Secondary structure in the 5′-leader or 3′-untranslated region reduces protein yield but does not affect the functional interaction between the 5′-cap and the poly(A) tail

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Abstract The 5′-cap structure and poly(A) tail of eukaryotic mRNAs cooperate to promote translation initiation but whether this functional interaction benefits certain classes of mRNAs has not been investigated. In this study, we investigate whether a structured 5′-leader or 3′-untranslated region (UTR) affects the cap/poly(A) tail interaction. A structured leader reduced the degree to which the 5′-cap promoted translation in plant cells and inhibited translation from capped and uncapped mRNAs equally in yeast. Secondary structure within the 3′-UTR reduced translational efficiency when adjacent to the stop codon but had little effect on the cap/poly(A) tail synergy. The functional interaction between the cap and poly(A) tail was as important for an mRNA with a structured leader or 3′-UTR as it was for an unstructured mRNA in either species, suggesting that these structures can reduce translation without affecting the functional interaction between the cap and poly(A) tail. However, the loss of Xrn1p, the major 5′→3′ exoribonuclease in yeast, abolished cap-dependent translation and the functional interaction between the cap and poly(A) tail, suggesting that the cap/poly(A) tail synergy is of particular importance under conditions of active RNA turnover.

Key words: Translation; Cap; Poly(A) tail; RNA structure

1. Introduction

Translation initiation of eukaryotic mRNAs is promoted by the 5′-cap structure which serves as the binding site for the eukaryotic initiation factor (eIF) 4E, the small subunit of eIF4F. eIF4G, the large subunit of eIF4F, interacts with eIF3 which promotes the binding of 40S ribosomal subunits to an mRNA (reviewed in [1]). eIF4G also recruits eIF4A and eIF4B to assist in the unwinding of secondary structure within the 5′-leader that would otherwise impede the scanning of the 40S ribosomal subunit in its search for the initiation codon [2]. When sufficiently stable, however, secondary structure represses translation [3,4] either through inhibiting 40S ribosomal subunit binding or scanning [5,6]. Introduction of secondary structure within 40 nucleotides (nt) of the 5′-cap did not prevent binding of eIF4F, eIF4B, or eIF4A in rabbit reticulocyte lysate but did inhibit binding of eIF3 and the 40S ribosomal subunit [7]. Introduction of secondary structure sufficiently distal to the 5′-cap to allow binding of the 40S ribosomal subunit halted 40S scanning in yeast but was less inhibitory in mammalian lysate [5,6,8], demonstrating a difference between yeast and animal translational machinery. Much less is known about the effect of a structured leader on translation in plants and little is known about the effect that a structured 3′-untranslated region (UTR) has on translation.

Although the 5′-cap is necessary, it is not sufficient for translation. Instead, translation is promoted by a functional interaction between the cap and poly(A) tail, mediated by a physical interaction between eIF4G and the poly(A) binding protein (PABP) [9–12]. Whether structural elements present within the untranslated regions of an mRNA influence the functional synergy between the cap and the poly(A) tail, and consequently, the contribution that this interaction makes to the translational efficiency of an mRNA, has not been investigated. In this study, we observed that structure within a 5′-leader or 3′-UTR can reduce the yield of protein from an mRNA but has little effect on the functional interaction between the cap and the poly(A) tail. However, disruption of Xrn1p, the major 5′→3′ cytoplasmic exoribonuclease in yeast [13,14], resulted in the loss of cap-dependent translation, abolished the functional interaction between the cap and the poly(A) tail, and increased poly(A) tail-dependent translation, suggesting that the functional interaction between the cap and poly(A) tail is most important when the degradatory apparatus is fully active.

2. Materials and methods

2.1. mRNA constructs and synthesis

pT7-*luc*-A_{50} has been described previously [15]. A *Bgl*II site was introduced immediately downstream of the open reading frame, with the last adenine of the TAA stop codon being the first base of the restriction site. This construct was used to generate the following constructs.

pT7-SL_{24}-*luc*-A_{50} (containing a 24 bp stem-loop structure of $\Delta G = -42.9$ kcal/mol introduced in the 5′-leader sequence) was produced by inserting the following 52 bp palindromic oligonucleotide into the *Hin*dIII site, allowing the formation of a stem loop positioned 4 nt downstream of the cap of the mRNA:

HindIII ApaI BglII MluI SnaBI MluI BglII ApaI HindIII
AAGCTTGGGCCCAGATCTACGCGTACGTACGCGTAGATCTGGGCCCAAGCTT

To produce pT7-SL_{19}-*luc*-A_{50}, pT7-SL_{13}-*luc*-A_{50}, or pT7-SL_7-*luc*-A_{50} (containing a 19, 13, or 7 bp stem-loop structure of $\Delta G = -31.8$, -21.3, or -4.5 kcal/mol, respectively), pT7-SL_{24}-*luc*-A_{50} was digested with *Mlu*I, *Bgl*II, or *Apa*I, respectively.

pT7-*luc*-SL_{19}-A_{50} (containing a 19 bp stem-loop structure of $\Delta G = -33.4$ kcal/mol) was produced by inserting the following 42 bp palindromic oligonucleotide into the *Bgl*II site, allowing the for-

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Abbreviations: eIF, eukaryotic initiation factor; *luc*, luciferase; nt, nucleotide; PABP, poly(A) binding protein; RRL, rabbit reticulocyte lysate; SL, stem-loop; UTR, untranslated region; WGL, wheat germ lysate

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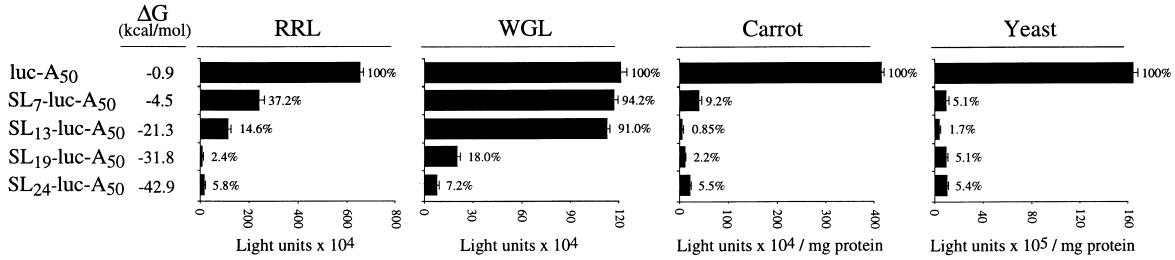


Fig. 1. Introduction of secondary structure inhibits translation to a greater extent in vivo than in vitro. A stem-loop (SL) with a 7, 13, 19, or 24 bp stem was introduced 4 nt downstream of the 5′-terminus of *luc*-$(A)_{50}$ mRNA. The free energy (ΔG) of the control leader and each stem-loop is indicated. The mRNA constructs were synthesized in vitro as capped, poly$(A)^+$ mRNAs and delivered to carrot or yeast or translated in either WGL or RRL. Equal cell numbers or amounts of lysate were assayed for luciferase activity. The level of expression from each construct as a percentage of the control is indicated to the right of each histogram. Each mRNA was electroporated in triplicate, and each luciferase assay was performed in duplicate. An error bar representing one standard deviation is shown for each histogram.

mation of a stem loop that included the last A of the stop codon:

Bam HI *Sma I* *Nde I* *Sma I* *Bam HI*
GGATCCTACGTACCCGGGCATATGCCCGGGTACGTAGGATCC

pT7-*luc*-SL_{43}-A_{50} (containing a 43 bp stem-loop structure of $\Delta G = -86.6$ kcal/mol) was obtained by cloning the following 54 bp sequence into the unique *Nde*I site of pT7-*luc*-SL_{19}-A_{50}:

Nde I *Apa I* *Kpn I* *Kpn I* *Apa I* *Nde I*
CATATGACGCGTGGGCCCGGTACCCTCGAGGGTACCGGGCCCACGCGTCATATG

pT7-*luc*-SL_{31}-A_{50} (containing a 31 bp stem-loop structure of $\Delta G = -55.6$ kcal/mol) was produced by digesting pT7-*luc*-SL_{43}-A_{50} with *Apa*I (thus excising the central 24 bp of the second oligonucleotide) followed by ligation.

The same series of stem-loops positioned either 20 or 100 nt downstream of the *luc* termination codon were constructed by introducing either one or five copies of a random, previously described 20 nt sequence [16] between the stop codon and the stem-loop. This resulted in pT7-*luc*-20nt-A_{50}, pT7-*luc*-20nt-SL_{19}-A_{50}, pT7-*luc*-20nt-SL_{31}-A_{50}, and pT7-*luc*-20nt-SL_{43}-A_{50} containing a stem-loop with a 0, 19, 31, or 43 bp stem, respectively, positioned 20 nt downstream of the *luc* termination codon. A similar series, pT7-*luc*-100nt-A_{50}, pT7-*luc*-100nt-SL_{19}-A_{50}, pT7-*luc*-100nt-SL_{31}-A_{50}, and pT7-*luc*-100nt-SL_{43}-A_{50} contained the same series of stem-loops positioned 100 nt downstream of the stop codon. In vitro synthesis of the mRNAs was carried out as described previously [15].

2.2. In vitro and in vivo translation

Equal amounts of mRNA were translated in vitro using wheat germ lysate (WGL) or rabbit reticulocyte lysate (RRL) as described by the manufacturer except all amino acids were unlabeled. The reactions were incubated for 1 h prior to assaying.

One μg of each mRNA construct was delivered to carrot protoplasts or yeast spheroplasts by electroporation as described previously [15]. For time course experiments, aliquots of cells were taken at the indicated time points and assayed. For end-point experiments, the cells were incubated for 6 h (yeast) or 12 h (carrot). For each experiment, an mRNA was delivered to triplicate samples and each sample assayed in duplicate. Each experiment was repeated a minimum of three times. The average value and standard deviation for the constructs of a typical experiment is reported. Cell extracts were assayed for luciferase activity following injection of 0.5 mM luciferin using a Monolight 2010 Luminometer as described previously [15].

3. Results and discussion

3.1. Structure within the 5′-leader or proximal to the stop codon reduces protein yield

In order to investigate the effect that secondary structure may have on the functional interaction between a cap and a poly(A) during translation in plants and yeast, it was neces-

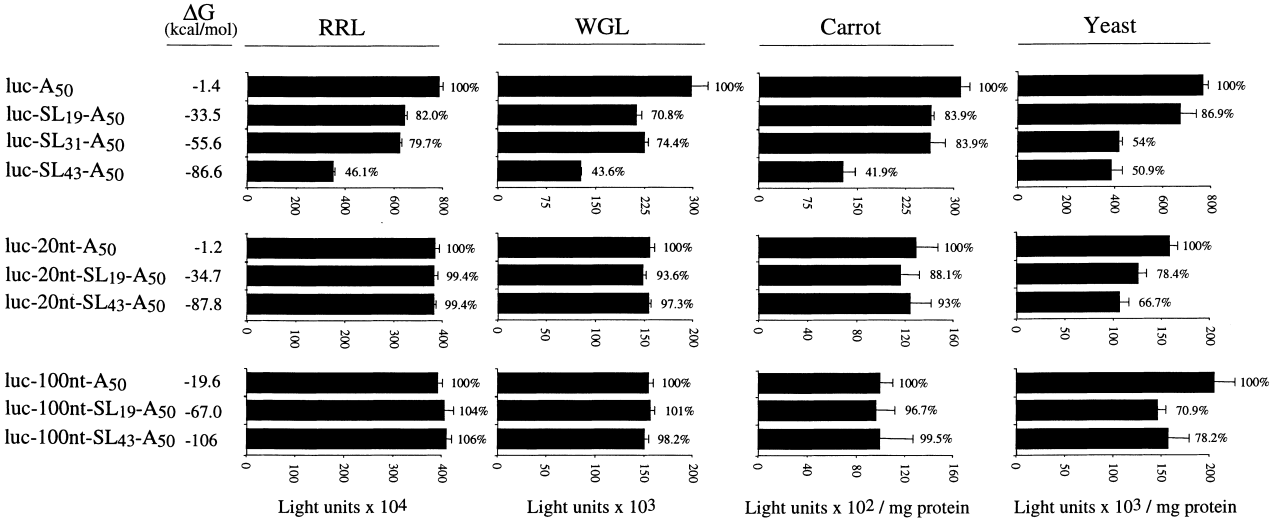
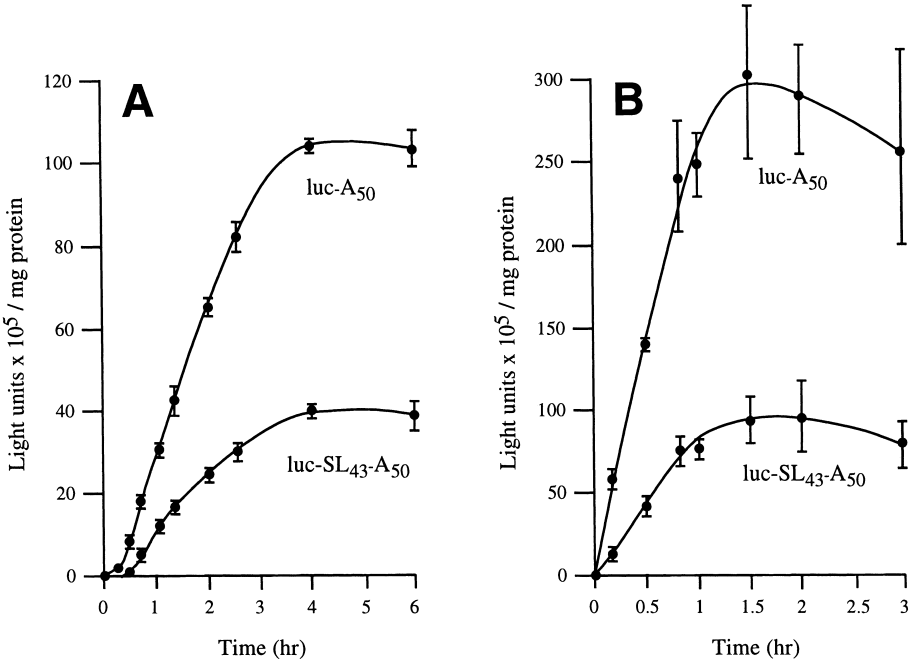


Fig. 2. Introduction of secondary structure adjacent to the stop codon inhibits translation. A stem-loop (SL) with a 19, 31, 43 bp stem was introduced adjacent to (top row), 20 nt (middle row), or 100 nt (bottom row) downstream of the termination codon of *luc*-$(A)_{50}$ mRNA. The free energy (ΔG) of the control 3′-UTR and each stem-loop is indicated. The mRNA constructs were translated as described in Fig. 1.



mRNA	Translational efficiency (Light units/min/ mg protein)	Relative rate of translation	Functional mRNA half-life (min)
WGL			
luc-A_{50}	87,600	1.0	75
luc-SL_{43}-A_{50}	39,400	0.45	80
Yeast			
luc-A_{50}	50,200	1.0	30
luc-SL_{43}-A_{50}	20,800	0.41	30

Fig. 3. Secondary structure adjacent to the stop codon affects translation efficiency but not mRNA stability. A stem-loop (SL) with a 43 bp stem was introduced adjacent to the termination codon of *luc*-$(A)_{50}$ mRNA and synthesized in vitro as capped, poly(A)$^+$ mRNAs. Aliquots of (A) WGL programmed with the *luc* mRNAs shown or (B) yeast spheroplasts in which the same constructs had been delivered 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 (see the table). The functional half-life is determined from the curves as the amount of time needed to complete a 50% decay in the capacity of an mRNA to synthesize protein.

sary to establish the degree to which a structure of increasing stability within the leader or 3′-UTR represses translation. A series of *luc* mRNA constructs, in which stem-loops of different stabilities were introduced 4 nt downstream of the cap, were delivered to carrot protoplasts and yeast spheroplasts and their translational competence measured. The introduction of a stem-loop with a $\Delta G = -4.5$ kcal/mol (i.e. SL_7-*luc*-A_{50}) reduced translation to 9.2% in plants or 5.1% in yeast relative to that of the control mRNA, i.e. *luc*-A_{50} (Fig. 1). This same stem-loop was less inhibitory in vitro: SL_7-*luc*-A_{50} was translated at 94.2% or 37.2% of the control mRNA in WGL or RRL, respectively (Fig. 1). Increasing the stability of the stem-loop to a free energy of −21.3 kcal/mol (i.e. SL_{13}-*luc*-A_{50}) further reduced in vivo translation to 0.85% or 1.7% of the control in plants or yeast, respectively (Fig. 1). A substantially more stable secondary structure was required to achieve a similar level of repression in vitro (Fig. 1). These results demonstrate that a 5′-proximal stem-loop with a free energy of only −4.5 kcal/mol was sufficient to inhibit translation substantially in vivo whereas a stem-loop with a free energy between −21.3 and −31.8 kcal/mol was required to achieve a similar reduction in RRL and a stem-loop with a free energy between −31.8 and −42.4 kcal/mol was required in WGL. The requirement for the increased stability of the secondary structure for in vitro translation may be a consequence of the higher concentration of unused translational machinery although neither lysate was treated to remove endogenous mRNA.

A 19 bp stem-loop with a free energy of −33.4 kcal/mol introduced immediately downstream of the *luc* stop codon (i.e. *luc*-SL_{19}-A_{50}) reduced in vivo translation to 83.9% or 86.9% of the control mRNA (i.e. *luc*-A_{50}) in plants or yeast,

respectively (Fig. 2). Increasing the stability of the stem-loop to a free energy of −55.6 kcal/mol (i.e. *luc*-SL_{31}-A_{50}) or −86.6 kcal/mol (i.e. *luc*-SL_{43}-A_{50}) resulted in a statistically significant reduction to 54% or 50.9% of the control mRNA, respectively, in yeast and 83.9% or 41.9% of the control mRNA, respectively, in plants (Fig. 2). A similar trend was observed in WGL and RRL (Fig. 2).

To determine whether the repressive effect of a structured 3′-UTR affected the translational efficiency or the stability of an mRNA, the translational kinetics of *luc*-SL_{43}-A_{50} were compared to *luc*-A_{50} mRNA following its translation in WGL or yeast. *luc*-SL_{43}-A_{50} mRNA was translationally active over the same period of time as the control (Fig. 3) and exhibited no difference in its physical half-life as determined by Northern analysis (data not shown) but was translated to only 45% or 41% of the control mRNA in WGL or yeast, respectively (Fig. 3). The reduction in translational efficiency was sufficient to account for the difference in expression observed between *luc*-SL_{43}-A_{50} and *luc*-A_{50} in Fig. 2.

To determine whether the inhibitory effect of the secondary structure was specific to its position adjacent to the stop codon, the effect of the same stem-loop structures used in the above constructs was examined when positioned further downstream of the termination codon. As the distance from the center to the leading edge of a ribosome covers 12–15 nucleotides [17], each stem-loop was introduced either 20 nt or 100 nt downstream of the termination codon, thus allowing sufficient distance between the stop codon and the stem-loop for a ribosome to terminate translation without steric hindrance. The repressive effect that a stem-loop exerted when adjacent to the stop codon was lost in plants, WGL and RRL (but not entirely in yeast) when it was positioned further downstream (Fig. 2). These data suggest that the presence of secondary structure can reduce translational efficiency when adjacent to the termination codon. However, a stem-loop of substantially greater stability was required in the 3′-UTR to affect expression than was required within the 5′-leader to achieve a repressive effect, demonstrating that initiation is considerably more sensitive to secondary structure than is termination. The proximal effect of secondary structure in the 3′-UTR may be a result of slowing translational termination through the steric hindrance that the structure presents to a ribosome as it approaches the stop codon. The observation that positioning a stem-loop proximal to the termination codon reduced the rate of translation but did not affect the functional mRNA half-life (see Fig. 3) and that the repressive effect was lost in plants, or in vitro when the stem-loop was positioned sufficiently downstream to allow ribosomes unhindered access to the termination codon, is consistent with this conclusion.

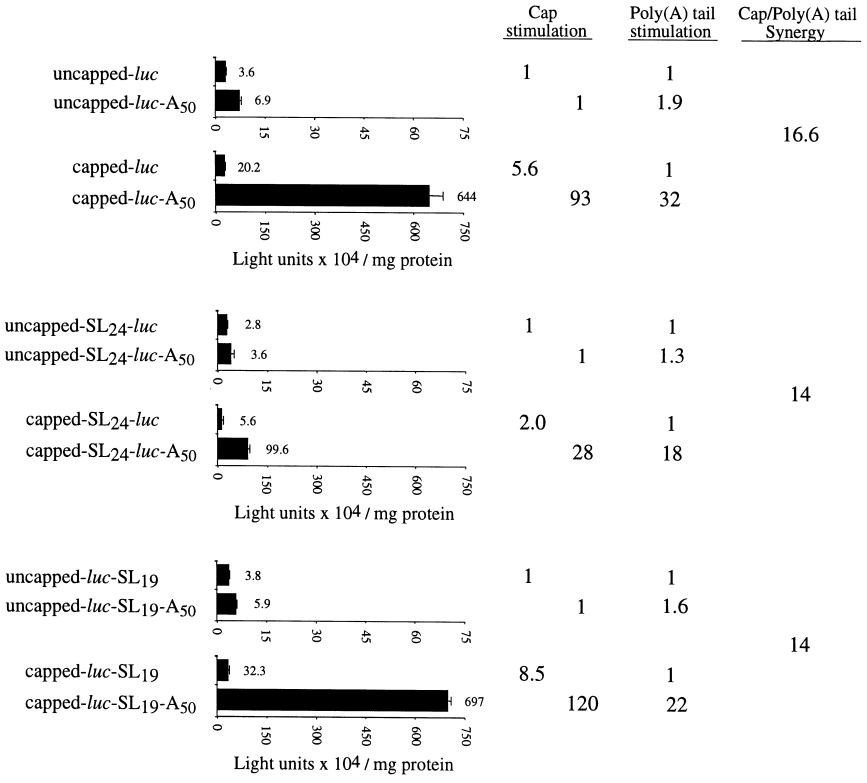


Fig. 4. A structured 5′-leader reduces cap-dependent translation but not the synergy between a cap and a poly(A) tail. Control *luc* mRNA or constructs containing a stem-loop (SL) with a 24 bp stem 4 nt downstream of the 5′-terminus or a stem-loop with a 19 bp stem 20 nt downstream of the termination codon were synthesized in vitro with or without a cap and as poly(A)$^-$ or poly(A)$^+$ mRNAs and delivered to carrot protoplasts. Each mRNA was electroporated in triplicate, and each luciferase assay was performed in duplicate and the final level of expression with an error bar representing one standard deviation is shown for each histogram. Note that the scale for capped mRNAs is 10-fold greater than the scale for uncapped mRNAs. The fold stimulation by the addition of a cap (where an uncapped mRNA is designated with a value of 1) or a poly(A)$_{50}$ tail (where an poly(A)$^-$ mRNA is designated with a value of 1) is indicated to the right of the histograms. The degree of synergy between the cap and poly(A) tail is also indicated to the right of the histograms.

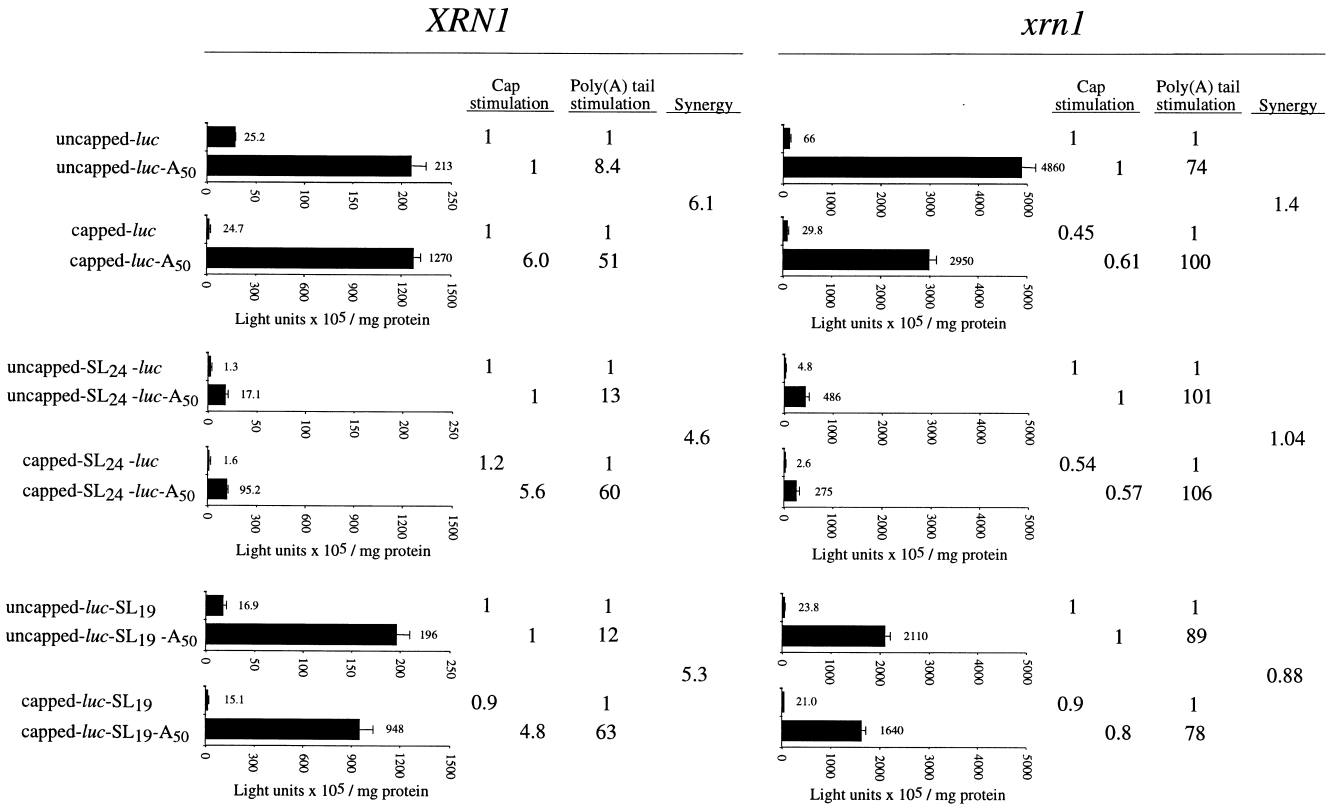


Fig. 5. Cap-dependent translation and the synergy between the cap and poly(A) tail are abolished in *xrn1* yeast. Control *luc* mRNA or constructs containing a stem-loop (SL) with a 24 bp stem 4 nt downstream of the 5′-terminus or a stem-loop with a 19 bp stem 20 nt downstream of the termination codon were synthesized in vitro with or without a cap and as poly(A)$^-$ or poly(A)$^+$ mRNAs and delivered to *XRN1* and *xrn1* yeast. Each mRNA was electroporated in triplicate, and each luciferase assay was performed in duplicate. Note that the scale for capped mRNAs in *XRN1* yeast is 6-fold greater than the scale for uncapped mRNAs whereas the scales for capped and uncapped mRNAs in *xrn1* yeast are identical. The fold stimulation by the addition of a cap (where an uncapped mRNA is designated with a value of 1) or a poly(A)$_{50}$ tail (where an poly(A)$^-$ mRNA is designated with a value of 1) is indicated to the right of the histograms. The degree of synergy between the cap and poly(A) tail is also indicated to the right of the histograms.

3.2. *A structured leader reduces cap-dependent translation but not the functional interaction between the cap and the poly(A) tail*

To examine whether a structured leader or 3′-UTR affects the functional interaction between the cap and poly(A) tail, the translation from *luc* mRNA containing either a 24 bp stem-loop in the leader or a 19 bp stem-loop in the 3′-UTR was measured in carrot protoplasts either in the presence or in the absence of a cap or poly(A) tail. A cap stimulated expression from the control mRNA 93-fold (compare capped-*luc*-A$_{50}$ to uncapped-*luc*-A$_{50}$, Fig. 4) but increased expression only 28-fold when the mRNA contained a structured leader (compare capped-SL$_{24}$-*luc*-A$_{50}$ to uncapped-SL$_{24}$-*luc*-A$_{50}$, Fig. 4). The repressive effect of a structured leader was exerted largely on capped mRNAs: expression from capped-SL$_{24}$-*luc* and capped-SL$_{24}$-*luc*-A$_{50}$ mRNA was substantially reduced relative to the control capped-*luc* and capped-*luc*-A$_{50}$ mRNA (28% and 15% relative to each capped mRNA without a stem-loop, respectively) whereas expression from uncapped-SL$_{24}$-*luc* and uncapped-SL$_{24}$-*luc*-A$_{50}$ mRNA was not repressed to the same extent relative to uncapped-*luc* and uncapped-*luc*-A$_{50}$ mRNA (78% and 52% relative to each uncapped mRNA without a stem-loop, respectively) (Fig. 4). Addition of a cap to *luc*-SL$_{19}$-A$_{50}$ (i.e. containing a structured 3′-UTR) resulted in a similar level of stimulation (120-fold) as that observed for the control mRNA (compare capped-*luc*-SL$_{19}$-A$_{50}$ to uncapped-*luc*-SL$_{19}$-A$_{50}$, Fig. 4), suggesting that a structured 3′-UTR does not affect cap-dependent translation.

The effect of secondary structure on poly(A) tail-dependent translation could be measured by comparing expression from a poly(A)$^+$ mRNA to that from the corresponding poly(A)$^-$ mRNA. Addition of a poly(A)$_{50}$ tail to the control *luc* mRNA stimulated expression 32-fold (compare capped-*luc*-A$_{50}$ to capped-*luc*, Fig. 4). The degree to which the addition of a poly(A)$_{50}$ tail stimulated expression was not substantially reduced when the mRNA contained a structured leader or 3′-UTR.

The synergy between the cap and poly(A) tail could be calculated from the degree to which a cap stimulated expression from poly(A)$^+$ versus poly(A)$^-$ mRNA. For example, the addition of a cap increased expression 93-fold from poly(A)$^+$ *luc* mRNA (the ratio of capped-*luc*-A$_{50}$/uncapped-*luc*-A$_{50}$ mRNA) but only 5.6-fold from poly(A)$^-$ *luc* mRNA (the ratio of capped-*luc*/uncapped-*luc* mRNA) (Fig. 4) resulting in a synergy of 16.6-fold (93 divided by 5.6). A similar level of synergy was observed for *luc* mRNA containing a structured leader or 3′-UTR (14-fold in each case). These

data suggest that although a structured leader can repress cap-dependent translation, it does not substantially affect the functional interaction between the cap and poly(A) tail.

3.3. The synergy between the cap and poly(A) tail is lost in an *xrn1* mutant

In good agreement with previous observations [15], addition of a cap stimulated translation of the control mRNA 6-fold in wild-type yeast (compare capped-*luc*-A_{50} to uncapped-*luc*-A_{50} in *XRN1* yeast, Fig. 5) compared to the 93-fold stimulation observed in plants (see Fig. 4) whereas addition of a poly(A) tail stimulated translation 51-fold in yeast (see *XRN1* yeast, Fig. 5) and 32-fold in plants (Fig. 4). The presence of a structured leader reduced the absolute level of expression from both uncapped and capped mRNAs, however, the degree to which the cap or poly(A) tail stimulated translation in yeast was largely unaffected by the presence of a structured leader or 3′-UTR (see *XRN1* yeast, Fig. 5). Moreover, the synergy between the cap and poly(A) tail was similar for an mRNA with a structured leader or 3′-UTR in the *XRN1* strain (see *XRN1* yeast, Fig. 5).

The 5′-cap structure serves an important protective role that maintains the integrity of the 5′-terminus from attack from Xrn1p, the major cytoplasmic 5′→3′ exoribonuclease in yeast [13,14]. mRNAs undergo decapping by Dcp1p [18] resulting in uncapped mRNAs that are normally quickly degraded by Xrn1p but accumulate in the *xrn1* mutant. As a consequence, the protective function of the 5′-cap structure becomes superfluous in the absence of 5′→3′ exoribonucleolytic activity. Therefore, we examined how the alteration in the requirement for the cap in *xrn1* yeast might affect the extent to which a structured leader represses translation and might affect the synergistic interaction between the cap and poly(A) tail. When the same mRNAs tested in the *XRN1* parent were translated in the *xrn1* mutant, a loss in cap-dependent translation was observed: addition of a cap did not stimulate expression from an mRNA whether or not it contained a structured leader (Fig. 5). In spite of the loss in cap dependence, translation remained 5′-end-dependent as the introduction of a stem-loop close to the 5′-terminus inhibited equally translation from capped and uncapped mRNAs (compare *luc* mRNAs containing the 24 bp stem-loop in the leader to the control *luc* mRNAs in *xrn1* yeast, Fig. 5). Coordinate with the loss in cap function, poly(A) tail-dependent translation increased, particularly for uncapped mRNAs: addition of a poly$(A)_{50}$ tail to the uncapped-*luc* mRNA increased expression 74-fold in the *xrn1* mutant versus 8.4-fold in the *XRN1* parent. The increase in poly(A) tail-dependent translation in *xrn1* yeast can be best explained if the role that the PABP/poly(A) tail complex plays during translation is to promote re-initiation. In contrast to *XRN1* yeast, in which uncapped mRNAs generated through the action of Dcp1p are rapidly degraded thus ensuring that they are not recruited for translation, the persistence of an uncapped mRNA in the *xrn1* mutant increases its opportunity for re-initiation and the protein yield from such an mRNA would reflect the contribution of the increased occurrence of re-initiation. One prediction from such a model is that translation from an uncapped, polyadenylated mRNA should increase to a greater extent in the *xrn1* mutant than would translation from a capped, polyadenylated mRNA. Moreover, any preferential increase in translation from an uncapped mRNA in the *xrn1* mutant when polyadenylated should not be observed to the same extent for an uncapped mRNA lacking a poly(A) tail. Both predictions were borne out by the observations from the *xrn1* mutant: the poly(A) tail dependence of uncapped-*luc*-A_{50} mRNA increased approximately 9-fold in the *xrn1* mutant relative to that observed in *XRN1* yeast (compare the 74-fold increase in translation following the addition of a poly-$(A)_{50}$ tail to an uncapped mRNA in the *xrn1* mutant versus the 8.4-fold increase in *XRN1* yeast) and this increase was substantially more than the increase in the poly(A) tail dependence of capped mRNAs (approximately 2-fold) or the increase in expression from poly$(A)^-$ *luc*, either when capped (a 1.2-fold increase) or when uncapped (a 2.6-fold increase) in *xrn1* versus *XRN1* yeast. As a consequence, translation from uncapped-*luc*-A_{50} mRNA in the *xrn1* mutant increased to a level similar to that observed from capped-*luc*-A_{50} mRNA, resulting in a loss in cap-dependent translation and synergy between the cap and the poly(A) tail. These results suggest that the functional interaction between the cap and the poly-(A) tail is of particular importance under conditions in which the RNA degradatory machinery is fully active.

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