The effect of the length of the 3′-untranslated region on expression in plants

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Abstract The effect of increasing the length of the 3′-untranslated region (UTR) on expression of luciferase mRNA was examined in transiently transfected carrot protoplasts. The effect of the 3′-UTR on both poly(A)$^-$ and poly(A)$^+$ mRNA was examined. A nested set of constructs in which the 3′-UTR increased from 4 to 104 bases was generated by the introduction and reiteration of a 20 base sequence downstream of the *luc* stop codon. For poly(A)$^-$ mRNA, there was a consistent increase in expression when the length of the 3′-UTR was increased from 4 to 104 bases. For poly(A)$^+$ mRNA, expression increased 18-fold when the length of the 3′-UTR was increased from 7 to 27 bases. Further increases in the length of the 3′-UTR did not affect expression. The increase in expression was largely due to an increase in translational efficiency. These data suggest that the length of the 3′-UTR plays an important role in determining the extent to which a poly(A) tail can stimulate the translation of an mRNA.

Key words: Gene expression; mRNA; Message stability; Translation; 3′-Untranslated region

1. Introduction

The poly(A) tail is a bifunctional regulator of expression from an mRNA, increasing both mRNA translational efficiency and stability in plant and animal cells. Its role as a regulator of translation is quantitatively greater than its role as a regulator of message stability [1]. The poly(A) tail is functionally dependent on the 5′-cap structure (m^7GpppN) in order to stimulate translational efficiency both in vivo [1] and in vitro [2], observations suggesting communication between the termini of an mRNA. Recent evidence has implicated the cap-associated initiation factors as candidates that mediate the interaction [3]. Virtually all polyadenylated mRNAs contain sequences of variable length between the coding region and the poly(A) tail which in several mRNAs can play an active role in determining the translational efficiency or stability of the mRNA (reviewed in [4–7]). Several studies have suggested that the extent to which a poly(A) tail stimulates translation may be gene dependent [5,8]. Expression did increase with the length of the 3′-untranslated region (3′-UTR) in Chinese hamster ovary cells [9] although whether this is true in plant species has not been examined. In this report, we examine the effect of the length of the 3′-noncoding region on the regulation of expression from poly(A)$^-$ and poly(A)$^+$ luciferase (*luc*) reporter mRNAs in transiently transfected carrot protoplasts. Increasing the length of the 3′-noncoding region increased the translational efficiency of poly-(A)$^-$ mRNA but had little effect on expression from poly(A)$^+$ mRNA. However, positioning a poly(A) tail immediately downstream of a stop codon either failed to stimulate translation in vivo or resulted in repression in vitro which may be a consequence of the poly(A) tail/poly(A)-binding protein complex functioning as a stearic block to translocating ribosomes as they approach the termination codon.

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2. Materials and methods

2.1. Plasmid constructs and in vitro transcription

The pT7-*luc*-WT-3′-UTR constructs in which the firefly luciferase (*luc*) is under the control of the T7 promoter has been described previously [8]. To remove the *luc* 3′-UTR, an *Xba*I site was engineered 8 bases downstream of the *luc* stop codon. Taking advantage of a second *Xba*I site downstream of the *luc* 3′-UTR, a 139 base fragment containing the *luc* 3′-UTR could be excised, resulting in *luc*-19b-3′-UTR which contains a 19 base 3′-UTR. The *luc*-4b-3′-UTR was produced by introducing a *Bgl*II site at the *luc* stop codon (*TAA*-GATCT, where TAA represents the *luc* stop codon and the *Bgl*II site is underlined). When linearized with *Bgl*II, this luciferase construct produces an mRNA with only a 4 base 3′-UTR. The *luc*-7b-3′-UTR-A_{50} construct was made by introducing the *Hin*dIII/*Bgl*II *luc* gene fragment from the *luc*-4b-3′-UTR construct into the *Hin*dIII/*Bam*HI sites of the pT7-A_{50} vector.

An oligonucleotide cassette was constructed that could be inserted, one copy at a time, in the *Bgl*II site of the *luc*-4b-3′-UTR construct. When the cassette was reiterated up to five times, a nested set of constructs was generated in which the 3′-UTR length increased in increments of 20 bases, [(GGATCATCTACAGCATATCT)$_n$], producing constructs *luc*-24b-3′-UTR through *luc*-104b-3′-UTR. Each construct was introduced as an *Hin*dIII/*Bgl*II fragment into the *Hin*dIII/*Bam*HI sites of the pT7-A_{50} vector to produce the equivalent constructs which terminate in a poly$(A)_{50}$ tail. In vitro transcription was carried out as described [10].

2.2. Electroporation conditions and luciferase assay

Protoplasts were isolated from a carrot cell suspension as described [11]. Two μg of each *luc* mRNA construct was mixed with 0.8 ml of protoplasts immediately before electroporation (500 μF capacitance, 350 V) using an IBI GeneZapper. For time course experiments, aliquots of protoplasts were taken at the time intervals indicated. Luciferase assays were carried out as described [1]. Each mRNA construct was assayed in duplicate and the average reported.

3. Results and discussion

3.1. The luciferase 3′-UTR modulates poly(A) tail function

The presence of a poly$(A)_{50}$ tail increased expression from β-glucuronidase (*uidA*) mRNA 70-fold but only 13-fold from *luc* mRNA in plant protoplasts [8]. This observation illustrated that expression from *luc* mRNA was less responsive to the addition of a poly(A) tail than it was from *uidA* mRNA. In order to examine whether the *luc* 3′-UTR influenced the effect of a poly(A) tail on stimulating expression, poly(A)$^-$ and poly(A)$^+$ *luc* mRNA constructs in which the *luc* 3′-UTR was present or absent were generated. To remove the

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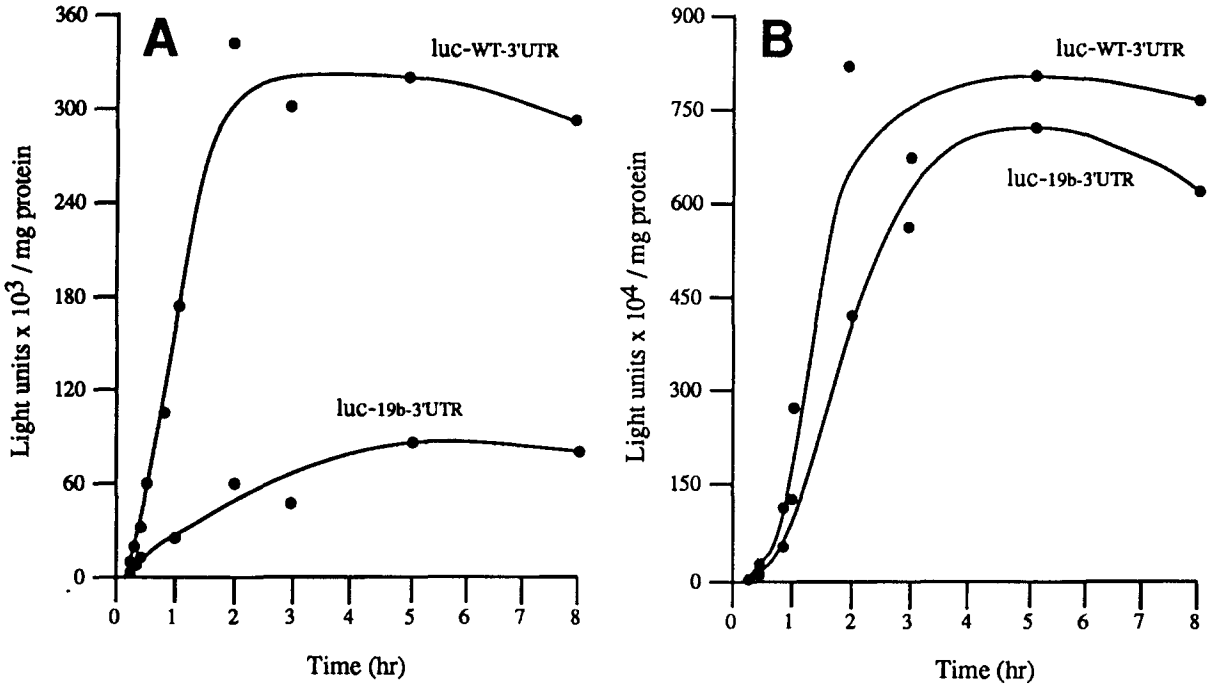

mRNA	Translational efficiency (Light units/min/ mg protein)	Relative rate of translation	Functional mRNA half-life (min)	Maximum accumulation (Light units/ mg protein)	Relative level of expresstion
Poly(A)⁻					
luc-WT 3'UTR	3,700	6.9	60	318,000	3.5
luc-19b 3'UTR	533	1	72	90,000	1
Poly(A)⁺					
luc-WT 3'UTR	85,000	1.7	78	8,000,000	1.1
luc-19b 3'UTR	50,000	1	108	7,000,000	1

Fig. 1. The effect of the *luc* 3′-UTR on the translational efficiency of *luc* mRNA and functional half-life in carrot cells. Aliquots of carrot protoplasts electroporated with *luc* mRNAs without (A) and with (B) a poly(A)$_{50}$ tail were taken at time intervals, assayed, and the luciferase activity plotted as a function of time of incubation of the cells. The translational efficiency for each mRNA construct was measured from the maximum slope of each curve and is shown in the table. The functional half-life is determined as the amount of time needed to complete a 50% decay in the capacity of an mRNA to synthesize protein and is shown in the table. The maximum accumulation is the final level of expression reached following the degradation of the mRNA and represents the combined impact that the translational efficiency and stability of an mRNA makes towards expression. Note that the *y*-axis scale in (B) is 10 times greater than in (A).

luc 3′-UTR, an *Xba*I restriction site was engineered 8 bases downstream of the *luc* stop codon of the wild-type construct, *luc*-WT-3′-UTR, to create the construct, *luc*-WT$_{XbaI}$. A 139 base region that included the *luc* 3′-UTR could then be deleted resulting in a *luc* mRNA that terminated in a 19 base 3′-UTR (*luc*-19b-3′-UTR). In a fourth construct, the putative polyadenylation signal, 5′-AAUAAUAAA-3′, was changed to 5′-AAGAAGAAA-3′ to result in *luc*-WT$_{AAGAAG}$. These pT7-based constructs were then introduced into a second pT7-based vector that contained a poly(A)$_{50}$ tract which allowed the in vitro synthesis of transcripts with a uniform poly(A)$_{50}$ tail. To measure the effect of the *luc* 3′-UTR on expression, *luc*-WT-3′-UTR and *luc*-19b-3′-UTR mRNAs were synthesized in triplicate in vitro as poly(A)$^-$ and poly(A)$^+$ and delivered in triplicate to carrot protoplasts by electroporation. The cells were then incubated for 7 h, time sufficient to allow translation and degradation of the introduced mRNAs, and the resulting amount of luciferase protein used to quantitate the level of expression from each mRNA.

Addition of a poly(A) tail to *luc* mRNA containing the *luc* 3′-UTR increased luciferase expression by 7.19-fold (Table 1) which is in good agreement with our previous observations [8]. In contrast, the addition of a poly(A) tail to *luc*-19b-3′-UTR mRNA increased expression by 39.2-fold. The presence or absence of the *luc* 3′-UTR had little effect on expression when the mRNAs were polyadenylated. However, expression from the poly(A)$^-$ mRNA was lower following the removal of the *luc* 3′-UTR. This accounts for the increased responsive-

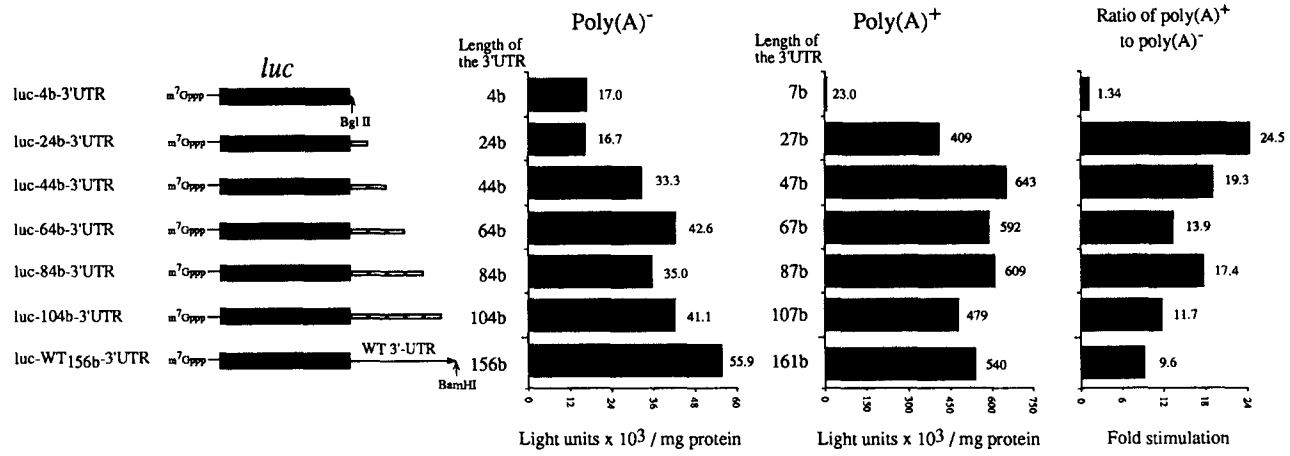

Fig. 2. The effect of the length of the 3'-UTR on translation in vivo. The *luc* mRNA constructs, shown on the left, were synthesized in vitro as poly(A)$^-$ and poly(A)$^+$ mRNAs and electroporated into duplicate samples of carrot protoplasts. Open boxes in the 3'-UTR represent copies of the oligonucleotide cassette, and the corresponding length of each 3'-UTR is indicated. Following incubation, the cells were assayed in duplicate for luciferase activity. The final level of expression for each construct is indicated to the right of each histogram.

ness of the *luc* mRNA to the addition of a poly(A) tail in the absence of the 3'-UTR (see the expression ratio of poly(A)$^+$ to poly(A)$^-$ in Table 1). The introduction of the *Xba*I site or the mutation of the polyadenylation signal within the *luc* 3'-UTR had only a small impact on expression from either poly(A)$^+$ or poly(A)$^-$ mRNA. These data demonstrate that the presence of the *luc* 3'-UTR diminishes the effect of the poly(A) tail on expression by increasing the level of expression from the poly(A)$^-$ form of the mRNA.

The effect of the *luc* 3'-UTR on translational efficiency and message stability could be separately quantitated by following the kinetics of *luc* mRNA translation in carrot protoplasts. The rate of luciferase protein production was used as a measure of translational efficiency and the length of time over which luciferase protein continued to accumulate was used to calculate message stability. Luciferase protein is stable in carrot cells: a 6% loss over a 24 h period was measured (data not shown). Following delivery of each mRNA construct, aliquots of cells were removed at time intervals and luciferase assays were performed. The kinetics of *luc* mRNA translation were determined by following the appearance of protein as measured by enzyme activity plotted as a function of time (Fig. 1). Recruitment begins immediately following mRNA delivery and once the mRNA has been loaded onto polysomes, translation proceeds at a rate (i.e. the slope of each curve) that is dictated by its translational efficiency and 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 luciferase protein ceases, represented by the plateau of each curve at the later time points in Fig. 1. Between the loading of the mRNA onto the polysomes and its eventual degradation, there is a phase of steady-state translation in which the rate of luciferase production is both maximal and constant and the slope in this region of the curve represents the translational efficiency for each mRNA. The translational efficiency was measured during this period. By comparing the rates for each *luc* mRNA construct, the impact that the *luc* 3'-UTR has on translational efficiency can be determined separate from any impact it might have on the functional mRNA stability.

The translational characteristics of *luc*-WT-A_{50} and *luc*-19b-3'-UTR-A_{50} were very similar in both the rate of translation and length of time over which they were translationally active (Fig. 1B). As with the previous experiment, these data suggest that the *luc* 3'-UTR does not impact expression from poly(A)$^+$ mRNA. In contrast, the presence of the *luc* 3'-UTR increased the translational efficiency of *luc* mRNA by 6.9-fold when the mRNA was poly(A)$^-$ (compare capped *luc*-WT-3'-UTR to capped *luc*-19b-3'-UTR in Fig. 1A). The *luc* 3'-UTR had little impact on the stability of the reporter mRNA. This can be seen by the length of time over which

Table 1
The effect of the *luc* 3'-UTR on the degree to which a poly(A) tail enhances expression

mRNA	Luciferase activity (light units/mg protein)	$\frac{\text{Poly(A)}^+}{\text{Poly(A)}^-}$
luc-WT-3'-UTR	3 586 258 ± 188 891	7.19
luc-WT-3'-UTR-A_{50}	25 789 587 ± 729 556	
luc-WT$_{XbaI}$-3'-UTR	3 866 351 ± 1 105 062	11.4
luc-WT$_{XbaI}$3'-UTR-A_{50}	44 006 405 ± 7 105 064	
luc-WT$_{AAGAAG}$	3 068 535 ± 226 988	12.9
luc-WT$_{AAGAAG}$-A_{50}	39 599 260 ± 2 219 464	
luc-19b-3'-UTR	924 189 ± 65 959	39.2
luc-19b-3'-UTR-A_{50}	36 247 280 ± 3 506 283	

the mRNAs were translationally active. Translation from all four mRNAs ceased 2–3 h following RNA delivery. The functional half-life of an mRNA is defined as the amount of time needed to complete a 50% decay in the capacity of an mRNA to synthesize protein [12,13] measured at the point at which 50% of the maximum yield of protein is reached (Fig. 1). As the functional half-life measures the stability of only that mRNA which is undergoing active translation, it more accurately describes the stability of message that is polysome-associated than does physical half-life. Removal of the *luc* 3′-UTR did not reduce the stability of the *luc* mRNA regardless of whether the mRNA was poly(A)$^+$ or poly(A)$^-$ (see Fig. 1, table). These data demonstrate that the presence of the *luc* 3′-UTR specifically increases the rate of translation of poly(A)$^-$ mRNA.

3.2. *Increasing the length of the 3′-noncoding region increases expression*

The effect of the *luc* 3′-UTR on the extent to which the addition of a poly(A)$_{50}$ tail could stimulate expression could be specific to the *luc* 3′-UTR or could be simply a consequence of its length. To investigate whether increasing the length of the 3′-UTR impacts the stimulatory effect of a poly(A) tail, we made a nested set of *luc* mRNA constructs which contained a reiterated 20 base sequence such that the 3′-UTR varied in length (from 4 to 104 bases) but not in sequence. The *luc*-WT-3′-UTR mRNA was included as a positive control. Each mRNA could be synthesized as a poly(A)$^-$ or poly(A)$^+$ mRNA and expression from each mRNA construct was measured following their delivery and translation in protoplasts. Addition of a poly(A)$_{50}$ tail had virtually no effect on the expression from the *luc* mRNA containing a 7 base 3′-noncoding region (Fig. 2). However, when the length of the 3′-UTR was increased to 27 bases, the addition of a poly(A) tail stimulated expression 24.5-fold. Increasing the length of the 3′-UTR further resulted in a progressive decrease in the extent to which the addition of a poly(A)$_{50}$ tail could stimulate expression. Expression increased with the length of the 3′-UTR from poly(A)$^-$ but not poly(A)$^+$ mRNA which accounts for the progressive decrease in responsiveness to the poly(A) tail (see the expression ratio of poly (A)$^+$ to poly(A)$^-$ in Fig. 2). The correlation between the length of the 3′-UTR and the degree to which the addition of a poly(A) tail was reproducible in several independent experiments. The presence of the poly(A) tail substantially reduced the length effect of the 3′-noncoding region. The only case in which this did not hold true was for the 7 base 3′-UTR construct. Moreover, translation from the poly(A)$^+$ form of the 7 base 3′-UTR construct was actually repressed by 17-fold compared to the poly(A)$^-$ form when translated in vitro using a lysate derived from wheat germ (data not shown). The low expression from the poly(A)$^+$ form of the *luc* mRNA containing only a 7 base 3′-UTR may be explained by the poly(A) tail/PAB complex forming a stearic block to translocating ribosomes as they approach the stop codon. Ribosomes prevented from reaching the termination codon may stall and/or release a truncated luciferase protein molecule, and thereby reduce luciferase expression. C-terminal truncation of luciferase protein by as few as 9 amino acids is known to result in the complete loss of its activity [14]. Eukaryotic ribosomes are estimated to cover approximately 30–35 nucleotides of an mRNA (reviewed in [15,16]). A terminating ribosome would therefore cover approximately 15–20 bases downstream of the stop codon. Of the nested set of constructs in Fig. 2, expression from *luc*-24b-3′-UTR was stimulated to the greatest extent by the addition of a poly(A)$_{50}$ tail. Expression from *luc*-19b-3′-UTR in Table 1 was stimulated to an even greater extent by the addition of a poly(A)$_{50}$ tail. A minimum requirement of approximately 19 bases between the poly(A) tail and the stop codon may be an optimal distance for the poly(A) tail to function as a stimulator of translation without interfering with ribosomes as they terminate translation. The spacing between the stop codon and the poly(A) tail, therefore, may be used for some genes as a means to regulate the appropriate level of expression.

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