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In Vivo Restoration of Biologically Active 3′ Ends of Virus-Associated RNAs by Nonhomologous RNA Recombination and Replacement of a Terminal Motif

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Sequences at the 3′ ends of plus-strand RNA viruses and their associated subviral RNAs are important *cis* elements for the synthesis of minus strands in vivo and in vitro. All RNAs associated with turnip crinkle virus (TCV), including the genomic RNA (4,054 bases) and satellite RNAs (sat-RNAs) such as sat-RNA D (194 bases), terminate with the motif CCUGCCC. While investigating the ability of in vivo-generated recombinants between sat-RNA D and TCV to be amplified in planta, we discovered that sat-RNA D, although truncated by as many as 15 bases in the chimeric molecules, was released from the chimeric transcripts and amplified to high levels. The "new" sat-RNA D molecules nearly all terminated with the motif (C_{1-2})UG(C_{1-3}) (which may begin with 1 or 2 cytosines and end with 1, 2, or 3 cytosines), which was similar or identical to the natural sat-RNA D 3′ end. The new sat-RNA D also contained between 1 and 22 bases of heterogeneous sequence upstream from the terminal motif, which, in some cases, was apparently derived from internal regions of either the plus or minus strand of the TCV genomic RNA. Since most of these internal genomic RNA sequences within TCV were not adjacent to (C_{1-2})UG(C_{1-3}), at least two steps were required to produce new sat-RNA D 3′ ends: nonhomologous recombination with the TCV genomic RNA followed by the addition or modification of the terminus to generate the (C_{1-2})UG(C_{1-3}) motif.

Replication of plus-strand RNA viruses is a multistage process that likely begins with the recognition of a promoter sequence and/or structure on the plus-strand template by the replicase complex. In vivo and in vitro studies using numerous plus-strand viruses have led to the identification of 3′-terminal sequences, of between 19 and 200 bases, that are required for synthesis of the minus strand (1, 3, 15–17, 20, 21, 23, 26, 32, 40). Minus-strand synthesis has been experimentally shown to depend upon one or more structural elements within the 3′-terminal region, including tRNA-like structures, pseudoknots, and small hairpins (4, 16, 18, 40–42).

While tRNA-like structures have been postulated to represent tags at the end of the genomic RNA, which in a primordial RNA world may have been necessary for recognition of the 3′ end by primitive RNA replicating enzymes (27), it is possible that the amino acid covalently linked to the 3′ end of modern viruses with tRNA-like structures serves to protect essential 3′-end sequences from degradation. Viruses lacking tRNA-like structures, and consequently not aminoacylated, may have evolved other means for protection against loss of 3′-terminal nucleotides. An example is the recent finding that the poliovirus 3D polymerase contains terminal adenylyl transferase activity that may facilitate the repair of deletions in the 3′-end poly(A) tail in vivo (29, 31). In addition, the restoration of deleted or altered 3′-terminal CCC residues in genomic or subviral templates of cymbidium ringspot tombusvirus was proposed to be catalyzed by either the virus-encoded polymerase or a terminal transferase host enzyme (12, 13).

RNA-RNA recombination mediated by the viral replicase has also been implicated in the in vivo repair of transcripts containing alterations in the 3′ untranslated regions of brome mosaic virus and cucumber mosaic virus (3, 5, 35). For these viruses, recombination occurred between the highly related sequences at the 3′ ends of the three genomic RNAs. The ability of the brome mosaic virus and cucumber mosaic virus replicases to promote this type of recombination, known as homologous or aberrant homologous depending on the location of the crossover junctions (22), may facilitate the strong sequence conservation in the untranslated 3′ ends of the tripartite genomes, which may be an important factor in maintaining replication-competent templates.

The evolutionary significance of intermolecular nonhomologous recombination (recombination between unrelated RNAs), such as that found associated with turnip crinkle virus (TCV), is less clear. In the TCV system, recombination occurs between virus-associated satellite RNAs (sat-RNAs) and between sat-RNAs and the single viral genomic RNA (7, 8, 10, 43). Recombination between sat-RNA D (194 bases) and the 3′ region of the TCV genomic RNA gives rise to chimeric sat-RNA D/TCV molecules of apparently limited importance that may not be able to replicate further, possibly because of size or other constraints (7, 25, 44). While investigating whether these chimeric RNAs were able to replicate, we discovered that sat-RNA D, present at the 5′ end of chimeric molecules and missing its 3′-terminal 13 or 15 nucleotides, is liberated from the inoculated chimeric RNA transcript and amplified to very high levels in nearly every inoculated plant. Sequence analysis of the newly amplified sat-RNA D revealed that, in many cases, the 3′ ends of sat-RNA D had been reformed from sequence derived from either the plus or minus strand of the viral genomic RNA, joined to a short motif (5 to 7 bases) similar or identical to the motif at the 3′ terminus of wild-type sat-RNA D. These results, and others presented in this paper, suggest a new purpose for nonhomologous RNA recombination: the restoration of templates with 3′-terminal deletions to biological activity.

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MATERIALS AND METHODS

RNA synthesis and plant inoculations. cDNA clones of sat-RNA D (pT7D), sat-RNA C (pT7CAM), and TCV isolate TCV-M (pT7TCVms) have been described elsewhere (7, 39). RNA was synthesized in vitro by using T7 RNA polymerase as previously described (7). All transcripts generated, with the exception of TCV, contained the exact 5′ end and seven plasmid-derived bases at the 3′ end (GGGGAUC-3′). Transcripts of TCV contained the exact 5′ and 3′ ends. For this study, approximately 3 μg of TCV transcripts and 3 μg of either sat-RNA D, sat-RNA C, CX9, CX10, or CX10-S transcripts or truncated versions of sat-RNA D or CX10-S transcripts were inoculated onto each of six turnip cv. Just Right plants as previously described (24). Total RNA was isolated from one or two uninoculated leaves of each plant 2 weeks postinoculation as previously described (36).

Construction of full-length cDNA clones of CX9 and CX10. CX9 (D_{181}/TCV_{3811}; subscript numbers denote junction points) was the most prevalent chimeric RNA found in plants assayed at two to three weeks postinoculation (7, 9) (see Fig. 1A). CX10 (D_{179}/TCV_{3902}) was a prevalent small chimeric RNA cloned from plants at six weeks postinoculation (9) (see Fig. 1A). The reverse transcription (RT)-PCR method used to clone CX9 has been described elsewhere (8). The primers used were complementary to the 3′-end 20 bases of TCV and homologous to the 5′-end 19 bases of sat-RNA D. Full-length cDNA was cloned into the *Sma*I site of pUC19, and clones matching the CX9 sequence were confirmed by chain termination sequencing (Sequenase; Amersham). The CX9 full-length *Sma*I fragment was recloned into the modified pT7E19(+) vector previously described (7, 33) from which in vitro transcripts were produced.

Partial cDNA for CX10 was produced by RT-PCR with primers complementary to bases 3950 to 3970 of TCV and homologous to the 5′-end 19 bases of sat-RNA D. The resultant amplified cDNA was digested with *Nco*I (position 105) and *Spe*I (position 3959 in the TCV homologous region). The fragment containing the CX10 junction sequence was used to replace the corresponding fragment in the pT7E19(+)-CX9 plasmid described, producing pT7E19(+)-CX10. Ligation junctions were confirmed by DNA sequencing.

Site-specific mutagenesis of CX10 cDNA to produce CX10-S. pT7E19(+)-CX10 was linearized with *Sca*I, treated with shrimp alkaline phosphatase (Amersham), and annealed to the modified pT7E19(+) vector, which had been digested with *Hin*dIII and phosphatase treated. Included in the annealing reaction mixture was an oligonucleotide complementary to the junction sequence of CX10 (5′-CCGTTTTTGGAGCTCTTTCGAG) with the desired changes (underlined). The remaining steps were performed as described by Morinaga et al. (28).

Generation of deletions in sat-RNA D and CX10-S. Deletions of sat-RNA D were generated with the plasmid pT7D described above. pT7D was digested with *Bam*HI, which cleaves 7 nucleotides downstream from the corresponding 3′ end of the cDNA insert, and treated with *Bal* 31 (New England Biolabs) for various lengths of time, and then the ends were made flush by using DNA polymerase I large fragment (New England Biolabs). Following digestion with *Sca*I, the resultant fragment was recloned into the modified pT7E19(+) vector previously digested with *Sma*I and *Sca*I. The number of bases deleted was determined by sequencing the resultant plasmids.

Deletions in CX10-S were obtained by using the same procedure, except that the restriction site from which the *Bal* 31 deletions were generated was the *Sac*I site at the junction between the sat-RNA D and TCV sequences.

RNA gel blots. Two and one-half micrograms of plant total RNA was subjected to electrophoresis on 5% polyacrylamide–8 M urea gels. The gels were stained with ethidium bromide and photographed before treatment with 6% formaldehyde for 20 min. Following standard overnight capillary transfer to a reinforced nitrocellulose membrane (Nitroplus 2000; MSI), the blots were sequentially hybridized (43) with oligonucleotides complementary to positions 175 to 199 of sat-RNA C, 4035 to 4054 of TCV, and 44 to 59 of sat-RNA D. Final posthybridization wash conditions were 0.1% sodium dodecyl sulfate (SDS) and 0.1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) at 34°C.

Analysis of recombinants between TCV and sat-RNA D with new 3′ ends. Crossover locations were determined by RT-PCR amplification and cloning of the recombinant molecules as previously described (8).

Analysis of 3′-terminal sequences of sat-RNA D. Thirty micrograms of total RNA, extracted from plants 2 weeks after inoculation with transcripts of TCV and either sat-RNA D or various chimeric molecules, was subjected to electrophoresis on a 5% acrylamide–8 M urea gel. After being stained with ethidium bromide, the region of the gel corresponding to the size of sat-RNA D was excised and the RNA was extracted by soaking the crushed gel in 100 μl of 0.6 M ammonium acetate–0.1% SDS–100 μl of phenol-chloroform (1:1 [vol/vol]) overnight at 37°C. After recovery of the aqueous phase, the RNA was precipitated and resuspended in 25 μl of H_2O. Eight microliters of this RNA was subjected to polyadenylation in a 25-μl reaction mixture containing 0.5 mM ATP, 165 U of yeast poly(A) polymerase (Amersham), and buffer supplied by the manufacturer. After incubation at 37°C for 10 min, the enzyme was heat inactivated at 75°C for 5 min. The RNA was then phenol-chloroform extracted, precipitated, and resuspended in 20 μl of H_2O. Four microliters of poly(A)-tailed RNA was used as the template in a 20-μl cDNA synthesis reaction mixture that included 1 μg of primer [5′-GGACTCGAGTCGACATCGA$(T)_{17}$], 0.5 mM each deoxynucleoside triphosphate (dNTP), 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies), and buffer supplied by the manufacturer. After incubation for 1 h at 42°C, 6 μl of this reaction mixture was subjected to PCR amplification in a 100-μl reaction mixture that contained 85 ng of sat-RNA D-specific oligonucleotide (homologous to positions 104 to 118), 400 ng of a primer identical to that used for cDNA synthesis without the terminal poly(T), 0.25 mM each dNTP, 1 U of Pyrostase thermal stable polymerase (Molecular Genetic Resources), and buffer supplied by the manufacturer. The enzyme was added following an initial denaturation at 95°C. After 15 min at 72°C, PCRs were carried out for 30 cycles of 45 s at 95°C, 1 min at 40°C, and 1 min at 72°C. The resultant PCR products were cloned into the *Sma*I site in pUC19 and sequenced.

RESULTS

Generation of sat-RNA D with heterogeneous 3′ ends. TCV is naturally associated with a highly infectious chimeric RNA species, sat-RNA C, which shares 89% similarity with 191 bases of sat-RNA D at the 5′ end and two regions of TCV at the 3′ end (36) (Fig. 1A). Inoculation of plants with full-length transcripts of sat-RNA D (194 bases) and TCV genomic RNA (4,054 bases) leads to the accumulation of a large heterogeneous population of de novo-generated recombinant molecules that differ from sat-RNA C mainly by being the products of single recombination events (7). Junctions in the sat-RNA D sequence were nearly always within 3 bases of the 3′ end or 12 to 15 bases upstream from the 3′ end. Approximately 60% of the crossover points in TCV (references to TCV are to the viral genomic RNA) were between positions 3810 and 3816, at the base of a stable hairpin that mutagenesis studies determined was required for recombination at the hot spot location (7). Crossover sites in TCV outside positions 3810 to 3816 varied, depending on the length of time following inoculation when the recombinants were assayed (9).

Since the population of recombinants appeared to be "evolving" (i.e., potentially increasing in fitness) over time, it was necessary to determine if, like sat-RNA C, the de novo-generated recombinant molecules were able to be amplified in plants. Two recombinant species were chosen for this analysis on the basis of the high prevalence at which these particular recombinants were recovered in plants either 2 to 3 weeks postinoculation (CX9) or 6 weeks postinoculation (CX10) with sat-RNA D and TCV (Fig. 1A). Full-length cDNAs of CX9 (D_{181}/TCV_{3811}) and CX10 (D_{179}/TCV_{3902}) were cloned downstream from a T7 RNA polymerase promoter. Transcription in vitro with T7 RNA polymerase resulted in transcripts of the chimeric constructs containing the exact 5′ end and 7 plasmid-derived bases at the 3′ end; previous results have demonstrated that additional 3′-end bases do not affect the biological activity of TCV-associated RNA transcripts (37). Since subviral sat- or chimeric RNAs have no biological activity unless coinoculated with a helper virus, transcripts of TCV (30) were included in all inocula containing the subviral RNAs.

Two weeks after the inoculation of turnip plants with transcripts of TCV and CX9, CX10, or sat-RNA C, total RNA was extracted and subjected to RNA gel blot analysis (Fig. 2). Plants inoculated with TCV and sat-RNA C accumulated normal levels of sat-RNA C. However, plants inoculated with CX9 (data not shown) or CX10 did not accumulate any detectable RNA the size of the inoculated transcripts. Rather, nearly all plants inoculated with CX9 or CX10 accumulated an RNA species that differed slightly in size among plants but was approximately the size of sat-RNA D (194 bases) and hybridized to the sat-RNA D-specific probe. This result was unexpected, since the sat-RNA D portions of CX9 and CX10 were missing the 3′-terminal 13 and 15 bases, respectively, including the terminal CCUGCCC-OH that is present at the 3′ ends of all genomic and subviral RNAs associated with TCV (24, 30, 36).

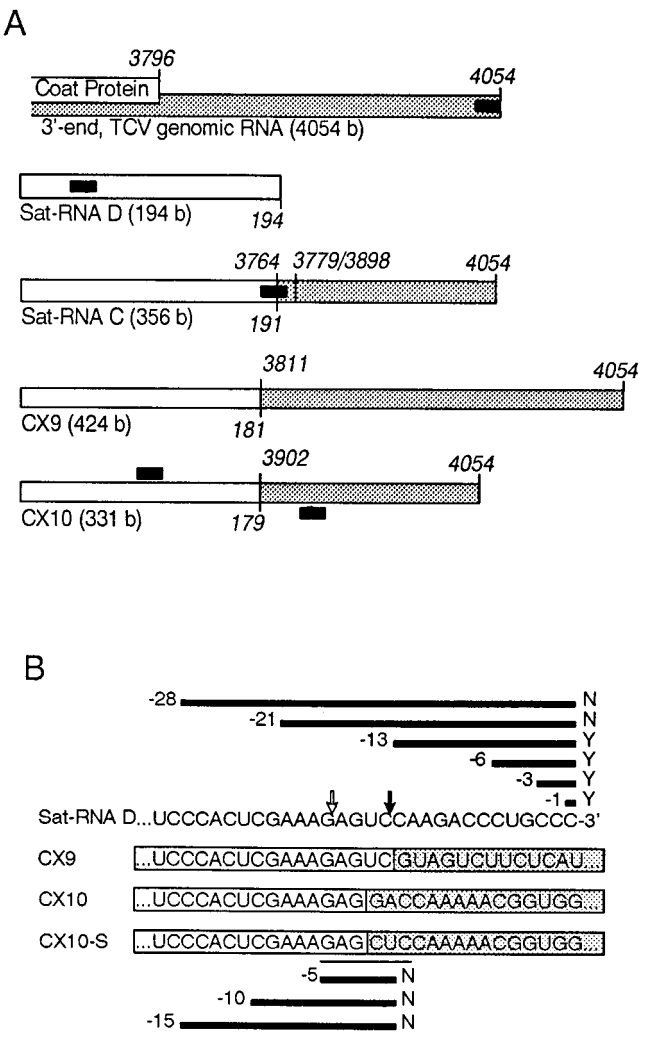

FIG. 1. Representations of the TCV-associated RNAs used in this study. (A) Relationship among sat-RNA D, the 3′ end of TCV, and the chimeric RNAs. Similar regions of molecules are shaded alike. Numbers above and below the diagrams denote positions in TCV genomic RNA and sat-RNA D of the related sequence, respectively. CX9 and CX10 are recombinant RNAs present in populations of recombinants in turnip plants previously inoculated with TCV and sat-RNA D. Short black bars within the RNAs denote the locations of oligonucleotide probes used in this study. Short black bars above and below CX10 denote the positions of oligonucleotides used to clone recombinants accumulating in infected plants. (B) Sequence at the 3′ end of sat-RNA D and the junctions of the chimeric RNAs. Open boxes in the chimeric RNAs enclose the sat-RNA D sequence, while shaded boxes enclose the TCV sequence. CX10-S is identical to CX10 except for the alteration of 2 bases in the TCV junction sequence that produces a *Sac*I restriction site in the corresponding cDNA (underlined). Bars beneath the CX10-S sequence represent the extent of deletions introduced into CX10-S from the *Sac*I site. Bars above the sat-RNA D sequence represent the extent of deletions generated from the 3′ end of sat-RNA D. Numbers denote the number of bases deleted. Y, transcripts containing the deletions produced sat-RNA D molecules with new 3′ ends in planta. N, no confirmed sat-RNA D species were detected by RNA gel blot analysis (see the text). Filled arrow denotes location of the major recombination site in over 2,000 sat-RNA D/TCV recombinants sequenced (7, 9). Open arrow indicates the location of the 5′-most junction in these recombinants.

The sat-RNA D-like species from individual plants was purified, cloned, and sequenced according to a protocol that allows for the determination of an RNA's 3′ end. 3′-end sequences of the sat-RNA D-like species from nine plants inoculated with CX9 or CX10 transcripts are presented in Table 1. Nearly all the molecules contained a distinctive CX9 or CX10 sequence that terminated at or near the junction point with TCV (in the original chimeric transcripts), joined to a sequence not present in the original transcripts. The new sequences at the 3′ ends of the sat-RNA D-like molecules were heterogeneous between plants. However, within a plant, variations of only one or two sequences were found (6 to 10 clones were sequenced per plant). Most of the variation within a plant was in the terminal nucleotides, which contained variable numbers of C residues and occasionally contained additional U residues (e.g., Table 1, CX10 plants E and F). Surprisingly, nearly every new sat-RNA D sequence, and all sequences present in multiple clones (and presumably present at higher levels in the RNA population within a plant), terminated with a motif, $(C_{1-2})UG(C_{1-3})$ (which may begin with 1 or 2 cytosines and end with 1, 2, or 3 cytosines), that is similar to the natural 3′ terminus, CCUGCCC (underlined in Table 1). Of the three clones that did not contain this motif, two terminated within the sat-RNA D sequence near the junction of the original chimeric transcript (Table 1, CX9 plant B and CX10 plant F) and one contained a new 3′-end sequence that terminated in four uracil residues (Table 1, CX9 plant C). Interestingly, within the same plant as the latter clone, multiple clones in which the 4 uracil residues were replaced with CUGCCC(C) were found, suggesting a further maturation of this species. Taken together, these results suggest that new sat-RNA D sequences are generated from the original chimeric molecules and efficient amplification is apparently dependent on the acquisition of new 3′-end sequences that contain terminal residues similar to natural sat-RNA D.

Similarity of new 3′-end sequences in sat-RNA D to internal TCV sequence. Most of the new sat-RNA D sequences were not sufficiently extensive to allow unambiguous determination of the possible origin. Sequence comparison analysis with three of the longest new 3′-end sequences suggested that these sequences derived from internal regions of plus-strand TCV (Table 2). One to two residues at the junction of the new sequences did not correspond to the internal TCV sequence and may be nontemplate nucleotide additions that are frequently found at the junctions of recombinant molecules associated with TCV (6, 10, 11). In addition, two of the new sat-RNA D sequences (from CX9 plant C and CX10 plant A) originated from the same internal TCV sequence. These findings suggest that nonhomologous recombination may be responsible for the generation of at least some of the new sat-RNA D species.

Chimeric sat-RNA D/TCV transcripts containing further truncations in sat-RNA D. As described above and shown in Fig. 1, CX9 and CX10 contained truncations of the sat-RNA D sequence of 13 and 15 bases, respectively. To determine if chimeric transcripts containing more extensive truncations in the sat-RNA D sequence could still generate new sat-RNA D species, a *Sac*I restriction site was engineered into the CX10 cDNA at the junction between the sat-RNA D and TCV sequences (Fig. 1B). This construct, designated CX10-S, required two nucleotide modifications in the TCV sequence just downstream from the junction. Total RNA extracted and analyzed by gel electrophoresis 2 weeks after inoculation of the turnip plants with transcripts of CX10-S and TCV again revealed the presence of new species of approximately the size of sat-RNA D in each plant (data not shown). Sequences of clones of the sat-RNA D-like species were comparable to CX9 and CX10-derived sat-RNA D sequences (Table 1).

Deletions of 5, 10, and 15 bases were generated in CX10-S cDNA (Fig. 1B). These deletions included 2 nucleotides of the TCV sequence and the remainder from sat-RNA D. Therefore, the 5-base deletion removed only an additional 3 bases of the sat-RNA D sequence from CX10-S. However, unlike

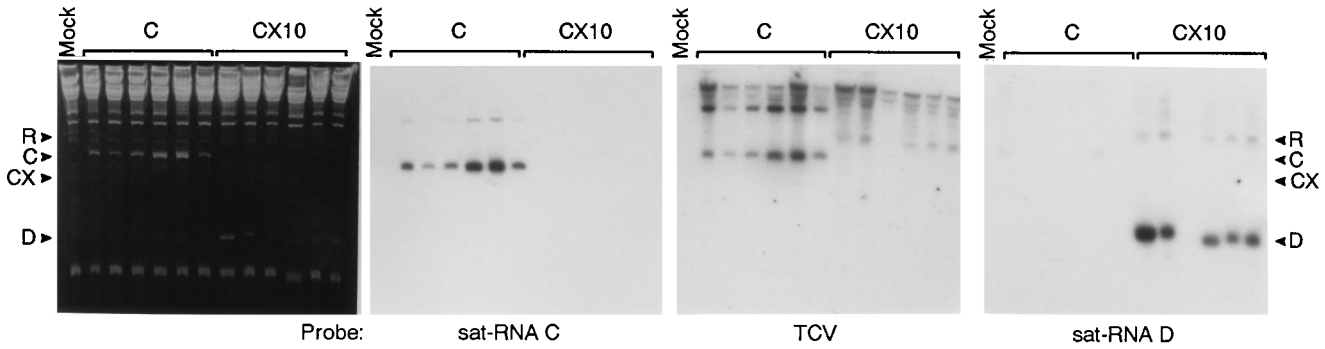

FIG. 2. Plants inoculated with CX10 and TCV transcripts accumulate new sat-RNA D-like species. (Left panel) Ethidium bromide-stained, denaturing polyacrylamide gel of total RNA (2.5 μg per lane) extracted from uninoculated leaves 2 weeks after treatment with buffer (Mock) or inoculation with transcripts of TCV and either sat-RNA C (C) or CX10. (Right three panels) RNA blots of the gel shown at left. Probes were oligonucleotides specific for the RNAs indicated below each panel. The sat-RNA C probe detects sat-RNA C monomers and dimers. The TCV probe detects the TCV genomic RNA and two subgenomic RNAs (1.45 and 1.7 kb) and cross-hybridizes with sat-RNA C. The migration positions of sat-RNA C (C), sat-RNA D (D), CX10 (CX), and the major sat-RNA D/TCV recombinant RNA species at 2 weeks postinoculation (R) are indicated.

CX10-S, no sat-RNA D-like species was detected by RNA gel blot analysis in RNA isolated from plants previously inoculated with any of the CX10-S deletion transcripts (data not shown). These deletions all extended beyond the furthest truncation yet found for the sat-RNA D portion of the more than 2,000 sat-RNA D/TCV recombinant molecules previously sequenced (Fig. 1B). These results suggest that genesis of new sat-RNA D species can occur only if the sat-RNA D sequence in the chimeric transcript is not truncated by more than 15 to 17 bases.

Generation of new 3′ ends with sat-RNA D transcripts truncated at the 3′ end. To determine if the generation of sat-RNA D with new 3′ ends requires prior linkage to the TCV 3′-end sequence as found in CX9 and CX10, deletions of between 1 and 28 bases were constructed in a full-length cDNA clone of sat-RNA D. Transcripts synthesized in vitro contained the exact 5′ end and 7 plasmid-derived bases at the 3′ end (GGG GAUC). RNA gel blot analysis of total RNA isolated from turnip plants 2 weeks after inoculation with transcripts of TCV and wild-type sat-RNA D or sat-RNA D containing deletions of between 1 and 13 bases from the 3′ end revealed the presence of sat-RNA D-like molecules in nearly every plant (Fig. 3). No sat-RNA D was detected in plants with deletions of 21 and 28 bases, with the exception of one plant (21-base deletion transcript) that contained a sat-RNA D-sized species that was faintly detectable upon overexposure of the autoradiogram with the sat-RNA D-specific probe (data not shown). Despite repeated efforts, we were unable to clone this sat-RNA D-like species.

Sequencing of clones derived from wild-type sat-RNA D transcripts indicated that 11 of 13 contained the exact wild-type 3′-end motif (CCUGCCC), 1 was missing the terminal C residue, and 1 contained a deletion of 14 3′-end bases (Table 3). Inoculation of plants with transcripts with deletions of the terminal C residue resulted in sat-RNA D molecules with heterogeneity in the number of terminal C residues at the 3′ end similar to the wild type. Of the 13 clones sequenced, 11 had the terminal C residue replaced and contained the wild-type 3′ end (CCUGCCC), 1 was missing the terminal residue (i.e., the sequence was identical to the input transcript, CCUGCC), and 1 clone ended with CCUGCCCU, where the nontranscript bases are underlined. Transcripts with deletions in the 3′-terminal CCC produced sat-RNA D with two or three of the C residues restored (10 of 11 clones); however, 1 clone contained an additional 11-base deletion (14-base deletion total) with a new 3′-end sequence apparently derived from positions 3063 to 3086 of plus-strand TCV (Table 3, Δ3 plant B). The internal TCV sequence did not contain the $(C_{1-2})UG(C_{1-3})$ motif found at the 3′ end of the new sat-RNA D species, suggesting that an additional step(s) is necessary following nonhomologous recombination.

Plants inoculated with transcripts containing deletions of 6 and 13 bases accumulated species of greater size heterogeneity (between plants) than plants inoculated with full-length or nearly full-length transcripts (Fig. 3; Table 3). Transcripts containing the 6-base deletion resulted in sat-RNA D sequences that each contained an additional 8-base deletion (14-base deletion total) attached to various sequences that differed for each of the two plants from which clones were analyzed. Transcripts containing 13-base deletions also resulted in a similar variety of sat-RNA D species with new 3′ ends. Unlike transcripts with the 6-base deletion, no additional bases of the original transcript sequence were deleted. As with the sat-RNA D derived from CX9 and CX10, most new sat-RNA molecules, especially those present in greater frequency in the population, ended with the $(C_{1-2})UG(C_{1-3})$ motif. The origin of at least some of the new 3′-end sequences was likely either the plus or minus strand of TCV genomic RNA. However, like some of the other new sat-RNA D sequences described above, the terminal motif was not a contiguous part of the TCV internal sequence. These results suggest that the mechanism resulting in the formation of new sat-RNA D 3′ ends does not require the input transcript to be a chimeric molecule and that at least two steps are likely required for formation of most of the new sat-RNA D 3′ ends.

Recombinant RNAs derived from new sat-RNA D and TCV. Although Fig. 2 showed that CX10 was not amplified to detectable levels in turnip plants, discrete RNA species of approximately 400 bases that hybridized to both sat-RNA D and TCV probes were present in all CX10-inoculated plants (and CX9-inoculated plants [data not shown]) that accumulated detectable levels of new sat-RNA D (Fig. 2). RNAs of the same size that hybridized to the sat-RNA D probe were also detected in plants inoculated with sat-RNA D containing deletions of 1 to 13 bases at the 3′ end (data not shown). To determine if the species found in plants inoculated with CX10 represented de novo-generated recombinant molecules derived from the new sat-RNA D and TCV, RT-PCR with sat-RNA D- and TCV-specific primers was used to amplify potential chimeric species. Sequencing the resultant cDNAs

TABLE 1. Generation of sat-RNA D with new 3′ ends from chimeric sat-RNA D/TCV transcripts

Name	Plant[a]	3′-end sequence[b]
CX9	A	CGAAAGAGUCG*UCGACUG*(C_{2-3})
CX9	A	CGAAAGAGUCG*UUGACUG*(C_{1-3})
CX9	A	CGAAAGAG*CCAAACCGACUGCC*
CX9	A	CGAAAGAGUC*ACCACCCUGCC*
CX9	B	CGAAAGAGUC*AUUGACUGCC*
CX9	B	CGAAAGAGU*UUAGUCUAACUGCCC*
CX9	B	CGAAAGAGUCG*UCGACUGCCC*
CX9	B	CGAAAGAGUC*CACGACCCUG*(C_{2-3})
CX9	B	CGAAAGAGU*UUUCUCUCUGCC*
CX9	B	CGAAAGAGUCG*UUGACUGCCC*
CX9	B	CGAAAGAG
CX9	C	CGAAAGAGU*UAGGUGAUUAUUCCGACUGCCC*
CX9	C	CGAAAGAGU*UUAGUCUAAUUUU*
CX9	C	CGAAAGAGU*UUAGUCUAACUG*(C_{3-4})
CX10	A	CGAAAGAG*UUGCAAUUAUGCCCAGUGC*
CX10	A	CGAAAGAG*UUGCGAUUAUGCCGACUG*(C_{2-4})
CX10	B	CGAAAGAG*UCCUCGACCCUG*(C_{2-3})
CX10	B	CGAAAGAGG*UCCACGACCCUG*(C_{2-3})
CX10	B	CGAAAGAG*AACAUCUGCC*
CX10	C	CGAAAGAGGAC*UUUCCCUG*(C_{2-3})
CX10	C	CGAAAGAGGAC*UACCCCUGCCC*
CX10	D	CGAAAGAG*AGUUCCAUAACUGCU*
CX10	D	CGAAAaAG*AGUUCCAUAUCUGCC*
CX10	D	CGAAAGAG*AGUUCCAUAUCUGCC*
CX10	D	CGAAAGAG*UUUUUAUCCUGCC*
CX10	E	CGAAAGAGGAC*AUUCCUGCCC*
CX10	E	CGAAAGAGGAC*AUUCCUGCCCU*
CX10	E	CGAAAGAGGAC*GUUCCUG*(C_{2-3})
CX10	E	CGAAAGAGGAC*GUUCCUGCCCU*
CX10	E	CGAAAGAGG*UCUCUCCUGCCC*
CX10	E	CGAAAGAGG*UUCUUCCUGCCC*
CX10	F	CGAAAGAGG*UCUCUCCUGCCC*
CX10	F	CGAAAGAGG*UCUCUCCUGCCCU*
CX10	F	CGAAAGAGG*UCUCUCCUGCCCUU*
CX10	F	CGAAAGAGG*UCUCUCCUGU*
CX10	F	CGAAAGAGG*UUAUUCCUGCCC*
CX10	F	CGAAAGAGG
CX10-S	A	C*CGAGGACGGUUUUAUAAU*
CX10-S	A	C*CGAAGACGGUUUAUAAUCUG*(C_{1-3})
CX10-S	A	C*CGAAGACGGUUUAUAAUCUGCCU*
CX10-S	A	C*CGAAGACGGUUUUAUAAUCU*
CX10-S	A	C*CAAAAGCGGUUUGUUCUC*
CX10-S	A	C*CGAAGACGGU*
CX10-S	B	CGAAAGAGCUCCAAAAACGGUA*CUGCC*
CX10-S	B	CGAAAGAG*UUUCUUCUG*(C_{3-4})
CX10-S	B	CGAAAGAGCU*AUUUGCUGCCC*
CX10-S	B	CGAAAGAGCU*AUUCGCUGCC*
CX10-S	B	CGAAAGAGC

[a] CX10 plants C to F are from four of the six RNA preparations shown in Fig. 2.

[b] Only the sequence downstream from sat-RNA D position 172 to the 3′ terminus is shown. This sequence was followed by poly(A) tails added during cloning. Sequences in boldface and italic type diverge from the original sequence in the chimeric CX9, CX10, and CX10-S transcripts (Fig. 1B). Sequences similar to the terminal motif found at the 3′ ends of all TCV-associated RNAs [(C_{1-2})UG(C_{1-3})] are underlined. Clones differing only in the number of C residues at the 3′ termini were grouped together. Between 6 and 10 clones were sequenced from each plant. Lowercase letter (CX10, plant D) denotes additional sequence alteration from the original transcript.

revealed that, in many cases, the amplified recombinant molecules were clearly derived from new sat-RNA D molecules previously identified in the same plants and TCV (Table 4). Other recombinants contained the sat-RNA D sequence derived from species not yet found in the population within the plant (e.g., Table 4, plant C). Although RT-PCR with these primers would have amplified any CX10 molecules present, no recombinants that were identical to the original chimeric transcript were found, supporting the probability that recombinant molecules like CX9 and CX10 are not amplified extensively in planta. Since the crossover points in TCV were similar to those previously found (7), these results also indicate that the wild-type sequence adjacent to the 3′-terminal motif in sat-RNA D is not required for normal recombination with TCV.

DISCUSSION

We have presented evidence that sat-RNA D missing 3′-end sequences, either as a separate transcript or as the 5′ portion of a chimeric construct, is able to be amplified in plants, probably because of the restoration of 3′-end sequences. The new 3′-end sequences are heterogeneous among sat-RNA D populations in different plants but are similar in populations within a plant. This suggests that while many different sequences can replace the 3′ ends of sat-RNA D, either the event(s) leading to the sequence replacement occurs infrequently within a plant or the new sat-RNA D's are under selective pressure, resulting in the amplification of only a few selected sequences. In addition, 90% (133 of 148) of the new sat-RNA D clones sequenced for this study contained (C_{1-2})UG(C_{1-3}) at their 3′ termini, motifs similar or identical to the CCUGCCC at the 3′ terminus of wild-type sat-RNA D. The average size of the new sat-RNA D species containing the 3′-terminal motif was 194.8 ± 3.3 bases, very similar to the size of wild-type sat-RNA D (194 bases). These results suggest that the sat-RNA D species most efficiently amplified in plants are approximately 194 bases and contain a 3′-terminal motif similar to that found in wild-type sat-RNA D. In addition, the sequence directly preceding the terminal motif can be highly variable.

The smallest new 3′-end addition (excluding the terminal motif) was 1 base, which was added onto the longest sequence derived from CX10-S transcripts (192 bases), producing a sat-RNA of 199 bases (Table 1, CX10-S plant B). The longest addition of new 3′-end sequence (including the terminal motif) was 22 bases, resulting in a sat-RNA of 202 bases (Table 1, CX9 plant C). The origin of at least some of the new 3′-end sequences is apparently the plus or minus strand of TCV. A strong possibility of a TCV origin was considered to be at least 8 consecutive nucleotide matches. Many of the new sequences, while probably originating from TCV, also contained a number of mismatches within an otherwise conserved region, most likely the consequence of errors that accumulate in variable regions during RNA replication by RNA replicases. In addition, 10 of 14 new sat-RNA D sequences clearly derived from TCV had between 1 and 8 residues at the junction that differed

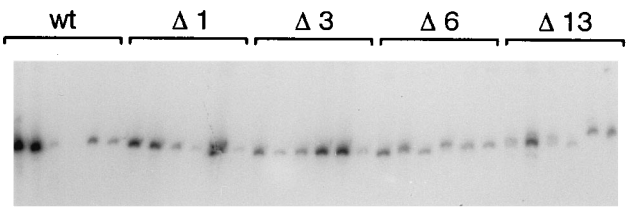

FIG. 3. RNA gel blot of sat-RNA D accumulating in plants inoculated with TCV and sat-RNA D with or without 3′-terminal deletions. RNA was extracted from plants 2 weeks after inoculation with TCV and sat-RNA D transcripts containing deletions of from 0 to 13 bases. The probe was an oligonucleotide specific for sat-RNA D. Δ1 to Δ13 denote the number of bases deleted. wt, wild-type sat-RNA D. Note that inoculation of plants with sat-RNA D transcripts containing deletions of 6 and 13 bases results in the accumulation of sat-RNA D of various sizes.

TABLE 2. Possible origin of some new sat-RNA D 3′-end sequences[a]

Name	Plant	New 3′-end sequence	Similar sequence in TCV genome (orientation, position)
CX9	C	UAGGUGAUUAUUCCGACUGCCC	gAGGcGAUUAUgCCGACUGCCu (+, 765–786)
CX10	A	UUGCGAUUAUGCCGACUGCC	agGCGAUUAUGCCGACUGCC (+, 766–785)
CX10	D	AGUUCCAUAUCUGCC	ucUUCCAUAUCUGug (+, 920–934)

[a] Three of the new 3′-end sequences shown in Table 1 were subjected to BESTFIT analysis (Genetics Computer Group package; University of Wisconsin) with the plus and minus strands of TCV isolate TCV-M (30). Lowercase letters do not match between the new sat-RNA D and TCV sequences. Orientation refers to whether the matched sequence is from the plus or minus strand. Positions refer to numbering on the TCV plus strand.

from the sat-RNA D transcript or TCV sequence (Table 3, Δ6 plant B [several examples]), suggesting the possibility of more than one recombination event. Furthermore, several new 3′-end sequences originating from TCV contained an additional 1 to 8 residues at the 3′ terminus, or preceding the terminal $(C_{1–2})UG(C_{1–3})$ motif, of unknown origin.

Any mechanism hypothesized to explain the genesis of new sat-RNA D 3′ ends must take into account the apparent multistage nature of the event. For example, in plant C inoculated with CX9 transcripts (Table 1), four clones contained the new sequence UUAGUCUAA; however, one clone terminated with UUUU while three terminated with the motif CUGC CC(C). This example and others (e.g., Table 1, CX10-S plant A) suggest that the addition of a new sat-RNA D 3′-end sequence and acquisition of the terminal motif may not be occurring in the same step. In addition, any model must explain the addition of sequence from internal regions of either plus- or minus-strand TCV, the loss of sequence present in transcripts of the chimeric construct (and the similar loss of 7 plasmid-derived bases in the sat-RNA D deletion transcripts), and the acquisition of the $(C_{1–2})UG(C_{1–3})$ terminal motif.

A possible model for the introduction of a new sat-RNA D

TABLE 3. New 3′-end sequences in sat-RNA D generated in vivo from transcripts of sat-RNA D with 3′-end deletions

Δ[a]	Plant(s)	3′-end sequence[b]	Similar sequence in TCV genome (orientation, position)[c]
0	A&B	CCUGCCC (11), CCUGCC (1), Δ14 bases (1)	
1	A&B	CCUGCCC (6), CCUGCC (1), CCUGCCCU (1)	
3	A&B	CCUGCCC (7), CCUGCC (3)	
3	B	Δ11-UGCUUAACCCAAGCGAACCUGCCC	UGCUcAACCCAAGCGAACCcGgaa (+, 3063–3086)
6	A	Δ8-UCAAGUUUGAACCUGCUUUCGG	gaAgGUUUGAACCUcaggguaG (−, 136–123)
6	A	Δ8-UCAAGUUUGACU	
6	A	Δ8-UCAAGUUUGACUGCCC	
6	A	Δ8-UUAGCUGGCUAUUU	
6	A	Δ8-UUAGCUCUGCC	
6	A	Δ8-UUAAGUUUAACUG	
6	A	Δ8-UUUUAAACUGCCC	
6	A	Δ8-UUAGCCCUGCCC	
6	B	Δ8-UUAACACUGCCC	
6	B	Δ8-UUGUAGUCUUCUCAUCUUAGUAGUUAGC	gUGUAGUCUUCUCAUCUUAGUAGUUAGC (+, 3810–3837)
6	B	Δ8-UUUCCGACUGUGACAUGAAGAGAUUCGACCAGAG	cgauaGgaUuUGACAUGAAGAGAUUCGACCAGAG (+, 1487–1518)
13	A	UUAACCCAAGUAAACUGCCC	aUAACCCAAGUAcccccgug (−, 2531–2514)
13	A	UUAACCCAAGCGUUGCCC	
13	A	UUAACCCAAGCGUUGCC	
13	B	UUGAAUGUCAACUGCCC	UUGuugGUCAACUGCCu (−, 1619–1603)
13	B	UUGAAUGUCAACUGCU	
13	B	UUAUCUCAAUCAACCUGCC	aagUCUCAAUCAACuUcaa (+, 2551–2571)
13	B	UAUUACUCUGCC	
13	B	UAUUAAACUGCC	
13	B	UUAUCAAACUGCCC	
13	B	UUAUAUCUGCC	
13	B	UCACUCCUGCC	
13	B	UUAUAUUCUGCC	
13	B	AACUGAGGAGCAGCCAAAGGGUAAAUGGCAAU	AACUGAGGAGCAGCCAAAGGGUAAAUGGCAAU (+, 3754–3784)
13	C	UACAAAAGUGGCUGCCC	cACAAAAGUGcagcuuu (−, 481–465)
13	C	UGCAAAAGUGGCUGCCC	
13	C	UGCAAAAGUGGUU	
13	C	UACAGAAGUGGCUGCC	
13	C	UACAAUCAUAUUGUUCCUGCCC	UACAAUCAUAUUaUUCuUGCCu (+, 2081–2102)
13	C	UACACAUCAGAACGAUGUCGACUGCCC	gcCACAUCAGAACGAUagucaauacgg (+, 568–592)
13	C	U	

[a] Number of 3′-end sat-RNA D bases deleted.

[b] For wild-type (0-base deletion) and nearly all 1- and 3-base deletion constructs, only the sequence of the terminal motif is shown (the remaining sequence was invariant) and the number of clones containing each of the sequences is given. For all other deletion constructs, only the new 3′-end sequence located downstream from the original deletions is shown. All sequences were followed by poly(A) tails added during cloning. Sequences similar to the terminal motif found at the 3′ ends of all TCV-associated RNAs $[(C_{1–2})UG(C_{1–3})]$ are underlined. Between 8 and 16 clones were sequenced for each plant. Both plants analyzed that were inoculated with transcripts containing 6-base deletions produced new sat-RNA D with additional 8-base deletions.

[c] Sequence in TCV plus or minus strand that is similar to the new sat-RNA D 3′-end sequence. Lowercase letters do not match between the sat-RNA D and TCV sequences. Orientation refers to whether the matched sequence is from the plus or minus strand. Positions refer to numbering on the plus strand of TCV isolate TCV-M.

TABLE 4. Recombinants generated between new sat-RNA D, derived from CX10 transcripts, and TCV genomic RNA

Plant[a]	3′-end sat-RNA D parental molecule[b]	sat-RNA D junction[c]	TCV junction[d]
C	nc	CGAAAGAGGACCAAAAACGGU***AGC***	3827
C	nc	CGAAAGAGGACCAAAAACGGU***AG***	3840
C	nc	CGAAAGAGGACCAAAAACGGU***AG***	3838
C	nc	CGAAAGAGGACCAAAAACGGU***AC***	3825
C	nc	CGAAAGAGGACCAAAAACGGU***A***	3817
C	nc	CGAAAGAGGACCAAAAACGGU***A***	3836
C	nc	CGAAAGAGGACCAAAAACGGU	3826
C	nc	CGAAAGAG***UUAUUCCUGCCC***	3799
C	CGAAAGAGGAC***UACCCCUGCCC***	CGAAAGAGGAC***UACCCCUGCC***	3807
C	CGAAAGAGGAC***UACCCCUGCCC***	CGAAAGAGGAC***UACCCCUGCC***	3659
C	nc	CGAAAGAGG***UUUCCCCUGCCC***	3751
C	nc	CGAAAGAGG***UUAUUCU***	3814
C	nc	CGAAAGAGG***AAGCC***	3805
C	nc	CGAAAGAGG	
D	CGAAAAAG***AGUUCCAUAUCUGCC***	CGAAAAAG***AGUUCCAUAUCUGC***	3732
D	CGAAAAAG***AGUUCCAUAUCUGCC***	CGAAAAAG***AGUU***uu	3717
D	nc	CGAAAGAuuuuu	3812
D	nc	CGAAAGAuuu	3850
E	CGAAAGAGGAC***GUUCCUGCCC***	CGAAAGAGGAC***GUUCCUGCCC***	3694
E	nc	CGAAAGAGGAC	3806
E	nc	CGAAAGAGG	
E	CGAAAGAGGAC***AUUCCUGCCC***	CGAAAAAGGAC***AUUCCU***	3810
E	CGAAAGAGGAC***AUUUCCUGCCC***	CGAAAAAGGAC***AUUCC***	3822
E	nc	CGAAAGAG***ACUUCCUG***	3810
E	nc	CGAAAGAG***AACGA***	3826
F	CGAAAGAGG***UCUCUCCUGCCC***	CGAAAGAGG***UCUCUCCUG***	3813
F	CGAAAGAGG***UCUCUCCUGCCC***	CGAAAGAGG***UCUCUC***	3810
F	CGAAAGAGG***UUAUUCCUGCCC***	CGAAAGAGG***UUAUUCC***	3809
F	nc	CGAAAGAG	3810

[a] Origin of CX10 plants is the same as described in Table 1.

[b] Sequence from position 172 to the 3′ end of the new sat-RNA D species that are the likely parental molecules for the recombinants shown at right. Sequences in boldface and italic type diverge from the original sequence in the chimeric CX10 transcripts. nc, no clones that were apparently parental molecules were sequenced.

[c] Sequence from position 172 until the junction with TCV sequence in recombinants amplified by PCR with sat-RNA D- and TCV-specific primers (sat-RNA D sequence is located 5′ of the junction). Lowercase U residues at the junction are not derived from either the sat-RNA D or the TCV sequence. Sequences in boldface and italic type diverge from the original sequence in the chimeric CX10 transcripts.

[d] Position of the junction sequence in TCV genomic RNA. Numbering is from TCV isolate TCV-M.

3′-end sequence is presented in Fig. 4. In this model, step 1 involves recombination with TCV during plus- or minus-strand synthesis of the viral genomic RNA and minus-strand synthesis of either truncated sat-RNA D or the chimeric constructs. Synthesis of full-length complementary strands of the recombinant molecule (Fig. 4, step 2) or synthesis of a partial product by premature termination resulting in a loss of the majority of added TCV sequence (step 2a) occurs next. If a full-length product is produced (step 2), then internal initiation near the junction with the TCV sequence would produce minus strands of truncated, mainly sat-RNA D sequence recombinants (step 3). This would be followed by plus-strand synthesis (step 4) and then, in step 5, which could also directly follow step 2a, maturation of the 3′ ends by the addition of the terminal $(C_{1-2})UG(C_{1-3})$ motif or modification of existing sequences (see below).

This model assumes that nonhomologous recombination with TCV is the mechanism by which the new 3′ ends of sat-RNA D are generated and the plasmid sequence on the 3′ ends of the transcripts is lost. We have previously shown that recombination can occur between sat-RNA D and both plus and minus strands of TCV (8) or between sat-RNA D and plus-strand sat-RNA C (10, 11). Interestingly, the sat-RNA D portion of recombinant molecules is never deleted by more than 17 bases from the 3′ end (7). In the present study, we have found that deletion of between 16 and 18 bases of the 3′-end sat-RNA D sequence abolishes the ability to repair the sat-RNA D 3′ end. Since our model for 3′-end repair suggests that it involves nonhomologous recombination, it is possible that the dispensable nature of the sat-RNA D terminal 17 bases in recombinants and the inability to repair a deletion longer than this sequence are related. Computer secondary-structure predictions suggest that the 3′-terminal 15 nucleotides in plus-strand sat-RNA D are single stranded while the remaining sequence is mostly base paired. It is possible that the base-paired region that adjoins the single-stranded terminus is required for RNA accumulation in both recombinants and in sat-RNA D and cannot be replaced by random sequences.

In most recombinants derived from crossing over within TCV (defective interfering RNAs) or between TCV and the sat-RNAs, the sequence 3′ of the junction resembled one of three motifs that were similar to sequences found at the 5′ ends of TCV-associated RNAs, including genomic, subgenomic, and sat-RNAs (7, 10, 43). However, most of the recombination junctions in this study did not contain these motif sequences. In addition, not all new 3′-end sequences can be traced unambiguously to TCV plus or minus strands. In some cases, insufficient new sequence [outside of the terminal $(C_{1-2})UG(C_{1-3})$ motif] is present to definitively attribute to TCV, and in other cases, mutations during amplification of new sat-RNA species may obscure a TCV origin. It is also possible that some new 3′-end sequences, such as the sequence in clones from CX10-S plant A (Table 1), originate by recombination with an RNA species other than TCV. The model also suggests that recombination is occurring during minus-strand synthesis of sat-RNA D. For recombination to occur during plus-strand synthesis,

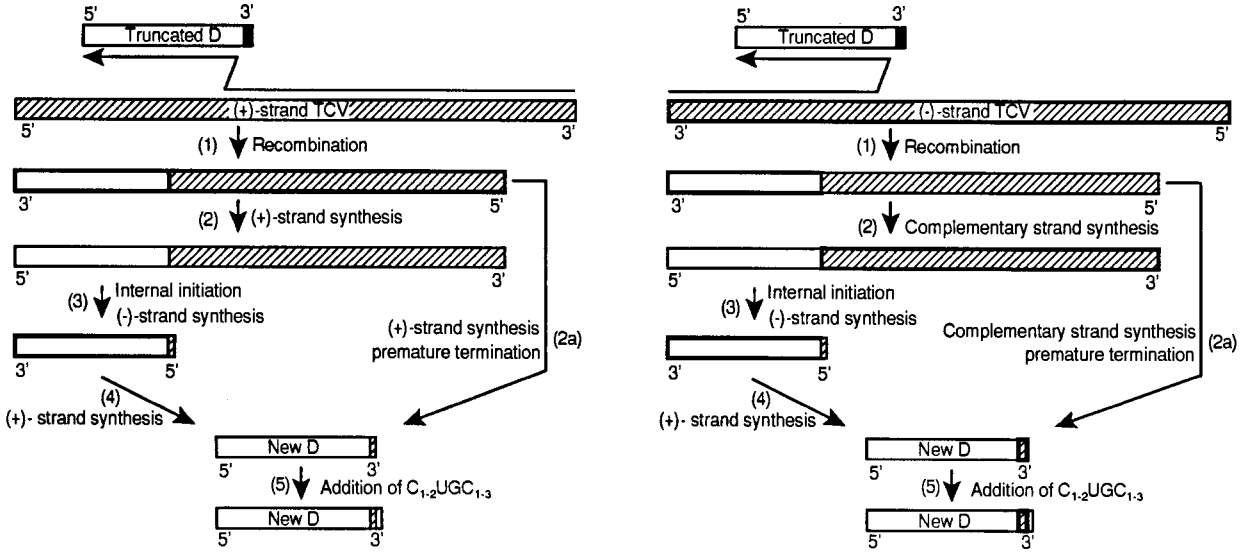

FIG. 4. Model for the generation of sat-RNA D with new 3′ ends. (Left) Incorporation of new sequence from plus-strand TCV. (Right) Incorporation of new sequence from minus-strand TCV. Plus-strand molecules are boxed in thick lines. Step 1: recombination between TCV and sat-RNA D containing 3′-terminal deletions occurring during the synthesis of either plus-strand TCV and minus-strand sat-RNA D (left) or minus-strand TCV and minus-strand sat-RNA D (right). This step would remove most or all of the TCV sequence from the chimeric CX9 or CX10 transcripts (not shown) or the 7 terminal plasmid-derived bases (filled rectangles) from the sat-RNA D transcripts. Steps 2 and 2a: synthesis of full-length (step 2) or truncated (step 2a) complementary strands of the recombinant molecules. If premature termination occurs, this would remove most of the TCV sequence. Step 3: synthesis of complementary strands with internal initiation. If following step 2, this would be required to remove most of the TCV sequence. Step 4: synthesis of full-length plus strands. Step 5: addition of the 3′-terminal motif, $(C_{1-2})UG(C_{1-3})$, or modification of existing nucleotides. This step may or may not be catalyzed by the TCV replicase (see the text).

the truncated sat-RNA D transcripts (and the chimeric transcripts) must be active templates for minus-strand synthesis. Using an RNA transcription system prepared from extracts of TCV-infected turnip plants (38), we should be able to determine which nucleotides present at the 3′ end of sat-RNA D are required for efficient synthesis of minus strands in vitro.

sat-RNA D transcripts truncated by 1 or 3 3′-terminal C residues generated sat-RNA D with the C residues restored (Table 3). Replacement of the C residues may have occurred independently of replication by a terminal transferase activity. We previously showed, using minus-strand sat-RNA C templates and active replicase fractions from TCV-infected turnip plants, that a major product of in vitro transcription was a species generated from elongation of the template's 3′ end in a self-priming reaction. The product of the reaction is in the form of a panhandle, containing the 356-base template and approximately 190 bases of newly synthesized sequence complementary to the 5′ end of the template (39). Eighty-eight percent of the resultant panhandle-like products contained between 1 and 4 nontemplated residues at the junction between template and nascent complementary sequence. Seventy-six percent of the nontemplate sequences were C or CCC. These nontemplate sequences must have been added prior to template-directed RNA synthesis, indicating that the TCV replicase can likely polymerize nucleotides, with a preference for C residues, onto an existing 3′ end. In addition, nontemplate residues, mainly C or U, have been frequently found at the junction of sat-RNA C multimers (6) and junctions of sat-RNA D/sat-RNA C and sat-RNA D/TCV recombinant molecules (7, 10, 11).

Two groups have also reported the repair of altered or missing viral 3′-terminal nucleotides. Rapid and efficient repair of alterations in the 3′-terminal CCA of the brome mosaic virus RNA3 tRNA-like structure in vivo is thought to occur by action of nucleotidyltransferase rather than the slower process of RNA recombination (34). Brome mosaic virus 3′ ends are efficient substrates for in vitro nucleotidyltransferase activity (19), and nucleotidyltransferase and other terminal transferases have been implicated in the maturation and repair of tRNA following nuclease-mediated removal of 3′-terminal nucleotides (14, 45). There is also evidence for 3′-end repair of cymbidium ringspot tombusvirus, a virus related to TCV (12, 13). Alteration or deletion of the 3′-terminal CCC on the viral genomic RNA resulted in the restoration of the terminal C nucleotides, with some RNAs also containing 1 or 2 additional nontemplate G residues upstream of the C's (13). Transcripts of the cymbidium ringspot tombusvirus sat-RNA containing 3′-end deletions of up to 8 bases still were biologically active, generating sat-RNAs with 3′ ends either identical to those of the wild type or containing variable nucleotides in the −5 and −6 positions (12).

The repair mechanisms for viral 3′-terminal sequences may be analogous to the repair mechanism that prevents the loss of telomeric sequences from chromosomes (2). The reformation of the $(C_{1-2})UG(C_{1-3})$ motif at the 3′ terminus of sat-RNA D may occur by a mechanism similar to that used by telomerases, the enzymes that repair cellular telomeres. These enzymes contain an RNA component that acts as a template to direct the correct synthesis of telomeric sequences (2). Besides the possible use of a template to direct the synthesis of the sat-RNA D terminal motif, the motif might be formed by a terminal transferase activity associated with a cellular enzyme or an activity that is part of the TCV replicase. Alternatively, this motif might be added by additional recombination with the 3′ end of TCV.

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