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5′-Coterminal Subgenomic RNAs in *Citrus Tristeza Virus*-Infected Cells

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Three unusual 5′ coterminal positive-stranded subgenomic (sg) RNAs, two of about 0.8 kb and one of 10 kb (designated LMT1, LMT2, and LaMT, respectively), from *Citrus* spp. plants and *Nicotiana benthamiana* protoplasts infected with *Citrus tristeza virus* (CTV) were characterized. The 5′ termini of the LMT RNAs were mapped by runoff reverse transcription and found to correspond with the 5′ terminus of the genomic RNA. The LMT 5′-coterminal sgRNAs consisted of two modal lengths of 744–746 and 842–854 nts. The 3′ of the LaMT RNAs terminated near the junction of ORF 1b and ORF 2 (p33). None of the 5′ sgRNAs had detectable amounts of corresponding negative-sense RNAs, as occurs with the genomic and 3′ coterminal subgenomic RNAs of CTV. The abundance of the short and long 5′ sgRNAs differed considerably in infected cells. The LMT RNAs were considerably more abundant than the genomic RNAs, while the larger LaMT RNA accumulated to much lower levels. The kinetics of accumulation of LMT1 and LMT2 in synchronously infected protoplasts differed. The larger RNA, LMT1, accumulated earlier with a strong hybridization signal at 2 days postinfection, a time when only traces of genomic and 3′ sgRNAs were detected. The lack of corresponding RNAs, that could be 3′ cleavage products corresponding to the 5′ coterminal sgRNAs and the lack of complementary negative strands, suggest that these sgRNAs were produced by termination during the synthesis of the genomic positive strands.

INTRODUCTION

Citrus tristeza virus (CTV), the largest (19.3 kb) known positive-stranded RNA virus of plants, belongs to the genus *Closterovirus*, family *Closteroviridae* (Bar-Joseph *et al.,* 1979; Dolja *et al.,* 1994; Agranovsky, 1996; German-Retana *et al.,* 1998). CTV occurs in most of the citrus trees throughout the world, causing one of the most economically significant diseases of this important fruit crop (Bar-Joseph *et al.,* 1989). The CTV genome is organized into 12 open reading frames (ORFs), which potentially encode at least 19 protein products (Pappu *et al.,* 1994; Karasev *et al.,* 1995; Mawassi *et al.,* 1996; Karasev, 2000). The 5′ proximal ORF 1a encodes a 349-kDa polyprotein that includes two cysteine papain proteinase-like (P-Pro) domains and methyltransferase- (MT) and helicase-like (HEL) domains. ORF 1b, which is thought to allow the continued translation of the polyprotein by a + 1 frameshift, encodes an RNA-dependent RNA polymerase (RdRp)-like domain. The remaining 3′ half of the genome contains a set of 10 ORFs expressed via 3′ coterminal subgenomic RNAs (Hilf *et al.,* 1995). Infected plants contain relatively large amounts of double-stranded (ds) replicative form (RF) RNA molecules corresponding to the genomic RNA, and nine or ten 3′ coterminal subgenomic RNAs which do not share a common 5′ terminus (Karasev *et al.,* 1997). Additionally, many CTV isolates contain one or more defective RNAs (dRNAs) of various sizes, composed of the 5′ and 3′ termini of the genomic RNA, with extensive internal deletions (Mawassi *et al.,* 1995b,c; Yang *et al.,* 1997a,b; Ayllón *et al.,* 1999).

Closteroviruses have properties that appear to place them intermediately between the animal Nidovirales, which include the *Arteriviridae* and *Coronaviridae* (Cavanagh, 1997; De Vries *et al.,* 1997), and viruses of the alphavirus supergroup, which includes several plant virus groups such as Tobamoviruses and Bromoviruses (Rozanov *et al.,* 1992). Similar to Nidovirales, Closteroviruses have large genomes, replicase-associated polyproteins with large interdomain regions, and translational frameshifts required to produce the RdRp proteins, and a large number of subgenomic mRNAs with large amounts of corresponding dsRNAs (Lai, 1990; Dolja *et al.,* 1994; Hilf *et al.,* 1995; Karasev *et al.,* 1995; Klaassen *et al.,* 1995). However, other properties are characteristic of the alphavirus supergroup. The amino acid sequences of replicase-associated domains of Closteroviruses are more similar to those of viruses in the alphavirus group (Koonin and Dolja, 1993; Dolja *et al.,* 1994). Closterovirus-subgenomic RNAs, similar to those of viruses in the alphavirus supergroup, do not contain a common leader sequence (Ou *et al.,* 1982; Levis *et al.,*

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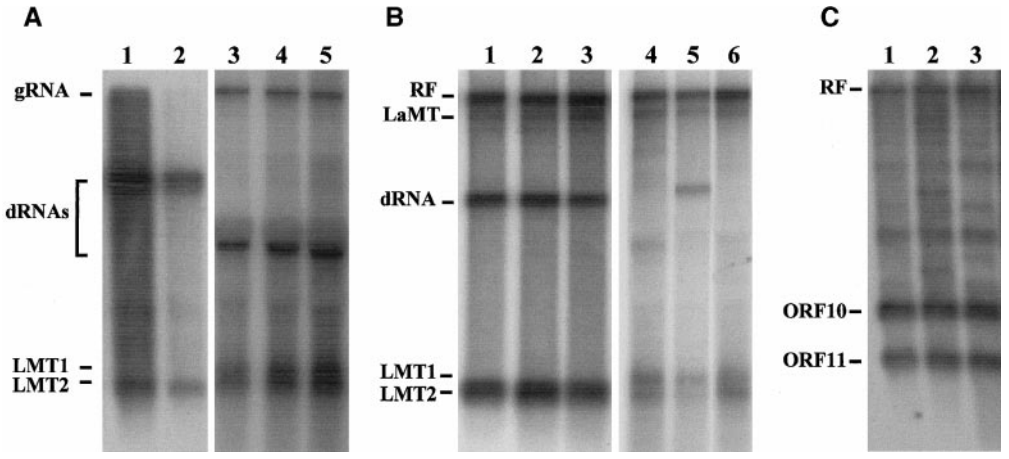

FIG. 1. Northern blot analysis showing 5′ and 3′ coterminal sgRNAs of CTV. Northern blot hybridizations of total ssRNAs (A) and dsRNA-enriched (B and C) extracts from different individual plants (numbered above the lanes) using a 5′ positive-stranded RNA-specific probe (A and B) or a 3′ negative-stranded RNA-specific probe (C). gRNA, genomic RNA; dRNA, defective RNA; LMT, low molecular weight tristeza; RF, replicative form; LaMT, large molecular weight tristeza; ORF, open reading frame.

1990; Karasev *et al.,* 1997), while the subgenomic RNAs of viruses in the *Coronaviridae* share an identical 5′ terminal leader sequence of about 60–80 nucleotides (nts) (Spaan *et al.,* 1988; Lai, 1990).

Previously, we demonstrated that an abundant CTV RNA species, designated LMT (low molecular weight tristeza), of approximately 800 nts, is a positive-stranded RNA that reacted with a hybridization probe to the 5′ region of the genomic RNA (Mawassi *et al.,* 1995c). Although the LMT molecules appeared to be single-stranded, a large proportion of these molecules was found in fractions enriched in dsRNAs (Mawassi *et al.,* 1995c; Yang *et al.,* 1999). The present paper reports that CTV LMT RNAs, which make up a major proportion of the total virus-associated RNAs, consisted of a population of RNAs mainly of two modal lengths of 744–746 and 842–854 nts. We found no evidence that these RNAs were replicated independently (lack of dsRNAs) or were cleavage products from full-length genomic RNAs (no corresponding 3′ cleavage products), which suggests that these highly abundant RNAs were produced by termination during production of genomic RNAs. Additionally, a second class of heterogeneous 5′ coterminal subgenomic RNAs of ~10.0 kb, designated LaMT (large molecular weight tristeza), were present in infected plants in much smaller amounts; some of these molecules were found to extend beyond the termination codon of ORF 1b. The sizes and abundance of these 5′ coterminal sgRNAs of Closteroviruses, LMT and LaMT, appear to be unique for positive-sense RNA viruses.

RESULTS

Small 5′ coterminal sgRNAs are found in infected plants and synchronously infected protoplasts

Northern blot hybridizations of total RNAs from CTV-infected plants or RNAs fractionated by CF11 chromatography for enrichment of dsRNAs (Dodds and Bar-Joseph, 1983), using a 5′ plus-stranded-specific probe, consistently revealed a population of small subgenomic RNAs of ~800 nts, in addition to other RNA molecules including the large genomic RNA and one or more dRNA molecules (Figs. 1A and 1B). The LMT RNA molecules from infected plants usually migrated as broad and diffuse bands after analysis by Northern blot hybridization (Fig. 1A, lanes 1–2; Fig. 1B, lanes 1–3). However, in some hybridizations the LMT appeared as two bands designated as LMT1 and LMT2 (Fig. 1A, lanes 3–5; Fig. 1B, lanes 4–6). The amounts of LMT RNAs usually appeared to be greater than that of the genomic RNA. The accumulation level of LMT was approximately equal to those of the two most abundant 3′ coterminal subgenomic mRNAs corresponding to ORFs 10 and 11 (Fig. 1C). The LMT RNA molecules persisted in chronically infected plants and even survived in plants that underwent a prolonged heat treatment (6 weeks at about 35°C) that considerably reduced the virus titers (data not shown).

To compare the time course of LMT synthesis with those of other CTV RNAs, protoplasts of *Nicotiana benthamiana* were inoculated with freshly extracted sap from plants infected with a range of CTV isolates (T36, T68, T3). Production of the genomic RNA, dRNAs, and 5′ coterminal subgenomic RNAs were monitored by Northern blot hybridizations. The 5′ coterminal subgenomic RNAs from infected protoplasts separated into two bands, referred to as LMT1 and LMT2 (Fig. 2). The larger RNA, LMT1, accumulated earlier with a strong hybridization signal at 2 days postinfection (dpi), at a time when only traces of the genomic RNA molecules and dRNAs were detected. LMT1 also accumulated earlier than the 3′ coterminal subgenomic mRNAs, which accumulated in parallel to the genomic RNA (data not shown). At 3 dpi or later, the accumulation of the smaller

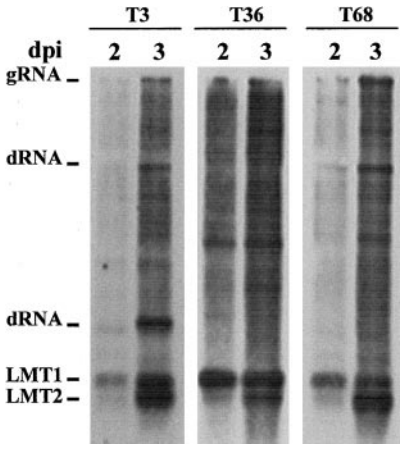

FIG. 2. LMT RNAs in CTV-infected protoplasts. Northern blot analysis of CTV RNAs from *Nicotiana benthamiana* protoplast inoculated with sap extracts of citrus plants infected with different CTV isolates, using a T36 5′ positive-stranded RNA-specific probe. Source isolates are indicated at the top. The numbers at the top indicate the time postinoculation in days (dpi). gRNA, dRNA, and LMT are as in Fig. 1.

RNA, LMT2, was as high as, or higher than, that of LMT1 (Fig. 2).

Mapping of the 3′ and 5′ termini of the LMT RNAs

To examine the 3′ termini of the LMT molecules, LMT RNA from plants infected with CTV-VT was separated on polyacrylamide gel after electrophoresis, and poly(A) tails were added to the 3′ termini with poly(A) polymerase. The 3′ portion of the RNA was amplified by RT-PCR with oligo(dT) and a specific primer complementary to nts 506 to 541 of the CTV-VT genome, followed by cDNA cloning and sequencing. Figure 3 shows the distribution of the 3′ termini of 23 different cDNA clones. The RNAs terminated between positions 724 and 886 of the CTV-VT genome. Two modal lengths of LMT2 and LMT1 molecules were found to have termini at positions 744–746 and 842–854, respectively. The 3′ terminus was found to be located at position 745 in five LMT2 cDNA clones that were obtained from three separate cloning events. Most of the 3′ termini of the LMT1 molecules were positioned at the basal parts of two closely spaced stems predicted in the RNA by the MFOLD program (Zuker, 1989) (Fig. 3B).

To examine whether LMT RNAs are coterminal with 5′ of CTV genomic RNA, the 5′ termini of LMT RNAs were mapped by runoff reverse transcription. A population of agarose-separated LMTs was used for primer extension with [γ-^{32}P]ATP end-labeled primer complementary to nts 54–75 of CTV-VT genome. The 5′ end of LMT RNAs corresponded to nt 1 was found to be an A as reported previously for the 5′ terminal nucleotide of genomic RNA of CTV (Fig. 4) (Karasev *et al.,* 1995; Mawassi *et al.,* 1996).

Origin of the LMT RNAs

One possible mechanism for production of these LMT RNAs is their replication through dsRNA intermediates after production of a minimal amount of the first template. The 3′ coterminal subgenomic RNAs have large amounts of corresponding dsRNAs, suggesting that, after the initial production of the mRNA or its complement, multiple copies might be amplified by replication. However, no complementary negative strands or dsRNAs corresponding to the LMT RNAs were detected from CTV-infected plants (Mawassi, 1995c), suggesting that this possibility is unlikely.

If LMT RNAs were produced by cleavage of full-length genomic RNAs, a population of corresponding 3′ RNAs of ~18.4 kb (or smaller RNAs adding up to that size if there were more processing) should be produced that would hybridize with probes targeted to sequences located downstream of the LMT. These molecules should be present in a 1:1 ratio with LMT, unless there were specific degradation of the other cleavage products. Because it is difficult to resolve the putative 18.4 kb cleavage product from the 19.3 kb genomic RNA, we chose to examine the production of the putative cleavage product from the smaller (~11 kb) efficiently replicating CTV deletion mutant CTV-ΔCla (Satyanarayana *et al.,* 1999). CTV-ΔCla contains the 5′ nontranslated region (NTR) plus the entire sequence of the replicase genes (ORFs 1a and 1b) and the first 105 nts of ORF 2 (p33) joined to the 768 nts of 3′ genomic RNA, which includes most of ORF 11 (p23) and the 3′ NTR. If cleavage of the CTV-ΔCla RNA into LMT and other RNAs occurs, the largest possible cleavage product of this mutant would be ~10 kb, which should be resolved from the ~11 kb RNA of CTV-ΔCla. Figure 5 shows Northern hybridization analysis of RNAs extracted from *N. benthamiana* protoplasts infected with CTV-ΔCla using 5′ or 3′ RNA-specific probes. Although both probes reacted approximately equally with the CTV-ΔCla RNA, we did not detect any cleavage product which could account for cleavage resulting in the LMT RNAs. The only sgRNA found hybridizing with the 3′ CTV-specific probes is the one accounted for the 3′ sgRNA (0.87 kb), which began 5′ of p33 ORF. Thus, these data suggest that the LMT RNAs are not produced as a result of a cleavage process of genomic RNA.

The Northern hybridization blots presented in Fig. 5A, obtained with the 5′ RNA-specific probes, demonstrated that LMT RNAs produced from CTV-ΔCla RNA are positive single-stranded RNA molecules. Because CTV-ΔCla RNA has the ten 3′ genes of CTV genome deleted, the production of LMT RNA by this mutant indicated that none of the products of these deleted genes was involved in LMT RNA synthesis.

Detection of a large 5′ coterminal subgenomic RNA

Hybridization of dsRNA-enriched extracts from plants infected with the VT isolate with riboprobe complementary to the 5′ terminus of the CTV genomic RNA revealed an additional RNA band with a size of about 10 kb (Fig.

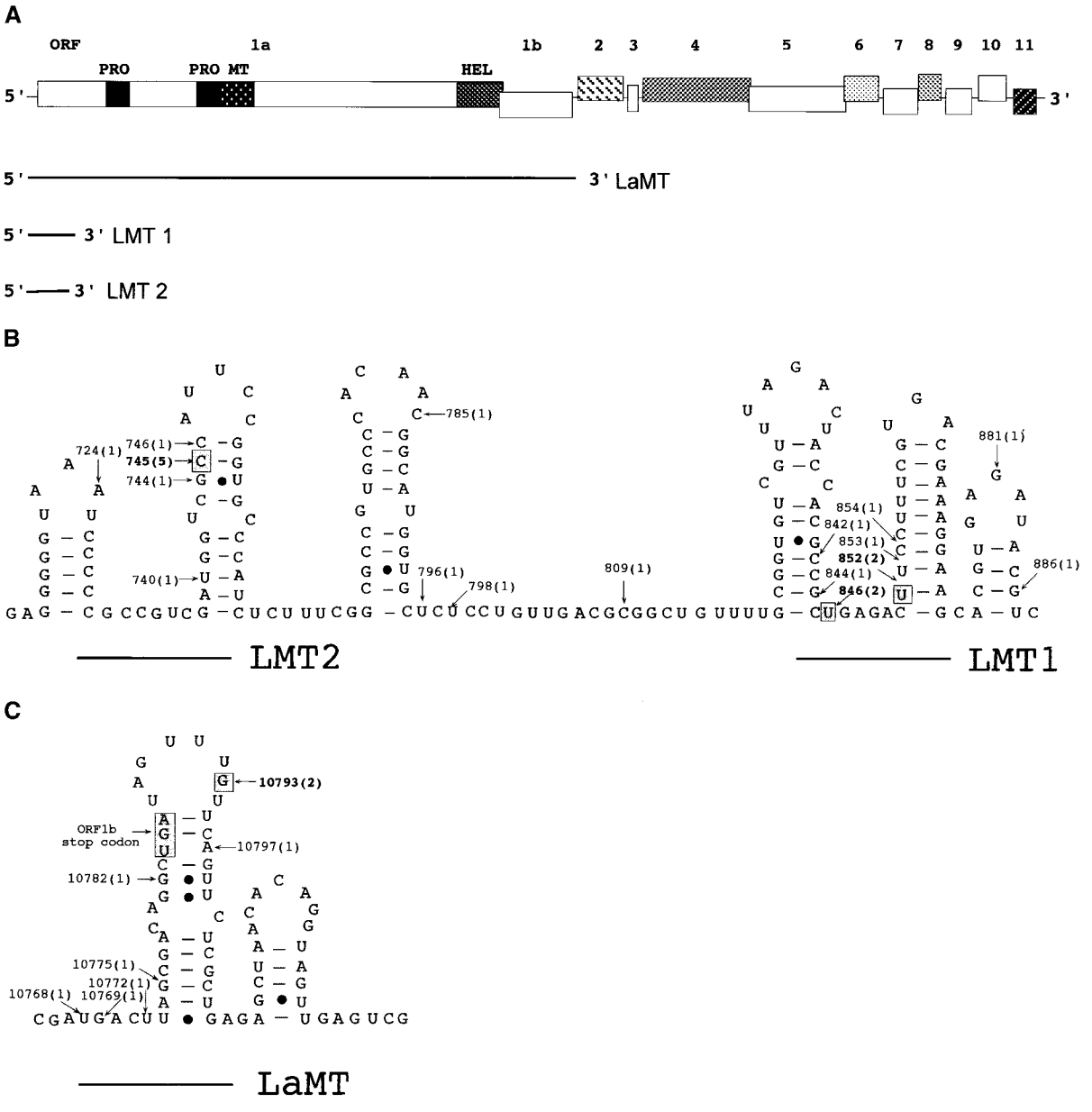

FIG. 3. (A) Diagram of the genomic organization of CTV. The putative domains of papainlike protease (PRO), methyltransferase (MT), helicase (HEL), and ORFs (open reading frames) with respective numbers are indicated. Lines shown below the genomic map are indicated 5′ coterminal subgenomic RNAs (LMT1, LMT2, and LaMT). (B) and (C) show the predicted secondary structure of the 3′ termini of LMT1 and LMT2, and LaMT RNAs from CTV-infected cells, respectively. The positions of the nucleotides in genomic RNA and the numbers (in parentheses) of clones having similar 3′ end termini are indicated by arrows. The single nucleotide with shadow indicates that more than one clone were found at this position, and three nucleotides together with shadow (C) point ORF 1b stop codon of CTV-VT. The lines shown below the secondary structure graphics indicate the range where the 3′ termini of LMT1, LMT2, and LaMT were determined.

1B). This RNA was also present in most total RNA extracts from infected plants (not shown). The ~10 kb RNA occurred in much smaller amounts than that the LMT RNA. This RNA was detected upon hybridization with the 5′ end of CTV genomic RNA probe (nts 1–755) (Fig. 6A), and ORF 1b probe complementary to nts 10,263–10,693 of CTV genome (Fig. 6B), but was absent in blots hybridized with the probe specific to nts 11,893–12,626 (Fig. 6C). It was not hybridized with negative-stranded specific 5′ probe, nor was this RNA detected with either the plus- or the minus-strand 3′ probes (not shown). These results suggested that these large molecules consisted of positive-polarity, single-stranded RNAs that extended from the 5′ terminus to approximately the junction of ORF 1b and ORF 2. These molecules, designated as large molecular weight tristeza RNAs, were found less consistently in tissue from chronically infected plants, but were found more often in RNA obtained from recent infections (not shown). Figure 3C shows the distribution of the 3′ termini from eight different cDNA clones obtained by

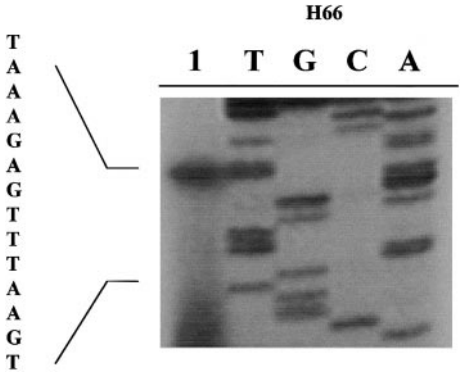

FIG. 4. Analysis of 5′ termini of LMT RNAs. [γ-^{32}P]ATP end-labeled primers positioned to nts 54–75 of CTV-VT genome were used for runoff transcription (lane 1). Reaction products were resolved on a 6% sequencing polyacrylamide gel next to the DNA sequencing ladder of CTV-VT-specific clone h66, with the same primer. The 3′ terminal sequence of genome-size dsRNA minus strand is present at left, and the 5′ to 3′ direction is from bottom upward.

poly(A) tailing of populations of LaMT molecules. The location of the 3′ terminal nucleotides from the tailed LaMT molecules varied between positions 10768 and 10797. Three of these molecules showed 3′ termini, which extended a few bases beyond the termination codon (10784) of CTV-VT-ORF 1b. Thus, sequence analyses showed that neither LMTs nor LaMTs had uniform 3′ termini. Because of the low concentration of the LaMT RNA and its large size, which made it difficult to isolate it from the genomic RNA, we were unable to determine the exact 5′ terminus of this RNA.

DISCUSSION

CTV, along with many other RNA viruses, has 3′ coterminal subgenomic RNAs. However, CTV appears to be unusual in also producing 5′ coterminal subgenomic

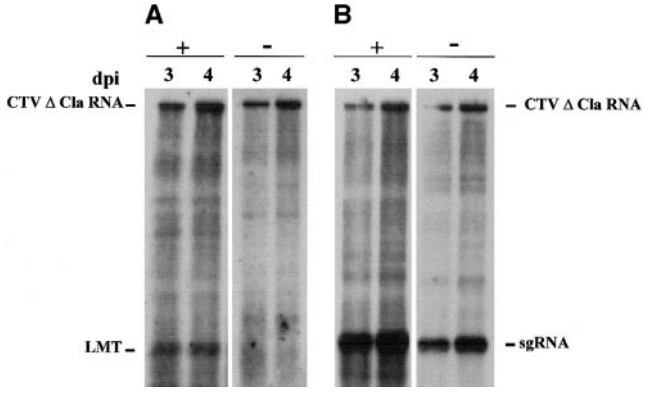

FIG. 5. Northern blot analysis of total RNAs from *N. benthamiana* protoplasts inoculated with CTV-ΔCla RNA, 3 and 4 days postinoculation (dpi). The hybridizations were done with the 5′- (A) or the 3′- (B) positive- (+) or negative- (−) stranded RNA-specific probes. The positions of CTV-ΔCla, LMT, and the subgenomic RNA (sgRNA) are indicated.

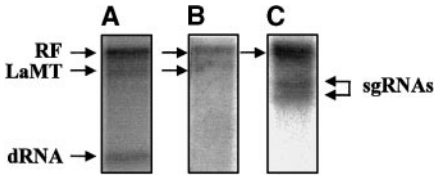

FIG. 6. Northern blot hybridization of dsRNA enriched extracts from Alemow plants infected with CTV-VT using riboprobes corresponding to the 5′ 755nts (A), ORF 1b nts 10,263 to 10,693 (B), and ORF 3 nts 11,893 to 12,626 (C) of the CTV-VT genome. RF, LaMT, and dRNA as in Fig. 1 are indicated.

RNAs in high abundance, and apparently at greater amounts than that of the genomic RNA. Hybridization results demonstrated that they were positive-sense RNAs of approximately 800 nts, without corresponding complementary negative strands. Most RNA preparations from asynchronously infected plants contained a population of the 5′ RNAs, migrating as broad bands during electrophoretic separations. The LMT RNAs found in synchronously infected protoplasts appeared mainly as two bands with temporal variation, the larger LMT RNAs appearing earlier than the smaller LMT RNAs. Sequence analysis revealed a variable population with termini ending between nts 724 and 886. LMT1 and LMT2 had modal sizes of 850 and 745, respectively. Similar prominent 5′ subgenomic RNAs are apparently present in plants infected with other members of the *Closteroviridae* (He *et al.,* 1997; Rubio *et al.,* 2000).

The LMT molecules did not appear to result from specific cleavage from the genomic RNA, because cleavage products corresponding to the rest of the genomic RNA molecules which would be expected to be produced in a 1:1 ratio were not found. Also, LMTs did not appear to be amplified by replication through a negative-stranded intermediate of a corresponding size, or a dsRNA. A more plausible explanation appears to be the presence of certain termination signals that cause plus-strand RNA transcription to terminate. The finding of two distinct sizes of LMT molecules in infected plants and protoplasts and the differing times of their appearance could result from two separate termination sites with temporally differing activation, starting at LMT1 and following to the LMT2 termination site. An alternative possibility would be a termination mechanism that produced LMT1, followed by cleavage to produce LMT2 and a small RNA fragment too small to resolve. CTV has ten 3′ genes, most with unknown functions. None of these genes appeared to be involved in the synthesis the 5′ coterminal subgenomic RNAs, since both of the latter appear to be produced normally in CTV mutants with all of the 3′ genes deleted, suggesting that all the viral functions needed for this process are encoded by ORF 1.

Cells infected with viruses of several families were previously found to contain distinct 5′ coterminal subgenomic RNAs. The leader region of coronaviral RNA is

found in a free form as well as is incorporated into 5′ terminal regions of the sgRNAs (Baric *et al.,* 1985). A different type of 5′ end transcript of 320 nts was found in cells infected with the double-stranded RNA *Leishmania-virus* (Chung *et al.,* 1994). These short LRV RNAs are generated by a different mechanism of capsid protein-mediated cleavage of full-length positive sense RNA transcripts. The resulting short transcripts have been implicated in a variety of regulatory functions in viral gene expression (MacBeth and Patterson, 1995).

The function of the CTV LMT molecules is unknown. One possibility is that they are alternative messenger RNA molecules that express the most 5′ domain of ORF 1 into a small protein. Cell-free translation in a wheat-germ system of RNA transcripts from a LMT1 cDNA clone showed a translation product of approximately 28 kDa (X. Che, unpublished data). However, we do not know if a similar product is also synthesized *in vivo*. Other possible roles of LMTs in virus-infected cells, including the enhancement of replicase access to subgenomic promoters by blocking genomic RNA synthesis or their possible encapsidation by the minor coat protein to produce "rattlesnake-like" polar virions (Febres *et al.,* 1996), are being investigated.

The small 5′ coterminal subgenomic RNAs (LMT1 and LMT2) of CTV appear to be unique among RNA viruses. No function has been associated with either molecule and it is possible that they also are artifacts of the replication process or rudiments of an earlier process with no apparent function. However, this would be surprising since the LMT molecules are almost the most abundant RNAs made during CTV replication.

The larger but less abundant 5′ coterminal subgenomic RNAs (LaMT) found in CTV-infected plants are also unusual. One possibility is that this RNA could serve as an alternative messenger for most of the replicase-associated polyproteins. The large size of the genomic RNA (20 kb) might cause it to be an inefficient mRNA, whereas the smaller RNA might be more efficient. However, the LaMT RNA did not have the 3′ nontranslated sequences that are generally thought to contain translation enhancers (Gallie, 1998). Another possibility is that this RNA is nonfunctional, an accidental product due to the interruption of a processive replicase complex by a stationary complex initiating subgenomic RNA synthesis at the p33 promoter. The CTV *cis*-acting elements that function as subgenomic RNA promoters might also cause termination of transcription from the 5′ terminus. A similar 5′ coterminal RNA was previously found in Sindbis virus-infected cells. The 5′ subgenomic RNAs that terminated at a site within the subgenomic RNA promoter were produced during replication and levels of production correlated with the intensity of the transcription of the 3′ subgenomic mRNA (Wielgosz and Huang, 1997). A similar strategy for the synthesis of 5′ coterminal RNAs that are displayed as dsRNAs in *Apple chlorotic leaf spot virus* infections was suggested by German *et al.* (1992). In contrast to the ACLSV system, dsRNA molecules were not found corresponding to any of the 5′ coterminal sgRNA of CTV.

MATERIALS AND METHODS

Virus sources and propagation

The biological, serological, and genomic characteristics of Israeli CTV isolate VT and the Florida isolates T36, T68, and T3, were described previously (Karasev *et al.,* 1995; Mawassi *et al.,* 1996; Hilf *et al.,* 1999). The viruses were propagated in Alemow (*Citrus macrophylla*), Volkamer (*C. volkameriana*), Citron (*C. medica*), or sweet orange (*C. sinensis*) seedlings. Infected plants were maintained in a glasshouse with temperatures ranging between 15 and 35°C. *N. benthamiana* protoplasts were prepared and inoculated with freshly extracted sap of CTV-infected tissue according to Mawassi *et al.* (2000). The sequence and nucleotide numbering are according to the CTV-VT strain described by Mawassi *et al.* (1996) (GenBank Accession No. U56902).

RNA extraction and Northern blotting

Total single-stranded RNAs were obtained from infected plants with Tri-Reagent (Molecular Research Center Inc.). RNA preparations enriched in dsRNA molecules were obtained according to Dodds and Bar-Joseph (1983) using CF11 columns. The RNAs were separated by electrophoresis in formamide-formaldehyde denaturing 1.1% agarose gels in MOPS buffer and transferred to Hybond N+ membranes according to Mawassi *et al.* (1995a). Detection of the membranes either with nonradioactive DIG-labeled or with radioactive [α-^{32}P]-labeled riboprobes was carried out according to Mawassi *et al.* (1995a, 2000). The 5′ terminal 755 nts, 3′ terminal 611 nts, 430 nts from ORF 1b, and 633 nts from ORF 3 of CTV genome were used to synthesize positive- or negative-stranded RNA-specific probes using T7 RNA polymerase.

3′ RACE analyses of LMTs and LaMTs

To determine the 3′ termini of LMTs and LaMTs, total dsRNAs from infected Alemow plants were separated by polyacrylamide gel. The gel was sliced in several pieces, and the electroeluted RNAs (Mawassi *et al.,* 1996) were denatured and poly(A) tailed with yeast poly(A) polymerase (USB) according to the manufacturer's instructions. The tailed RNA was amplified, cloned, and sequenced as described by Mawassi *et al.* (1996).

Primer extension of LMTs

The ~800 nts RNA reacting with the RNA probe specific to 5′ terminus of CTV genome was eluted from a low-melting agarose gel. After phenol-chloroform extrac-

tion and ethanol precipitation, a primer extension reaction was performed with a [γ-^{32}P]ATP end-labeled primer complementary to nts 54–75 of CTV genome and as previously described by Karasev *et al.* (1995). The resulting products were analyzed on a 6% urea/polyacrylamide sequencing gel side by side with a sequencing reaction of the CTV-VT 5′ terminus cloned in PUC57/T vector (Fermentas) using the same primer.

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