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# RNA-dependent RNA polymerase from plants infected with turnip crinkle virus can transcribe (+)- and (−)-strands of virus-associated RNAs

(virus replication/*in vitro* transcription/satellite RNAs/viral polymerase)

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**ABSTRACT** **RNA-dependent RNA polymerase (RdRp) was solubilized from membranes of turnip infected with turnip crinkle virus (TCV), a single-stranded, monopartite RNA virus. The RdRp activity could be separated into three peaks by Sephacryl S500HR chromatography. RdRp from peak I, which contained substantial amounts of endogenous TCV genomic RNA, and peak II were template-specific, synthesizing full-length complementary strands of exogenous TCV subviral RNAs but not control RNA templates. Peak III RdRp was nonspecific, synthesizing full-sized products for all added RNA templates. Peak II RdRp transcribed several different TCV satellite (sat) and defective interfering RNA templates in both (+)- and (−)-sense orientations but did not transcribe (+)-strands of satellite RNAs associated with unrelated viruses. Monomeric-length sat-RNA C was synthesized from a template containing as many as 220 nonsatellite bases at the 3′ ends of either (+)- or (−)-strands, indicating that the RdRp was able to recognize 3′-end sequences in an internal location. Deletion of 95–242 bases from the 3′ end of (+)-strand sat-RNA C abolished the synthesis of template-length product. However, transcription of template-length products was not affected by the deletion of at least 257 bases from the 3′ end of (−)-strand sat-RNA C template (leaving only the 100 5′-terminal residues), implying that different mechanisms exist for synthesis of (+)- and (−)-strand satellite RNA *in vitro*.**

(+)-Strand RNA viruses rely on RNA-dependent RNA polymerases (RdRp), composed of virus- and host-encoded subunits, for a two-stage replication process: (−)-strand RNA is synthesized from the uncoated (+)-strands followed by synthesis of progeny (+)-strands from (−)-strand intermediates. Despite differences in host range, in genomic RNA organization and expression, in sequences of structural proteins, and in virion morphology, striking similarities exist in amino acid sequences of known or putative viral RdRp that have led to the delineation of three large supergroups of viruses (1, 2): supergroup I, which comprises picorna-, noda-, como-, nepo-, poty-, bymo-, sobemo-, and some luteoviruses; supergroup II, which comprises carmo-, tombus-, diantho-, and the remaining luteoviruses; supergroup III, which includes tobamo-, tobra-, hordei-, and tricornaviruses. The replication of viruses belonging to supergroups I and III has been extensively studied (3); with the exception of Qβ bacteriophage, little is known about the replication of members of supergroup II.

Several groups have succeeded in preparing extracts from infected tissues or cells that can synthesize viral RNA *in vitro*. *In vitro* replication systems, which can reproduce the complete cycle of virus replication (4–7), and transcription systems, which can complete only the first stage of viral replication in a template-dependent (8–12) or template-independent (13–16) manner, have been described. *In vitro* transcription or replication systems for plant or animal RNA viruses have not been reported to synthesize full-length (+)-strand copies from (−)-strand templates in a template-dependent manner. This has led to suggestions of fundamental differences between (+)- and (−)-strand synthesis and/or suggestions that the presence of (+)-strands interacting with (−)-strands may be required for synthesis of (+)-strands (17–20). With the exception of Qβ, all of the RNA viruses whose genomes have been transcribed *in vitro* are members of supergroups I and III.

Turnip crinkle virus (TCV) is a small icosahedral virus with a monopartite genome that infects a broad range of dicotyledonous plants. On the basis of sequence comparison studies, TCV has been classified as a member of the carmoviruses in supergroup II (1). The genomic RNA, which is capped at the 5′ end and contains a free hydroxyl group at the 3′ end, consists of 4054 bases specifying five open reading frames (21). The p88 polypeptide, produced by translational readthrough of an amber termination codon, contains the polymerase domain and is required for replication of the viral genome. To study the replication properties of RdRp from TCV-infected plants, we have solubilized an RdRp activity from plants infected with TCV genomic RNA transcripts. This RdRp activity is dependent on TCV-associated RNA templates and is able to synthesize complementary full-length molecules from both (+)- and (−)-strand RNAs.

## MATERIALS AND METHODS

**Plant Inoculations.** Two-week-old turnip cv. Just Right seedlings were inoculated with 30 μl of infection buffer (22) per leaf containing 4 μg of total plant RNA extracted from leaves infected with full-length TCV genomic RNA transcripts (C. D. Carpenter, C.S., and A.E.S., unpublished data). Systemically infected leaves were collected after 7 days and stored at −80°C.

**RdRp Preparation.** All steps were conducted at 4°C. Frozen leaves (10 g) were ground with 10 g of white quartz sand in 30 ml of buffer A [50 mM Tris·HCl, pH 8.2/15 mM $MgCl_2$/10 mM KCl/2 mM EDTA/20% (vol/vol) glycerol/90 mM 2-mercaptoethanol/1 μM each pepstatin A and leupeptin]. After centrifugation at 300 × *g* for 10 min, the supernatant was subjected to further centrifugation at 35,000 × *g* for 30 min. The pellet (particulate fraction) was resuspended in 8 ml of buffer B (50 mM Tris·HCl, pH 8.2/10 mM $MgCl_2$/20 mM 2-mercaptoethanol/6% glycerol/1 μM each pepstatin A and leupeptin) containing 1.2 M NaCl and stirred for 20 min.

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Abbreviations: TCV, turnip crinkle virus; DI RNAs, defective interfering RNAs; sat-RNAs, satellite RNAs; RdRp, RNA-dependent RNA polymerase(s); MDV, midivariant.

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After centrifugation at 43,000 × *g* for 20 min, the pellet was resuspended in 1.5 ml of buffer B containing 6% (vol/vol) Nonidet P-40, 0.6% (wt/vol) *n*-dodecyl β-D-maltoside, and 1.5 M LiCl. The mixture was stirred for 45 min and then centrifuged at 43,000 × *g* for 20 min. The supernatant was further cleared by centrifugation at 100,000 × *g* for 1 hr and then was loaded onto a Sephacryl S500HR (Pharmacia) column (1.5 × 50 cm) preequilibrated with buffer B and 0.5% Nonidet P-40. To assay for RdRp activity, 7.5 μl from each fraction was mixed with 7.5 μl containing 50 mM Tris·HCl (pH 8.2); 10 mM $MgCl_2$; 10 mM dithiothreitol; 160 μg of actinomycin D per ml; 2 mM each ATP, GTP, and CTP; 1 μCi of [α-$^3$H]UTP (43 Ci/mmol; 1 Ci = 37 GBq; ICN), 0.5 unit of RNasin (Promega); and 2 μg of total RNA from TCV-infected turnip plants. After incubation at 20°C for 30 min, the incorporation of radioactive nucleotides was determined according to Rackwitz *et al.* (23). Active fractions were aliquoted and stored at −80°C.

**Preparation of RNA Templates.** PCR was used to place full-length cDNAs of satellite (sat)-RNA D, sat-RNA C, and defective interfering (DI) RNA G (22, 24–26; C. D. Carpenter and A.E.S., unpublished data) immediately downstream of a T7 RNA polymerase promoter in both orientations [sequence corresponding to the 5′ end of the (+)- or (−)-strand was adjacent to the promoter]. PCR products were cloned into the *Sma* I site of pUC19. The resultant plasmids, when digested with *Sma* I and transcribed using T7 RNA polymerase, generated RNA containing the exact wild-type sequences. The transcripts were extracted with phenol/chloroform (1:1; vol/vol) and precipitated twice. The plasmid DNA did not interfere with the RdRp reaction (data not shown) and was therefore not separated from the RNA transcripts.

To generate transcripts of sat-RNA C containing additional bases at the 3′ end, full-length sat-RNA C cDNA was cloned in both orientations into the *Sma* I site of pT719E(+) (27) that had been altered by deletion of the *Kpn* I and *Sst* I sites (C. D. Carpenter and A.E.S., unpublished data). Linearization of the plasmid by *Bam*HI, *Hin*dIII, or *Pvu* II followed by *in vitro* transcription using T7 RNA polymerase resulted in transcripts containing 7, 34, or 220 bases of vector sequence, respectively, at the 3′ ends of the molecules. To generate transcripts of sat-RNA C containing 3′-end deletions, plasmid with the cDNA in the (+) orientation [that is, T7 transcription produces (+)-strands of sat-RNA C] was digested with *Spe* I, *Ava* II, or *Dra* I. (+)-Strand transcripts produced *in vitro* using T7 RNA polymerase contained 3′-terminal deletions of 95, 146, or 242 bases, respectively. (−)-Strand transcripts with 3′-terminal deletions of 8, 101, 114, or 257 bases were produced by digesting the plasmid containing sat-RNA C cDNA in the (−) orientation with *Dde* I, *Nco* I, *Dra* I, or *Spe* I, respectively. The concentration of RNA products generated in the T7 *in vitro* transcription reactions was estimated by measuring the $A_{260}$ value of the total reaction and subtracting the value contributed by the known amount of DNA template.

pT7MDV plasmid DNA (28) was a gift of V. D. Axelrod (American Cyanamid). Midivariant (MDV) RNA was produced by T7 *in vitro* transcription of *Sma* I-digested pT7MDV DNA. Satellite RNAs CARNA 5 (29) of cucumber mosaic virus (strain S) and PARNA 5 of peanut stunt virus (30) were isolated from infected plants and were the gift of G. Wu and J. M. Kaper (U.S. Department of Agriculture, Agricultural Research Service).

***In Vitro* Transcription Using RdRp-Active Fractions and Product Analysis.** Optimized RdRp reactions were carried out in a 25-μl volume containing 50 mM Tris·HCl (pH 8.2), 10 mM $MgCl_2$; 10 mM dithiothreitol; 100 mM potassium glutamate; 80 μg of actinomycin D per ml; 350 μg of yeast tRNA per ml; 40 μg of RNA template per ml; 1 mM each ATP, GTP, and CTP; 10 μM UTP; 10 μCi of [α-$^{32}$P]UTP (800 Ci/mmol; NEN); and 12.5 μl of active RdRp fraction. Reaction mixtures were incubated at 20°C for 1.5 hr, terminated by extraction with phenol/chloroform/EDTA/SDS emulsion (31), and precipitated with isopropanol. Radiolabeled products, either with or without subsequent S1 nuclease treatment (31), were subjected to electrophoresis through 42-cm 5%

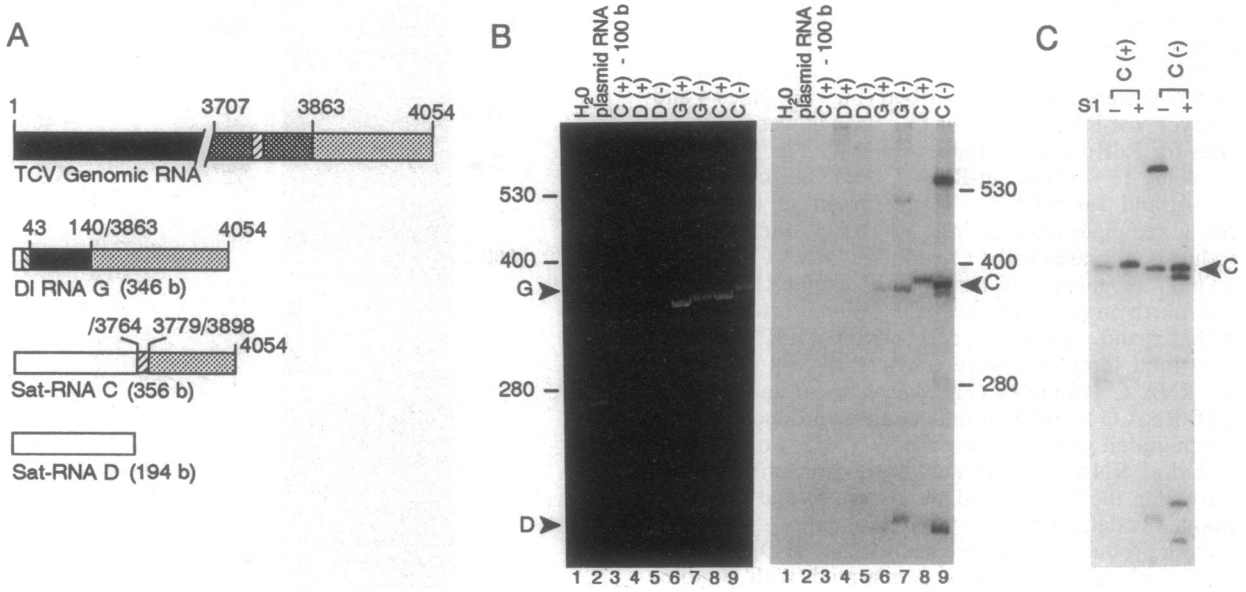


FIG. 1. *In vitro* transcription of (+)- and (−)-strand TCV subviral RNA templates using peak II RdRp. (*A*) Sequence similarities among the subviral RNAs and the TCV genomic RNA. Similar sequences are shaded alike. Numbered positions correspond to location in the TCV genomic RNA. (*B*) $^{32}$P-labeled products were produced by *in vitro* transcription and analyzed by denaturing PAGE. (*Left*) Ethidium bromide-stained gel indicating migration positions of the templates. (*Right*) Autoradiograph of the dried gel. Positions of (+)-strand sat-RNA C, sat-RNA D, and DI RNA G and RNA markers (in bases) are indicated. (*C*) Products from (+)- and (−)-strand sat-RNA C templates were subjected to digestion with S1 nuclease before electrophoresis through a denaturing polyacrylamide gel (see text). Position of (+)-strand sat-RNA C is shown. Templates are indicated above each lane. $H_2O$, no exogenous template; D, sat-RNA D; G, DI RNA G; C, sat-RNA C. Template orientation (+) or (−) is shown. Control plasmid RNA template was generated from a noncoding region of pT719E(+), adjacent to the T7 RNA polymerase promoter. C(+)-100 b, sat-RNA C (+)-strand with a 100-base 3′-terminal deletion.

polyacrylamide/8 M urea gels or nondenaturing 5% polyacrylamide gels. After electrophoresis, all gels were stained with ethidium bromide, photographed under UV illumination, dried, and exposed to x-ray film for autoradiography.

## RESULTS

**Preparation and Properties of RdRp from TCV-Infected Turnip Plants.** The particulate fraction from TCV-infected turnip leaves, but not mock-infected leaves, was able to synthesize apparently full-length TCV genomic and subgenomic RNAs in a template-independent manner (data not shown). Treatment of the particulate fraction from infected plants with either 0.1 M EDTA, 1.5 M NaCl, or 1.5 M LiCl did not release the RdRp activity, indicating that the RdRp complex was tightly membrane bound. RdRp activity was solubilized by treatment of the particulate fraction with 6% Nonidet P-40, 0.6% *n*-dodecyl β-D-maltoside, and 1.5 M LiCl. After chromatography with Sephacryl S500HR, aliquots from each fraction were used to determine radiolabeled nucleotide incorporation into products using either total RNA from infected plants or TCV subviral RNA [sat-RNA C (+)-strand] as template. Regardless of template, three peaks of RdRp activity could usually be detected (data not shown). Peak I was associated with endogenous genomic and subgenomic RNAs. Peak II contained low levels of endogenous TCV genomic RNA. Peak III did not contain, or did not detectably extend, endogenous templates. The RdRp activities in peaks I and II were able to synthesize full-length products by using exogenous sat-RNA C (+)-strand as template and did not synthesize full-length products for yeast 5S rRNA or sat-RNA C (+)-strands deleted by 100 bases at the 3′ end. RdRp activity in peak III did not exhibit template specificity and produced full-length products regardless of the template origin.

**The RdRp from Infected Turnip Plants Transcribes Both (+)- and (−)-Strands of TCV-Associated Small RNAs but Not sat-RNAs of Unrelated Viruses.** The sequence relationships among TCV subviral RNAs have been determined (Fig. 1*A*; refs. 22 and 24). The ability of the peak II RdRp activity to transcribe (+)- and (−)-strands of sat-RNA D, sat-RNA C, and DI RNA G was examined (Fig. 1*B*). (+)- and (−)-strands of sat-RNA D were poorly transcribed (Fig. 1*B*, lanes 4 and 5). However, sat-RNA C (+)- or (−)-strands were efficient templates for RdRp activity (lanes 8 and 9). The prominent product using (+)-strand sat-RNA C template comigrated with (−)-strand sat-RNA C. Transcription of (−)-strand sat-RNA C template resulted in synthesis of four products: one product comigrated with (+)-strand sat-RNA C; a second product migrated slightly faster than (+)-strand RNA C; the third and fourth products migrated at positions corresponding to ≈545 bases and ≈180 bases, respectively. DI RNA G (+)- and (−)-strand templates were transcribed less efficiently than sat-RNA C templates (Fig. 1*B*, lanes 6 and 7). (−)-Strand DI RNA G template produced three products, one of which comigrated with (+)-strand DI RNA G. For all three TCV subviral RNAs, (−)-strands were transcribed more efficiently than their (+)-strand counterparts, and no cyclic replication was detected.

One major product synthesized using sat-RNA D, sat-RNA C, and DI RNA G templates comigrated with double-stranded monomeric forms on nondenaturing polyacrylamide gels (data not shown). To further demonstrate the double-stranded nature of the full-length RdRp reaction products, RNAs synthesized using (+)- and (−)-strand sat-RNA C templates were digested with S1 nuclease prior to electrophoresis. Staining of the resultant gel indicated that the S1 nuclease treatment completely digested the single-stranded template RNA (data not shown). The product synthesized using (+)-strand template was not a substrate for S1 nuclease, while ≈50% of the full-length product generated from (−)-strand template migrated slightly faster than the full-length position after S1 nuclease treatment (Fig. 1*C*). This result suggests that terminal sequences from at least one end of the latter full-length product can be found in single-stranded form. The 545-base product produced from (−)-strand template was also digested by S1 nuclease into two species that migrated near the position of sat-RNA D (194 bases).

To further determine the specificity of the RdRp activity for TCV-associated templates, three additional RNAs were subjected to transcription: CARNA 5 and PARNA 5 are sat-RNAs associated with cucumber mosaic virus and peanut stunt virus, respectively (29, 30). MDV is an RNA that is replicated by RdRp from Qβ bacteriophage (28). Unlike (+)- and (−)-strand sat-RNA C transcripts, which were efficiently transcribed by the RdRp activity (Fig. 2, lanes 1 and 2), CARNA 5, PARNA 5, and MDV RNA were poor templates, resulting in little or no template-length products (lanes 3–5).

**Transcription of sat-RNA C Templates with Plasmid-Derived Sequences on the 3′ Ends.** To determine whether the RdRp activity could recognize sat-RNA C 3′-end sequences if located internally in the template, (+)- and (−)-strand transcripts containing 0, 7, 34, or 220 bases of plasmid-derived sequence on the 3′ ends were prepared. *In vitro* transcription using these templates resulted in products that comigrated with the complementary strands of wild-type length sat-RNA C (Fig. 3, lanes 3–5 and 10–12). This result indicates that the RdRp was able to initiate transcription at (or near) the wild-type 3′ end even when located distal to the 3′ end of the template. Substantially more sat-RNA monomer-length product was produced from (+)-strand templates when compared with synthesis from (−)-strand templates (compare Fig. 3 lanes 3–5 with lanes 10–12). (+)-Strand

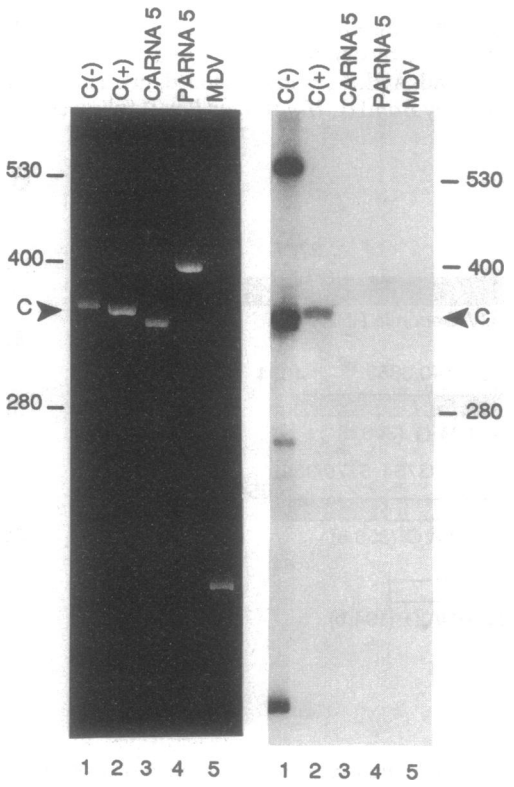


FIG. 2. *In vitro* transcription of different virus- or phage-associated small RNAs using peak II RdRp. Products were analyzed by denaturing PAGE. Gel was stained with ethidium bromide to reveal the template positions (*Left*), dried, and exposed to x-ray film to show the product positions (*Right*). Templates are indicated above each lane. Positions of RNA markers are shown (in bases). C, sat-RNA C.

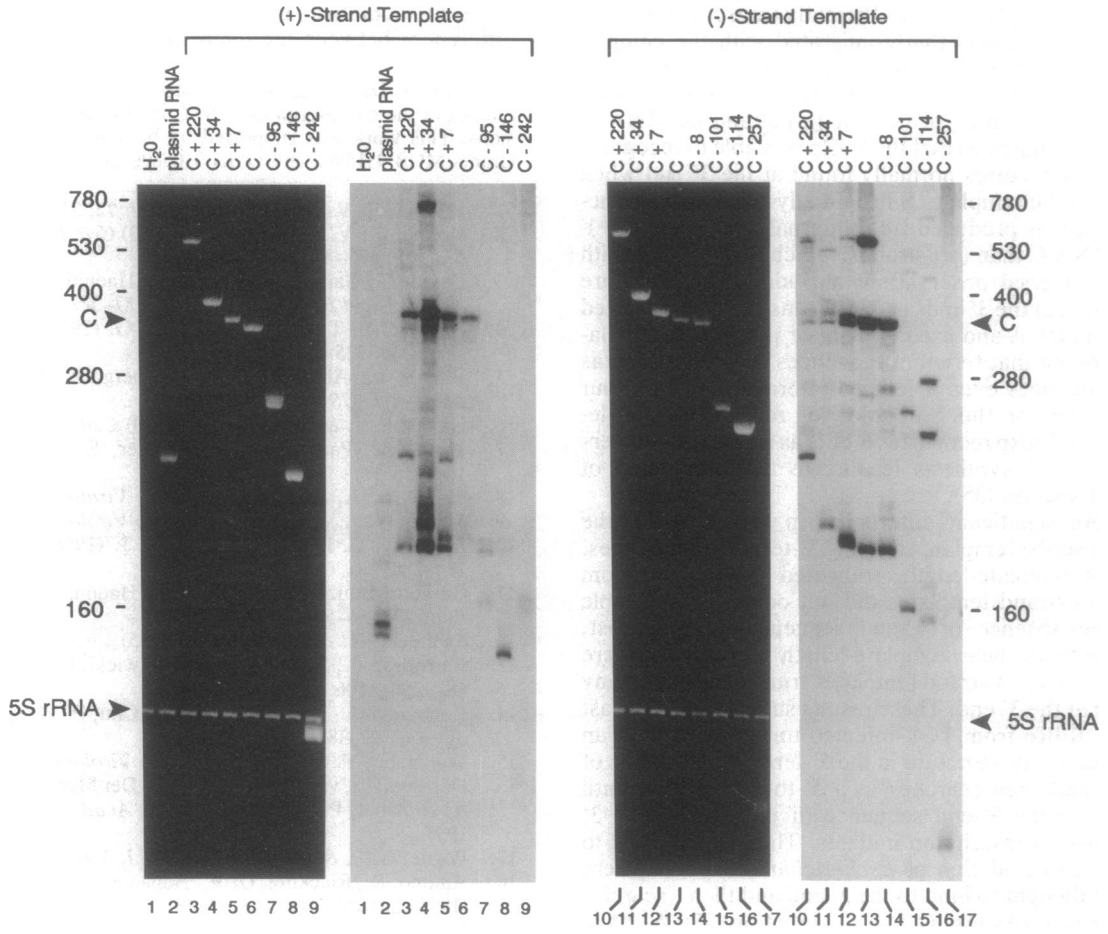


FIG. 3. *In vitro* transcription of (+)- and (−)-strand sat-RNA C templates with 3′-terminal additions and deletions using peak II RdRp. Templates and products were separated on denaturing polyacrylamide gels. Ethidium bromide-stained gels, indicating the migration positions of the templates, are shown on the left of the autoradiograms. Transcripts contained exact 5′ and 3′ ends (C), additional plasmid-derived sequence at the 3′ end [C + (number of added bases)], or deletions from the 3′ end [C − (number of deleted bases)]. Positions of RNA markers (in bases), (+)-strand sat-RNA C (C), and the yeast 5S rRNA are shown. Plasmid RNA control is described in the legend to Fig. 1.

templates with nonviral sequences at the 3′ ends also resulted in synthesis of numerous additional products that were greater than and less than full length, indicating less preference of the RdRp for initiating and/or terminating at the proper sequences.

**Transcription of sat-RNA C Templates with 3′-Terminal Deletions.** To determine the importance of 3′-terminal sequences for transcription of sat-RNA C *in vitro*, templates with 3′-terminal deletions of between 0 and 257 bases were tested for synthesis of complementary strands by the peak II RdRp-active fractions (Fig. 3). Synthesis of full-length, truncated (−)-strands from truncated (+)-strand templates did not occur to detectable levels in the absence of 3′-terminal sequences (lanes 7–9). In contrast, full-sized (+)-strands were synthesized from (−)-strand templates truncated by as many as 257 bases (lanes 14–17). This result suggests that the RdRp from TCV-infected turnip leaves recognizes internal sequences in the 5′ 100 bases of (−)-strand transcripts and then initiates transcription at (or near) the 3′ end. (−)-Strand template with a deletion of 114 nucleotides from the 3′ end produced a second major product that migrated to a position ≈40 bases larger than the template. The identity of this larger-than-full-length product has not yet been determined.

## DISCUSSION

We have established an *in vitro* transcription system for TCV subviral RNAs by using a template-dependent RdRp activity (peak II) prepared from infected turnip leaves. Several properties of this RdRp activity argue against its being a host-encoded RdRp induced by virus infection. (*i*) Peak II RdRp activity is tightly membrane bound, while host RdRps are generally soluble (2, 32). (*ii*) Peak II RdRp activity was eluted from the Sephacryl S500HR column before the major chlorophyll-containing peak, implying that the enzyme is a large, multisubunit complex. This is in contrast with all known host-encoded RdRps, which are composed of a single 120- to 130-kDa polypeptide (2, 33); proteins of this size would elute much later under our chromatographic conditions. (*iii*) Peak II RdRp transcribed TCV-associated small RNAs or RNA fragments from the 3′ end of the viral genome into full-length products (up to 494 bases) with a high degree of specificity, while host RdRp appear to have little or no specificity and produce RNA transcripts of only ≈100 bases (32, 34).

**Specificity of the Peak II RdRp Activity for TCV Genomic and Subviral RNAs.** The RdRp activity in peak II efficiently transcribed full-length products using (+)- or (−)-strands of most TCV subviral RNAs. In contrast, little or no full-length products were obtained using (+)-strand sat-RNA templates from unrelated plant and bacterial viruses. This result, combined with the initiation of transcription at or near the natural 3′-end sequence even when located internally in the template (see below), indicates that the RdRp activity in TCV-infected leaves is able to specifically recognize TCV promoter sequences or structures.

**Different Mechanisms May Exist for the Transcription of (+)- and (−)-Strands.** Both (+)- and (−)-strand sat-RNA C

and DI RNA G templates were efficiently transcribed *in vitro* into full-length products that comigrated with the complementary strand. Normal monomeric-sized products were also synthesized despite the addition of plasmid-derived sequence to the 3′ ends of (+)- or (−)-strand sat-RNA C templates. This indicates that the RdRp was able to recognize sequences or structures normally found at the 3′ end when located within the template. Substantially more monomeric-sized product was produced from internal initiation on (+)-strand sat-RNA C than (−)-strands, which is consistent with the nearly universal observation that additional bases are better tolerated at the 3′ ends of RNA transcripts synthesized *in vitro* from cDNA and used in plant or protoplast inoculation. The finding that terminal sequences are recognized as initiation sequences even if located internally supports our previous model for the generation of recombinant molecules—i.e., the RdRp recognizes motifs as internal promoters for reinitiation of synthesis leading to the production of recombinant species (35).

There were significant differences in the ability of the RdRp to transcribe templates missing 3′-terminal sequences. Synthesis of template-length, truncated (−)-strands from truncated (+)-strand templates did not occur to detectable levels in the absence of 3′-end sequences. In contrast, template-length or near template-length (+)-strands were synthesized from (−)-strand templates truncated by as many as 257 bases at the 3′ end. These results suggest that, at least *in vitro*, the RdRp from TCV-infected turnip recognizes an internal sequence or structure in the 5′-terminal 100 bases of (−)-strands and then searches in a 5′-to-3′ direction until reaching either the 3′-end sequence (if present) or the 3′ terminus where transcription initiates. This is analogous to the model for degradation of *Escherichia coli* RNA where RNase III is thought to bind to the 5′ end and then "search" for specific cleavage sites in a 5′-to-3′ direction (36). We have determined that truncation of 46 bases from the 5′ end of (−)-strand sat-RNA C abolished the synthesis of template-sized product (unpublished observations), suggesting that one border of the promoter element is within the 5′-terminal 46 bases.

**Synthesis of Non-Template-Length Products.** In addition to synthesis of full-length products, transcription of many (+)- and (−)-strand subviral RNA templates resulted in the production of discrete species that migrated slower or faster than full length. Several groups have also reported longer-than-full-length products in *in vitro* transcription/replication systems using viral RdRp. For flock house virus, such products could be suppressed by altering the reaction conditions (5). Poliovirus RdRp produces double-sized products using related and unrelated viral templates, which were determined to be generated by foldback priming and not multimer synthesis (37). With the exception of sat-RNA C (+)-strands with seven extra 3′-end bases, none of the larger-than-full-length products that we detected was twice the template size, and synthesis of these species was not affected by altering the reaction conditions (data not shown). Preliminary experiments suggest that the 545-base species produced using (−)-strand sat-RNA C template (Fig. 1*B*, lane 9; Fig. 3, lane 13) is synthesized by terminal elongation of the 3′ end of the template from the region that is crucial for RNA–RNA recombination between sat-RNAs C and D (ref. 38; unpublished data). The identities of the fast-migrating products are not yet known but may reflect either internal initiation and/or premature termination. Although these products shifted in mobility according to the size of the template (see Fig. 3), the shifts in mobilities did not absolutely correspond to size differences between the templates.

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