Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency

Daniel R. Gallie

Department of Biochemistry, University of California, Riverside, California 92521 USA

The cap structure and the poly(A) tail are important regulatory determinants in establishing the translational efficiency of a messenger RNA. Although the mechanism by which either determinant functions remains poorly characterized, the interaction between the poly(A) tail–poly(A)-binding protein complex and events occurring at the 5′ terminus during translation initiation has been an intriguing possibility. In this report, the mutual dependence of the cap and the poly(A) tail was studied. Poly(A)$^+$ and poly(A)$^-$ luciferase (*Luc*) mRNAs generated in vitro containing or lacking a cap were translated in vivo in tobacco protoplasts, Chinese hamster ovary cells, and yeast following delivery by electroporation. The poly(A) tail-mediated regulation of translational efficiency was wholly dependent on the cap for function. Moreover, cap function was enhanced over an order of magnitude by the presence of a poly(A) tail. The relative differences in stability between the mRNAs could not account for the synergism. The synergism between the cap and poly(A) tail was not observed in yeast cells in which active translation had been disrupted. In addition, the synergism was not observed in in vitro translation lysates. These data demonstrate that the cap and the poly(A) tail are interdependent for optimal function in vivo and suggest that communication between the two regulatory determinants may be important in establishing efficient translation.

[*Key Words*: Translational control; mRNA; poly(A) tail; cap; gene expression]

Received July 22, 1991; revised version accepted August 11, 1991.

Since their discovery, both the cap and the poly(A) tail have been implicated as being involved in the regulation of translational efficiency and message stability (Furuichi et al. 1977; Filipowicz 1978; Banerjee 1980; Brawermann 1981; Green et al. 1983; Rhoads 1988; Sonenberg 1988; Bernstein and Ross 1989; Jackson and Standart 1990; Munroe and Jacobson 1990a,b). Recently, we demonstrated that although the addition of a poly(A) tail to poly(A)$^-$ mRNA increased both its translational efficiency and stability in plant and animal cells, the poly(A) tail was predominantly a regulator of translational efficiency (Gallie et al. 1989, 1991). Considerable evidence has accumulated recently to support this view. The poly(A)-binding (PAB) protein, a highly conserved protein in eukaryotes (Grange et al. 1987), mediates the regulation associated with the poly(A) tail and is essential for viability in yeast (Sachs et al. 1987). Suppressor mutants which overcome the loss of PAB protein affect 60S ribosomal subunit biogenesis or function (Sachs and Davis 1989). In an in vitro study, exogenous oligo(A) reduced the translation of capped, poly(A)$^+$ mRNA in rabbit reticulocyte lysate, possibly by competing for PAB protein. Moreover, at certain concentrations, exogenous oligo(A) could stimulate the translation of capped, poly(A)$^-$ mRNA in *trans* (Munroe and Jacobson 1990a). It has been postulated, therefore, that interaction between the poly(A) tail (as mediated by PAB protein) and the 60S subunit is important in controlling 80S initiation complex formation (Sachs and Davis 1989). We hypothesized that the poly(A) tail–PAB protein complex may not be limited to interaction with the 60S subunit, but may be involved in earlier events in translation initiation, such as cap recognition. To test this possibility, we synthesized mRNAs in vitro as poly(A)$^+$ or poly(A)$^-$ that were either capped or uncapped and determined their translational competence in vivo in tobacco protoplasts, Chinese hamster ovary (CHO) cells, and yeast following delivery using electroporation. Using the firefly luciferase (*Luc*) reporter gene, the kinetics of translation can be followed, and for the first time, the synergism between the cap and the poly(A) tail in their role as regulators of translational efficiency can be demonstrated in vivo.

Results

The function of the cap and poly(A) tail are mutually dependent as regulators of translational efficiency in tobacco

The debate concerning the functional role of the poly(A) tail in increasing expression has not yet been resolved. In

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

plants, however, the poly(A) tail has a dual cytoplasmic regulatory role: mRNAs containing a poly(A)$_{50}$ tail are two- to threefold more stable than their poly(A)$^-$ counterparts and translational efficiency is markedly stimulated (Gallie et al. 1989). Because of this demonstrated involvement in regulating translation, we wished to examine whether there might be a functional interaction between the cap at the 5′ terminus of an mRNA and the poly(A) tail. To directly assess the effect of the cap on poly(A) tail function, *Luc* mRNA constructs were synthesized in vitro as four different species: uncapped poly(A)$^-$, uncapped poly(A)$^+$, capped poly(A)$^-$, and capped poly(A)$^+$ mRNA. The poly(A)$^+$ *Luc* mRNAs were synthesized from a T7–*Luc* vector containing a poly(A)$_{50}$ sequence, thereby ensuring production of *Luc* mRNA with a uniform poly(A) tail length. Moreover, the conditions used for the in vitro synthesis of capped RNA result in mRNA that is uniformly capped (Yisraeli and Melton 1989).

To test the in vitro synthesized mRNAs in vivo, 1 μg each of the four *Luc* mRNAs was electroporated into tobacco protoplasts and the cells incubated overnight, sufficient time to ensure complete degradation of the *Luc* mRNAs. In tobacco protoplasts, luciferase expression was 1.5-fold greater from uncapped, poly(A)$^+$ *Luc* mRNA than from uncapped, poly(A)$^-$ *Luc* mRNA (Table 1). An excellent correlation can be made between the impact of the poly(A) tail on LUC expression and its effect on the physical half-life of the *Luc* transcript. The half-life of poly(A)$^+$ *Luc* mRNA was 1.4-fold longer than that for the corresponding poly(A)$^-$ mRNA (Fig. 1), in good agreement with earlier measurements (Gallie et al. 1989, 1991). In contrast to the small impact of the poly(A) tail on uncapped *Luc* mRNA, the addition of a poly(A) tail increased expression dramatically when added to the capped form of the mRNA. Expression from the poly(A)$^+$ mRNA was 21-fold greater than the poly(A)$^-$ mRNA (Table 1). The poly(A) tail, however, increased capped *Luc* mRNA half-life only 1.9-fold (Fig. 1), which is insufficient to account for the effect of the poly(A) tail on capped *Luc* mRNA translation. Although the poly(A) tail does stabilize *Luc* mRNA whether it is capped or not, it is only when the mRNA is capped that its second and more important function, that is increasing translational efficiency, is apparent.

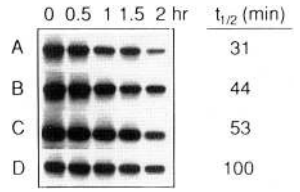


Figure 1. In vivo message stabilization conferred by a cap and a poly(A) tail in tobacco. (*A*) uncapped *Luc* mRNA; (*B*) Uncapped *Luc*-A$_{50}$ mRNA; (*C*) capped *Luc* mRNA; (*D*) capped *Luc*-A$_{50}$. Aliquots of protoplasts electroporated with 5 μg of each mRNA were removed at the time indicated at the *top* of each lane. RNA half-life analysis was carried out as described in Materials and methods.

Examination of the data from the perspective of the cap demonstrates the requirement for a poly(A) tail for the cap to function optimally. Addition of a cap increased expression 21-fold for poly(A)$^-$ *Luc* mRNA but increased expression 297-fold for poly(A)$^+$ *Luc* (Table 1). Since the addition of a cap stabilized poly(A)$^-$ and poly(A)$^+$ *Luc* mRNA to approximately the same extent, the effect of the cap on *Luc* mRNA half-life could not account for the difference in cap activity. We conclude, therefore, that although a cap does increase translational efficiency in the absence of a poly(A) tail, its function is enhanced by an order of magnitude by a poly(A) tail.

The monomethylated form of the cap (m^{7}GpppG) was used throughout these experiments. To examine the role of the methyl group in the synergistic interaction with the poly(A) tail, capped (GpppG) *Luc* mRNAs were tested in tobacco protoplasts. The interdependence between the cap and the poly(A) tail was observed with these mRNAs, illustrating that, although the methyl group increases the synergism, it is not absolutely required (Table 1). We have repeated these experiments more than 15 times to rule out potential variation in protoplast quality. With each repetition, the interdependence was observed. We conclude, therefore, that the cap and poly(A) tail interact synergistically: In its capacity as a regulator

Table 1. *Synergism of cap and poly(A) during translation in tobacco protoplasts electroporated with in vitro-synthesized* Luc *mRNA*

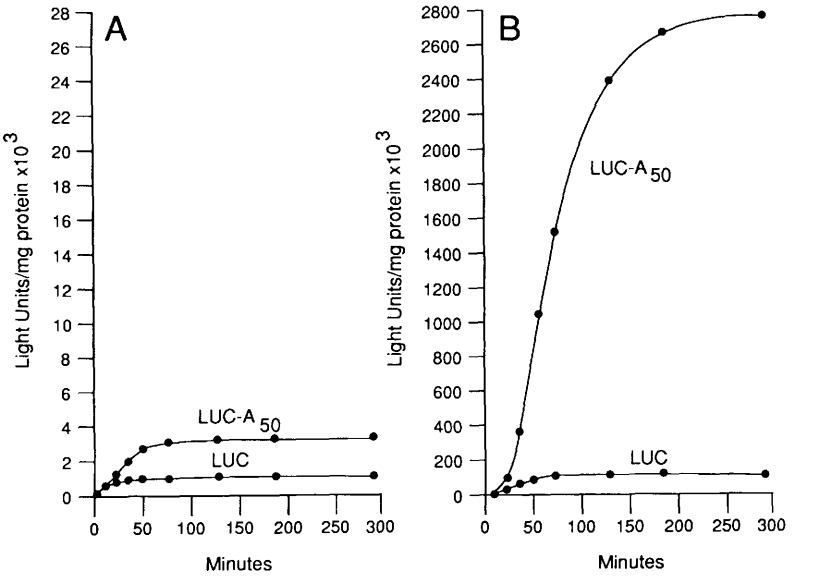
mRNA	Luciferase activity (light unit/mg protein)	Relative effect of poly(A) tail on expression	Relative effect of cap on expression
Uncapped			
Luc	2,941	1	1
Luc–A$_{50}$	4,480	1.5	1
Capped (GpppG)			
Luc	35,442	1	12
Luc–A$_{50}$	406,878	11.5	91
Capped (m^{7}GpppG)			
Luc	62,595	1	21
Luc–A$_{50}$	1,331,917	21	297

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

of translational efficiency, the poly(A) tail is dependent on the cap for function; the activity of the cap is not entirely dependent on, but is enhanced by, a poly(A) tail.

If regulation of translational efficiency by the poly(A) tail is stimulated by a cap, an increase in the rate of appearance of luciferase should be observed. A similar increase in the translational efficiency should be detected for the poly(A) stimulation of cap function. To address this possibility, the same four *Luc* mRNA constructs tested above were electroporated into tobacco protoplasts, aliquots of cells were taken at time intervals, and assayed for luciferase activity. Luciferase activity was plotted as a function of time (Fig. 2) and the translational efficiency for each construct determined from the first derivative of each curve (Fig. 2). Luciferase is a stable protein under these conditions; $t_{1/2}$ = 20 hr at 24°C in tobacco (D.R. Gallie, unpubl.); therefore, its enzyme activity accurately reflects LUC protein production. The translational efficiency of uncapped, poly(A)$^+$ *Luc* mRNA was equivalent to that of the uncapped, poly(A)$^-$ mRNA. In contrast, the addition of a poly(A) tail to capped *Luc* mRNA increased translational efficiency 15-fold, demonstrating that the poly(A) tail not only regulates translational efficiency in tobacco but requires a cap for function. In this experiment, as before, cap function was stimulated by a poly(A) tail. Although capped, poly(A)$^-$ *Luc* mRNA was translated 23-fold more efficiently than the uncapped form; the translational efficiency of the poly(A)$^+$ *Luc* mRNA was 365-fold greater for the capped versus the uncapped form (Fig. 2).

The physical half-life of an mRNA may not be an accurate measurement of its functional half-life. The loss of a few nucleotides at either terminus would go undetected by Northern analysis. For example, a transcript which had lost just the cap through exonuclease attack could not be resolved from the "full-length" transcript population in physical half-life determinations but would be significantly compromised in its translational competency. As the physical half-life for uncapped *Luc* mRNA was 31 min, such decapped transcripts are not immediately degraded. A better measure of the longevity of a message is its functional half-life, defined as the time required, following mRNA delivery, to reach 50% of the final level of protein produced from a given mRNA construct. Functional half-life, therefore, measures the length of time over which the mRNA is actively engaged in translation, in contrast to the physical half-life, which



mRNA	Translational Efficiency (Light units/ mg min)	Relative rate of translation	Functional mRNA Half-life (min)	Relative mRNA Half-life	Maximum Accumulation (Light units/ mg protein)	Relative level of expression
Uncapped						
Luc	92	1	7	1	940	1
Luc-A$_{50}$	88	0.96	26	3.7	3200	3.4
Capped						
Luc	2160	1	33	1	100,000	1
Luc-A$_{50}$	32100	15	63	1.9	2,800,000	28

Figure 2. The effect of a cap and poly(A) tail on the translation efficiency and functional half-life of *Luc* mRNA in tobacco. Aliquots of protoplasts electroporated with uncapped (*A*) or capped (*B*) *Luc* mRNAs were taken at time intervals and assayed; data were plotted as luciferase sp. act. vs. time. The translational efficiency for each mRNA construct was determined from maxin.um rate of the first derivative of each curve. The functional half-life is defined as the time required to reach 50% of the maximum accumulation of luciferase activity.

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

serves only as a measure of an mRNA's physical longevity without regard to its translational competence.

The functional half-life of the uncapped poly(A)$^+$ *Luc* mRNA was 3.7-fold longer than the corresponding poly(A)$^-$ mRNA (Fig. 2). Increased message stability should not affect the rate of translation but will directly affect the final level of protein produced. This 3.7-fold increase in half-life correlated well with the 3.4-fold increase in the maximum accumulation of luciferase produced from the uncapped, poly(A)$^+$ mRNA when compared to its poly(A)$^-$ counterpart (Fig. 2). The translational efficiencies for the uncapped mRNAs, it will be recalled, were equivalent. The functional half-life for the capped, poly(A)$^+$ mRNA was 1.9-fold greater than for the corresponding poly(A)$^-$ mRNA. However, a 28-fold difference in the maximum accumulation of luciferase activity was measured (Fig. 2). The effect that the poly(A) tail has on the maximum accumulation of a protein is a function of its impact on the translational efficiency multiplied by its impact on mRNA stability. The difference in the translational efficiencies between the capped, poly(A)$^+$ and poly(A)$^-$ mRNAs (14.8-fold) and their functional half-life measurements (1.9-fold) accounts completely for this 28-fold difference.

The functional half-life for three of the *Luc* mRNAs was just 60% of the physical half-life, while for uncapped, poly(A)$^-$ Luc mRNA, it was only 23%, results reproducibly obtained in subsequent half-life measurement experiments. These data suggest that functional inactivation of the mRNA was occurring more quickly than could be detected by the physical half-life measurements. The functional half-life of a message, therefore, was a more rigorous assessment of the inherent stability of a message than physical half-life measurements. However, neither the physical nor functional half-life measurements could account for the synergism between the cap and poly(A) tail.

Synergism between the cap and poly(A) tail is present in vivo in animal cells but not in an in vitro lysate

To determine whether the interdependence between the cap and the poly(A) tail was common among higher eukaryotes, we examined *Luc* mRNA translation in Chinese hamster ovary (CHO) cells. In electroporated CHO cells, addition of a poly(A) tail increased expression 8.5-fold for uncapped *Luc* mRNA but 156-fold when *Luc* mRNA was capped (Table 2), demonstrating that although the poly(A) tail is partially functional in the absence of a cap, its function is stimulated by more than an order of magnitude when the transcript is capped. As in tobacco, the poly(A) tail in CHO cells increases *Luc* mRNA half-life only 1.7-fold (Gallie et al. 1991). The cap was almost entirely dependent on the poly(A) tail for function. Addition of a cap increased translation of poly(A)$^-$ *Luc* mRNA 2.7-fold but increased by 50-fold the translation of the poly(A)$^+$ counterpart.

The translational efficiency and functional half-life were also measured for the *Luc* mRNAs in CHO cells (Fig. 3). As seen in tobacco, the presence of poly(A) tail did little to increase the rate of translation or the functional half-life of uncapped *Luc* mRNA. Only when the transcript was capped was the poly(A) tail effective in increasing the translational efficiency. Similarly, the cap required a poly(A) tail to function as a regulator of translational efficiency. The synergism was observed for a second gene, the *uidA* gene from *Escherichia coli* (data not shown), demonstrating that the synergism is not specific to eukaryotic genes in general or the luciferase gene in particular.

An in vitro translation lysate was used to examine whether the synergism observed in vivo could occur in vitro. The same preparations of *Luc* mRNAs used for the in vivo analysis in CHO cells were translated in reticulocyte lysate. Cap stimulation of translation was the same for both poly(A)$^+$ and poly(A)$^-$ *Luc* mRNAs and the poly(A) tail had virtually no effect (Fig. 4). Neither the cap nor the poly(A) tail increased message stability in vitro, verifying earlier observations for this lysate (Furuichi et al. 1977). Moreover, no synergism was observed between the cap and the poly(A) tail in vitro. The lack of synergism may be a consequence of the failure of the lysate to reflect an in vivo environment in a number of ways: Translation was an order of magnitude less cap-dependent in vitro than in vivo; the poly(A) tail failed to function in vitro; and the cap and poly(A) tail did not increase message stability in vitro.

Synergism between the cap and the poly(A) tail in Saccharomyces cerevisiae *is influenced by the state of translational competency*

To extend our observations to lower eukaryotes, we established the appropriate conditions for mRNA delivery

Table 2. *Synergism between cap and poly(A) tail during translation in CHO 001 cells electroporated with* Luc *mRNA*

mRNA	Luciferase activity (light unit/mg protein)	Relative effect of poly(A) tail on expression	Relative effect of cap on expression
Uncapped			
Luc	160,000	1	1
Luc–A_{50}	1,360,000	8.5	1
Capped			
Luc	437,000	1	2.7
Luc–A_{50}	68,000,000	156	50

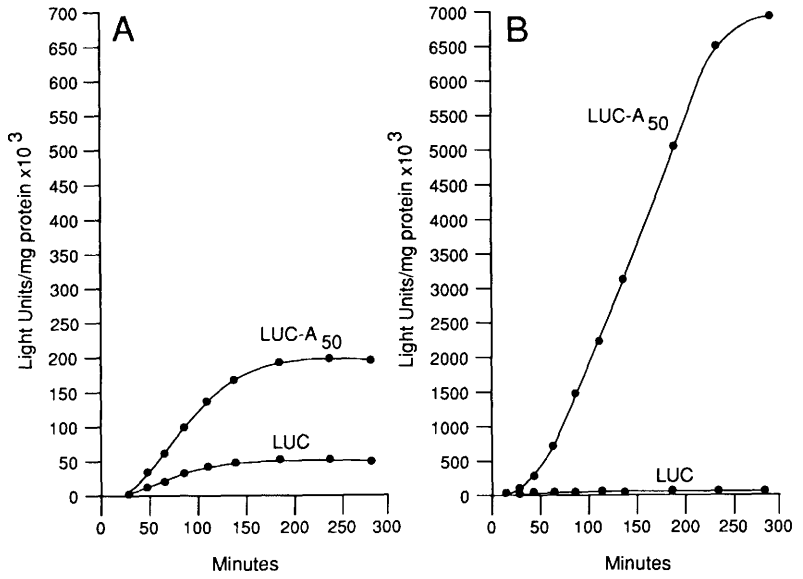
Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

into yeast using electroporation (J.G. Everett and D.R. Gallie, in prep.). The yeast cells were first spheroplasted and allowed to recover for 90 min before electroporation to allow active translation to resume. The addition of a poly(A) tail to uncapped *Luc* mRNA resulted in a 31-fold increase in LUC expression (Table 3). However, expression was increased by 201-fold when a poly(A) tail was added to capped *Luc* mRNA. These data illustrate that the poly(A) tail can increase expression in the absence of the cap but is functionally stimulated by a cap. In yeast, the cap is functionally active in *Luc* mRNA lacking a poly(A) tail, but is enhanced when the mRNA is polyadenylated. The synergism observed in higher eukaryotes is, therefore, also present in a lower eukaryote such as *S. cerevisiae*, but interestingly, to a lower extent.

Because spheroplasting disrupts active translation in yeast, it was of interest to determine whether the synergism between the cap and poly(A) tail would be similarly disrupted. LUC expression from yeast electroporated immediately following spheroplasting was significantly reduced when compared to the same spheroplast preparation allowed a 90 min recovery before electroporation (Table 3), verifying that active translation is disrupted by spheroplasting. The function of a poly(A) tail was not adversely affected when added to uncapped *Luc* mRNA, nor was cap function affected when added to poly(A)$^-$ *Luc* mRNA. However, the synergism normally observed in spheroplasts engaged in active translation was not detected in the newly spheroplasted yeast. These data suggest that the synergism between the cap and the poly(A) tail is lost when active translation is disrupted by spheroplasting and is only regained when active translation resumes.

Discussion

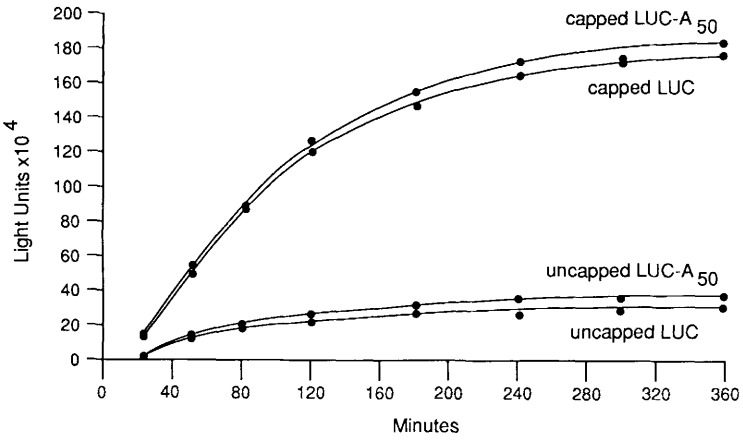
Since the discovery that RNAs terminate in a poly(A) tail (Darnell et al. 1971; Edmonds et al. 1971; Lee et al. 1971) and the subsequent discovery that the 5′ terminus is capped (Reddy et al. 1974), the central role that these regulatory elements play in establishing efficient expression has been well documented (Filipowicz 1978; Brawermann 1981; Rhoads 1988; Sonenberg 1988; Bernstein and Ross 1989; Jackson and Standart 1990; Munroe and Jacobson 1990b). The role of the cap as the recognition site for binding the eukaryotic initiation factor (eIF) 4E, a subunit of eIF-4F, has been demonstrated as an early and essential step in translation initiation (Filip-



mRNA	Translational Efficiency (Light units/ mg min)	Relative rate of translation	Functional mRNA Half-life (min)	Relative mRNA Half-life	Maximum Accumulation (Light units/ mg protein)	Relative level of expression
Uncapped						
Luc	700	1	71	1	48,000	1
Luc-A$_{50}$	2,140	3.1	82	1.2	195,000	4.1
Capped						
Luc	925	1	56	1	60,000	1
Luc-A$_{50}$	32,800	35	145	2.6	6,850,000	114

Figure 3. The effect of a cap and poly(A) tail on the translation efficiency and functional half-life of *Luc* mRNA in CHO cells. Aliquots of protoplasts electroporated with uncapped (*A*) or capped (*B*) *Luc* mRNAs were taken at time intervals, assayed, and the values determined as described in Fig. 2.

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press



mRNA	Translational Efficiency (Light units/min)	Relative rate of translation	Functional mRNA Half-life (min)	Relative mRNA Half-life	Maximum Accumulation (Light units)	Relative level of expression
Uncapped						
Luc	3,300	1	72	1	280,000	1
Luc-A_{50}	3,400	1.03	72	1	330,000	1.2
Capped						
Luc	16,200	1	75	1	1,720,000	1
Luc-A_{50}	16,300	1	78	1.04	1,790,000	1.04

Figure 4. Analysis of the synergism between a cap and poly(A) tail in reticulocyte lysate. The translation of 200 ng of each *Luc* mRNA construct was followed by assaying aliquots for luciferase activity.

owicz 1978; Banerjee 1980; Altmann et al. 1987, 1989; Rhoads 1988; Sonenberg 1988). Genetic and biochemical evidence suggests that the PAB protein, which binds to the poly(A) tail, may interact with the 60S ribosomal subunit as a means by which the translational efficiency of a message may be regulated (Sachs and Davis 1989; Munroe and Jacobson 1990a).

Our in vivo studies in both higher and lower eukaryotes suggest that an interdependence exists between the cap and the poly(A) for efficient function at the level of regulating translational efficiency. Because of their dual regulatory roles, translational efficiency and message stability were both increased by the addition of either the cap or poly(A) tail. However, the synergism was observed only as a function of the translational efficiency.

The stabilizing effect of a cap and poly(A) tail together, although not synergistic, was additive. The cap increased message stability two- to fourfold, in good agreement with other studies which examined the stabilization afforded by a cap. Reovirus mRNA was fourfold more stable when capped than was uncapped mRNA in microinjected *Xenopus* oocytes and threefold more stable in wheat germ lysate (Furuichi et al. 1977). Chicken lysozyme mRNA was stabilized fourfold by the addition of a monomethylated cap and twofold by a dimethylated cap in microinjected oocytes (Drummond et al. 1985).

Table 3. *Synergism between cap and poly(A) tail during translation in yeast is dependent on translational competency*

	Yeast electroporated after 90 min of recovery following spheroplasting			Yeast electroporated immediately following spheroplasting		
mRNA	luciferase activity (light unit/mg protein)	relative effect of poly(A) tail on expression	relative effect of cap on expression	luciferase activity (light unit/mg protein)	relative effect of poly(A) tail on expression	relative effect of cap on expression
Uncapped						
Luc	26,930	1	1	3,700	1	1
Luc–A_{50}	844,410	31	1	160,730	43	1
Capped						
Luc	100,170	1	3.7	41,620	1	11
Luc–A_{50}	20,141,220	201	24	1,854,320	45	12

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

Polyhedrosis viral mRNA was twofold more stable when capped than was uncapped mRNA in wheat germ lysate (Shimotohno et al. 1977). The poly(A) tail increased mRNA stability approximately two- to threefold, in good agreement with our previous observations (Gallie et al. 1989, 1991). The functional half-life measurements for the *Luc* constructs were shorter in plant cells than in animal cells, as was reported previously for the physical half-life measurements (Gallie et al. 1991). In this regard, it is interesting to note that addition of a cap or a poly(A) tail had a greater effect on *Luc* mRNA stability in plant cells.

One explanation for the low functional activity of the cap or poly(A) tail when either was present alone in a construct might have been that the *Luc* mRNA is too rapidly degraded to allow the cap or poly(A) tail an opportunity to function. However, the four *Luc* mRNAs were translationally active for 30–200 min in tobacco and 150 to over 300 min in CHO cells, depending on the construct. Since luciferase activity can be detected within 3 min following electroporation (D.R. Gallie, unpubl.), one cycle of translation is completed within this time. Translation of capped *Luc* mRNA was markedly enhanced over that seen for uncapped mRNA, even at the earliest time point, demonstrating that the cap is recognized and functional even during the first round of translation. As the *Luc* mRNAs are sufficiently stable to allow multiple rounds of translation, the cap and poly(A) tail have ample opportunity to function before mRNA degradation occurs.

If cap function is dependent on a poly(A) tail for optimal function, how does the cap function in naturally occurring poly(A)$^-$ mRNAs? In plants, the only known poly(A)$^-$ mRNAs are plant viral mRNAs, for example, tobacco mosaic virus (TMV). We have demonstrated that the TMV 3′-untranslated region (3′ UTR) is the functional equivalent of a poly(A) tail, serving to markedly increase the translational efficiency of chimeric mRNA constructs (Gallie and Walbot 1990). We have observed the same interdependence between the TMV 3′ UTR and the cap that we have seen between the cap and poly(A) tail (D.R. Gallie, in prep.). These data suggest that although efficient translation of TMV mRNA is not dependent on a poly(A) tail, the synergism between the terminal regulatory elements is nevertheless involved.

Much of our understanding of the regulatory function of the cap–eIF-4F and poly(A) tail–PAB protein complexes has been made using cell lysates. Although an in vitro approach has been useful in analyzing the binding of these regulatory proteins with their target sites, it has not demonstrated the interdependence between the cap and the poly(A) tail. As we illustrate in this report, the reason for this is that an in vitro lysate does not exhibit the synergism. What might be the nature of this deficiency? The impact of the cap or the poly(A) tail on expression in vivo is not reflected in vitro. Translation was an order of magnitude less cap-dependent in vitro (reticulocyte lysate) than in vivo (CHO cells). The poly(A) tail had virtually no impact on translation in vitro. Studies demonstrate, however, that eIF-4F and PAB protein do bind to the cap and poly(A) tail, respectively, in vitro (Sachs et al. 1987; Rhoads 1988; Sonenberg 1988). If in fact the poly(A) tail–PAB protein and cap–eIF-4F do interact so that they are functionally activated, a factor mediating the interaction may be missing or nonfunctional in the in vitro systems. The factor might be a single protein, such as an initiation factor, or might be a framework, such as the cytoskeleton. Cytoskeleton-associated polysomes have been observed (Lenk et al. 1977; Cervera et al. 1981; Howe and Hershey 1984) and it has been shown that eIF-4E preferentially associates with intermediate filaments (Zumbe et al. 1982). Association of mRNA with the cytoskeleton may be a necessary prerequisite for the synergism between the two terminal regulatory determinants to take place.

The synergism observed between the cap and poly(A) tail suggest that these two regulatory determinants work in concert to mediate the regulation associated with each. One function of the poly(A) tail–PAB protein complex may be to facilitate initiation factor or 40S subunit interaction with the mRNA. The potential for interaction between PAB protein and the 60S subunit (Sachs and Davis 1989, 1990) supports the idea that the poly(A) tail–PAB protein complex might be involved in several early events during translation initiation. Although the mechanism remains to be determined, the 3′ terminus certainly plays a pivotal role in the regulation of events occurring at the 5′ terminus. Current models concerning translation initiation are often decribed as a series of steps (Rhoads 1988; Sonenberg 1988). A model describing efficient initiation of translation, however, may be one not so much sequential, as one in which the participants, for example, the cap, eIF's, ribosomal subunits, and the PAB protein–poly(A) tail, function in concert.

Materials and methods

Plasmid constructs and in vitro transcription

The *Luc* and *uidA* constructs have been described previously (Gallie et al. 1989, 1991). The 3′-untranslated region from the *Luc* construct has been removed for this study. The T7-based constructs were linearized with the appropriate restriction enzyme and in vitro transcription of the DNA was carried out as described (Yisraeli and Melton 1989). For poly(A)$^+$ mRNAs, plasmid linearization with *Dra*I results in a poly(A)$_{50}$ tail terminating in three uridylate residues. Virtually 100% of the mRNA synthesized as capped mRNA is capped (Yisraeli and Melton 1989). 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.

In vitro translation

Two hundred nanograms of each *Luc* mRNA construct was translated in rabbit reticulocyte lysate according to the recommendations of the supplier (Boehringer Mannheim), with the exception that a complete mix of nonradiolabeled amino acids was used. Aliquots were removed at time intervals and frozen on dry ice. The extent of translation was determined by assaying each aliquot for luciferase activity as described below.

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

Preparation and electroporation conditions

Protoplast media and isolation methods for tobacco (cv. Xanthi) were as described (Fromm et al. 1987), except that 1.0% Cytolase (Genecor) was used in place of Rhozyme. Tobacco protoplasts were electroporated with a GeneZapper (IBI) by using 300 V and 500 μF capacitance.

CHO cells were collected from flasks by brief trypsinization, washed twice with phosphate-buffered saline (PBS), and electroporated in PBS with 400 V and 250 μF capacitance.

Electroporation of yeast has been described in detail elsewhere (J.G. Everett and D.R. Gallie, in prep.). Strain CRY1 Mata *can 1-100 ade 2-1 his 3-11,15 leu 2-3, 112 trp 1-1 ura 3-1*, a derivative of W303a, was generated by R. Fuller and provided by A. Sachs. Briefly, mid-log-phase *S. cerevisiae*, grown in YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose), was spheroplasted with Zymolyase −100T (ICN) in 50 mM Tris (pH 7.5), 1 mM $MgCl_2$, 30 mM dithiothreitol, 15 mM β-mercaptoethanol, 1 M sorbitol for 30 min, and subsequently washed twice in the same buffer. The spheroplasts, allowed to recover in YPD medium supplemented with 1 M sorbitol for 90 min, were washed twice with 1 M sorbitol. One-hundred and eighty microliters of yeast was mixed with the test mRNA and electroporated using 800 V, 21 μF capacitance, 1000Ω.

For time-course experiments, aliquots of cells were removed at time intervals, collected by centrifugation, and frozen until assayed. Error in these experiments is 15%. Dose-response analysis of RNA electroporation is linear through 30 μg of input mRNA (Gallie et al. 1989).

mRNA stability

Aliquots of tobacco protoplasts electroporated with 5 μg of each mRNA were removed at time intervals, the cells collected by centrifugation, and total RNA purified as described (Chomczynski and Sacchi 1987). The RNA was displayed on a formaldehyde–agarose gel, followed by Northern transfer, and probed with anti-*Luc* RNA. The region of the membrane representing the full-length form of *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 $t½ = 0.693/k$.

Analysis of luciferase activity

Following incubation, the cells were harvested and resuspended in luciferase assay buffer [25 mM Tricine (pH 7.5), 15 mM $MgCl_2$, 7 mM β-mercaptoethanol, 2 mM ATP], sonicated for 5 sec, and the cell debris pelleted. Luciferase activity was measured using 0.5 mM luciferin in luciferase assay buffer and a Monologht 2010 Luminometer (Analytical Luminescence Laboratory). Protein concentration was determined using the Bio-Rad protein assay kit.

Acknowledgments

We thank J. Traugh for critical reading of the manuscript, C. Byus for the CHO cell line, and M. Kobayashi for technical assistance.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Altmann, M., C. Handschin, and H. Trachsel. 1987. mRNA cap-binding protein: Cloning of the gene encoding protein synthesis initiation factor eIF-4E from Saccharomyces cerevisiae. *Mol. Cell. Biol.* **7:** 998–1003.

Altmann, M., N. Sonenberg, and H. Trachsel. 1989. Translation in Saccharomyces cerevisiae: Initiation factor 4E-dependent cell-free system. *Mol. Cell. Biol.* **9:** 4467–4472.

Banerjee, A.K. 1980. 5′-Terminal cap structure in eucaryotic messenger ribonucleic acids. *Bacteriol. Rev.* **44:** 175–205.

Bernstein, P. and J. Ross. 1989. Poly(A), poly(A) binding protein and the regulation of mRNA stability. *Trends Biochem. Sci.* **14:** 373–377.

Brawermann, G. 1981. The role of the poly(A) sequence in mammalian messenger RNA. *CRC Crit. Rev. Biochem.* **10:** 1–38.

Cervera, M., G. Dreyfuss, and S. Penman. 1981. Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa cells. *Cell* **23:** 113–120.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.

Darnell, J.E., R. Wall, and R.J. Tushinski. 1971. An adenylic acid-rich sequence in messenger RNA of HeLa cells and its possible relationship to reiterated sites in DNA. *Proc. Natl. Acad. Sci.* **68:** 1321–1325.

Drummond, D.R., J. Armstrong, and A. Colman. 1985. The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNAs in Xenopus oocytes. *Nucleic Acids Res.* **13:** 7375–7394.

Edmonds, M., M.H. Vaughan Jr., and H. Nakazato. 1971. Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: Possible evidence for a precursor relationship. *Proc. Natl. Acad. Sci.* **68:** 1336–1340

Filipowicz, W. 1978. Functions of the 5′-terminal m^7G cap in eukaryotic mRNA. *FEBS Lett.* **96:** 1–11.

Fromm, M.E., J. Callis, L.P. Taylor, and V. Walbot. 1987. Electroporation of DNA and RNA into plant protoplasts. *Methods Enzymol.* **153:** 351–366.

Furuichi, Y., A. LaFiandra, and A.J. Shatkin. 1977. 5′-Terminal structure and mRNA stability. *Nature* **266:** 235–239.

Gallie, D.R. and V. Walbot. 1990. RNA pseudoknot domain of tobacco mosaic virus can functionally substitute for a poly(A) tail in plant and animal cells. *Genes & Dev.* **4:** 1149–1157.

Gallie, D.R., W.J. Lucas, and V. Walbot. 1989. Visualizing mRNA expression in plant protoplasts: Factors influencing efficient mRNA uptake and translation. *Plant Cell* **1:** 301–311.

Gallie, D.R., J.N. Feder, R.T. Schimke, and V. Walbot. 1991. Post-transcriptional regulation in higher eukaryotes: The role of the reporter gene in controlling expression. *Mol. Gen. Genet.* **228:** 258–264.

Grange, T., C. Martins de Sa, J. Oddos, and R. Pictet. 1987. Human mRNA polyadenylate binding protein: Evolutionary conservation of a nucleic acid binding motif. *Nucleic Acids Res.* **15:** 4771–4787.

Green, M.R., T. Maniatis, and D.A. Melton. 1983. Human β-globin pre-mRNA synthesized *in vitro* is accurately spliced in Xenopus oocyte nuclei. *Cell* **32:**681–694.

Howe, J.G. and J.W.B. Hershey. 1984. Translational initiation factor and ribosome association with the cytoskeletal framework fraction from HeLa cells. *Cell* **37:** 85–93.

Jackson, R.J. and N. Standart. 1990. Do the poly(A) tail and

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

3′-untranslated region control mRNA translation? *Cell* **62:** 15–24.

Lee, S.Y., J. Mendecki, and G. Brawerman. 1971. A polynucleotide segment rich in adenylic acid in the rapidly-labeled polyribosomal RNA component of mouse sarcoma 180 ascites cells. *Proc. Natl. Acad. Sci.* **68:** 1331–1335.

Lenk, R., L. Ransom, Y. Kaufmann, and S. Penman. 1977. A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell* **10:** 67–78.

Melton, D.A., P.A. Kreig, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12:** 7035–7056.

Munroe, D. and A. Jacobson. 1990a. mRNA poly(A) tail, a 3′ enhancer of translational initiation. *Mol. Cell. Biol.* **10:** 3441–3455.

———. 1990b. Tales of poly(A): A review. *Gene* **91:** 151–158.

Reddy, R., T.S. Ro-Choi, D. Henning, and H. Busch. 1974. Primary sequence of U-1 nuclear ribonucleic acid of Novikoff hepatoma ascites cells. *J. Biol. Chem.* **249:** 6486–6494.

Rhoads, R.E. 1988. Cap recognition and the entry of mRNA into the protein synthesis initiation cycle. *Trends Biochem. Sci.* **13:** 52–56.

Sachs, A.B. and R.W. Davis. 1989. The poly(A) binding protein is required for poly(A) shortening and 60S ribosomal subunit-dependent translation initiation. *Cell* **58:** 857–867.

———. 1990. Translation initiation and ribosomal biogenesis: Involvement of a putative rRNA helicase and RPL46. *Science* **247:** 1077–1079.

Sachs, A.B., R.W. Davis, and R.D. Kornberg. 1987. A single domain of yeast poly(A)-binding protein is necessary and sufficient for RNA binding and cell viability. *Mol. Cell. Biol.* **7:** 3268–3276.

Shimotohno, K., Y. Kodama, J. Hashimoto, and K.-I. Miura. 1977. Importance of 5′-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis. *Proc. Natl. Acad. Sci.* **74:** 2734–2738.

Sonenberg, N. 1988. Cap-binding proteins of eukaryotic messenger RNA: Functions in initiation and control of translation. *Prog. Nucleic Acid Res. Mol. Biol.* **35:** 173–207.

Yisraeli, J.K. and D.A. Melton. 1989. Synthesis of long, capped transcripts in vitro by SP6 and T7 RNA polymerases. *Methods Enzymol.* **180:** 42–50.

Zumbe, A., C. Stahli, and H. Trachsel. 1982. Association of a M_r 50,000 cap-binding protein with the cytoskeleton in baby hamster kidney cells. *Proc. Natl. Acad. Sci.* **79:** 2927–2931.

Downloaded from genesdev.cshlp.org on August 25, 2022 - Published by Cold Spring Harbor Laboratory Press

The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency.

D R Gallie

Genes Dev. 1991, **5:**
Access the most recent version at doi:10.1101/gad.5.11.2108

References This article cites 34 articles, 14 of which can be accessed free at:
http://genesdev.cshlp.org/content/5/11/2108.full.html#ref-list-1

License

Email Alerting Service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here.**

Copyright © Cold Spring Harbor Laboratory Press