 per ml in incubation medium. Samples (20 μl) were diluted with an equal volume of disruption buffer, boiled, and centrifuged as above. Proteins from the leaf or protoplast extracts were separated by electrophoresis in 10% polyacrylamide gels containing SDS (26) and transferred to nitrocellulose (27). Following transfer, the nitrocellulose membrane was blocked with 5% nonfat dry milk (28) in Tris-buffered saline (50 mM Tris-HCl, pH 7.5/200 mM NaCl) and incubated with antiserum against TVMV CP, C1 or HC (29) for 16 hr. The membrane was then washed with Tris-buffered saline and incubated for 1–3 hr with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Immune complexes were detected by the method of Leary et al. (30).

**RNA Dot Hybridization.** Samples containing 5 × 10^3 electroporated protoplasts were collected at 2, 24, 48, and 72 hr after inoculation and frozen in liquid nitrogen. The frozen protoplasts were lysed in 4 M guanidinium isothiocyanate (31) and the extracts were applied to nitrocellulose. The blots were baked, prehybridized, hybridized, and washed as described by Thomas (32). A (+)-sense TVMV RNA probe was prepared by transcription of a full-length cDNA derived from pBS1220, with [α-32P]UTP as the labeled nucleotide.

**RESULTS**

**Construction of Full-Length cDNA Copies of the TVMV Genome.** Since the presence of 5' nonviral sequences diminishes the infectivity of in vitro transcripts of BMV cDNA (33), efforts were made to minimize the length of plasmid-derived sequences at the 5' termini of the in vitro transcripts. Both T7 and T3 RNA polymerases initiate transcription primarily at guanosine residues (34). The second and third nucleotides of transcripts initiated by these polymerases are also guanosine residues. As TVMV RNA begins with an adenosine residue, it was necessary to introduce guanosine residues at the 5' terminus of TVMV cDNA for effective transcription by T7 and T3 RNA polymerases. For the construction of pXB7 (Fig. 1A), an oligonucleotide that introduced a single guanosine between the T7 promoter and the 5' terminus of the TVMV sequence was used to prime second-strand cDNA synthesis (Fig. 1B). When this clone was linearized with Sst I and transcribed, the transcripts theoretically contained a single guanosine at the 5' terminus and a single cytidine at the 3' terminus not derived from the TVMV sequence (Fig. 1B). A full-length clone containing a T7 promoter with adenosines at positions +1, +2 and +3 was not transcribed by T7 RNA polymerase (data not shown), indicating that at least one guanosine residue is required for transcription.

In the construction of pBS1220 (Fig. 2A), oligonucleotide-directed mutagenesis was used to remove the plasmid sequences between the T3 promoter and the start of the TVMV sequence. The primer used in the in vitro mutagenesis reaction was a mixture of two oligonucleotide sequences. One sequence contained a guanosine residue at position +3, which conformed to the reported consensus sequence for T3 promoters (34). The other contained an adenosine at position +3, which would produce a variant T3 promoter and leave two guanosines and one extra adenosine at the 5' termini of the transcripts. Clones were isolated with either a guanosine or an adenosine in position +3. Both were actively transcribed by T3 RNA polymerase. Since clones with two guanosine residues produce transcripts more similar to the TVMV RNA sequence, these were used in the construction of pBS1220. pBS1220 represents a full-length cDNA copy of TVMV, of which nt 796–9500 were derived from pXB7 (Fig. 1A). In addition to the two 5' nonviral guanosine residues, transcripts from pBS1220 should have a poly(A) tail of 96 nt, and a 3' nonviral cytidine residue (Fig. 2B).

**Protoplast Infectivity Assays.** Evidence that the transcripts were infectious was initially obtained when TVMV CP was detected in extracts of tobacco mesophyll protoplasts that had been electroporated in the presence of in vitro transcripts of either of the full-length clones (Fig. 3). These protoplasts, however, produced much less CP than those inoculated with native TVMV RNA. In a separate experiment, a dilution series of TVMV RNA indicated that the amount of CP produced in response to 100 μl of transcription reaction mixture was approximately equal to that produced by 0.05 μg of TVMV RNA (data not shown). Since the transcription reactions contained ~25 μg of product RNA, the in vitro transcripts were therefore about 500 times less infectious.
than an equivalent amount of native TVMV RNA. No CP was produced in protoplasts when m'GpppG was omitted from the transcription reaction. Similarly, CP was not detected in protoplasts electroporated with linearized plasmid DNA alone (data not shown), indicating that, as has been shown with other RNA plant viruses (5–8), the full-length cDNA was not infectious.

The accumulation of viral RNA in protoplasts inoculated with in vitro transcripts was assayed by dot blot hybridization (Fig. 4). The positive reactions in extracts of protoplasts collected 2 hr after inoculation with transcripts were due to the presence of residual inoculum. The hybridization signal increased between 24 and 48 hr in transcript-inoculated protoplasts, indicating that replication of transcript RNA had occurred. DNA in protoplasts inoculated with linearized plasmid DNA gave a low but fairly constant signal.

**Tobacco Seedling Infectivity Assays.** In four separate experiments, aliquots of in vitro transcription mixtures were applied by standard mechanical inoculation procedures to the leaves of small tobacco seedlings. Typical vein mottling symptoms appeared on an average of 5.5% of the seedlings 7–10 days after inoculation (Table 1). Other seedlings were inoculated with an amount of native RNA estimated to be 10–20% of the amount of transcript RNA applied to each seedling; an average of 60% of these displayed symptoms after 4–5 days.

Seedlings were also mock-inoculated or were inoculated with pXBS7 DNA (Table 1). These control plants were maintained for the same period of time and in the same growth room as the seedlings that had been inoculated with native or transcript RNA. At no time during these or many other experiments involving infectivity assays with TVMV or TVMV RNA have any of the control seedlings become infected. We are therefore confident that none of the transcript-inoculated plants became infected as a result of adventitious mechanical or vector-mediated inoculation with native virus or viral RNA.

As noted above, symptoms in transcript-infected seedlings developed more slowly than did those infected with native viral RNA. After 3 weeks, however, the two types of plants were indistinguishable and had accumulated CP, HC, and CI to essentially equal levels (Fig. 5).

**Infectivity of Progeny Virus.** To determine whether the initial lag in the development of symptoms in tobacco seedlings was due to the number of infectious RNA molecules in the inoculum or to a difference in nucleotide sequence in the cloned cDNA, extracts of plants inoculated 3 weeks previously with either in vitro transcripts or TVMV RNA were used to inoculate a second set of seedlings. There was no lag in the development of symptoms in plants inoculated with sap from transcript-infected plants, indicating that those RNA molecules capable of producing an infection were not phenotypically different from viral RNA. Aphids efficiently transmitted virus from either TVMV RNA- or in vitro transcript-infected plants (data not shown).

**DISCUSSION**

We have constructed full-length TVMV cDNA clones that, when transcribed with either bacteriophage T7 or T3 RNA polymerase, produced genomic-length RNA transcripts that were infectious in tobacco seedlings and mesophyll protoplasts. Transcripts derived from an initial set of cDNA clones with very short (12-nt) poly(A) tails were not infectious (data not shown). The substitution of 3'-terminal fragments containing longer poly(A) tails was sufficient to produce infectious transcripts. The 3'-terminal fragments used to construct these clones contained 1300 nt of coding sequence, the 3'

**Table 1. Infectivity of in vitro transcripts of cloned TVMV cDNA**

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Infectivity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0/60</td>
</tr>
<tr>
<td>Linearized pXBS7</td>
<td>0/30</td>
</tr>
<tr>
<td>TVMV RNA</td>
<td>24/40</td>
</tr>
<tr>
<td>In vitro transcripts</td>
<td>11/200</td>
</tr>
</tbody>
</table>

* Tobacco seedlings were mock-inoculated or were inoculated with linearized pXBS7 plasmid DNA (1 μg per plant), TVMV RNA (0.5 μg per plant), or in vitro transcription reaction mixtures (20 μl per plant).
† Total number of plants in four separate experiments that developed TVMV symptoms/total number of plants inoculated. The results with in vitro transcripts in the individual experiments were 3/60, 2/50, 1/40, and 3/50.

**Fig. 4.** Detection of TVMV RNA in tobacco mesophyll protoplasts inoculated with in vitro transcription reaction mixtures. Protoplasts were electroporated with pXBS7 transcripts, pBS1220 transcripts, pXBS7 DNA, or inoculation medium alone and were sampled 2, 24, 48, and 72 hr thereafter. Total nucleic acid extracts from the protoplasts were dotted onto nitrocellulose and hybridized to a 32P-labeled (–)sense TVMV RNA probe. All samples were analyzed on the same nitrocellulose filter.

**Fig. 5.** Detection of TVMV proteins in tobacco seedlings inoculated with in vitro transcripts. Proteins in extracts of leaves inoculated with pXBS7 transcripts (T7), pBS1220 transcripts (T3), or native TVMV RNA (R) or mock-inoculated (M) were separated in 10% polyacrylamide gels, transferred to nitrocellulose, and probed with anti-TMV CP, HC, and CI antibodies.
noncoding sequence, and either a 37- or a 96-nt poly(A) tail. It is therefore possible that the lack of infectivity of the original transcripts was due to a lethal mutation in one of these regions in the original clones. However, the presence of a poly(A) tail of 140 nt was found to be optimal for the production of infectious transcripts of the comovirus cowpea mosaic virus (35). Thus, there may be a threshold length of poly(A) below which TVMV RNA transcripts are unstable after introduction into plant cells.

Picornaviruses, like potyviruses, have a VPg at the 5′ terminus of the encapsidated RNA. The in vitro transcription of poliovirus cDNA yields infectious RNA that possesses a 5′ m7GpppG cap in place of the VPg. This indicates that the VPg is not absolutely required for infectivity of picornaviral RNA (36). In the protoplast studies reported here, the addition of 5′ m7GpppG was required to produce infectious in vitro transcripts from TVMV-derived cDNA. Since the genomic organization of the picorna- and potyviruses is very similar (37), this result was not unexpected. Similarly, it has been noted that the addition of 5′ m7GpppG to in vitro transcripts of BMV (5) and TMV (6, 7) cDNAs greatly enhances their infectivity. It is not clear whether the cap functions to enhance infectivity by facilitating the translation of the transcripts or by protecting the transcripts from degradation.

The ability to produce infectious in vitro transcripts will enable us to begin to analyze the TVMV genome genetically. Potyviruses produce a number of nonstructural proteins, the functions of which are not completely understood. It will now be possible to introduce defined alterations into the cistrons encoding the various viral proteins and to assay the effects of these alterations on virus replication, symptom development, cell-to-cell movement, and aphid transmissibility.

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