5'-Terminal capping of RNA by guanylyltransferase from HeLa cell nuclei
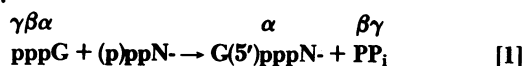
(mRNA/7-methylguanosine/RNA processing)

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ABSTRACT   A soluble extract prepared from HeLa cell nuclei has been shown to catalyze the 5'-terminal modification of RNA and synthetic polyribonucleotides to form $m^7G(5')pppA$- and $m^7G(5')pppG$- structures referred to as *caps*. The reaction involves the transfer of a GMP moiety from GTP to the 5' end of an RNA molecule containing at least two terminal phosphates. Significantly, neither the β nor the γ phosphates of GTP are transferred and polynucleotides with no 5'-terminal phosphate or only one are not acceptors. In the absence of methyl donor, $G(5')pppA$- and $G(5')pppG$- structures were synthesized, indicating that methylation is not required for guanylylation. Cap formation was considered to occur by the following mechanism:

$$\overset{\gamma\beta\alpha}{pppG} + (p)ppN- \overset{\alpha}{\longrightarrow} G(5')pppN- + \overset{\beta\gamma}{PP_i} \quad [1]$$

$$G(5')pppN- + AdoMet \longrightarrow m^7G(5')pppN- + AdoHcy \quad [2]$$

in which AdoMet is S-adenosylmethionine, AdoHcy is S-adenosylhomocysteine, and (p)ppN- represents either the original 5' end of an RNA molecule or an internal site to which one or more phosphates were added after processing.

Structures of the type $m^7G(5')pppN^mp(N^m)$-, known as *caps*, are present at the 5' termini of viral (1–4) and cellular (5–9) mRNAs. A comprehensive review of the occurrence and function in translation of cap structures has recently been published (10). Information regarding the mechanism of formation of these structures has been obtained only for those viruses that contain the capping and methylating enzymes within the purified virus particles. Two general mechanisms have been found. With vaccinia virus (11–13), cytoplasmic polyhedrosis virus (14), and reovirus (15), a GMP residue from GTP is transferred to the 5' terminus of mRNA, while with vesicular stomatitis virus (16), a GDP residue is transferred. The first mechanism is suitable for capping the initial triphosphate end of an RNA molecule, while the second seems appropriate for capping monophosphate ends formed by cleavage.

Elucidation of the mechanism of capping cellular mRNA species is important for understanding RNA processing. The presence of caps in heterogeneous nuclear RNA (17, 18) implies that this modification occurs within the nucleus. Moreover, subcellular fractions containing nuclei have been shown to synthesize capped mRNA *in vitro* (19, 20). The requirement for the presence of all four ribonucleoside triphosphates and the sensitivity to α-amanitin suggested that cap formation is closely coupled to transcription in this system (20). The experiments described here, which utilize soluble enzymes obtained from HeLa cell nuclei and defined RNA substrates, provide evidence regarding a mechanism of capping cellular mRNA.

MATERIALS AND METHODS

Preparation of Guanylyltransferase from HeLa Cells. HeLa S3 cells were harvested from suspension culture at 3×10^5 cells per ml and washed three times with 20 mM Tris·HCl (pH 7.6)/146 mM NaCl/11 mM glucose at 4°. The cells were then swollen in three volumes of 20 mM Tris·HCl (pH 7.9)/10 mM $MgCl_2$/10 mM KCl/0.25 mM spermidine at 0° and disrupted by Dounce homogenization. An equal volume of cold 20 mM Tris·HCl (pH 7.6)/50% glycerol was immediately added to the lysate. The nuclei were pelleted by centrifugation, resuspended in buffer A [50 mM Tris·HCl (pH 7.9)/25% glycerol/1 mM dithiothreitol/0.1 mM EDTA], and layered over 50 mM Tris·HCl (pH 7.9)/10 mM $MgCl_2$/50% glycerol. The nuclei were pelleted again by centrifugation at 37,400 × g for 30 min, resuspended in buffer A, and lysed by adding 3 M $(NH_4)_2SO_4$ (adjusted to pH 7.9) to a final concentration of 0.3 M. The nuclear lysate was dispersed by six 10-sec bursts with a Branson sonifier at 0°, and the supernatant obtained by centrifugation at 234,000 × g for 50 min was used as the enzyme source.

Guanylyltransferase Assay. The standard reaction mixture (0.1 ml) containing 50 mM Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM ATP, 1 mM dithiothreitol, 12.5% glycerol, 10 μg of RNA substrate, 20 μl of enzyme extract, 25–50 μCi of $[\alpha\text{-}^{32}P]GTP$ (100–200 Ci/mmol) and 100 μM S-adenosylmethionine (AdoMet) (50 μM added at 0 min and 50 μM at 30 min) or 200 μM S-adenosylhomocysteine (AdoHcy) was incubated at 30° for 1 hr. The reaction was stopped by addition of 1 ml of 50 mM Tris·HCl (pH 7.9), 0.5% sodium dodecyl sulfate, 0.1 mM GTP, 1 mM EDTA, and 0.3 mg of yeast tRNA carrier. The mixture was extracted with chloroform/buffer-saturated phenol (1:1), and the labeled RNA was isolated by precipitation of the aqueous phase with 5% trichloroacetic acid. The precipitate was redissolved in minimal Tris·HCl (pH 8.5) and the precipitation procedure was repeated three times. The final precipitate was washed with 95% ethanol. Alternatively, the RNA, after chloroform/phenol extraction, was separated from the majority of unreacted $[\alpha\text{-}^{32}P]GTP$ by gel filtration. The labeled RNA was digested in 0.1 ml of 10 mM sodium acetate (pH 6) with 50 μg of nuclease P_1 at 37° for 2 hr and then in 50 mM Tris·HCl (pH 8.5)/5 mM $MgCl_2$ with 20 μg of *Escherichia coli* alkaline phosphatase at 37° for 2 hr. The digests were analyzed by paper electrophoresis.

Electrophoresis and Chromatography. Paper electrophoresis was performed on 57-cm sheets of Whatman 3 MM paper in 0.5% pyridine/5% acetic acid (pH 3.5)/1 mM EDTA buffer. Ascending paper chromatography was in isobutyric acid/0.5 M NH_4OH (5:3).

Abbreviations: m^7G, 7-methylguanosine; A^m, 2'-O-methyladenosine; G^m, 2'-O-methylguanosine; m^6A^m, N^6,2'-O-dimethyladenosine; N^m, any 2'-O-methylnucleoside; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine.

Biochemistry: Wei and Moss

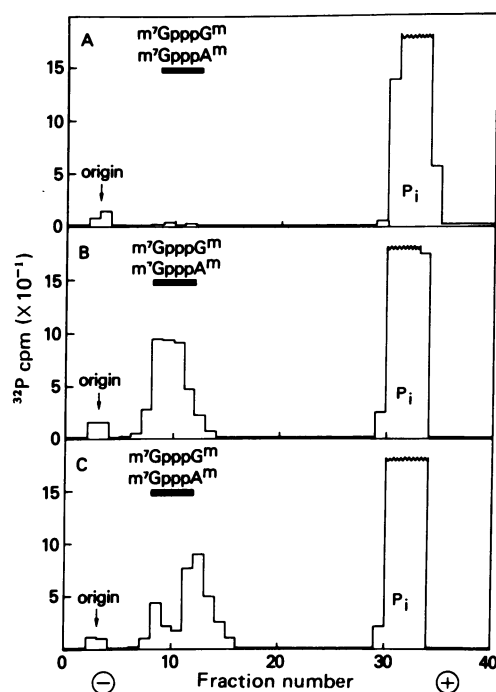*Proc. Natl. Acad. Sci. USA 74 (1977)* 3759

FIG. 1. Paper electrophoresis of nuclease P_1- and phosphatase-resistant products of guanylyltransferase reaction. Guanylyltransferase was measured with [α-^{32}P]GTP and unmethylated vaccinia mRNA in the presence of AdoMet or AdoHcy. After phenol extraction and trichloroacetic acid precipitation, the RNA was digested with nuclease P_1 and alkaline phosphatase and the products were analyzed by paper electrophoresis at pH 3.5. *Methyl*-^{3}H-labeled m^{7}GpppG^m and m^{7}GpppA^m used as internal markers were derived from *methyl*-^{3}H-labeled vaccinia mRNA synthesized by virus cores. (A) Minus vaccinia RNA plus AdoMet; (B) plus vaccinia RNA and AdoMet; (C) plus vaccinia RNA and AdoHcy.

Materials. Vaccinia mRNA was synthesized as described by Ensinger *et al.* (11). [α-^{32}P]GTP (150 Ci/mmol) was purchased from either New England Nuclear Co. or Amersham/Searle Corp.; [*ribose*-^{3}H]GTP (23 Ci/mmol) from Amersham/Searle Corp.; [β,γ-^{32}P]GTP (24 Ci/mmol) from International Chemical and Nuclear Corp.; poly(A) lot no. 341101 and methylated nucleotide markers from P-L Biochemicals; *E. coli* alkaline phosphatase from Worthington Biochemical Corp.; nuclease P_1 from Yamasa Shoyu Co.; and nucleotide pyrophosphatase from Sigma Chemical Corp. A second phosphate was added to the 5′ terminus of poly(A), as described by Martin and Moss (12).

RESULTS

Demonstration of Guanylyltransferase Activity. Previous studies from our laboratory (11, 12) demonstrated that guanylyltransferase and methyltransferase activities isolated from vaccinia virus cores could be assayed using [α-^{32}P]GTP, AdoMet, and unmethylated vaccinia virus mRNA containing predominantly ppG and ppA termini. A similar approach was therefore used to detect guanylyltransferase activity in a high-speed supernatant fraction from HeLa cell nuclei. Fig. 1*B* shows the incorporation of isotope into nuclease P_1- and alkaline phosphatase-resistant oligonucleotides that comigrated with authentic m^{7}G(5′)pppG^m and m^{7}G(5′)pppA^m on paper electrophoresis. This analytical procedure is highly specific for caps since all 3′,5′-phosphodiester linkages in RNA or DNA are cleaved by nuclease P_1 and the released nucleotides are subsequently hydrolyzed by alkaline phosphatase. The dependence on added RNA is shown by the absence of incorporation of
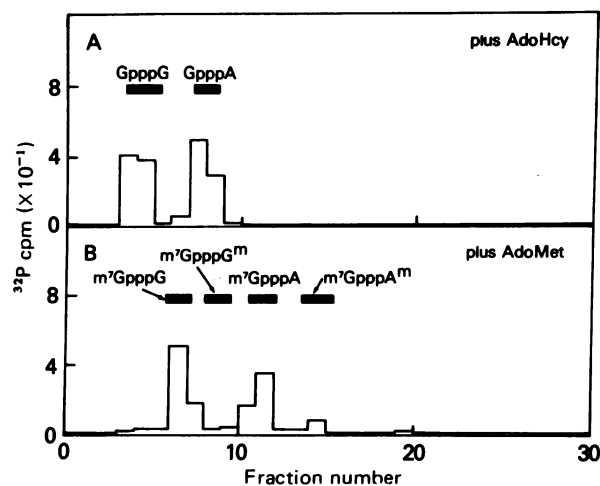

FIG. 2. Identification by paper chromatography of methylated and unmethylated caps. The experiment was carried out as in Fig. 1 except that labeled RNA was separated from unreacted [α-^{32}P]GTP by gel filtration instead of acid precipitation. (A) The material migrating with and slightly ahead of the marker, as in Fig. 1C, was eluted and chromatographed in isobutyric acid/NH$_4$OH. (B) The material migrating with the marker, as in Fig. 1B, was eluted and chromatographed.

[α-^{32}P]GTP into nuclease P_1- and alkaline phosphatase-resistant material when RNA was omitted from the reaction (Fig. 1A). In the presence of AdoHcy, a methylation inhibitor, [α-^{32}P]GTP was incorporated predominantly into material migrating slightly ahead of the methylated cap markers (Fig. 1C). The difference in migration is consistent with the absence of the positive charge associated with methylation at the 7 position of guanine.

The Products of Guanylyltransferase. Nuclease P_1- and alkaline phosphatase-resistant oligonucleotides labeled with [α-^{32}P]GTP were isolated by paper electrophoresis as described in the preceding section. For further characterization, the labeled oligonucleotides were chromatographed in an isobutyric acid/NH$_4$OH system. As shown in Fig. 2A, the material synthesized in the presence of AdoHcy cochromatographed with G(5′)pppG and G(5′)pppA. However, the material synthesized in the presence of AdoMet cochromatographed with m^{7}G-(5′)pppG and m^{7}G(5′)pppA (Fig. 2B). Since the unmethylated vaccinia virus mRNA contains both ppA and ppG ends, the above results were consistent with the transfer of the GMP moiety of GTP to the ends of the substrate to form the appropriate caps.

Although resistant to the combined actions of nuclease P_1 and alkaline phosphatase, cap structures are susceptible to nucleotide pyrophosphatase. When the material synthesized in the presence of AdoHcy was digested with the latter enzyme, the ^{32}P label comigrated upon electrophoresis with pG (Fig. 3A). When the material labeled in the presence of AdoMet was subjected to this analysis, the ^{32}P label comigrated with pm^{7}G (Fig. 3B). Thus, the nuclear lysate is capable of capping and specifically methylating added RNA.

Substrate Specificity of Guanylyltransferase. Upon treatment with alkaline phosphatase, vaccinia virus mRNA lost its ability to serve as a substrate for the HeLa cell guanylyltransferase (not shown). Since unmethylated vaccinia virus mRNA contains primarily diphosphate-terminated molecules, we considered that a 5′ di- or triphosphate might be essential. In agreement with this, poly(A) synthesized with polynucleotide phosphorylase, which has a single terminal phosphate (21), was totally ineffective as a substrate (Fig. 4A). After reaction of
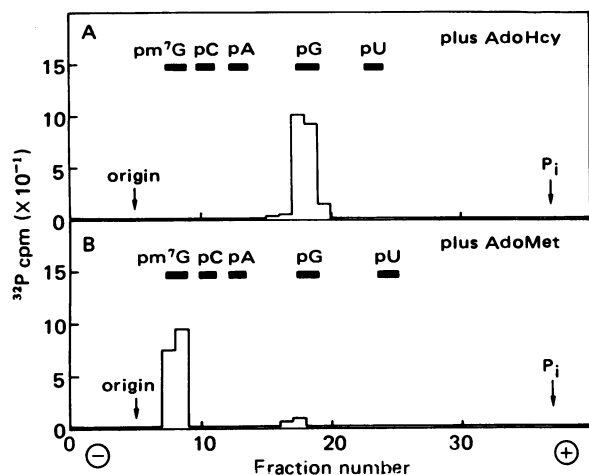
FIG. 3. Analysis of labeled ribonucleotides released by nucleotide pyrophosphatase. A second portion of ^{32}P-labeled material used for analysis in Fig. 2 was digested by 0.05 unit of nucleotide pyrophosphatase in 0.1 ml of 20 mM Tris·HCl (pH 7.6)/1 mM MgCl$_2$ at 37° for 30 min. The digest of cap synthesized in the presence of (*A*) AdoHcy and (*B*) AdoMet was analyzed by electrophoresis along with authentic markers.

poly(A) with the diimidazolidate of orthophosphate, which reacts exclusively with primary phosphates to form diphosphate-terminated molecules (22, 23), however, the synthetic polynucleotide became an effective substrate. In the presence of AdoHcy, a single nuclease P$_1$- and alkaline phosphatase-resistant product, identified as G(5′)pppA, was formed (Figs. 4C and 5A). In the presence of AdoMet, two products were formed (Fig. 4B). The faster migrating species was identified as G(5′)pppA (not shown), while the slower was predominantly
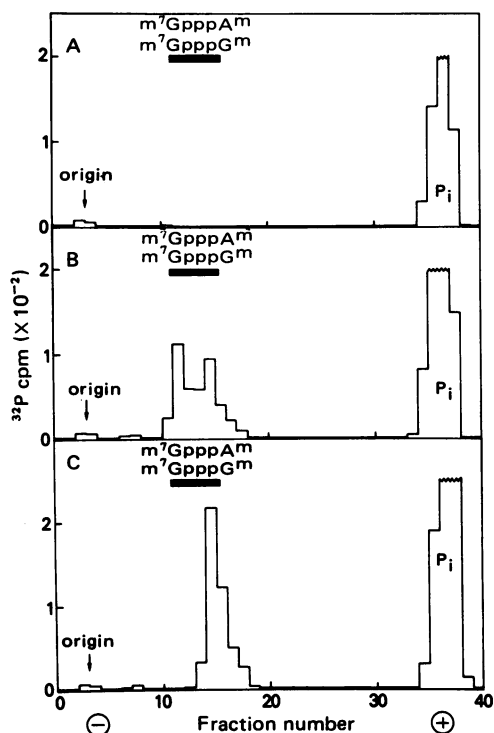

FIG. 4. Paper electrophoresis of nuclease- and phosphatase-resistant products of guanylyltransferase reaction with poly(A) substrates. The experimental procedure is similar to that of Fig. 1 except for the use of poly(A) with one or more 5′-terminal phosphates as substrate. (*A*) Plus pA(pA)$_n$ and AdoMet; (*B*) plus ppA(pA)$_n$ and AdoMet; (*C*) plus ppA(pA)$_n$ and AdoHcy.
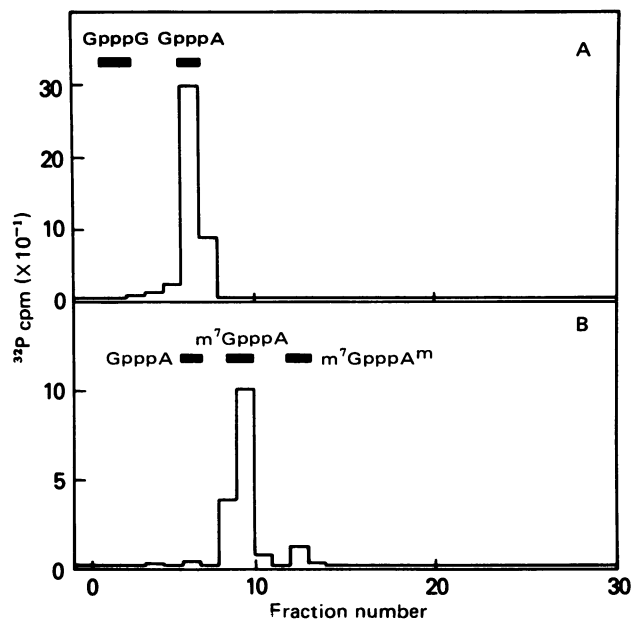

FIG. 5. Identification of methylated and unmethylated caps from poly(A) substrate by paper chromatography. (*A*) The ^{32}P-labeled material synthesized in the presence of AdoHcy with poly(A) as substrate, as in Fig. 4C, was eluted and analyzed by paper chromatography. (*B*) Material, as in Fig. 4B, made in the presence of AdoMet and migrating just behind the product of Fig. 4C was eluted and analyzed by paper chromatography.

m^{7}G(5′)pppA (Fig. 5B). A small amount of the more highly methylated cap m^{7}G(5′)pppA^m may also have formed (Fig. 5B).

β,γ-Phosphates of GTP Are Not Transferred. The above experiments carried out with [α-^{32}P]GTP demonstrated that the α phosphate was transferred to form the cap structure. To determine whether additional phosphates are also transferred, we carried out experiments with [β,γ-^{32}P]GTP. As a positive control for cap formation, [*ribose*-^{3}H]GTP was added to the same reaction mixture. The results indicated that although [^{3}H]guanosine was incorporated into caps using both unmethylated vaccinia virus mRNA and modified poly(A) as substrates, no significant incorporation of [β,γ-^{32}P]GTP occurred (Table 1). Since the number of pmol of [α-^{32}P]GTP incorporated in previous experiments was similar to the number of pmol of [*ribose*-^{3}H]GTP incorporated, we conclude that only the α-phosphate of GTP is transferred into the cap structure.

DISCUSSION

This report provides evidence for a mechanism of capping cellular RNA. A guanylyltransferase activity present in a soluble

Table 1. Absence of incorporation of β,γ-phosphates of GTP into caps

RNA substrate	Incorporation into caps, dpm	
	[*ribose*-^{3}H]GTP	[β,γ-^{32}P]GTP
None	52	<20
Unmethylated vaccinia mRNA	944	<20
ppA(pA)$_n$	5508	<20

The reaction was performed under standard conditions in a total volume of 1 ml containing 100 μCi of [*ribose*-^{3}H]GTP (23 Ci/mmol) and 100 μCi of [β,γ-^{32}P]GTP (24 Ci/mmol). The radioactivity resistant to nuclease P$_1$ and alkaline phosphatase was determined after electrophoresis at pH 3.5.

(p)pp⁵'N-N --

              ⌐ GTP

              ↓➤ PP$_i$

G⁵'ppp⁵'N-N ---

              ⌐ AdoMet

              ↓➤ AdoHcy

m⁷G⁵'ppp⁵'N-N ---

              ⌐ AdoMet

              ↓➤ AdoHcy

m⁷G⁵'ppp⁵'Nᵐ-N --
(Aᵐ)

              ⌐ AdoMet

              ↓➤ AdoHcy

m⁷G⁵'ppp⁵'Nᵐ-N --
(m⁶Aᵐ)

              ⌐ AdoMet

              ↓➤ AdoHcy

m⁷G⁵'ppp⁵'Nᵐ-Nᵐ --------------------------------------
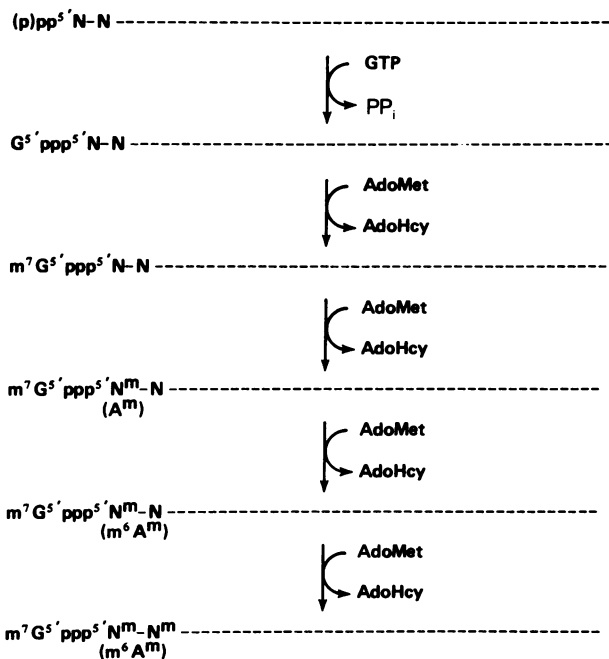(m⁶Aᵐ)

FIG. 6.   Proposed model for modification of 5' termini of HeLa cell heterogeneous nuclear RNA or mRNA.

extract from HeLa cell nuclei was able to cap unmethylated vaccinia viral mRNA or synthetic polyribonucleotides. A polyribonucleotide with only a single 5'-terminal phosphate could not be capped unless a second phosphate was chemically added. Furthermore, of the three phosphates of GTP, only the α phosphate was incorporated into the cap structure. In both regards, the HeLa cell enzyme is similar to the guanylyltransferase purified from vaccinia virus cores (12) suggesting the reaction:

$$\gamma\beta\alpha \qquad\qquad \alpha \quad\ \beta\gamma$$
$$pppG + (p)ppN\text{-} \rightarrow G(5')pppN\text{-} + PP_i$$
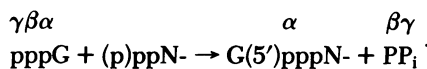
It will be of interest to learn whether a guanylyltransferase recently isolated from calf thymus also has a similar mechanism (24).

The specificity of di- or triphosphate-terminated RNA molecules makes the guanylyltransferase described here appropriate for capping RNA at initiation sites. If this enzyme is responsible for modifying all mRNA species, then the 5' ends of RNA must be preserved during processing or, as proposed by Schibler and Perry (25), a kinase must add additional phosphates to ends generated by cleavage. Alternatively, a second cellular capping enzyme, analogous to the activity within vesicular stomatitis virus (16), may exist. An activity that can effect the net transfer of a GDP residue to an RNA chain containing a single terminal phosphate would be difficult to detect in nuclear extracts if transcription, processing, and capping are obligatorily coupled.

The HeLa cell nuclear extract used for this study is also capable of methylating the newly formed cap at the 7 position of guanine. Nevertheless, methylation is not required for capping since G(5')pppN- termini were formed in the presence of the inhibitor AdoHcy. The guanylyltransferase and guanine-7-methyltransferase are separable activities since the latter has been partially purified from the cytoplasmic fraction of HeLa cells (26). The guanine-7-methyltransferase has a strict specificity for cap structures and will not catalyze the methylation

of GTP, indicating that methylation must follow capping. Presumably the methylation of the penultimate nucleoside of cell mRNA in the 2' position is the next step, as has been shown with the purified vaccinia virus enzyme (27). An enzyme capable of further methylating 2'-O-methyladenosine of caps at the N-6 position to form m⁷G(5')pppm⁶Aᵐ has been isolated from HeLa cells and characterized (28). This enzyme also appears to be entirely specific for cap structures and will methylate m⁷G(5')pppAᵐ- at higher rates than m⁷G(5')pppA-. Kinetic evidence that methylation of the third nucleoside at the 2' position to form m⁷G(5')pppNᵐ-Nᵐ- cap structures occurs within the cytoplasm has been reported (29, 30). A proposed sequence of events leading to the 5'-terminal modification of cellular mRNA is outlined in Fig. 6.

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