

RNA determinants of junction site selection in RNA virus recombinants and defective interfering RNAs

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ABSTRACT

RNA recombination plays an important role in the diversification and evolution of RNA viruses. Most of these events are believed to be mediated by an actively copying viral replicase switching from a donor template to an acceptor template, where it resumes synthesis. In addition, intramolecular replicase-mediated events (i.e., rearrangements) can lead to the generation of replicable deleted forms of a viral genome, termed defective interfering (DI) RNAs. To gain further insight into the recombination process, the effect of various primary and secondary structures on recombination site selection *in vivo* was examined using plant RNA tombusviruses. The effect of sequence identity and complementarity on deletion events that generate DI RNAs was also investigated. Our results suggest that (1) 5' termini and strong hairpin structures in donor templates represent preferred sites for recombination, (2) junction sites in acceptor templates do not occur in double-stranded regions, (3) nucleotide homology can shift donor and acceptor recombination sites closer to regions of identity and, (4) both sequence identity and complementarity can direct deletion sites in DI RNAs. These results further define RNA determinants of tombusvirus RNA recombination and rearrangement.

Keywords: plant virus; RNA evolution; RNA recombination; RNA replication; RNA structure

INTRODUCTION

Viruses containing RNA genomes constitute an extremely diverse group of pathogens. This high degree of variability is the result of a combination of factors including very high mutation rates, genetic recombination, genome reassortment, gene duplication, and *de novo* gene origin (Keese & Gibbs, 1993). Despite such diversity, related genomic elements can be found in quite remote groups of viruses. A unifying element in all RNA viruses is the gene for RNA-dependent RNA polymerase (RdRp), which is proposed to have a monophyletic origin (reviewed in Koonin & Dolja, 1993). In addition, the analyses of genome structures of distinct RNA viruses suggest that shuffling of genomic elements has occurred (Gibbs, 1987; Goldbach & Wellink, 1988). This has led to the proposed modular theory of RNA virus evolution, which suggests that

segments, or modules, of genetic information have been exchanged between different viruses (Gibbs, 1987). Furthermore, cellular genomic elements such as poly(A) tails and tRNA structures have been identified in various RNA virus genomes (reviewed in Dolja & Carrington, 1992), suggesting the acquisition of sequences from host cells via recombination.

Despite its apparent ubiquity, the mechanism(s) of viral RNA recombination remains poorly understood. It has been proposed that most RNA recombination is mediated by the viral replicase (Kirkegaard & Baltimore, 1986; Cascone et al., 1990; Nagy et al., 1995). In the so-called copy choice model, noncontiguous segments are joined by an actively copying viral RNA replicase switching from one template (i.e., the donor template) to another (i.e., the acceptor template), where it resumes RNA synthesis (Kirkegaard & Baltimore, 1986). In some cases, genome rearrangements are thought to result from erroneous replication, and many RNA viruses generate defective interfering (DI) RNAs (Roux et al., 1991). These molecules represent replicable deletion mutants of viral genomes that do not appear to play any legitimate role in the infectious

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process. In other cases, replicase hopping is thought to represent a normal event in the RNA virus life-cycle. For instance, the synthesis of subgenomic (sg) mRNAs in coronaviruses involves the joining of noncontiguous sequences via discontinuous transcription (Makino et al., 1986).

The two major types of RNA recombination, homologous and nonhomologous, have been observed in various viral systems (Lai, 1992). Homologous recombination occurs between identical sequences at precisely corresponding positions, whereas nonhomologous recombination occurs between sequences that do not share significant identity (Lai, 1992). Recombination junctions, derived from identical sequences, that do not occur at precisely corresponding positions (i.e., plus or minus one or a few residues) are referred to as aberrant homologous recombinants (Lai, 1992).

A great deal of our present knowledge on viral RNA recombination has stemmed from studies on plant viruses. Both brome mosaic bromovirus (BMV) and turnip crinkle carmovirus (TCV) have proven to be useful systems for examining this process (Simon & Bujarski, 1994). Plant tombusviruses also provide an excellent system for studying RNA recombination (White & Morris, 1994b). Tomato bushy stunt virus (TBSV) and cucumber necrosis virus (CNV) are closely related plant tombusviruses that contain single-stranded, messenger-sense RNA genomes of approximately 4.8 kb and encode five similarly organized open reading frames (ORFs; Fig. 1A; Rochon & Tremaine, 1989; Hearne et al., 1990). The 5'-proximal ORF encodes a 33-kDa protein, and read-through suppression of its termination codon allows for the production of a fusion protein of 92 kDa; both are required for genome replication (Scholthof et al., 1995). Coat protein is encoded just downstream of the polymerase ORFs, and 3' to it are two overlapping ORFs encoding proteins that mediate the spread of the infection throughout the plant (Rochon & Johnston, 1991; Scholthof et al., 1993). These 3'-located ORFs are expressed from two sg mRNAs that are synthesized during infections.

When either TBSV or CNV are serially passaged in plants at high concentrations, DI RNAs containing short noncontiguous segments of the viral genome accumulate de novo (Fig. 1A; Knorr et al., 1991; Rochon, 1991). These molecules are amplified because they maintain promoter elements that are recognized by the viral polymerase, which is supplied by the parental genome. Interestingly, TBSV DI RNAs are amplified in coinfections with CNV (White & Morris, 1994a), and it has been demonstrated that these molecules can recombine intermolecularly with nonreplicating 3'-truncated CNV genomes to generate replicable and fully functional CNV-TBSV hybrid genomes (White & Morris, 1994b). The analysis of sequences at the junction sites in these chimeric molecules, as well as those in DI RNAs, suggested that upstream and downstream seg-

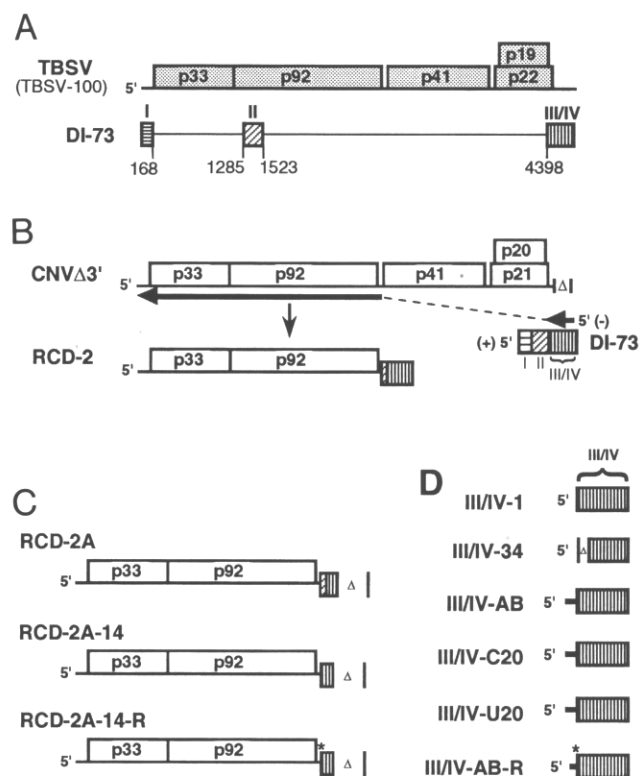


FIGURE 1. A: Schematic representation of the TBSV RNA genome and DI-73. Coding regions in the genome are shown as shaded boxes with the approximate M_r values of the encoded proteins (Hearne et al., 1990). Below, noncontiguous genomic segments present in DI-73 are shown as hatched boxes (denoted by roman numerals) and deleted regions are depicted as lines. B: Proposed replicase-driven mechanism to describe the generation of RCD-2 recombinants (White & Morris, 1994b). C: Schematic representation of the structures of RCD-2-derived acceptor RNA templates. D: Schematic representation of the structures of the structures of DI-73-derived donor RNA templates. Asterisks in C and D indicate the positions of introduced hairpin structures.

ments that share sequence identity and/or complementarity may represent preferred sites for recombination (White & Morris, 1994a, 1994b, 1994c).

The present study examines the effect of various primary and secondary RNA structures on recombination and deletion events. Our data indicate that both types of structure, present in either the same or different RNA templates, can markedly influence the location of junction sites. These results further define RNA determinants of genome recombination and rearrangement in tombusviruses.

RESULTS

Recombination occurs with defective viral RNAs

We have shown previously that coinoculation of protoplasts with a nonreplicating 3'-truncated form of the CNV genome (CNV $\Delta 3'$) and a TBSV DI RNA (DI-73) yields replicable chimeric recombinants (Fig. 1B; White & Morris, 1994b). One class of recombinants, referred

to as RCD-2, contained the 3' terminus of the DI RNA (i.e., region III/IV) fused to a 5' segment of CNV Δ 3' encoding the p33/92 replicase components (Fig. 1B). It was proposed that formation of these molecules occurred through a replicase-driven mechanism in which the replicase-nascent strand complex switched from copying the DI RNA to copying CNV Δ 3' during negative strand synthesis (Fig. 1B, White & Morris, 1994b). The analysis of junctions present in recovered recombinants suggested a relatively unbiased distribution of both 5' and 3' junction sites within limited regions in the donor and acceptor templates (White & Morris, 1994b).

To extend our study of this recombination event, we have constructed various acceptor and donor derivatives of CNV Δ 3' and DI-73, respectively (Fig. 1C,D). The previous observation that replicable RCD-2 molecules contained region III/IV at their 3' termini suggested that this segment alone contains the negative strand promoter (White & Morris, 1994b). To test whether region III/IV on its own could act as a substrate for recombination, a donor molecule containing all but the 5'-terminal residue of region III/IV was constructed (i.e., III/IV-1). This molecule contained a C to G substitution at the second position from its 5' end, which served as a marker. Various *in vitro*-generated viral RNA transcripts were inoculated into protoplasts and, after a 48-h incubation, total nucleic acids were analyzed by northern blotting. Inoculation with wild-type CNV genome (CNV-K2/M5) generated both genomic and sg mRNAs (Fig. 2A). When inoculated singly, no viral RNAs were detected for CNV Δ 3', and only a weak signal corresponding to residual III/IV-1 inoculum was detected after 48 h (Fig. 2A). However, coinoculation of these molecules lead to the consistent accumulation of RCD-2 recombinants (Fig. 2A). These molecules were gel-purified, their junctions amplified

by reverse transcription-PCR (RT-PCR), and the products cloned and sequenced. These and other recombinant RNAs were gel-purified in order to remove residual input molecules, the presence of which could lead to RT-PCR artifacts. The 5' junction sites in the CNV Δ 3' acceptor were distributed relatively randomly just downstream of the p92 stop codon, spanning a region of approximately 80 residues (Fig. 3A). Interestingly, all the 3' junctions in the donor mapped to the 5' end of III/IV-1 (Fig. 3A). These results confirm that region III/IV can serve as a recombination substrate for the generation of replicable RCD-2-type molecules.

Effect of primary sequence on recombination

To see if sequences near the regions of recombination could influence the distribution of junction sites, additional nonreplicating acceptor molecules were constructed. RCD-2A is a derivative of a RCD-2 recombinant molecule containing a 3'-terminal 271-nt truncation (Fig. 1C). This deletion removed approximately three-quarters of region III/IV, leaving a 107-nt segment of identity between the 3'-terminal region of RCD-2A and the 5'-terminal region of III/IV-1. Coinoculation of RCD-2A and III/IV-1 resulted in the formation of RCD-2-sized recombinants (Fig. 2B) with 3' junction sites mapping to the 5' terminus of III/IV-1 (Fig. 3B). Interestingly, we observed a marked clustering of 5' junction sites in RCD-2A closer to the corresponding site of identity (Fig. 3B). A comparable distribution of 5' junction sites was also observed when a derivative of RCD-2A, RCD-2A-14, was coinoculated with III/IV-1 (Fig. 3D). RCD-2A-14 is similar to RCD-2A, except that the small segment of region II (diagonal hatching; Fig. 3B) was deleted so as to remove any potential positive selective influence it might have on recombinant accumulation. The 5' junctions tended to cluster near

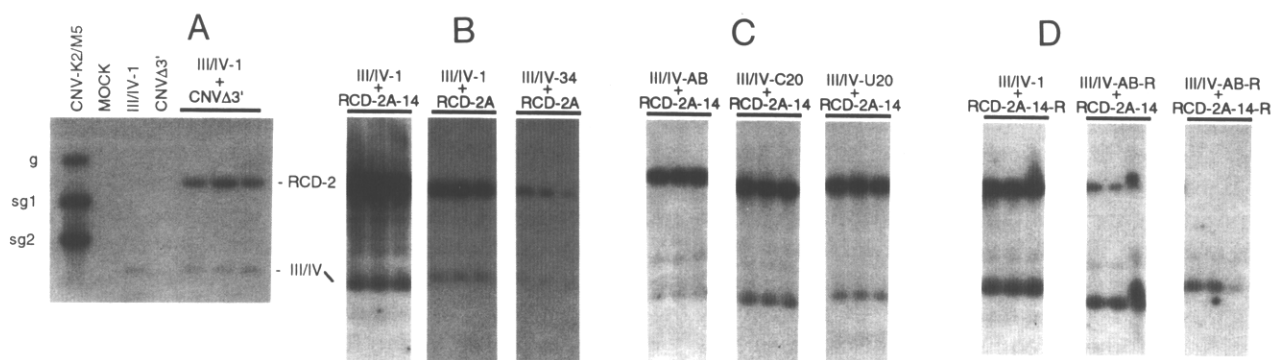


FIGURE 2. Northern blots showing the accumulation of RCD-2 recombinants. Protoplasts (3×10^5) were inoculated with 10 μ g each of acceptor and/or donor transcripts and total nucleic acids were harvested 48-h post-inoculation, separated in a 1.4% agarose gel, and analyzed by northern blotting using [32 P]-end-labeled oligo 9 complementary to the 3' terminus of the TBSV genome. Samples in each of the three lanes denoted by horizontal bars are derived from separate coinoculations. Viral RNAs present in the inoculum are indicated above the lanes in A–D. Positions of genomic (g) and subgenomic (sg) RNAs of CNV are indicated on the left in A, and positions of accumulating RCD-2 recombinants and residual III/IV-1 inoculum are indicated.

Effect of 5' extensions on recombination

To determine if the invariant 3' junctions observed were due to selection for recombinants maintaining the 5'-terminal donor sequences, a nonviral 23-nt-long 5' extension was added to III/IV-1, creating III/IV-AB. Coinoculation of RCD-2A and III/IV-AB yielded RCD-2-sized recombinants (Fig. 2C) with identifiable 5' junction sites clustering just downstream of the stop codon for p92 (Fig. 3E). The majority of the corresponding 3' junctions mapped to the 5' terminus of III/IV-AB and included the nonviral sequence. Similar recombinants were generated when III/IV-C20 and III/IV-U20, possessing 5' extensions with poly(C) or poly(U) tracts, respectively, were coinoculated with RCD-2A (Fig. 2C). The majority of the identifiable 5' junctions in these molecules again mapped to positions downstream of the p92 stop codon and most of the corresponding 3' junctions mapped to the 5' termini of III/IV-C20 (Fig. 3F) and III/IV-U20 (data not shown). These recombinants did, however, maintain different lengths of the poly(C) or poly(U) tracts, which may be due to replicase stuttering (Carpenter et al., 1991). The presence of the 5'-terminal G residues from donors in most of these molecules indicates that the replicase is able to copy poly(C) and poly(U) tracts, and that these tracts do not appear to represent hot-spots for recombination. A small number of homologous recombinants were also observed in coinoculations with donors containing 5' extensions (Fig. 3E,F).

Effect of secondary structure on recombination

Intramolecular secondary structure has been shown to be important in directing recombination sites in TCV (Cascone et al., 1993; Carpenter et al., 1995), although it was not found to have any appreciable effect on homologous recombination in BMV (Nagy & Bujarski, 1995). To determine its effect on recombination in our system, we introduced sequences that formed stable hairpin structures at target regions in our RNA templates. These hairpins were predicted to form by analysis of the corresponding regions with the computer program MFOLD (Zuker, 1989), and their presence was supported by analysis of the respective transcripts with structure-specific endoribonucleases (Fig. 4).

A hairpin structure was introduced into RCD-2A-14 11 nt 3' to the p92 stop codon, creating RCD-2A-14-R (Fig. 4A). This was accomplished by inserting a segment complementary to the 5' portion of region III/IV just upstream of this region. Coinoculation of RCD-2A-14-R with III/IV-1 yielded recombinants (Fig. 2D) with 3' junctions corresponding primarily to the 5' terminus of III/IV-1, and all but one of the 5' junctions mapped upstream of the hairpin structure (Fig. 4A). Interestingly, the upper two-thirds of the hairpin was deleted

in the single recombinant that had its 5' junction site downstream of the hairpin (Fig. 4A, recombinant 204).

To test whether intramolecular secondary structure in III/IV-1 could also affect observed junction sites, a derivative of III/IV-AB was constructed with a hairpin structure at its 5' terminus (III/IV-AB-R; Fig. 4B). Formation of this hairpin sequesters the 5' end of region III/IV into a double-stranded region with the base of the hairpin mapping to a position corresponding approximately to the 5' end of III/IV-34. Coinoculation of RCD-2A-14 and III/IV-AB-R lead to the formation of molecules with 5' junctions corresponding approximately to those in recombinants from RCD-2A+III/IV-34 coinfections (cf. Fig. 4B with 3C), whereas 3' junctions in these molecules mapped to the 3' side of the hairpin structure (Fig. 4B). In addition to these nonhomologous recombinants, a large proportion (53%) of the molecules were the result of homologous recombination occurring downstream of the marker C residue in III/IV-AB-R.

In control RT-PCR reactions using the RCD-2A-14-R transcript as template, the sequence corresponding to the hairpin was deleted from approximately half of the products, but this sequence was maintained in PCR products amplified from a cloned DNA corresponding to RCD-2A-14-R (data not shown). This result indicated that deletion of the hairpin in some of the control RT-PCR products likely occurred during the reverse transcription step. As a consequence of this result, we wanted to confirm our experimental RT-PCR data (which suggested the absence of hairpin-containing recombinants) by a more direct approach. Northern blots of the recombinant RNAs were probed with an oligonucleotide (oligo 48) complementary to the inserted segment forming the 5' half of the hairpins (data not shown). This oligonucleotide hybridized efficiently to control transcripts containing the hairpin (i.e., RCD-2A-14-R) but not to control transcripts lacking the hairpin (i.e., RCD-2A-14). None of the RCD-2 recombinants were detected with oligo 48, indicating that these molecules did not contain the hairpin. These results are consistent with our experimental RT-PCR data.

When III/IV-AB-R and RCD-2A-14-R were coinoculated, no detectable RCD-2-sized recombinants were observed (Fig. 2D). In III/IV-1+RCD-2A-14-R coinoculations, there was decreased levels of accumulation of RCD-2-type recombinants compared to those from III/IV-1+RCD-2A-14 coinfections (cf. Fig. 2D with 2B). Also, recombinant accumulation from III/IV-AB-R+RCD-2A-14 coinfections were dramatically reduced compared to III/IV-AB+RCD-2A-14 coinfections (cf. Fig. 2D with 2C). The consistent inability to detect recombinants in III/IV-AB-R+RCD-2A-14-R coinoculations is likely due, in part, to the cumulative inhibitory effect of the hairpins. Furthermore, any recombinants with junctions 5' and 3' to the hairpins in RCD-2A-14-R and III/IV-AB-R, respectively, would exclude the 5'-

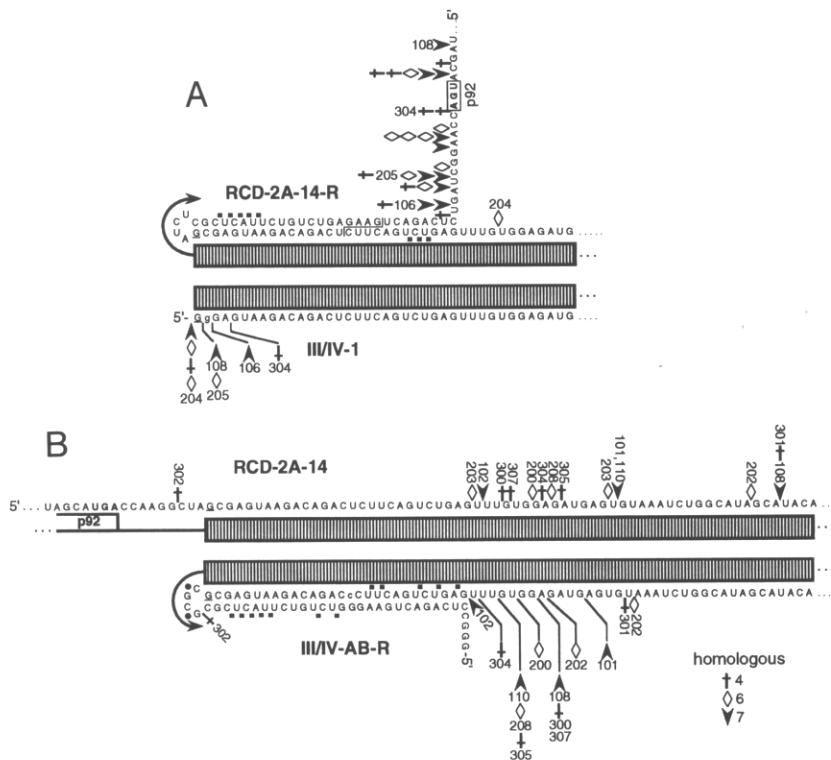


FIGURE 4. Schematic representation of recombination sites in acceptor and donor templates containing hairpin structures. RNA templates involved are labeled in **A** and **B**. Marker nucleotide substitutions are indicated in lowercase and G residues at positions of identity are underlined in acceptor and donor templates. Recombination sites are indicated by arrowheads, diamonds, and daggers, and represent junctions from three separate coinoculations. Corresponding junction sites are designated by three-digit numbers, and the number of homologous recombinants is indicated to the right of the panels. In **A**, the p92 stop codon is boxed and the thin line within the hairpin indicates the portion deleted in 204. Calculated free energies of hairpins shown in **A** and **B** are -47.7 and -50.0 kcal/mol, respectively. Nuclease-sensitive sites within the hairpins are indicated by small solid boxes (ribonuclease V1) or small solid circles (ribonuclease T1).

terminal portion of region III/IV, and this may reduce their viability.

Effect of sequence identity and complementarity on junction sites in DI RNAs

Analysis of deletion borders in TBSV DI RNAs led us to propose that either sequence identity and/or complementarity at the upstream and downstream sites may facilitate selection of junctions during the natural formation of these molecules (White & Morris, 1994a, 1994c). To directly test this concept, we constructed artificial precursor DI RNA molecules containing segments of identity or complementarity. We have described previously a precursor DI RNA molecule, DI-82, in which the segment deleted between regions I and II in DI-72 (Fig. 5A) was reintroduced (White & Morris, 1994a). When this molecule was coinoculated with infectious transcripts of wild-type CNV (CNV-K2/M5; which serves as helper), it transformed after a single passage to DI-72-like molecules via the deletion of sequences between regions I and II (White & Morris, 1994a). A derivative of DI-82, DI-82XP, was constructed with *Xba* I and *Pst* I sites flanking region II (Fig. 5A). A 191-nt-long segment derived from just downstream of region I was then inserted in the minus- and plus-sense orientation at the *Xba* I site in DI-82XP to create DI-82XP-191(-) and DI-82XP-191(+), having segments with either complementarity or identity, respectively, to the segment just 3' to region I (Fig. 5A). These mol-

ecules were individually coinoculated with helper CNV-K2/M5 into protoplasts and, after 24 h, total nucleic acids were isolated and analyzed by northern blotting. DI-82XP and DI-82XP-191(+) were easily identified, however, DI-82XP-191(-) was not detectable (Fig. 5B). Aliquots of these isolated nucleic acids were then used to inoculate a new preparation of protoplasts, and the nucleic acids from these infections were analyzed. In all cases, small DI-72-sized molecules were identified (Fig. 5C). These results are consistent with previous studies that showed that not all precursor DI RNAs accumulate to detectable levels and that DI-82 evolves to DI-72-sized molecules (White & Morris, 1994a).

The smaller viral RNAs generated were gel-purified and their structures were determined by sequencing their corresponding cloned RT-PCR products. The sequences confirmed that segments between regions I and II were deleted to varying degrees, resulting in molecules with structures similar to that of DI-72. These molecules did not contain any helper CNV sequences, which indicated that they arose from either rearrangement or intermolecular recombination involving precursors and/or their deleted forms. Distinct junction sites were observed in the products generated from each of the three different precursors (Fig. 6). Molecules derived from DI-82XP contained nonhomologous junctions with 5' sites mapping just downstream of the 3' end of region I and 3' sites mapping in the 5' portion of region II (Fig. 6A). The junctions in DI-82XP-191(-) were also nonhomologous and were of two dis-

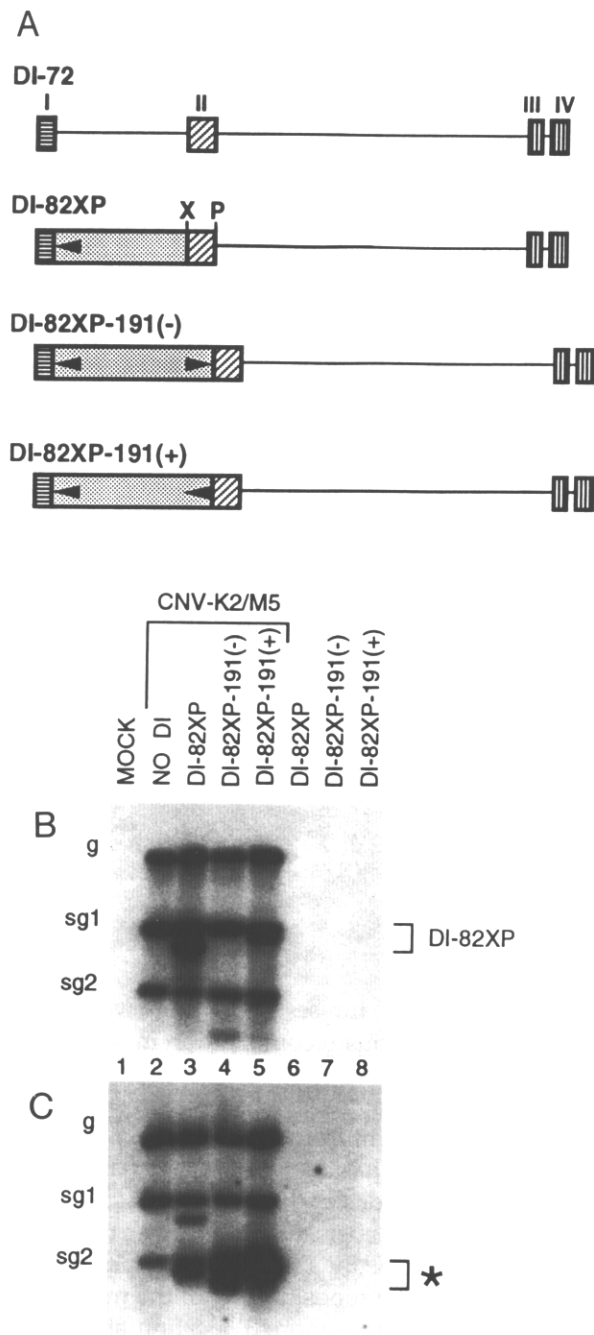


FIGURE 5. **A:** Schematic representation of DI-72 and precursor DI RNAs. Positions of engineered *Xba*I (X) and *Pst*I (P) restriction sites in DI-82XP are indicated. In DI-82XP, the 191-nt-long segment located just 3' to region I is indicated by an arrowhead. In DI-82XP-191(-) and DI-82XP-191(+), this segment was inserted at *Xba*I in the minus and plus orientations, respectively. **B:** Northern blot analysis of precursor DI RNAs in protoplasts. Protoplasts (3×10^5) were inoculated with transcripts of CNV-K2/M5 (2 μ g) and/or precursor DIRNAs (2 μ g). Total nucleic acids isolated 24-h post-infection were separated in neutral 1.4% agarose gels, transferred to nylon, and hybridized with [32 P]-end-labeled oligo 9. Transcripts present in the inoculum are indicated above the lanes. Positions of genomic (g) and subgenomic (sg) RNAs of CNV are indicated on the left and the position of the DI-82XP-type molecules is indicated on the right. **C:** Northern blot analysis of viral RNAs following a single passage of total nucleic acids isolated in B. Position of newly generated small viral RNAs is indicated to the right by an asterisk.

tinct size classes. The larger molecules, which comigrated with sg mRNA 2 (Fig. 5C), had similarly located junction sites to those derived from DI-82XP, whereas the smaller size-class molecules had junction sites located just 5' and 3' to the upstream and downstream 191-nt segments, respectively (Fig. 6A). Junctions in these smaller molecules mapped near the base of a double-stranded region that could potentially form between the two complementary 191-nt-long segments (Fig. 6B). The absence of sequence corresponding to the 191-nt segment in these smaller molecules was confirmed by northern blot analysis using the 191-nt segment as a probe (data not shown). Most of the DI-82XP-(+)-derived molecules (86%) contained homologous junctions within the 191-nt segments of identity.

DISCUSSION

5' termini can represent hot-spots for recombination

In CNV Δ 3'+III/IV-1 coinfections, all the 3' junction sites in the recovered recombinants corresponded to the 5' terminus of III/IV-1. Comparable 3' junction sites were also observed when different acceptor molecules were used, suggesting that the 5' terminus of III/IV-1 represents a preferred site for recombination. An alternative explanation may be that the invariant 3' junctions were the result of selection for molecules that were more replicable or stable. This possibility seems less plausible because III/IV-1 derivatives containing nonviral 5' extensions, or 5' truncations, produced recombinants with 3' junction sites corresponding primarily to the new 5' termini. The data, therefore, are more consistent with the concept that 5' termini in donor molecules do represent hot-spots for recombination.

A replicase-mediated "run-off recombination" mechanism is proposed to explain the biased location of 3' junction sites (Fig. 7A). After initiating synthesis of a negative strand on a donor molecule, the polymerase continues copying until it reaches the extreme 5' end, where it dissociates along with the nascent strand. The complex then rebinds to an internal site in an acceptor template and continues synthesis of the nascent strand. We suggest run-off recombination as an appropriate term to describe this event because the release of the viral polymerase-nascent strand complex from the donor template is similar to the release of bacteriophage RNA polymerases during *in vitro* run-off transcription (Melton et al., 1984).

In BMV, heteroduplex-mediated nonhomologous recombination involves base pairing between sequences at 5' and 3' recombination sites (Nagy & Bujarski, 1993). This base pairing brings the two sites into close proximity and promotes template switching by the replicase. It is unlikely that this mechanism is responsible for generating the nonhomologous junctions observed

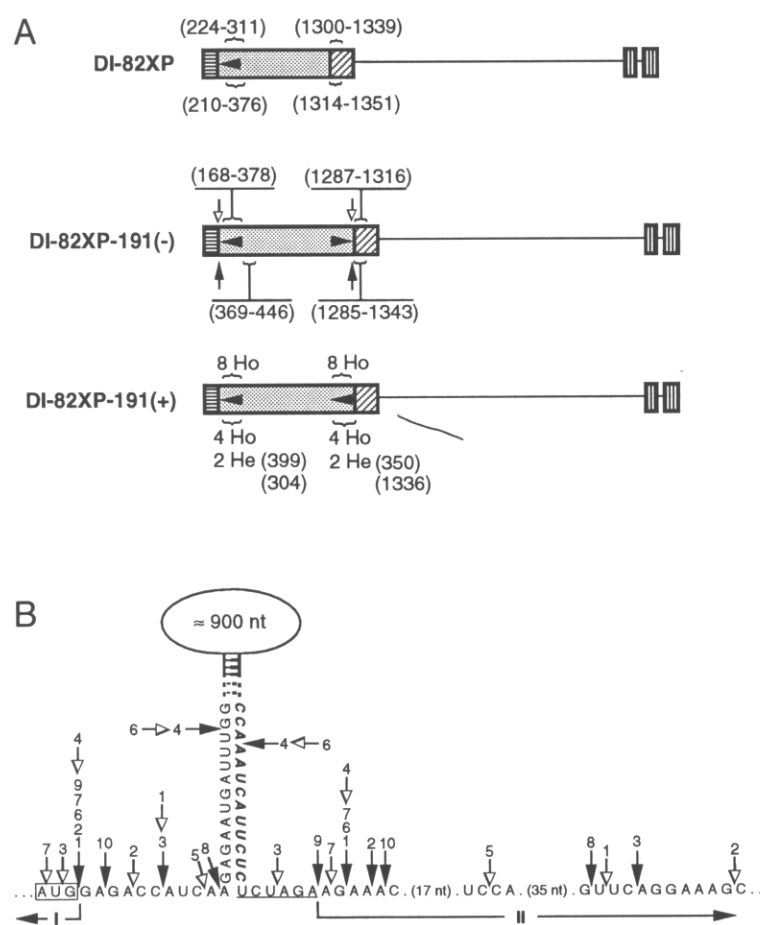


FIGURE 6. A: Schematic representation of the positions of junction sites in precursor DI RNAs. Observed junction sites from two separate experiments are indicated above and below the respective DI RNAs. Locations of 5' and 3' junction sites are indicated by horizontal brackets and, where applicable, the corresponding range of coordinates is given in parentheses. Short arrows (open and solid) show the approximate positions of 5' and 3' junctions in the smaller size class molecules derived from DI-82XP-191(-). The number of homologous (Ho) and heterologous (He) junction sites in DI-82XP-191(+) are indicated. **B:** Junction sites in the smaller size class molecules derived from DI-82XP-191(-). The nucleotide sequence surrounding the 3' end of region I and the 5' end of region II in DI-82XP-191(-) is shown. A portion of the base paired region that could potentially form between the inserted 191-nt segment (italicized) and the complementary upstream sequence is shown. Positions of junction sites in molecules from two separate experiments are mapped as open and solid arrows, and corresponding 5' and 3' sites are indicated by number. Start codon for p33/92 is boxed, *Xba* I site is underlined, and regions I and II are delineated.

with III/IV-1 because there is no sequence upstream of the 5'-terminus of this molecule available to base pair with the acceptor template. This suggests that other mechanisms, such as the proposed run-off recombination, are capable of generating nonhomologous recombinants.

At present, it is unclear whether run-off recombination occurs during natural RNA virus infections, but one can imagine that breaks in viral RNAs occurring *in vivo* could promote recombination. This idea is supported by the proposal that breaks in retroviral RNAs may promote recombinational repair *in vivo* (Coffin, 1979) and this so-called forced copy choice mechanism has been shown to occur *in vitro* with Moloney murine leukemia virus (Luo & Taylor, 1990).

Nucleotide identity changes the distribution of 5' junction sites

Recombinants derived from coinfections with acceptor and donor RNAs that shared no significant sequence identity exhibited a relatively random distribution of 5' junction sites, whereas those from coinoculations with molecules that shared stretches of identity showed a biased distribution of 5' sites closer to corresponding

positions of identity. Interestingly, there were few examples of homologous recombination in coinfections of either RCD-2A or RCD-2A-14 with III/IV-1. One explanation for this may be that the marker substitution in III/IV-1, located 1 nt from its 5' terminus, interfered with this process. It has been suggested that misincorporation of nucleotides at the 3' end of nascent donor strands may lead to aberrant homologous recombination events (Nagy & Bujarski, 1995; Pilipenko et al., 1995). However, the repositioning of the mismatched marker in III-IV-34 to a more 3' location also yielded few homologous recombinants, suggesting that homologous recombination occurs rather infrequently under these experimental parameters.

In coinoculations with III/IV-1, approximately 66% of the junctions in either RCD-2A or RCD-2A-14 were within 10 nt of the corresponding position of identity, whereas the comparably located regions in CNV Δ 3' contained approximately 36% and 40%, respectively. This shift in 5' sites suggests that the stretch of identity may assist in the alignment of these sequences, perhaps via base pairing of the nascent strand with complementary sequence in the template (Fig. 7A). The actual sites of reinitiation may, however, be somewhat imprecise and not necessarily correspond to iden-

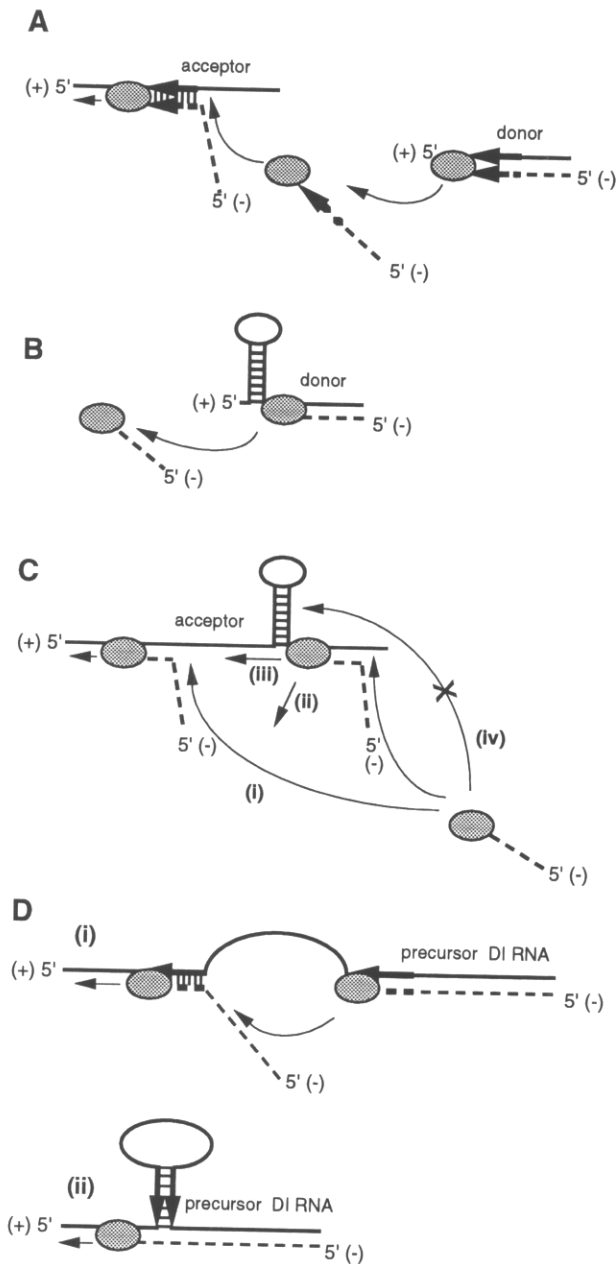


FIGURE 7. Proposed replicase-mediated mechanisms for the formation of various recombinant viral RNAs (see text for details). RNA templates are depicted as solid lines and nascent strands are shown as dashed lines. Viral replicase is represented by shaded ovals and its path is indicated by thin arrows. **A:** Run-off recombination mechanism. Bold arrows indicate segments of identity that may be involved with alignment of recombination sites. **B:** Dissociation of replicase-nascent strand complex promoted by a hairpin structure in donor template. **C:** Influence of hairpin structure in acceptor template on reinitiation sites. **D:** Deletion of large segments from precursor DI RNA molecules directed by tracts (bold arrows) of sequence (i) identity or (ii) complementarity.

tical positions (as observed previously; White & Morris, 1994b). Factors such as the length and composition of the stretches of identity, as well as their secondary structure, may influence the frequency of homologous recombination.

Secondary structure influences junction sites in recombinants

Intramolecular RNA secondary structure plays an important role in recombination in the TCV system (Cascone et al., 1993; Carpenter et al., 1995), although it has not been demonstrated to significantly affect homologous recombination events in BMV (Nagy & Bujarski, 1995). When a hairpin structure was introduced near the 5' terminus of our donor molecule (i.e., III/IV-AB-R), there was a downstream shift in 3' junction sites in recombinant molecules to positions corresponding to the 3' side of the hairpin. Recombinants derived from donor templates containing similarly sized 5' extensions that were not predicted to form strong secondary structure (e.g., III/IV-AB and III/IV-C20) had 3' junctions that mapped primarily to donor 5' termini. These results support the concept that the 3' base of a hairpin structure in the donor molecule can represent a preferred site for recombination, however, selection for replicable molecules may also be contributing to these results. We suggest that generation of these 3' junction sites is due to the hairpin acting as an obstacle, which causes the replicase to dissociate before reaching the 5' terminus of the template (Fig. 7B). Alternatively, the formation of recombinant 302 in Figure 4B can be better explained by a heteroduplex-mediated mechanism, whereby the 5' extension in III/IV-AB-R base pairs with the 5'-terminal segment of III/IV in RCD-2A-14. This suggests that the heteroduplex-mediated mechanism for recombination (Nagy & Bujarski, 1993) may also operate in our system.

When a similar hairpin structure was introduced into an acceptor molecule (i.e., RCD-2A-14-R), 5' junction sites were predominantly located upstream of the structure (Fig. 7C-i). It is possible that reinitiation of the polymerase downstream of the structure causes it to dissociate when it encounters the hairpin (Fig. 7C-ii), and our results with III/IV-AB-R where junctions mapped to the 3' side of the hairpin support this idea. There was, however, one 5' junction in a RCD-2A-14-R-derived recombinant that mapped downstream of the hairpin, but sequence corresponding to a large portion of the hairpin was deleted in this molecule. This suggests that, in some instances, the polymerase is able to bypass the hairpin and, in doing so, deletes a portion of it (Fig. 7C-iii). Similar results have been reported in BMV (Bujarski et al., 1994). Interestingly, there were no 5' junction sites located within the predicted double-stranded stem region in RCD-2A-14-R-derived recombinants, suggesting that this section in the acceptor template is not a preferred site for recombination (Fig. 7C-iv). If, as suggested, these recombination events are replicase-mediated, the polymerase may be unable to reinitiate synthesis within the double-stranded stem region and instead may have to target less structured regions located either upstream or

downstream. Reinitiation of RNA synthesis by the replicase may therefore require unpaired residues in the acceptor template.

The clustering of recombination sites at the base of hairpins in our templates is likely related primarily to secondary structure and does not involve specific-sequence motifs such as those required for recombination in TCV (Cascone et al., 1993; Carpenter et al., 1995). In BMV, recombination via the heteroduplex-mediated mechanism also does not appear to require a particular primary sequence (Nagy & Bujarski, 1993; Dzianott et al., 1995), and it has been suggested that it is the inability of the replicase to unwind secondary structure that causes template switching (Bujarski & Dzianott, 1991; Nagy & Bujarski, 1993). In support of this concept, it was found that mutations in the helicase-like domain of the BMV polymerase shifted junction sites to less energetically stable regions of the heteroduplex (Nagy et al., 1995). Interestingly, the viral RNA polymerases of tombusviruses do not contain any identifiable helicase-like motifs (Koonin & Dolja, 1993) and this may, in part, explain their apparent non-progressive nature.

RNA sequence and structure direct junctions sites in DI RNAs

Our results on the transitions of precursor DI RNAs support a role for both sequence identity and complementarity in the selection of deletion sites during the generation of these molecules. Analysis of junctions in DI-72-like molecules derived from DI-82XP indicated a relatively random distribution of nonhomologous junctions. However, when segments of identity were introduced, as in DI-82XP-190(+), primarily homologous junctions were observed. It is unlikely that the exact maintenance of the 190-nt segment confers a selective advantage to recombinant molecules because this region is deleted in highly competitive DI RNA molecules (Knorr et al., 1991; White & Morris, 1994a). Instead, homologous junctions within this region likely reflect mechanistic features of the deletion events. The tracts of identity could be directing the selection of junction sites by providing an opportunity for base pairing between the 3' sequence of the replicase-complexed nascent strand and the complementary sequence upstream in the template (Fig. 7D-i). This proposed mechanism is similar to that suggested for intermolecular recombination events (Fig. 7A), however, it is would most likely involve only one template (as depicted in Fig. 7D-i). Unlike the recombinants that arose from the III/IV-1+RCD-2A and similar coinoculations, the junctions in the DI RNAs appear to be more precise, occurring primarily at corresponding positions. The reason for the observed difference in overall precision is unclear, but is likely related to differences in size, composition, secondary structure, and/or location of the duplications.

Nonetheless, our data do indicate that sequence identity in precursor DI RNAs can promote homologous deletion events.

The examination of junction sites in TBSV DI RNAs led us to suggest that intramolecular secondary structure may also be directing the sites of deletions in TBSV DI RNAs (White & Morris, 1994a, 1994c). Similar suggestions have been made for the formation of DI RNAs from other plant (broad bean mottle bromovirus; Romero et al., 1993) and animal (flock house nodavirus; Li & Ball, 1993) viruses. Our results with DI-82XP-191(-) provide experimental evidence in support of this concept. The junction sites in the smaller size class of recombinants were found to map close to the base of the double-stranded tract predicted to form between the complementary 191-nt segments positioned approximately 900-nt apart. These data are consistent with a mechanism for the generation of deletions in DI RNAs whereby the replicase bypasses copying an intramolecularly base paired sequence (Fig. 7D-ii) in a manner similar to the proposed heteroduplex-mediated mechanism for intermolecular recombination in BMV (Nagy & Bujarski, 1993).

The deletion events that lead to the formation of DI RNAs appear to share some elements with RNA recombination. Both types of processes are influenced by sequence identity and complementarity, and similar types of RNA interactions, which are consistent with the proposed mechanisms for junction site selection, can be predicted. These studies provide further insight into the role of RNA structure in the selection of junction sites in RNA virus recombination and rearrangements and they provide a foundation for more detailed investigations of these processes.

MATERIALS AND METHODS

Materials

Plasmid K2/M5 (Rochon & Johnston, 1991), TBSV-100 (Hearne et al., 1990), CNV Δ 3' (White & Morris, 1994b), DI-82, DI-73, and DI-72 (White & Morris, 1994a) have been described. The following deoxyoligonucleotides (oligos) were used in this study (underlined residues correspond to nonviral sequence; residues not underlined correspond to viral sequence, with residues in lowercase representing marker substitutions):

7F, 5'-GGCGGAGCTCTAATACGACTCACTATAGGAAA

TTCTCCAGGATTTCTC [TBSV, (+)sense, 1-20];

9, 5'-GGCGGCCCGCATGCCCGGGCTGCATTCTGCA

ATGTTCC [TBSV, (-)sense, 4754-4776];

12, 5'-GATGGTCCGGAACGTGACAACAGCC [CNV,

(+)sense, 2200-2224];

30, 5'-GGCGTCTAGATAATACGACTCACTATAGGAG

TAAGACAGACTCTTCAG [TBSV, (+)sense,

4399-4420];

- 34, 5'-GGCGTCTAGATAATACGACTCACTATAGGA
GATGAGTTTAAATCTGGC [TBSV, (+)sense,
4432-4452];
- 36, 5'-GCGGCGTCTAGATAATACGACTCACTATA
GGGCCCTAACTGCAGAATTGCGCGCGAGTAA
GACAGACcCTTCAGTC [TBSV, (+)sense,
4399-4422];
- 37, 5'-CGCGCGGAGTAAGACAGACcCTTCAGTCTG
AGGGCC [TBSV, (+)sense, 4399-4425];
- 38, 5'-CTCAGACTGAAGgGTCTGTCTTACTCGCG
[TBSV, (-)sense, 4399-4425];
- 39, 5'-GCGGCGTCTAGATAATACGACTCACTATA
GGGC₍₂₀₎GCGAGTAAGACAGACTCTTCA
GTC [TBSV, (+)sense, 4399-4422];
- 40, 5'-GCGGCGTCTAGATAATACGACTCACTATA
GGGT₍₂₀₎GCGAGTAAGACAGACTCTTCAGT
C [TBSV, (+)sense, 4399-4422];
- 41, 5'-GCGGCGGCTAGCCTTGGTCATGCTACGGCGG
AGTC [CNV, (-)sense, 2591-2613];
- 42, 5'-GCGGCGGCTAGCGAGTAAGACAGACTCTTCA
GTCTG [TBSV, (+)sense, 4399-4424];
- 44, 5'-GGCCCTCTAGACTCTACGCAGAAGACTCTCTC
CAC [TBSV, (-)sense, 1284-1261];
- 45, 5'-GGCCTCTAGAGAGAATGATTTGGCCTAAGAAA
GAG [TBSV, (+)sense, 180-204];
- 46, 5'-GGCCGGCCGGCTAGCCAGCACAATCAGTTTT
GAGTAATTC [TBSV, (-)sense, 346-370];
- 47, 5'-CTAGTCTCAGACTGAAGAGTCTGTCTTACTC
GCT [TBSV, (-)sense, 4398-4426]; and
- 48, 5'-CTAGAGCGAGTAAGACAGACTCTTCAGTCTG
AGA [TBSV, (+)sense, 4398-4426].

Plasmid construction

RCD-2A is a 3'-truncated derivative of RCD-2-721 (an RCD-2 recombinant) and was generated by digestion of pRCD-2-721 with *Sal* I (position 4501, TBSV) and *Sma* I (position 4774, TBSV) followed by replacement of the smaller virally derived fragment with a *Sal* I/*Pvu* II fragment from pUC19. RCD-2A-14, which contains a modified CNV-TBSV DI RNA junction, was created by PCR amplification of two segments, seg1 and seg2, respectively, of pRCD-2A with the oligo sets 12 and 41, and 42 and M13/pUC primer #1224 (New England Biolabs) under conditions described previously (White & Morris, 1994a). Oligos 12 and 41 were engineered to include *Msc* I and *Nhe* I restriction sites, respectively, whereas oligo 42 contained a *Nhe*

I site, and a *Sal* I site was located near the #1224 priming site. Seg1 and seg2 products were digested with *Msc* I/*Nhe* I and *Nhe* I/*Sal* I, respectively, and ligated simultaneously into a *Msc* I/*Sal* I-digested pRCD-2A, thus creating RCD-2A-14. RCD-2A-14-R was created by digestion of RCD-2A-14 with *Nhe* I, followed by ligation to annealed oligos 47 and 48.

III/IV-1, corresponding to region III/IV, was created by PCR amplification of region III/IV of DI-73 with oligos 9 and 30 using conditions described previously (White & Morris, 1994a). Oligo 30 included a T7 RNA polymerase promoter to allow for the synthesis of in vitro transcripts of III/IV-1. Derivatives of III/IV-1 containing various 5' modifications (i.e., III/IV-34, III/IV-AB, III/IV-C20, and III/IV-U20), were generated in a similar manner using oligo 9 in combination with oligos 34, 36, 39, and 40, respectively. III/IV-AB-R was generated by digestion of III/IV-AB with *Apa* I and *Bss*H II (which were sites introduced into the 5' extension in III/IV-AB) followed by ligation to annealed oligos 37 and 38.

DI-82XP, a derivative of DI-82 (White & Morris, 1994a), was created by PCR amplification of residues 1-1284 from clone TBSV-100 (Hearne et al., 1990) using oligos 7F and 44. Oligo 7F included a *Sac* I site and a T7 RNA polymerase promoter and oligo 44 included an *Xba* I site. The product was digested with *Sac* I/*Xba* I and ligated into *Sac* I/*Xba* I-digested DI-72XP (White & Morris, 1994c). The resulting construct, DI-82XP, contained a contiguous 5' TBSV terminus up to region II, and possessed *Xba* I and *Pst* I sites at the 5' and 3' ends of region II. DI-82XP was used to make DI-82XP-191(-) and DI-82XP-191(+) by inserting a 191-nt segment (positions 180-370) at the *Xba* I site of DI-82XP in the minus- or plus-sense orientation, respectively. The 191-nt insert was generated by PCR amplification using oligos 45 and 46, both of which contained *Xba* I sites. The authenticity of all constructs was verified by restriction enzyme analysis and/or dideoxynucleotide sequencing.

In vitro transcription, protoplast infection and RNA analysis

In vitro transcription was conducted on *Xba* I-digested RCD-2 derivatives and *Sma* I-digested III/IV-1 or DI-82XP derivatives using the Ampliscribe™ T7 RNA polymerase transcription kit (Epicentre Technologies). The transcripts were further processed as described previously (White & Morris, 1994a). Hairpin-containing transcripts (III/IV-AB-R and RCD-2A-R) were digested with structure-specific ribonucleases and analyzed by primer extension with oligo 47 as described previously (Shelness & Williams, 1985). Protoplasts, prepared from cucumber (variety Straight 8) cotyledons, were inoculated with in vitro-generated viral transcripts, and total nucleic acids were prepared 24- and 48-h post-inoculation as described previously (White & Morris, 1994a). Aliquots (a tenth) were separated in neutral 1.4% agarose gels and viral RNAs were detected by electrophoretic transfer to nylon (Hybond-N, Amersham) followed by northern blot analysis using [³²P]-5'-end-labeled oligo 9.

cDNAs corresponding to junctions in different recombinant viral RNAs were prepared by first purifying the molecules from 1% low melting temperature agarose gels using Gelase (Epicentre Technologies). The eluted RNAs were subsequently subjected to RT-PCR as described previously (White & Morris, 1994a). RCD-2-type recombinants were amplified with oligos

9 and 12 and DI RNAs were amplified with oligos 9 and 7F. These products were then digested with *Xba* I and *Sph* I or *Sac* I and *Sph* I, respectively, gel purified (Geneclean, Bio 101), and ligated into *Xba* I/*Sph* I- or *Sac* I/*Sph* I-digested pUC19. Clones were sequenced by the dideoxynucleotide chain termination method, some of which was performed by the Center of Biotechnology DNA Sequencing Facility (University of Nebraska-Lincoln).

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