

Regulation of ribonucleotide reductase M2 expression by the upstream AUGs

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ABSTRACT

Ribonucleotide reductase catalyzes a rate-limiting reaction in DNA synthesis by converting ribonucleotides to deoxyribonucleotides. It consists of two subunits and the small one, M2 (or R2), plays an essential role in regulating the enzyme activity and its expression is finely controlled. Changes in the M2 level influence the dNTP pool and, thus, DNA synthesis and cell proliferation. M2 gene has two promoters which produce two major mRNAs with 5'-untranslated regions (5'-UTRs) of different lengths. In this study, we found that the M2 mRNAs with the short (63 nt) 5'-UTR can be translated with high efficiency whereas the mRNAs with the long (222 nt) one cannot. Examination of the long 5'-UTR revealed four upstream AUGs, which are in the same reading frame as the unique physiological translation initiation codon. Further analysis demonstrated that these upstream AUGs act as negative *cis* elements for initiation at the downstream translation initiation codon and their inhibitory effect on M2 translation is eIF4G dependent. Based on the findings of this study, we conclude that the expression of M2 is likely regulated by fine tuning the translation from the mRNA with a long 5'-UTR during viral infection and during the DNA replication phase of cell proliferation.

INTRODUCTION

Ribonucleotide reductase (RR) is an enzyme that catalyzes the reaction converting ribonucleotides to their corresponding deoxyribonucleotides, the precursor of DNA synthesis and DNA repair. This reaction is a rate-limiting step of the synthesis of dNTP and, thus, of DNA (1). Because DNA synthesis is an essential event of cell cycle progression, down-regulating the level or decreasing the activity of RR also reduces the

dNTP pool, and consequently decreases the DNA synthesis which in turn results in reduced cell growth (2,3).

Mammalian RR consists of two different subunits, M1 and M2 (or R1 and R2), which are both required for the RR activity. The expression levels of these two subunits are regulated differently during cell cycle progression. While the level of M1 appears to be constant throughout the cell cycle in proliferating cells (4,5), the level of M2 oscillates with cell cycle and peaks in S-phase (6,7). Thus, the level of the M2 subunit plays an essential role in regulating the active RR and, therefore, DNA synthesis and cell proliferation (6).

It has been found previously that over-expression of M2 increased the malignancy of H-ras transformed fibroblasts and enhanced the invasive potential of human cancer cells (8,9). Several anti-proliferation agents, such as hydroquinone, orotic acid and hydroxyurea, were reported to inhibit DNA synthesis by inhibiting the expression or activity of M2 and consequently reducing the dNTP pool (10–13). Down regulating the level of M2 with antisense oligonucleotides or DNA specific to M2 has been shown to cause the decrease in RR activity, cell proliferation, tumorigenicity and metastasis of a variety of human cancer cells (14,15). Clearly, the activity or the expression level of M2 subunit is closely related to the proliferation of cells and may be a malignancy determinant critically involved in mechanisms controlling malignancy progression. Thus, delineating the regulatory mechanism of M2 expression is very important for understanding the control of cell proliferation and cancer and for designing better cancer therapeutics by targeting M2.

The regulation of M2 expression occurs at both transcriptional and post-transcriptional levels. It has been reported that the mRNA level of M2 oscillates with cell cycle with being undetectable in G₀/G₁, rising at the G₁-S border and peaking in S phase, and finally declining in G₂-M (16–18). The promoter activity of M2 could be induced up to 10-fold by UV irradiation in a dose-dependent manner (19). However, in growth-arrested Caski cells treated with ionizing radiation, the protein level of M2 increased 17-fold without any change in the mRNA level (20), suggesting that the M2 expression is also controlled at the

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translational level (6). Recently, we also found that the *de novo* synthesis of M2 is decreased without any change in its mRNA level by mimosine, a plant non-protein amino acid, and that the eIF3 p170 may be a mediator of the mimosine effect (21,22).

It has been reported that the M2 gene has two promoters responsible for the production of two major transcripts with 5'-untranslated regions (5'-UTRs) of 63 and 222 nt, respectively (23,24), and it is unknown whether both mRNA species can be translated and how their translations are regulated. In this study, we investigated the translational regulation of these two M2 mRNA species. We found that the long 5'-UTR contains four AUGs that are in the same reading frame as the physiological translation initiation codon and these AUGs are not used as translation initiation sites to generate M2 with an extended N-terminus but instead they act as negative *cis*-regulatory elements for translation at the physiological initiation codon.

MATERIALS AND METHODS

Materials

Monoclonal antibody YL1/2 against RR M2 was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Plasmid pCMV2A is a kind gift from Dr Robert G. Korneluk (Apoptogen Inc, Ottawa, Ontario, Canada). Mimosine and monoclonal antibody against β -actin were purchased from Sigma (St Louis, MO). Soluble Trail and the luciferase assay system were purchased from BioMol Research Labs (Plymouth Meeting, PA) and Promega (Madison, WI) respectively. The enhanced chemiluminescence and [³⁵S]methionine were from Amersham (Arlington Heights, IL) and PerkinElmer (Boston, MA) respectively. The Sequi-Blot™ PVDF membrane and concentrated protein assay dye reagent were from BioRad (Hercules, CA). pCRM2 containing M2 cDNA was purchased from ATCC (Manassas, VA) and cell culture media and reagents, vector pCDNA3 were obtained from Invitrogen (Carlsbad, CA). All other reagents were of molecular biology grade and purchased from Sigma (St Louis, MO) or Fisher Scientific (Chicago, IL).

Anti M2 antibody preparation

The polyclonal antibodies against human M2, antiSM2 and antiLM2, were generated commercially by Alpha Diagnostic International Company (San Antonio, TX). Briefly, peptides with the N-terminal sequence of the putative long form of M2 (MGRVGGMAQPMGRAG) and an internal sequence of the short M2 (TDPQQQLQLSPLKGLSLVDKE) were synthesized chemically and conjugated to KLH. Rabbits were immunized with these conjugated peptides and sera were prepared and characterized against the peptides by ELISA.

Engineering of the M2 constructs

M2 has two major mRNA species with different lengths of 5'-UTRs. The long and short 5'-UTRs of M2 gene were cloned by PCR using genomic DNA as templates and the following primers: 5'-GCGCCTCGAGCGCGCCCCGCGGCCA-3' (long 5'-UTR, forward); 5'-GCGCCTCGAGCCCCGTGCACCTGTGCCA-3' (short 5'-UTR, forward); and 5'-CGTGTTCTCCTTGTCGACCAAGCTGA-3' (reverse). The M2 cDNA

constructs containing the different lengths of 5'-UTRs, pCRLM2 and pCRSM2, were generated by inserting the long and short 5'-UTRs into the sites of Sall and XhoI of pCRM2, respectively.

The reporter constructs were generated by subcloning the luciferase gene from pGL3 (Promega) directly into the EcoRI and BamHI sites of PCR II (Invitrogen) to create pCRL. The 5'-UTRs of M2 gene and the poly(A) tail from pSP64-Poly(A) vector (Promega) were released and cloned into the NcoI/XhoI and BamHI/HindIII sites of pCRL, respectively, resulting in the constructs pCRL-Luc-A30 and pCRS-Luc-A30 that contain the long and short M2 5'-UTRs, respectively. In these constructs, the luciferase gene with 5'-UTRs of M2 was under the control of T7 promoter and there is a poly(A) sequence at the 3' end of the coding sequence.

The mutants of reporter constructs were engineered using PCR as described previously (25). The primers used for deletion mutations are as follows: LM2D1: 5'-CGCTCGAGGG-AAGGGTCGGAGGCAUGGCA-3'; LM2D2: 5'-CGCTCGA-GCAGCAAUGGGGAAGGGCCGGGGCACCA-3'; LM2D3: 5'-CGCTCGAGAGGGCCGGGGCACCAAAGCCA-3'; LM2D4: 5'-GCCGCTCGAGGGAAGGGCCGGGAGCGCG-3'; LM2D5: 5'-GCCGCCGAGAAAGGCTGTGGAGTGAGG-GGT-3'; and LM2D6: 5'-GCCGCTCGAGAGTGAGGGGTC-GCCCGTGCACCCT-3'. The primers used for point mutations are as follows: 5'-CAGCCATTCGGAAGGGTTCGGAGGCA-UGGCA-3' (forward, uAUG1); 5'-TCCGAAUGGCTGCGC-CCTGCCCTACC-3' (reverse, uAUG1); 5'-GTCGGAGGC-TTCGCACAGCCAAUG-3' (forward, uAUG2); 5'-TGGC-TGTGCGAAGCCTCCGACCCTTCC-3' (reverse, uAUG2); 5'-CCATTCGGAAGGGCCGGGGCACCAAAG-3' (forward, uAUG3); 5'-CCTTCCGAAUGGCTGTGCGAAGCCTCCCT-3' (reverse, uAUG3); 5'-TTCGGAAGGGCCGGGAGCGCGCG-GCGC-3' (forward, uAUG4); and 5'-GGCCCTTCGAAU-GGCTTTGGTGCCCCG-3' (reverse, uAUG4). All constructs were confirmed by DNA sequencing.

Cell lines, treatment and transfection

HeLa cells were maintained in modified Eagle's medium supplemented with 10% fetal bovine serum and in humidified atmosphere of 5% CO₂ at 37°C. HeLa cells were seeded at 6 × 10⁵ cells in 100 mm dishes and grown for 3 days before treatment with 250 ng/ml Trail.

For transient transfection, 1.5 × 10⁶ cells were seeded into 100 mm cell culture dishes and grown for 24 h before transfection with 4–10 μg pCMV2A or pCDNA3 vector using Lipofectamine/Plus reagent (Invitrogen). Cells were harvested at 24–48 h after transfection and the cell lysates were prepared for further analysis. Transfection with *in vitro* RNA transcripts was performed 24 h after transfection with pCMV2A or pCDNA3 as described below.

In vitro transcription, translation and RNA transfection

In vitro transcription and translation were performed as described previously (25). Briefly, DNA templates of pCRLM2 and pCRSM2 were linearized with BamHI, and the transcripts with 5'-cap were synthesized using T7 RNA polymerase in the presence of 1 mM m⁷GpppG and purified using the Qiagen RNeasy mini kit. A 20–40 ng of the capped RNA transcripts were used to program cell-free translation

in rabbit reticulocyte lysate (RRL) in a final volume of 5 μ l containing 3.5 μ l of RRL and 3–4 μ Ci [35 S]methionine. The products of the translation were separated by SDS–PAGE for autoradiography analysis.

RNA transfection was performed using the cationic liposome-mediated method as described previously (25) using RNA transcripts containing both 5'-cap and 3'-poly(A) tail. Briefly, the reporter constructs were linearized with EcoRI and the transcripts with 5'-cap and 3'-poly(A) tail were synthesized using T7 RNA polymerase in the presence of 1 mM m⁷GpppG and purified using the Qiagen RNeasy mini kit. Approximately 3×10^5 HeLa cells/well were seeded into six-well plates on the day before transfection. Opti-MEM1 medium (1 ml) was mixed with 12.5 μ g of Lipofectin reagent and 5–10 μ g RNA transcripts. The liposome–RNA medium was immediately added to cells. Control RNA transcripts of β -galactosidase was co-transfected to monitor transfection efficiency. The cells were harvested at 8 h after the transfection and lysed for analysis of luciferase activity using the luciferase assay systems. The activity of β -galactosidase was measured as described previously (26).

Metabolic labeling

HeLa cells transfected with pCMV2A or vector were trypsinized and re-suspended in serum-free and methionine-free medium 30 h following transfection. A total of 4×10^5 cells/well were seeded in six-well plate and pulse-labeled for 1 h with 20 μ Ci/ml [35 S]methionine. The cells were washed for three times and precipitated with 10% TCA. The acid-insoluble material was collected on a filter by rapid filtration and the radioactivity was determined by scintillation counting.

For precipitation of pulse-labeled products, the cells were washed twice with phosphate-buffered saline (PBS) and once with DMEM lacking methionine followed by incubation for 2 h in the same medium supplemented with 75 μ Ci/ml [35 S]methionine. The pulse-labeled cells were then washed three times with PBS and harvested for cell lysate preparation and immunoprecipitation.

Sample preparation, western blot and immunoprecipitation

Sample preparation, western blot and immunoprecipitation analyses were performed as described previously (22). Briefly, cell lysates were prepared by lysis of cells with TNN-SDS buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 0.1% SDS and 2 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min followed by centrifugation (10 000 g for 10 min at 4°C) and protein concentration measurement using Bradford method (27). The cell lysates were separated by 8% SDS–PAGE and transferred to a PVDF membrane for western blot analysis with actin monoclonal antibody (1:3000 dilution), M2 monoclonal antibody YL1/2 (1:500 dilution), and M2 polyclonal antibodies antiLM2 (1:1000) and antiSM2 (1:1000). For immunoprecipitation, SDS and DTT were added to cell lysates of 200–500 μ g proteins to final concentrations of 0.5% and 10 mM, respectively. The samples were boiled for 15 min, diluted 10-fold with TNN buffer containing 2% BSA but without SDS and DTT, and then mixed with 30 μ l 50% protein

G-Sepharose 4B slurry. The mixture was incubated at 4°C for 1 h and centrifuged to remove Sepharose beads together with non-specifically bound proteins. To the supernatant, 10 μ l of antibody was added and incubated at 4°C for 3 h before mixing with 30 μ l 50% protein G-Sepharose 4B slurry. The mixture was further incubated overnight at 4°C with agitation. The precipitate was collected by centrifugation and washed six times with TNN-SDS buffer. The final pellet was solubilized in 15 μ l sample buffer for SDS–PAGE and autoradiography.

RNA purification and RNase protection assay

The cells were harvested and the total RNA was extracted using RNeasy Mini Kit (Qiagen). RNase protection assay was performed as previously described with 10–20 μ g total RNAs (22) by using RPA-III kit (Ambion, Austin, TX) according to the instructions of the supplier. The constitutively expressed human GAPDH gene obtained from Ambion (Austin, TX) was used as a control for RNase protection assay.

Real-time PCR analysis of RNA stability

Ten micrograms *in vitro* transcripts of reporter constructs containing 5'-cap and 3'-poly(A) tail were co-transfected into HeLa cells with 10 μ g of β -galactosidase transcripts as described above. Eight hours following transfection, total RNAs were extracted and the levels of luciferase RNAs and β -galactosidase RNAs were determined using real-time quantitative PCR analysis as described previously (28). Briefly, 2 μ g of total RNAs were reverse transcribed using AMV Reverse Transcriptase and Oligo(dT)_{12–18} primer (Invitrogen). The PCRs were carried out in ABI Prism@7000 Sequence Detection System (Applied Biosystems) using SYBR Green diction according to the manufacturer's instructions. The primers used for reporter luciferase RNAs are 5'-GCGAA-GGTTGTGGATCTGGAT-3' (forward) and 5'-CACACAC-AGTTCGCCTCTTTG-3' (reverse). The primers used for β -galactosidase RNAs are 5'-TGCTGCACGCGGAAGAA-3' (forward) and 5'-AGTCGTCGCCACCAATCC-3' (reverse). The threshold cycle (Ct) was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold reflecting a statistically significant point above the calculated baseline. The Ct of each target product was determined and normalized against that of co-transfected β -galactosidase control. The relative luciferase RNA level = $2^{\Delta Ct}$.

RESULTS

Effect of the 5'-UTR sequence on the translation of M2 RNA transcripts

It has been reported that the M2 gene possesses two promoters that are responsible for the production of two major transcripts with 5'-UTRs of 63 and 222 nt, respectively (23,24) (Figure 1A). Northern blot analysis also showed that two populations of M2 mRNA exist in HeLa cells (Figure 1B), consistent with the reported ones (23,24). Examining the sequence of the 222 nt 5'-UTR of the known M2 sequence showed that it has four extra AUG triplets, all in the same reading frame as the believed physiological translation initiation codon AUG (Figure 1A).

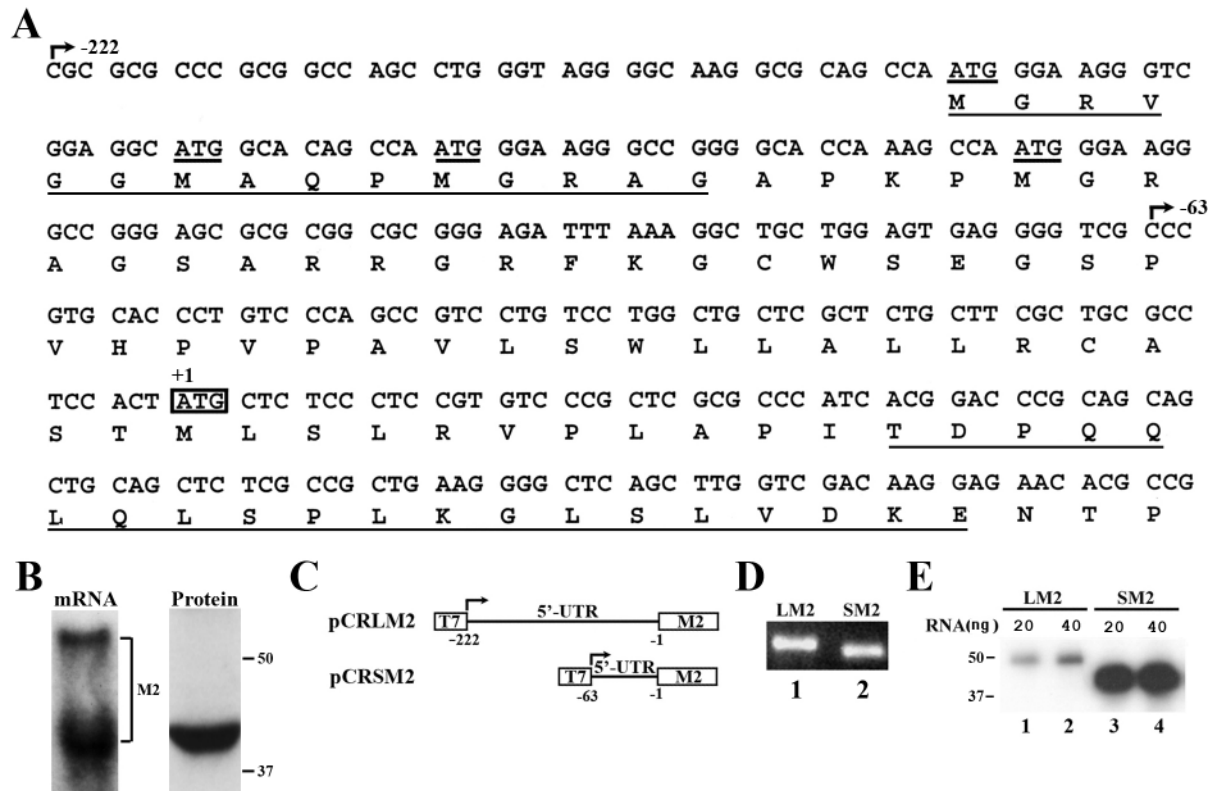


Figure 1. *In vitro* translation of M2 mRNA with different 5'-UTRs. (A) The DNA sequence encoding the 5'-UTR of M2 mRNA. The upstream AUG codons were underlined and the physiological start codon was boxed. The transcription start sites for mRNAs with both long and short 5'-UTRs were marked with arrows. The protein sequence encoded by the 5'-UTR is also shown with the underlined sequence used for antibody production. (B) Expression of endogenous M2 mRNA and protein in HeLa cells. Northern and western blot analyses were conducted to determine the mRNA and protein species, respectively. (C) Schematic representation of expression constructs with two different 5'-UTRs of M2 cDNA. T7 promoter and the M2 ORF are boxed. Arrows indicate the transcription start site. (D) RNA transcripts. The RNAs were generated from linearized M2 expression constructs by *in vitro* transcription using T7 RNA polymerase. (E) M2 protein generated by *in vitro* translation in RRL using the *in vitro* RNA transcripts as template shown in (D). The products were labeled with [³⁵S]methionine and detected as described in Materials and Methods. LM2, M2 with long 5'-UTR; SM2, M2 with short 5'-UTR.

To determine whether these upstream AUGs are used as translation initiation codons to produce a M2 protein with extended N-terminus, we first produced two antibodies against the predicted N-terminus of the putative long form of M2 (antiLM2) and an internal sequence of the short form of M2 (antiSM2) (see underlined amino acid sequence in Figure 1A). These antibodies were then used to probe a western blot of HeLa cell lysate. As shown in Figure 1B, only M2 protein of 43 kDa which is consistent with the product translated from the presumed physiological initiation site was detected by the antiSM2 antibody. No bigger protein between the size of 43 and 49 kDa (corresponding to the size of the longest form of M2) was detected by this antibody, suggesting that the longer forms of M2 with an extended N-terminus may not exist in HeLa cells. The use of antiLM2 antibody also did not detect any M2 protein of 49 kDa (data not shown), consistent with the results of antiSM2. These observations are also consistent with previous reports where no long form of M2 was found (23,24).

The finding that HeLa cells express a longer form of M2 mRNA, yet do not express the detectable level of a longer form of M2 protein, makes it of interest to determine whether the longer form of M2 mRNA is translationally competent. For this purpose, we engineered M2-expression constructs,

pCRLM2 and pCRSM2 (Figure 1C), containing the long and short 5'-UTR sequences, respectively, for *in vitro* translation analysis. *In vitro* transcripts with 5'-caps were generated from these constructs using T7 RNA polymerase at similar levels (Figure 1D) and were then used to program cell-free translation in RRL. As shown in Figure 1E, the RNA transcripts containing the short (63 nt) 5'-UTR (SM2) directed the abundant production of the expected M2 protein (lanes 3 and 4). The RNA with the long (222 nt) 5'-UTR (LM2) produced a larger protein, presumably due to the use of an upstream AUG as a translation initiation codon (lanes 1 and 2). However, the translation efficiency using this upstream codon is extremely low compared with the physiological AUG codon in the SM2 construct (Figure 1E, compare lanes 1 and 2 with lanes 3 and 4). It also appears that the physiological AUG codon in the longer construct LM2 was not used for translation initiation *in vitro* to generate the short form of M2 protein.

We next investigated the effect of the long 5'-UTR on translation of M2 *in vivo*. For this purpose, both the long and short 5'-UTR sequences of M2 were cloned at the upstream of the open reading frame (ORF) of luciferase reporter gene, resulting in constructs pCRL-Luc-A30 and pCRS-Luc-A30 (Figure 2A), respectively. *In vitro* transcripts with both 5'-caps and 5'-poly(A) tails were generated at similar levels

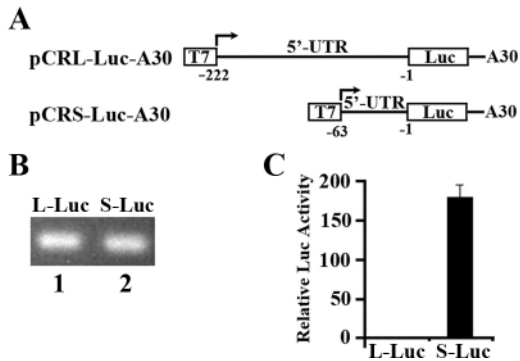


Figure 2. Translation of RNA transcripts with two different 5'-UTRs of M2 mRNA in HeLa cells. (A) Schematic representation of the reporter constructs containing the M2 5'-UTR. T7 promoter and the luciferase reporter gene are boxed. The poly(A) tail is shown as A30. Arrows indicate the transcription start site. (B) RNA transcripts of luciferase with different M2 5'-UTRs. The RNAs were generated from linearized reporter expression constructs by *in vitro* transcription using T7 RNA polymerase. (C) Translation of luciferase reporter with different M2 5'-UTRs. The translation efficiency of luciferase reporter was measured by the determination of the relative luciferase activity in HeLa cells following transfection of the RNA transcripts shown in (B) and normalization to the activity of the co-transfected β -galactosidase.

from these two constructs (Figure 2B) using T7 RNA polymerase and were then used to transfect HeLa cells. Eight hours following transfection with the RNA transcripts, cell lysates were prepared for luciferase assay. As shown in Figure 2C, the transcripts with the long 5'-UTR of M2 generated negligible luciferase activity, whereas the transcripts with the short 5'-UTR of M2 generated abundant luciferase activity. This observation is consistent with that shown in the *in vitro* studies (compare Figure 2C with Figure 1E). Together, the results from both the *in vitro* and *in vivo* analyses suggest that the long 5'-UTR of M2 mRNA inhibits the translation initiation from the physiological AUG initiation codon and the upstream in-frame AUGs are poor initiation sites for translation.

The upstream AUG triplets in the long 5'-UTR of M2 mRNA inhibits translation initiation at the physiological initiation codon

The results in Figures 1 and 2 suggest that not only the upstream AUGs are poor initiation sites for translation, but also the long 5'-UTRs with these AUGs inhibit translation initiation at the downstream physiological initiation codon. We thought that the inhibitory effect of the long 5'-UTR on translation initiation at the physiological AUG initiation codon is possibly due to its length and/or the secondary structure. To test this hypothesis and to localize the *cis* translation regulatory elements in the 5'-UTR of M2 mRNA, we performed deletion mapping analysis. The long 5'-UTRs of M2 were deleted at the cDNA level from the 5' end, and the various truncated 5'-UTRs were inserted in front of luciferase reporter, resulting in a set of deletion mutant constructs (Figure 3A). *In vitro* transcripts with 5'-caps and 3'-poly(A) tails were then generated from these constructs (Figure 3B) and were introduced into HeLa cells for translation efficiency analysis by measuring luciferase activity. As shown in Figure 3C, the luciferase expression increased with the 5'-deletions and

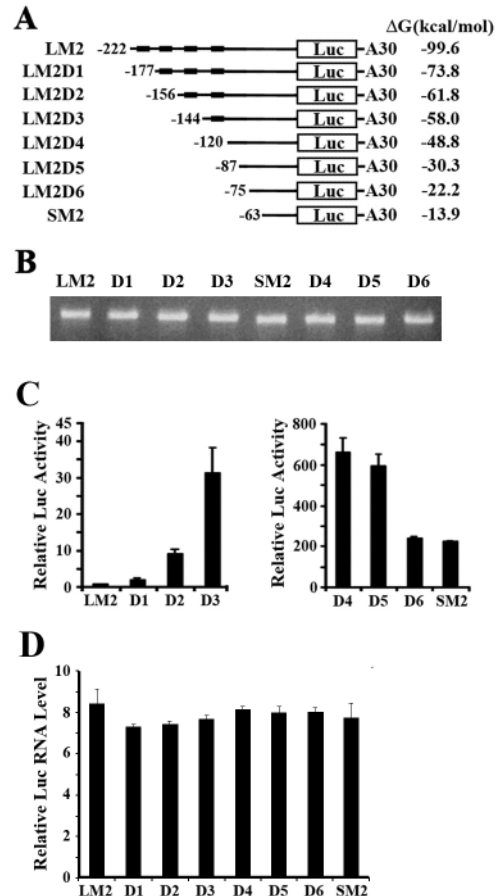


Figure 3. The long 5'-UTR of M2 contains negative *cis* regulatory elements. (A) Schematic representation of reporter constructs with wild-type and truncated M2 5'-UTRs. The solid and open boxes indicate the upstream AUGs and luciferase genes, respectively. A30 represents the poly(A) tail of the *in vitro* RNA transcripts generated from these constructs. The free energy of the 5'-UTR in each construct was predicted using the mfold 3.1 algorithm (<http://www.bioinfo.rpi.edu/~zukerm/mfa/>). (B) RNA transcripts of luciferase with different M2 5'-UTRs. The RNAs were generated from linearized reporter expression constructs by *in vitro* transcription using T7 RNA polymerase. (C) Translation of luciferase reporter with different M2 5'-UTRs. The translation of luciferase reporter was measured by the determination of the relative luciferase activity in HeLa cells following transfection of the RNA transcripts shown in (B) and normalization to the activity of the co-transfected β -galactosidase. (D) Real-time PCR analysis of RNA levels of luciferase reporter with different M2 5'-UTRs. *In vitro* transcripts of luciferase reporters with different M2 5'-UTRs were co-transfected into HeLa cells with transcripts of β -galactosidase. Total RNAs were then isolated 8 h following transfection for real-time PCR analysis and the relative levels of luciferase RNAs were determined as described in Materials and Methods.

reached maximum with a deletion of the first 100 nt in the construct LM2D4. The luciferase expression then decreased with further deletions and reached plateau with deletion construct of LM2D6, which has a 5'-UTR of only 75 nt. These observations suggest that the long 5'-UTR of M2 contains translational regulatory elements and the inhibitory effect is not simply due to the length of the 5'-UTR. Likely, the 5' region (-222 to -120) contains an inhibitory element whereas the middle region (-120 to -63) contains an enhancer element for translation of the luciferase reporter gene. Alternatively, the deletion may have changed the secondary structure of the 5'-UTR and, thus, affects the translation

efficiency. Analysis of the secondary structures of the 5'-UTRs of the deletion mutants showed that the free energy of these constructs decreased with the deletion (Figure 3A), which suggests that with the decreased complexity in secondary structure the translation efficiency should increase. Yet, we observed initially an increase and later a decrease in translation efficiency. Hence, it is unlikely that the change in translation efficiency is due to the simple change in secondary structures of the 5'-UTR sequence.

To rule out the possibility that the effect of deletion of the 5'-UTRs on the luciferase activity is due to their effect on the stability of these transcripts, we conducted an experiment to quantify the remaining transcripts of all constructs in the cells 8 h following transfection. As shown in Figure 3D, the remaining quantity of all transcripts with deletion appears to be similar to that of the wild-type sequence (LM2). Hence, it is unlikely that the deletion of the 5'-UTR changed the stability of the transcripts which then affected the luciferase activity level.

However, it is interesting to note that the inhibitory element in the 5' region (-222 to -120) possesses all the four upstream AUG triplets. It is possible that these AUG triplets function as *cis* elements to inhibit translation initiation at the downstream physiological AUG initiation codon. To test this possibility, we engineered reporter constructs with point mutations to eliminate the AUG codons by mutating the AUGs to UUCs while maintaining the length of the 5'-UTRs (Figure 4A). Again, *in vitro* transcripts with 5'-caps and 3'-poly(A) tails were generated from these constructs (Figure 4B) and the same amount of RNA transcripts were introduced into HeLa cells for translation efficiency analysis by measuring the luciferase activity. As shown in Figure 4C, the luciferase expression increased with the mutation of the upstream AUG triplets and reached maximum (similar to the shorter 5'-UTR, SM2) with the mutation of all four upstream AUG triplets (LM2U4). Hence, the upstream AUG triplets in the 5'-UTR sequence of M2 mRNA likely act as inhibitors of the translation initiation at the physiological initiation codon of the long M2 mRNA. Interestingly, all AUG mutant RNA transcripts have similar complexity of secondary structures at their 5'-UTRs (Figure 4A, similar ΔG) and, yet, their translation efficiencies are very different, supporting our argument that the secondary structure at the 5'-UTR and its length is not responsible for the inhibition of the translation efficiency. We also tested the stability of the transcripts with mutated AUGs using real-time PCR as described above. As shown in Figure 4D, the remaining quantity of all transcripts with point mutations appears to be similar to that of the wild-type sequence (LM2) 8 h following transfection. Hence, the point mutations of upstream AUGs did not change the stability of the transcripts which then affected the luciferase activity level.

The inhibition of translation by upstream AUG triplets requires intact eIF4G

It has been reported that AUG triplets in the 5'-UTR of mRNAs could act as translation regulator by inhibiting the cap-dependent translation at the physiological AUG initiation codon (29). It has also been shown previously that poliovirus 2A^{pro} cleaves both forms of translation initiation factor eIF4G,

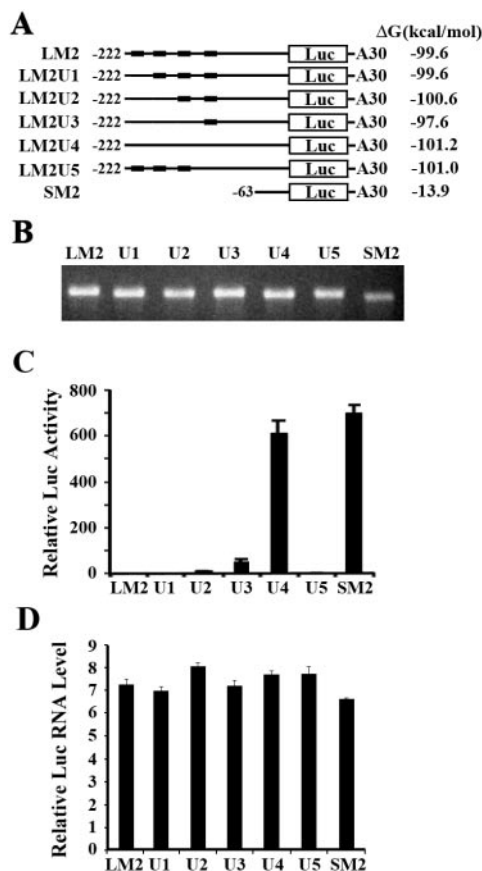


Figure 4. The upstream AUG triplets act as negative *cis* regulatory elements. (A) Schematic representation of reporter constructs with wild-type and mutant M2 5'-UTRs. The solid and open boxes indicate the upstream AUGs and luciferase genes, respectively. A30 represents the poly(A) tail of the *in vitro* RNA transcripts generated from these constructs. The free energy of the 5'-UTR in each construct was predicted using the mfold 3.1 algorithm (<http://www.bioinfo.rpi.edu/~zukerm/rna/>). In the mutants, the upstream AUGs were mutated to UUCs. (B) RNA transcripts of luciferase with different M2 5'-UTRs. The RNAs were generated from linearized reporter expression constructs by *in vitro* transcription using T7 RNA polymerase. (C) Translation of luciferase reporter with different M2 5'-UTRs. The translation of luciferase reporter was measured by the determination of the relative luciferase activity in HeLa cells following transfection of the RNA transcripts shown in (B) and normalization to the activity of the co-transfected β -galactosidase. (D) Real-time PCR analysis of RNA levels of luciferase reporter with different M2 5'-UTRs. *In vitro* transcripts of luciferase reporters with different M2 5'-UTRs were co-transfected into HeLa cells with transcripts of β -galactosidase and then real-time PCR analysis was performed as described in Figure 3 legend.

causing extensive inhibition of cap-dependent mRNA translation (30,31). To determine whether the upstream AUG triplets in the long 5'-UTR sequence of the M2 mRNA may inhibit the cap-dependent translation initiation at the physiological AUG codon, we analyzed the expression profile of various reporter constructs following inhibition of cap-dependent translation by transiently expressing poliovirus 2A^{pro} in HeLa cells. A mammalian expression vector that carries the poliovirus 2A^{pro} gene was transiently transfected into HeLa cells, and the global protein synthesis was monitored by measuring [³⁵S]methionine incorporation. As shown in Figure 5A, the [³⁵S]methionine incorporation decreased about half by expressing poliovirus 2A^{pro}, suggesting that the global protein synthesis was inhibited by 2A^{pro} cleavage

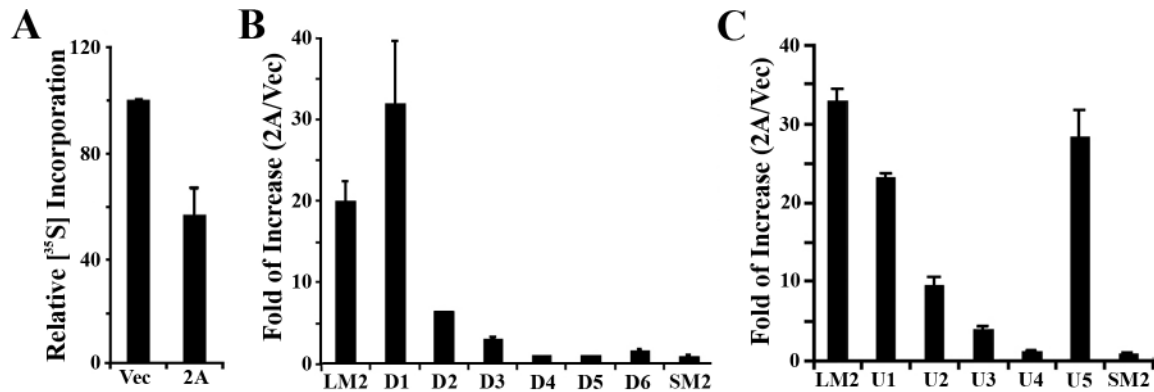


Figure 5. Effect of poliovirus protease 2A on the translation of luciferase reporter transcripts with different M2 5'-UTRs. (A) Effect of poliovirus protease 2A ($2A^{pro}$) on global translation. The effect of $2A^{pro}$ on global protein synthesis was determined by measuring total incorporation of [35 S]methionine following transfection of HeLa cells with $2A^{pro}$ as described in Materials and Methods. (B) Effect of poliovirus $2A^{pro}$ on the translation of luciferase reporter with wild-type and truncated M2 5'-UTRs. The translation of luciferase reporter was measured by the determination of the luciferase activity in HeLa cells by first transfecting the cells with $2A^{pro}$ followed by transfection with the RNA transcripts containing the wild-type and truncated M2 5'-UTRs as described in Materials and Methods. The relative luciferase activity was calculated by normalizing to the activity of the co-transfected β -galactosidase. (C) Effect of poliovirus $2A^{pro}$ on translation of luciferase reporter with M2 5'-UTRs containing mutated AUG triplets. The translation of luciferase reporter was measured as described in (B).

of eIF4G (data not shown). We next tested the effect of poliovirus $2A^{pro}$ on the translation efficiency of the RNA transcripts with or without the upstream AUG triplets in the 5'-UTR of M2. As shown in Figure 5B and C, the expression of $2A^{pro}$ increased the translation of luciferase reporter with the long M2 5'-UTR (LM2) by 20- to 30-fold, whereas the translation of the luciferase with the short M2 5'-UTR (SM2) was not affected significantly. The effect of the $2A^{pro}$ expression on the translation of luciferase reporter with various AUG mutants of M2 5'-UTRs decreased with the increase in the number of AUG triplets removed either by deletion (Figure 5B) or by site-specific mutations (Figure 5C). We next determined whether the expression $2A^{pro}$ affects the RNA stability of luciferase reporter transcripts with different M2 5'-UTRs using real-time PCR as described above in Figures 3D and 4D. As shown in Figure 6, the remaining quantity of all RNA transcripts did not change significantly 8 h following transfection both in the presence of the control vector (Figure 6A and C) and the $2A^{pro}$ expression plasmid (Figure 6B and D). These results suggest that the inhibitory effect of upstream AUG triplets on translation initiation at the physiological AUG were released by $2A^{pro}$ expression and, thus, the inhibitory effects likely require intact eIF4G.

$2A^{pro}$ in HeLa cells increased the expression level of endogenous M2

Based on the above observation that expressing $2A^{pro}$ increased the translation of transcripts with long 5'-UTRs but had little effect on the translation of transcripts with short 5'-UTRs of M2, we thought that the endogenous M2 protein level should increase due to the activated translation from the M2 mRNA with long 5'-UTRs by expressing poliovirus $2A^{pro}$. To test this possibility, we transfected HeLa cells with $2A^{pro}$ and determined the endogenous M2 level. As shown in Figure 7A, the protein level of M2 was increased following $2A^{pro}$ transfection as determined by western blot. It is noteworthy that the level of β -actin was decreased $\sim 50\%$ following $2A^{pro}$ transfection, consistent with the observation that $2A^{pro}$ decreased the global protein synthesis (Figure 5A).

However, the total mRNA level of M2 was not changed significantly as determined by RNase protection assay (Figure 7B). To determine whether the increase in endogenous M2 protein was due to the increased protein synthesis, a pulse-labeling by [35 S]methionine followed by immunoprecipitation was performed following $2A^{pro}$ transfection. As shown in Figure 7C, the newly synthesized M2 protein was indeed increased drastically following $2A^{pro}$ transfection. Hence, the increased level of endogenous M2 protein following $2A^{pro}$ transfection is likely due to the increased synthesis rate of M2 protein and it may be by activating the translation of M2 transcripts with the long 5'-UTRs.

Trail treatment increased the expression level of endogenous M2

Because poliovirus $2A^{pro}$ cleaves eIF4G and, thus, inhibits cap-dependent translation, it is possible that the increase in endogenous M2 protein synthesis following $2A^{pro}$ transfection was due to the decreased inhibition of translation of M2 mRNA with long 5'-UTRs. To confirm that the eIF4G is involved, we took another approach by treating cells with Trail. It has been reported that treating HeLa cells with Trail for 4 h would cause cleavage of eIF4G, thus, impair the cap-dependent translation initiation and decrease the incorporation of [35 S]methionine (32). To test the effect of Trail on the expression of M2, we treated HeLa cells with Trail for 4 h and then detected the protein level of M2 using western blot. As shown in Figure 8A, the endogenous M2 protein level was increased following treatment with Trail. However, the M2 mRNA level was not changed significantly as determined by RNase protection assay (Figure 8B). The result of pulse-labeling by [35 S]methionine and immunoprecipitation following Trail treatment showed that the synthesis rate of M2 protein was increased (Figure 8C). Hence, the increase in the level of endogenous M2 protein following Trail treatment is likely due to the increased synthesis rate of M2 protein and it may be by activating the translation of M2 transcripts with long 5'-UTRs. This result is consistent with that shown by transiently expressing $2A^{pro}$ in HeLa cells (Figure 7).

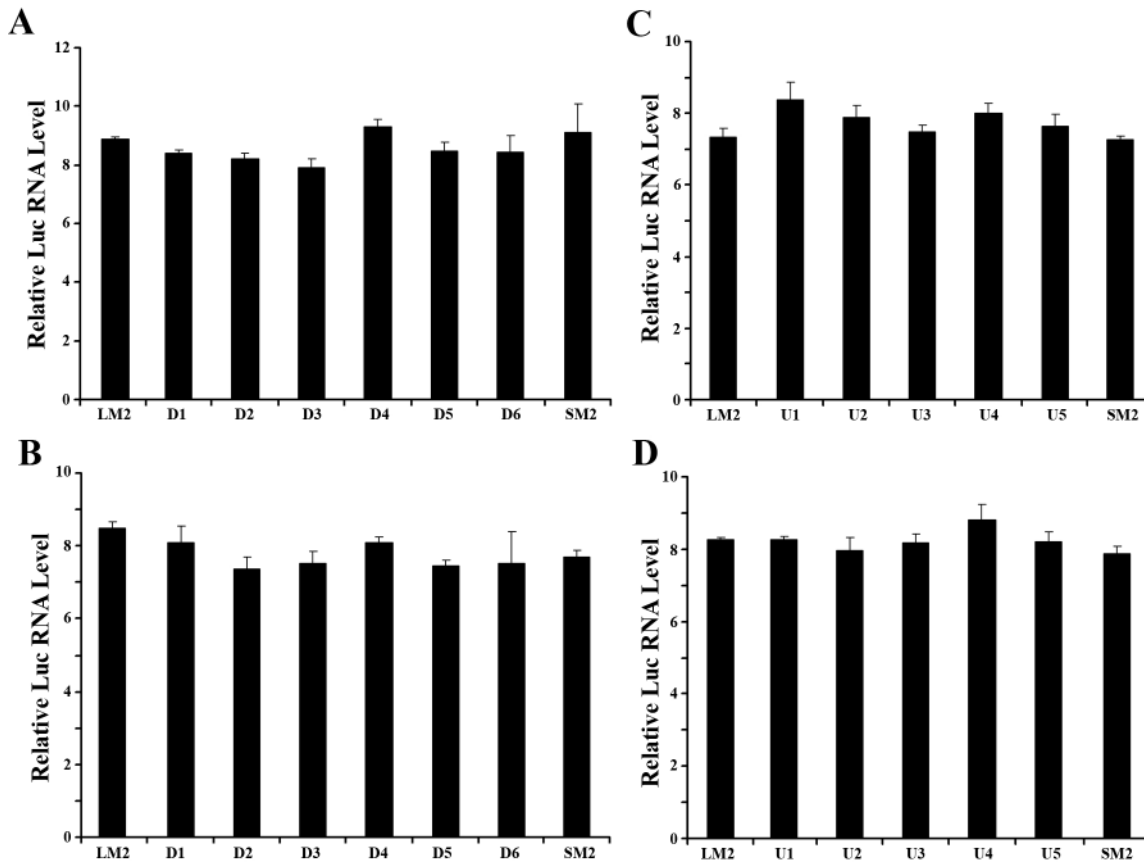


Figure 6. Effect of poliovirus protease 2A on stability of luciferase reporter transcripts with different M2 5'-UTRs. *In vitro* transcripts of luciferase reporters with different M2 5'-UTRs carrying deletions (A and B) or point mutations (C and D) were co-transfected into HeLa cells with transcripts of β -galactosidase 24 h following transfection with pcDNA3 (A and C) or 2A^{pro} expression plasmid (B and C). Total RNAs were then isolated 8 h following RNA transfection for real-time PCR analysis and the relative levels of luciferase RNAs were determined as described in Materials and Methods.

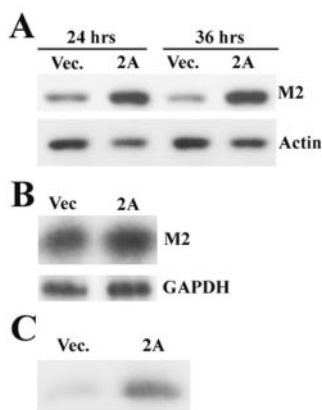


Figure 7. Effect of poliovirus protease 2A on the expression of endogenous M2. (A) Effect of 2A^{pro} on the level of endogenous M2 protein. The expression level of endogenous M2 protein in HeLa cells was determined using western blot at 24 and 36 h following transfection of the cells with 2A^{pro}. (B) Effect of 2A^{pro} on the endogenous M2 mRNA. The expression level of endogenous M2 mRNA in HeLa cells was detected using RNase protection assay 36 h following transfection of the cells with 2A^{pro}. (C) Effect of 2A^{pro} on the synthesis of endogenous M2 protein. HeLa cells were first transfected with 2A^{pro} or control vector followed by pulse labeling of newly synthesized proteins with [³⁵S]methionine. M2 protein was then immunoprecipitated and separated by SDS-PAGE for autoradiography as described in Materials and Methods.

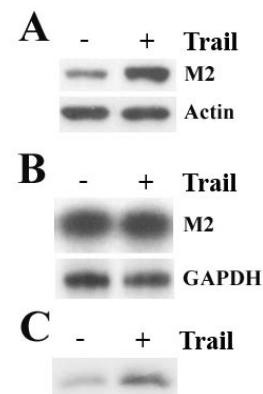


Figure 8. Effect of Trail on the expression of endogenous M2. (A) Effect of Trail on the level of endogenous M2 protein. The expression level of endogenous M2 protein in HeLa cells was determined using western blot 4 h following Trail (250 μ g/ml) treatment. (B) Effect of Trail on the endogenous M2 mRNA. The expression level of endogenous M2 mRNA in HeLa cells was detected using RNase protection assay 4 h following Trail (250 μ g/ml) treatment. (C) Effect of Trail on the synthesis of endogenous M2 protein. HeLa cells were first treated with 250 μ g/ml Trail followed by pulse labeling of newly synthesized proteins with [³⁵S]methionine. M2 protein was then immunoprecipitated and separated by SDS-PAGE for autoradiography as described in Materials and Methods.

DISCUSSION

Post-transcriptional controls including translational regulation are major regulatory steps for gene expression. Alteration in the expression level of translation initiation factors may cause tumorigenesis or increase the malignancy of cancer cells (21,33–36). Increasing evidence suggests that both the 5′- and the 3′-UTRs of mRNAs are major *cis* regulatory elements for the translational control of mRNAs.

In eukaryotes, the translation initiation uses a cap-dependent scanning mechanism (37,38), with which the 5′-cap structure of mRNAs is first recognized by cap-binding factor eIF4E followed by binding of the 40S ribosome and scanning downstream of the 5′-UTR to the initiation codon. However, the existence of upstream AUG codons or ORFs in the 5′-UTRs will likely affect the translation initiation at the physiological initiation codon if the scanning mechanism is to be used and they may play an important role as *cis* elements regulating the translation of the mRNA [reviewed in (39,40)].

Mammalian RR M2 has two mRNAs with different 5′-UTRs generated by transcription at two different start sites (23,24). In order to investigate the translation regulation of these two kinds of mRNA, we employed both *in vitro* translation and RNA transfection (*in vivo*) techniques. The advantage of using the RNA transfection technique to study the translation regulation is to avoid the influence of unknown factors in the transcription procedure if DNA plasmids were to be used. We found that while the mRNA with a short 5′-UTR (63 nt) can be translated efficiently likely using the ribosome scanning mechanism, the mRNA with a long 5′-UTR (222 nt) cannot be translated efficiently either *in vitro* or *in vivo*. Although the long 5′-UTR has four AUG triplets, which are all in frame with the physiological AUG initiation codon, they are not used efficiently for translation to generate a protein with an extended N-terminus. Hence, these AUG triplets in the long 5′-UTR are not functional as translation initiation codons to generate M2 *in vivo*. However, we found instead that these AUG triplets act as translation regulators to control the translation of the M2 mRNA with the long 5′-UTR sequence. These AUG triplets inhibit the efficient translation initiation at the physiological AUG initiation codon and this inhibition appears to be dependent on the presence of intact cellular eIF4G. We also ruled out the possibility that the stability of luciferase reporter transcripts with different M2 5′-UTRs is different, which could affect the final expression level of luciferase activity by performing real-time PCR analysis (Figures 3D and 4D).

Under various conditions, such as during viral infection and apoptosis, the cap-dependent translation initiation is inhibited due to the cleavage of eIF4G by proteases. eIF4G serves as a scaffold molecule bridging eIF4E (the 5′-cap binding protein) and eIF4A (the RNA helicase) and help recruit eIF3, poly(A)-binding protein, and 40S ribosome complex for the formation of preinitiation complexes. With the cleavage of eIF4G by 2A^{Pro} during viral infection (30,31) or by caspases during apoptosis (32), the recruitment of ribosomes and eIF3 to the 5′ end of mRNAs cannot be accomplished and, thus, the cap-dependent translation will be inhibited. The finding that expressing 2A^{Pro} drastically reduced the inhibitory effect of the upstream AUGs on the translation from the physiological start codon AUG of M2 suggests that this inhibition requires

intact eIF4G and it is likely by the cap-dependent translation initiation mechanism.

The regulation by the upstream AUGs or ORFs has been observed for other eukaryotic mRNAs (39,40). It has been thought that the small peptide encoded by the upstream ORF may be involved in regulation. While the mechanism of translational regulation by the upstream AUGs or ORFs varies with different transcripts, most of these AUGs or ORFs are inhibitory for the translation initiation at the physiological start codon. However, the upstream ORFs in yeast GCN4 (41) and mouse ATF4 (42) are known to be stimulatory under stressed conditions. Because the upstream AUGs in the M2 transcript are in the same reading frame as the physiological start codon and no additional stop codons exist in this frame, no separate small peptides are encoded by these upstream sequences. Only a protein with an extended N-terminus was produced by initiating at the upstream AUG *in vitro* as shown in Figure 1. Hence, it is unlikely that the translational regulatory effect of these AUG triplets in the M2 transcripts is due to the production of a small peptide or by reinitiation (39). Considering that the inhibitory effect is cap-dependent, it is more likely that the translation elongation initiated at any of the upstream AUGs by the cap-dependent mechanism may be stalled by the existence of potential complex secondary structures in the 5′-UTR sequence of the long M2 transcript (40). Hence, no M2 protein with extended N-terminus can be produced.

Analysis of the sequence between the upstream AUGs and the physiological initiation codon AUG using an online software (<http://www.bioinfo.rpi.edu/~zukerm/rna/>) showed that there is a stable secondary stem-loop structure of -52.5 kcal/mol (Figure 9A and B). This structure likely serves as a barrier for the moving of ribosomes during elongation after initiation at one of the upstream AUGs (Figure 9C). When the upstream AUGs are removed by deletion or mutation, the preinitiation complex formed at the 5′ end of the mRNA may be able to scan through the 5′-UTR for the physiological AUG initiation codon while the eIF4A in the complex help unwind the secondary stem-loop structures of the 5′-UTR sequence. This unwinding process cannot be accomplished during translation elongation if the upstream AUGs are used for initiation because eIF4A are released together with other factors following the initiation at these upstream AUGs (43). This hypothesis is in contradiction to the conclusion from *in vitro* studies, which showed that the elongating 80S ribosome has more melting power than the 40S ribosome (43). In the Kozak study (43), an AUG initiation codon engineered upstream of a -61 kcal/mol hairpin resulted in the production of CAT reporter, whereas the transcript lacking the upstream AUG could not be translated. However, completely opposite observation was made with the M2 transcript in this study where transcripts with upstream AUGs could not be translated and removal of these AUGs drastically enhanced the translation of the transcript. The reason for the difference between these studies is currently unknown.

Further close examination of the 5′-UTR sequence showed that three of the four upstream AUGs share completely identical flanking sequences (Figure 9D). It is, thus, also possible that these consensus sequences in the 5′-UTR causes instability of the mRNA with these 5′-UTRs and, thus, the production of proteins from these mRNAs are drastically reduced.

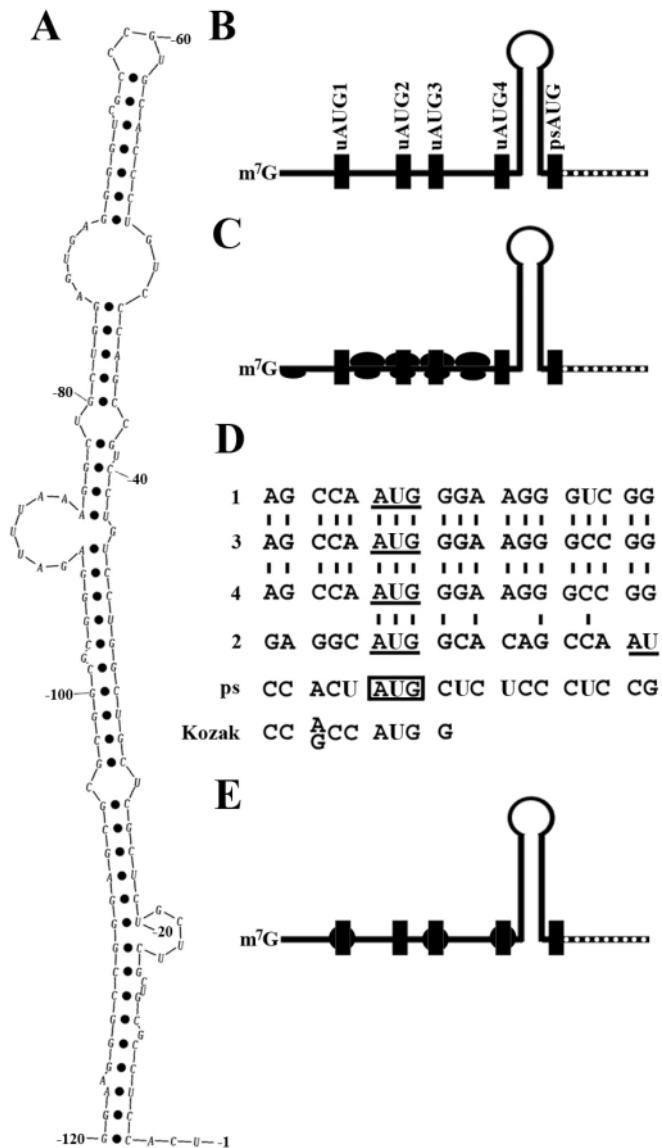


Figure 9. Model for translational regulation of M2 mRNA with the long 5'-UTR. (A) Secondary structure of 5'-UTR sequence between -120 and the physiological start codon AUG. The secondary structure was predicted using an online program (<http://www.bioinfo.rpi.edu/~zukerm/rna/>). (B) Schematic representation of the secondary structure (hairpin) relative to the upstream and the physiological start AUGs (filled boxes) in the long 5'-UTR of M2 mRNA. (C) Model of inhibition of translation initiation at the physiological start codon. The secondary structure serves as a barrier for the moving of ribosomes during elongation with initiation at one of the upstream AUGs. (D) Alignment of the AUG triplets with their flanking sequences. The consensus Kozak sequence is also shown. (E) Model of protein-bound upstream AUG triplets. An hypothetical protein (filled circle) binds to an upstream AUG triplet with conserved flanking sequences, which blocks the movement of ribosomes during the elongation of translation initiated at an upstream AUG.

Consistent with this possibility, it has been shown previously that the 5'-UTR of mouse M2 contains a sequence that can bind to a cytosolic protein to destabilize the mRNA (44,45). It is thus possible that these consensus sequences may bind a destabilizing factor that causes degradation of the mRNA. However, this possibility is unlikely as we have clearly shown that the stability of transcripts with altered upstream AUGs did not increase (Figures 3D and 4D).

It is still also possible that these consensus sequences may bind to a protein factor, which blocks the movement of ribosomes during scanning process (Figure 9E). Under stressed conditions such as during viral infection or apoptosis when eIF4G is cleaved and the cap-dependent translation is inhibited, another mechanism such as cap-independent initiation (such as the putative IRES-mediated initiation) may be used. It is tempting to speculate that, under these conditions, the proteins bound to these consensus sites may also be cleaved similarly as eIF4G and, thus, these sites become exposed for recruiting factors required for cap-independent initiations of translation at the physiological AUG start codon of the M2 mRNA with a long 5'-UTR. We are currently testing this possibility.

The finding that the translation of the M2 mRNAs with a long 5'-UTR is highly regulated is very important. During viral infections when the translation of cellular mRNAs are stopped due to hijacking of the translational machinery by virus for its own use, some cellular mRNAs still need to be translated for the viral replication. RR is one of the enzymes that would be required to make deoxyribonucleotides for replication of the viral genome. Activated translation from the M2 mRNA with the long 5'-UTR under such conditions will help provide sufficient enzymes for the increased requirement by virus. In this study, we indeed found that the endogenous M2 was increased by expressing 2A^{pro} or by treating cells with Trail and this increase was due to the elevated synthesis of M2 protein (Figures 7 and 8). We speculate that this increase in M2 synthesis is likely due to the activated translation of the mRNA with a long 5'-UTR because the translation of the mRNA with a short 5'-UTR is not affected by 2A^{pro} (Figure 5). In addition, during the S phase of cell cycle more RRs are needed and M2 has been shown to increase. Translation of the M2 mRNA with long 5'-UTRs may also be activated during S phases for DNA synthesis.

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Conflict of interest statement. None declared.

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