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The histone 3′-terminal stem–loop is necessary for translation in Chinese hamster ovary cells

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ABSTRACT

The metazoan cell cycle-regulated histone mRNAs are the only known cellular mRNAs that do not terminate in a poly(A) tail. Instead, mammalian histone mRNAs terminate in a highly conserved stem–loop structure which is required for 3′-end processing and regulates mRNA stability. The poly(A) tail not only regulates translational efficiency and mRNA stability but is required for the function of the cap in translation (m^7GpppN). We show that the histone terminal stem–loop is functionally similar to a poly(A) tail in that it enhances translational efficiency and is co-dependent on a cap in order to establish an efficient level of translation. The histone stem–loop is sufficient and necessary to increase the translation of reporter mRNA in transfected Chinese hamster ovary cells but must be positioned at the 3′-terminus in order to function optimally. Mutations within the conserved stem or loop regions reduced its ability to facilitate translation. All histone mRNAs in higher plants are polyadenylated. The histone stem–loop did not function to influence translational efficiency or mRNA stability in plant protoplasts. These data demonstrate that the histone stem–loop directs efficient translation and that it is functionally analogous to a poly(A) tail.

INTRODUCTION

The vast majority of eukaryotic mRNAs are polyadenylated. The poly(A) tail regulates both the translational efficiency (1) and the stability (2) of mRNAs *in vivo*. It and the cap (m^7GpppN, where N represents any nucleotide) are co-dependent in their role as regulators of translational efficiency (3). Those mRNAs that naturally lack a poly(A) tail present an apparent paradox in that, in the absence of the poly(A) tail, the cap should be virtually non-functional and, consequently, the mRNA should be rendered translationally incompetent. The cell cycle-regulated histone mRNAs represent the only known class of cellular mRNAs that are naturally non-polyadenylated. Instead of a poly(A) tail, these histone mRNAs terminate in a stem–loop structure that is highly conserved from *Caenorhabditis elegans* to humans (4,5). Expression of the cell cycle-regulated histones is tightly coupled to nuclear DNA synthesis during the S phase of the mitotic cell cycle (6). Expression is regulated both at the levels of transcription and mRNA stability (7–9). Changes in transcription and 3′-end processing account for histone mRNA regulation during the G_1 phase, whereas the mRNA is specifically destabilized during the G_2 phase (10). The 3′-terminal stem–loop structure facilitates several steps of histone gene expression. The stem–loop and a downstream purine-rich region that forms a duplex with a complementary sequence at the 5′-end of U7 snRNA are required for 3′-end processing (11–16). The stem–loop is also required for nucleocytoplasmic transport (17,18), cytoplasmic mRNA stability (19–21) and localization to polysomes (22). Mutations within the stem–loop established that the phylogenetically conserved sequences are required for histone regulation (23). Proteins have been identified that specifically recognize the histone stem–loop structure that may mediate the post-transcriptional regulation associated with this structure (24,25).

Although the histone stem–loop is required for multiple steps in histone gene expression, there has been no direct demonstration that the structure is important during translation. In this study, we investigate this aspect of histone gene expression by determining the impact of the histone stem–loop structure on the translational efficiency and mRNA stability of luciferase mRNA transfected into Chinese hamster ovary (CHO) cells. We find that the histone stem–loop was necessary and sufficient to enhance the stability and the translational efficiency of the reporter mRNA and, like a poly(A) tail, was co-dependent on the cap in its function as a facilitator of translation.

MATERIAL AND METHODS

mRNA constructs and *in vitro* transcription reaction conditions

The pT7-*luc* and pT7-*luc*-A_{50} constructs, in which the firefly luciferase coding region is under the control of the T7 promoter, have been described previously (3). The histone and related sequences were introduced from synthetic oligonucleotides into the *Bam*HI/*Kpn*I sites of the pT7-*luc* construct. Restriction sites (either *Afl*II or *Avr*II) immediately downstream of the stem–loop were used to linearize the plasmid prior to *in vitro* transcription.

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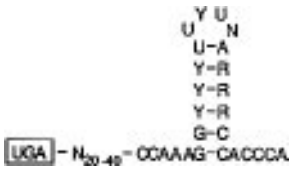


Figure 1. The consensus primary and secondary structure of the 3′-terminal stem–loop of histone mRNAs derived from metazoans. The sequences of over 200 metazoan histone mRNAs were compared for the consensus. Y, pyrimidine; R, purine, N, any nucleotide.

The pT7-*luc* and pT7-*luc*-A_{50} constructs were linearized with *Bam*HI and *Dra*I, respectively, prior to *in vitro* transcription. *In vitro* transcription and the integrity of the RNA were determined as described (26,27).

Preparation and electroporation of carrot protoplasts and CHO cells

Protoplasts were isolated and electroporated from a carrot cell suspension as described (28).

CHO cells were grown to ~80% confluence in Ham's 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 phosphate-buffered saline (PBS) solution and electroporated as described (3). The error resulting from RNA delivery via electroporation is ~±15% (29).

Luciferase assay and RNA half-life measurements

Luciferase and protein assays were performed as described (3,30).

The half-life was determined from the kinetic analysis of each mRNA in transfected CHO cells. The functional half-life is calculated by measuring the decay of the capacity to synthesize proteins following RNA delivery and recruitment onto polysomes using the following equation: $E_{rc} = E_m - E_t$, where E_m is the maximum level of expression achieved, E_t is the level of expression at a given point in time and E_{rc} is the remaining capacity of the mRNA.

RESULTS

The histone stem–loop increases reporter gene expression in transiently-transformed CHO cells

As the histone 3′-terminal stem–loop structure is highly conserved in metazoans (reviewed in 5), a consensus stem–loop can be generated from a phylogenetic comparison (see Fig. 1). The conserved elements of the stem–loop are: a 6 bp stem consisting of two GC base pairs at the base; a set of three pyrimidine/purine bases forming the central portion of the stem and a UA base pair at the top; a four base loop in which the first and third positions are U (except in nematodes, where the first position is a C; 31), the second position is usually a U and the fourth position varies. Three A residues usually precede and four C or A residues follow the stem. In contrast to metazoans, histone mRNAs from yeast, fungi, protists and plants, with the exception of those from *Volvox* and *Chlamydomonas* (32,33), are polyadenylated and do not contain this stem–loop structure.

To examine whether the histone 3′-terminal stem–loop structure could direct efficient expression from reporter mRNA, a 32 bp

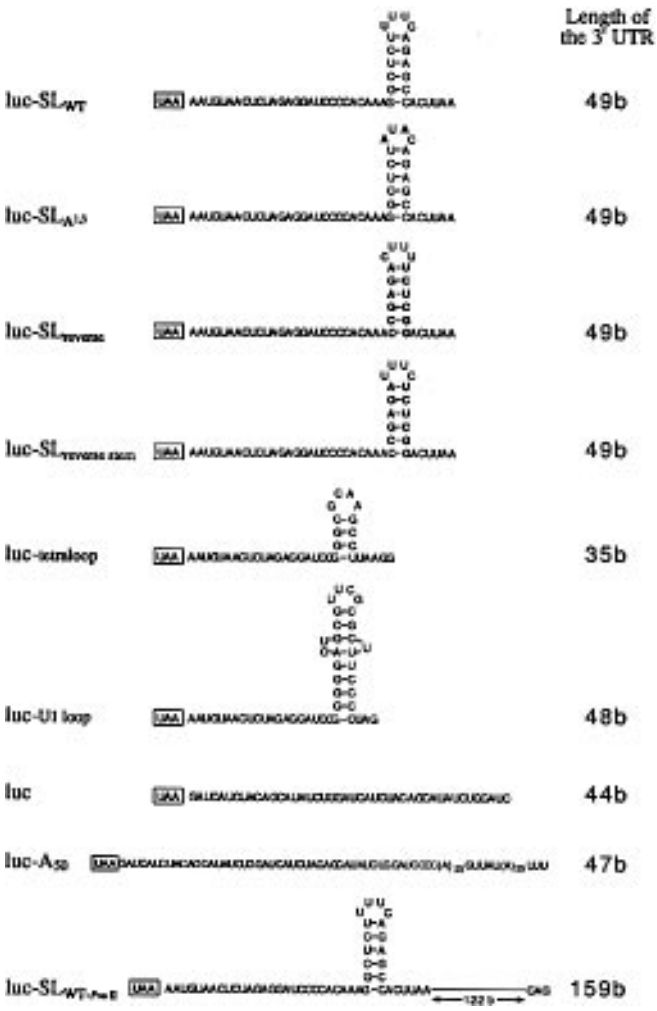


Figure 2. The sequence and structure of the 3′-UTR for each mRNA construct used in this study. The sequence of the wild-type construct (*luc*-SL_{WT}) is shown at the top and the exact length of the 3′-UTR is shown to the right. In the *luc*-$SL_{A1,3}$ construct, the two conserved uridines in the loop have been changed to adenosines; in *luc*-$SL_{reverse}$, the entire stem–loop has been inverted; in *luc*-$SL_{reverse\ stem}$, the stem has been inverted but the loop has been maintained in the wild-type configuration. *luc*-tetraloop contains a synthetic stem–loop composed of a 4 bp stem and a tetraloop. *luc*-U1 contains the terminal 26 bases of the U1 snRNA. The *luc* and *luc*-A_{50} mRNAs served as negative and positive controls, respectively. For the *luc*-$SL_{WT\text{-}PvuII}$ mRNA, the histone stem–loop was internalized by restricting the *luc*-SL_{WT} DNA construct at a *Pvu*II site 116 bases downstream of the stem–loop sequence before mRNA synthesis. The stop codon of the *luc* coding region is shown boxed.

fragment containing the consensus histone stem–loop sequence was introduced downstream of the *luc* reporter gene in a T7-based vector (3). A restriction site incorporated into the construct immediately downstream of the stem–loop allowed the *in vitro* production of capped-*luc* mRNA terminating in the histone stem–loop (*luc*-SL_{WT}, Fig. 2). The stem–loop was positioned 27 bases downstream of the *luc* stop codon, which is similar to the spacing present in histone mRNAs. The total length of the 3′-untranslated region (3′-UTR) was 49 bases. A polyadenylated *luc* mRNA construct containing 47 bases between the *luc* stop codon and the poly(A) tail (*luc*-A_{50}) served as a positive control and the same *luc* mRNA construct with a 47 base 3′-UTR but

Length of the 3′UTR	Light units / mg protein					
	0.03 μg mRNA	Rel. level	0.5 μg mRNA	Rel. level	2.0 μg mRNA	Rel. level
44b	3,319,569	1	62,114,459	1	200,391,132	1
47b	65,877,138	19.8	1,046,048,732	16.8	4,615,769,410	23.0
44b	41,852,615	12.6	804,072,426	12.9	2,723,932,003	13.6

Figure 3. The impact of the histone stem–loop on expression from luciferase reporter mRNA in transiently-transfected CHO cells. Aliquots of 0.03, 0.5 or 2μg of each capped mRNA, synthesized *in vitro*, were delivered to cells using electroporation. For the exact 3′-UTR sequence of each construct see Figure 2. Each mRNA was delivered into triplicate samples of cells and the luciferase assays were performed in duplicate. The standard deviation for each construct is shown.

without the poly(A) tail served as a negative control. Each mRNA, synthesized *in vitro* as capped mRNA, was delivered in triplicate to CHO cells by electroporation and the resulting level of luciferase protein produced, as measured by its enzymatic activity, was used as a measure of the extent of translation from each construct. Once the cells were transformed with the mRNA, they were incubated for 8 h in order to allow sufficient time for the mRNA to be fully degraded. Therefore, with this approach, we measured the impact that the histone stem–loop makes on the translational efficiency as well as the stability of the *luc* mRNA.

Using 0.5 μg of each mRNA construct for delivery, we observed that the presence of the histone stem–loop increased expression from *luc* mRNA 13-fold with respect to the poly(A)$^-$ control mRNA (Fig. 3). This was comparable with the effect of a poly(A) tail, the addition of which increased luciferase expression 16.9-fold. We then examined whether the amount of mRNA delivered influenced the degree to which the histone stem–loop affected reporter gene expression. When 0.03 μg of each mRNA construct was delivered, expression from *luc*-SL$_{WT}$ was 12.6-fold greater than that from the poly(A)$^-$ *luc* mRNA. Similar results were obtained when 2 μg of each mRNA construct was delivered: expression from *luc*-SL$_{WT}$ was 13.6-fold greater than that from the poly(A)$^-$ *luc* mRNA. These data demonstrate that the histone stem–loop is sufficient and necessary to increase reporter gene expression and the extent to which it increases expression remains similar over a 60-fold range of input mRNA. In subsequent experiments, the amount of RNA used for delivery was maintained within this range. The stimulation afforded by the histone stem–loop is not a consequence of introducing a structured sequence at the 3′-terminus, as previous studies have demonstrated that even considerably more stable 3′-terminal structures than the histone stem–loop do not substantially increase expression in CHO cells (34).

As the stability of those histone mRNAs terminating in the conserved stem–loop structure are cell cycle regulated, we examined whether cell density affected the extent to which the histone stem–loop could impact gene expression under our assay conditions. CHO cells were harvested during low (~25% confluent) or high (~80% confluent) cell density and the same constructs shown in Figure 3 were delivered. The presence of the histone stem–loop increased expression to the same extent in the cells regardless of whether they had been grown to low or high density (data not shown).

We had previously determined that a poly(A) tail must be positioned at the 3′-terminus in order to function (28). When additional sequence was present downstream of the poly(A) tail, up to a 90% decrease in poly(A) tail function resulted. To determine whether the histone stem–loop must be present at the 3′-terminus in order to stimulate expression, the *luc*-SL$_{WT}$ construct was linearized at a restriction site in the transcription vector that was 116 bp downstream of the stem–loop structure. This resulted in an mRNA in which the histone stem–loop was internalized by 116 bases (*luc*-SL$_{WT\text{-}Pvu\,II}$). The expression from this mRNA was compared with *luc*-SL$_{WT}$ in transiently-transformed CHO cells. Internalization of the stem–loop resulted in a 60% decrease in expression compared with the wild-type configuration (Fig. 4), data suggesting that, like a poly(A) tail, the histone stem–loop must be positioned at the 3′-terminus in order to function optimally. This observation is in good agreement with previous studies in which the stem–loop-mediated regulation of histone mRNA stability was lost upon internalization of the stem–loop (35,36). Interestingly, even when the stem–loop was positioned internally, there was still a 6.4-fold increase in expression relative to the poly(A)$^-$ *luc* mRNA control. This increase could be due to several, non-exclusive possibilities. First, the stem–loop may exhibit residual activity when present in an internal position. Second, removal of the 116 base extension by 3′ exonucleases (37) would result in a transcript with the histone stem–loop present at the 3′-terminus. Third, expression from poly(A)$^-$ mRNA increases with the length of the 3′-UTR: an increase in the 3′-UTR from 44 to 156 bases increases expression 4.1-fold through increases in both translational efficiency and mRNA stability (38). Therefore, the residual activity from *luc*-SL$_{WT\text{-}Pvu\,II}$ may not be due to the presence of the histone stem–loop but rather a result of its 159 base 3′-UTR.

The histone stem–loop and the cap are co-dependent regulators of expression

We have shown that the cap (m^{7}GpppN) and poly(A) tail cooperate to form the basis for efficient translation in plants and animals (3). As a regulator of translation, the poly(A) tail requires the cap for function: for uncapped messages, the translational efficiency of poly(A)$^+$ mRNA is not substantially greater than poly(A)$^-$ mRNA (3). Moreover, the degree to which a cap stimulates translation is an order of magnitude greater for poly(A)$^+$ than it is for poly(A)$^-$ mRNA (3). Therefore, the cap and poly(A) tail are not functionally separate but work in concert to direct efficient translation.

If efficient translation of conventional mRNAs is dependent on a synergy between the cap and poly(A) tail, what then is the translational strategy employed by histone mRNAs? To examine

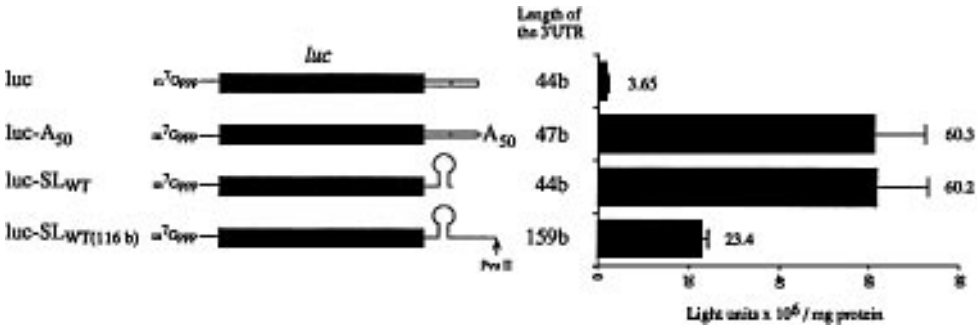


Figure 4. The effect of positioning the histone stem–loop internally in an mRNA on its ability to regulate translation. Expression is compared with *luc*-SL_{WT}, in which the histone stem–loop is positioned at the 3′-terminus. Capped mRNAs were synthesized *in vitro* and delivered in triplicate using electroporation. Each luciferase assay was performed in duplicate. The resulting level of expression is shown as a histogram to the right of each construct. The standard deviation for each construct is shown as an error bar.

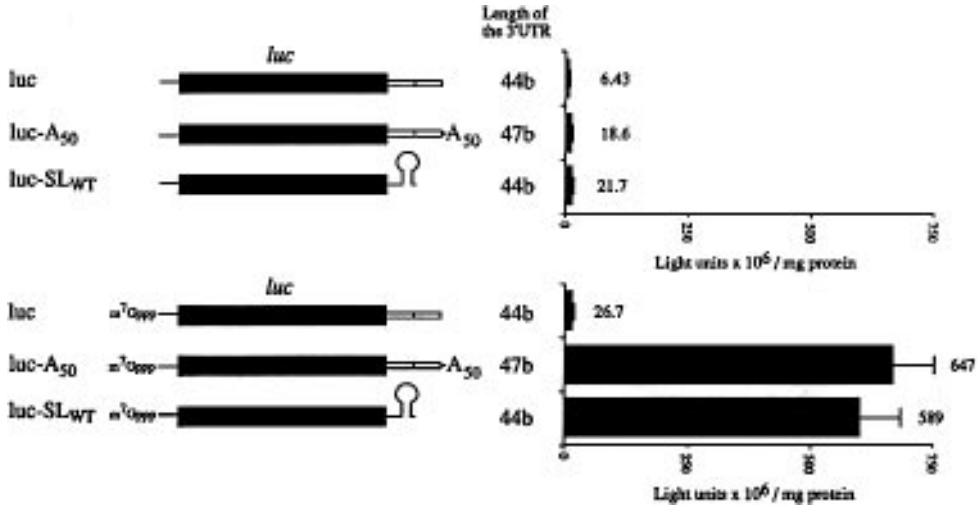


Figure 5. Analysis of the co-dependency between a cap and the histone stem–loop as regulators of translation. The three constructs used in Figure 1 were synthesized *in vitro* as capped and uncapped mRNAs and delivered in triplicate using electroporation. Each luciferase assay was performed in duplicate. The resulting level of expression is shown as a histogram to the right of each construct. The standard deviation for each construct is shown as an error bar.

whether the cap and the histone stem–loop are co-dependent regulators of translation, *luc*-SL_{WT} mRNA was synthesized *in vitro* as capped or uncapped mRNA and the level of expression from these mRNAs in CHO cells was compared with that from *luc* and *luc*-A_{50} mRNA as capped and uncapped mRNAs. The presence of the histone stem–loop increased expression only 3.4-fold relative to the *luc* mRNA when the mRNAs were uncapped, but increased expression 22.2-fold when the mRNAs were capped (Fig. 5). This is a similar effect to that observed for a poly(A) tail: addition of a poly(A) tail increased expression from uncapped *luc* mRNA 2.9-fold but increased expression from capped *luc* mRNA 24.4-fold. Therefore, the histone stem–loop is dependent on the presence of a cap for full function. The data can also be analyzed to determine whether the function of the cap is affected by the histone stem–loop. Addition of a cap to *luc* mRNA without the histone stem–loop or poly(A) tail increased expression 4.1-fold (compare the expression from capped *luc* mRNA with uncapped *luc* mRNA) but increased expression from *luc*-SL_{WT} 27.2-fold (compare the ratio of expression from capped *luc*-SL_{WT} mRNA with uncapped *luc*-SL_{WT} mRNA). Therefore, the impact that a cap makes on expression is enhanced by the presence of the histone stem–loop. The level of co-dependency between the cap and histone stem–loop, calculated as the ratio of the impact that the histone stem–loop makes on the translation of uncapped versus capped mRNA, is 6.5-fold (i.e. 22.2 ÷ 3.4 = 6.5-fold). This is similar to the 8.4-fold level of co-dependency between a cap and poly(A) tail (i.e. 24.4 ÷2.9 = 8.4-fold). These data demonstrate, therefore, that although histone messages have evolved an alternative to the poly(A) tail, they have nevertheless maintained a co-dependency between the terminal regulatory elements that is presumably a result of an interaction between *trans*-acting factors, e.g. the cap binding initiation factors and the histone stem–loop binding protein.

Mutations within the histone stem–loop affect its function

The phylogenetically conserved bases within the histone stem–loop suggest that primary sequence within this structure is important for its function. Mutations within the conserved positions of the loop or stem disrupt 3′-end processing (16,23,39,40). Changing the

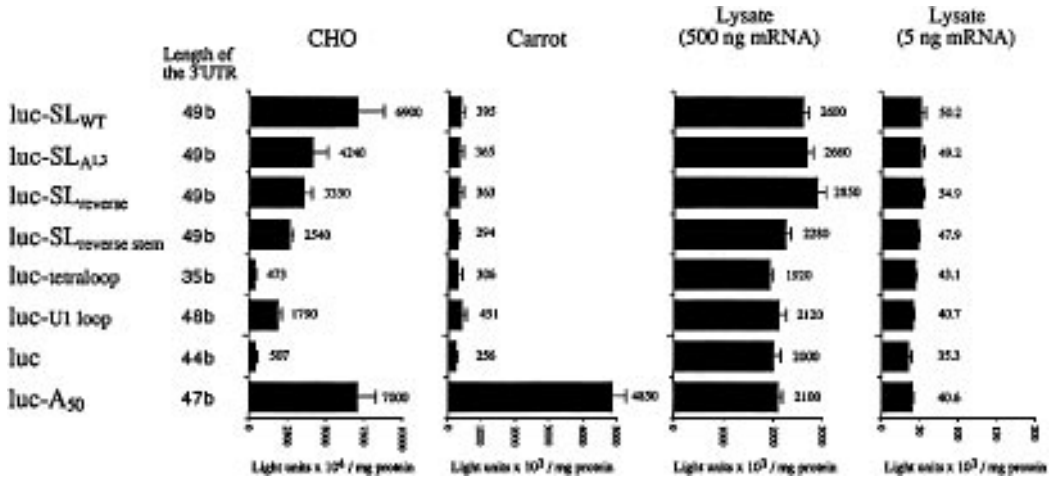


Figure 6. Mutational analysis of the histone stem–loop on its ability to function in animal and plant cells and *in vitro*. Capped mRNAs were synthesized *in vitro* and delivered to either CHO cells or carrots protoplasts using electroporation. The same mRNAs were used to program *in vitro* translation in a lysate derived from rabbit reticulocytes using either 500 or 5 ng of input mRNA. For the exact 3′-UTR sequence of each construct see Figure 2. The resulting level of expression is shown as a histogram to the right of each construct. Each mRNA was delivered into triplicate samples of cells and the luciferase assays were performed in duplicate. The standard deviation for each construct is shown as an error bar.

conserved uridine residues in the loop to adenosines also abolished binding of the nuclear and polyribosomal proteins that specifically recognize the histone stem–loop (24). We therefore examined the effect of mutations within the histone stem–loop on its ability to enhance expression from reporter mRNA. Three different mutants were examined: in *luc*-$SL_{A1,3}$ the two conserved uridines in the loop have been changed to adenosines; in *luc*-$SL_{reverse}$ the entire stem–loop has been inverted; in *luc*-$SL_{reverse\ stem}$ the stem has been inverted but the loop has been maintained in the wild-type configuration (Fig. 2). In addition to the wild-type construct, *luc*-SL_{WT} and the *luc* and *luc*-A_{50} control mRNAs, two additional control constructs were made. *luc*-tetraloop contains a synthetic stem–loop composed of a 4 bp stem and a tetraloop that makes use of an unusual base pair for increased thermodynamic stability (41,42) which is recognized by tetranucleotide loop binding proteins (43). This construct, therefore, serves as a control for the effect of a stable 3′-terminal stem–loop on expression. *luc*-U1 contains the terminal 26 bases of the U1 snRNA which forms a stem–loop (44) composed of a 9 bp stem with a four base loop which itself is also an example of a stable tetraloop.

The effect of the mutations on histone stem–loop function was examined in CHO cells following translation of the test mRNAs in transiently-transformed CHO cells. The presence of the histone stem–loop increased expression 13.6-fold relative to the *luc* mRNA control construct, which was similar to the impact of adding a poly(A)$_{50}$ tail to the reporter mRNA (Fig. 6). Changing the two conserved uridines in the loop to adenosines resulted in a 39% drop in stem–loop function. Inverting the entire stem–loop or just the stem resulted in a drop of 49 or 63% in function, respectively. The tetranucleotide stem–loop structure had no impact on reporter expression, whereas the U1 sequence increased expression by 3.5-fold. The impact of the histone stem–loop mutations on reporter mRNA translation was consistent with the observation that mutation of the conserved uridines in the loop structure affects 3′-end processing and/or transport but not localization to polysomes (23,18). The failure of the tetranucleotide stem–loop to affect expression and the low level of stimulation observed for the U1 stem–loop construct suggests that the histone stem–loop-mediated increase in expression is specific to this sequence.

The histone stem–loop does not function in plant protoplasts

Plant histone mRNAs are polyadenylated and therefore do not contain the stem–loop structure present in animal histone mRNAs. Either the histone stem–loop evolved after the divergence of plants and animals or was lost from plants during their subsequent evolution. The observation that histone mRNAs of *Chlamydomonas* (32) and the multicellular alga *Volvox* (33) are not polyadenylated but contain the phylogenetically-conserved stem–loop supports the latter hypothesis. The histone stem–loop serves as a binding site for specific nuclear and polysomal proteins (24,25). If the histone stem–loop regulatory mechanism had once been present in the evolution of plants, the stem–loop binding protein genes may still be present in plant genomes, particularly if they were required to facilitate other cellular processes.

To examine whether the histone stem–loop can function to enhance expression from reporter mRNA in plants, the set of mRNA constructs used in the previous experiment were introduced into carrot protoplasts using electroporation. Carrot is a rapidly growing cell culture like CHO cells and ~25–30% of the cells are in S phase at any one time. The same batch of mRNAs used in the transient transfection of CHO cells was used for transient protoplast transformation to rule out any variation in RNA preparations. Expression from *luc*-A_{50} mRNA was 18.9-fold greater than that from the poly(A)$^-$ *luc* construct (Fig. 6), demonstrating that the poly(A) tail is functionally active in carrot protoplasts, as previously reported (28). In contrast to the observations in CHO cells, the levels of expression from the constructs containing either the wild-type or mutant histone stem–loops were not significantly greater than that from the *luc* mRNA control construct. Moreover, neither the tetranucleotide stem–loop nor U1 sequence affected expression from reporter mRNA. The

failure of the histone stem–loop to function in plant protoplasts further supports the conclusion that its ability to function in CHO cells is specific to the histone stem–loop sequence. The observation that 3′ structured sequences from plant viral mRNAs are specifically recognized and stimulate translation in plant protoplasts (45,46) demonstrates that these cells are competent to recognize specific 3′ regulatory elements. Their failure to functionally recognize the histone stem–loop supports the idea that this mechanism has been lost in the evolution of higher plants.

The histone stem–loop is not required for translation in a reticulocyte lysate

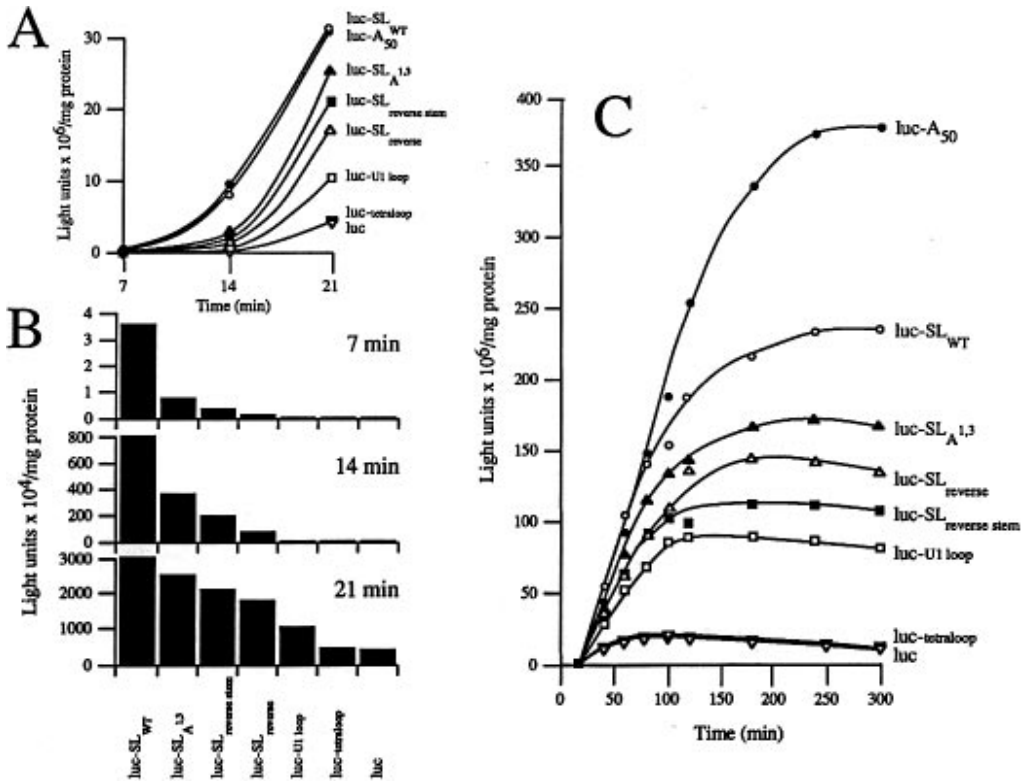
To examine whether the histone stem–loop can function *in vitro*, a lysate derived from rabbit reticulocytes was programed with the set of mRNA constructs used in the previous experiment. Once again, the same batch of mRNAs used in the previous experiments was used for the *in vitro* translation. The lysate was programed with both a high level (500 ng) and low level (5 ng) of mRNA. At neither the high nor low mRNA levels did the histone stem–loop function to significantly increase translation *in vitro*. Likewise, the poly(A) tail also failed to increase expression, which we had observed previously with this lysate (3). Moreover, other mRNAs ending in the stable tetraloop or U1 stem–loop were translated equally well in the lysate. These data demonstrate that the histone stem–loop structure does not increase expression in an *in vitro* lysate derived from rabbit reticulocytes when translated under standard conditions. The observation that 3′ regulatory elements in general, including the poly(A) tail and 3′ regulatory structures from plant viral mRNAs, do not function well *in vitro* (45,46,47) suggests that *in vitro* lysates fail to fully reflect the *in vivo* environment.

The histone stem–loop increases both the translational efficiency and stability of reporter mRNA

Although the effect of the histone stem–loop on mRNA stability has been documented (19–21), its impact on translation has been suggested (22) but has not been previously quantitated. The effects of the histone stem–loop on translational efficiency and message stability could be separately quantitated, therefore, by following the kinetics of *luc* mRNA translation in transiently transfected CHO cells. Following delivery of each mRNA construct, aliquots of cells were removed at time intervals following mRNA delivery 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. 7). Once the mRNA has been initially loaded onto polysomes, translation proceeds at a rate 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, represented by the plateau of each curve at the later time points in Figure 7C. By comparing the rates for each *luc* mRNA construct, the impact that the wild-type or mutant histone stem–loops have on the translational efficiency can be determined.

We were interested in determining whether the histone stem–loop influenced translational efficiency in addition to mRNA stability. In order to measure the effect of the histone stem–loop early during translation, i.e. the phase of loading onto polysomes, luciferase measurements were made every 7 min following mRNA delivery for the first hour. The amount of luciferase produced within the first 21 min following delivery was then plotted as a function of time (Fig. 7A). The *luc*-SL_{WT} mRNA construct performed equally well as the *luc*-A_{50} mRNA. Mutations within the histone stem–loop had a substantial impact on the ability of the mRNA to be recruited quickly for translation. At 7 min following delivery, expression from the *luc*-$SL_{A1,3}$ mRNA was reduced 5-fold, that from *luc*-$SL_{reverse\ stem}$ was reduced 12-fold and that from *luc*-$SL_{reverse}$ was reduced 38-fold compared with *luc*-SL_{WT} control mRNA (Fig. 7B). Interestingly, these trends correlate well with the affinity of the stem–loop binding protein for the same wild-type and mutant histone stem–loop structures (25). Expression from *luc* mRNA constructs terminating in the U1 loop or tetraloop was 200- to 500-fold lower than that from *luc*-SL_{WT} 7 min following the introduction of the mRNAs into the cytoplasm, suggesting that these structures do not support rapid polysome loading. At 14 min, expression from the mutant histone stem–loop mRNA constructs was still substantially lower than from the wild-type construct and expression from the U1- or tetraloop-containing constructs was still 60- to 100-fold lower than that from *luc*-SL_{WT}. By 21 min, the phase of steady-state translation was reached and the histone stem–loop mutants had improved relative to the wild-type construct. Figure 7C shows the translational characteristics of each mRNA over its lifetime. The translational efficiency of the mRNA is measured during the transient steady-state phase of translation. The presence of the histone stem–loop increased the translational efficiency of *luc*-SL_{WT} mRNA by 5.9-fold over that measured for the *luc* construct terminating in the tetraloop (Fig. 7C). This rate of translation was virtually identical to that observed for the *luc*-A_{50} construct. The increase in the final yield of luciferase protein produced from *luc*-A_{50} relative to *luc*-SL_{WT} was due to the greater stability of the *luc*-A_{50} mRNA (discussed below). Changing the two conserved uridines in the loop to adenosines, inverting the entire stem–loop or inverting just the stem all resulted in a drop in translational efficiency that was consistent with their effect in Figure 6. The tetranucleotide stem–loop structure had no impact on translation compared with the *luc* mRNA construct with a random 44 base 3′-UTR. The U1 sequence increased translational efficiency relative to the tetraloop-containing construct by 2.9-fold. We conclude, therefore, that the histone stem–loop structure increases translational efficiency and the effect on translation is specific to the histone stem–loop sequence, as the mutants were compromised in their ability to facilitate translation, particularly in the early stages of polysome association.

As luciferase protein accumulates over time only for as long as there is intact *luc* mRNA present for translation, the length of time over which luciferase protein accumulates reflects the stability of the mRNA. The data from Figure 7C can be used to determine the functional mRNA stability, which is a measure of the integrity of the message as determined by the length of time over which it is translationally active and is defined as the amount of time needed to complete a 50% decay in the capacity of an mRNA to synthesize protein (48,49). Using this approach, the stability of polysome-associated mRNA can be specifically measured. The functional mRNA half-life of the control *luc* mRNA was 33 min, whereas the addition of a poly$(A)_{50}$ tail increased the half-life to 105 min, results that are in good agreement with previous measurements (3). The presence of the histone stem–loop increased the mRNA half-life to 69 min, similar to histone mRNA



mRNA	Translational efficiency (Light units/min mg protein)	Relative rate of translation	Functional mRNA half-life (min)	Relative mRNA half-life
Capped				
luc-SL WT	2,230,000	5.9	69	2.1
luc-SL A 1,3	1,780,000	4.7	67	2.0
luc-SL reverse	1,430,000	3.8	69	2.1
luc-SL reverse stem	1,680,000	4.4	53	1.6
luc-U1 loop	1,120,000	2.9	57	1.7
luc-tetraloop	380,000	1.0	33	1.0
luc	380,000	1.0	33	1.0
luc-A50	2,220,000	5.8	105	3.2

Figure 7. Kinetic analysis of the translation of the wild-type and mutant histone stem–loop mRNA constructs in CHO cells. Capped mRNAs were synthesized *in vitro* and delivered using electroporation. Following delivery, aliquots of cells were removed at time intervals and assayed. The resulting luciferase activity was plotted as a function of time. (**A**) Expression for each construct is plotted for the first 21 min following mRNA delivery. (**B**) The levels of expression resulting from each construct at 7, 14 and 21 min following mRNA delivery are displayed as histograms. (**C**) In an independent experiment, the translational characteristics of each mRNA were followed for 6 h following delivery. The translational efficiency was determined from the slope of each line during the transient steady-state phase of translation and the values are shown in the table. The functional mRNA half-life was determined as the amount of time required to complete a 50% decay in the capacity of the *luc* mRNA to synthesize luciferase. The relative rate of translation or functional stability is expressed in the table relative to the poly(A)$^-$ *luc* control mRNA.

in CHO cells (8,10,50). This represents a 2.1-fold increase over that observed for the control *luc* mRNA, but is less than the stabilizing effect of a poly(A)$_{50}$ tail. The mutations within the histone stem–loop had little effect on the stem–loop-mediated increase in stability. The tetraloop did not significantly change mRNA stability compared with the control *luc* mRNA. The U1 stem–loop increased the mRNA half-life 1.7-fold, relative to the control *luc* mRNA, which partly accounts for the effect of this sequence on expression from *luc* mRNA. Visual inspection of the length of time over which each mRNA construct is translationally active confirms these measurements. *luc*-SL$_{WT}$ is translationally active for a period of time (the end point determined by the plateau for each curve) that is approximately twice as long as the control *luc* mRNA. The mutations within the histone stem–loop resulted in a period of translational activity which was intermediate between that for *luc*-SL$_{WT}$ and *luc* mRNAs. From these data, we conclude that the histone stem–loop increases both the translational efficiency as well as the stability of an mRNA.

DISCUSSION

Studies focusing on the 3′-terminal stem–loop structure of the cell cycle-regulated histone mRNAs have demonstrated its involvement in multiple steps of histone gene expression, including 3′-end processing (11–16), nucleocytoplasmic transport (17,18) and cytoplasmic mRNA stability (19–21). The observation that mRNA constructs terminating in the histone stem–loop were both efficiently processed and localized to polysomes suggested that mRNAs containing the histone stem–loop were translationally competent (22). However, the role of the histone stem–loop as a facilitator of translational efficiency has not been directly addressed. In this study, we have shown that the histone stem–loop does enhance the translational efficiency of reporter mRNA in transfected CHO cells. It is functionally similar to a poly(A) tail in that it increases both the translational efficiency as well as the stability of the reporter mRNA and that it must be positioned at the 3′-terminus in order to function optimally. It is also similar to a poly(A) tail in that it is co-dependent on a cap in order to function as a facilitator of translation. It is interesting to note that although the cell cycle-regulated histone mRNAs have evolved as an alternative to polyadenylated mRNAs, they have nevertheless maintained a functional co-dependency between the terminal regulatory elements, i.e. between the cap and the histone stem–loop.

We have shown recently that some initiation factors (eIFs), such as eIF-4F and eIF-4B, bind not only to the cap structure but to poly(A) as well (47). If this bi-functional binding constitutes part of the basis for the co-dependency between a cap and poly(A) tail, how could this mechanism function in a cell cycle-regulated histone mRNA that has a cap but does not terminate in a poly(A) tail? A 45 kDa stem–loop binding protein (SLBP) associated with polysomes has been identified that specifically recognizes the histone stem–loop structure (23–25). It is possible that the SLBP may mediate the interaction between the termini, perhaps through protein–protein interactions with eIFs.

We observed the histone stem–loop-mediated enhancement of translation and mRNA stability in animal cells but not in plant cells, even though other 3′ regulatory elements, such as a poly(A) tail (Fig. 6) or the 3′-UTR of the non-polyadenylated tobacco mosaic virus RNA (46), do function. That the histone stem–loop does not function in higher plants is not surprising, as plant histone mRNAs are polyadenylated and therefore plant cells would not be expected to contain factors that specifically recognize the histone stem–loop structure. The observation that *Volvox* (33) and *Chlamydomonas* (32) histone mRNAs terminate in the conserved stem–loop structure does suggest that plant histone mRNAs may have been regulated by the stem–loop mechanism early in their evolution and that this mechanism was subsequently lost in the evolution of higher plants. The failure of the histone stem–loop to function in rapidly dividing plant cells demonstrates, as we have shown previously for other structured RNA sequences (34), that the introduction of structured sequences alone at the 3′-terminus of an mRNA is not sufficient to increase expression. Such observations provide additional support for the conclusion that the effect of the histone stem–loop on translation and mRNA stability is specific to this sequence in CHO cells.

The degree to which the histone stem–loop enhances translational efficiency and mRNA stability was individually quantitated and compared with a poly(A) tail. The addition of the histone stem–loop to reporter mRNA increased the translational efficiency of the reporter mRNA to the same extent as the addition of a poly(A) tail. Moreover, although both the histone stem–loop and a poly(A) tail increased mRNA stability, the extent to which the histone stem–loop increased mRNA half-life was less than that for a poly(A) tail. This may be a reflection of the changing length of histone mRNA half-life throughout the cell cycle, which varies from ~60 min during the G_1 and S phases to <15 min during the G_2 phase of the cell cycle (9,10). As the mRNA constructs were translationally active for only 5–6 h, at most, and the CHO cell cycle under our growth conditions is <20 h, the impact of the histone stem–loop on reporter mRNA half-life must be viewed as an average value for the entire cell cycle. If the histone stem–loop increases translational efficiency only during the S phase, represented by 30–40% of the cells at any given time, then our measurements would underestimate the stimulation of translation afforded by the histone stem–loop by 2- to 3-fold.

Based on several criteria, we conclude that the observed increase in translation efficiency was specific to the histone stem–loop sequence. First, expression from *luc*-SL_{WT} was higher than that from mutant and control constructs, although the length of the 3′-UTR was similar, ruling out an effect of 3′-UTR length on expression. Second, the presence of a tetraloop structure did not increase expression, suggesting that the machinery that recognizes and mediates the effect of the histone stem–loop is specific to this structure and not just a general tetraloop binding protein (43). In this regard, it may be more than fortuitous that the U1 stem–loop has a small but measurable impact on translation. Inspection of the U1 stem–loop (51) reveals some shared characteristics with the histone stem–loop. This includes a four base loop that contains uridines in the first and second positions and two GC base pairs at the base of the stem. These features may contribute to the low level of stimulation of translation and mRNA stability observed for reporter mRNA terminating in the U1 stem–loop. In contrast, the tetraloop structure neither contains the features present in the histone stem–loop nor does it enhance translation. Third, the wild-type histone stem–loop had no effect on expression in plant protoplasts, although addition of a poly(A) tail did substantially increase expression from reporter mRNA. The failure of the histone stem–loop structure to significantly increase expression *in vitro* is not altogether surprising. The increase in expression resulting from the addition of a poly(A) tail has been well documented *in vivo* in both animal and plant cells (3,28), however, its ability to function is greatly reduced *in vitro* (47). Moreover, the 3′-UTRs from several viral mRNAs which, like the cell cycle-regulated histone mRNAs, naturally lack a poly(A) tail and contain higher order structures that facilitate translational efficiency *in vivo* also failed to function *in vitro* (45,46).

Mutations within the histone stem–loop did reduce its function, although they did not completely abolish it. Their most dramatic impact was on the speed with which the mRNA was recruited for translation. Expression from the mutant constructs was reduced up to 38-fold early in translation when compared with the wild-type construct. Following this delay, the mutant mRNAs were translated with moderate efficiency. Similar or identical mutations within the histone stem–loop have been previously shown to substantially reduce 3′-end processing (23). The mRNA from those mutants that allowed a low but measurable level of 3′-end processing nevertheless localized to polysomes (18,23), results which are in good agreement with our observations that mutations within the stem–loop delay and reduce but do not completely abolish translational competence. It is not known whether SLBP mediates the translation of histone mRNA, but its association with polysomes is suggestive

of such a role. We recently demonstrated that SLBP associated with actively translating histone mRNA is the same form which is involved in histone 3′-end processing in the nucleus and which accompanies the mRNA to the cytoplasm (52). Therefore SLBP appears to remain associated with histone mRNA during all steps of its existence. The degree to which the binding affinity of SLBP for mutant histone stem–loop structures is reduced (23–25) correlates with the effect of the mutants on translational recruitment (Fig. 7B). Binding of SLBP to the histone stem–loop may be necessary to mediate the stimulatory effect of the stem–loop on translation. Those mRNAs without the histone stem–loop, therefore, would not bind SLBP and would not be efficiently recruited to polysomes for translation. One possible explanation for why the mutations cause a delay in translational recruitment but have a smaller impact on the efficiency of translation once the mRNA is actively being translated is that the histone stem–loop may be composed of multiple recognition motifs, e.g. the loop and the stem, the loss of either of which might reduce the binding affinity of histone SLBP. Of the three mutants tested, the wild-type stem was present in the *luc*-$SL_{A1,3}$ construct, whereas the wild-type loop remained present in the *luc*-$SL_{reverse\ stem}$ and *luc*-$SL_{reverse}$ constructs (the C in the first position of the loop is found in nematode histone mRNAs; 31). Once bound, however, histone SLBP may be stabilized through interaction with other proteins involved in translation. Further analysis of the translational strategy used by the cell cycle-regulated histone mRNAs will contribute to our understanding of these protein–RNA interactions *in vivo*.

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REFERENCES

1 Jackson,R.J. and Standart,N. (1990) *Cell*, **62**, 15–24.
2 Bernstein,P. and Ross,J. (1989) *Trends Biochem. Sci.*, **14**, 373–377.
3 Gallie,D.R. (1991) *Genes Dev.*, **5**, 2108–2116.
4 Hentschel,C.C. and Birnstiel,M.L. (1981) *Cell*, **25**, 301–313.
5 Marzluff,W.F. (1992) *Gene Expression*, **2**, 93–97.
6 Robbins,E. and Borun,T.W. (1967) *Proc. Natl. Acad. Sci. USA*, **58**, 1977–1983.
7 Graves,R.A. and Marzluff,W.F. (1984) *Mol. Cell. Biol.*, **4**, 351–357.
8 Heintz,N., Sive,H.L. and Roeder,R.G. (1983) *Mol. Cell. Biol.*, **3**, 539–550.
9 Sittman,D.B., Graves,R.A. and Marzluff,W.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1849–1853.
10 Harris,M.E., Bohni,R., Schneiderman,M.H., Ramamurthy,L., Schumperli,D. and Marzluff,W.F. (1991) *Mol. Cell. Biol.*, **11**, 2416–2424.
11 Bond,U.M., Yario,T.A. and Steitz,J.A. (1991) *Genes Dev.*, **5**, 1709–1722.
12 Melin,L., Soldati,D., Mital,R., Streit,A. and Schumperli,D. (1992) *EMBO J.*, **11**, 691–697.
13 Mowry,K.L., Oh,R. and Steitz,J.A. (1989) *Mol. Cell. Biol.*, **9**, 3105–3108.
14 Mowry,K.L. and Steitz,J.A. (1987) *Mol. Cell. Biol.*, **7**, 1663–1672.
15 Mowry,K.L. and Steitz,J.A. (1987) *Science*, **238**, 1682–1687.
16 Vasserot,A.P., Schaufele,F.J. and Birnstiel,M.L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4345–4349.
17 Eckner,R., Ellmeier,W. and Birnstiel,M.L. (1991) *EMBO J.*, **10**, 3513–3522.
18 Williams,A.S., Ingledue,T.C., Kay,B.K. and Marzluff,W.F. (1994) *Nucleic Acids Res.*, **22**, 4660–4666.
19 Capasso,O., Bleecker,G.C. and Heintz,N. (1987) *EMBO J.*, **6**, 1825–1831.
20 Levine,B.J., Chodchoy,N., Marzluff,W.F. and Skoultchi,A.I. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6189–6193.
21 Pandey,N.B. and Marzluff,W.F. (1987) *Mol. Cell. Biol.*, **7**, 4557–4559.
22 Sun,J., Pilch,D.R. and Marzluff,W.F. (1992) *Nucleic Acids Res.*, **20**, 6057–6066.
23 Pandey,N.B., Williams,A.S., Sun,J.-H., Brown,V.D., Bond,U. and Marzluff,W.F. (1994) *Mol. Cell. Biol.*, **14**, 1709–1720.
24 Pandey,N.B., Sun,J.-H. and Marzluff,W.F. (1991) *Nucleic Acids Res.*, **19**, 5653–5659.
25 Williams,A.S. and Marzluff,W.F. (1995) *Nucleic Acids Res.*, **23**, 654–662.
26 Yisraeli,J.K. and Melton,D.A. (1989) *Methods Enzymol.*, **180**, 42–50.
27 Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
28 Gallie,D.R., Lucas,W.J. and Walbot,V. (1989) *Plant Cell*, **1**, 301–311.
29 Gallie,D.R., Feder,J.N. and Walbot,V. (1992) In Gallagher,S.R. (ed.), *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression*. Academic Press, San Diego, CA, pp. 181–188.
30 Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
31 Roberts,S.B., Sanicola,M., Emmons,S.W. and Childs,G. (1987) *J. Mol. Biol.*, **196**, 27–38.
32 Fabry,S., Muller,K., Lindauer,A., Park,P.B., Cornelius,T. and Schmitt,R. (1995) *Curr. Genet.*, **28**, 333–345.
33 Muller,K., Lindauer,A., Bruderlein,M. and Schmitt,R. (1990) *Gene*, **93**, 167–175.
34 Gallie,D.R., Feder,J.N., Schimke,R.T. and Walbot,V. (1991) *Nucleic Acids. Res.*, **19**, 5031–5036.
35 Alterman,R.M., Sprecher,C., Graves,R., Marzluff,W.F. and Skoultchi,A.I. (1985) *Mol. Cell. Biol.*, **5**, 2316–2324.
36 Graves,R.A., Pandey,N.B., Chodchoy,N. and Marzluff,W.F. (1987) *Cell*, **48**, 615–626.
37 Ross,J., Kobs,G., Brewer,G. and Peltz,S.W. (1987) *J. Biol. Chem.*, **262**, 9374–9381.
38 Tanguay,R.T. and Gallie,D.R. (1996) *Mol. Cell. Biol.*, **16**, 146–156.
39 Eckner,R. and Birnstiel,M.L. (1992) *Nucleic Acids Res.*, **20**, 1023–1030.
40 Streit,A., Koning,T.W., Soldati,D., Melin,L. and Schumperli,D. (1993) *Nucleic Acids Res.*, **21**, 1569–1575.
41 Cheong,C., Varani,G. and Tinoco,I.,Jr (1990) *Nature*, **346**, 680–682.
42 Heus,H.A. and Pardi,A. (1991) *Science*, *253*, 191–194.
43 Zwieb,C. (1992) *Nucleic Acids Res.*, **20**, 4397–4400.
44 Steitz,J.A., Black,D.L., Gerke,V., Parker,K.A., Kramer,A., Frendewey,D. and Keller,W. (1988) In Birnstiel,M.L. (ed.), *Structure and Function of Small Ribonucleoprotein Particles*. Springer-Verlag, Berlin, Germany, pp. 115–154.
45 Gallie,D.R. and Kobayashi,M. (1994) *Gene*, **142**, 159–165.
46 Leathers,V., Tanguay,R., Kobayashi,M. and Gallie,D.R. (1993) *Mol. Cell. Biol.*, **13**, 5331–5347.
47 Gallie,D.R. and Tanguay,R. (1994) *J. Biol. Chem.*, **269**, 17166–17173.
48 Kepes,A. (1963) *Biochim. Biophys. Acta*, **76**, 293–309.
49 Pedersen,S., Reeh,S. and Friesen,J.D. (1978) *Mol. Gen. Genet.*, **166**, 329–336.
50 Stimac,E., Groppi,V.E.,Jr and Coffino,P. (1984) *Mol. Cell. Biol.*, **4**, 2082–2087.
51 Epstein,P., Reddy,R. and Busch,H. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1562–1566.
52 Dominski,Z., Sumerel,J., Hanson,R.J. and Marzluff,W.F. (1995) *RNA*, **1**, 915–923.