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Frameshift mutations in infectious cDNA clones of *Citrus tristeza virus*: a strategy to minimize the toxicity of viral sequences to *Escherichia coli*

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Abstract

The advent of reverse genetics revolutionized the study of positive-stranded RNA viruses that were amenable for cloning as cDNAs into high-copy-number plasmids of *Escherichia coli*. However, some viruses are inherently refractory to cloning in high-copy-number plasmids due to toxicity of viral sequences to *E. coli*. We report a strategy that is a compromise between infectivity of the RNA transcripts and toxicity to *E. coli* effected by introducing frameshift mutations into “slippery sequences” near the viral “toxicity sequences” in the viral cDNA. *Citrus tristeza virus* (CTV) has cDNA sequences that are toxic to *E. coli*. The original full-length infectious cDNA of CTV and a derivative replicon, CTV- Δ Cla, cloned into pUC119, resulted in unusually limited *E. coli* growth. However, upon sequencing of these cDNAs, an additional uridylylate (U) was found in a stretch of U's between nts 3726 and 3731 that resulted in a change to a reading frame with a stop codon at nt 3734. Yet, in vitro produced RNA transcripts from these clones infected protoplasts, and the resulting progeny virus was repaired. Correction of the frameshift mutation in the CTV cDNA constructs resulted in increased infectivity of in vitro produced RNA transcripts, but also caused a substantial increase of toxicity to *E. coli*, now requiring 3 days to develop visible colonies. Frameshift mutations created in sequences not suspected to facilitate reading frame shifting and silent mutations introduced into oligo(U) regions resulted in complete loss of infectivity, suggesting that the oligo(U) region facilitated the repair of the frameshift mutation. Additional frameshift mutations introduced into other oligo(U) regions also resulted in transcripts with reduced infectivity similarly to the original clones with the +1 insertion. However, only the frameshift mutations introduced into oligo(U) regions that were near and before the toxicity region improved growth and stability in *E. coli*. These data demonstrate that, when hosts are sufficiently susceptible for infection by transcripts of reduced specific infectivity, introduction of frameshift mutations at “slippery sequences” near toxic regions of viral cDNAs can be used as an additional strategy to clone recalcitrant viral sequences in high-copy-number plasmids for reverse genetics. © 2003 Elsevier Science (USA). All rights reserved.

Introduction

Reverse genetics, the ability to produce specific mutations followed by examination of phenotype, revolutionized the study of RNA viruses. The first successful attempts initiated infection of cells by inoculation with bacterial plasmids containing the viral cDNA inserts (Taniguchi et al., 1978; Racanicillo and Baltimore, 1981). However, production of in vitro RNA transcripts that were identical to the

virion RNA (except for designed mutations and perhaps a few extra nucleotides at either terminus) allowed genetic manipulation using the standard inoculation procedures previously in use for virion RNAs (Ahlquist et al., 1984). The current ideal is to clone a full-length viral cDNA in a high-copy-number bacterial plasmid behind a promoter for a commercially available RNA polymerase. Most viruses with relatively small RNA genomes have been amenable for such a genetic system. However, some viruses are inherently difficult to clone due to the instability of the viral cDNA, often because the viral sequences are toxic to the bacterium (Boyer and Haenni, 1994; Lai, 2000).

The cloning difficulties of some recalcitrant viral genomes have been surmounted by a variety of methods. In

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in vitro ligation of individually cloned cDNA fragments followed by in vitro transcription and transfection was used to overcome the cloning difficulties of *Yellow fever virus*, *Mouse hepatitis virus*, *Transmissible gastroenteritis virus* (TGEV), and *Beet necrotic yellow vein virus RNA2* (Quillet et al., 1989; Rice et al., 1989; Yount et al., 2000, 2002). The disadvantages of this approach, though, are relatively low levels of full-length cDNA and reduced levels of RNA transcripts compared to that of in vitro transcripts from a complete clone in one plasmid. Another approach is to reduce the toxicity of viral sequences in high-copy-number plasmids by inserting introns into the viral sequences. Recently, cDNA clones of TGEV, *Japanese encephalitis flavivirus*, *Pea seedborne mosaic virus*, and *Plum pox virus* were stabilized by this method (Johansen, 1996; López-Moya and García, 2000; Yamshchikov et al., 2001; González et al., 2002). This strategy, however, requires cytoplasmic viruses to have a nuclear phase and also requires that the viral sequences not have endogenous intron sequences, which has been suggested as a possible problem of large cytoplasmic RNA viruses (Lai, 2000). In fact, *Tobacco mosaic virus* (TMV) appears to be an example of such a virus with an endogenous intron, based on the fact that the infectivity of 35S:cDNA constructs are several orders of magnitude less than RNA transcripts and often are infectious in only certain plant species (W.O. Dawson, unpublished data). Moreover, most cells of tobacco plants with transgenes of the infectious cDNA of TMV behind the 35S promoter do not become infected from the transgene, although they do become infected via virus movement from other cells (Turpen, 1992). Yet another approach is to avoid the use of high-copy-number plasmids. The reticent RNA genome of TGEV could be propagated in *Escherichia coli* when cloned into a bacterial artificial chromosome, a low-copy-number plasmid (Almazán et al., 2000). Other approaches avoid the use of bacteria. Vaccinia virus was used as a vector to clone the coronaviruses, *Avian infectious bronchitis virus* (IBV) and *Human coronavirus* (Casais et al., 2001; Thiel et al., 2001). Although this approach allowed the cloning of the full-length coronavirus cDNA, the viral cDNA in the vaccinia virus vector is not very amenable for mutational analysis, requiring multiple cycles of ligation and recombination. Here we report an additional strategy to allow cloning of recalcitrant viral cDNAs in high-copy-number plasmids in *E. coli* that can be added to the repertoire of strategies available.

Citrus tristeza virus (CTV), a member of the *Closterovirus* genus of the *Closteroviridae* family, has a single-stranded positive-sense RNA genome of ~20.0 kb that is organized into 12 open reading frames (ORFs) (Fig. 1A) (Pappu et al., 1994; Karasev et al., 1995). ORF 1a encodes a polyprotein containing two papain-like protease domains, plus methyltransferase-like (MT) and helicase-like (HEL) domains, and a large interdomain region (IDR) between the MT and HEL domains (Fig. 1A). ORF 1b encodes an RNA-dependent RNA polymerase-like domain that is

thought to be translated by a +1 frameshift from ORF 1a (Karasev et al., 1995). The ten 3' ORFs are expressed by 3'-coterminally sgRNAs (Hilf et al., 1995) that encode an assembly gene block of four proteins (Satyanarayana et al., 2000), a protein that controls asymmetry of CTV RNAs (p23) (Satyanarayana et al., 2002a), and five proteins of unknown function.

The development of infectious cDNA clones of CTV was difficult due to the large size of the genomic RNA and its toxicity to *E. coli* (Satyanarayana et al., 1999). We were able to maintain the plasmids containing the viral cDNA in only one strain of *E. coli*, which after transformation grew very slowly into minute colonies. However, we found that this level of growth was due to a fortuitous frameshift in the CTV cDNA that allowed its cloning by reducing the toxicity to *E. coli*. The corrected cDNA exhibited substantially greater toxicity to *E. coli*, now requiring 3 days to see visible colonies. Examination of other frameshift mutations demonstrated that 6–7 U's functioned as "slippery" frameshift sites, reducing toxicity to *E. coli* if positioned appropriately near the toxicity region in the viral cDNA. Thus, purposely introduced frameshift mutations into oligo(U) regions in a toxic viral cDNA can provide a compromise between infectivity and toxicity to *E. coli*. If hosts cells are susceptible enough to allow a reduction in infectivity, frameshift mutations can be inserted in the viral cDNA to allow cloning in high-copy-number plasmids of *E. coli*.

Results

Characteristics of the original infectious cDNA clones of CTV

The full-length infectious cDNA clones of CTV, pCTV9, and its derivatives were constructed in pUC119 behind the SP6 RNA polymerase promoter and grown in *E. coli* strain JM109 (Satyanarayana et al., 1999). CTV- Δ Cla contains the 5'-nontranslated region (NTR) plus ORFs 1a and 1b and the first 105 nts of ORF 2 (nts 1–11,005) fused to the 3' 768 nts of the genomic RNA that includes part of the p23 ORF and the 3'-NTR (nts 18,526–19,293). The cDNA of this small replicon with the ten 3' genes (7.5 kb) deleted is much easier to manipulate than the full-length virus (Satyanarayana et al., 2002b). The in vitro generated RNA transcripts were biologically active as evidenced by their replication in *Nicotiana benthamiana* protoplasts and synthesis of sgRNAs (Fig. 1, CTV9 and CTV- Δ Cla), and production of viable CTV9 virions that passaged with a specific infectivity similar to that of the parental wild-type virus (Gowda et al., 2001; Satyanarayana et al., 1999, 2000, 2001, 2002a, 2002b). The RNA transcripts of full-length CTV and the replicon replicated similarly in citrus protoplasts (data not shown). The recombinant virus had a phenotype identical to that of the parental wild-type virus in citrus trees (Satyanarayana et al., 2001).

pCTV9 and pCTV- Δ Cla were equally toxic to *E. coli*,

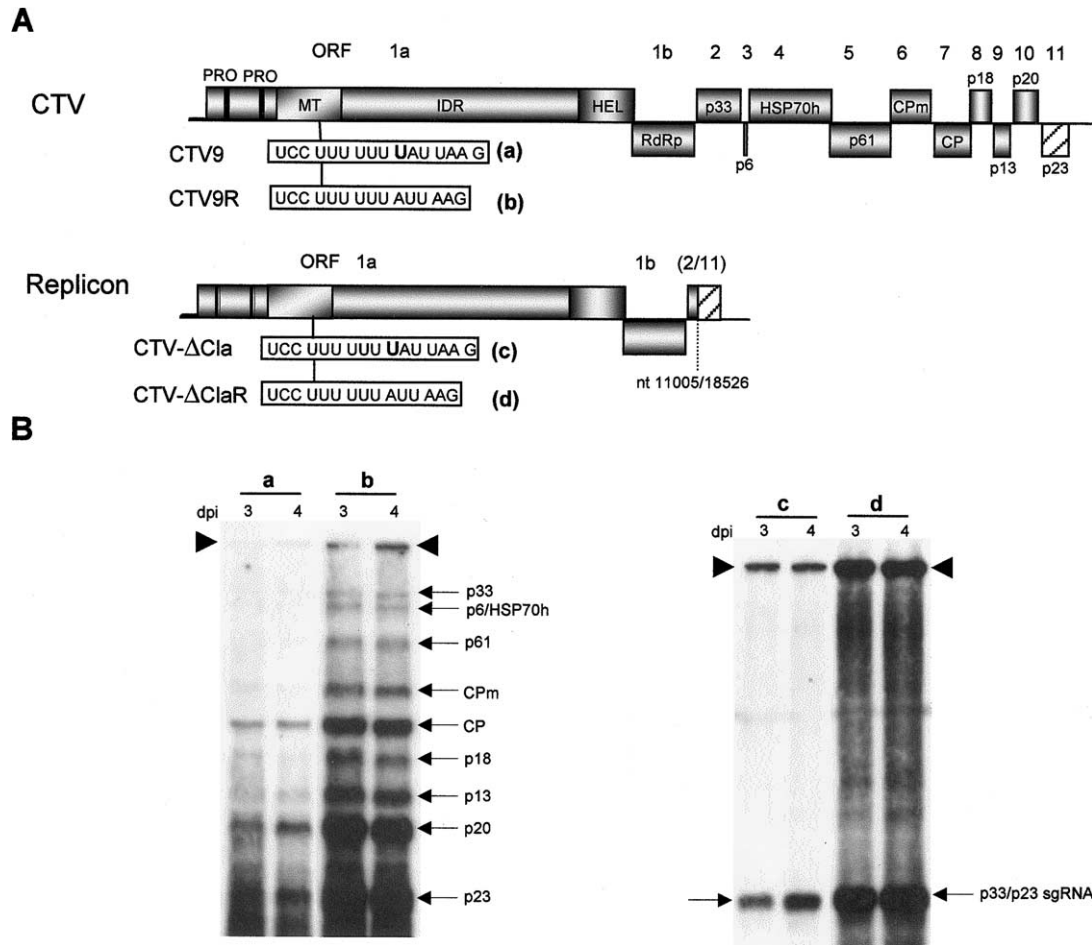


Fig. 1. Replication of wild-type CTV and replicon with and without a +1 insertion at nt 3732 in *Nicotiana benthamiana* mesophyll protoplasts. (A) Schematic diagram of the genome organization of wild-type CTV and derivative replicon showing the putative domains of papain-like proteases (PRO), methyltransferase (MT), helicase (HEL), a large interdomain region (IDR), and an RNA-dependent RNA polymerase (RdRp). The ORFs (boxes) with their numbers and corresponding translation products are also indicated. HSP70h, HSP70 homolog; CPm, minor coat protein; CP, major coat protein; (2/11), fusion of ORFs 2 and 11 with the deletion of internal sequence. The fortuitous frameshift found at nt 3732 (nt in bold) in CTV9 (a) and CTV-ΔCla (c) was corrected in CTV9R (b) and CTV-ΔClaR (d). (B) Northern blot hybridization analysis of total RNA isolated from protoplasts transfected with CTV9 (a), CTV9R (b), CTV-ΔCla (c), and CTV-ΔClaR (d) at 3 and 4 days postinoculation (dpi). The position of genomic and subgenomic (sg) RNAs corresponding to ORFs 2–11 are indicated by arrowheads and arrows, respectively. The blots were hybridized with 3' 900-nt positive-stranded RNA-specific digoxigenin-labeled probe.

but grew enough on LB agar-amp plates for minute colonies to be picked for propagation after 24 h, compared to 1.8-mm-diameter colonies of the vector pUC119 after overnight incubation at 37°C (Fig. 2). The cDNA clones grew marginally in 2xYT liquid medium, but failed to grow in LB liquid medium. From numerous *E. coli* strains examined, the original cDNA clones of CTV in pUC119 were stable only in JM109.

Sequence analysis of pCTV9

To compare the sequence of the full-length infectious cDNA clone with that of the consensus sequence from the native quasispecies of parental CTV T36 isolate (GenBank Accession No. NC_001661), both strands of pCTV9 were sequenced completely (GenBank Accession No. AY170468). Although we found numerous nucleotide differences, we ob-

served an additional “U” inserted at nt 3732 into a homopolymeric run of 6 U’s that resulted in a shift to a reading frame with a stop codon at nt 3734. A similar frameshift mutation was also found in the derivative replicon pCTV-ΔCla. However, the sequence of the progeny virion RNA of CTV9 from infected protoplasts or citrus plants did not contain the additional nt at position 3732 that occurred in the cDNA clones, suggesting that the frameshift mutation present in the original cDNA clone was repaired either during the in vitro transcription or during replication in the protoplasts.

Repair of the +1 insertion in original cDNA clones resulted in increased replication, but increased toxicity to E. coli

The +1 insertion found at nt 3732 of the cDNA clones was corrected in pCTV9 and pCTV-ΔCla by exchanging a

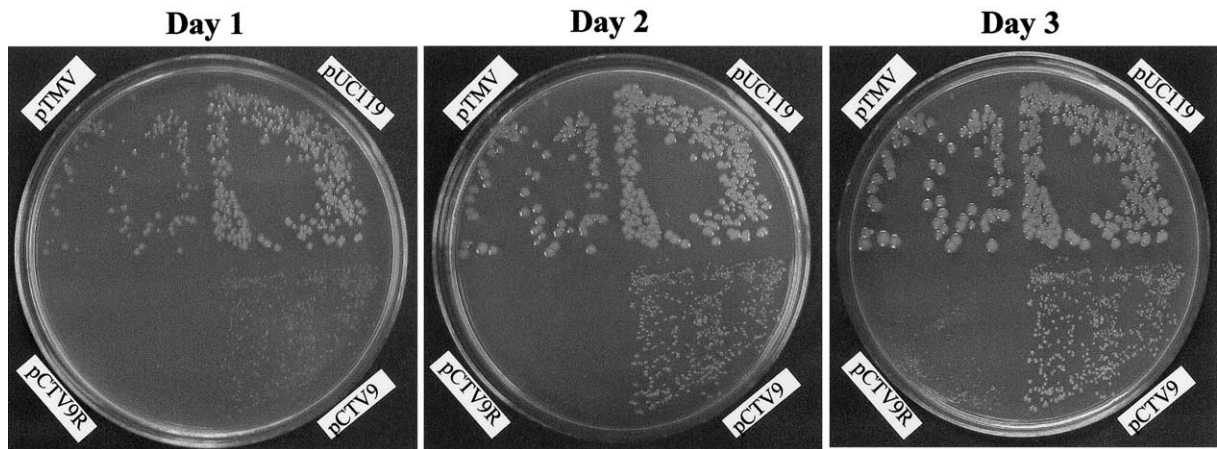


Fig. 2. *E. coli* colonies on LB-agar plates transformed with pCTV9 (with a +1 insertion at nt 3732) and pCTV9R (without a frameshift at nt 3732) at 1, 2, and 3 days posttransformation. The parental plasmid pUC119 and the cDNA clone of TMV in pUC19 (pTMV) (Dawson et al., 1988) were used as controls for comparison of the size of *E. coli* colonies to evaluate the toxicity of CTV sequences.

RT-PCR amplified product between the unique *Nco*I (nt 3685) and *Bsu*36I (nt 4430) restriction endonuclease sites creating pCTV9R and pCTV- Δ ClAR (Fig. 1A). Replication of the full-length and replicon transcripts from the original and corrected cDNA clones was compared in *N. benthamiana* protoplasts. The RNA transcripts from the repaired clones accumulated genomic and sgRNAs about 30 to 40-fold more than those from the original cDNAs (Fig. 1B, compare a and b, and c and d). We then examined the number of protoplasts infected in the presence or absence of a frameshift mutation by introducing green fluorescent protein (GFP) ORF into CTV using mutants CTV-GFP-p23 (with a +1 insertion at nt 3732) and CTVR-GFP-p23 (no frameshift at nt 3732) (Fig. 3A). The number of protoplasts infected with CTVR-GFP-p23 that exhibited fluorescence under UV light was approximately 18- to 30-fold more than those inoculated with CTV-GFP-p23 (Fig. 3C), again with the accumulation of approximately 30 to 40-fold more genomic and sgRNAs than those of CTV-GFP-p23 (Fig. 3B).

The original CTV clones were toxic to *E. coli*, resulting in substantially reduced colony sizes (Fig. 2). Bacteria transformed with the repaired clones pCTV9R or pCTV- Δ ClAR grew much slower than those containing the original clones, producing visible colonies only 3 days after transformation with ligation mixtures. However, *E. coli* transformed with purified plasmid DNAs of pCTV9R or pCTV- Δ ClAR produced visible colonies slightly earlier. The yields of plasmid DNA from *E. coli* strain JM109 harboring pCTV9 and pCTV9R after overnight growth in 2xYT liquid medium were 2.5–3.0 and 1.5–2.0 μ g of DNA per milliliter of culture, respectively, compared to 8–10 μ g DNA per milliliter of culture for pTMV (Dawson et al., 1988). Thus, although the original CTV clones with the frameshift mutation reduced the growth of *E. coli* and plasmid DNA yields, the correction of the +1 insertion in pCTV9R further reduced the growth of *E. coli* to a level below the range of expectations for routine laboratory work. Although not

quantified here, the degree of difficulty of ligating and isolating intact cDNA clones was proportionally increased with the increased toxicity of the repaired constructs.

Examination of other frameshift mutations in ORF 1a

We expected frameshift mutations in CTV to be lethal. We were surprised that cDNA clones of CTV with a frameshift mutation would produce transcripts with an appreciable amount of infectivity in protoplasts. We further examined this phenomenon by introducing additional frameshift mutations into CTV- Δ ClAR. We introduced +1 insertion mutation in other regions of ORF 1a to examine whether the frameshift mutations would result in a viable virus similar to that of original cDNA clones that contained a +1 insertion at 3732 nt. Two mutations were introduced separately at nt 4079 in the IDR (GCC AAG CUU UGU AAG, frameshift nt in bold) and at nt 8390 in the HEL domain (AGG AAG CUU AGC UGC, frameshift mutation in bold), creating Δ ClAR-M1 and Δ ClAR-M2, respectively (Fig. 4A, e and h). The in vitro RNA transcripts from these mutants failed to replicate to detectable levels in protoplasts (Fig. 4B, e and h).

Examination of frameshifts in oligo(U) stretches

In contrast to the frameshifts examined above, the +1 insertion found in the original infectious cDNA clones that allowed infectivity and greatly reduced the toxicity of the cDNA in *E. coli* was in a run of 6 U's, which is known to be a "slippery sequence" associated with frameshifting in other viral systems (Wilson et al., 1988; Brierley et al., 1992). However, frameshifting also has been correlated with downstream structures (Jacks et al., 1988; Chamorro et al., 1992). It is possible that the 6 U's at 3732 had an adventitious accessory structure downstream, and the frameshifting at this position was unusual. To examine

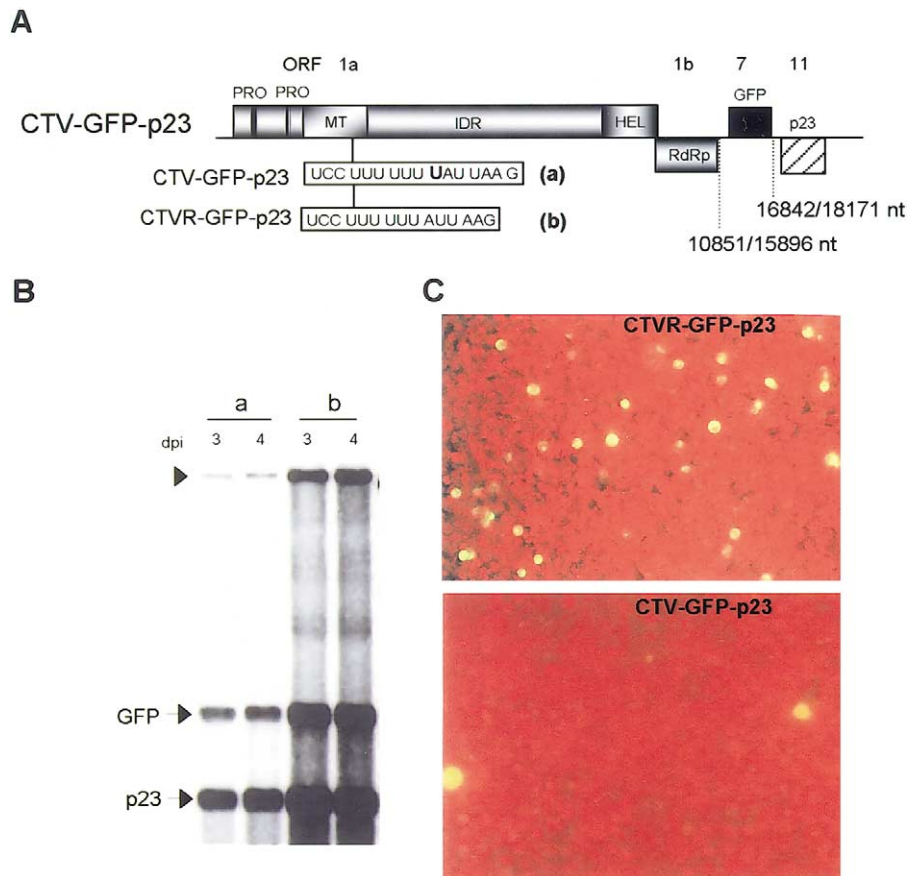


Fig. 3. Expression of GFP in *N. benthamiana* protoplasts transfected with CTV with and without a +1 insertion at nt 3732. (A) Schematic diagram of CTV-GFP-p23. The CP ORF was precisely exchanged with GFP ORF in CTV-CP/p23 (Satyanarayana et al., 2002a). CTV-GFP-p23 contained a +1 insertion at nt 3732 (nt in bold) (a), and the +1 insertion was corrected in CTVR-GFP-p23 (b). (B) Northern blot analysis of accumulation of positive-stranded genomic, and GFP and p23 sgRNAs from *N. benthamiana* protoplasts transfected with CTV-GFP-p23 and CTVR-GFP-p23 at 3 and 4 dpi using a 3' positive-stranded RNA-specific riboprobe. The genomic and sgRNAs are indicated with an arrowhead and arrows, respectively. (C) A representative field of protoplasts expressing GFP transfected with CTVR-GFP-p23 or CTV-GFP-p23 at 4 dpi at approximately the same density viewed by a fluorescent microscope.

whether other U-rich regions of CTV would similarly function as slippery-sequence frameshifting signals to recover infectivity and reduce toxicity in *E. coli*, we created frameshifts in other oligo(U) sites. A run of 6 U's occurs at four different places in ORF 1a at nts 1103–1108, 3726–3731, 4957–4962, and 7078–7083. Mutations to alter the reading frame of ORF 1a in each of the U-rich regions were created (Fig. 4A). The plasmids were evaluated for toxicity to *E. coli*, and the RNA transcripts were evaluated for replication in protoplasts.

A +1 insertion mutation was introduced at nt 1109 in the L-ProI region to obtain Δ ClaR-M3 (Fig. 4A, c). A +2 insertion mutation was introduced at nt 3732 in the MT domain of ORF 1a to obtain Δ ClaR-M4 (Fig. 4A, d). These mutants replicated in protoplasts and produced genomic and sgRNAs at reduced levels, 30- to 50-fold lower when compared to that of CTV- Δ ClaR, but with approximately comparable levels to that of CTV- Δ Cla, which contained a +1 insertion at nt 3732 (Fig. 4B, b–d). Similarly, a +1 insertion was introduced at the 6 U's at nt 4963 in the 5' half of IDR to obtain Δ ClaR-M5, and an adenylate was deleted after the

6 U's at nt 7084 in the 3' half of IDR to create Δ ClaR-M6 (Fig. 4A, f and g). Mutant Δ ClaR M6 replicated at levels comparable with that of the original CTV- Δ Cla, while mutant Δ ClaR-M5 replicated slightly less. Both accumulated 30- to 50-fold less viral RNAs than that of the wild-type CTV- Δ ClaR (Fig. 4B, f and g). These results suggested that the homopolymeric stretch of U's in ORF 1a are shift-prone sequences.

We examined the requirements of U-rich regions as shift-prone sequences by introducing silent mutations plus a +1 insertion to disrupt the U-run of the original U-rich region between nts 3726 and 3732 (UUU UUU U changed to UUC UUC U), resulting in mutant CTV- Δ Cla-M7 (Fig. 5A, b). As a control, similar silent mutations were introduced in CTV- Δ ClaR between nts 3726 and 3731 without the reading frame mutation (UUU UUU A changed to UUC UUC A), resulting in CTV- Δ ClaR-M8 (Fig. 5A, d). Mutant CTV- Δ Cla-M7 failed to replicate to detectable levels in protoplasts, suggesting that the homopolymeric stretch of U's facilitated frameshifting (Fig. 5B, b). As expected, CTV- Δ ClaR-M8 replicated approximately similarly to that

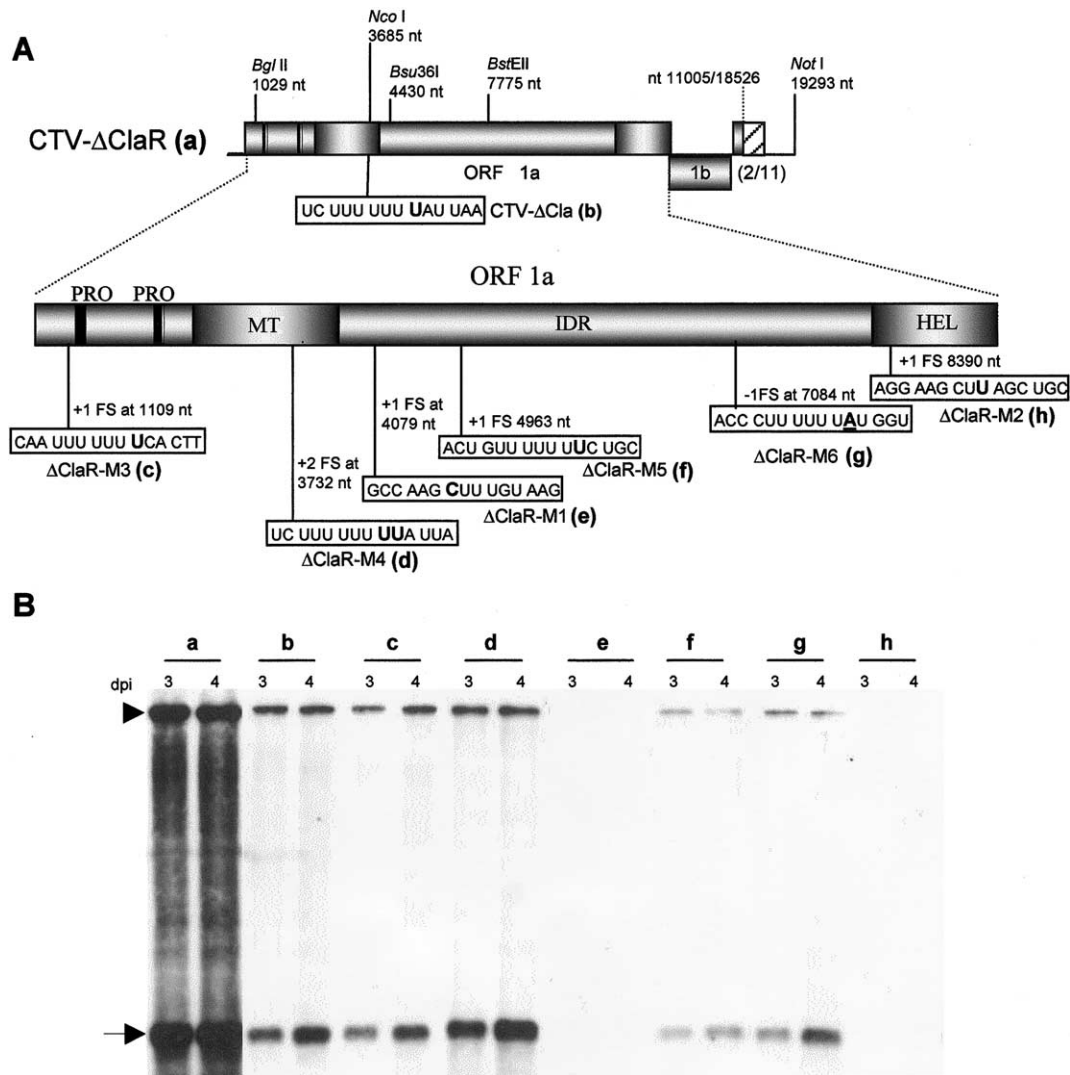


Fig. 4. Replication of insertion (+1 and +2) and deletion (–1) mutants in ORF 1a of CTV in *N. benthamiana* protoplasts. (A) The schematic diagram of CTV replicon, CTV- Δ ClaR, containing ORF 1a and 1b plus portions of ORFs 2 and 11 (a). Selected unique restriction sites used to generate mutants in CTV- Δ ClaR are indicated above the diagram. The +1 insertion found at nt 3732 (nt in bold) in CTV- Δ Cla (b) is shown below the CTV- Δ ClaR. The expanded view of ORF 1a is shown below CTV- Δ ClaR with the location of +1 or +2 insertion, and –1 deletion mutations in ORF 1a (c–h). The inserted nucleotides are shown in bold. The nucleotide in bold and underlined was deleted in Δ ClaR-M6 (g). The different domains of ORF 1a are as outlined in the legend for Fig. 1A. (B) Northern blot analysis of accumulation of positive-stranded genomic and sgRNAs from *N. benthamiana* protoplasts transfected with insertion or deletion mutants at 3 and 4 dpi. The blots were hybridized with a 3' positive-stranded RNA-specific riboprobe. The position of the genomic and sgRNAs are indicated by an arrowhead and an arrow, respectively.

of CTV- Δ ClaR, demonstrating that the silent mutations did not affect the replication (Fig. 5B, d).

Effect of frameshift mutations in ORF 1a on toxicity to *E. coli*

E. coli transformed with intact, supercoiled plasmids containing the original cDNA clones (pCTV9 and pCTV- Δ Cla) with the +1 insertion mutation produced visible colonies after overnight incubation, whereas 2–3 days were required to see visible colonies with plasmids containing the repaired clones CTV9R and CTV- Δ ClaR (Figs. 2 and 6), suggesting that the frameshift mutation present in the orig-

inal cDNA clones reduced the toxicity to *E. coli*. Since the original full-length (CTV9) and replicon (CTV- Δ Cla) cDNA clones were equally toxic to *E. coli* (the repaired clones equally more toxic) (Fig. 6), the toxic region appeared to be located within ORFs 1a or 1b. The frameshift at nt 3732 reduced the toxicity of the original clones, suggesting that the toxicity domain was between nts 3732 and 11011. This location is supported by the observation that, when constructing cDNA clones for assembly of the full-length cDNA clone, we were able to clone the sequences between *Nco*I (nt 3685) and *Bst*EII (nt 7775) only in the reverse orientation in pUC119 (pT36-Nco-Bst; Satyanarayana et al., 1999).

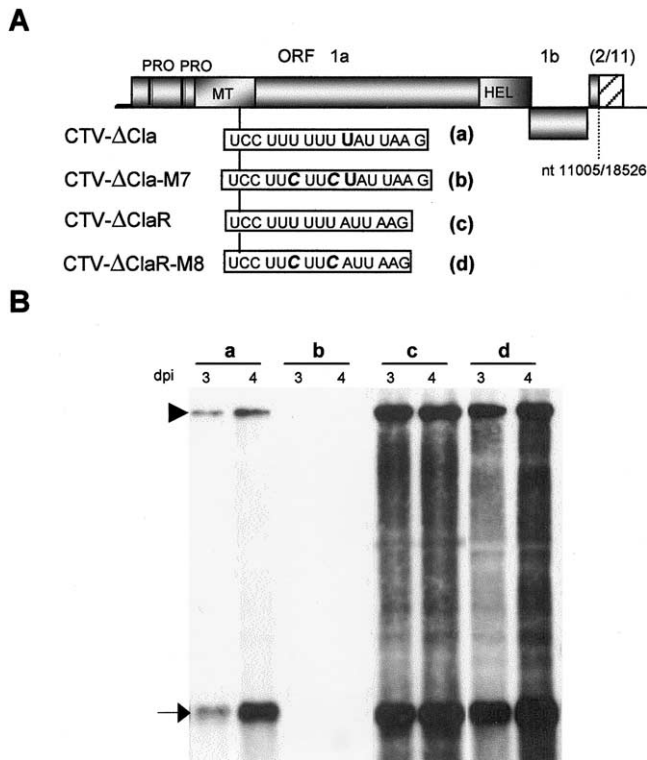


Fig. 5. The oligo (U) stretch in ORF 1a of CTV facilitated frameshifting in vivo. (A) The schematic diagram of CTV- Δ Cla showing the sequence around nt 3732 containing an additional nt "U" (in bold) (a), the codons UUU UUU in CTV- Δ Cla encoding phenylalanine were changed to UUC UUC without changing the amino acid sequence (nts in bold and italics) to create CTV- Δ Cla-M7 (b). As a control the condons UUU UUU in CTV- Δ ClaR (corrected clone: without a +1 U insertion) (c) were also changed to UUC UUC (nts in bold and italics) in CTV- Δ ClaR-M8 (d). The domains of ORF 1a are as outlined in the legend for Fig. 1A. (B) Northern blot analysis of accumulation of positive-stranded RNAs from protoplasts transfected with CTV- Δ Cla and CTV- Δ ClaR and their silent mutants at 3 and 4 dpi. The blots were hybridized with a 3' positive-stranded RNA-specific riboprobe. The genomic and sgRNAs are indicated with an arrowhead and an arrow, respectively.

We examined the effects of the other frameshift mutations on toxicity to *E. coli*. The purified plasmids containing the frameshift mutations were used to transform *E. coli* strain JM109, and bacterial growth on LB agar plates was compared with that of *E. coli* transformed with pCTV9, pCTV- Δ Cla, pCTV9R, pCTV- Δ ClaR, pUC119, and pTMV (Dawson et al., 1988). Mutants with frameshift mutations between nts 3732 and 7084 (p Δ ClaR-M1, -M4, -M5, -M6, pCTV9, and pCTV- Δ Cla) produced colonies of 0.2–0.9 mm diameter at 1 day posttransformation (dpt) and 0.9–1.6 mm diameter at 2 dpt, respectively (Figs. 2 and 6), compared to 1.5–1.8 and 2.6–2.7 mm diameter colonies containing pUC119 and pTMV at 1 and 2 dpt, respectively. However, we failed to observe visible colonies under the microscope with the p Δ ClaR-M3 (with a frameshift mutation at nt 1109), p Δ ClaR-M2 (with a frameshift at nt 8390), pCTV9R, or pCTV- Δ ClaR after overnight incubation at 37°C; however, the colonies grew to 0.2–0.3 mm diameter

after 2 days of incubation (Fig. 6). Thus, the frameshift mutations in ORF 1a around the IDR (p Δ ClaR-M1, -M4, -M5, -M6) minimized the toxicity to *E. coli*, while the mutations introduced before (p Δ ClaR-M3) or after (p Δ ClaR-M2) failed to minimize the toxicity (Fig. 6), suggesting that the toxicity region was located between nts 3732 and 8390.

Discussion

We found that frameshifts within U stretches in CTV were repaired during transcription or replication, resulting in infection of approximately 2–3% as many protoplasts as the repaired transcripts. Oligo(U) stretches have been identified as slippery sequences that are involved in frameshifting of other viruses (Wilson et al., 1988; Brierley et al., 1992; Honda et al., 1995; Kim et al., 2001). However, frameshifting also is often associated with downstream structures in the RNA (Jacks et al., 1988; Brierley et al., 1992; Chamorro et al., 1992; Vickers and Ecker, 1992; Chen et al., 1995; Marczinke et al., 1998; Barry and Miller, 2002). In HIV, a run of 6 U's is required for efficient -1 frameshifting to produce the gag-pol proteins with an extended run of U's resulting in slightly increased levels of frameshifting (Wilson et al., 1988; Honda et al., 1995). However, Parkin et al. (1992) reported that a stem-loop structure 3' of the HIV -1 shift site was important for wild-type levels of frameshifting in vivo. Nevertheless, downstream structures appeared not to be needed in the observed repair of the frameshift mutations after replication in *N. benthamiana* protoplasts. We did not identify any common structures by examining the sequences with the MFOLD program (data not shown). Also, since we examined all of the 6 U-regions, none of which are thought to naturally be involved in frameshifting, there is no expectation that all would conserve some accessory structure needed for frameshifting.

Slippery sequences identified in retrovirus frameshifting include AAAAAAC (*Mouse mammary tumor virus*); AAAUUUA (*Rous sarcoma virus*); UUUUUUA (HIV-1 and HIV-2); and UUUAAAC in coronavirus frameshifting (IBV) (Jacks et al., 1987, 1988; Wilson et al., 1988; Brierley et al., 1992). Additionally, numerous other slippery sequences have been identified (Brierley et al., 1992; Marczinke et al., 1998; Kim et al., 2001) and some of them occur throughout the CTV genome. We did not examine whether other potentially slippery sequences effect frameshifting in CTV. However, we examined six oligo(U) sites that allowed frameshifting when a mutation was created to destroy the reading frame. It would be expected that each of these sites also shift to an incorrect reading frame in the absence of mutations. If there are additional slippery sequences scattered throughout the CTV genome that also occasionally shift the reading frame, it is fortunate that sufficient full-length translation product is produced to allow efficient

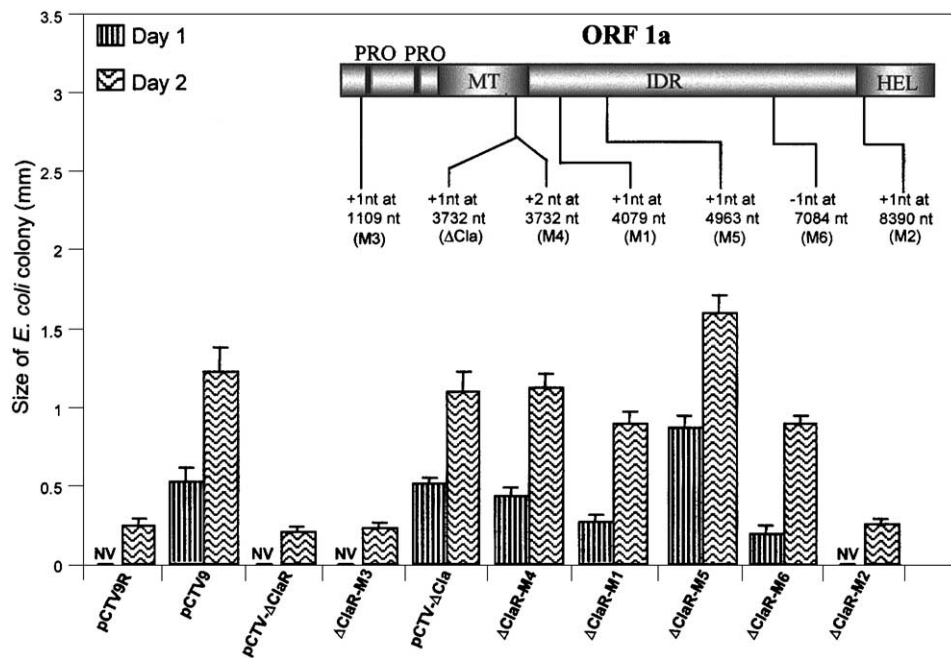


Fig. 6. Effect of insertion (+1 and +2) and deletion (–1) mutations in ORF 1a of CTV on *E. coli* strain JM109 colony size on LB-agar plate. The purified plasmid DNAs of pCTV9, pCTV9R, pCTV-ΔC1a, and pCTV-ΔC1aR, and frameshift mutants were used to transform *E. coli*. The location of frameshift mutations introduced in ORF 1a are indicated in upper right corner of the graph. The domains of ORF 1a are as described in the legend for Fig. 1A. The data represent mean and standard deviation of 25–30 colonies at 1 and 2 days posttransformation. NV, colonies not visible.

replication. Yet it is possible that slippery sequences serve as a survival mechanism for large RNA viruses. Viral RNA polymerases are known to be error prone (Drake and Holland, 1999) without an error repair mechanism, defining viruses as pseudospecies of sequence variants (Domingo et al., 1995). It has been argued that recombination and selection are the viral mechanisms to repair errors. Slippery sequences could serve as an additional repair mechanism. If a mutation occurs upstream of a slippery sequence, a frameshift could repair the mistake if the intervening altered amino acids are tolerated. Perhaps there is an advantage for CTV to have slippery sequences scattered throughout ORF 1a. This could be one of the reasons some CTV isolates have been observed to have a high degree of evolutionary stasis (Albiach-Martí et al., 2000).

The frameshifts inserted into the oligo(U) regions of CTV created a compromise between infectivity and toxicity of the cDNA in high-copy-number plasmids in *E. coli*. The number of protoplasts that became infected with RNA transcripts from the frameshifted clone was reduced by 30- to 50-fold when compared to that of the corrected clone. However, the toxicity in *E. coli* was greatly reduced. Bacterial colonies were visible after 1 day, even though they were minute, compared to 2–3 days with the repaired clone. The progeny virus from the frameshifted clone was repaired; it did not contain the original frameshift caused by the inserted nucleotide and had a phenotype identical to the parental wild-type virus (Satyanarayana et al., 2001). Thus, the frameshift mutation appeared to have no negative impact on the value of the clones other than the initial reduc-

tion in infectivity. Developing an infectious, full-length cDNA clone of CTV was difficult. It is unlikely that we would have obtained an infectious full-length clone without a fortuitous frameshift mutation in the original full-length cDNA clone.

These results suggest that purposely inserting frameshifts at slippery sequences of viral cDNAs that are toxic to *E. coli* could be an additional strategy for cloning these viruses in convenient high-copy-number plasmids. The frameshift provides a compromise: a reduction of infectivity for a decrease in toxicity in *E. coli*. If the host system is susceptible enough to provide infection with a couple of magnitudes of decrease in infectivity, the frameshift should allow growth of an infectious clone in *E. coli*. If the host cells are even more susceptible, it is possible that two frameshifts could be used. As is evident from this work, all frameshifts in slippery sequences will not minimize the toxicity to *E. coli*. The frameshift must be positioned to prevent expression of the toxicity domain, similar to the insertion of introns for the same purpose. We examined only stretches of U's, but if the frameshifting occurred during translation, it is likely that other identified slippery sequences would also work. Since the repair of the frameshift mutations was due to in vitro transcription or translation in protoplasts, neither of which are virus-specific functions, the purposeful introduction of frameshift mutations into frameshift-prone sequences in front of toxic regions should work for any recalcitrant positive-stranded RNA virus. Thus, this strategy provides an additional approach in the virologist's reper-

toire for developing reverse-genetic systems for difficult viruses.

Materials and methods

Plasmid construction

The assembly of a full-length infectious cDNA clone of CTV T36, pCTV9, and a replicon, pCTV- Δ Cla, behind the SP6 RNA polymerase promoter in pUC119 was reported previously (Satyanarayana et al., 1999). The +1 insertion mutation found at nt 3732 in pCTV9 and pCTV- Δ Cla was corrected by reverse transcription of the region between nts 3559 and 4630 followed by amplification by PCR from the double-stranded RNA extracted from CTV T36 isolate-infected bark tissue. The amplified cDNA fragment was digested with *Nco*I (nt 3685) and *Bsu*36I (nt 4430) restriction endonucleases and followed by ligation into similarly digested pCTV9 and pCTV- Δ Cla to obtain pCTV9R and pCTV- Δ ClaR, respectively (Fig. 1A). CTV-GFP-p23 was obtained by precisely exchanging the CP ORF with that of GFP in CTV-CP/p23 (Satyanarayana et al., 2002a). Subsequently, the +1 insertion mutation found at nt 3732 in CTV-GFP-p23 was removed by exchanging a DNA fragment between *Nco*I and *Bsu*36I from pCTV9R to obtain CTVR-GFP-p23 (Fig. 3A).

The frameshift mutations in ORF 1a were introduced by PCR using a set of positive- and negative-sense oligonucleotides that contained the intended mutation, followed by overlap-extension PCR (Ho et al., 1989) of the region between unique restriction sites in pCTV- Δ ClaR (Fig. 4A). The +2 and +1 insertion mutations at nts 3732 and 4079, respectively, were introduced by amplifying DNA fragments that contained the mutations between nts 3559 and 4630 and then ligated between *Nco*I (nt 3685) and *Bsu*36I (nt 4430) restriction sites into pCTV- Δ ClaR to obtain Δ ClaR-M4 and Δ ClaR-M1, respectively (Fig. 4A). Insertion mutations (+1) were introduced at nt 8390 by amplifying a PCR product between nts 7747 and 19293 using pCTV- Δ ClaR as a template and ligated between *Bst*EII (nt 7775) and *Not*I (nt 19293) restriction sites into pCTV- Δ ClaR to obtain Δ ClaR-M2, and at nt 1109 by overlap-extension PCR, and the PCR product was ligated between *Bg*/II (nt 1029) and *Nco*I (nt 3685) restriction sites into pCTV- Δ ClaR to obtain Δ ClaR-M3 (Fig. 4A). The PCR products amplified between nts 3559 and 7803 that contained +1 and -1 mutations at nts 4963 and 7084 were ligated between *Nco*I and *Bst*EII sites into pCTV- Δ ClaR to obtain Δ ClaR-M5 and Δ ClaR-M6, respectively (Fig. 4A). Silent mutations in homopolymeric run of U's between nts 3726 and 3732 (UUU UUU U changed to UUC UUC U) were introduced by overlap-extension PCR using mutagenized primers between nts 3559 and 4630 and then ligated into pCTV- Δ Cla between *Nco*I and *Bsu*36I restriction sites to obtain pCTV- Δ Cla-M7 that contained a +1

insertion mutation at nt 3732 (Fig. 5A). pCTV- Δ ClaR-M8 contained silent mutations in wild-type pCTV- Δ ClaR between nts 3726 and 3731 (UUU UUU changed to UUC UUC) (Fig. 5A).

Protoplasts transfection and Northern blot analysis of viral RNAs

SP6 RNA polymerase-derived transcripts from *Not*I-linearized CTV cDNAs were generated in vitro as described by Satyanarayana et al. (1999). The procedures for the isolation of mesophyll protoplasts from *N. benthamiana* leaves and polyethylene glycol mediated transfections were carried out as described previously (Satyanarayana et al., 1999). The total RNA isolated from 3 and 4 day post inoculated (dpi) protoplasts were analyzed by Northern blot hybridization using a 3' positive-stranded RNA-specific riboprobe (Satyanarayana et al., 1999). Bands from different exposures of Northern hybridization blots of total RNA from protoplasts at 4 dpi were quantified by scanning and densitometry with the OS-SCAN program (Oberlin Scientific, Oberlin, OH). Results represented at least three to four independent protoplasts transfections using two to three independent clones for each construct.

E. coli strain, media, and plasmid DNA isolation

Competent cells of *E. coli* strain JM109 prepared as described by Hanahan (1995) were transformed and plated on LB agar-amp plates (1.0% tryptone, 0.5% yeast extract, and 1.0% sodium chloride in water, pH 7.0 plus 1.5% bacto-agar containing 100 μ g/ml ampicillin). The plates were incubated overnight at 37°C followed by 2 days incubation at room temperature. The size of *E. coli* colonies was measured with a microscope at 1 and 2 dpt. 2xYT liquid medium (1.6% tryptone, 1.0% yeast extract, and 0.5% sodium chloride in water, pH 7.0) was used to grow *E. coli* harboring recombinant DNAs for the plasmid DNA isolation (Sambrook and Russell, 2001).

Nucleotide sequencing

Sequencing of the full-length infectious clone pCTV9 and the frameshift mutants to confirm the mutations was performed with an automatic sequencer (Applied Biosystems, model 373) at the Interdisciplinary Center for Biotechnology Research DNA sequencing core facility of the University of Florida, Gainesville, FL.

Nucleotide sequence accession number

The complete nucleotide sequence of the infectious full-length cDNA clone of CTV T36 isolate, pCTV9R, was deposited in the GenBank database under the Accession No. AY170468. The nucleotide numbering and positions men-

tioned in this investigation correspond to the pCTV9R sequence.

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