Functional analysis of the tobacco mosaic virus tRNA-like structure in cytoplasmic gene regulation

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ABSTRACT

The 3'-untranslated region (UTR) of tobacco mosaic virus (TMV), which terminates in a tRNA-like structure, functionally substitutes for a poly(A) tail in both plant and animal cells. The addition of the TMV 3'-UTR to chimeric mRNA constructs increases their expression up to 100-fold, increasing both translational efficiency and mRNA stability. The domain largely responsible for the regulation maps to a 72 base region immediately upstream of the tRNA-like structure, however, the 3'-terminal, tRNA-like structure is required for full function. Its contribution is lost if separated from the upstream pseudoknot domain by as few as 5 bases or if 6 bases are removed from the 3'-terminus. Sequence addition to the 3'-terminus of the TMV 3'UTR or the upstream pseudoknot domain inhibits function in both tobacco and Chinese hamster ovary cells.

INTRODUCTION

The tobacco mosaic virus (TMV) 3'-UTR forms, by means of secondary and tertiary interactions, a tRNA-like structure at its 3'-terminus. Among several RNA plant viruses, including TMV, brome mosaic virus, and turnip yellow mosaic virus, the tertiary structure of the tRNA-like terminus is conserved. Although there is little or no conservation at the primary or secondary levels, in its tertiary form, these tRNA-like structures are similar enough to true tRNAs to be recognized by many of the tRNA-specific enzymes such as aminoacyl tRNA synthetase (1, 2, 3). For each tRNA-like structure, the aminoacylation is specific; for example, TMV RNA is specifically charged with histidine (4, 5, 6). As is the case for authentic tRNAs, if the last three nucleotides of the tRNA-like structure (CCA-3') are partially or entirely missing, they can be replaced by nucleotidyl transferase (1, 7, 8); also mutations involving the three terminal bases can be corrected *in vivo* (9). Additional tRNA-specific enzymes that recognize the tRNA-like structures include peptidyl hydrolase (8), tRNA methyltransferase (10), and RNase P (11, 12). The purpose of this tRNA-mimicry in the virus life cycle remains unknown, however the tRNA-like structure is important for recognition by the viral replicase (13, 14, 15).

Recently, we demonstrated that the non-polyadenylated 3'-UTR of TMV stabilizes reporter mRNA and substantially increases gene expression in higher eukaryotes (16). Dissection of the TMV 3'-UTR determined that a domain immediately upstream to the tRNA-like structure, (referred to as the upstream pseudoknot domain (UPD)) was largely responsible for the regulation of expression (16). This domain is 72 bases in length and is solely composed of three pseudoknots (17). The tRNA-like structure also plays a role in the regulation associated with the TMV 3'-UTR as it is necessary for full function of the UPD and serves as the 3'-terminus not only for the genomic RNA but for the subgenomic mRNAs as well.

Because TMV is a positive strand, cytoplasmic virus, the genomic RNA functions as an mRNA and faces the same challenges as cellular mRNAs, i.e., maintaining RNA integrity and interacting efficiently with the translational machinery. As virtually all eukaryotic mRNAs are polyadenylated, a feature essential for efficient expression, TMV RNA must have evolved a means by which it can overcome the lack of a poly(A) tail. We report here the functional requirements of the tRNA-like domain as part of our examination of how the TMV 3'-UTR acts as the functional equivalent of a poly(A) tail.

MATERIAL AND METHODS

Plasmid constructs and *in vitro* transcription

The GUS mRNA constructs containing either the complete TMV 3'-UTR or subdomains have been described previously (16). The T7-based GUS constructs were linearized with the appropriate restriction enzyme, and *in vitro* transcription of the DNA was carried out using bacteriophage T7 RNA polymerase (18). Capped transcripts were synthesized by modifying the published reaction conditions to include 200 μ M GTP and 1.5 mM m⁷G(5')ppp(5')G (New England Biotabs). RNA integrity was determined by formaldehyde–agarose gel electrophoresis (19).

Preparation and electroporation of plant protoplasts

Protoplast media and isolation methods for cell suspensions of tobacco (Xanthi) have been described (20) except that 1.0% Cytolase (Genencor) was used in place of 0.5% Rhozyme. The cells were digested for approximately 1.5 hr before harvesting.

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Electroporation conditions for tobacco were 180 V, 8 msec pulse, 1550 μ F capacitor using an X-cell TM 450 Electroporation System (Promega Biotec). mRNA was added to 1×10^6 protoplasts followed by immediate electroporation on ice. After 10 min, the protoplasts were removed from the ice and allowed to recover for 18 hr before collection for analysis.

Preparation and electroporation of CHO cells

CHO cells were cultured in Ham's F12 medium (Gibco) containing 10% fetal bovine serum and were released from the plate by trypsinization. The cells were washed once in phosphate-buffered saline and used immediately. Standard electroporation conditions for the CHO cells were 450 V and 250 μ F capacitance using a BioRad Gene Pulser. Electroporation of 5×10^6 cells was carried out at room temperature and after 10 min, the cells were added to fresh Ham's F12 serum-supplemented medium, and allowed to recover for 18 hr.

Analysis of GUS activity

Plant protoplasts or CHO cells collected by centrifugation were resuspended and sonicated in 0.5 ml of buffer (50 mM sodium phosphate, pH 7.0, 10 mM β -mercaptoethanol, 1 mM EDTA). For GUS assays, 4-methylumbelliferyl- β -D-glucuronide (Sigma Co.) was added to 1 mM to aliquots which were then incubated at 37°C for 15 to 120 min; the reaction was terminated by the addition of 0.2 M sodium carbonate. Fluorescence was measured by excitation at 365 nm and emission at 455 nm in a TKO 100 DNA Fluorometer (Hoefer Scientific Inc.). GUS-specific activity was determined as nanomoles of substrate per minute per milligram of protein. Protein was determined using the BioRad protein assay kit.

RESULTS

Structural disruption of the tRNA-like domain results in loss of function

Although the upstream pseudoknot domain (UPD) is largely responsible for the regulation associated with the TMV 3'-UTR (16), the tRNA-like domain is required for full function. To further elucidate the role of the tRNA-like domain, we have examined the impact of additional mutations within this domain. The structure of the TMV 3'-UTR as described by van Belkum *et al.* (17) is shown in Figure 1. The 5 base insertion mutation that introduces a *Bgl*II site between the UPD and tRNA-like domain was designed so that the tertiary structure of the pseudoknots on either side of the site of insertion would not be disrupted. The 3'-terminus of the TMV 3'-UTR created by plasmid linearization with *Apa*I, *Nde*I, or *Eco*RI is indicated with an arrow.

When the T7-based Ω -GUS-TMV 3'-UTR plasmid constructs are linearized with *Nde*I, the resulting mRNA contains the complete TMV 3'-UTR terminus. The DNA sequence of the 3'-terminal pseudoknot of the tRNA-like domain with the relevant restriction sites is shown below. Small case letters represent vector sequence.

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-----3' pseudoknot-----
...CCGTTACCCCCGGTAGGGGCCCAtatg....
...GGCAATGGGGGCCATCCCCGGGTatac....
                                ApaI  NdeI

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The RNA 3'-terminus predicted from linearization with *Nde*I is:

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... CCGUUACCCCCGGUAGGGGCCCAua-3'
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Linearization with *Apa*I results in the deletion of the 3'-terminal 6 bases of the tRNA-like domain. Because of the 3' overhang generated by *Apa*I results in strand switching by the T7 RNA polymerase (18) additional sequence will be present at the 3'-terminus. The RNA 3'-terminus predicted for *Apa*I-digested constructs is:

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... CCGUUACCCCCGGUAGGnnnnnnnnn.....3'
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Linearization with *Apa*I, followed by the removal of the 3' overhang with T4 DNA polymerase to prevent T7 RNA polymerase strand-switching not only prevents formation of the 3'-terminal pseudoknot but also prevents sequence addition to the 3'-terminus. It should be noted that the TRNA nucleotidyl transferase can only repair the terminal three bases of the tRNA-like structure (9), therefore RNA resulting from *Apa*I-digested DNA should not be a substrate for the nucleotidyl transferase. The RNA 3'-terminus predicted from the *Apa*I-linearized constructs in which the 3' overhang has been removed by T4 DNA polymerase is:

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... CCGUUACCCCCGGUAGG-3'
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Expression from the Ω -GUS mRNA construct containing the complete TMV 3'-UTR was maximal in both cell types (Figure 2). Upon deletion of six bases by *Apa*I digestion and T4 DNA polymerase processing, only 28% and 54% of the wild type activity was observed in tobacco and CHO cells, respectively. For constructs not processed by T4 DNA polymerase, the resulting activity was only 10% and 31% that of wild type in tobacco and CHO cells, respectively. Similar impairment of activity was observed for the TMV 3'-UTR-containing construct in which the 5 base insertion mutation separated the two domains or the construct in which only the tRNA-like domain was present (Figure 2). These data demonstrate that the deletion of just 6 bases from the 3'-terminus of the tRNA-like domain can profoundly affect the function of the TMV 3'-UTR, reducing activity up to 70% in tobacco. Because deletion of the entire tRNA-like domain is less detrimental, we propose that the UPD can tolerate a tRNA-like domain that has been separated from it only if the tRNA-like domain is intact — deletions that destroy higher-order interactions and result in a mutated tRNA-like domain, inhibit UPD function.

Using the data from the first two GUS constructs (Figure 2) we can assess the effect of a 5-base insertion (resulting from the creation of a *Bgl*II site) when the 3'-terminal pseudoknot of the tRNA-like domain is intact or has been disrupted. The 5-base insertion reduced the TMV 3'-UTR activity to 62% of the wild type TMV 3'-UTR in tobacco. The same 5-base insertion had little effect, however, on the construct in which the 3'-terminal six bases were missing (*Apa*I/T4 DNA polymerase) or when extra sequence was present at the 3'-terminus (*Apa*I). These data suggest that the contribution of the tRNA-like domain can be disrupted in several ways: by an insertion that separates this domain from the UPD or by deletions or sequence additions to the 3'-pseudoknot. The effect of these mutations is quite distinct. The 5-base insertion prevents the contribution of the tRNA-like domain; however, the presence of the separated tRNA-like domain is neutral to UPD function since the level of expression for this construct is the same as when the tRNA-like domain has been deleted. In contrast, deletion of the six 3'-terminal bases has far more drastic consequences for TMV 3'-UTR activity. This suggests that 3'-terminal deletion not only prevents the

positive contribution of the tRNA-like domain, but also results in inhibition of UPD function.

If the tRNA-like domain functions by increasing message stability, this should be reflected in a longer half-life for the GUS mRNA constructs terminating in the tRNA-like structure. The physical half-life measurements were made by following the kinetics of decay for the GUS mRNA constructs in electroporated tobacco protoplasts. Aliquots of cells were taken at time intervals, total RNA extracted, and Northern analysis performed (16). The

half-life of Ω -GUS-TMV 3'-UTR mRNA (98 min) was 1.7-fold longer than the control Ω -GUS mRNA (57 min), a relative increase in agreement with our previous measurements (16). Ω -GUS-tRNA mRNA exhibited a half-life of 92 min whereas Ω -GUS-UPD mRNA was not as stable (76 min). These data suggest that the presence of the tRNA-like domain increases message stability and that the UPD, which primarily regulates translational efficiency, can have a small stabilizing effect if the tRNA-like domain is absent.

Figure 1. The sequence and higher-order structure of the TMV 3'-UTR (17). The site of insertion for the *Bgl*II site is indicated. The UPD is immediately upstream and the tRNA-like domain immediately downstream of the *Bgl*II insertion site. Small case letters represent vector sequence.

	Specific Activity (nmoles/min-mg)					
	TOBACCO			CHO CELLS		
	NdeI	Apal-T4	Apal	NdeI	Apal-T4	Apal
GpppG Ω AUG GUS UGA TMV-3'-UTR	95.8	26.9	9.8	62.1	33.8	19.2
GpppG Ω AUG GUS UGA [5 base insertion] A N	59.1	26.0	9.2	49.7	29.4	26.3
GpppG Ω AUG GUS UGA [tRNA-like domain] A N	1.0	0.6	0.6	30.6	21.0	16.1
GpppG Ω AUG GUS UGA [UPD] N	56.4	n.a.	n.a.	42.1	n.a.	n.a.
GpppG Ω AUG GUS UGA PL-3'-UTR	0.4	n.a.	n.a.	14.8	n.a.	n.a.

Figure 2. Mutations of the tRNA-like domain affecting GUS expression in electroporated tobacco and CHO cells. The UPD is represented by the stippled box and the tRNA-like domain is represented by the black box. The GUS construct containing the wild type TMV 3'-UTR is represented at the top. The second construct contains a 5 base insertion (generating a *Bgl*II site) between the UPD and tRNA-like domain. The third and fourth constructs contain the tRNA-like domain or the UPD alone. The last construct contains pBluescript sequence as its 3'-UTR. Each DNA construct was linearized with the appropriate restriction enzyme, either *Nde*I, *Apal*I, or *Apal*I followed by processing with T4 DNA polymerase. For the GUS construct containing the PL-3'-UTR, the DNA was linearized with *Bgl*II. n.a.; not assayed because the DNA construct does not contain an *Apal* site. A, *Apal*I; N, *Nde*I; B, *Bgl*II; BI, *Bgl*III.

Figure 3. The effect of additional sequence 3' to the TMV 3'-UTR or a poly(A)₅₀ tail. Ω -GUS-TMV 3'-UTR, Ω -GUS-A₅₀, and Ω -GUS-PL-3'-UTR mRNAs were electroporated into tobacco (panels A, C, E) and CHO (panels B, D, F) cells. In each case, the X-axis corresponds to the number of nucleotides extending from the stop codon of the GUS reading frame which is drawn to the scale of the relevant panel. The Y-axis is the GUS activity resulting from each electroporated mRNA construct. Restriction sites used to produce mRNAs with various 3'-UTRs are S, *Sac*I; N, *Nde*I; D, *Dra*I; RI, *Eco*RI; Pv, *Pvu*II; Bg, *Bgl*II; Sc, *Sca*I.

The effect of additional downstream sequence on the ability of the UPD, tRNA-like structure, or the TMV 3'-UTR to function

A poly(A) tail and the TMV 3'-UTR function best when positioned at the 3'-terminus of mRNAs (16, 21). To investigate the effect of additional sequence present 3' to either domain of the TMV 3'-UTR, GUS mRNAs containing either the UPD, the tRNA-like domain or the TMV 3' UTR were synthesized with increasing lengths of sequence 3' to the respective domain. This was accomplished by linearizing the respective plasmids at various restriction sites downstream of the GUS coding region (see Figure 3) and electroporating the *in vitro* synthesized mRNAs into plant and animal cells. For comparison, poly(A)⁺ and poly(A)⁻ GUS mRNAs with the same 3'-UTRs were synthesized and assayed in the same way as the TMV 3'-UTR-containing constructs. When GUS activity resulting from the construct containing the TMV 3'-UTR was analyzed, the pattern was similar in both tobacco and CHO cells. Linearization with *Nde*I exhibited maximal activity; however, when the construct was linearized with *Eco*RI, resulting in a 12 base addition to the 3'-terminus, activity decreased 25% in CHO cells and 48% in tobacco. A 102 base addition caused a 44% decrease in expression

in CHO cells and 85% in tobacco. Larger additions resulted in a greater decrease in the CHO cells. These observations demonstrate that there is a functional requirement for the TMV 3'-UTR to be positioned at, or very near the 3'-terminus.

When the GUS construct containing the poly(A)₅₀ tract was analyzed, some differences were observed between tobacco and CHO cells (compare Figure 3C with 3D). In tobacco, a 12 base addition to the 3'-terminus resulted in a 53% drop in expression (when compared to the construct with the shortest additional sequence, i.e., three uridylyte residues). In contrast, in CHO cells, a 12 base addition resulted in a 40% increase in expression (Figure 3); this increase was reproducible in subsequent experiments (data not shown). Greater lengths of sequence, however, were inhibitory. In the absence of a poly(A) tail or the TMV 3'-UTR, the presence of additional downstream sequence resulted in a moderate increase in expression in tobacco (Figure 3E), possibly by acting as a buffer through which an exonuclease must pass before reaching the GUS coding region. In CHO cells, the addition of up to 163 bases increased expression (Fig. 3F) as in tobacco, however, lengths of sequence greater than this decreased expression.

Because additional sequence downstream of the TMV 3'-UTR adversely affected function, we next investigated the effect of

Figure 4. The effect on function of additional sequence 3' to the UPD, the tRNA-like domain, or the TMV 3'-UTR mutant in which the two domains are uncoupled by a 5-base insertion. Ω -GUS-UPD, Ω -GUS-tRNA (tRNA-like domain), and Ω -GUS-TMV 3'-UTR (5 base insertion) mRNAs were electroporated into tobacco (panels A, C, E) and CHO (panels B, D, F) cells. In each case, the X-axis corresponds to the number of nucleotides extending from the stop codon of the GUS reading frame which is drawn to the scale of the relevant panel. The GUS coding region is not drawn to scale. The UPD is represented by a stippled box; the tRNA-like domain is represented by a black box. The Y-axis is the GUS activity resulting from each electroporated mRNA construct. Restriction sites present within the 3'-region used to produce mRNAs with the various 3'-UTRs are S, *SacI*; B, *BglII*; N, *NdeI*; RI, *EcoRI*; Pv, *PvuI*; Bg, *BglI*; Sc, *SalI*.

adding sequence downstream of each domain individually. Because the UPD is largely responsible for the regulation observed, the tRNA-like domain can be viewed, at a first approximation, as additional downstream sequence. Would replacing the tRNA-like domain with unrelated sequence have a different effect? Sequence additions to the 3'-terminus of GUS containing just the UPD were detrimental in both tobacco and CHO cells (Figures 4A and 4B), even when sequence of approximately equivalent length was employed. It can be concluded that the presence of additional sequence 3' to the UPD has an inhibitory effect on UPD function. In this respect, the tRNA-like domain functions quite differently. Its effect on UPD function was either positive, i.e., when present in its wild type configuration, or neutral, i.e., when separated by five bases from the UPD.

For GUS mRNAs containing only the tRNA-like domain, a 12 base addition is detrimental. As greater lengths of sequence are added, however, this effect is generally moderated in both cell types (Figures 4C and 4D). This observation may reflect a non-specific 'protective effect' as discussed above.

In the construct in which the 5 base insertion separates the UPD and tRNA-like domains, it might be predicted that the presence

of additional downstream sequence would have little effect on expression. We found, however, that sequence addition to the 3'-terminus inhibited function in both cell types (Figures 4E and 4F). This demonstrates that even when the two domains are uncoupled, UPD function is still sensitive to the presence of additional sequence 3' to the tRNA-like structure.

DISCUSSION

The observation that the TMV 3'-UTR functions in mammalian as well as plant cells suggests that if any cellular factor(s) is required for TMV 3'-UTR function, it must be common in higher eukaryotes. This raises the intriguing question of the role of such a factor(s) in normal cellular processes. The TMV 3'-UTR is a sequence which seems to have evolved as an functional alternative to a poly(A) tail; however, it is unlikely that the TMV 3'-UTR interacts with PAB protein, as the binding of PAB protein is specific for poly(A). Moreover, exogenous oligo A is inhibitory to the translation of poly(A)⁺ mRNA but not TMV RNA *in vitro* (24).

Although the UPD is largely responsible for the regulation associated with the TMV 3'-UTR, the tRNA-like domain is

required for full function and was the focus of the present work. The 2-fold effect on UPD function associated with the tRNA-like domain was lost either by deleting the tRNA-like domain altogether or by the less drastic approach of separating the two domains with a 5-base insertion. The uncoupling of the tRNA-like domain was sufficient to destroy its contribution completely, however, the presence of this separated domain was not inhibitory to UPD function. Deletion of the tRNA-like domain which leaves five additional bases at the UPD 3'-terminus also had little effect on expression. On the other hand, removal of the terminal six bases of the tRNA-like domain, which results in the loss of the 3'-terminal pseudoknot and should prevent this domain from assuming a tRNA-like conformation, seriously impaired UPD function. This suggests that an incomplete tRNA-like domain acts in an as yet unknown inhibitory fashion on the UPD. Separating this mutated tRNA-like domain by a 5-base insertion between it and the UPD did not rectify its inhibitory action. Similarly, additional sequence present 3' to the TMV 3'-UTR terminus was detrimental. Downstream sequence, therefore, whether derived from the plasmid vector sequence or from an incomplete tRNA-like domain had a markedly deleterious effect on UPD function. It is possible that all of these mutations interfere with correct pseudoknot formation within the UPD.

How does the tRNA-like structure make its contribution to the regulation of the TMV 3'-UTR? We propose several possible mechanisms by which the tRNA-like domain could function and help to explain the tRNA mimicry. Message stabilization in prokaryotes and chloroplasts can be achieved by specific secondary structures at the 3'-terminus blocking 3'→5' exonuclease activity (25, 26, 27). The secondary and tertiary structure of the tRNA-like domain may function in a similar manner, providing no free single-stranded terminus for exonuclease attack. Moreover, if the CCA-3' terminus undergoes attack, it can be repaired by nucleotidyl transferase. Thus, the integrity of the 3'-terminus would be maintained (9, 28), and resistance to exonuclease attack would result in a longer half-life. Aminoacylation of the tRNA-like structure may also help to protect the RNA from exonucleases. Aminoacylation requires an intact CCA sequence at the 3'-terminus, and therefore provides a role for nucleotidyl transferase as well. And finally, as a result of tRNA mimicry, several interactions with tRNA-specific, RNA-binding proteins are possible. The presence of these protein(s) may also serve to block exonuclease attack.

Because the TMV 3'-UTR not only increases message stability but also influences translational efficiency, the 3'-UTR may be involved in ribosome loading or translation initiation. There are several examples of determinants in the 3'-UTR that are involved in both mRNA stabilization and regulation of translational efficiency. The AUUUA element found in the 3'-UTR of many oncogenes, in addition to its well-documented role as a destabilizer of mRNA (29, 30, 31), can also repress translation (32, 33). Moreover, sequences within the 3'-UTRs of ferritin M subunit (34) and human β -interferon (33) mRNAs act as translational repressors. There are conflicting observations on the question of whether the poly(A) tail functions by increasing message stability or translational efficiency. Our data demonstrating that a poly(A) tail increased both the half-life and translational efficiency of reporter mRNA (21) in plant protoplasts would support the hypothesis of a dual role. Thus, the TMV 3'-UTR would share this property with the poly(A) tail.

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