RNA pseudoknot domain of tobacco mosaic virus can functionally substitute for a poly(A) tail in plant and animal cells

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The genomes of many RNA viruses terminate in a tertiary structure similar to the L-conformation of tRNAs and this structure is recognized by many tRNA-specific enzymes such as aminoacyl-tRNA synthetase. Virtually the entire 3'-untranslated region (UTR) of tobacco mosaic virus (TMV) RNA is involved in an extended tertiary structure containing, in addition to a tRNA-like structure, a pseudoknot domain that lies immediately upstream. Although the functions of these structures are not well understood, they are essential to the virus. We demonstrate that the addition of the 204-base TMV 3'-untranslated region to foreign mRNA constructs can increase gene expression up to 100-fold compared to nonadenylated mRNA. The 3'-UTR of TMV was equal to or greater than a polyadenylated tail in enhancing gene expression in electroporated dicot and monocot protoplasts. The TMV 3'-UTR is functionally similar to a polyadenylated tail in that it increases mRNA stability and translation and must be positioned at the 3' terminus to function efficiently. Similar effects on expression were observed in Chinese hamster ovary cells, demonstrating that the sequence functions in a wide range of eukaryotes. When the extended tertiary structure was dissected, the upstream pseudoknot domain was found to be largely responsible for increasing expression. The inclusion of the tRNA-like structure, however, was important for full regulation.

[Key Words: RNA pseudoknot; mRNA; translational efficiency; message stability; poly(A); tobacco mosaic virus]

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Two important properties of the post-transcriptional steps in the quantitative control of eukaryotic gene expression are the rate of mRNA decay in the cytoplasm and the rate of translation initiation. Most mRNAs contain a polyadenylated tail, a feature that protects the RNA from degradation (Brawerman 1987). A few additional determinants influencing mRNA stability have been discovered. For example, nonpolyadenylated histone mRNAs contain sequences in the 3'-untranslated region (UTR) that link the mRNA steady state to the cell cycle (Pandey and Marzluff 1987), and several proto-oncogenes contain AU-rich sequences that promote rapid turnover (Miller et al. 1984; Shaw and Kamen 1986; Brewer and Ross 1988). Several determinants important in establishing the stability of a transcript have also been implicated in influencing translational efficiency. A number of examples demonstrate the positive effect that the poly(A) tail has on translation initiation (Jacobson and Favreau 1983; Palatnik et al. 1984; Shapiro et al. 1988). The rapid degradation of human interferon, granulocyte-macrophage colony-stimulating factor, and *c-fos* mRNAs is mediated by an AUUUA element in the 3'-UTR, and this element can also regulate their translational efficiencies (Kruys et al. 1987, 1989). These ex-

amples demonstrate that the 3'-UTR contains determinants that, in conjunction with the poly(A) tail, control the level of expression of protein from a message. In general, however, our understanding of the role of the 3'-UTR in influencing expression remains rudimentary. Moreover, little is known about the role that the determinants within the 3'-UTR of nonpolyadenylated mRNAs play in establishing the stability of an mRNA or its translational efficiency. Electroporation of in vitro-synthesized mRNA into mammalian and higher plant cells, however, allows a direct test for the impact of these specific features (Callis et al. 1987; Gallie et al. 1989).

The genomes of many RNA viruses are not polyadenylated but terminate with a tRNA-like structure (Hall 1979; Haenni et al. 1982), which, although deviating from the cloverleaf model of tRNA (Florentz et al. 1982; Rietveld et al. 1983), can be strikingly similar to the L-shaped tertiary conformation proposed for tRNAs (Rietveld et al. 1983; Joshi et al. 1983). Moreover, the tRNA-like RNAs can be recognized specifically by enzymes that normally require tRNAs as their substrate. Specific aminoacylation of the tRNA-like structures has been demonstrated for several viral RNAs (Hall 1979; Dreher and Hall 1988a); aminoacylation kinetics are comparable to those of the corresponding tRNA (Giege et al.

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1978), and only the aminoacylated form of the tRNA-like RNAs interacts with elongation factors (Litvak et al. 1973; Bastin and Hall 1976; Giege et al. 1978). These tRNA-like structures are recognized by ribonuclease P (Silberklang et al. 1977; Guerrier-Takada et al. 1988), nucleotidyl transferase (Litvak et al. 1970; Yot et al. 1970; Dreher and Hall 1988b), and a ribothymidine (rT)-forming tRNA methyltransferase that specifically methylates a uridine residue found in a stem-loop structure of TMV which is similar to the D-loop of tRNAs (Lesiewicz and Dudock 1978). Although evidence suggests that the tRNA-like structures are involved in viral replication (Ahlquist et al. 1984; Miller et al. 1986; Dreher and Hall 1988b), their full purpose is not defined. They are, however, essential: deletion of 5–10 bases from the 3' terminus of TMV results in the loss of infectivity (Salomon et al. 1976), and certain point mutations are lethal within the tRNA-like structure of brome mosaic viral RNA (Dreher and Hall 1988b) as is the acetylation of the aminoacylated form of the RNA (Hall 1979). Because the integrity of the 3' terminus must be maintained, it has been suggested that the tRNA-like structure acts in a telomeric fashion (Weiner and Maizels 1987; Rao et al. 1989).

Immediately upstream of the 105-base region comprising the tRNA-like structure of TMV lies a 72-base region in which three consecutive pseudoknots are found (van Belkum et al. 1985). These pseudoknots are not required for the recognition of the tRNA-like structure by the tRNA-aminoacyl synthetase or nucleotidyl-transferase (Joshi et al. 1985). Although no function has been determined for them, all capped nonpolyadenylated plant viral mRNAs examined to date contain one or more pseudoknots in the 3'-UTR (Pleij et al. 1987).

Replication of TMV and other RNA viruses does not pass through a DNA stage. Evidence suggests that the site of replication for TMV is in the cytoplasm rather than in the nucleus (Saito et al. 1987), therefore, the

TMV 3' region interacts only within the cytoplasmic milieu. As the TMV genome is also an mRNA, we hypothesized that its unusual 3'-UTR may act as a functional equivalent to a poly(A) tail to increase gene expression. We initiated this study of the TMV 3'-UTR, therefore, to gain a greater understanding of the alternative mechanisms by which nonpolyadenylated mRNAs regulate mRNA stability and translational efficiency and to gain a greater appreciation of the contribution that the 3'-UTR makes to controlling gene expression.

Results

The TMV 3'-UTR increases gene expression in plant protoplasts

To measure accurately the effect of the TMV 3'-UTR on gene expression, a cDNA clone containing the 247-base 3'-terminal sequence of TMV was introduced downstream of the β -glucuronidase (GUS) reporter gene (Jefferson et al. 1986; Fig. 1). A 260-bp *SacI*–*EcoRI* DNA fragment from the TMV cDNA clone, p3F1 (in which the *PstI* site had been modified to an *EcoRI* site; a generous gift from W. Dawson) was introduced into the *SacI*–*EcoRI* sites of pT7-GUS, a T7-based transcription vector described previously (Gallie et al. 1989). The TMV 3'-UTR was also introduced downstream of the GUS reporter gene in which the TMV 5'-UTR [called Ω (Mandel 1968); Fig. 1] was substituted for the 17-base polylinker leader of the pT7-GUS vector. Ω has been shown to enhance substantially the translation of many mRNAs (Gallie et al. 1987, 1989). Control GUS mRNA constructs contained either a poly(A)₅₀ tail, previously shown to be sufficient in plant cells (Gallie et al. 1989), or a sequence derived from the T7-based vector of approximately the same length as the TMV 3'-UTR (PL 3'-UTR, Fig. 1). GUS mRNAs synthesized *in vitro* were introduced, via electroporation, into tobacco, carrot,

Figure 1. mRNA constructs used to test the effect of the TMV 3'-UTR on gene expression. All the constructs contain precisely the same GUS gene (not drawn to scale) and the first 43 bases of the native GUS 3'-UTR. Two 5'-UTRs were employed: a 17-base polylinker and a 67-base sequence representing the TMV 5'-UTR (Ω). Three 3'-UTRs (downstream of the 43-base native GUS 3'-UTR to the *BglI* site of pUC19; a 194-base sequence PL-3'-UTR representing sequence from the junction with the 43-base native GUS 3'-UTR to the 6150 to 6396 of the TMV genome (Goelet et al. 1982)—the first 43 bases of this contain the 3' terminus of the coding region for the TMV coat protein gene; and a synthetic 82-base 3'-UTR containing a functional poly(A)₅₀ tail. The constructs containing the PL-3'-UTR and the poly(A)₅₀ tail have been described previously (Gallie et al. 1989). The PL-3'-UTR, TMV 3'-UTR, and poly(A)₅₀ sequences shown are those resulting from transcription of each vector when linearized with *BglI*, *NdeI*, and *DraI*, respectively. For the positions of the restriction sites, see Fig. 3.

maize, and rice protoplasts, and the resulting GUS enzyme activity measured. In tobacco, the presence of the TMV 3'-UTR resulted in a 35- to 50-fold increase in GUS expression compared to the PL-3'-UTR and 2-fold relative to the poly(A) tail (Table 1), demonstrating that the TMV 3'-UTR sequence has a substantial effect on expression. In the presence of Ω , expression increased 80- to 120-fold when the TMV 3'-UTR was present compared to the PL-3'-UTR and 7-fold when compared to the poly(A) tail, suggesting that a small synergistic interaction may exist between the 5'- and 3'-UTR sequences of TMV in the usual viral host, tobacco. In carrot, there was an equally large effect (>80-fold increase in expression), whereas in maize and rice, >40- and 50-fold increases, respectively, were observed compared to the PL-3'-UTR, demonstrating that the TMV 3'-UTR is functional in plant species that are not hosts for TMV. As was found with tobacco, in the nonhost maize, the TMV 3'-UTR was approximately twofold more effective than a poly(A) tail, independent of the presence of Ω at the 5' terminus of the mRNA. Absolute levels of expression were increased in all species when Ω was present as is expected from its role as a translational enhancer (Table 1).

The upstream pseudoknot domain is the primary determinant for the regulation associated with the TMV 3'-UTR sequence

To elucidate whether the upstream pseudoknot domain and the tRNA-like structure contribute individually to the regulation associated with the TMV 3'-UTR, each of these domains was tested separately in tobacco protoplasts. This was made possible by creating a *Bgl*III site between the two domains (Fig. 2). The introduction of the *Bgl*III site, an insertion of only 5 bases, resulted in a 38% drop in GUS expression (Fig. 2, line 2). When the

domain containing the three upstream pseudoknots was present, ~65% of full activity remained (Fig. 2, line 3). The tRNA-like structure, in the absence of the upstream pseudoknot domain, only marginally increased GUS expression (Fig. 2, line 4). When the Ω -containing GUS constructs were analyzed, similar results were obtained. The introduction of the *Bgl*III site between the domains of the TMV 3'-UTR resulted in a drop of 44%. When the pseudoknot domain was present alone, 52% of wild-type activity remained, whereas the tRNA-like structure alone did little to increase expression. The presence of Ω in the GUS constructs, therefore, did not affect the contributions that the individual domains make towards increasing expression. These data demonstrate that it is the upstream pseudoknot domain that is largely responsible for the regulation observed for the TMV 3'-UTR but that the tRNA-like structure is also required for full function. The contribution that the tRNA-like structure makes was lost when the 5-base insertional mutation was made between it and the pseudoknot domain, suggesting that the tRNA-like structure must be positioned in the correct relationship with respect to the pseudoknot domain to function. Whether the disruption caused by the *Bgl*III site depends on its sequence or the additional spacing introduced between the two domains remains to be elucidated. It is interesting to note, however, that the 5-base insert interrupts a 35-bp region of coaxial stacking of which the pseudoknot domain contributes 25 bp and the tRNA-like structure contributes 10 bp (van Belkum et al. 1985).

The TMV 3'-UTR functions efficiently only if present at the 3' end of a message

We have shown recently that the poly(A) tail must be positioned at the 3' terminus to function efficiently (Gallie et al. 1989). As the poly(A) tail forms the 3' ter-

Table 1. Effect of the TMV 3'-UTR on GUS mRNA expression in electroporated dicot and monocot protoplasts

mRNA	Specific activity							
	tobacco (<i>n</i> = 3)		carrot (<i>n</i> = 2)		maize (<i>n</i> = 2)		rice (<i>n</i> = 1)	
Polylinker 5'								
GUS	0.08	0.03	0.04	0.05	0.10	0.16	0.02	
GUS-TMV 3'-UTR	2.79	1.62	3.30	5.61	4.70	5.59	1.13	
GUS-A ₅₀	1.28	0.77	NA	NA	2.24	2.67	NA	
TMV 5'								
Ω -GUS	2.06	1.68	1.21	1.77	0.96	1.07	0.11	
Ω -GUS-TMV 3'-UTR	170	208	90.1	142	42.9	56.6	5.71	
Ω -GUS-A ₅₀	27.1	41.0	NA	NA	21.6	27.1	NA	

Specific activity is reported in nanomoles of substrate metabolized per minute per milligram of protein. *n* represents the number of times an experiment was repeated. For tobacco, values from two independent experiments giving the highest and lowest expression are reported to illustrate the range observed for each construct. The GUS and Ω -GUS constructs were linearized with *Bgl*II (see Fig. 3 for location of restriction sites), which, after in vitro transcription, results in an mRNA containing a 237-base 3'-UTR. The Ω -GUS-TMV 3'-UTR constructs were linearized with *Nde*I resulting in a 323-base 3'-UTR, the terminal 247 bases of which is the TMV 3'-UTR sequence. The poly(A)-containing constructs were linearized with *Dra*I, resulting in a 125-base 3'-UTR. The 5'-UTR consisted of either a 17-base polylinker sequence or the TMV 5'-UTR (Ω). Five micrograms of capped mRNA was used for each electroporation, and the protoplasts were incubated for 20 hr following electroporation before analysis.

(NA) Not assayed.

Figure 2. Delineation of the structural feature responsible for the regulation associated with the TMV 3'-UTR. The light stippled box represents the upstream pseudoknot domain and the black box represents the tRNA-like domain. GUS mRNAs were constructed such that the following were positioned downstream of the GUS gene: the entire TMV 3'-UTR (line 1); an insertional mutation introducing a *Bgl*II site between the two domains of the TMV 3'-UTR (line 2); the pseudoknot domain alone (line 3); the tRNA-like domain alone (line 4); the TMV OAS sequence (line 5); or the vector-derived sequence, PL-3'-UTR (line 6). GUS mRNAs with Ω or without Ω (i.e., the leader was the PL-5'-UTR) were used for the above constructions. mRNAs were delivered into both tobacco protoplasts and Chinese hamster ovary cells using electroporation. Specific activity is reported in nanomoles of substrate metabolized per minute per milligram of protein. The values are the average from two independent experiments.

minus for cellular mRNAs, so the tRNA-like structure forms the 3' terminus of the viral RNA. To test whether sequences 3' to the TMV 3'-UTR compromise its function, Ω-GUS mRNAs with increasing lengths of sequence downstream of the TMV 3'-UTR were analyzed (Fig. 3). In tobacco protoplasts, the presence of just 12 extra bases (Fig. 3, vector linearized with *Eco*RI) proved to have a detrimental effect (20% loss of expression) on the capacity of the TMV 3'-UTR to boost expression. A decrease was consistently observed in subsequent repetitions and varied from 10 to 50%. Expression dropped 80% when 102 bases were added 3' to the TMV 3'-UTR, but longer 3' sequences (up to 1100 bases) had little additional effect over that seen for the first 102 bases. Similar results were observed in carrot, maize, and rice protoplasts (Fig. 3; similar results were obtained with constructs containing the PL-5'-UTR, data not shown). We hypothesize that additional bases interfere with formation of the higher-order structure and thereby compromise the function of the TMV 3'-UTR.

The TMV 3'-UTR increases gene expression in animal cells

To determine whether the TMV 3'-UTR could function in other eukaryotes, the GUS mRNA constructs illustrated in Figure 2 were introduced into Chinese hamster ovary (CHO) cells using electroporation. In these cells, expression was increased by the TMV 3'-UTR (4.5-fold) although to a lesser extent than that observed in tobacco

protoplasts (Fig. 2, line 1). A similar increase in expression was observed when a poly(A)₅₀ tail was added to a GUS mRNA construct in CHO cells (data not shown), suggesting that structural features at the 3' terminus of an mRNA may have less of an impact on increasing expression in CHO cells than in tobacco. The introduction of the *Bgl*II site between the two domains of the TMV 3'-UTR resulted in a 27% reduction in activity in CHO cells (Fig. 2, line 2). As observed in tobacco protoplasts, the upstream pseudoknot domain had the largest effect on increasing expression (64% activity of the intact TMV 3'-UTR; Fig. 2, line 3), but in contrast to its marginal effect in tobacco protoplasts, the tRNA-like domain had a greater effect in CHO cells, resulting in a 1.9-fold increase in expression above that observed for the PL-3'-UTR control (Fig. 2, line 4). Similar results were obtained when GUS constructs containing Ω were analyzed, demonstrating that, as in tobacco protoplasts, Ω does not alter the contribution that the individual domains make to increasing expression. It is interesting to note that the level of expression of the construct containing both domains (Fig. 2, line 1) can be viewed as the sum of the individual contributions made by each domain separately. This is not observed for the construct in which the two domains are separated by the *Bgl*II site, demonstrating that the tRNA-like domain does play a role in the function of the TMV 3'-UTR, but only when positioned in the correct relationship with respect to the upstream pseudoknot domain. The contribution of the appropriate positional relationship of the tRNA-like

Figure 3. Effect of additional sequences 3' to the TMV 3'-UTR on gene expression. Ω -GUS-TMV 3'-UTR and Ω -GUS constructs are illustrated. The region downstream of the GUS coding region is drawn to the scale (shown directly above the constructs). The GUS coding region is not drawn to scale. The TMV 3'-UTR is represented by the solid region. Restriction sites present within the 3' region used to produce mRNAs with 3'-UTRs differing in length are: (S) *SacI*; (N) *NdeI*; (R) *EcoRI*; (Pv) *PvuII*; (Bg) *BglI*; (Sc) *ScaI*. Species of protoplasts tested were: (A) tobacco; (B) carrot; (C) maize; (D) rice. (●) Ω -GUS-TMV 3'-UTR-based constructs (left scale); (○) Ω -GUS-based constructs (right scale). Five micrograms of each capped GUS mRNA construct was used for electroporation. Virtually 100% of the viable protoplasts receive and express the mRNA delivered during electroporation (Gallie et al. 1989).

structure to the pseudoknot domain is independent of cell type, as it was observed in both tobacco and CHO cells. These data demonstrate that the TMV-3'-UTR sequence is capable of functioning in a wide variety of eukaryotic cells, suggesting that if any interactions with cellular factors are involved, those factors are common to both higher plant and mammalian cells.

The presence of higher-order structure at the 3' terminus does not account for the increase in expression

One plausible mechanism to explain why the highly structured TMV 3'-UTR influences expression is that it acts to block 3' $\rightarrow$ 5' exonuclease degradation. This mechanism has been demonstrated for chloroplast and *Escherichia coli* mRNAs; that is, stem-loop structures present in the 3'-UTRs of certain mRNAs are responsible for the relative stability of those messages (Burton

et al. 1983; Belasco et al. 1986; Stern and Grissem 1987). To test whether a stable secondary structure was sufficient to increase gene expression, a reporter mRNA was constructed with the origin of assembly sequence (OAS; Butler 1984) of TMV at its 3' terminus. Consisting of a 237-base region within TMV, the OAS acts as the nucleation site for encapsidation of the RNA as a result of its extensive stable structure; this structure is recognized by the coat protein during virion assembly (Turner and Butler 1986; Turner et al. 1988). A 440-bp cDNA clone containing the TMV OAS was positioned downstream of the GUS gene so that GUS mRNA could be produced that terminated with this higher-order structure. Because the tobacco protoplasts used for the transient assay were not infected with TMV, no TMV coat protein was present, eliminating any potential for interaction between the coat protein and the OAS region. The expression resulting from this construct was

equivalent to GUS mRNA constructs containing the PL-3'-UTR (Fig. 2, line 5). The inability of the OAS sequence to increase expression demonstrates that the mere presence of higher-order structure at the 3' terminus of a message does not guarantee increased expression. Similar results were observed in CHO cells where the presence of the OAS sequence did not result in an increase in expression (Fig. 2, line 5). The observation that the stable, higher-order, tRNA-like structure does not substantially increase expression when present alone at the 3' terminus of the message reinforces the conclusions of the OAS experiment.

The TMV 3'-UTR increases mRNA half-life to the same extent as a poly(A)₅₀ tail

To determine whether the presence of the TMV 3'-UTR actually resulted in mRNA stabilization, the $t_{1/2}$ of GUS mRNAs containing either the TMV 3'-UTR or an Ω , or both, was measured in tobacco protoplasts (Fig. 4). In good agreement with previous determinations (Gallie et al. 1989), the $t_{1/2}$ for GUS and Ω -GUS mRNAs was 27 and 24 min, respectively. The TMV 3'-UTR increased the $t_{1/2}$ of GUS mRNA 2.8-fold (75 min) and that of Ω -GUS mRNA by 2.5-fold (60 min). These effects are similar to the threefold increase conferred by a poly(A) tail ($t_{1/2}$ of 72 min). As demonstrated previously for a poly(A) tail (Gallie et al. 1989), the increase in mRNA stability by the TMV 3'-UTR does not fully account for the large stimulatory effect observed on gene expression. It is possible, however, that there are multiple pools of a given mRNA, for example, polysome associated or not, with different rates of turnover. By analyzing the total mRNA pool, only an average $t_{1/2}$ is determined; this approach may underestimate the full effect of a stabilizing factor such as the TMV 3'-UTR. What can be concluded from this experiment, though, is that the magnitude of stabilization mediated by the TMV 3'-UTR is approximately equivalent to that of a poly(A)₅₀ tail.

Because the stability data can not completely account for the regulation associated with the TMV 3'-UTR, it seems likely that the TMV 3'-UTR has a secondary role involved in controlling translational efficiency. Moreover, the TMV 3'-UTR appears to act in a synergistic manner with Ω . If these two determinants had functioned independently when both were present in a GUS mRNA construct, the resulting level of expression would be the multiplication of their individual effects. The resulting level of expression, however, is consistently greater than this theoretical amount, suggesting that an interaction between these two determinants exists that is either direct or mediated by a cellular factor. Although further work will be required to establish the mechanism of such proposed interactions, the synergism observed supports the idea that the TMV 3'-UTR influences translation efficiency at least in tobacco.

Discussion

In this report, we have demonstrated that the TMV 3'-

Figure 4. The effect of 3'-UTR sequence on GUS mRNA half-life. Tobacco protoplasts were electroporated with 5 μ g of in vitro-synthesized, capped GUS mRNAs and total RNA was extracted at the time intervals indicated above each lane of the Northern hybridization. The half-life of each mRNA was determined as described in Methods. (A) GUS mRNA; (B) Ω -GUS mRNA; (C) GUS-TMV 3'-UTR mRNA; (D) Ω -GUS-TMV 3'-UTR mRNA; (E) Ω -GUS-A₅₀ mRNA.

UTR acts post-transcriptionally to increase substantially gene expression in both plant and animal cells. Work in progress has demonstrated that the TMV 3'-UTR increases the expression of a second reporter gene, that is, luciferase, demonstrating that the effect is reporter gene independent in all cell types tested (D.R. Gallie, J. Feder, and V. Walbot, unpubl.). We have demonstrated that a 177-base region, which contains extensive tertiary interactions and encompasses virtually the entire 204-base 3'-untranslated region of TMV, is required for full regulation. The 72-base region that comprises the upstream pseudoknot domain, however, is largely responsible for the regulation associated with the TMV 3'-UTR. The precise requirements for this control of expression remain to be elucidated, but we have ruled out the mere presence of higher-order structure in the mRNA. Both the TMV OAS sequence, known to contain stable secondary structure, and the TMV tRNA-like structure, containing both secondary and tertiary structure, failed to increase expression substantially. Because the domain upstream of the tRNA-like structure is primarily responsible for the increase in gene expression, it may be hypothesized that it is the presence of RNA pseudoknots in general that is important for the regulation. As the tRNA-like structure itself contains RNA pseudoknots, this hypothesis is not sufficient. Further dissection of the 72-base upstream pseudoknot domain will determine whether any of the three pseudoknots are

nonessential for function. Whether the secondary and tertiary interactions required for pseudoknot formation are essential for the domain's regulatory function will be established either by point mutations that disrupt, or by compensatory mutations that restore, the higher-order interactions. It is also possible that a protein factor(s) interacts specifically with this domain and is required for regulatory function. If such a cellular factor exists, it is likely to be universal in higher eukaryotes as the TMV 3'-UTR does function in both plant and animal cells.

There are functional similarities between the TMV 3'-UTR and a poly(A) tail. Both serve to stabilize mRNA and increase gene expression; each is present at the 3' terminus, and the functions of both are inhibited by additional 3' sequence. Moreover, like a poly(A) tail, the TMV 3'-UTR functions in a variety of cell types, and in the absence of TMV, demonstrating that it acts independently of any specific viral factors and that any host factors that are involved are common to both plant and animal cells. The TMV 3'-UTR and a poly(A) tail do not necessarily operate through the same mechanism, however. The inhibitory effect of exogenous poly(A) on translation of polyadenylated mRNA (Jacobson and Favreau 1983; Grossi de SA et al. 1988) suggests that free poly(A) successfully competes with the polyadenylated mRNA for the poly(A)-binding protein, a factor required for efficient expression. In contrast, exogenous poly(A) has no effect on the expression of TMV mRNA (Grossi de SA et al. 1988), suggesting that the pseudoknot domain and tRNA-like structure do not require poly(A) binding protein for efficient function.

The upstream pseudoknot domain is the major factor in controlling expression for the TMV 3'-UTR, although its activity is increased twofold by the presence of the tRNA-like domain immediately downstream. By analogy, polyadenylated mRNAs may contain 3'-UTR determinants in addition to the poly(A) tail, which establish the level of expression of specific messages, leaving the poly(A) tail a more generic "protective" function.

Why should the TMV 3'-UTR have evolved as an alternative to a poly(A) tail in stabilizing mRNA and regulating gene expression? One possibility is that, in serving as a recognition site for the viral replicase, the TMV 3'-UTR promotes the selective amplification of viral RNA over the host mRNA population while it also fulfills the same role as a poly(A) tail. Because of the need to be unique in structure but similar in function, the study of nonpolyadenylated viral RNAs will continue to increase our understanding of the alternative means by which mRNAs have evolved to overcome the twofold problem of message stability and translational efficiency.

Methods

Plasmid constructs and in vitro transcription

The Ω -containing and poly(A)-containing GUS constructs have been described previously (Gallie et al. 1989). 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 as described (Schenborn and Mierendorf 1985). Capped transcripts were synthesized by modification of the published reaction conditions to include 200 μ M GTP and 1.5 mM m⁷G(5')ppp(5')G (New England BioLabs), which results in reproducible capping efficiency as measured by gene product production. The integrity and quantitation of RNA were determined by formaldehyde-agarose gel electrophoresis as described (Melton et al. 1984). Equivalent amounts of each mRNA construct were used for the *in vivo* analyses. The mRNAs produced from these constructs are illustrated in Figure 1.

Preparation and electroporation of plant protoplasts and animal cells

Protoplast media and isolation methods for cell suspensions of maize (Black Mexican Sweet) were as described (Fromm et al. 1987) except that 1.0% Cytolase (Genecor) was used in place of 0.5% Rhozyme. The cells were digested for ~2.5 hr before being harvested. Preparation of tobacco (Xanthi) and carrot (RCWC) protoplasts was identical except the cells were digested for 1.5 hr before harvesting. Rice (Yamabiko) protoplasts were prepared as described (Kyoizuka et al. 1987).

All electroporations used a 1550 μ F capacitor and an X-cell TM 450 Electroporation System (Promega Biotec). The condition for tobacco and maize was a 180-volt, 8-msec pulse, whereas a 270-volt, 10-msec pulse was used for carrot and rice. 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.

Standard electroporation conditions for the CHO cells were set with a 450-volt and 250- μ F capacitor using a Bio-Rad Gene Pulser. Electroporation of 5×10^6 cells was carried out at room temperature, and after 10 min, the cells were allowed to recover for 18 hr in 10% fetal calf serum medium before collection for analysis. Typical error associated with electroporation of RNA was less than $\pm 15\%$.

mRNA stability

Aliquots of tobacco protoplasts electroporated with 5 μ g of each mRNA were removed at specific time intervals, the cells collected by centrifugation, resuspended in 200 μ l of 50 mM Tris (pH 7.0), 50 mM EDTA, and 1% SDS, mixed with a 1:1 phenol/chloroform mixture, and the aqueous phase precipitated. After resuspension, the RNA was denatured and loaded onto a 1.2% formaldehyde-agarose gel, followed by Northern transfer to Nytran membrane (Schleicher & Schuell, Inc.), and probed with GUS antisense RNA. The region of the membrane representing the full-length form of the GUS mRNA was cut from the membrane, counted, and the natural log (ln) of the values plotted against time. k , the slope of the best-fit line through the data points, was used to calculate the half-life for each construct according to the equation $t_{1/2} = 0.693/k$ (Kabnick and Housman 1988).

Analysis of GUS activity

Plant or CHO cells were collected by centrifugation and resuspended and sonicated in 0.5 ml of buffer (50 mM sodium phosphate (pH 7.0), 10 mM β -mercaptoethanol, and 1 mM EDTA). 4-methylumbelliferyl- β -D-glucuronide (Sigma) was added to a final concentration of 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 Sci. Instr.). GUS-specific activity was determined as nanomoles of substrate per minute per milligram of protein. Protein concentration was determined using the Bio-Rad assay kit.

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RNA pseudoknot domain of tobacco mosaic virus can functionally substitute for a poly(A) tail in plant and animal cells.

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