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The tobacco etch viral 5′ leader and poly(A) tail are functionally synergistic regulators of translation

(Gene regulation; mRNA; cap; potyvirus; translational efficiency; electroporation; protoplasts; luciferase reporter gene)

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SUMMARY

The 5′ cap (m^7GpppN) and the poly(A) tail of eukaryotic mRNAs work in concert to establish an efficient level of translation in vivo. Nevertheless, several mRNAs naturally lack a cap or a poly(A) tail. Determining how these messages effectively compete for the translational machinery not only reveals alternative mechanisms for translational competence, but can also underscore similarities between alternative mechanisms and the standard cap/poly(A) tail interaction. The genomic RNA of tobacco etch virus (TEV), a potyvirus, is a polyadenylated mRNA that naturally lacks a cap (m^7GpppN) at the 5′-terminus and yet is a highly competitive mRNA during translation. The 144-nt 5′-leader is largely responsible for directing efficient translation and can greatly increase the translational competence of reporter mRNAs. We have examined the synergy between the TEV 5′-leader and the poly(A) tail in transfected plant and animal cells. The TEV 5′-leader functioned optimally as a regulator of reporter mRNA translation only when a poly(A) tail was present. The effect of the TEV 5′-leader on the translation of capped transcripts was significantly less than that for uncapped mRNAs, suggesting that the TEV 5′-leader and the cap may promote similar steps in translation.

INTRODUCTION

The majority of cellular mRNAs employ a cap (m^7GpppN) and a poly(A) tail to promote the initiation of translation. As regulators of translation, the cap and the poly(A) tail are mutually dependent (Gallie, 1991). Consequently, the cap and poly(A) tail are not functionally separate but work in concert, in conjunction with associated proteins, to direct efficient translation.

Not all mRNAs have or even require a cap or poly(A) tail for efficient translation. Several positive strand RNA viruses that infect plants are naturally capped but terminate in a tRNA-like structure instead of a poly(A) tail. For example, the 204-nt 3′-*UTR* of TMV is composed of two domains containing a total of five RNA pseudoknots (van Belkum et al., 1985). The pseudoknot immediately upstream from the terminal tRNA-like domain is responsible for directing efficient translational initiation (Leathers et al., 1993). Like a poly(A) tail, the 3′-*UTR* from TMV and BMV function optimally only when the mRNA is capped (Leathers et al., 1993; Gallie and Kobayashi, 1994). The 3′ terminal stem-loop of metazoan histone mRNAs not only regulates translation but requires a cap in order to do so (D.R.G., N.J. Lewis and W.F. Marzluff, data not shown). STNV mRNA is an example of a message which contains neither a cap or

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Abbreviations: bp, base pair(s); BMV, brome mosaic virus; BSA, bovine serum albumin; CHO, Chinese hamster ovary; eIF, eukaryotic initiation factor; kb, kilobase(s) or 1000 bp; Luc, firefly luciferase; *luc*, gene (DNA, RNA) encoding Luc; nt, nucleotide(s); PBS, phosphate-buffered saline (1.4 mM KH_2PO_4/3 mM Na_2HPO_4/150 mM NaCl); STNV, satellite tobacco necrosis virus; TEV, tobacco etch virus; TMV, tobacco mosaic virus; *uidA*, gene (DNA, RNA) encoding β-glucuronidase; *UTR*, untranslated region(s).

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poly(A) tail. For STNV mRNA, the 5′-leader and the 3′-*UTR* stimulate translation but only do so optimally when both are present in an mRNA (Danthinne et al., 1993; Timmer et al., 1993).

Other plant viruses naturally lack a cap but are polyadenylated. The 5′-leader of members of the potyvirus, comovirus, and luteovirus families can promote cap-independent translation (Tacke et al., 1990; Carrington and Freed, 1990; Thomas et al., 1991). Whether the 5′-leader functions more effectively to enhance the translation of poly(A)$^+$mRNA vs. poly(A)$^-$mRNA has not been investigated. To address this question, we examined the leader from TEV, which is a member of the picornavirus-like group of viruses (Goldbach and Wellink, 1988). The 144-nt leader of TEV RNA, the genomic RNA of which is naturally uncapped but polyadenylated, confers cap-independent translation on reporter mRNAs in plants (Carrington and Freed, 1990). In vitro synthesized reporter mRNAs were constructed to contain the TEV leader, a cap, a poly(A) tail, or any combination thereof. Examination of their effect on translation in vivo demonstrated that the TEV leader stimulated translation from poly(A)$^+$mRNA to a greater extent than it did from poly(A)$^-$mRNA in both plant and animal cells suggesting an interaction between the viral leader and poly(A) tail.

EXPERIMENTAL AND DISCUSSION

(a) The TEV 5′-leader stimulates expression from poly(A)$^+$mRNA in vivo but not in vitro

To determine whether the TEV 5′-leader preferentially stimulates expression from poly(A)$^+$mRNA, we measured the effect of the TEV 5′-leader and poly(A) tail on translation from firefly luciferase mRNA (*luc*) in vivo and in vitro. The *luc* open reading frame was introduced into a pT7-based vector between the TEV 5′-leader and a poly(A)$_{50}$ tract. Similar constructs without either the TEV leader or poly(A)$_{50}$ tract were also made. Following linearization of the vectors, eight *luc* mRNAs with every combination of the TEV leader, a poly(A) tail and a cap were synthesized in vitro (see Fig. 1) and delivered to carrot protoplasts using electroporation. The resulting Luc activity, assayed following incubation of the protoplasts, was used as a measure of the extent of expression from each construct. The effect of the TEV 5′-leader on expression could be determined by comparing the level of expression from the TEV 5′-leader-*luc* mRNA to that from the corresponding *luc* mRNA control. This can be done for the *luc* poly(A)$^+$mRNA, as well as the poly(A)$^-$mRNA. For uncapped transcripts, the addition of the TEV leader to *luc* poly(A)$^+$mRNA increased translation 260-fold (compare the TEV 5′-leader-*luc*-A$_{50}$ mRNA construct to the *luc*-A$_{50}$ mRNA control in Fig. 1) whereas the addition of the TEV leader to poly(A)$^-$mRNA increased translation only 44.9-fold (compare the TEV 5′-leader-*luc* mRNA construct to the *luc* mRNA control). The extent to which the TEV leader stimulated translation for each *luc* mRNA construct is summarized in Fig. 2A. The synergy is calculated as the ratio of the stimulatory effect that the TEV leader has on *luc* poly(A)$^+$mRNA vs. poly(A)$^-$mRNA (260:44.9 = 5.8). The TEV leader also increased translation from capped mRNA (Fig. 1); however, the degree to which it did so was virtually identical for both the poly(A)$^-$ and poly(A)$^+$ forms of the mRNA (33.6- and 36.2-fold, respectively, Fig. 2A). Consequently, there was a loss in the synergy between the TEV leader and the poly(A) tail when a cap was present. These results confirm that the TEV leader does increase translation in a cap-independent manner as was previously demonstrated (Carrington and Freed, 1990) and that it does so in plant protoplasts regardless of whether the mRNA is capped or polyadenylated. However, in addition to this basal effect on translation, the stimulatory effect of the TEV leader in an uncapped mRNA is almost 6-fold greater for those mRNAs with a poly(A) tail vs. nonpolyadenylated mRNAs. In their study, Carrington and Freed (1990) did not examine the synergy between the TEV 5′ leader and the poly(A) tail as they limited their analysis to polyadenylated mRNAs only. Their use of the *uidA* reporter gene also precluded an accurate measurement of the level of expression from the *uidA* mRNA without a cap or TEV 5′ leader as it was not significantly different from background. Our use of *luc* has allowed an accurate quantitation of the TEV 5′ leader on expression, as well as its synergistic interaction with the poly(A) tail.

A similar trend was observed in CHO cells into which the same *luc* mRNA constructs had been introduced via electroporation (Fig. 1). When the mRNA was uncapped, the TEV leader significantly increased translation only when the mRNA was polyadenylated (14-fold, Fig. 2B). The leader did not appreciably increase translation of capped mRNAs even when the mRNAs were polyadenylated. The data from the animal cells parallel those made in plant protoplasts: the TEV 5′-leader preferentially stimulates expression from uncapped but not capped poly(A)$^+$mRNA.

The same batch of *luc* mRNA constructs used for the above in vivo studies were translated in in vitro translation lysates to determine whether the preferential stimulation by the TEV leader on expression from poly(A)$^+$mRNA could be observed in vitro. The TEV leader failed to increase translation in wheat germ lysate regardless of whether the *luc* mRNA was capped or poly-

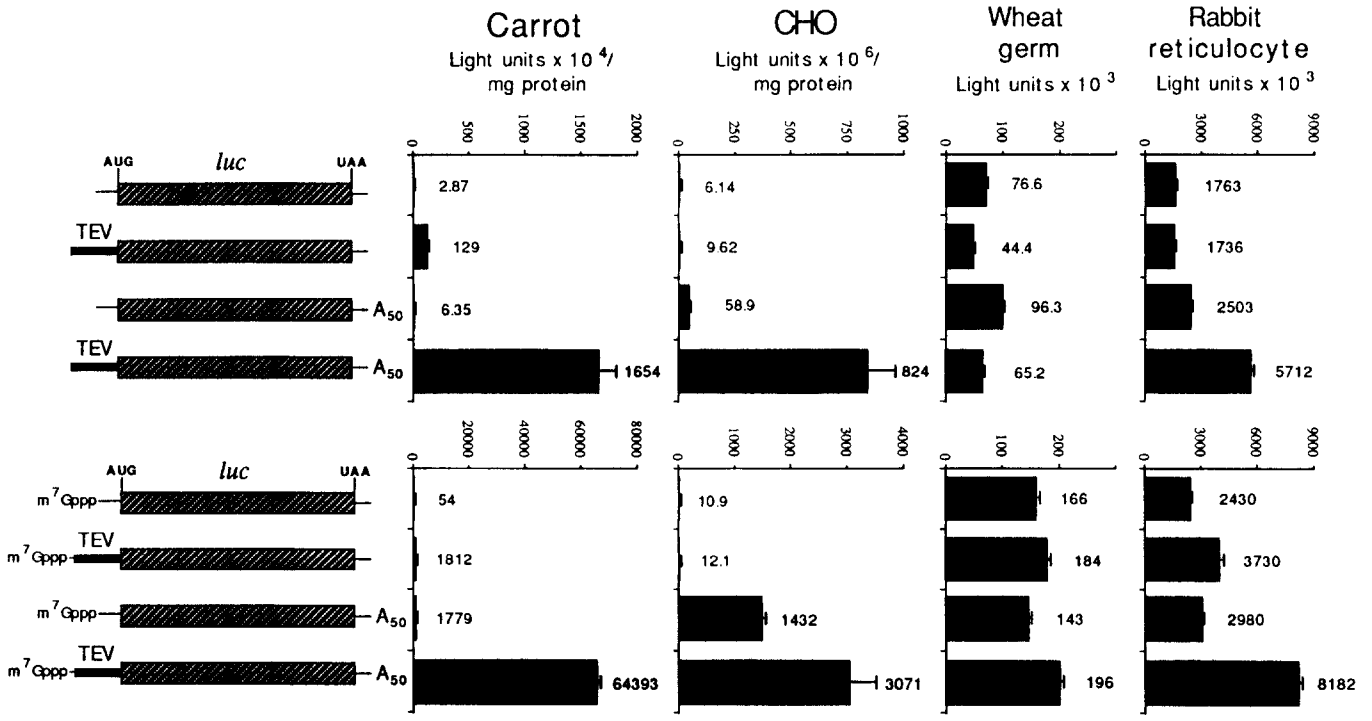


Fig. 1. The effect of the TEV leader on translation of *luc* mRNAs with or without a cap or poly(A) tail. A 133-bp *Eco*RI-*Nco*I fragment from the 144-nt TEV 5′-leader was introduced between the *Stu*I and *Nco*I sites of pT7-*luc*. A poly(A)$_{50}$ tract, introduced downstream from the *luc* coding region as a uninterrupted stretch of poly(A), allowed the in vitro synthesis of poly(A)$^+$mRNA following digestion of the template plasmid with *Nde*I which cuts at a site immediately downstream from the poly(A) tract. The *luc* mRNA constructs, shown on the left, were synthesized in vitro as described (Leathers et al., 1993) and delivered to carrot and CHO cells using electroporation. The same preparation of mRNAs was used for translation in vitro using lysates derived from wheat germ and rabbit reticulocytes. Following incubation of the cells or lysates, Luc assays were performed. The level of synthesis resulting from each mRNA construct for each translation system is shown as a histogram directly to the right of the respective mRNA. The numerical value for each histogram is indicated immediately to the right of each histogram. Note the difference in scale in the panels for the capped and uncapped mRNAs for the carrot and CHO cells. Under our transcription conditions, >95% of the mRNA is capped as determined by the shift in size on a 20% polyacrylamide gel of a small radiolabeled RNA synthesized either as uncapped and capped RNA. Each mRNA construct was synthesized in triplicate in separate transcription reactions so that any variability in RNA yield would be reflected in the standard deviation calculated as part of the expression data for each construct. The integrity and relative quantity of RNA were determined by formaldehyde-agarose gel electrophoresis as described (Melton et al., 1984). Each Luc assay was performed in duplicate as described previously (Leathers et al., 1993). Error bars representing one standard deviation are shown for each histogram. **Methods:** Protoplasts were isolated from a carrot cell suspension by digestion with 0.25% CELF cellulase (Worthington Biochemicals)/1% cytolase M103S (Genencor)/0.05% pectolyase Y23 (Seishin)/0.5% BSA/7 mM β-mercaptoethanol in isolation buffer (12 mM Na-acetate pH 5.8/50 mM $CaCl_2$/0.25 M mannitol) for 75 min. Protoplasts were washed once with isolation buffer, once with electroporation buffer (10 mM Hepes pH 7.2/130 mM KCl/10 mM NaCl/4 mM $CaCl_2$/0.2 M mannitol), and resuspended in electroporation buffer to a final concentration of 1.0×10^6 cells/ml. Each *luc* mRNA construct (2 µg) was mixed with 0.8 ml of protoplasts immediately before electroporation (500 µF capacitance, 350 V) using an IBI GeneZapper. CHO cells were grown to approx. 80% confluence in Hams F-12 medium supplemented with 10% fetal calf serum. Cells were collected from flasks by a brief incubation with 4 mM EDTA and washed twice with PBS. Cells (1×10^6) in 0.4 ml were mixed with 1 µg test mRNA and electroporated in PBS (200 µF capacitance, 400 V). Following electroporation, the cells were incubated for 6 h in Hams F-12 medium supplemented with 10% fetal calf serum before assaying. For in vitro translations, 50 ng of each *luc* mRNA construct was added to untreated rabbit reticulocyte or wheat germ lysate according to the recommendations of the supplier (Promega, Madison, WI, USA) with the exception that a complete mix of nonradiolabeled amino acids was used. The extent of translation was determined by assaying each aliquot for Luc activity.

adenylated (Fig. 1). In lysate derived from rabbit reticulocytes, the TEV leader increased translation marginally only when the mRNA was polyadenylated. This was observed for both capped and uncapped forms of the mRNA. Therefore, the activity of the TEV leader is greatly reduced in in vitro lysates compared to its effect in vivo, data suggesting that the in vitro translation systems do not fully reflect the conditions that prevail in vivo. Carrington and Freed (1990) had concluded that the TEV 5′ leader does enhance *uidA* expression in vitro. However, their construct containing the TEV 5′-leader also contained the TEV 3′-*UTR*. The 3′-*UTR* of several viral mRNAs has been shown to regulate translation (Leathers et al. 1993; Timmer et al., 1993; Danthinne et al., 1993; Gallie and Kobayashi, 1994) although the 3′-*UTR* from TEV has not been investigated. Consequently, the impact on expression attributed to the TEV 5′ leader by Carrington and Freed (1990) was complicated by the presence of the TEV 3′-*UTR*. As our constructs did not contain any other TEV sequence other

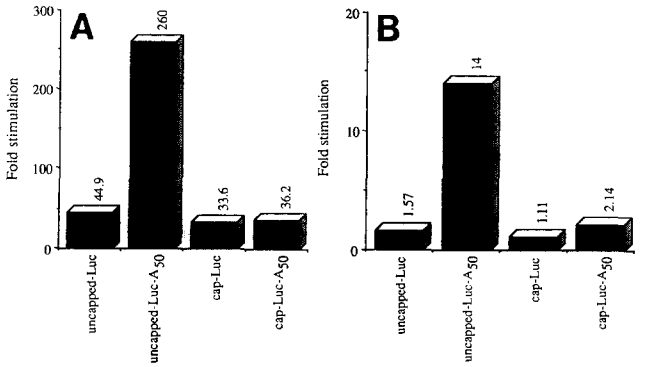


Fig. 2. Quantitative analysis of the extent to which the TEV leader stimulates the translation of *luc* mRNA in the presence or absence of a cap or poly(A) tail in (**A**) carrot protoplasts and (**B**) CHO cells. The histograms were derived from the data in Fig. 1 and represent the ratio of expression from each form of the mRNA with and without the TEV leader. Each histogram, therefore, represents the increase (-fold stimulation) in translation due to the addition of the TEV leader. The numerical values are indicated above each histogram.

than the TEV 5′ leader, we can attribute the observed changes in translation specifically to the TEV 5′ leader. We conclude, therefore, that the presence of the TEV 5′ leader alone in a reporter mRNA is insufficient to enhance translation in vitro.

(b) The TEV 5′-leader functions by stimulating the translational efficiency of an mRNA

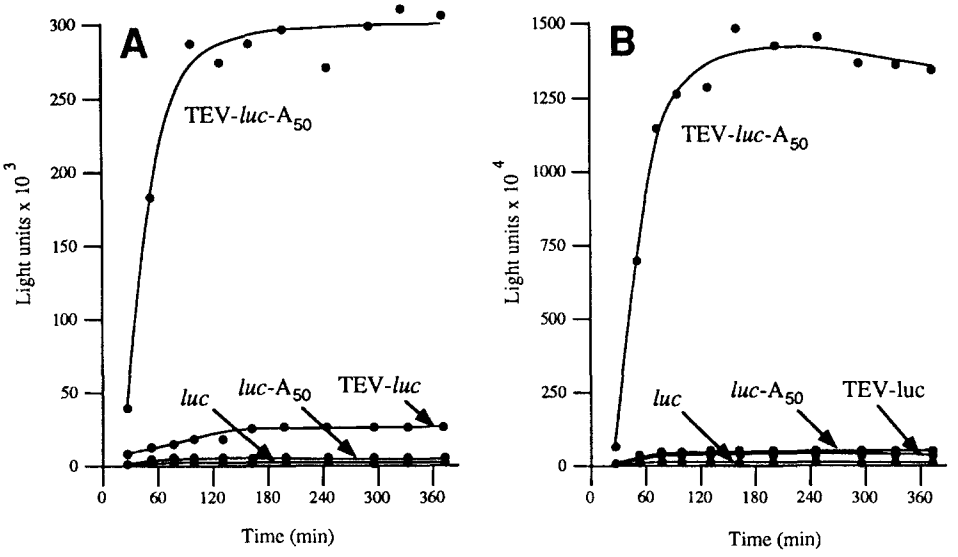
The effect of the TEV leader on expression might be the result of an increase in translational efficiency or mRNA stability. These can be separately quantitated by following the kinetics of translation for each mRNA construct in transiently-transfected carrot and CHO cells (Fig. 3). The rate of Luc production was used as a measure of translational efficiency and the length of time over which Luc continued to accumulate was used to calculate message stability. For the uncapped *luc* mRNAs, addition of the TEV leader increased the translational efficiency of poly(A)$^+$mRNA 322-fold but increased the translational efficiency of poly(A)$^-$mRNA only 36-fold (Fig. 3A). This represents a synergy of 8.9-fold (compare the ratio of uncapped-TEV 5′-leader-*luc*-A_{50}/uncapped-*luc*-A_{50} to the ratio of uncapped-TEV 5′-leader-*luc*/uncapped-*luc*, i.e., 322:36 = 8.9-fold). The data can also be viewed from the perspective of the degree to which a poly(A) tail stimulates translational efficiency. Addition of a poly(A) tail stimulated expression from uncapped *luc* mRNA only 2.2-fold. We have shown previously that the addition of a poly(A) tail only marginally increases the translational efficiency of an uncapped mRNA (Gallie, 1991). However, addition of a poly(A) tail to *luc* mRNA containing the TEV leader increased its translational efficiency 20-fold. This represents a synergy of nine fold (compare the ratio of uncapped-TEV 5′-leader-*luc*-A_{50}/uncapped-TEV 5′-leader-*luc* to the ratio of uncapped-*luc*-A_{50}/uncapped-*luc*, i.e., 20:2.2 = 9-fold). These data illustrate that, in the absence of a cap, the poly(A) tail can stimulate translation if the mRNA contains the TEV 5′-leader. Similarly, the TEV 5′-leader stimulates the translational efficiency of poly(A)$^+$mRNA to a much greater extent than it does for poly(A)$^-$mRNA.

In contrast to the synergy between the TEV leader and the poly(A) tail in an uncapped mRNA, when the mRNAs are capped, the TEV leader increases equally the translational efficiency of poly(A)$^-$ and poly(A)$^+$mRNAs (Fig. 3B). These data illustrate that the synergy between the TEV leader and the poly(A) tail is observed only in the context of an uncapped mRNA, such as TEV mRNA itself. We conclude, therefore, that as with a cap and poly(A) tail, or a cap and the TMV 3′-*UTR*, the TEV leader and the poly(A) tail function synergistically.

(c) The impact of the TEV 5′ leader on mRNA stability

The stability of each mRNA construct was determined two ways: the functional mRNA half-life (determined from Fig. 3) and the physical half-life (Fig. 4). As the functional mRNA half-life measurements focus on only that pool of mRNA actively engaged in translation, it is a more specific measure of transcript stability than is the physical half-life. Each mRNA construct will be translated until it is finally degraded (represented by the plateau of each curve in Fig. 3) at which point no more Luc will be produced. The kinetic curves all plateau 90–150 min following delivery demonstrating that the period over which a *luc* mRNA is translationally active, i.e., its functional stability, is only moderately affected by the addition of a cap, a poly(A) tail, or the TEV leader. The half-life for *luc* mRNA without a cap, poly(A) tail, or the TEV leader was 39 min. Addition of the TEV leader increased the half-life only marginally, if at all, and can not account for the effect of the TEV leader on expression from the *luc* mRNA constructs tested in Fig. 1, suggesting that the stimulatory effect of the TEV leader on expression is due to an increase in translational efficiency alone.

The physical half-life of a message is a measure of the physical integrity of the total amount of a given mRNA within a cell without regard to whether the mRNA is polysome-associated or present in a translationally inactive state. It represents, therefore, an average of all the pools of an mRNA present in a cell. The decay of the full-length form of each mRNA construct introduced into carrot was followed over time. Addition of a cap or poly(A) tail increased *luc* mRNA half-life (Fig. 4A–D) as observed previously (Gallie, 1991). The presence of the TEV leader had no impact on the half-life, regardless if the *luc* mRNA was capped or polyadenylated



mRNA	Translational efficiency (Light units/min)	Relative rate of translation		Functional mRNA half-life (min)	Maximum accumulation (Light units)	Relative level of expresstion	
Uncapped							
luc	5.4	1		39	550	1	
TEV-*luc*	196	36		51	22,000	40	
luc-A_{50}	12.1		1	51	1,300		1
TEV-*luc*-A_{50}	3,900		322	51	292,000		225
Capped							
luc	113	1		55	9,800	1	
TEV-*luc*	8,270	73		30	344,000	35	
luc-A_{50}	3,500		1	84	320,000		1
TEV-*luc*-A_{50}	233,000		67	54	13,600,000		43

Fig. 3. The effect of the TEV leader on translational efficiency and functional mRNA half-life in carrot. Aliquots of protoplasts electroporated with uncapped (**A**) or capped (**B**) *luc* mRNAs were taken at time intervals, assayed, and the data plotted as a function of the time of incubation. Note the difference in scale in the panels for the capped and uncapped mRNAs. Between loading of the mRNA onto polysomes and its eventual degradation, there is a phase of steady-state translation in which the rate of Luc production is both maximal and constant and is dictated by its translational efficiency. The efficiency of translation of each mRNA is measured during this steady-state phase. The mRNA is then translated for a period of time that is determined by the stability of the mRNA. The eventual degradation of the mRNA results in a decreased rate of protein accumulation. Following degradation of the mRNA, further accumulation of Luc ceases, represented by the plateau of each curve at the later time points. The length of time over which Luc accumulated was used to calculate message stability. The functional half-life is defined as the amount of time necessary to complete a 50% decay in the capacity of an mRNA to synthesize protein (Kepes, 1963; Pedersen et al., 1978). Luc is stable over the length of the kinetic analysis in both cell types. The maximum accumulation is the final level of expression achieved once the mRNA has been degraded.

(Fig. 4E–H). We conclude that the TEV leader does not have a substantial impact on mRNA stability as measured by the physical half-life.

(d) Conclusions

(*1*) The TEV 5′ leader stimulates expression from poly(A)$^+$mRNA to a greater extent than from poly(A)$^-$mRNA. Consequently, the TEV 5′ leader and the poly(A) tail are functionally synergistic in a way similar to the synergy previously described for a cap and a poly(A) tail. Although the TEV 5′ leader may represent an alternative mechanism to promote efficient translation in the absence of a cap, a functional synergy between the termini of the mRNA nevertheless has been maintained.

(*2*) The synergy between the TEV 5′ leader and poly(A) tail was not observed when the mRNA was capped. The presence of the cap may override the functional interaction between the TEV 5′ leader and the poly(A) tail. Moreover, the TEV 5′ leader and a cap may affect similar steps in translation. A 10–15-fold level of synergy between the cap and poly(A) tail was previously determined (Gallie, 1991), whereas that between the TEV 5′ leader and poly(A) tail was only 6–9-fold. As the synergy between a cap and a poly(A) tail is stronger, this may

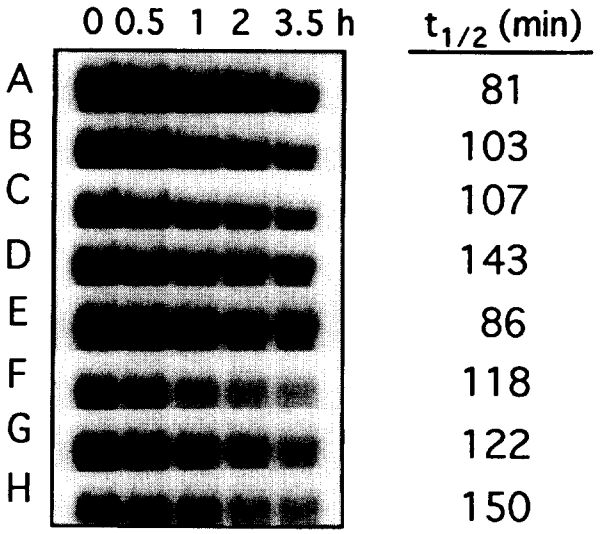


Fig. 4. The effect of the TEV leader on the physical *luc* mRNA half-life in carrot. (**A–D**) without the TEV leader; (**E–H**) with the TEV leader. (**A** and **E**) uncapped mRNA; (**B** and **F**) poly(A)$^+$mRNA; (**C** and **G**) capped mRNA; (**D** and **H**) capped and poly(A)$^+$mRNA. **Methods:** Following *luc* mRNA delivery via electroporation, aliquots were taken at time intervals and total RNA purified as described (Chomczynski and Sacchi, 1987). The RNA was displayed on a denaturing 2 M formaldehyde-1.5% agarose gel, followed by Northern transfer, and probed with anti-*luc* mRNA. The region of the membrane representing the full-length form of the *luc* mRNA was cut from the membrane, counted, and the $\log_{10}$ of the values plotted as a function of time. k, the slope of the best-fit line through the data points, was used to calculate the half-life according to the equation $t_{1/2}=0.693/k$.

explain the absence of synergy between the TEV 5′ leader and the poly(A) tail in a capped mRNA.

(*3*) The stimulation afforded by the TEV 5′ leader was not observed in vitro, data suggesting that lysates may lack some critical component required for the function of the TEV leader in promoting translation.

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