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Cap-Independent Translational Enhancement of Turnip Crinkle Virus Genomic and Subgenomic RNAs

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Cap-Independent Translational Enhancement of Turnip Crinkle Virus Genomic and Subgenomic RNAs

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The presence of translational control elements and cap structures has not been carefully investigated for members of the *Carmovirus* genus, a group of small icosahedral plant viruses with positive-sense RNA genomes. In this study, we examined both the 5′ and 3′ untranslated regions (UTRs) of the turnip crinkle carmovirus (TCV) genomic RNA (4 kb) as well as the 5′ UTR of the coat protein subgenomic RNA (1.45 kb) for their roles in translational regulation. All three UTRs enhanced translation of the firefly luciferase reporter gene to different extents. Optimal translational efficiency was achieved when mRNAs contained both 5′ and 3′ UTRs. The synergistic effect due to the 5′-3′ cooperation was at least fourfold greater than the sum of the contributions of the individual UTRs. The observed translational enhancement of TCV mRNAs occurred in a cap-independent manner, a result consistent with the demonstration, using a cap-specific antibody, that the 5′ end of the TCV genomic RNA was uncapped. Finally, the translational enhancement activity within the 5′ UTR of 1.45-kb subgenomic RNA was shown to be important for the translation of coat protein in protoplasts and for virulent infection in *Arabidopsis* plants.

Several novel mechanisms by which RNA plant viruses regulate gene expression at the level of translation have been reported. The enhancement of the translation of specific viral mRNAs leading to high levels of protein synthesis of specific genes in plants may well be a fundamental mechanism by which viral mRNAs outcompete their cellular counterparts. Central to understanding this process is the observation that many viral mRNAs have evolved alternative strategies of translational enhancement that are different from those used by most cellular mRNAs. Typically, cellular mRNAs have a 5′-terminal cap and a poly(A) tail which interact synergistically and function as codependent regulators of translation by promoting interaction between the termini of the mRNAs (16, 17). Tobacco mosaic virus (TMV) represents a well-studied example of a naturally capped, nonpolyadenylated mRNA that has a complex 3′ untranslated region (UTR) consisting of a pseudoknot domain and tRNA-like structure. The pseudoknot domain appears to substitute functionally for the poly(A) tail to promote 5′-3′ interaction and enhance translation in a cap-dependent manner (24). In addition, the 5′ UTR of TMV also contains a CAA-rich translational enhancer (TE) element (termed Ω) which dramatically enhances translation of the downstream genes in both animal and plant cells (10, 11, 14, 16). The genome of tobacco etch potyvirus (TEV) represents a second example whose RNA is polyadenylated but has a covalently linked VPg at the 5′ end instead of a cap structure. Interestingly, the 5′ UTR of TEV confers cap-independent enhancement of the translation of reporter genes (4) by promoting interaction between the leader and the poly(A) tail (15). Yet another distinct mechanism of translational enhancement appears to have evolved for a number of plant viral RNAs that lack both a cap structure and a poly(A) tail. It has been demonstrated that the RNAs of both satellite tobacco necrosis virus (STNV) and the PAV strain of barley yellow dwarf luteovirus (BYDV) contain TE sequences in the 3′ UTR that are essential for efficient translation in vitro (6, 36). In the latter case, it has been shown that the 3′ TE sequence, located more than 4.5 kb downstream of the 5′ end of the mRNA, functions in vivo to significantly enhance translation initiation in a cap-independent manner (1, 37). In this report, we describe results showing that the translation of turnip crinkle virus (TCV) RNAs is coordinately enhanced by both the 3′ and 5′ UTRs in the cap-independent manner.

TCV is a small icosahedral plant virus with a positive-sense RNA genome of 4,054 bases encoding five open reading frames (ORFs). The two 5′-proximal genes (P28 and a readthrough product of P88) are translated directly from the genome (41). The remaining three genes are translated from two subgenomic RNAs (sgRNAs). Two small nested ORFs in the middle of the genome encode two proteins (P8 and P9) that are both required for cell-to-cell movement of the virus (25). Both are translated from a 1.7-kb sgRNA by the process of leaky scanning. The coat protein (p38 or CP) is encoded by the most 3′-proximal ORF and is translated from a 1.45-kb sgRNA. TCV replicates to very high levels in infected cells, and both of the sgRNAs appear to accumulate to levels approaching that of the viral genomic RNA. We have also observed that there is marked difference in the levels of the translation products detected in infected cells, with the CP accumulating to concentrations more than 100-fold higher than those of the other gene products (25; W.-Z. Li and T. J. Morris, unpublished data). These observations indicated that some form of translational enhancement was likely responsible for the elevated expression of the CP gene. The absence of poly(A) on the genome of TCV is well established (3), but evidence for the presence of a cap at the 5′ end of the genomic RNA of TCV and other related carmoviruses has been equivocal. In this study, we have reevaluated the status of the cap structure on the genomic RNA and initiated a comprehensive assessment of the role of the 5′ and 3′ UTRs in translational regulation of TCV. Our results show that TCV gene expression is regulated in a cap-independent manner similar to that reported recently for luteoviruses (1).

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MATERIALS AND METHODS

Plasmid construction. Standard procedures (30) were followed. The wild-type TCV infectious clone (T1d1) was as previously described (25). Mutants mpr2, mpr2a, mpr2615A, mpr3, mpr4, mpr5, and mprNco were made by using a commercial mutagenesis kit (Transformer site-directed mutagenesis kit; Clontech, Palo Alto, Calif.) and appropriate oligodeoxyribonucleotides. The sgUTR-luc-3′TCV construct was made as follows: (i) the cDNA of the 1.45-kb sgRNA was PCR amplified from mutant mprNco by using one oligodeoxyribonucleotide containing the T7 promoter sequence as its 5′ half and TCV sequence from nucleotide (nt) 2607 to 2630 as its 3′ half and another oligodeoxyribonucleotide with sequence complementary to TCV nt 4054 to 4030; (ii) the PCR-amplified fragment was cloned into pUC19, and a *Sna*BI site was introduced at the 3′ end of the TCV CP coding region; (iii) the resulting plasmid was cut with *Nco*I and *Sna*BI, and the CP coding region was replaced with the firefly luciferase gene (*luc*) obtained by cutting pSP-luc+ (Promega, Madison, Wis.) with *Nco*I and *Xba*I. All other *luc*-containing constructs were derived from the sgUTR-luc-3′TCV by manipulating either the 5′ or 3′ UTR region or both. Plasmids Ω-luc-3′TMV and TEV5′UTR-luc-A50 were provided by Daniel Gallie. Another poly (A) (A_{30})-containing plasmid, pSP64polyA, was kindly provided by Allen Miller.

In vitro transcription. All RNAs were transcribed from respective T7 promoter-containing constructs by using an Ampliscribe T7 kit (Epicentre Technologies, Madison, Wis.). The transcripts were then precipitated with ammonium acetate, and the concentration was determined by UV spectrophotometry and gel electrophoresis.

Infection of protoplasts and plants; analysis of RNA, virion, and CP. Preparation and inoculation of protoplasts, infection of plants, and sample analysis were carried out as described elsewhere (25, 29). All infectious transcripts used were uncapped. Western blot analysis was performed with the ECL (enhanced chemiluminescence) system (Amersham International, Little Chalfont, England).

Luciferase assays. Cucumber protoplasts were electroporated with transcripts as described by Gallie et al. (12) except that 20 μg of each transcript was used. Except for the data presented in Fig. 3, where capped and uncapped transcripts were compared for their effect on translation, the transcripts used were all uncapped. Protoplasts were harvested 20 h after electroporation and handled as instructed for the Promega luciferase assay system; 20 μl of the 500-μl total extract was removed from each sample and applied to a 96-slot Costar plate. Luciferase activity was measured with a LUMIstar (BMG LabTechnologies, Durham, N.C.) luminometer. Light unit readings of the extracts of mock-inoculated protoplasts were used as background. Transcripts from the plasmid constructs of the sgUTR-luc-3′TCV and luc-3′TCV transcripts were included in every experiment. After the readings of individual samples were obtained, the background readings were subtracted and the relative activity of each sample was calculated as a percentage of the value for the positive control, sgUTR-luc-3′TCV. This permitted direct comparison between experiments for two standard constructs, which we felt important because of significant variability (ca. 50%) in absolute values of light units between experiments. Each experiment was repeated at least three times with different batches of protoplasts, and the percentage data were averaged for all experiments. The functional half-life of the electroporated RNAs in protoplasts was determined essentially as described by Gallie et al. (15). The electroporated protoplasts were divided into several equal aliquots, and activity in light units was determined for each time point.

RESULTS

Evidence for a TE in the 5′ UTR of the 1.45-kb sgRNA. We suspected that the disproportionately high level of accumulation of TCV CP in comparison to the other viral gene products was likely regulated at the level of translation rather than transcription. This was suggested from the observation that the viral genomic and 1.45-kb sgRNAs normally accumulate to similar levels in infected cells, whereas the level of the 1.7-kb sgRNA is only about fivefold lower (Fig. 1B, lane 2). This inference was also generally supported by data from in vitro translation experiments with TCV (Li and Morris, unpublished data) and other carmoviruses (40). Mapping of the transcription initiation sites on the genome for both subgenomic RNAs (3, 35) revealed that the 5′ UTR of the 1.7-kb sgRNA is relatively short (26 nt extending from nt 2331 to 2356) in comparison to the 137-nt leader (from nt 2606 to 2742) of the 1.45-kb sgRNA that encodes the CP. We therefore focused attention on the role of the longer 5′ UTR sequence of the 1.45-kb sgRNA on the translation of the CP gene.

In an effort to identify possible translational enhancement elements in this 5′ UTR region, we produced the comprehensive set of mutations diagrammed in Fig. 1A and examined the replication and sgRNA transcription levels of each mutant by Northern blot analysis of protoplast infections at 20 h postinoculation (Fig. 1B). We also evaluated the relative levels of accumulation of viral CP by Western blot analysis and virus particle formation by gel analysis as described by Qu and Morris (29) for the same batch of infected cells. Although deletion of 120 nt of the 137-nt leader sequence (mutant mpr2 deletion from nt 2619 to 2739) had little effect on the synthesis of either the genomic or subgenomic RNA, it did result in a very marked reduction in the accumulation of viral CP and a concomitant absence of detectable virions. To eliminate a possible functional role of the upstream AUG (nt 2614 to 2616) in the truncated leader in the mpr2 mutant, we converted the AUG to an AUA codon (mutant mpr2a [Fig. 1B, lane 5]). The absence of a functional role for this upstream AUG in regulating expression of the CP in a wild-type background was also confirmed by converting the upstream AUG to AAG (mutant mpr2615A [Fig. 1B, lane 3]). We concluded from these results that the upstream AUG served no role in regulating CP expression. One alternate possibility that remained was that the mpr2 deletion eliminated a region (nt 2618 to 2623, UUUC UA) that might have been involved in secondary structure interactions with the sequence encompassing the genuine CP start codon at nt 2743 (AUGGAAA). To test this, we constructed mutant mpr3 in which the sequence from nt 2618 to 2623 was changed to ACCGGU to preclude potential base pairing. This mutant behaved like the wild type (Fig. 1B, lane 6), thus eliminating a role for possible structural interaction between these regions in regulating CP production.

We next confirmed that the inability of mpr2 and mpr2a mutants to form virus particles was most likely the result of reduced CP synthesis and not an indirect effect of the mutations on the ability of the viral RNA to recognize CP and assemble into virus. We felt it important to evaluate this possibility because the identified origin of assembly for TCV, an RNA structural element located at the 3′ end of the CP coding sequence (29), could possibly be disrupted by upstream structural changes in this region of the viral RNA. In a previous study in which we identified the origin of assembly, we produced a mutant strain of TCV that contained a small region of foreign sequence inserted into the P8 gene (TCR mutant). This enabled detection of the mutant genome in virus particles resulting from mixed infections with helper wild-type TCV and permitted the assay of deletion mutants for the ability to assemble into virus by using CP provided in *trans*. The mpr2 and mpr2a mutants were transferred into the TCR background (mutants TCR-mpr2 and TCR-mpr2a) and inoculated into protoplasts with and without helper wild-type TCV (T1d1). The results presented in Fig. 1C clearly show that although the mutants alone are unable to produce virions, the mutant RNA is fully capable of being assembled into virus particles in infections in which the TCV CP is provided in *trans* by wild-type virus. Hence the mutant RNAs are not defective with respect to the ability to recognize viral CP and assemble into virions; rather, they are most likely defective in the ability to produce enough CP in cells to reach the threshold level needed to initiate assembly.

The previous experiments suggested that the large region of deleted sequence in mpr2 probably contained an enhancer element responsible for the elevated level of CP synthesis. To further delineate this putative TE, we produced two additional deletion mutants. Deletion of 53 nt between nt 2623 and 2676 in the wild-type background (mutant mpr4) did not have any significant negative effect on the ability of virus to accumulate CP and assemble into virus particles. In contrast, deletion of 61 nt between nt 2676 and 2737 (mutant mpr5) resulted in the inability to synthesize sufficient CP to produce any detectable

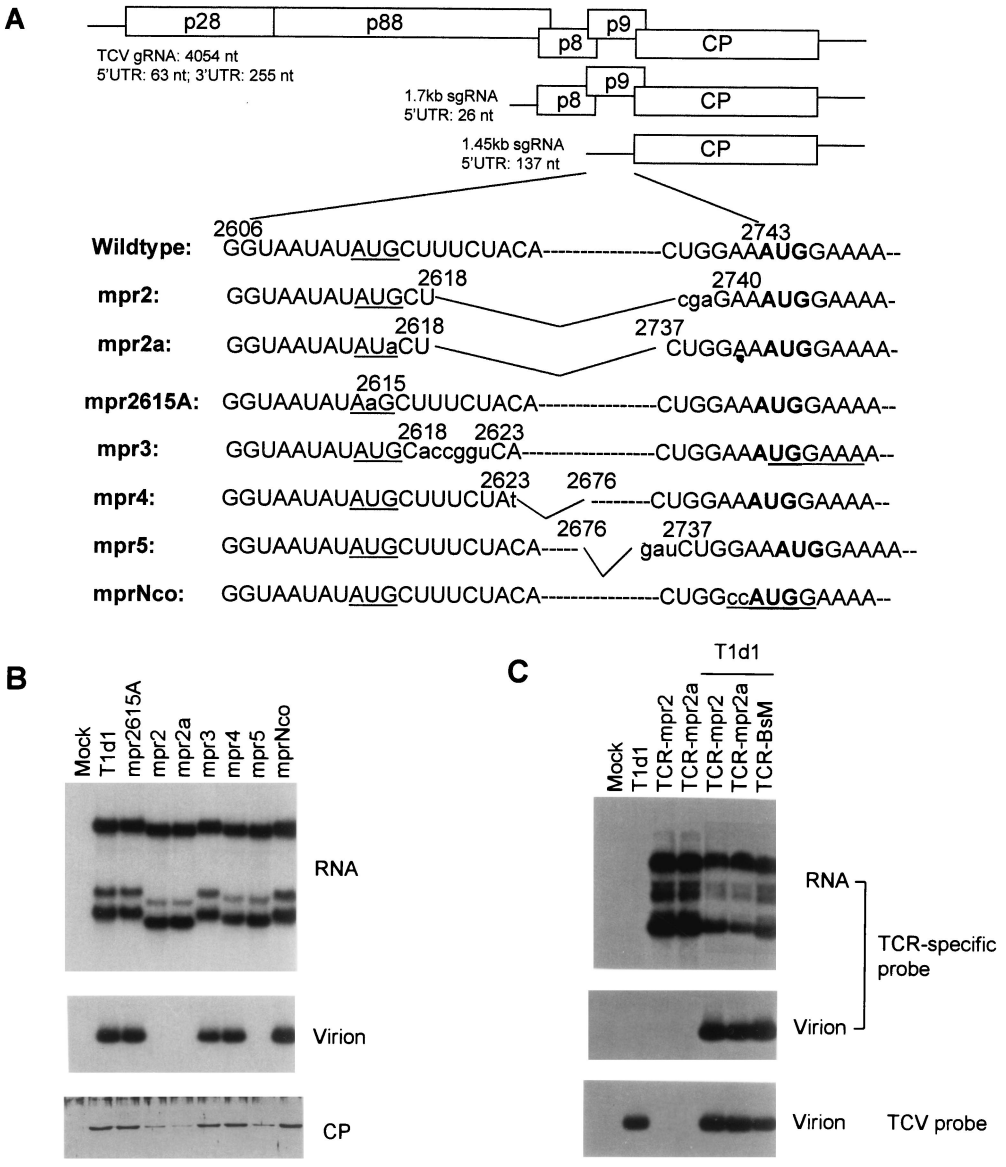

FIG. 1. Effects of modifications in the 5′ UTR of the 1.45-kb sgRNA of TCV on viral RNA synthesis, CP production, and virion accumulation in protoplasts. (A) Diagram showing the genome map of TCV and selected portions of the 5′ UTR sequence. Mutations made in each of the designated mutants are indicated along with the relative location of the 5′ UTR of CP sgRNA. (B) (Top) Northern blot analysis of TCV specific RNAs isolated from protoplast infections showing the minimal effect of the mutations on accumulation of genomic and subgenomic viral RNAs. (Middle) Northern analysis showing accumulation of virions purified from protoplasts and separated in agarose gels; the Northern hybridizations were done with a ^{32}P-labeled TCV-specific probe. (Bottom) Accumulation of viral CP as measured by Western blot analysis of proteins from the inoculated protoplasts, using antiserum raised against TCV CP. (C) Demonstration that mutants mpr2 and mpr2a are capable of assembly into virions. (Top) Northern blot analysis of TCR-specific RNAs isolated from protoplast infections to show accumulation of the individual mutants in the marked TCR background. (Middle) Northern analysis showing accumulation of TCR-specific virions purified from protoplasts and separated in agarose gels; the Northern hybridizations of the top two panels were done with a ^{32}P-labeled TCR-specific probe that did not recognize wild-type TCV sequence. (Bottom) Northern analysis showing accumulation of TCV-specific virions purified from protoplasts and separated in agarose gels; the Northern hybridizations were done with a ^{32}P-labeled TCV-specific probe. Note that the accumulation of TCR-specific sequence in virions (lanes 5 and 6) occurred only in protoplasts coinfected with helper TCV that supplied viral CP in *trans*.

virions. Interestingly, this same region of sequence contained a CA-rich element similar to that reported in the 5′ UTRs of many other RNA viruses (see Discussion).

Assessment of translational enhancement by 5′ UTR sequences in luciferase assays. We next chose to quantitatively evaluate the importance of the different sequence elements in the 5′ UTR of the 1.45-kb sgRNA in translational enhancement by using a luciferase assay system. To facilitate this, we produced the mprNco mutant in which the two AA nucleotides preceding the AUG were changed to CC. This created an *Nco*I site so as to permit precise insertion of the *luc* reporter gene just behind the 5′ UTR of 1.45-kb sgRNA. This mutant behaved similarly to the wild type in its ability to produce CP and accumulate virus (Fig. 1B, lane 9). The mprNco mutant was then used to produce the constructs depicted in Fig. 2, in which the *luc* gene was placed precisely between a series of 5′ UTR sequences and the complete 3′ UTR of TCV. The 3′ UTR was included in these experiments because we anticipated from previous reports that there would likely be a coordinated effect on translation between the 5′ and 3′ UTRs (13, 15, 36). Tran-

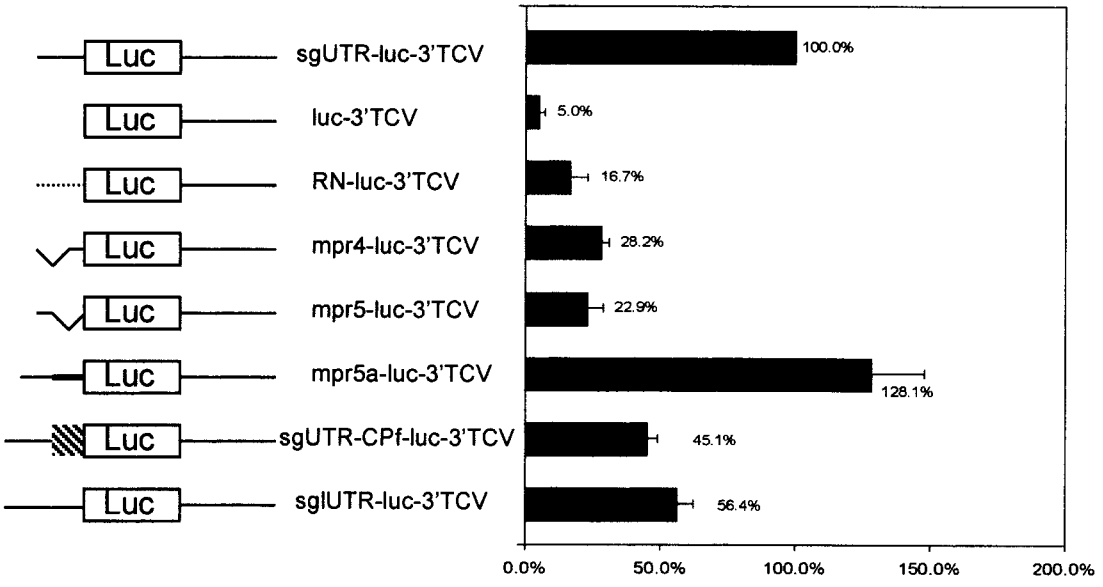

FIG. 2. Examination of translational enhancement activity of the 5′ UTR of 1.45-kb sgRNA in a luciferase assay. Luciferase expression was measured in protoplasts 20 h after electroporation with transcripts containing the various 5′ UTR constructs diagrammed to the left. The data are expressed as a percentage of the control construct, sgUTR-luc-3′TCV. This construct consisted of the entire 5′ UTR of the 1.45-kb sgRNA followed by the *luc* gene and the intact 3′ UTR of TCV. Modifications made to the 5′ UTR are depicted in the diagrams for each of the named constructs and described in the text.

scripts of each of the constructs shown in Fig. 2 were electroporated into protoplasts and analyzed for luciferase activity at 20 h. The results are reported as a percentage of activity relative to the control construct (sgUTR-luc-3′TCV) containing the 5′ and 3′ UTRs of the 1.45-kb sgRNA.

Deletion of the entire 5′ UTR, to produce a construct with a leader of only six vector nucleotides (GGATCC; luc-3′TCV), had the expected effect of markedly reducing translation, as measured by a 20-fold reduction of luciferase activity (5% of the control level). Replacing the TCV leader with a randomly selected region of TCV (nt 2359 to 2498) of similar size to produce RN-luc-3′TCV also resulted in reduced activity to about 16% of the control (sixfold reduction). We next tested the effect of the two deletions in the 5′ UTR derived from the mpr4 and mpr5 mutants. Interestingly, both deletions resulted in comparably lower levels of translation, as measured by reduced luciferase activities of 28 and 23%, respectively. The small difference between the two was somewhat unexpected given the higher level of CP accumulation observed for the mpr4 mutant in the protoplast infections. Additional experiments confirmed the reliability of the result and consistently showed only a slightly greater expression of luciferase in the mpr4 mutant background than in the mpr5 background. To further investigate the relative importance of the CA-rich sequence deleted in mpr5, we replaced the deleted region with a similar-size segment of the genomic 5′ UTR of TCV which did not contain a CA-rich region. Surprisingly, this construct (mpr5a-luc-3′TCV) generated luciferase activity that was higher than the control level (ca. 128%), suggesting that the CA-rich sequence deleted in the mpr5 mutant did not comprise the optimal TE element and that it could be replaced with an element of similar function. This result also suggested that 5′ UTR of the genomic RNA contained its own functional TE element.

Previous work on Sindbis virus CP translation demonstrated that the TE for the CP gene of this animal virus consists of sequence that occurs after the start codon and within the CP coding region (8, 9). To test this possibility for TCV, we made a construct (sgUTR-CPf-luc-3′TCV) in which the luciferase coding region was fused in frame to the first 41 amino acids of the CP gene. This extended the 5′ leader beyond the normal 5′ UTR to include the first 123 nt of the CP coding region. In a second construct, a stop codon was introduced into sgUTR-CPf-luc-3′TCV after the fifth codon of the CP. This effectively extended the 5′ UTR with 123 nt of CP sequence without producing a fusion of the CP and luciferase gene products. Both clones produced lower than optimal luciferase activity (45 and 56%, respectively), indicating that the translational enhancing activity of the TCV CP gene probably does not extend into the coding region.

Comparison of the TCV 1.45-kb sgRNA TE with other plant viral TEs in the presence and absence of a cap. The relative strength of the TE identified in the 1.45-kb sgRNA of TCV was next evaluated in comparison to other known TEs derived from TMV, TEV, and alfalfa mosaic virus (AMV), with and without a 5′ m^7GpppG cap (Fig. 3). The TMV construct (Ω-luc-3′TMV) consisted of the Ω fragment, the *luc* gene, and the 3′ UTR of TMV (10, 11, 16). The AMV construct (AMV 5′UTR-luc-3′TCV) included the 5′ UTR of the AMV RNA4 (22) synthesized by annealing two complementary oligodeoxyribonucleotides with the sequence of the 5′ UTR of the AMV RNA4 fused to *luc* and the TCV 3′ UTR at the 3′ end. The TEV construct (TEV5′UTR-luc-A50) consisted of 5′ UTR and a poly(A) tail of 50 nt (4, 15).

The results reported in Fig. 3 show the relative level of luciferase activity for each of the constructs compared to the uncapped control TCV construct (sgUTR-luc-3′TCV). The effect of adding a cap to the complete TCV construct enhanced translation about twofold (188%). The enhancement derived from adding a cap was greater for both of the defective TCV constructs tested. Although the overall translational enhancement was lower than the control level for the construct that lacked the 5′ UTR (6 and 160%) and for the construct with the random UTR sequence (16 and 111% over the control level), the net benefit of capping the defective TCV transcripts was greater overall. The most significant increase in translation that resulted from adding a cap was noted for the TMV construct. Although the uncapped TMV construct was less effi-

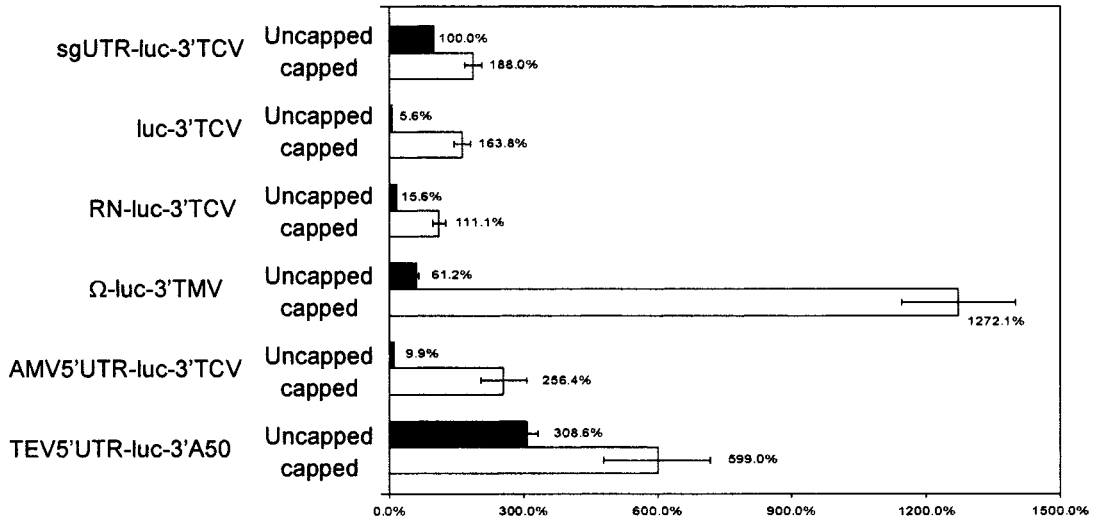

FIG. 3. Effect of capping on the translational activity of the TCV TE compared to several other viral TEs in a luciferase assay. Luciferase expression was determined essentially as described in the legend to Fig. 2; activity is reported relative to that of the uncapped control construct sgUTR-luc-3′TCV. The constructs that contain TE elements from TMV, AMV, and TEV are detailed in the text.

cient than TCV, it showed about a 20-fold increase in translational efficiency when capped. This result is consistent with the cap-dependent nature of TMV translation as described by Gallie and Walbot (14). A similar level of cap-dependent translational enhancement was observed for the AMV construct in our experiments as well. In contrast, the uncapped TEV construct showed significant enhancement over the uncapped control TCV construct (300%), but as for TCV, the effect of adding the cap increased the translational enhancement by only about twofold. These results were consistent with previous work demonstrating the cap-independent nature of translational enhancement for TEV (4). Our conclusion from these experiments is that translational enhancement in TCV is mediated in a cap-independent manner.

The TCV genomic RNA is not capped in vivo. In view of our results suggestive of cap-independent enhancement of translation of the 1.45-kb sgRNA of TCV, we chose to reexamine whether TCV RNAs are capped in vivo. The literature is equivocal on this issue. To address this question directly, we acquired an antibody (Ab H20) that had been developed to specifically detect an m^7GpppG cap structure on small nuclear RNAs (2, 7). TCV virion RNA was extracted from freshly purified virus particles, and both capped and uncapped full-length TCV RNAs were prepared by in vitro transcription and purified by ammonium acetate precipitation. The quantities of the virion RNA and transcripts were then equalized, spotted on nylon membranes, and subjected to either hybridization using a TCV-specific probe (Fig. 4B) or immunodetection using the cap-specific Ab H20 (Fig. 4A). Only the artificially capped full-length transcripts in dots 2 and 5 reacted positively to Ab H20. We concluded from this experiment that the genomic RNA packaged into virus particles is not capped.

The 5′ UTR of genomic RNA also enhances translation. It might also be expected that 5′ UTR of the TCV genomic RNA would contain sequences responsible for translational enhancement of the polymerase gene (*pol*). This was suggested from the result of the construct mpr5a-luc-3′TCV (Fig. 2), in which we observed elevated translation relative to the control when the CA-rich region of the sgRNA 5′ UTR was replaced with 5′ UTR of the genomic RNA. To test this further, we fused the 63-nt 5′ UTR of the genomic RNA to the 5′ end of the *luc* gene to produce gUTR-luc-3′TCV. The sgUTR-luc-3′TCV construct was included as a positive control. Constructs lacking a 5′ UTR (luc-3′TCV) or with the long random 5′ leader (RN-luc-3′TCV) were included as well (Fig. 5). The results show that the construct with the 63-nt UTR derived from the genomic RNA was translated much less efficiently than the positive control (16% of control) and at about the same level as the longer construct with the random 5′ leader. This result, and the observation that the first 30 nt of *pol* contained CA-rich regions, prompted us to examine the coding region for translational enhancement activity. To test this, we made a construct (gUTR-POLf-luc-3′TCV) in which the luciferase coding region was fused inframe to the first 10 amino acids of the *pol* gene so as to extend the 5′ leader to include the first 30 nt of the polymerase coding region. In the other construct, a single-base deletion in the AUG of the *pol* gene was made such that translation initiated at the genuine *luc* gene. This effectively extended the 5′ UTR with *pol* sequence without resulting in a fusion of the polymerase and luciferase coding regions (glUTR-luc-3′TCV). Both clones produced luciferase activity lower (34 and 52%, respectively) than that of the control sgUTR construct but higher than that of the gUTR sequence alone. We conclude that the 5′ UTR of TCV genomic RNA also enhances translation and that the sequence needed for this activity extends into polymerase coding region.

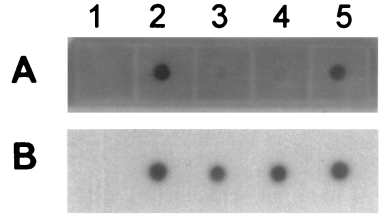

FIG. 4. Direct evidence for absence of an m^7G cap on the 5′ end of the genomic RNA of TCV. Two nylon membranes were each spotted with 0.5 μg of the following RNA preparations: lane 1, blank control, no RNA; lanes 2 and 5, full-length TCV transcript capped in vitro with m^7GpppG; lane 3, uncapped full-length TCV transcript; lane 4, TCV RNA isolated from virions. (A) Immunodetection of the cap structure with the cap-specific Ab H20; (B) RNA hybridization with a ^{32}P-labeled TCV-specific probe.

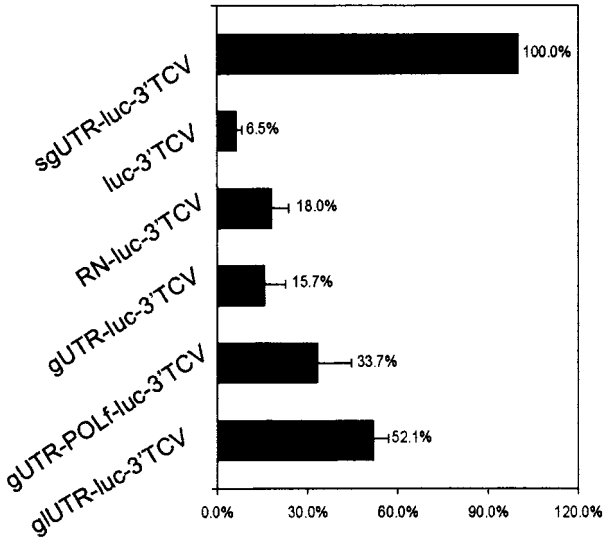

FIG. 5. Examination of translational enhancement activity of the 5′ UTR of the TCV genomic RNA in a luciferase assay. Luciferase expression was determined as described in the legend to Fig. 2, and activity is expressed relative to that of the sgUTR-luc-3′TCV control. Modifications made to the 5′ UTR are described in the text.

The 3′ UTR contributes more significantly to translational enhancement than the 5′ UTR. The data supporting the cap-independent nature of translational enhancement with TCV, and the comparable results of Wang et al. (37) for BYDV, prompted an examination of the role of the 3′ UTR of TCV for translational enhancement activity. Previously, we showed that removal of the 5′ UTR of 1.45-kb sgRNA reduced luciferase activity to 5% (20-fold) of the control construct (sgUTR-luc-3′TCV) containing the intact 3′ UTR. In this reciprocal experiment, the intact 5′ UTR was retained and the 3′ UTR was removed, leaving only nine bases of vector sequence after the *luc* gene (sgUTR-luc [Fig. 6]). This caused a 35-fold reduction in translation to about 2.7% of the control. We consider this to be the basal level of translational enhancement contributed by the 5′ UTR alone.

To test the effect of the length of the 3′ UTR on translation (34), the entire region was replaced by a similar-size random sequence (213 nt versus 255 nt of the TCV 3′ UTR) derived from the plasmid vector by cutting the construct sgUTR-luc with *Nde*I (sgUTR-luc-NdeI). Translation of this construct was 7.2% of the control level. These data support the notion that the 3′ UTR is a stronger contributor to the translational enhancement than the 5′ UTR of 1.45-kb sgRNA. The contribution of random sequences to luciferase translation was examined by testing three additional constructs: one with 140 nt of random sequence (RN) as the 5′ UTR, another with 213 nt of random sequence as the 3′ UTR, and third with both sequences. All of these constructs had luciferase activities not significantly above background levels (data not shown). The fact that the translational efficiency of sgUTR-luc-3′TCV is fourfold greater than the combined activities of the RN-luc-3′TCV and sgUTR-luc-NdeI constructs suggests that the 5′ sgUTR and the 3′ UTR must complement each other to enhance translation in a synergistic manner.

The loss of activity due to the absence of the 3′ UTR was also partially restored by the addition of a poly(A) tail. A shorter 30-nt addition was less effective (24% of control) than a 50-nt tail (35% of the control level). The presence of a poly(A) tail in the absence of a 5′ UTR failed to restore a significant level of translational activity (1% of the control level [data not shown]). These results suggested that while the 5′ UTRs of TCV likely confer cap-independent translation, the 3′ UTR contributes in a general way to translation, probably by mimicking the role of a poly(A) tail.

To further delineate the putative 3′TE, we tested a construct (sgUTR-luc-3′TCV/SpeI) in which the intact 255-nt 3′ UTR was truncated at the *Spe*I site at nt 3953. This produced a 155-nt 3′ UTR that lacked the hairpin structure identified as the promoter of minus-strand transcription at the 3′ end of the genome (32). This large truncation had a relatively minor effect, reducing translation to about 80% of the control level. This localized the enhancement activity to within the first 150 nt of the 3′ UTR and confirmed that the 3′ UTR sequence of TCV likely contains the most important element responsible for translational enhancement in this virus.

Modification of the TCV UTRs does not significantly alter RNA stability. We used the procedure of Leathers et al. (24) to investigate the possible effect that modifying the different UTRs might have on RNA stability. This permitted measurement of the functional half-life of an mRNA, defined as the time needed to complete a 50% decay in the capacity of the mRNA to synthesize protein. The very low activity associated with constructs completely lacking 5′ or 3′ ends precluded effective measurement of some constructs in this experiment. However, it is quite clear that all of the constructs tested showed a peak of translational activity between 240 and 280 minutes postinoculation (mpi) (Fig. 7), suggesting that each of the mRNAs had a functional half-life of approximately 120 to 140 min. This suggests that modifications made to either the 5′ or 3′ ends of the mRNAs had no significant effect on their stability in protoplasts during the time interval of testing. It should be noted that all assays showed an abrupt decrease in luciferase activity after 280 min followed by a gradual decrease over 24 h.

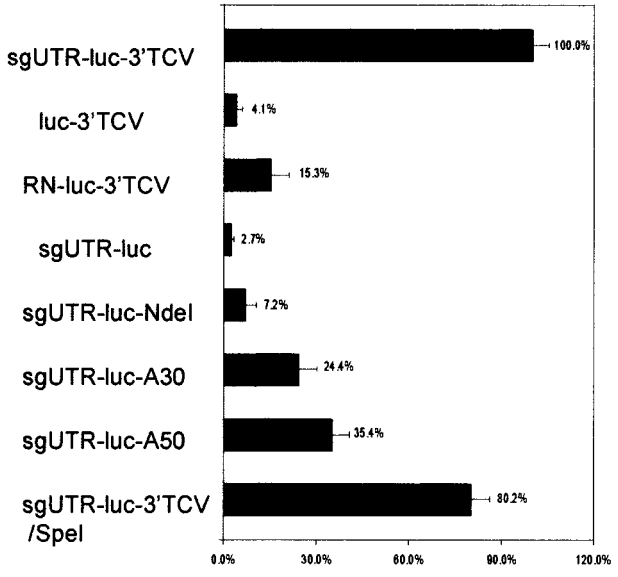

FIG. 6. Examination of translational enhancement activity of the 3′ UTR of the TCV genomic RNA in a luciferase assay. Luciferase expression was determined as described in the legend to Fig. 2, and activity is expressed relative to that of the sgUTR-luc-3′TCV control. Modifications made to the 3′ UTR are described in the text.

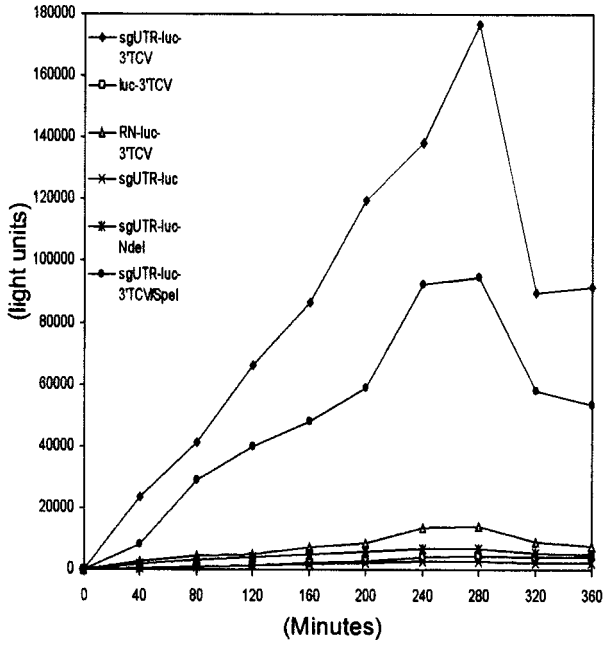

FIG. 7. Effects of modifications in the 5′ and 3′ UTR sequences of TCV on the functional mRNA half-life of transcripts in protoplasts. The various constructs tested are described in the text. The extent to which luciferase accumulated in protoplasts was measured for 360 min, and the functional half-life was calculated.

DISCUSSION

We have investigated the role of the 5′ UTRs in the major sgRNA encoding the CP gene and the genomic RNA encoding the viral polymerase and shown that both 5′ UTR regions contain sequences important in enhancing translation of the RNAs in vivo. More significantly, we explored the role of the same 3′ UTR present on both RNAs and demonstrated that this sequence has a more pronounced effect on enhancing translation of the reporter luciferase gene than either of the 5′ UTR regions alone. Our results with the sgRNA message for the CP also demonstrate that the 5′ UTR and 3′ UTR function coordinately to enhance translational efficiency to a level that is at least fourfold greater than the additive effect of either the 5′ or 3′ region alone. These results support a model by which optimal interaction between the 5′ and 3′ UTRs of the mRNA has a synergistic effect on translation. We have also provided the first direct evidence for the absence of a cap on the TCV genomic RNA, an observation consistent with the cap-independent nature of the observed translational enhancement results. We have also observed that the translational enhancement activity mediated by the 5′ UTR of the CP mRNA has a marked effect on the ability of the virus to invade a plant host systemically (data not shown), presumably an effect resulting primarily from differential regulation of the level of translation of the viral CP. This report provides the first evidence that synergistic enhancement of translation in carmoviruses occurs in a cap-independent manner mediated by optimal interaction between both the 5′ and 3′ UTRs of the viral genomic and subgenomic RNAs.

The coordinated interaction between 5′ and 3′ UTRs of mRNAs has been demonstrated in numerous plant and animal viruses as well as cellular mRNAs (17). Most recently, physical evidence of circularized mRNAs has also been reported (39). In the case of cellular RNAs, it is believed that the 5′ and 3′ UTR interaction is mediated by translational factors like eukaryotic initiation factor 4F and poly(A)-binding protein, which interact with the 5′ cap and 3′ poly(A), respectively, to promote initiation (5, 39). In plant viruses, such protein factors remain to be identified. Interestingly, it has been suggested for two defined examples of cap-independent translational enhancement (BYDV and STNV) that such host factors may be involved in promoting interaction of the 5′ and 3′ UTRs and thus facilitating translation. In both of these cases, a 3′ UTR enhancer similar to the type we have described here for TCV has been identified (27, 37).

We have not as yet been able to definitively identify any characteristic sequence motifs within the UTRs that could be assigned specific responsibility for translational enhancement. The initial results with the 5′ UTRs implicated the involvement of a CA-rich motif [CA(A)CAC(A/U)] that is repeated four times within a 60-nt region in the 5′ UTR of the 1.45-kb sgRNA (Fig. 8A). Two similar CA repeats were also present near genomic 5′ UTR within first 30 nt of the coding region of the *pol* gene (Fig. 8A). Deletion of the 60-nt segment of the 5′ UTR of the 1.45-kb sgRNA that contained the CA-rich motif (mpr5) had a more deleterious effect on CP accumulation than deletion of similar-size segment directly upstream of the CA-rich region (mpr4). Deletion of either region, however, had very comparable effects on reducing translation in luciferase expression assays. Moreover, the enhanced effect of replacing the CA-rich region with a similar-size region of the genomic 5′ UTR showed that the CA-rich region could be effectively replaced by an enhancer region lacking CA-rich sequences.

The presence of CA-rich motifs in proximity of the 5′ UTRs is a common occurrence in many plant viral RNAs (Fig. 8A), although their role in translational enhancement has not been well defined. In TMV, the $(CAA)_n$ motif occurs within the Ω element, where it has been shown to contribute to translational enhancement. Additional examples include the CA-rich motifs within the 5′ UTRs of TEV, potato virus X (23), and tobacco necrosis virus (26). Among members of the *Tombusviridae*, CA-rich motifs are found in proximity to the 5′ UTRs of both the genomic and subgenomic RNAs in tomato bushy stunt tombusvirus and in the 5′ UTRs of the CP sgRNAs of carnation mottle virus and cardamine chlorotic fleck carmovirus (31). Our data for TCV indicate that the CA-rich motifs may well contribute to translational enhancement in both subgenomic and genomic RNAs, but they are not essential for efficient translation of either message. Part of the explanation for the improved translation of RNAs containing CA-rich regions could be that they contribute both additional length and reduced secondary structure to the UTR region. Both have been implicated as critical factors for effective translational enhancement (16).

Another identifiable sequence element present in both genomic and subgenomic 5′ UTRs and 3′ UTR of TCV are tracts of CU-rich sequence of variable length. These CU-rich regions in TCV show strong sequence similarity to a pyrimidine-rich domain identified in hepatitis C virus (HCV) (Fig. 8B) that is implicated in specific high-affinity binding to a host factor called polypyrimidine tract binding protein (20). The HCV genome is also a single-stranded, plus-sense RNA which is neither capped nor 3′-polyadenylated. In this example, binding of polypyrimidine tract binding protein to the 3′ UTR (X region) of HCV enhances translation initiated from the 5′ internal ribosomal entry site, whereas binding to an internal pyrimidine-rich domain attenuates translation (21). It will be interesting to find out if similar protein factors are present in plant cells and if they act in a similar manner in TCV.

The significant role of the 3′ UTR of the TCV RNA in enhancing translation was somewhat unexpected but not un-

A:

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CA-rich elements are in or near the 5′UTR of CP sgRNAs in the following viruses:
TCV:   CACACATCCTACAACACACA-16nt-CAACACATAAGCATCAACAC-5nt-AUG
CCFV:              CAACACCACTGACAACACAAAAAGCCACACA--18nt--AUG
CarMV:                       CAACAACACATTTCAATAAGTACACCAA-AUG
TNV:                      CAAUCAACAA--36nt--CAACACA--19nt--AUG--14nt--CAACAACAA
TBSV:                                    CACACAC--13nt--AUG--8nt--AAACAACAACAC

CA-rich elements are in or near genomic 5′UTRs in the following viruses:
TCV:                                                  AUG--9nt--CACACACUCAACACA
TMV:                                CAACAACAACAAACAACA--24nt--AUG
TNV:                                                  AUG--7nt--CAAACCAACACAAGCAAAC
TBSV:                                         CAACAAACAAGCGACAUG
TEV:                     CAACACAACAUAUACAAAACAAAC---106nt---AUG
PVX:               CAAACACCACCAACACA--24nt--CACACAC--25nt--AUG
```

B:

```
TCV:   2617  CUUUCUACAA CUCUCUCUCACUGGUCCUCCUACUUUGUCAUCUGAUUCCU 2666
             *** **     **** ****  *  **** **          ***  * ***
HCV:    846  CUUCCUGGUUGCUCUUUCUCUAUCUUCCUUCUGGCCCUGC UCUCUUGCCU 895
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FIG. 8. (A) CA-rich elements in the 5′ UTRs of plant viral (and subgenomic) RNAs. (B) Comparison of the CU-rich regions of TCV and HCV.

precedented given the recent identification of functional TE elements in the 3′ UTRs of several phylogenetically related viruses including BYDV and STNV (6, 37). More recently, an analogously functioning region has been proposed to exist within the 3′ UTR of an even more closely related tombusvirus (28). Our search for sequence similarities to these other elements in the 155-nt TE region of TCV failed to identify any obvious motifs. However, the similarity of the observed phenomena of cap-independent translational enhancement involving regions of sequence in the 3′ UTR of these relatively closely related viruses suggests that a common and somewhat unique mechanism of translational enhancement may have evolved in these viruses. This possibility is supported by our unpublished results indicating that a chimeric construct that included the 5′ UTR of TCV 1.45-kb sgRNA and the 3′ UTR of tomato bushy stunt tombusvirus promoted translation of luciferase to levels that were 50% higher than that of the homologous (sgUTR-luc-3′TCV) construct. Additional work will be needed to further delineate the specific region of 3′ UTR sequence responsible for this synergistic TE activity.

Our results demonstrate that the 5′ UTR of the viral genomic and CP subgenomic RNAs differ in the ability to enhance translation of their respective messages. We think this likely has biological relevance that could fit the following model. All viral messages produced by TCV have the same 3′ UTR containing the strong 3′ TE element, which suggests that the primary role of the 3′ TE is to ensure efficient translation of all viral products in competition with cellular mRNAs. The presence of a strong TE element in the CP sgRNA would ensure successful competition of the CP message with the genomic RNA message for the viral polymerase. Such a highly competitive CP mRNA is necessitated by the fact that the subgenomic mRNAs appear later in the virus replication cycle and therefore must compete efficiently for translation factors with the newly amplified pool of genomic RNA. If this prediction is credible, then we might speculate that the primary switch between early gene functions (e.g., replication) and late gene functions (e.g., movement and virus assembly) is mediated principally at the level of translational regulation rather than transcription. This model is consistent with the observation that compromising CP translation efficiency has a nonlethal but otherwise dramatic effect on viral invasiveness in the host plant (data not shown). A recent report (38) describing the novel characteristics of the 3′ TE of BYDV proposed a more complex mechanism for the function of the 3′ TE of BYDV. In this system, the TE is located in the 3′ UTRs of the genomic RNA and sgRNA1 as well as in the 5′ UTR of sgRNA2. Interestingly, this enhancer element was found to enhance the translation of genomic RNA and sgRNA1 only during the early phases of virus multiplication. Later in infection, after substantial accumulation of sgRNA2, the same element is believed to function in *trans* to inhibit translation of the early mRNAs.

The cap-independent nature of the translational enhancement of the TCV sgRNA that we have described here suggests, but does not prove, that the sgRNAs are not capped in vivo. This suggestion is corroborated by the independent demonstration, using cap-specific antibody, that the genomic RNA is also uncapped. The literature regarding the cap status of the genomic RNA of TCV and other carmoviruses is equivocal. The first report on the sequencing of the related carnation mottle virus suggested that the genomic RNA was capped (18). In addition, doublet bands, suggestive of a cap, were found at the 5′ ends of TCV RNAs in primer extension experiments designed to map the locations of the sgRNAs (3, 35). However, in contrast to most other capped viral genomes, the infectivity of in vitro-transcribed TCV RNA was only modestly enhanced by capping (19). In contrast to our more definitive results with the genomic RNA of TCV, we have not been successful in convincingly demonstrating the absence of a cap on the

sgRNAs because they could not be purified in sufficient quantity for direct analysis with cap antibody. Hence, we can only suggest that the sgRNAs are uncapped based on the observation that their translation behaves in the cap-independent manner in vivo. Another observation in support of this conclusion is that the TCV genome does not encode an enzyme likely to be involved in capping activity. These observations prompt our speculation that the absence of cap may actually be advantageous for viruses with very small genomes like TCV. Structurally similar viruses with T=3 symmetry like brome mosaic virus encode domains for capping enzymes including methyltransferase and guanyltransferase (33) that are not present in TCV. The significantly larger genome (ca. 9 kb versus 4 kb) necessitated by these extra domains may well define the evolutionary constraint that requires packaging of such viral genomes into multiple virions. This idea is supported from our previous results that demonstrated an upper size constraint in the range of 4 to 5 kb for an icosahedral virion of T=3 symmetry composed of subunits of 35 to 40 kDa (29). Hence the constraint on genome size is defined by the size of the virion that can be assembled. The selective advantage to maintaining a small genome size is the higher specific infectivity afforded by the ability to package an entire functional genome into a single virus particle.

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