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Inhibition of Translation of mRNAs Containing γ-Monomethylphosphate Cap Structure in Frog Oocytes and in Mammalian Cells

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The γ-monomethylphosphate cap structure is found in several eukaryotic small RNAs including nuclear U6, U6atac, 7SK, plant nucleolar U3, and rodent cytoplasmic B2 RNAs. In the case of human U6 snRNA, the 5′ end sequence corresponding to nucleotides 1–25 serves as the capping signal and directs the formation of methylphosphate cap structure. In this study, we show that the U6 RNA capping signal, when introduced at the 5′ end of RNAs, can efficiently direct the methylphosphate cap formation in RNAs of up to 2.7 kb long, as well as in different mRNAs. These data show that the methylphosphate capping signal functions in mRNAs having different primary sequences and different lengths. Presence of the methylphosphate cap structure on the 5′ end of a luciferase mRNA with EMCV 5′ noncoding region, which is translated in an IRES-dependent pathway, resulted in a 6- to 100-fold inhibition of translation compared to the same mRNA with a 5′ triphosphate when microinjected into frog oocytes or expressed in mouse cells in tissue culture. Thus, conversion of the pppG structure to a methyl-pppG structure on the 5′ end of an mRNA, which is translated in an IRES-dependent pathway, results in severe inhibition of translation. These data show that the 5′ end motif of mRNAs plays an important role even in the IRES-mediated mRNA translation.

Methylphosphate cap structure RNA translation Frog oocytes Mammalian cells

THE 5′ triphosphate structure in all eukaryotic mRNAs is blocked by an inverted nucleotide linked through 5′–5′ linkage known as the cap structure. This capping of mRNAs is coupled to transcription, and all pre-mRNAs transcribed by RNA polymerase II are capped with 7-methylguanosine (m^7G). In most cases, the cap structure is m^7GpppN; however, in some instances the m^7G residue in the cap structure is further methylated to dimethylguanosine or trimethylguanosine [reviewed in (1,20,22)]. The m^7G cap structure in mRNAs has multiple functions and plays important role(s) in splicing, transport, stability, and translation (6,15,21,22). In addition to mRNAs, some small RNAs like U1 and U2 snRNAs transcribed by RNA polymerase II are synthesized with the m^7G cap and are further methylated to yield trimethylguanosine cap structure (3,17,19). In the case of a few eukaryotic small RNAs, a simple cap structure has been identified where the γ-phosphate of the 5′ pppN is methylated. These RNAs, which contain γ-monomethylphosphate cap structure, include U6 snRNA (28), U6atac RNA (30), 7SK RNA (9,25), plant nucleolar U3 RNA (23), and rodent cytoplamsic B2 RNA (25). The formation of the methylphosphate cap structure was shown to be posttranscriptional and the signal for the cap formation is within the RNA (27,29). In the case of mouse and human U6 snRNA, nucleotides 1–25, comprised of a stem–loop structure and a short single-stranded region, was found to be the capping signal (29). The methylphosphate cap

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structure in small RNAs was shown to enhance stability of small RNAs by up to ninefold in the frog oocyte system (26).

Expression of genes in mammalian cells has the advantage of producing proteins with appropriate posttranslational modifications, which are important for biological activity and antigenicity. To overcome the problem of low yields in mammalian cells, many investigators have successfully engineered mammalian cells to constitutively express T7 RNA polymerase, and have then introduced a T7 promoter in front of the cDNA to be expressed. Using this strategy, levels of the mRNA of interest as well as protein yields have been substantially increased (4,7,32). However, in these cells the mRNAs transcribed by the T7 RNA polymerase are not capped with m^7G and translation of these mRNAs is dependent on an internal ribosome entry site (IRES). While the translation of most eukaryotic mRNAs is cap dependent, some viral mRNAs do not contain the cap structure and their translation is IRES dependent (18); one of the well-characterized IRES is from encephalomyocarditis virus (EMCV) (5,13). Because the methylphosphate cap structure enhances the stability of small RNAs (26), we reasoned that the introduction of the U6 snRNA capping signal into the 5′ end of mRNAs would direct the formation of methylphosphate cap structure on the 5′ end of these mRNAs. These mRNAs with methylphosphate cap structure on their 5′ ends were expected to be more stable and thus lead to increased yields of recombinant proteins.

In our studies, we found that the U6 capping signal can drive the methylphosphate cap formation on mRNAs of different primary sequences and of different lengths. We also carried out studies to test our hypothesis that this methylphosphate cap structure would increase RNA stability, thus increasing protein yields. However, our results in both the frog oocyte system and in mammalian cells show that RNAs containing 5′ methylphosphate cap structure inhibit IRES-dependent translation of a luciferase mRNA. These data suggest that the 5′ end motif of mRNAs plays an important role even in mRNAs where translation is IRES dependent and that minimal modifications, such as a single methylation of the 5′ phosphate residue, result in significant inhibition of translation.

MATERIALS AND METHODS

Plasmids

T7-$U6_{1\text{-}28}$ fragment was PCR amplified and inserted into pUC19 vector between *Eco*RI and *Bam*HI sites. While nucleotides 1–25 of mammalian U6 snRNA were necessary and sufficient to direct methylphosphate cap formation (29), we used nucleotides 1–28 to facilitate cloning. Different cDNAs (HBV-X, HTLV-1-TAX, CAT, and λF) were inserted downstream of the U6 capping signal, as shown in Figure 2A. The EMCV-CAT plasmid containing the EMCV IRES sequence was a gift from Dr. N. Sonenberg. T7-$U6_{1\text{-}28}$ sequence was inserted upstream of this IRES by PCR. T7-$U6_{1\text{-}28}$-18 nt-IRES fragment was also amplified by PCR and inserted into the pGL3-Basic vector (Promega) between *Sac*I and *Bgl*II sites. A deletion-insertion mutation was introduced into the U6 capping signal ($mU6_{1\text{-}28}$) by PCR and the T7-$mU6_{1\text{-}28}$-18 nt-IRES fragment was inserted into pGL3-Basic vector between the *Sac*I and *Bgl*II sites. A spacer (120 bp) from the 3′ end of human p120 gene [*Eco*RI fragment; see (16)] was inserted between the U6 capping signal and the IRES sequence to increase the distance between these two motifs.

RNAs

For run-off in vitro transcription with T7 RNA polymerase, plasmids were linearized with appropriate restriction enzymes. T7-$U6_{1\text{-}28}$-pUC19 was linearized by different restriction enzymes as shown in Figure 1A to generate RNAs of different length. T7-$U6_{1\text{-}28}$-cDNAs were linearized as shown in Figure 2A to generate $U6_{1\text{-}28}$-RNA with different sequences downstream of the capping signal. Luciferase constructs were linearized by *Hpa*I to obtain the full-length luciferase mRNA, and linearized by *Bgl*II to obtain partial luciferase mRNA for studies involving RNA stability and cytoplasmic retention. T7-IRES-CAT was linearized by *Pst*I. In vitro transcription with T7 RNA polymerase was performed according to standard protocol (New England Biolabs). RNA samples were labeled by [γ-^{32}P]GTP for the in vitro capping reaction, and by [α-^{32}P]UTP for microinjection into oocytes. RNAs including mouse B2 RNA containing CH_3-pppG on their 5′ ends were prepared by in vitro transcription in the presence of large excess of CH_3-pppG (26). RNA products were purified by fractionation on a 10% polyacrylamide gel (for <500-nt-long RNAs) or a 5% polyacrylamide gel (for >500-nt-long RNAs).

In Vitro Capping Reaction

The capping reaction was carried out essentially as described by Gupta et al. (10). Briefly, approximately 1000 cpm of the in vitro synthesized uncapped RNA, labeled at the 5′ end by [γ-^{32}P]GTP, was incubated with the whole HeLa cell extract (33) in the capping buffer (50 mM Tris-HCl, pH 8.0, 150 mM KCl, 10 mM $MgCl_2$, and 4 mM AdoMet) at 30°C for 1 h.

The RNAs were extracted by phenol, purified, and digested by nuclease P1. The digestion products were fractionated by homochromatography on polyethyleneimine-cellulose sheet (24) and subjected to autoradiography.

Microinjection Into Oocytes

Internally labeled RNAs containing about 10,000 cpm (in 40–50 nl) were injected into the cytoplasm of oocytes (31). For the RNA transport assay, the nucleus and the cytoplasm were separated manually and the radioactivity in each compartment was determined in a scintillation counter. For the translation assays, at different time points, oocytes were lysed in 200 μl PBS buffer, centrifuged for 5 min at 10,000 rpm at room temperature, and the supernatants were used for CAT and luciferase activity analyses. For RNA stability analysis, RNAs were isolated from the oocytes according to the procedure described by Fischer et al. (8). After various periods of incubation, the oocytes were suspended in homomedium (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1.5% SDS, 300 mM NaCl), and proteinase K (1.5 mg/ml) was added, mixed, and incubated for 15 min at 37°C. Labeled RNAs were extracted using the phenol-chloroform procedure and purified by precipitation with ethanol. The purified RNAs were fractionated on a 5% polyacrylamide gel containing 7 M urea, and the dried gels were subjected to autoradiography.

Transfections

NIH3T3/T7 and OST7-1 mouse cell lines that constitutively express T7 RNA polymerase were used for all tranfections. NIH3T3/T7 and OST7-1 mouse cell lines were grown in 60-mm-diameter dishes and transfected by the calcium phosphate precipitation technique using a total of 8 μg of DNA, which included 3 μg of the CAT reporter and 5 μg of one of the luciferase constructs (pLuc-Basic, T7-mU6$_{1-28}$-120 nt-IRES, T7-U6$_{1-28}$-120 nt-IRES, T7-mU6$_{1-28}$-18 nt-IRES, T7-U6$_{1-28}$-18 nt-IRES). Cells were harvested 72 h posttransfection and resuspended in 400 μl of reporter lysis buffer (Promega). The cell pellet was disrupted by a single freeze–thaw cycle.

CAT and Luciferase Assays

Chloramphenicol acetyltransferase (CAT) assays were done by a single-phase extraction assay. Briefly, 25 μl of the total cell extract was added to a mixture of 50 μl of water, 10 μl of 1 M Tris (pH 7.4), 10 μl of 2.5 mM *n*-butyryl-coenzyme A, and 5 μl of xylene-extracted [^{3}H]chloramphenicol (NEN) at 0.2 μCi/reaction. The CAT assay mixture was incubated overnight at 37°C and extracted with 200 μl of 2,6,10,14-tetramethylpentadecane and xylene (2:1). The organic phase was then counted in a scintillation counter. For luciferase assays, 50 μl of the total cell extract was added to 50 μl of luciferase substrate (Promega). Luciferase activity was quantitated using a TopCount NXT scintillation counter (Packard).

RESULTS

RNAs of up to 2.7 kb Are Capped Efficiently In Vitro

All the known cellular RNAs with methylphosphate cap structure are relatively small in size, the longest known RNA being the 330-nt-long human 7SK RNA. Initially we wanted to test whether the U6 capping signal could direct the methylphosphate cap formation in long messenger RNAs. To test this, pUC 19 plasmid containing T7 promoter followed by human U6 snRNA capping signal was linearized with different restriction enzymes so that when these DNA templates were transcribed with T7 RNA polymerase, RNAs of different lengths of up to 2.7 kb could be obtained (Fig. 1A). All these RNAs contained on their 5′ end the human U6 snRNA capping signal with pppG where the γ-phosphate was labeled with [^{32}P]phosphate. These RNAs were incubated with HeLa cell extract under conditions optimal for methylphosphate cap formation in vitro (10). After incubation, the conversion of pppG to CH_3-pppG in RNAs of different lengths was analyzed and quantitated (Fig. 1B). Using human U6 snRNA as a standard reference, it was found that RNAs of up to 2.7 kb length (Fig. 1B, lane 6) were capped as efficiently as the human U6 snRNA (Fig. 1B, lane 1). RNAs shorter than 50 nucleotides were shown earlier to be capped poorly in vitro (29). Similar results were obtained in this study where 33-nt-long RNA was capped at low efficiency (Fig. 1B, lane 2). These data show that long RNAs with the capping signal at their 5′ end are suitable substrates for capping in vitro and that long RNAs can be capped with high efficiency similar to that observed for human U6 snRNA.

Messenger RNAs With Different Sequences Are Capped Efficiently In Vitro

Next we wanted to test whether the sequence variation inherent in different messenger RNAs had any effect on the methylphosphate cap formation in mRNAs directed by the U6 snRNA capping signal. Different mRNAs varying in length from 800 to 2700 nt were transcribed in vitro (Fig. 2A) and the mRNAs were purified. Again, all these mRNAs contained the

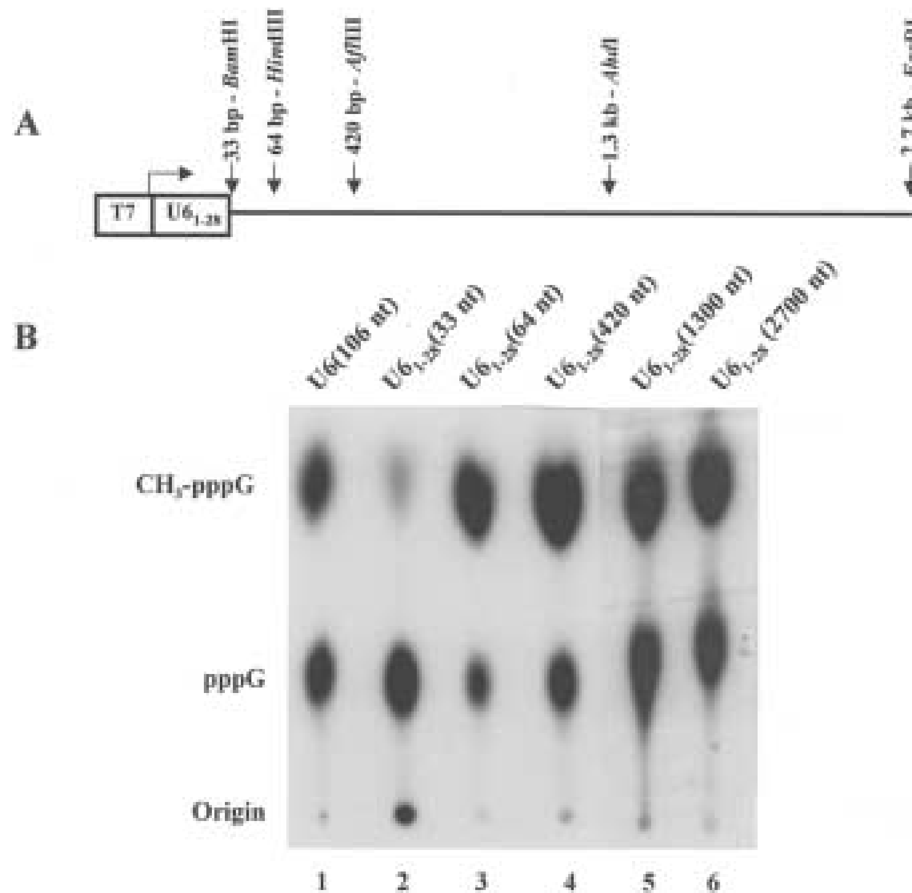

Figure 1. Effect of RNA length on methylphosphate capping efficiency. (A) Diagramatic representation of the T7-U6$_{1-28}$-pUC 19 plasmid that was linearized by different restriction enzymes to yield RNAs of different lengths upon transcription by T7 RNA polymerase. (B) RNAs with a 5′ [γ-^{32}P]pppG followed by the U6 capping signal were subjected to the in vitro capping reaction as described in Materials and Methods (10). RNAs were purified, digested by nuclease P1, and fractionated by homochromatography on a polyethyleneimine-cellulose sheet (24). The length of each RNA transcript is indicated on the top of each lane. The pppG and CH_3-pppG spots were quantitated by phosphorimager and the capping efficiency was calculated, taking the capping efficiency of wild-type U6 snRNA as 100%.

5′ human U6 snRNA capping signal with pppG where the γ-phosphate was labeled with [^{32}P]phosphate. These mRNAs were incubated with HeLa cell extract optimal for methylphosphate cap formation and the cap formation was analyzed and quantitated (Fig. 2B). Taking the efficiency of cap formation in human U6 snRNA as 100%, the efficiency of methylphosphate cap formation in different mRNAs of 800 nt or longer was between 82% and 130% (Fig. 2B, lanes 2–5). These data show that the U6 snRNA capping signal can direct the efficient formation of the methylphosphate cap in mRNAs of diverse sequences in vitro.

Translation of mRNAs With Methyphosphate Cap Structure in Frog Oocytes

Two mRNAs, one with CH_3-pppG and another with pppG as their 5′ ends were prepared by transcription of linearized plasmid DNA with T7 RNA polymerase. This plasmid (T7-mU6$_{1-28}$-18 nt-IRES) (Fig. 3A) contained a mutated U6 snRNA capping signal (29) so that the mRNA with pppG derived from this plasmid construct could not get converted to an mRNA with a CH_3-pppG during incubation in frog oocytes. The amount of mRNAs injected into the frog oocytes was quantitated by determining the optical density at 260 nm and the quality of RNA was evaluated by fractionation of RNAs on an agarose gel and staining with ethidium bromide (Fig. 3B). The amount of RNA injected into each oocyte was adjusted to be approximately the same in all cases. The translation of these mRNAs was IRES dependent, and the extent of translation was monitored by assaying the luciferase activity (Fig. 4A).

Our earlier results with small RNAs injected into frog oocytes showed that RNAs containing CH_3-pppN cap structure are 3–10 times more stable com-

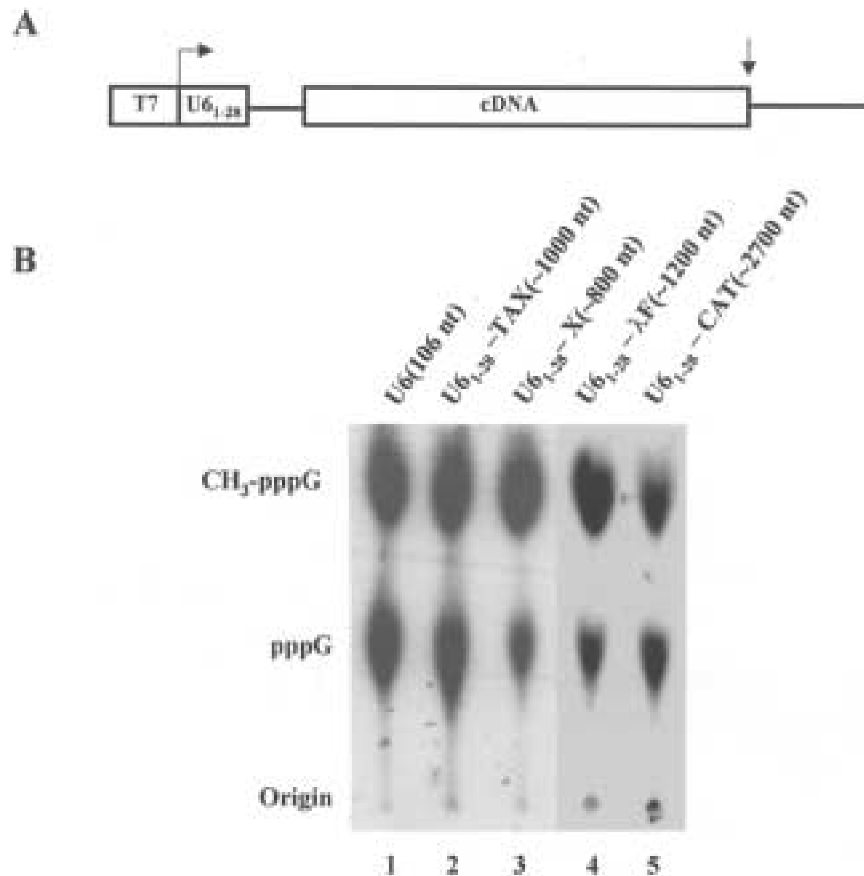

Figure 2. Effect of mRNA sequence on methylphosphate capping efficiency. (A) Diagramatic representation of the basic T7-$U6_{1\text{-}28}$-cDNA construct with different cDNA sequences. (B) Different mRNAs indicated on the top of each lane with a 5′ [γ-^{32}P]pppG followed by the U6 capping signal were subjected to in vitro capping (10). RNAs were purified, digested by nuclease P1, and fractionated by homochromatography on polyethyleneimine-cellulose sheet (24). The pppG and CH_3-pppG spots were quantitated by phosphorimager and the capping efficiency was calculated, taking the capping efficiency of the wild-type human U6 snRNA as 100%.

pared with RNAs containing pppN at the 5′ end (26). Based on these results, we predicted that mRNAs containing methylphosphate cap structure would be more stable and result in higher luciferase activity. However, the observed results were opposite to what we predicted and, at each time point from 1 to 10 h after injection of RNAs into frog oocytes, the amount of luciferase activity was 6- to 15-fold lower with mRNA containing CH_3-pppG at the 5′ end when compared with mRNA with pppG at its 5′ end (Fig. 4A).

Effect of B2 RNA on Translation of mRNA With Methylphosphate Cap Structure

We reasoned that one of the mechanisms that might be responsible for the inhibition of translation was that methylphosphate cap structure may bind a factor which in turn may inhibit IRES-dependent translation. If this were true, co-injection of an RNA having a methylphosphate cap structure may bind and titrate out this factor, thus relieving this inhibition of translation. We chose cytoplasmic B2 RNA because this RNA will remain in the cytoplasm and is the only characterized cytoplasmic small RNA known to contain CH_3-pppN cap structure (25). Injection of pppG-containing luciferase mRNA resulted in expression of luciferase with peak values observed 6 h postinjection (Fig. 4B). As shown earlier by Sarnow (11), introduction of m^7G cap structure into a viral mRNA translated in an IRES-dependent manner resulted in significant inhibition of translation. Our studies also showed that mRNA with m^7G cap structure is translated 5–13-fold less efficiently compared with the translation of mRNA with 5′ pppG (Fig. 4B).

Similarly, the mRNA with the CH_3-pppG cap structure instead of the pppG on the 5′ end translated in an IRES-dependent manner also showed dramatic (15- to 100-fold) inhibition of translation (Fig. 4B). Injection of methylphosphate-capped B2 RNA along with mRNA containing pppG at its 5′ end resulted in a modest increase in luciferase activity. The CH_3-

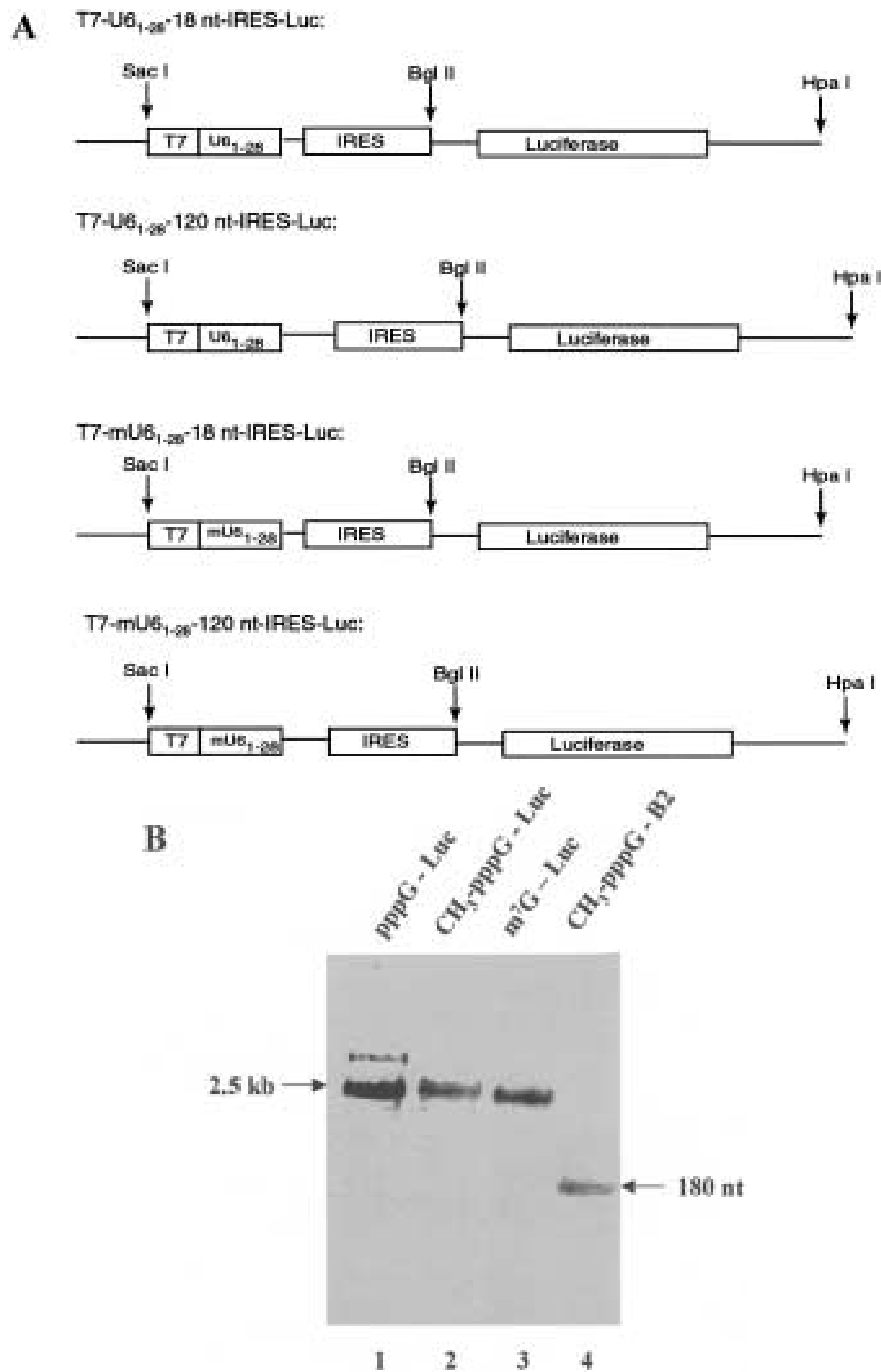

Figure 3. Diagrammatic representation of plasmid constructs and analysis of RNA transcripts. (A) Structure of all the constructs that were used for making RNAs and also for transfection into mouse cell lines. The T7-$U6_{1-28}$-18 nt-IRES was PCR amplified and cloned into the pGL3 basic vector using *Sac*I and *Bgl*II sites. The *Hpa*I site was used to linearize the plasmid and make T7 RNA transcripts. A 120-bp fragment from human p120 gene (16) was inserted into the *Eco*RI site to increase the distance between the IRES and the U6 capping signal. The constructs are not drawn to scale. T7, T7 promoter; m, mutant; $U6_{1-28}$, U6 snRNA capping signal; Luc, luciferase. (B) In vitro transcription was carried out in the presence of CH_3-pppG to get γ-monomethylphosphate-capped mRNAs and in the presence of m^7GpppG to get m^7G-capped RNAs. The RNAs were run on an agarose gel and stained with ethidium bromide to estimate quality and quantity of RNAs.

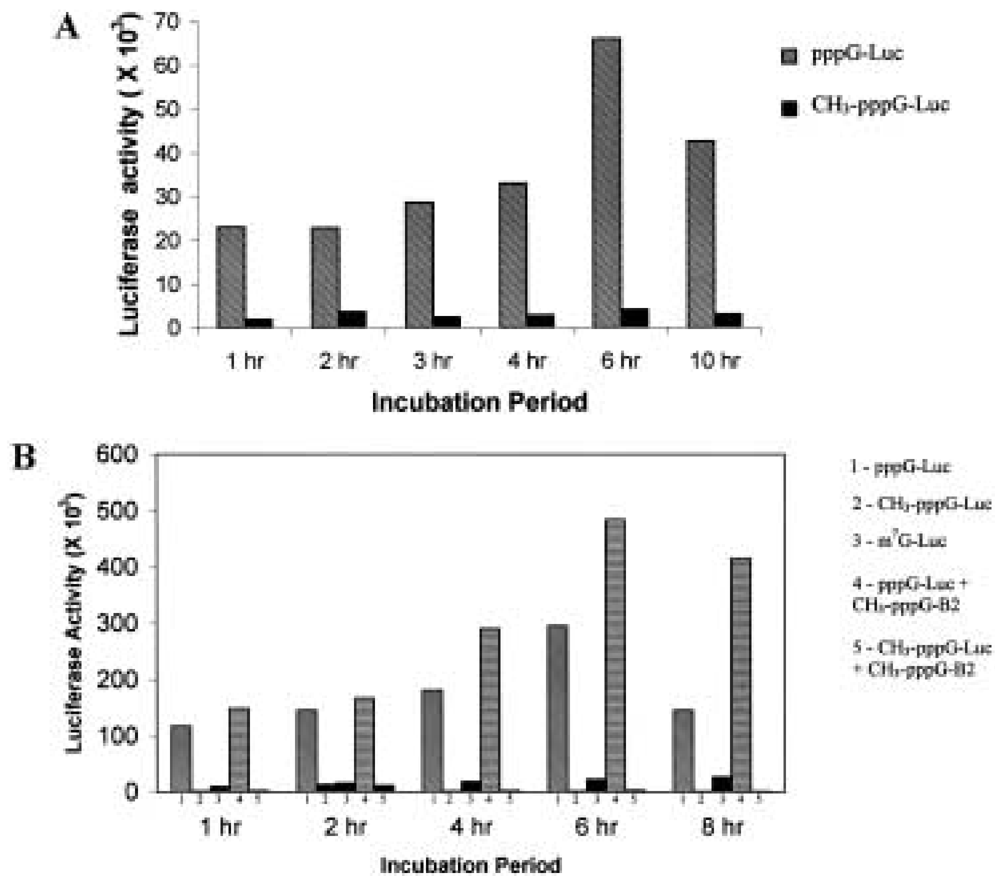

Figure 4. Translation of mRNAs with different 5′ caps in frog oocytes. (A) Similar amounts of T7-mU6$_{1-28}$-18 nt-IRES-Luc RNAs with or without methylphosphate cap structure were injected into the cytoplasm of oocytes, together with the CAT mRNA as an internal control. Oocytes were incubated for different time periods and then lysed in PBS buffer. Activities of luciferase and CAT were analyzed and the luciferase activity was normalized by the CAT activity. (B) Similar amounts of T7-mU6$_{1-28}$-18 nt-IRES-Luc RNAs with either no 5′ cap (pppG-Luc) or the 5′ methyl cap (CH_3-pppG-Luc) or m^7G capped Luc (m^7G-Luc) were injected into the cytoplasm of frog oocytes. 5′ methyl capped B2 RNA (CH_3-pppG-B2) was also co-injected with CH_3-pppG-Luc or with pppG-Luc. CAT mRNA was co-injected as an internal control in all injections. Oocytes were lysed in PBS buffer after different time periods and luciferase activity was analyzed. The luciferase activity was normalized by the CAT activity.

pppG-capped B2 RNA injected along with the mRNA containing CH_3-pppG at its 5′ end still showed inhibition and there was no detectable difference in inhibition in the presence of B2 RNA (Fig. 4B). These data show that co-injected B2 RNA, which is cytoplasmic and normally contains CH_3-pppN cap structure (25), cannot relieve the inhibition of translation observed in mRNA with the 5′ methylphosphate cap structure.

Fate of RNA Injected Into Frog Oocytes

Because these results with mRNA containing CH_3-pppG were unexpected, we wanted to test whether the mRNA with CH_3-pppG was degraded preferentially or rapidly transported to the nucleus, or both. Uniformly labeled mRNA from the same plasmid construct, T7-mU6$_{1-28}$-18 nt-IRES (Fig. 3A), was prepared with CH_3-pppG or with pppG at the 5′ end and injected into frog oocytes. After various periods of incubation, similar amounts of intact mRNAs with and without the methylphosphate cap structure were detected, indicating that there was no difference in stability between the two mRNAs (data not shown). In addition, after various periods of incubation the nucleus and cytoplasm were separated and it was found that virtually all the mRNA remained in the cytoplasm (Table 1). These data show that the significant inhibition of translation observed with the mRNA containing CH_3-pppG cap structure is not accounted for by either preferential degradation of mRNA with CH_3-pppG or migration of mRNA with CH_3-pppG on its 5′ end to the nuclear compartment.

Inhibition of Translation in Mammalian Cells

Next, we tested whether inhibition of translation observed in the *Xenopus* oocyte system also occurs

TABLE 1
FATE OF RNA INJECTED INTO FROG OOCYTES

RNA Injected	2 h (cpm)		4 h (cpm)		6 h (cpm)		9 h (cpm)	
	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic
pppG-Luc (*Bgl*II)	670 (3.60%)	19,000 (96.40%)	570 (3.20%)	18,000 (96.80%)	720 (4%)	18,000 (96%)	620 (3.30%)	19,000 (96.70%)
CH_3-pppG-Luc (*Bgl*II)	450 (1.50%)	31,000 (98.50%)	990 (3.20%)	31,000 (96.80%)	750 (2.50%)	30,500 (97.50%)	680 (3.10%)	22,000 (96.90%)

T7-mutU6$_{1\text{-}28}$-18 nt-IRES-Luc was linearized with *Bgl*II and in vitro transcription was carried out with or without CH_3-pppG, in the presence of [α-^{32}P]UTP, by T7 RNA polymerase. Approximately 10,000 cpm of 5′ CH_3-pppG-Luc or 5′ pppG-Luc was injected into the cytoplasm of the frog oocytes. At different time points, the nucleus and the cytoplasm were manually separated and the radioactivity in each compartment was determined in a scintillation counter.

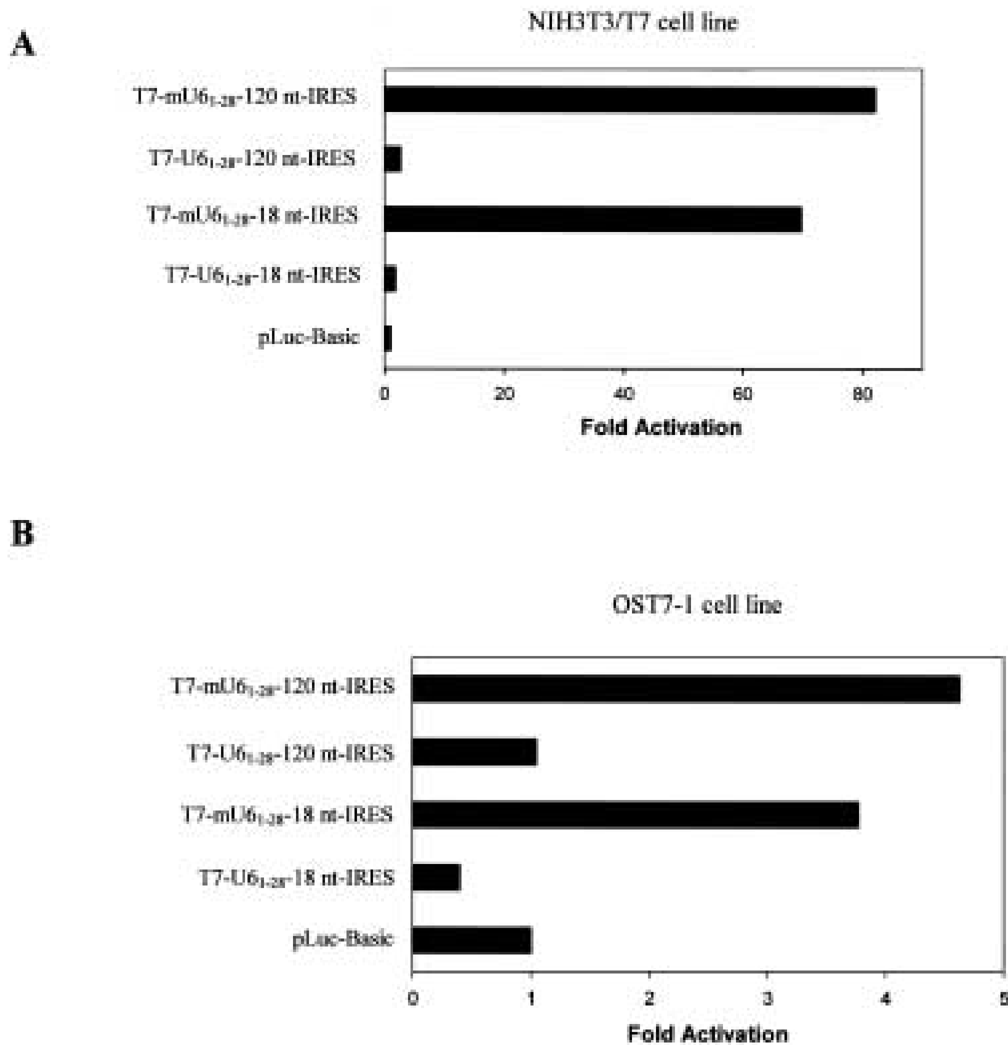

Figure 5. Suppression of translation of 5′ methyl-capped mRNAs in mouse cells in culture. 5 μg of pLuc-Basic, T7-mutU6$_{1\text{-}28}$-120 nt-IRES, T7-U6$_{1\text{-}28}$-120 nt-IRES, T7-mutU6$_{1\text{-}28}$-18 nt-IRES, T7-U6$_{1\text{-}28}$-18 nt-IRES plasmids were transfected into NIH3T3-T7 cells (A) and OST7-1 cells (B) along with the CAT reporter plasmid as an internal control. Cells were harvested and lysed after 72 h and the luciferase activity and CAT activity were analyzed. Luciferase activity was normalized to CAT activity to control for transfection efficiency. Fold activation was determined by dividing the sample luciferase activity by the basal luciferase activity in the presence of pLuc-Basic plasmid.

in the mammalian system. Several plasmid DNA constructs shown diagramatically in Figure 3A were transfected into mouse cell lines that constitutively express T7 RNA polymerase. The plasmid constructs contain the T7 promoter, and the mRNAs made from these constructs by T7 RNA polymerase contain pppG on their 5′ ends. These plasmid constructs along with a CAT expression plasmid were transfected into mouse cells. In the case of mRNAs containing the U6 snRNA capping signal, the cellular capping enzyme is likely to methylate the 5′ end and result in a CH_3-pppG structure. The mRNA containing the U6 RNA capping signal (T7-U6$_{1\text{-}28}$-18 nt-IRES) showed significant inhibition of translation compared to mRNA (T7-mU6$_{1\text{-}28}$-18 nt-IRES) containing mutated U6 snRNA capping signal in NIH3T3/T7 cell line (Fig. 5A) and also in OST7-1 cell line (Fig. 5B). We also altered the spacing by introducing a 120-bp fragment between the U6 snRNA capping signal and the IRES to rule out the possibility that the close proximity of the U6 snRNA capping signal and IRES might have some effect on IRES function. Results with RNAs derived from these two constructs (T7-U6$_{1\text{-}28}$-120 nt-IRES and T7-mU6$_{1\text{-}28}$-120 nt-IRES) were the same as those observed with the mRNAs containing the shorter spacer sequence. The mRNA with the capping signal gave significantly less luciferase activity compared with the mRNA with mutated U6 snRNA capping signal in both cell lines (Fig. 5A, B). These data show that

the inhibition initially observed with mRNAs containing CH_3-pppG on their 5′ end in the frog oocyte system is also true in the mouse cells.

DISCUSSION

The main observation made in this study is that IRES-dependent mRNAs containing the 5′ methylphosphate cap structure are translated in frog oocytes and in mouse cell lines very inefficiently compared with mRNAs containing pppG on their 5′ ends. Thus, even in the case of mRNAs translated in an IRES-dependent manner, the 5′ sequence motif plays a significant role. This inhibition of translation was observed in two different mouse cell lines and also in the *Xenopus* oocyte system, showing that the 5′ end of mRNA is important in many species.

Hambidge and Sarnow (11) showed the importance of the 5′ end in mRNAs containing IRES by introducing a m^7G cap on the 5′ end of these RNAs. Interestingly, they observed that m^7G cap inhibited the translation of IRES-containing mRNAs. In this study, we studied the most simple 5′ cap structure found in nature, namely, CH_3-pppN found in many eukaryotic small RNAs. We initiated this study with the prediction that CH_3-pppN-containing mRNAs dependent on IRES for translation would be more stable compared with identical RNAs with 5′ pppG, thus resulting in higher protein yields. To our surprise, we observed dramatic inhibition of translation when mRNAs contained CH_3-pppN instead of pppG in both mammalian cells, as well as in the *Xenopus* oocyte system. It is surprising that a single methyl group on the 5′ end of an mRNA, which does not use its 5′ end to bind and assemble functional ribosomes, can have such dramatic effect on translation. The mechanism responsible for this inhibition is not known.

We reasoned that CH_3-pppG might bind a protein factor and this might interfere with the IRES-dependent translation. Therefore, we tested whether or not co-injection of another RNA containing CH_3-pppN might titrate out this factor, thus relieving this inhibition of translation. Co-injection of methylphosphate-capped B2 RNA, which is normally cytoplasmic, did not relieve this inhibition. In fact, a slight stimulation of translation was observed in the presence of B2 RNA. While this experiment does not rule out factor(s) binding to the CH_3-pppG on the 5′ end of mRNA and interfering with translation, the experimental data do not support this possibility. More experiments with different IRES sequences and different reporter constructs are needed to determine the generality of this mechanism and also to understand the mechanism responsible for this inhibition of translation.

Maraia and his associates showed that the La protein binds to the 3′ U-tail as well as to the 5′ ppp of a precursor tRNA through two distinct domains (12). There are several reports showing that La protein is required for IRES-mediated translation (2,14). Therefore, it is possible that mRNAs containing IRES bind the La protein through both the IRES and the 5′ ppp, and when this 5′ ppp is methylated, the affinity to La protein is affected. This possibility has to be experimentally tested. Sarnow studied the effect of m^7G cap structure in mRNAs that are translated in an IRES-dependent pathway (11). The m^7G cap on the 5′ end of these mRNAs inhibited IRES-mediated translation. These data were interpreted to mean that there are common and limiting translation factors in the cell. The m^7G cap competes for these limiting factors, thus inhibiting the IRES-dependent translation (11). The methylphosphate cap structure is not known to bind factors common to m^7G cap structure or bind factors necessary for IRES-dependent translation. Thus, the mechanism of inhibition of translation has to be different. Further studies are needed to determine the effect of the methylphosphate cap structure in other mRNAs containing IRES motifs and with different 5′ end sequence motifs.

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