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Translational Efficiency Is Regulated by the Length of the 3′ Untranslated Region

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All polyadenylated mRNAs contain sequence of variable length between the coding region and the poly(A) tail. Little has been done to establish what role the length of the 3′ untranslated region (3′UTR) plays in posttranscriptional regulation. Using firefly luciferase (*luc*) reporter mRNA in transiently transfected Chinese hamster ovary (CHO) cells, we observed that the addition of a poly(A) tail increased expression 97-fold when the length of the 3′UTR was 19 bases but that its stimulatory effect was only 2.3-fold when the length of the 3′UTR was increased to 156 bases. The effect of the *luc* 3′UTR on poly(A) tail function was orientation independent, suggesting that its length and not its primary sequence was the important factor. Increasing the length of the 3′UTR increased expression from poly(A)$^-$ mRNA but had little effect on poly(A)$^+$ mRNA. To examine the effect of length on translational efficiency and mRNA stability, a 20-base sequence was introduced and reiterated downstream of the *luc* stop codon to generate a nested set of constructs in which the length of the 3′UTR increased from 4 to 104 bases. For poly(A)$^-$ reporter mRNA, translational efficiency in CHO cells increased 38-fold as the length of the 3′UTR increased from 4 to 104 bases. Increasing the length of the 3′UTR beyond 104 bases increased expression even further. Increasing the length of the 3′UTR also resulted in a 2.5-fold stabilization of the reporter mRNA. For poly(A)$^+$ mRNA, the translational efficiency and mRNA half-life increased only marginally as the length of the 3′UTR increased from 27 to 161 bases. However, positioning the poly(A) tail only 7 bases downstream of the stop codon resulted in a 39-fold reduction in the rate of translation relative to a construct with a 27-base 3′UTR, which may be a consequence of the poly(A) tail–poly(A)-binding protein complex functioning as a steric block to translocating ribosomes as they approached the termination codon. The optimal length of the 3′ noncoding region for maximal poly(A) tail-mediated stimulation of translation is approximately 27 bases. These data suggest that the length of the 3′UTR plays an important role in determining both the translational efficiency and the stability of an mRNA.

Cytoplasmic regulation of gene expression has received increased attention in recent years. The 3′ untranslated region (3′UTR) of an mRNA often plays an active role in determining translational efficiency or mRNA stability (reviewed in references 17, 18, 46, and 47). There are now a number of examples in which the 3′UTR specifically regulates expression: message stability is regulated by the 3′UTR of transferrin receptor mRNA (6, 30), granulocyte-monocyte colony-stimulating factor mRNA (41), and cell cycle-regulated histone mRNAs (34); translational efficiency is regulated by the 3′UTR of tobacco mosaic virus mRNA (12, 25), human cytokine mRNA (23), and 15-lipoxygenase mRNA (33). mRNAs in which the 3′UTR orchestrates developmental regulation include mouse protomine-1 mRNA (4), *Xenopus* c-*mos* and cyclin mRNAs (42), and *Caenorhabditis elegans tra-2* mRNA (15). mRNAs in which the 3′UTR is responsible for localization include *Drosophilia nanos* mRNA (13, 14), *Xenopus* vegetal mRNA (28), and chicken fibroblast β-actin mRNA (20).

Not only are specific regulatory elements present within the 3′UTR, but general regulatory elements, such as the poly(A) tail, are also associated. The poly(A) tail is an important regulator of expression that is common to virtually all mRNAs. Postulated to regulate message stability and translational efficiency, its precise role as a cytoplasmic regulatory element remains controversial. Initially, the poly(A) tail–poly(A)-binding (PAB) protein complex was thought to play a passive role by functioning as a steric block to 3′→5′ exoribonucleases. Recent evidence, however, suggests a more active role for the PAB protein-poly(A) tail complex in RNA metabolism (reviewed in reference 31). A PAB-dependent poly(A) RNase is responsible for poly(A) tail shortening in yeast cells (39). Paradoxically, the addition of a poly(A) tail increases mRNA half-life in higher eukaryotes (2, 5).

The role of the poly(A) tail as a regulator of translation is quantitatively greater than its role as a regulator of message stability (9). The poly(A) tail is functionally dependent on the 5′ cap structure (m^7GpppN) in order to stimulate translational efficiency both in vivo (9) and in vitro (32), observations suggesting communication between the termini of an mRNA. Recent evidence has implicated cap-associated initiation factors as candidates for mediating the interaction (11). The observation that exogenously added poly(A) repressed the translation of uncapped mRNAs to a greater extent than capped mRNAs in in vitro cell lysates (11, 32) suggests that exogenous poly(A) can sequester necessary translational components that also associate with the cap. The addition of the cap-associated eukaryotic initiation factors eIF-4F and eIF-4B could reverse the poly(A)-mediated repression of translation (11). Moreover, both eIF-4F and eIF-4B were found to specifically form complexes with poly(A) (11), supporting the idea that these initiation factors might associate with both the cap and poly(A) tail during translation.

Despite the interest in the roles of the 3′ noncoding region and poly(A) tail as regulators of translation, no study has examined whether the length of the 3′ noncoding region per se affects expression. We had observed previously that the addition of a $poly(A)_{50}$ tail had only a small stimulatory effect on the expression from luciferase (*luc*) mRNA when the reporter

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gene contained its native 131-base 3′UTR (10), whereas the stimulation provided by a poly(A)$_{50}$ tail was more than an order of magnitude greater when the 3′UTR was reduced to 19 bases (9). Either the sequence of *luc* 3′UTR or its length was responsible for modulating poly(A) tail function.

In this study, we examined the effect of the length of the 3′ noncoding region on the regulation of expression from poly(A)$^-$ and poly(A)$^+$ reporter mRNAs in transiently transfected Chinese hamster ovary (CHO) cells. Increasing the length of the 3′ noncoding region increased both the translational efficiency and the stability of poly(A)$^-$ mRNA. Twenty-seven bases was the optimal length of the 3′UTR for poly(A) tail-mediated enhancement of translation. For poly(A)$^+$ mRNA, increasing the length of the 3′UTR from 24 to 156 bases had only a small effect on expression. However, positioning a poly(A) tail immediately downstream of a stop codon either failed to stimulate translation in vivo or resulted in repression in vitro.

MATERIALS AND METHODS

Plasmid constructs. The pT7-*luc*-WT-3′UTR, pT7-*luc*-WT-3′UTR-A$_{50}$, and pT7-GUS constructs, in which the firefly *luc* or β-glucuronidase (GUS) coding region is under the control of the T7 promoter, have been described previously (10). The GUS-6b-3′UTR was produced by introducing a *Bam*HI site 1 base downstream from the GUS stop codon, by using site-directed mutagenesis (24), which allows the production of a GUS mRNA containing a 6-base 3′UTR. The second control GUS construct, GUS-64b-3′UTR, contains a 64-base 3′UTR (43 bases of the native GUS 3′UTR and 21 bases of polylinker) when linearized with *Bam*HI.

To remove the *luc* 3′UTR, an *Xba*I restriction site was introduced 8 bases downstream of the TAA stop codon. By taking advantage of a second *Xba*I site downstream of the *luc* 3′UTR, a 139-base fragment containing the *luc* 3′UTR could be excised to produce the *luc*-19b-3′UTR construct. When linearized with *Bam*HI, this luciferase construct produces an mRNA with a 19-base 3′UTR. The *Xba*I fragment containing the *luc* 3′UTR could be introduced in forward and reverse orientations in an *Xba*I site 9 bases downstream of the stop codon of the GUS-6b-3′UTR construct to produce GUS-WT$_{luc}$-3′UTR and GUS-WT$_{\text{reverse } luc}$-3′UTR, respectively. The introduction of two copies of the *luc* 3′UTR produced the GUS-WT$_{2\times\text{-}luc}$-3′UTR construct.

The *luc*-4b-3′UTR was produced by introducing a *Bgl*II site at the *luc* stop codon (TAA GATCT; TAA represents the *luc* stop codon, and the *Bgl*II site is underlined). When linearized with *Bgl*II, this luciferase construct produces an mRNA with only a 4-base 3′UTR. The *luc*-7b-3′UTR-A$_{50}$ construct was made by introducing the *Hin*dIII-*Bgl*II *luc* gene fragment from the *luc*-4b-3′UTR construct into the *Hin*dIII-*Bam*HI sites of the pT7-A$_{50}$ vector.

An oligonucleotide cassette that could be inserted, one copy at a time, in the *Bgl*II site of *luc*-4b-3′UTR was constructed. When the cassette was reiterated up to five times, a nested set of constructs in which the 3′UTR length increased in increments of 20 bases was generated, producing constructs *luc*-24b-3′UTR through *luc*-104b-3′UTR. Each construct was introduced as a *Hin*dIII-*Bgl*II fragment into the *Hin*dIII-*Bam*HI sites of the pT7-A$_{50}$ vector to produce the constructs *luc*-27b-3′UTR through *luc*-107b-3′UTR. Note that the polyadenylated mRNAs contain 3′UTRs that are 3 bases longer than their poly(A)$^-$ counterparts.

In vitro transcription. mRNAs were synthesized in vitro following linearization of the template plasmid immediately downstream of the poly(A) tract with the appropriate restriction enzyme. The concentration of each of the template DNAs was quantitated spectrophotometrically following linearization and brought to 0.5 mg/ml. In vitro transcription was carried out as described previously (48) in a mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 100 μg of bovine serum albumin per ml, 0.5 mM each ATP, CTP, and UTP, 160 μM GTP, 1 mM m^{7}GpppG, 10 mM dithiothreitol, 0.3 U of RNasin RNase inhibitor (Promega) per μl, and 0.5 U of T7 RNA polymerase (New England Biolabs) per μl. Under our transcription conditions, >95% of the mRNA is capped. The integrity and relative quantity of RNA were determined by formaldehyde-agarose gel electrophoresis as described previously (27).

Preparation and electroporation of CHO cells. CHO cells were grown to approximately 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, washed twice with phosphate-buffered saline (PBS), and used immediately. Cells (10^6) in 0.8 ml were mixed with 2 μg of test mRNA and electroporated in PBS (250-μF capacitance, 400 V). Following electroporation, the cells were incubated for 7 h in Ham's F-12 medium supplemented with 10% fetal calf serum before assaying. Capped *luc*-A$_{50}$ mRNA was translationally active for approximately 5 to 6 h in CHO cells before the mRNA was finally degraded. For endpoint analysis, cells were harvested by scraping after 6 to 8 h. For time course experiments, aliquots of cells were harvested at the indicated time intervals.

Assay for cytoplasmic polyadenylation. Radiolabeled *luc* RNAs were electroporated into CHO cells, aliquots were removed after 30 and 90 min, and total RNA was isolated by using guanidinium thiocyanate (7). Polyadenylated RNA was isolated from each sample by using the poly(A)Tract mRNA isolation system as instructed by the manufacturer (Promega). The amount of poly(A)$^+$ RNA in each sample was determined by scintillation counting.

Polysome association determination. Radiolabeled *luc* RNAs were electroporated into CHO cells, and aliquots were harvested after 20, 40, and 60 min. Cell pellets were resuspended in 200 μl of extraction buffer (200 mM Tris [pH 9.0], 400 mM KCl, 25 mM EGTA, 35 mM $MgCl_2$, 200 mM sucrose, 5 mM dithiothreitol, 100 μg of cycloheximide per ml, 50 μg of chloramphenicol per ml), and cytoplasmic extracts were prepared by using a Dounce homogenizer. The extract was centrifuged immediately at 12,000 × *g* for 3 min to remove nuclei and cellular debris. The supernatant was applied onto a 300-μl sucrose cushion (40 mM Tris [pH 9.0], 200 mM KCl, 5 mM EGTA, 30 mM $MgCl_2$, 1.75 M sucrose, 100 mM β-mercaptoethanol, 100 μg of cycloheximide per ml, 50 μg of chloramphenicol per ml) and centrifuged at 60,000 rpm at 4°C for 1 h. Polysome pellets were washed two times each with 100 μl of extraction buffer and then resuspended in water. The amount of *luc* mRNA present in the polysome pellet was determined by scintillation counting.

In vitro translation. A 100-ng aliquot of each luciferase mRNA was translated by using rabbit reticulocyte lysate as described by the manufacturer (Promega) except that all amino acids were unlabeled and used at 80 μM. The extent of translation was measured by luciferase activity.

Luciferase and GUS enzyme assays. Cells collected by centrifugation at 100 × *g* were sonicated for 5 s in 100 mM Tricine (pH 7.8)–2 mM dithiothreitol–2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid–10% glycerol–1% Triton X-100, and the cell debris was pelleted. Aliquots of the extract were added to 100 μl of luciferase assay buffer [20 mM Tricine (pH 7.8), 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, 500 μM ATP (Promega)], and the reaction was initiated with the injection of 100 μl of 0.5 mM luciferin in luciferase assay buffer. Photons were counted with a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). Each mRNA construct was assayed in duplicate, and the averages are reported. Protein concentration was determined by the method described by Bradford (3).

For GUS enzyme assays, cells were sonicated in 0.5 ml of GUS assay buffer (50 mM sodium phosphate [pH 7.9], 10 mM β-mercaptoethanol, 1 mM EDTA). After pelleting of cellular debris, 4-methylumbelliferyl-β-D-glucuronide was added to an aliquot to a final concentration of 1 mM. To aliquots that were incubated at 37°C for 10 to 60 min, sodium carbonate was added to 0.2 M to terminate the reaction. Fluorescence was measured by excitation at 365 nm and emission at 455 nm in a TKO 100 DNA Fluorometer (Hoefer Scientific Inc.). Specific activity is calculated as nanomoles of 4-methyllumbelliferone produced per minute per milligram of protein.

RESULTS

The luciferase 3′UTR modulates poly(A) tail function. To investigate the impact of the *luc* 3′UTR on poly(A) function, we needed to produce a set of poly(A)$^-$ and poly(A)$^+$ mRNA constructs in which the *luc* 3′UTR was present or absent. To delete the *luc* 3′UTR, an *Xba*I restriction site was engineered 8 bases downstream of the *luc* stop codon of the wild-type construct (*luc*-WT-3′UTR). A 139-base region that included the *luc* 3′UTR could then be deleted (see Materials and Methods), resulting in a *luc* mRNA that terminates in a 19-base 3′UTR (*luc*-19b-3′UTR). These two pT7-based constructs were then introduced into a second pT7-based vector that contained a poly(A)$_{50}$ tract that allowed the in vitro synthesis of transcripts with a uniform poly(A)$_{50}$ tail length. To measure the effect of the *luc* 3′UTR on expression, *luc*-WT-3′UTR and *luc*-19b-3′UTR mRNAs were synthesized in vitro in triplicate as poly(A)$^-$ and poly(A)$^+$ mRNAs and delivered to CHO cells by electroporation. The cells were then incubated for 7 h, time sufficient to allow translation and degradation of the introduced mRNAs. The resulting amount of luciferase protein produced was then measured and used to quantitate the extent of expression (Table 1). A second reporter mRNA, GUS, mRNA, was codelivered with each *luc* mRNA as an internal control. Expression from each of the *luc* mRNA contructs is also shown normalized to the GUS expression (Table 1).

The presence of the *luc* 3′UTR increased expression from

TABLE 1. Effect of the *luc* 3′UTR on the degree to which a poly(A) tail enhances expression

mRNA	Mean luciferase activity (light units/mg of protein) ± SD	Mean GUS activity (nmol/min/mg of protein) ± SD	Luciferase activity/ GUS activity	Expression from poly(A)$^+$ form/ expression from poly(A)$^-$ form
luc-WT-3′UTR	4,137,667 ± 1,124,621	4.35 ± 1.29	951,188	0.72
luc-WT-3′UTR-A_{50}	3,592,767 ± 568,613	5.24 ± 1.44	685,643	
luc-19b-3′UTR	86,896 ± 17,085	4.24 ± 1.33	18,799	71
luc-19b-3′UTR-A_{50}	8,729,311 ± 1,223,289	6.56 ± 1.59	1,330,688	

poly(A)$^-$ mRNA by 51-fold (compare *luc*-WT-3′UTR with *luc*-19b-3′UTR in Table 1). When the mRNA constructs were polyadenylated, expression from the construct containing the *luc* 3′UTR was not greater than when this 3′UTR was absent (compare *luc*-WT-3′UTR-A_{50} with *luc*-19b-3′UTR-A_{50}, in Table 1). When the impact of a poly(A) tail on expression was examined for each construct, the addition of a poly$(A)_{50}$ tail did not increase expression from the *luc*-WT-3′UTR constructs but increased expression from *luc*-19b-3′UTR mRNA 71-fold. These data demonstrate that the impact that a poly(A) tail has on expression is greatly reduced when the *luc* 3′UTR is present. Moreover, the *luc* 3′UTR affects expression by increasing expression from the poly(A)$^-$ form of the mRNA rather than by lowering expression from the poly(A)$^+$ form of the mRNA.

The effect of the *luc* 3′UTR on translational efficiency and message stability could be separately quantitated by monitoring the kinetics of *luc* mRNA translation in transiently transfected CHO cells. The rate of luciferase protein production was used as a measure of translational efficiency, and the length of time over which luciferase protein continued to accumulate was used to calculate message stability. Luciferase protein has a half-life of 14 h in CHO cells (10a). Following delivery of each mRNA construct, aliquots of cells were removed at time intervals and luciferase assays were performed. The kinetics of *luc* mRNA translation were determined by monitoring the appearance of protein as measured by enzyme activity plotted as a function of time (Fig. 1). Recruitment begins immediately following mRNA delivery, as there is detectable luciferase enzyme activity within 3 min following electroporation (data not shown). Once the mRNA has been loaded onto polysomes, translation proceeds at a rate (i.e., the slope of each curve) that is dictated by its translational efficiency and for a period of time that is determined by the stability of the mRNA. The eventual degradation of the mRNA results in a decreased rate of protein accumulation. Following degradation of the mRNA, further accumulation of luciferase protein ceases, represented by the plateau of each curve at the later time points in Fig. 1. Between the loading of the mRNA onto the polysomes and its eventual degradation, there is a phase of steady-state translation in which the rate of luciferase production (i.e., the maximum slope) is both maximal and constant. This represents the translational efficiency of each mRNA, which is quantitated separately from the stability of the mRNA. By comparing the rates for the various *luc* mRNA constructs, the impact that the *luc* 3′UTR had on translational efficiency could be determined.

The translational characteristics of three constructs were analyzed. In addition to the *luc*-WT-3′UTR and *luc*-19b-3′UTR constructs described above, we analyzed a third construct in which the putative polyadenylation signal (AAUA AUAAAA) present in the wild-type *luc* 3′UTR was changed to AAGAAGAAAA (*luc*-Mut_{AAGAAG}). This was done because cytoplasmic polyadenylation requires the polyadenylation sig-

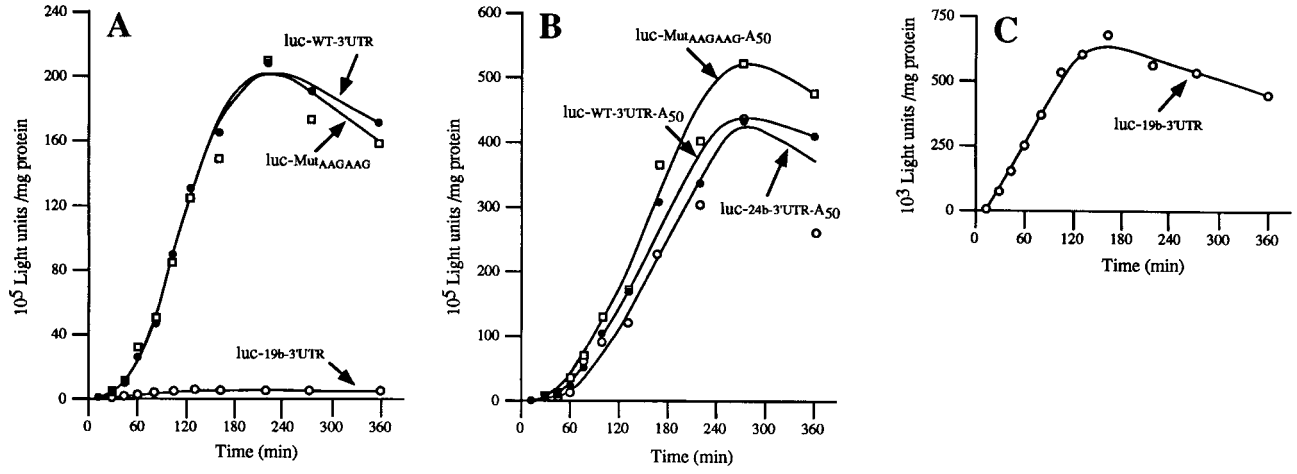

FIG. 1. The *luc* 3′ UTR increases translational efficiency and functional half-life of *luc* mRNA in CHO cells. Aliquots of CHO cells electroporated with *luc* mRNAs without (A) and with (B) a poly$(A)_{50}$ tail were taken at time intervals and assayed, and the luciferase activity was plotted as a function of time of incubation of the cells. (C) The curve representing the expression from *luc*-19b-3′UTR mRNA in panel A is redrawn with an expanded scale on the *y* axis to show clearly the kinetics of its translation. All mRNAs used were synthesized as capped mRNAs. The translational efficiency for each mRNA construct was measured from the maximum slope of each curve (see Table 2). 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 (see Table 2). The plateau of each curve is the maximum accumulation of expression following the degradation of the mRNA and represents the combined impact that the translational efficiency and stability of an mRNA have on expression.

TABLE 2. Translational efficiencies and mRNA stabilities of the mRNAs analyzed in Fig. 1

mRNA	Translational efficiency (light units/min/mg of protein)	Relative rate of translation	Functional mRNA half-life (min)	Maximum accumulation (light units/mg of protein)	Relative level of expression
Poly(A)$^-$					
luc-WT-3′UTR	125,000	21.2	122	20,400,000	29.1
luc-Mut$_{AAGAAG}$	119,000	20.2	120	21,400,000	30.6
luc-19b-3′UTR	5,900	1	78	700,000	1
Poly(A)$^+$					
luc-WT-3′UTR	225,000	1.05	146	43,200,000	0.99
luc-Mut$_{AAGAAG}$	267,000	1.25	156	53,200,000	1.21
luc-24b-3′UTR	214,000	1	158	43,800,000	1

nal and an upstream U-rich sequence (8, 26, 35), both of which are present in the *luc* 3′UTR sequence. If the *luc* 3′UTR can direct cytoplasmic polyadenylation, this might explain why expression from poly(A)$^-$ *luc* mRNA constructs containing its own 3′UTR was nearly as high as that from *luc*-WT-3′UTR-A$_{50}$ mRNA.

Following the delivery of *luc*-WT-3′UTR, *luc*-Mut$_{AAGAAG}$, and *luc*-19b-3′-UTR mRNAs, synthesized in vitro as capped mRNAs with and without a poly(A)$_{50}$ tail, aliquots of cells were taken at time intervals and assayed for luciferase activity. When the mRNAs were poly(A)$^-$, the rate of translation of *luc*-WT-3′UTR mRNA was 21.2-fold greater than that for *luc*-19b-3′UTR mRNA (Fig. 1A and Table 2). This difference is due specifically to differences in the translational efficiencies of the two mRNAs and does not include any differences in mRNA stability (see below). There was little difference in translational efficiency when the mRNAs were polyadenylated (Fig. 1B and Table 2). These data demonstrate that the presence of the *luc* 3′UTR increases the rate of translation of *luc* mRNA. The translational characteristics of the *luc*-Mut$_{AAGAAG}$ construct did not differ significantly from those of the *luc*-WT-3′UTR construct, data suggesting that changing the putative polyadenylation signal did not alter expression.

We could also measure the stability of each construct. Those forms of an mRNA that are more stable will be translationally active longer, represented in a kinetic analysis by a longer period of time over which the protein will continue to accumulate. From visual inspection of the data in Fig. 1, it is clear that the length of time over which the mRNAs are translationally active does not vary substantially and can not account for the 29-fold-greater amount of protein produced from *luc*-WT-3′UTR mRNA than from *luc*-19b-3′UTR mRNA (Table 2). We can quantitate the stability of each mRNA by measuring the functional mRNA half-life, which is a measure of the integrity of the message as determined by the length of time over which it is translationally active. Physical half-life measurements monitor the physical integrity of a message over time independent of its translational competence and do not distinguish between the pool of mRNA which is actively being translated and that which is not. As the functional half-life measures the stability of only that mRNA which is undergoing active translation, it more accurately describes the stability of message that is polysome associated than does physical half-life. The functional half-life is defined as the amount of time needed to complete a 50% decay in the capacity of an mRNA to synthesize protein (19, 36). For poly(A)$^-$ mRNA, the functional mRNA half-life of *luc*-WT-3′UTR was 1.6-fold longer than that of *luc*-19b-3′UTR (Fig. 1 and Table 2). The two parameters that contribute to the maximum yield of protein from an mRNA are the translational efficiency and the mRNA stability. The 29-fold difference in protein yield between the *luc*-WT-3′UTR and *luc*-19b-3′UTR mRNAs can be accounted for by the multiplication of the extent to which the *luc*-3′UTR increases translational efficiency (21.1-fold) with the extent to which it increases mRNA stability (1.6-fold). This 29-fold difference is less than the 71-fold difference in protein yield for these two mRNAs measured in Table 1. The kinetic analysis should be a more accurate reflection of the relative differences in expression, as there is less time for the luciferase protein produced from less stable *luc* mRNAs to be degraded.

Although the addition of a poly(A)$_{50}$ tail increased the half-life of each mRNA construct compared with its poly(A)$^-$ counterpart as had been observed previously (9), the half-life did not vary significantly among the poly(A)$^+$ mRNAs. These data suggest that, in addition to its effect on translational efficiency, the *luc* 3′UTR increases the stability of poly(A)$^-$ mRNA to a small extent, but the extent to which it does falls well short of the 29-fold difference in protein yield between the *luc*-WT-3′UTR and *luc*-19b-3′UTR mRNAs.

One explanation for the observed effect of the *luc* 3′UTR on the rate of expression in Fig. 1 is that the presence of the *luc* 3′UTR in an mRNA may cause a greater percentage of the introduced mRNA to be recruited onto polysomes. The greater the amount of *luc* RNA recruited onto polysomes, the higher the rate of luciferase protein production will be. If this were the case, the effect of the *luc* 3′UTR on expression would be on initial ribosomal recruitment rather than ribosomal reinitiation. To determine whether the *luc* 3′UTR has an effect on the extent to which an mRNA associates with polysomes, radiolabeled *luc* mRNA, with or without the *luc* 3′UTR, was synthesized in vitro as poly(A)$^+$ and poly(A)$^-$ mRNAs and delivered to CHO cells. Polysomes from cell extracts prepared at 20, 40, and 60 min following RNA delivery were isolated by pelleting through a sucrose cushion. The amount of radiolabeled *luc* mRNA present in the polysomal fraction was then quantitated by scintillation counting (Fig. 2). The presence of the *luc* 3′UTR did not increase the extent to which the *luc* mRNA associated with polysomes. This was also true for the poly(A) tail. These data suggest that neither the *luc* 3′UTR nor the poly(A) tail functions by increasing the total amount of mRNA associated with polysomes.

A second possible explanation for the observed effect of the *luc* 3′UTR on the translational efficiency of poly(A)$^-$ mRNA in Fig. 1 may be to direct cytoplasmic polyadenylation of the poly(A)$^-$ mRNA once it is delivered to cells. Cytoplasmic polyadenylation has been observed in developing oocytes of several species, and the 3′UTR of each of those mRNAs subject to this type of regulation is necessary to direct cytoplasmic polyadenylation (reviewed in references 18, 37, 45, and 47). We examined the polyadenylation status of in vitro-synthesized *luc*-WT-3′UTR-A$_{50}$, *luc*-WT-3′UTR, *luc*-Mut$_{AAGAAG}$, and *luc*-19b-3′UTR mRNAs by determining what percentage of

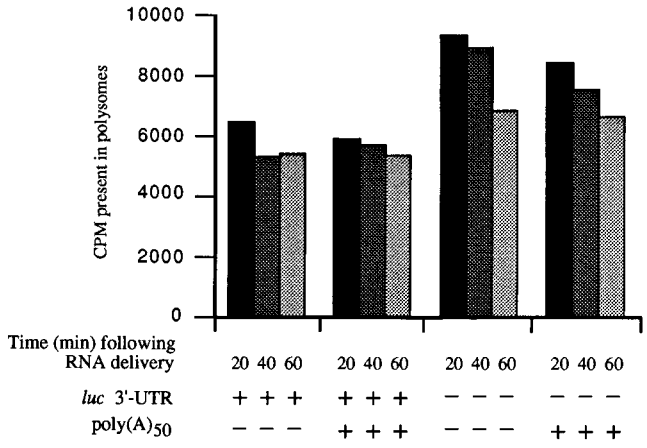

FIG. 2. The presence of the *luc* 3′UTR in an mRNA does not increase polysome loading. The amount of mRNA that is recruited onto polysomes following RNA delivery was determined for four mRNA constructs: *luc*-WT-3′UTR, *luc*-WT-3′UTR-A_{50}, *luc*-19b-3′UTR, and *luc*-19b-3′UTR-A_{50}. The mRNA constructs were synthesized in vitro as radiolabeled, capped RNAs and electroporated into CHO cells. The polysomal pellet was isolated from cells harvested at the indicated times by centrifugation through a sucrose cushion, and the *luc* mRNA was quantitated by scintillation counting.

introduced mRNA bound to oligo(dT) at 30 and 90 min after they were delivered to CHO cells. These times correspond to the period of active translation and little mRNA turnover which is illustrated in Fig. 1. Radiolabeled *luc* mRNAs were used in order to be able to quantitate the amount of RNA delivered to the cells and the percentage of that RNA that bound to oligo(dT). Thirty minutes after the introduction of *luc*-WT-3′UTR-A_{50} mRNA, 43% was recovered on oligo(dT) (Fig. 3). Similar results were obtained at 90 min following mRNA delivery. For the nonpolyadenylated form of this mRNA, i.e., *luc*-WT-3′UTR mRNA, only 0.6% of the introduced mRNA was recovered on oligo(dT) 30 min following delivery, with little change at 90 min, data suggesting that the nonpolyadenylated *luc*-WT-3′UTR did not undergo substantial polyadenylation in the cytoplasm of CHO cells. Equally low levels of mRNA were retained on oligo(dT) for the *luc*-Mut_{AAGAAG} and *luc*-19b-3′UTR mRNAs at both 30 and 90 min following mRNA delivery, data suggesting that the presence or absence of the *luc* 3′UTR in an mRNA had little effect on its polyadenylation status. These results demonstrate that the only mRNA to be substantially retained on oligo(dT) was an mRNA delivered as polyadenylated mRNA and that those mRNAs delivered as poly(A)$^-$ mRNAs remained so up to 90 min following introduction into the cells. The effect of the *luc* 3′UTR on translational efficiency during this same period, therefore, cannot be explained by cytoplasmic polyadenylation.

The effect of the *luc* 3′UTR on expression is gene and sequence independent. Addition of a poly(A)$_{50}$ tail to GUS mRNA increased its expression 22-fold in CHO cells, whereas little to no increase was observed for *luc* mRNA containing its wild-type 3′UTR (10) (Table 1). We wanted to examine whether the addition of the *luc* 3′UTR to GUS mRNA would alter the extent to which a poly(A)$_{50}$ tail could stimulate GUS expression and, if so, determine whether the effect was sequence specific or simply due to the length of the *luc* 3′UTR. The *luc* 3′UTR was introduced downstream of the GUS coding region to produce the construct GUS-WT_{luc}-3′UTR (Fig. 4). One control construct (GUS-6b-3′UTR) contained a 6-base 3′UTR, whereas a second GUS control construct, GUS-64b-3′UTR, contained a 64-base 3′UTR which was composed partly of the native GUS 3′UTR (43 bases) and partly of polylinker sequence (21 bases). The GUS construct with the 6-base 3′UTR was used to make the GUS-WT_{luc}-3′UTR construct.

When the mRNAs were poly(A)$^-$, the addition of the *luc* 3′UTR to the GUS mRNA increased protein expression 500-fold (compare GUS-WT_{luc}-3′UTR with GUS-6b-3′UTR in Fig. 4). Inversion of the *luc* 3′UTR (GUS-$WT_{reverse\ luc}$-3′UTR) resulted in a level of expression comparable to that of GUS-WT_{luc}-3′UTR, data suggesting that the effect of the *luc* 3′UTR may be a consequence of its length and not specific to its sequence. The 50-fold increase in expression resulting from increasing the length of the 3′UTR from 6 to 64 bases (compare GUS-64b-3′UTR with GUS-6b-3′UTR in Fig. 3) supports this possibility. Expression from a GUS construct containing two copies of the *luc* 3′UTR (GUS-$WT_{2\times\text{-}luc}$-3′UTR), which creates a 280-base 3′UTR, was similar to that from GUS-WT_{luc}-3′UTR.

For poly(A)$^+$ mRNAs, expression increased 19-fold when the 3′UTR was increased from 11 to 69 bases (compare GUS-69b-3′UTR-A_{50} with GUS-11b-3′UTR-A_{50}), whereas the levels of expression of GUS-69b-3′UTR-A_{50}, GUS-WT_{luc}-3′UTR-A_{50}, and GUS-$WT_{reverse luc}$-3′UTR-A_{50} were within 2-fold of each another. These data demonstrate that the introduction of the *luc* 3′UTR downstream of the GUS coding region converts the translational activity of this reporter mRNA from one which can be greatly stimulated by the addi-

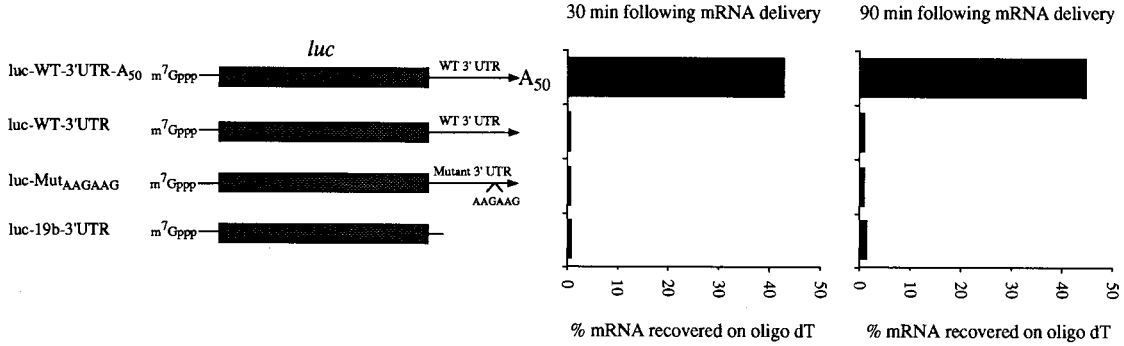

FIG. 3. The *luc* 3′UTR does not direct cytoplasmic polyadenylation in vivo. The polyadenylation status of each of four radiolabeled, capped constructs, *luc*-WT-3′UTR-A_{50}, *luc*-WT-3′UTR, *luc*-Mut_{AAGAAG}, and *luc*-19b-3′UTR mRNAs, was determined 30 and 90 min following RNA delivery into CHO cells. Total RNA was isolated from cells harvested at the times indicated and quantitated by scintillation counting. The amount of each mRNA construct present in the poly(A)$^+$ fraction was determined by measuring the percentage bound to oligo(dT).

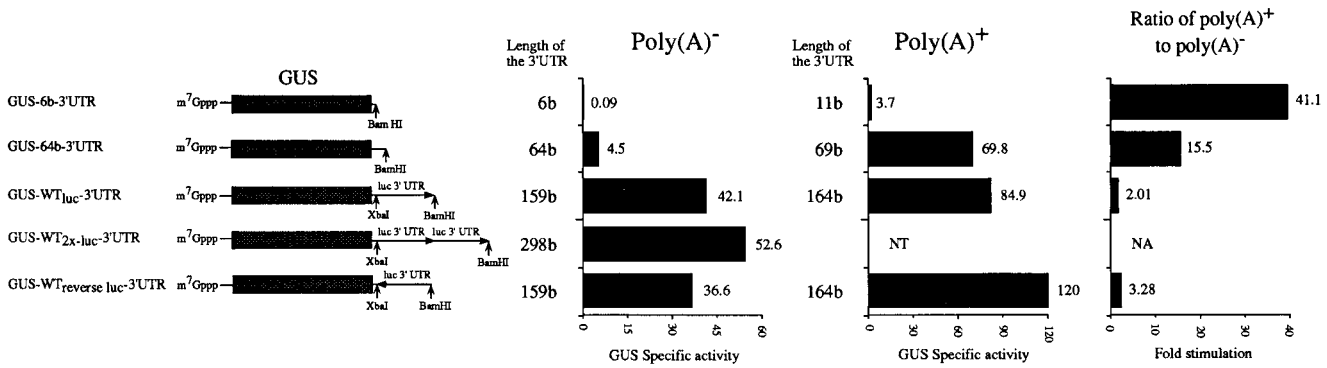

FIG. 4. The presence of the *luc* 3′UTR in GUS mRNA constructs reduces the extent to which a poly(A) tail enhances GUS expression. The GUS mRNA constructs indicated on the left were synthesized in vitro as capped, poly(A)$^-$, and poly(A)$^+$ mRNAs and electroporated into CHO cells. Restriction sites are indicated, and the direction of the arrow indicates the orientation of the inserted *luc* 3′UTR. Following incubation, the cells were assayed for GUS activity. The final level of expression for each construct is indicated to the right of each histogram. Each GUS assay was performed in triplicate. NT, not tested; NA, not applicable. b, bases.

tion of a poly(A)$_{50}$ tail to one that, like the wild-type *luc* construct, is only marginally stimulated by the addition of a poly(A)$_{50}$ tail. As with the *luc* mRNA constructs, the *luc* 3′UTR increased expression from the poly(A)$^-$ form of the GUS mRNA without greatly stimulating expression from the poly(A)$^+$ form of the mRNA. We conclude that the effect of the *luc* 3′UTR on expression is not specific to its primary sequence but that it was the length of the *luc* 3′UTR that reduced the stimulatory effect of a poly(A) tail on expression.

Increasing the length of the 3′ noncoding region increases expression. As the effect of the *luc* 3′UTR is both reporter gene independent and orientation independent, we hypothesized that the length of the 3′UTR, itself, was responsible for the increase in translational efficiency and stability seen for poly(A)$^-$ mRNA. To investigate this possibility, we made a nested set of *luc* mRNA constructs which contained a reiterated 20-base sequence such that the 3′UTR varied in length (from 4 to 104 bases) but not in sequence. Linearization of the longest of these constructs with a restriction enzyme (*Pvu*II) that cuts within the vector produced an even longer 3′UTR of 219 bases (Fig. 5). The *luc*-WT-3′UTR mRNA was included as a construct containing a 156-base 3′UTR.

Expression from these mRNA constructs was measured following mRNA delivery and incubation of the CHO cells. For poly(A)$^-$ mRNAs, increasing the length of the 3′UTR from 4 to 24 bases had little effect, but increasing the 3′UTR to 44 or 64 bases resulted in a 5- or 8.5-fold increase in expression, respectively (Fig. 5). Further increases in expression were observed for the longest 3′UTR tested. These data demonstrate that increasing the length of the 3′UTR dramatically improves expression when the mRNA is poly(A)$^-$.

Increasing the length of the 3′ noncoding region from 24 to 156 bases had little effect on expression from poly(A)$^+$ mRNAs, suggesting that the presence of the poly(A) tail largely overrides the length effect of the 3′ noncoding region. The exception to these observations was the poly(A)$^+$ construct with a 4-base 3′ noncoding region. The extent to which the poly(A)$_{50}$ tail stimulated expression could be measured as the ratio of expression from the poly(A)$^+$ form to expression from the poly(A)$^-$ form of each construct (Fig. 5). As the length of the 3′ noncoding region increased from 24 bases, the

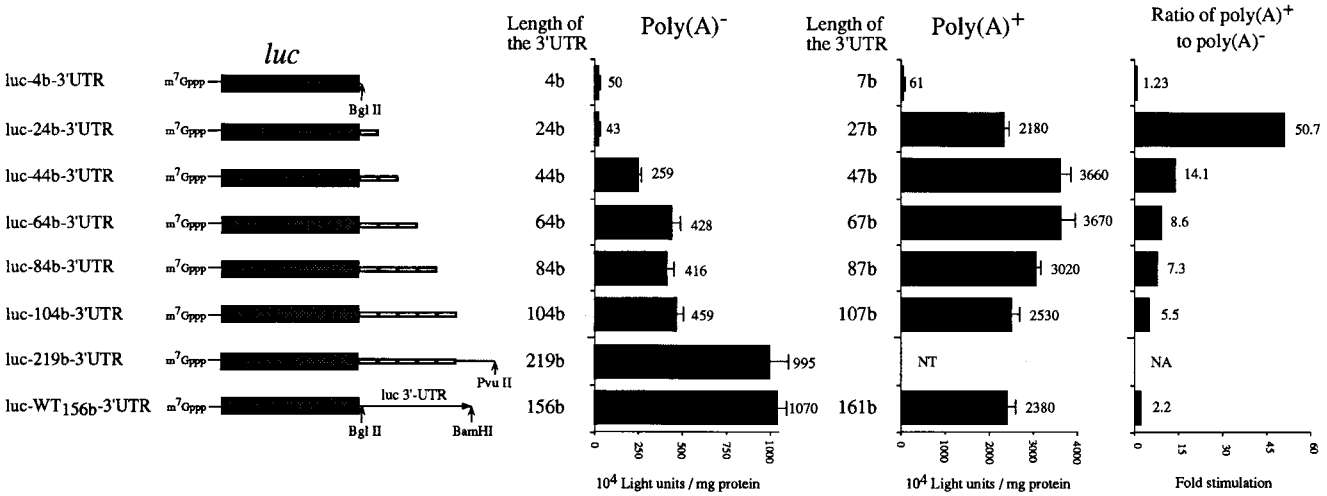

FIG. 5. Increasing the length of the 3′UTR increases luciferase expression in vivo. The *luc* mRNA constructs indicated on the left were synthesized in vitro as capped, poly(A)$^-$, and poly(A)$^+$ mRNAs and electroporated into CHO cells. Open boxes in the 3′UTR represent copies of the oligonucleotide cassette, and the corresponding length of each 3′ UTR is in bases [b]) indicated. Following incubation, the cells were assayed for luciferase activity. The final level of expression for each construct 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 1 standard deviation is shown for each histogram. NT, not tested; NA, not applicable.

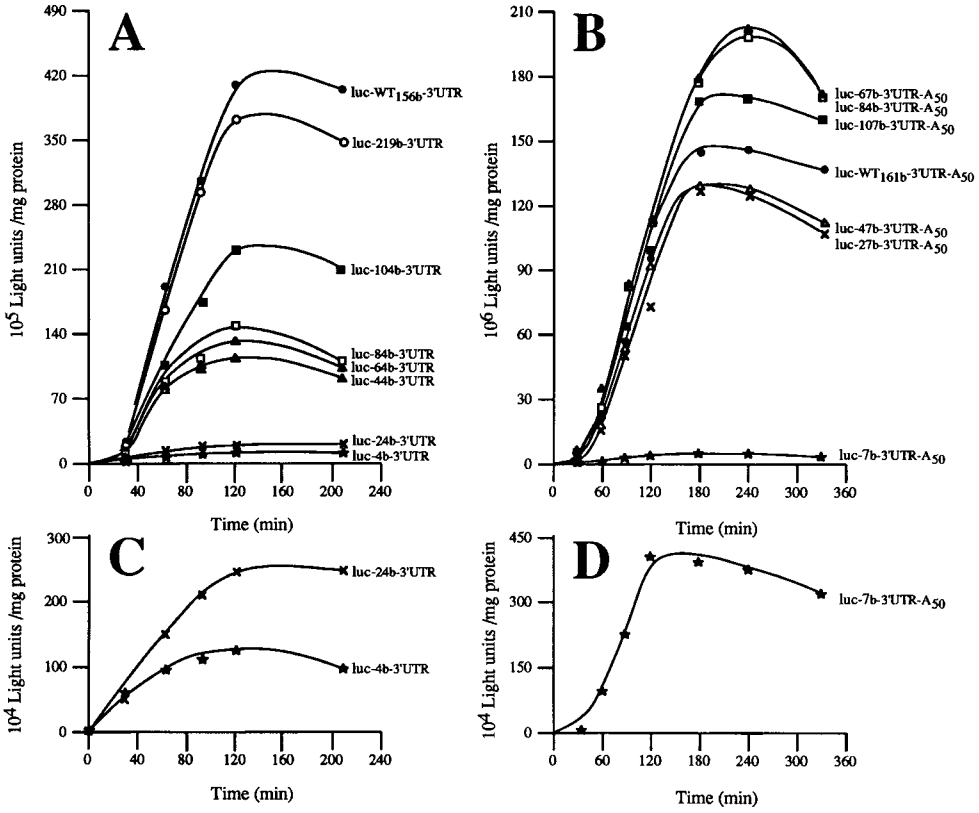

FIG. 6. Increasing the length of the 3′UTR increases the translational efficiencies and functional half-lives of *luc* mRNAs in CHO cells. Aliquots of CHO cells electroporated with capped luciferase mRNAs without (A) and with (B) a poly(A)$_{40}$ tail were taken at time intervals and assayed, and the luciferase activity was plotted as a function of time of incubation of the cells. In panels C and D, the curves representing expression from the poly(A)$^-$ *luc*-4b-3′UTR and *luc*-24b-3′UTR mRNAs from panel A (C) and the poly(A)$^+$ *luc*-7b-3′UTR mRNA in panel B (D) are redrawn with expanded scales on the *y* axis to show clearly the kinetics of their translation. Translational efficiencies and mRNA stabilities are quantitated in Table 3.

impact of the poly(A) tail on expression consistently decreased.

The functional half-life and translational efficiency for each construct could be measured separately by monitoring the kinetics of translation for each mRNA in vivo (Fig. 6 and Table 3). For poly(A)$^-$ mRNA constructs, there was a good correlation between translational efficiency and the length of the 3′UTR. For example, the translational efficiency of *luc*-104b-3′UTR (which is quantitated separately from the mRNA stability) was 37.8-fold greater than that from *luc*-4b-3′UTR (Table 3). Visual inspection of the length of time over which the mRNAs were translationally active demonstrates that although increasing the length of the 3′UTR increased the stability of the mRNA, the stabilization effect was small and fell far short of the 200-fold increase in protein yield from *luc*-WT$_{156b}$-3′UTR mRNA compared with that from *luc*-4b-3′UTR mRNA (Fig. 6). Functional half-life measurements confirm the visual inspection: mRNA half-life increased from 30 min for *luc*-4b-3′UTR mRNA up to 75 min for *luc*-WT$_{156b}$-3′UTR mRNA, only a 2.5-fold increase.

The translational efficiency and mRNA half-life did not vary substantially with the length of the 3′ noncoding region when the mRNAs were polyadenylated (Fig. 6B and Table 3). The exception was *luc*-7b-3′UTR-A$_{50}$, for which the translational efficiency was reduced 38.6-fold with respect to *luc*-27b-3′UTR-A$_{50}$ mRNA. Although positioning the poly(A) tail close to the stop codon affected translational efficiency, it did not significantly alter message stability.

By using the data from Table 3, the effects of the 3′UTR on translational efficiency and mRNA stability and the stimulatory effect of a poly(A)$_{50}$ tail could be plotted as a function of the length of the 3′UTR (Fig. 7). The translational efficiency of poly(A)$^-$ mRNA increased linearly with the length of the

TABLE 3. Translational efficiencies and mRNA stabilities of the mRNAs analyzed in Fig. 6

mRNA	Translational efficiency (light units/min/mg of protein)	Relative rate of translation	Functional mRNA half-life (min)	Relative functional half-life
Poly(A)$^-$				
luc-4b-3′UTR	6300	1	30	1
luc-24b-3′UTR	22,500	3.6	57	1.9
luc-44b-3′UTR	145,000	23.0	43	1.4
luc-64b-3′UTR	167,000	26.5	57	1.9
luc-84b-3′UTR	167,000	26.5	65	2.2
luc-104b-3′UTR	238,000	37.8	75	2.5
luc-219b-3′UTR	444,000	70.5	72	2.4
luc-WT$_{156b}$-3′UTR	491,000	77.9	75	2.5
Poly(A)$^+$				
luc-7b-3′UTR	28,000	1	80	1
luc-27b-3′UTR	1,080,000	38.6	106	1.3
luc-47b-3′UTR	1,430,000	51.1	115	1.4
luc-67b-3′UTR	1,440,000	51.4	109	1.4
luc-87b-3′UTR	1,280,000	45.7	111	1.4
luc-107b-3′UTR	1,040,000	37.1	96	1.2
luc-WT$_{161b}$-3′UTR	1,180,000	42.1	93	1.2

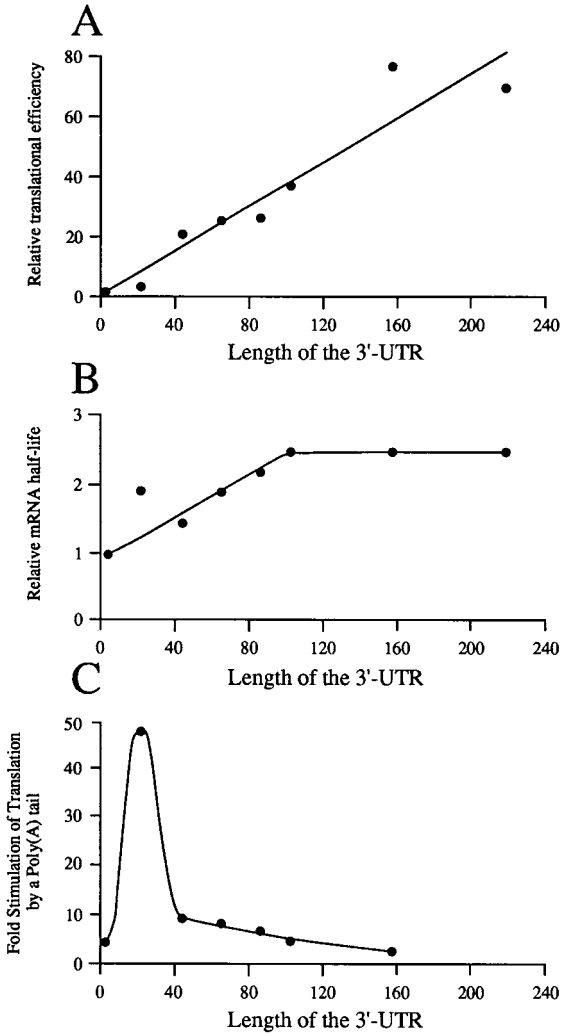

FIG. 7. Relative impact of increasing the length of the 3′UTR on translational efficiency (A), mRNA half-life (B), and the extent to which a poly(A)$_{50}$ tail increases the translational efficiency of *luc* mRNA in CHO cells (C). The data from Fig. 5 are plotted as a function of the length of the 3′UTR. The values in panels A and B are relative to the translational efficiency and mRNA half-life, respectively, of the *luc*-4b-3′UTR mRNA. In panel C, the fold stimulation of a poly(A)$_{50}$ tail is calculated as the ratio of the poly(A)$^+$ to poly(A)$^-$ form of each *luc* mRNA construct.

3′UTR (Fig. 7A). The half-life of poly(A)$^-$ mRNA also increased linearly with the length of the 3′UTR up to at least 104 bases, with little additional increase in half-life for longer 3′UTRs (Fig. 7B). The length of the 3′UTR that resulted in the greatest stimulation in the rate of translation upon addition of a poly(A)$_{50}$ tail was 27 bases (Fig. 7C). Expression from mRNA containing a 3′UTR that was either shorter or longer than this optimal length was stimulated markedly less by the addition of a poly(A) tail.

The same batch of *luc* mRNA constructs as used in the above-described kinetic analysis was translated in vitro in rabbit reticulocyte lysate to determine whether the effect of increasing the length of the 3′UTR on expression could be observed in vitro. For both the poly(A)$^-$ and poly(A)$^+$ constructs, the length of the 3′UTR did not have a substantial impact on translation in vitro with the exception of the *luc*-7b-3′UTR-A$_{50}$ mRNA (Fig. 8). As observed in vivo, translation from this poly(A)$^+$ construct was much less than that from the other poly(A)$^+$ constructs. Moreover, expression from the *luc*-7b-3′UTR-A$_{50}$ mRNA was actually eightfold less than that from the *luc*-4b-3′UTR mRNA. Interestingly, expression from the *luc*-4b-3′UTR was similar to that from the other poly(A)$^-$ constructs. This is in sharp contrast to the in vivo results in which expression from *luc*-4b-3′UTR (or *luc*-24b-3′UTR) was much less than that from constructs with longer 3′UTRs. Therefore, as a short 3′UTR does not impair translation from poly(A)$^-$ mRNA in vitro, it is probably not responsible for the poor level of translation when the construct contained a poly(A)$_{50}$ tail. More likely, it is the poly(A)$^+$ tail, positioned close to the stop codon, that impairs a step in translation such as termination.

The 3′UTR interacts synergistically with the cap to increase translational efficiency. The experiments described above suggest that increasing the length of the 3′UTR increases the efficiency of translation of poly(A)$^-$ mRNAs. Of the three steps that make up translation, i.e., initiation, elongation, and termination, it is more probable that the length of the 3′UTR on translation affects initiation and/or termination rather than elongation. That a 4-base 3′UTR does not reduce the in vitro translation from poly(A)$^-$ mRNA (Fig. 8) suggests that termination is not impaired by a short 3′UTR.

We have shown that the cap (m^{7}GpppN) and poly(A) tail cooperate to form the basis for efficient translation (9). 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 that of poly(A)$^-$ mRNA (9). Only when the transcript is capped does the addition of a poly(A) tail substantially stimulate translational efficiency. The synergy between the cap and poly(A) tail was greater than 10-fold. As a regulator of mRNA stability, the poly(A) tail does not functionally require the cap: reporter mRNA is stabilized by the addition of a poly(A) tail regardless of whether the mRNA is capped (9).

To determine whether the effect of a long 3′UTR on the rate of translation of poly(A)$^-$ mRNA is functionally dependent on the 5′ cap, *luc* mRNA with a short (*luc*-WT-19b-3′UTR) or long (*luc*-156b-3′UTR) 3′UTR was synthesized in vitro as capped or uncapped mRNA. A kinetic analysis of the expression from each mRNA was performed following mRNA delivery into CHO cells in order to measure separately the impact of the 3′UTR on the translational efficiency compared with the mRNA stability of capped and uncapped mRNAs (Fig. 9 and Table 4). Increasing the length of the 3′UTR from 19 to 156 bases increased translational efficiency only 7.3-fold for uncapped mRNA (compare uncapped-*luc*-WT$_{156b}$-3′UTR with uncapped *luc*-19b-3′UTR in Fig. 9A and Table 4) but 17-fold for capped mRNA (compare capped *luc*-WT$_{156b}$-3′UTR with capped *luc*-19b-3′UTR in Fig. 9B and Table 4). As the rate of translation and mRNA half-life can be separately measured in this assay, we can conclude that the synergy between the cap and the 3′UTR functions at the level of translational efficiency regardless of the changes in message stability. However, the extent of the synergy was only 2.3-fold (17 divided by 7.3 = 2.3-fold), which is substantially less than the 10-fold observed between a cap and poly(A) tail.

DISCUSSION

Since the discovery that eukaryotic mRNAs terminate in a poly(A) tail, there has been controversy concerning the extent to which a poly(A) tail enhances expression. Although there are probably several parameters that influence the effect that a poly(A) tail has on expression, the observations made in the

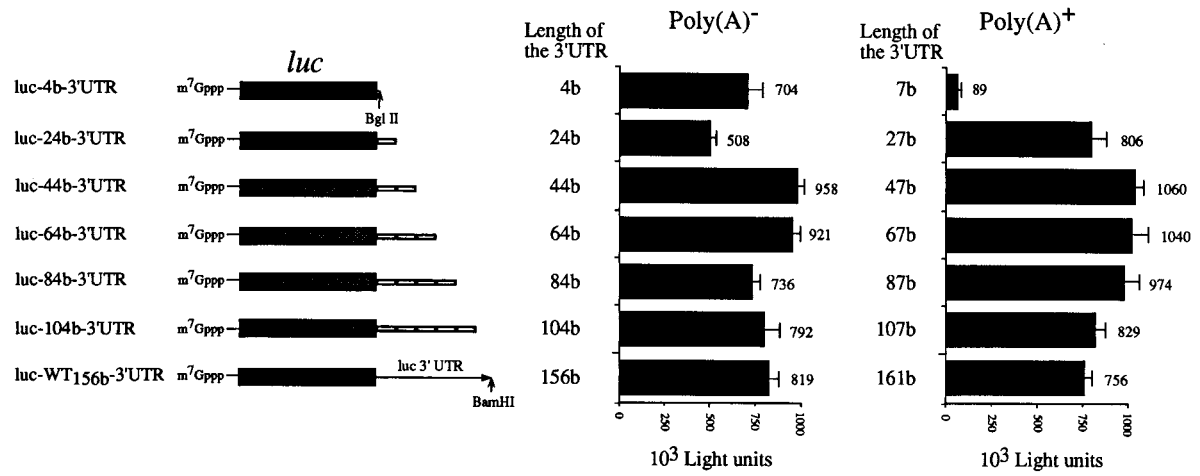

FIG. 8. Increasing the length of the 3′UTR does not increase luciferase expression in vitro. Rabbit reticulocyte lysate was programmed with 100 ng of each capped *luc* mRNA indicated at the left. Following incubation, the lysate was assayed for luciferase activity. Each mRNA was translated in vitro in triplicate. An error bar representing 1 standard deviation is shown for each histogram. b, bases.

present work suggest that the length of the 3′UTR is an important mRNA structural element that influences the effect of a poly(A) tail on expression. We have shown that expression from poly(A)$^-$ mRNA increases as a function of the length of its 3′UTR independent of its sequence but the length of the 3′UTR has a much smaller impact on expression from poly(A)$^+$ mRNA. Our conclusion that the effect is largely sequence independent is based on our observations of four 3′UTR sequences: the *luc* 3′UTR in forward and reverse orientations, the GUS 3′UTR, and the reiterated cassette of random sequence. The observation that the length of the 3′UTR was much less significant in the expression from poly(A)$^+$ mRNAs suggests that the 3′UTR length effect is largely overridden by the presence of a poly(A) tail. The effect of a longer 3′UTR on the stability of poly(A)$^-$ mRNA may be as simple as creating a buffer through which a putative 3′→5′ exoribonuclease involved in mRNA turnover would need to progress before reaching the coding region. The observation that the half-life increases in a linear relationship with the length of the 3′UTR from 4 to 104 bases supports this possibility.

Positioning the poly(A) tail close to the stop codon of reporter mRNA reduced its translational efficiency in vivo without substantially altering its half-life in comparison with the other poly(A)$^+$ constructs with longer 3′ noncoding regions. Positioning a poly(A) tail close to the stop codon actually reduced translation by eightfold in vitro compared with the poly(A)$^-$ form of the construct. These observations are most easily explained by the poly(A) tail-PAB complex forming a steric block to translocating ribosomes as they approach the stop codon. Ribosomes prevented from reaching the termination codon either may stall and thereby reduce luciferase expression or may release a truncated luciferase protein molecule. The loss of nine amino acids from the C-terminal end of luciferase is known to result in the complete loss of its activity (40). Packing density of PAB in yeast cells is one molecule per 25 A residues (38), and the ribosome protects approximately 30 to 35 nucleotides (reviewed in references 21 and 43). A

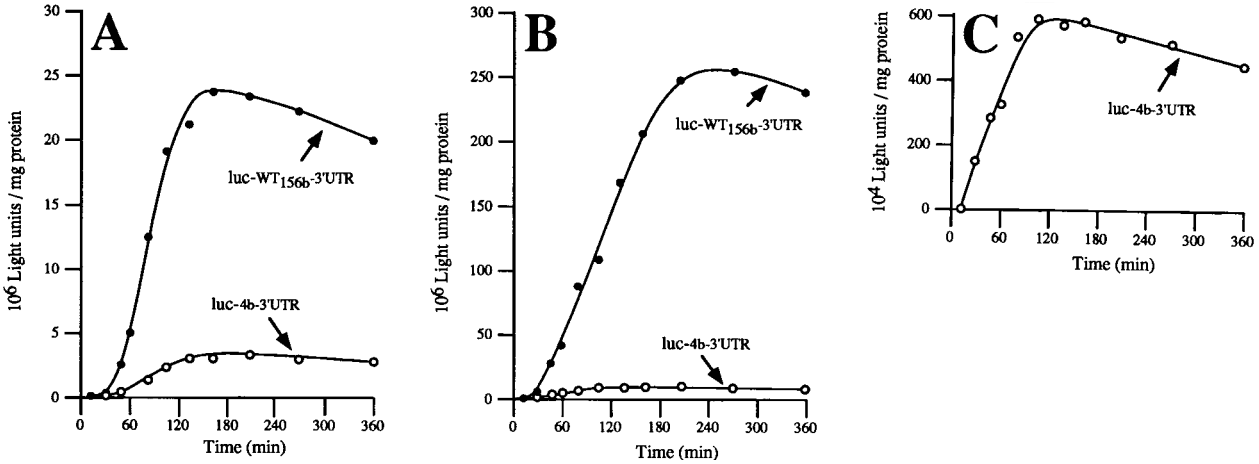

FIG. 9. Effect of the *luc* 3′UTR on cap function in CHO cells. Aliquots of CHO cells, electroporated with *luc* mRNAs synthesized as uncapped (A) or capped (B) RNAs, were taken at time intervals and assayed, and the luciferase activity was plotted as a function of time of incubation of the cells. (C) The curve representing the expression from capped *luc*-4b-3′UTR mRNA in panel B is redrawn with an expanded scale on the *y* axis to show clearly the kinetics of its translation. Translational efficiencies and mRNA stabilities are quantitated in Table 4.

TABLE 4. Translational efficiencies and mRNA stabilities of the mRNAs analyzed in Fig. 9

mRNA	Translational efficiency (light units/min/mg of protein)	Relative rate of translation	Functional mRNA half-life (min)	Maximum accumulation (light units/mg of protein)	Relative level of expression
Uncapped					
luc-4b-3′UTR	36,666	1	66	2,950,000	1
luc-WT_{156b}-3′UTR	266,667	7.3	82	22,000,000	7.5
Capped					
luc-4b-3′UTR	80,000	1	52	5,430,000	1
luc-WT_{156b}-3′UTR	1,388,889	17	114	230,000,000	42.4

ribosome at the stop codon would therefore cover approximately 20 bases downstream of the stop codon. Our observation that the poly(A) tail requires approximately 27 bases in order to stimulate translation agrees remarkably well with the predicted spacing needed for ribosomes to terminate translation efficiently.

In the absence of a poly(A) tail, does a short 3′UTR reduce termination efficiency? The observation that the in vitro expression was essentially the same between a construct with a 4-base 3′UTR and one with a 156-base 3′UTR suggests that a short 3′UTR does not reduce termination efficiency. Moreover, the observation that the rate of translation both in vivo and in vitro remained largely unaffected as the 3′ noncoding region increased from 27 to 161 bases in a poly(A)$^+$ mRNA suggests that the length of the 3′ noncoding region within these limits does not affect termination.

If, in the absence of poly(A) tail, the length of the 3′ noncoding does not affect termination, how might the 3′UTR affect the initiation step of translation? We observed that a longer 3′UTR had a greater effect on capped mRNA than on uncapped mRNA (Fig. 9 and Table 4). Similarly, the degree to which the addition of a cap stimulated the rate of translation was greater for mRNA with a longer 3′UTR. This synergy between the cap and the 156-base 3′UTR, however, was only 2.3-fold. We have previously demonstrated a synergy between a cap and a poly(A) tail of approximately 10-fold (9). One possible explanation for this difference is that a poly(A) tail has evolved to optimize the synergy with the cap. The reduced level of synergy between the cap and the 156-base 3′UTR nevertheless supports the possibility that the 3′UTR influences translation initiation. To explain how increasing the length of the 3′UTR can affect initiation, we propose a speculative model that is consistent with the observations presented in this study. Following termination, 40S subunits may remain associated with the mRNA and transit the 3′UTR. The continued association of a 40S subunit with the 3′UTR of a given mRNA molecule would raise the local concentration of ribosomes not actively engaged in translation for that mRNA and increase the likelihood that the 40S subunit would be re-recruited for translation. In the absence of secondary structure, the 40S subunit would transit the 3′UTR at a constant rate. Increasing the length of the 3′UTR would, therefore, increase the time over which the 40S subunit remains associated with a given mRNA molecule and consequently increase its chance for re-recruitment. What is the evidence for continued ribosomal association with an mRNA following termination? The length of the region between cistrons is critical in determining the efficiency of reinitiation (22). Reinitiation was poor with a short intercistronic region, but if the intercistronic region was at least 79 bases, translation from the downstream cistron was as efficient as if there were no upstream cistron at all. It was proposed that this minimum length was required for the 40S subunit to associate with factors required for reinitiation, such as initiator Met-tRNA (22). Recent work with the four small open reading frames present upstream in the yeast *GCN4* gene supports the idea that 40S subunits not only remain associated with the mRNA following termination but must scan a certain length of intercistronic sequence before regaining their competence for reinitiation (1, 16). *GCN4* is expressed only during conditions of cell starvation via a mechanism that requires reinitiation following the translation and termination of one of the upstream open reading frames (29, 44). The continued association of the 40S subunit with the mRNA following termination, therefore, has been used as the basis for the translational regulation of *GCN4* mRNA. Moreover, it is interesting that in both of these studies, the distance of the untranslated region is critical for ribosomes to regain their ability to participate in a subsequent initiation event.

If the 3′UTR serves as a transient reservoir of ribosomes (or 40S subunits) for re-recruitment, why does increasing the length of the 3′UTR have little impact on poly(A)$^+$ mRNAs? The high degree of functional codependence between the cap and poly(A) tail previously observed (9) suggests an interaction between these termini during translation. Recent evidence suggests that two eIFs known to bind to the cap structure, i.e., eIF-4F and eIF-4B, also bind to poly(A) and may form part of the basis for the interaction between the cap and poly(A) tail (11). A consequence of this model would be that the interaction maintains the physical proximity of the termini during translation and thereby increases even further the local concentration of ribosomes available for reinitiation. The poly(A) tail-mediated form of regulation might be an active mechanism in which a biochemically based interaction between the termini is used to promote reinitiation. In contrast, the increase in translation conferred by a long 3′UTR in a poly(A)$^-$ mRNA would be a passive mechanism in which those ribosomes (or 40S subunits) that transit the 3′UTR following termination are prevented from diffusing away from the mRNA and therefore are more likely to reinitiate on the same mRNA than a ribosome that has already dissociated from the mRNA. If the effect of a long 3′UTR serves to raise the local concentration of ribosomes, this model would predict that the effect of a long 3′UTR would be lost if the availability of ribosomes were no longer limiting. In other words, the extent to which the 3′UTR could raise the local concentration of ribosomes would be inconsequential in an environment in which the concentration of available ribosomes was already high. This is exactly the sort of conditions that prevail in an in vitro lysate in which endogenous mRNA levels are low and translation is relatively noncompetitive, e.g., less cap dependent than translation in vivo. This prediction of the model is borne out by the in vitro translation data (Fig. 8). An increase in the length of the 3′UTR did not result in an increase in translation in vitro. This observation supports the idea that the 3′UTR increases translation initiation by raising the local concentration of ribosomal machinery.

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REFERENCES

1. **Abastado, J.-P., P. F. Miller, B. M. Jackson, and A. G. Hinnebusch.** 1991. Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for *GCN4* translational control. Mol. Cell. Biol. **11:**486–496.
2. **Bernstein, P., and J. Ross.** 1989. Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem. Sci. **14:**373–377.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Braun, R. E., J. J. Peschon, R. R. Benringer, R. L. Brinster, and R. D. Palmiter.** 1990. Protamine 3′-untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. Genes Dev. **3:**793–802.
5. **Brawerman, G.** 1981. The role of poly(A) sequences in mammalian messenger RNA. Crit. Rev. Biochem. **10:**1–38.
6. **Casey, J. L., M. W. Hentze, D. M. Koeller, S. W. Caughman, T. A. Rouault, R. D. Klausner, and J. B. Harford.** 1988. Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. Science **240:** 924–928.
7. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
8. **Fox, C. A., and M. Wickens.** 1990. Poly(A) removal during oocyte maturation: a default reaction selectively prevented by specific sequences in the 3′UTR of certain maternal mRNAs. Genes Dev. **4:**2287–2298.
9. **Gallie, D. R.** 1991. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev. **5:**2108–2116.
10. **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.

10a. **Gallie, D. R., N. J. Lewis, and W. F. Marzloff.** Submitted for publication.

11. **Gallie, D. R., and R. Tanguay.** 1994. Poly(A) binds to initiation factors and increase cap-dependent translation *in vitro*. J. Biol. Chem. **269:**17166–17173.
12. **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.
13. **Gavis, E. R., and R. Lehmann.** 1992. Localization of *nanos* RNA controls embryonic polarity. Cell **71:**301–313.
14. **Gavis, E. R., and R. Lehmann.** 1994. Translational regulation of *nanos* by RNA localization. Nature (London) **369:**315–318.
15. **Goodwin, E. B., P. G. Okkema, T. C. Evans, and J. Kimble.** 1993. Translational regulation of *tra-2* by its 3′ untranslated region controls sexual identity in *C. elegans*. Cell **75:**329–339.
16. **Grant, C. M., P. F. Miller, and A. G. Hinnebusch.** 1994. Requirements for intercistronic distance and level of eukaryotic initiation factor 2 activity in reinitiation on *GCN4* mRNA vary with the downstream cistron. Mol. Cell. Biol. **14:**2616–2628.
17. **Jackson, R. J.** 1993. Cytoplasmic regulation of mRNA function: importance of the 3′ untranslated region. Cell **74:**9–14.
18. **Jackson, R. J., and N. Standart.** 1990. Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell **62:**15–24.
19. **Kepes, A.** 1963. Kinetics of induced enzyme synthesis determination of the mean life of galactosidase-specific messenger RNA. Biochim. Biophys. Acta **76:**293–309.
20. **Kislauskis, E. H., Z. Li, R. H. Singer, and K. L. Taneja.** 1993. Isoform-specific 3′-untranslated sequences sort α-cardiac and β-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. J. Cell Biol. **123:** 165–172.
21. **Kozak, M.** 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol. Rev. **47:**1–45.
22. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. Mol. Cell. Biol. **7:**3438–3445.
23. **Kruys, V., O. Marinx, G. Shaw, J. Deschamps, and G. Huez.** 1989. Translational blockade imposed by cytokine-derived UA-rich sequences Science **245:**852–854.
24. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
25. **Leathers, V., R. Tanguay, M. Kobayashi, and D. R. Gallie.** 1993. A phylogenetically conserved sequence within viral 3′ untranslated RNA pseudoknots regulates translation. Mol. Cell. Biol. **13:**5331–5347.
26. **McGrew, L. L., E. Dworkin-Rastl, M. B. Dworkin, and D. Richter.** 1989. Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. Genes Dev. **3:**803–815.
27. **Melton, D. A., P. A. Krieg, 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.
28. **Mowry, K. L., and D. A. Melton.** 1992. Vegetal messenger RNA localization directed by a 340-nt RNA sequence element in *Xenopus* oocytes. Science **255:**991–994.
29. **Mueller, P. P., and A. G. Hinnebusch.** 1986. Multiple upstream AUG codons mediate translational control of *GCN4*. Cell **45:**201–207.
30. **Mullner, E. W., and L. C. Kuhn.** 1988. A stem-loop in the 3′ untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. Cell **53:**815–825.
31. **Munroe, D., and A. Jacobson.** 1990. Tales of poly(A): a review. Gene **91:** 151–158.
32. **Munroe, D., and A. Jacobson.** 1990. mRNA poly(A) tail, a 3′ enhancer of translational initiation. Mol. Cell. Biol. **10:**3441–3455.
33. **Ostareck-Lederer, A., D. H. Ostareck, N. Standart, and B. J. Thiele.** 1994. Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3′ untranslated region. EMBO J. **13:**1476–1481.
34. **Pandey, N. B., and W. F. Marzluff.** 1987. The stem-loop structure at the 3′ end of histone mRNA is necessary and sufficient for regulation of histone mRNA stability. Mol. Cell. Biol. **7:**4557–4559.
35. **Paris, J., and J. D. Richter.** 1990. Maturation-specific polyadenylation and translational control: diversity of cytoplasmic polyadenylation elements, influence of poly(A) tail size, and formation of stable polyadenylation complexes. Mol. Cell. Biol. **10:**5634–5645.
36. **Pedersen, S., S. Reeh, and J. D. Friesen.** 1978. Functional mRNA half lives in *E. coli*. Mol. Gen. Genet. **166:**329–336.
37. **Richter, J. D.** 1993. Translational control in development: a perspective. Dev. Genet. **14:**407–411.
38. **Sachs, A. B., R. W. Davis, and R. D. Komberg.** 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.
39. **Sachs, A. B., and J. A. Deardorff.** 1992. Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast. Cell **70:**961–973.
40. **Sala-Newby, G. B., and A. K. Campbell.** 1994. Stepwise removal of the C-terminal 12 amino acids of firefly luciferase results in graded loss of activity. Biochim. Biophys. Acta **1206:**155–160.
41. **Shaw, G., and R. Kamen.** 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:**659–667.
42. **Sheets, M. D., C. A. Fox, T. Hunt, G. Vande Woude, and M. Wickens.** 1994. The 3′-untranslated regions of c-*mos* and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes Dev. **8:**926–938.
43. **Steitz, J. A.** 1980. RNA · RNA interactions during polypeptide chain initiation, p. 479–495. *In* G. Chambliss, G. R. Craven, J. Davies, K. Davies, L. Kahan, and M. Nomura (ed.), Ribosomes: structure, function and genetics. University Park Press, Baltimore.
44. **Tzamarias, D., D. Alexandraki, and G. Thireos.** 1986. Multiple *cis*-acting elements modulate the translational efficiency of *GCN4* mRNA in yeast. Proc. Natl. Acad. Sci. USA **83:**4849–4853.
45. **Wickens, M.** 1992. Forward, backward, how much, when: mechanisms of poly(A) addition and removal and their role in early development. Semin. Dev. Biol. **3:**399–412.
46. **Wilhelm, J. E., and R. D. Vale.** 1993. RNA on the move: the mRNA localization pathway. J. Cell Biol. **123:**269–274.
47. **Wormington, M.** 1994. Unmasking the role of the 3′ UTR in the cytoplasmic polyadenylation and translational regulation of maternal mRNAs. Bioessays **16:**533–535.
48. **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.