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Terminal 7-Methyl-Guanosine Cap Structure on the Normally Uncapped 5′ Noncoding Region of Poliovirus mRNA Inhibits Its Translation in Mammalian Cells

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We have used an RNA transfection assay to study the translation of cellular and viral mRNAs with and without 5′-terminal m^7GpppG cap structures in human tissue culture cells. HeLa cells were transfected with in vitro-transcribed hybrid RNA molecules containing the 5′ noncoding regions of either luciferase or poliovirus linked to the coding region of the firefly luciferase gene. Transcripts containing a capped luciferase 5′ noncoding region produced luciferase, while similar uncapped transcripts did not. In contrast, transcripts containing a capped 5′ noncoding region of poliovirus accumulated 10-fold-lower levels of luciferase than similar transcripts without a terminal cap structure. Inhibition of poliovirus mRNA translation by a 5′-terminal cap structure was not observed in in vitro translation systems. This finding indicates that factors involved in cap-independent translation of poliovirus RNA are quantitatively or qualitatively different in human tissue culture cells and in in vitro translation systems. Furthermore, this study emphasizes the importance of studying translational control of mRNAs in intact cells.

The translation of poliovirus RNA has been intensively studied for a number of years. As is the case with other picornaviruses, the 5′ noncoding region (5′NCR) of poliovirus is unusual in several respects. First, it does not have a 7-methyl-guanosine cap structure (m^7GpppN, where N is any nucleotide) at its 5′ terminus, as do all known eukaryotic cellular mRNAs, excluding organellar mRNAs (20). Instead, the viral RNA has a small polypeptide, VPg, covalently linked to its 5′ end (3, 11). VPg is removed prior to the translation of viral RNA, leaving pUpU as the 5′-terminal structure of polysomal viral RNA (5, 14). Second, the polioviral 5′NCR is very long (750 nucleotides [nt]) and highly conserved among the three poliovirus serotypes (7, 18, 23). Third, there are multiple AUG codons (eight in Mahoney type 1) that precede the AUG codon used for translation initiation. All of these characteristics render poliovirus RNA incompatible with the scanning model of translation initiation (9). In fact, it has been shown that initiation of translation in poliovirus RNA (17) and in two other picornavirus RNAs, encephalomyocarditis virus RNA (6) and foot-and-mouth disease virus RNA (10), occurs by the binding of ribosomes to internal sequences within the 5′NCRs.

Studies of the translation of poliovirus RNA in vivo have relied on transient transfection of DNA vectors into human cells. These vectors have typically contained simian virus 40 promoter sequences and a cDNA encoding the poliovirus 5′NCR cloned upstream of a reporter gene (17, 24). After transfection, these vectors are transcribed by cellular RNA polymerase II to produce capped hybrid RNA molecules. Therefore, studying the translation of the normally uncapped poliovirus mRNA in human cells has not proved feasible. We designed an experimental system to study the translation efficiencies of capped and uncapped RNAs after direct RNA transfection into mammalian cells. This method of RNA transfection permits the bypass of potentially confounding nuclear events such as transcription, RNA splicing, or RNA transport to the cytoplasm.

Direct transfection of RNA molecules into human HeLa cells. It has been shown that transfection of in vitro-transcribed full-length polioviral RNA into human cells results in the production of infectious virus (19, 25). More recently, a variety of techniques for the transient transfection of nonviral RNA molecules have been reported (2, 8, 13). We have introduced RNA molecules into tissue culture cells by a modified DEAE-dextran transfection method (22).

The vectors used for in vitro transcription of RNA molecules, T7-5′ncluc-LUC and T7-5′ncpolio-LUC, contain the 5′NCRs of luciferase or poliovirus, respectively, cloned between the bacteriophage promoter for T7 RNA polymerase and the coding region of luciferase (LUC) (12). After linearization downstream of LUC at a unique *Nhe*I site, both T7-5′ncluc-LUC and T7-5′ncpolio-LUC were used as templates for in vitro transcription of RNA molecules. Capped and uncapped luciferase RNAs containing the 5′NCRs of luciferase (m^7GpppG-5′ncluc-LUC and pppG-5′ncluc-LUC) and of poliovirus (m^7GpppG-5′ncpolio-LUC and pppG-5′ncpolio-LUC) were thus produced. RNA concentrations were quantified prior to transfection by Northern (RNA) analysis and densitometric scanning. RNAs from transcription reactions were then transfected without any further purification into HeLa cells by the use of DEAE-dextran.

Transfection efficiencies of hybrid RNA molecules. To measure the transfection efficiencies of the four hybrid RNA molecules, we transfected RNAs into HeLa cells and attempted to quantitate the intracellular RNAs by Northern blot analysis. Unfortunately, this method did not prove sensitive enough, and we subsequently monitored the uptake of in vitro-synthesized, radiolabeled RNAs into HeLa cells (Fig. 1). After the RNA–DEAE-dextran mix had been added to the cell monolayer for 15 min, the cells were washed multiple times with phosphate-buffered saline and cytoplas-

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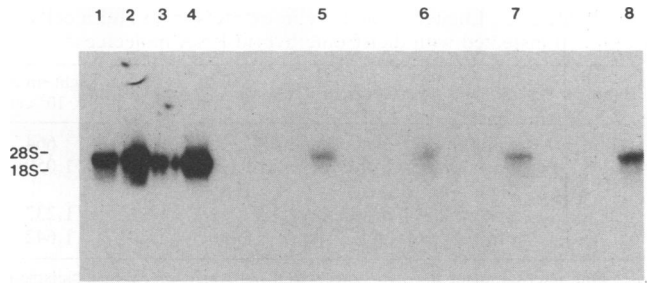

FIG. 1. Transfection efficiencies of in vitro-transcribed hybrid RNA molecules. Subconfluent HeLa cells were transfected with radioactively labeled RNAs as described in the text. Lanes 1 through 4, input RNA obtained directly from the transcription cocktail (diluted 1:1,000 prior to electrophoresis). Note that radiolabeled uncapped RNAs are synthesized more efficiently than radiolabeled capped RNAs. Lanes 5 through 8, cytoplasmic RNA. Lanes: 1 and 5, capped m^7GpppG-5'ncpolio-LUC RNA; 2 and 6, uncapped pppG-5'ncpolio-LUC RNA; 3 and 7, capped m^7GpppG-5'ncluc-LUC RNA; 4 and 8, uncapped pppG-5'ncluc-LUC RNA. The somewhat slower migration of the intracellular RNA species (lanes 5 through 8) may be due to polyadenylation of the RNAs after transfection.

mic extracts were prepared. The RNAs were then analyzed in formaldehyde-containing agarose gels. An autoradiograph of such a gel is shown in Fig. 1. Densitometric analysis of the bands in Fig. 1 showed that all the hybrid RNAs were introduced with similar efficiencies into HeLa cells, with approximately 0.05% of the input RNA being taken up into the cytoplasm of the cells.

Expression of luciferase from transfected RNA molecules. Next, we determined the efficiencies with which the transfected RNAs could be translated by cellular ribosomes. The accumulation of the translation product, luciferase, was used as an indicator of the translational efficiencies of transfected RNAs (12). Figure 2A shows that capped m^7GpppG-5'ncluc-LUC RNA supported the translation of luciferase protein, while uncapped pppG-5'ncluc-LUC RNA was translated at levels only slightly above background. Therefore, this system mimicked the translation of cellular RNAs, which require a 5' cap structure for efficient translation.

The translation of as little as 10 ng of RNA could be detected easily (Fig. 2A). Transfection of amounts of RNA from 0.01 to 10 μg revealed an approximately linear relationship between the amount of RNA transfected and the amount of luciferase translated (Fig. 2A). Figure 2B shows that the level of luciferase, accumulated from translation of transfected RNAs, reached a plateau at 1 to 2 h after transfection.

To show that the luciferase had been translated directly from the transfected RNA, the transfection mix was pretreated with DNase-free RNase, which abolished the production of luciferase (4). However, substantial luciferase activity was detected after pretreatment of the transcription reaction with RNase-free DNase (4).

A 5' cap structure greatly enhances translation in mammalian cells of RNAs containing the 5'NCR of luciferase but inhibits translation of RNAs containing the 5'NCR of poliovirus. Figure 2A shows that capped m^7GpppG-5'ncluc-LUC RNA supported the translation of luciferase, while uncapped pppG-5'ncluc-LUC did not. Similarly, only capped m^7GpppG-5'ncα-globin-LUC RNAs, containing 37 nt of human α-globin 5'NCR cloned upstream of the LUC gene, produced luciferase upon transfection into mammalian cells (4). In contrast, uncapped pppG-5'ncpolio-LUC RNA was translated approximately 10-fold more efficiently than either capped m^7GpppG-5'ncpolio-LUC or capped m^7GpppG-5'ncluc-LUC RNA (Fig. 2), suggesting that cap-independent translation initiated by the normally uncapped poliovirus 5' NCR is highly efficient in vivo and may be inhibited by the presence of a cap structure.

We employed an indirect test to ensure that uncapped pppG-5'ncpolio-LUC RNAs were not becoming capped after transfection. By deletion of nt 220 to 670 of the poliovirus 5'NCR in plasmid T7-5'ncpolio-LUC, the internal ribosome-binding site in the viral 5'NCR was removed. In vitro transcription of this plasmid produced capped m^7GpppG-5'ncpolio(Δ220-670)-LUC RNA, which produced luciferase after transfection into mammalian cells. However, it produced less than m^7GpppG-5'ncpolio-LUC RNA did.

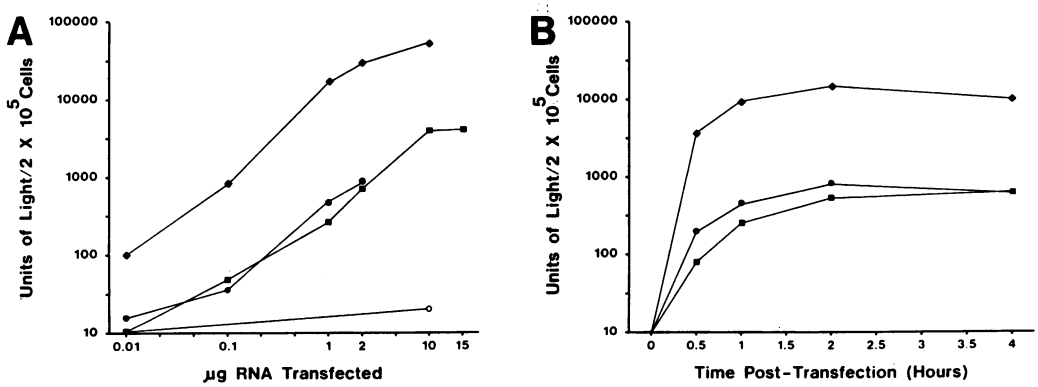

FIG. 2. (A) RNA concentration dependence of the accumulation of translation products from transfected hybrid RNA molecules. In vitro-transcribed RNAs were transfected into HeLa cells by a DEAE-dextran transfection method (22). Cellular extracts were prepared, and luciferase activity was measured 1 h after transfection (12). (B) Time course of accumulation of products from transfected hybrid RNA molecules. One microgram of RNA was transfected in each case. The level of translation products (units of light) for the following hybrid RNAs are shown: uncapped pppG-5'ncluc-LUC (○), capped m^7GpppG-5'ncluc-LUC (■), uncapped pppG-5'ncpolio-LUC (◆), and capped m^7GpppG-5'ncpolio-LUC (●). All values represent the means of two measurements of each extract with background luciferase activity (approximately 50 light units) subtracted from the mean.

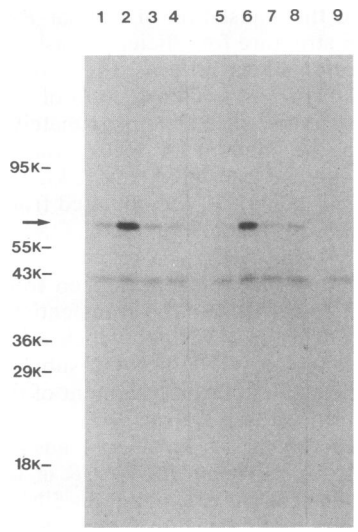

FIG. 3. In vitro translation of capped and uncapped hybrid RNA molecules. In vitro-transcribed hybrid RNA molecules were translated in the presence of radiolabeled methionine in a supplemented RRL as described previously (26). The translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An autoradiograph of the gel is shown. Lanes: 1 and 5, uncapped pppG-5'ncluc-LUC RNA; 2 and 6, capped m^7GpppG-5'ncluc-LUC RNA; 3 and 7, uncapped pppG-5'ncpolio-LUC RNA; 4 and 8, capped m^7GpppG-5'ncpolio-LUC RNA; 9, no RNA. Translations were initiated with 500 ng (lanes 1 through 5) or 100 ng (lanes 5 through 8) of RNA. Scanning by densitometry showed that incorporation of ^{35}S-methionine was still in the linear range in the translation reactions initiated with 100 ng of RNA. Molecular weights of known marker proteins are indicated. The arrow denotes the 62-kDa luciferase protein.

The same uncapped RNA did not produce luciferase, demonstrating that the 5'-proximal sequences in the viral RNA were not capped in mammalian cells (4).

It is unlikely that the production of luciferase in cells transfected with pppG-5'ncpolio-LUC RNA, which was 10-fold higher than that in cells transfected with m^7GpppG-5'ncpolio-LUC or m^7GpppG-5'ncluc-LUC RNA, was due to the shorter half-lives of the capped RNAs, since a 5' cap structure is known to stabilize RNA molecules, particularly by protecting them from 5' exonucleolytic degradation (20). Furthermore, the kinetics of luciferase expression after transfection of these three different RNAs were similar (Fig. 2B), suggesting that the three RNAs displayed similar half-lives. Therefore, it is most likely that the 5' cap structure on the normally uncapped poliovirus 5'NCR inhibited cap-independent translation in vivo.

A 5' cap structure greatly enhances translation in vitro of RNAs containing the 5'NCR of luciferase but not translation of RNAs containing the 5'NCR of poliovirus. It has been shown that RNAs containing capped and uncapped poliovirus 5'NCRs initiate translation with similar efficiencies in vitro (15, 16). This contrasts with the translational efficiencies of the RNAs observed in vivo (Fig. 2). To test whether the particular reporter gene, the luciferase gene, was influencing its own translational control, we assayed the four hybrid RNA molecules in an in vitro translation system using a rabbit reticulocyte lysate (RRL) supplemented with an S10 HeLa cell extract (kindly provided by Sandra Dildine and Bert Semler, University of California at Irvine) (26). Figure 3 shows that a 5' cap structure greatly enhanced the translation of pppG-5'ncluc-LUC RNA (compare lanes 1 and 2), mimicking the results seen in vivo (Fig. 2). Uncapped pppG-5'ncpolio-LUC RNA was a poor template for translation in vitro, in agreement with published reports of studies in which other reporter genes were used (15, 16). However, a 5' cap structure on pppG-5'ncpolio-LUC RNA did not affect its translational efficiency in vitro (Fig. 3, compare lanes 3 and 4). Therefore, the luciferase reporter gene did not confer an unusual translational regulation on these hybrid RNA molecules. Instead, the relative translational efficiencies of capped and uncapped pppG-5'ncpolio-LUC RNAs appear quite different in vivo and in vitro.

TABLE 1. Luciferase activity in extracts from HeLa cells transfected with dicistronic hybrid RNA molecules[a]

Expt.	Structure of RNA	Light units/ 2×10^5 cells
1	pppG-CAT-5'ncpolio-LUC	664
	m^7GpppG-CAT-5'ncpolio-LUC	1,050
2	pppG-CAT-5'ncpolio-LUC	1,232
	m^7GpppG-CAT-5'ncpolio-LUC	1,642

[a] One microgram of in vitro-synthesized uncapped or capped dicistronic chloramphenicol acetyltransferase (CAT)-5'ncpolio-LUC RNA (12) was transfected into HeLa cells, and luciferase activity was measured 1.5 h after transfection as described in reference 12.

The enhanced translational efficiency of uncapped pppG-5'ncpolio-LUC RNA in vivo could be explained by a number of reasons. First, the translation initiation factors available in HeLa cells may be qualitatively different from those in the supplemented RRL. Alternatively, factors necessary for cap-independent translation of the poliovirus 5'NCR may be present in lower relative concentrations in the supplemented RRL than in HeLa cells, allowing more effective competition by the cap-dependent translation apparatus. Finally, it is well documented that poliovirus RNA associates with cellular membranes during its life cycle and that poliovirus infection induces membrane proliferation in HeLa cells (1). One explanation for the highly efficient translation of the uncapped poliovirus 5'NCR in vivo may be that the transfected RNA has access to intact membranes and to membrane-associated proteins.

The translation of capped m^7GpppG-5'ncpolio-LUC RNA but not capped m^7GpppG-5'ncluc-LUC RNA was inhibited in vivo. One possible explanation for this inhibition is that the cap structure facilitates attachment of the cellular cap-binding protein complex eIF-4F, which either sterically or enzymatically prevents internal ribosome binding. Enzymatic interference could occur by the disruption of RNA secondary structure by eIF-4F and the subsequent migration of the 40S ribosomal subunit in a 5'-to-3' direction. Such a process could interfere with internal ribosome binding by changing RNA structures or masking RNA sequences that are part of the functional internal ribosome binding site. In this context, it is noteworthy that an RNA hairpin structure at the very 5' end of the viral RNA has been found to modulate the translational efficiency of the viral RNA in mammalian cells (21).

Interestingly, in dicistronic RNAs bearing an additional first cistron, 800 nt long and located upstream of the poliovirus 5'NCR, translation of the second cistron was not inhibited by a 5' cap structure (Table 1). Thus, the location of the viral internal ribosome binding site more than 1,000 nt away from the capped 5' terminus of the RNA did not

interfere with initiation of translation by internal ribosome binding in vivo.

In summary, we have shown that hybrid RNAs bearing a 5′-terminal cap structure and the 5′NCR of poliovirus are translated much less efficiently than the same uncapped RNAs in mammalian cells. However, the same capped and uncapped RNAs are translated with similar efficiencies in vitro in an RRL supplemented with a HeLa extract. This finding emphasizes the importance of studying the translational control of mRNAs in intact cells.

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ADDENDUM IN PROOF

Russell et al. (R. J. Russell, S. J. Hambidge, and K. Kirkegaard, Nucleic Acids Res., in press) have shown that similar hybrid luciferase RNA molecules can be successfully transfected into *Saccharomyces cerevisiae*.

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