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Biochemistry

"Pretranscriptional capping" in the biosynthesis of cytoplasmic polyhedrosis virus mRNA

(*S*-adenosylmethionine/mRNA methylation/initiation of transcripton/virus gene expression)

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ABSTRACT **The *in vitro* synthesis of cytoplasmic polyhedrosis virus (CPV) mRNA was previously shown to be dependent upon the presence of the methyl donor *S*-adenosylmethionine (AdoMet). We now find that the competitive inhibitor of methylation, *S*-adenosylhomocysteine (AdoHcy), also stimulates CPV mRNA synthesis efficiently, resulting in the synthesis of viral mRNAs containing 5′-terminal GpppA and ppA, rather than m^{7}GpppAm as observed with AdoMet. In addition to AdoHcy, other AdoMet analogues, including *S*-adenosylethionine and adenosine, also stimulate CPV mRNA synthesis but to a smaller extent than does AdoHcy or AdoMet. In order to study the relationship between cap formation and mRNA synthesis, nucleoside triphosphates were replaced in the RNA-synthesizing reaction mixture (containing AdoMet) by the corresponding β,γ-imido analogues, which are resistant to nucleotide phosphohydrolase, an enzyme involved in cap formation. Although mRNA synthesis occurred in the presence of UMP-pNHp or GMP-pNHp, none was observed when AMP-pNHp was substituted for ATP. Because the ATP molecule that becomes the 5′-terminal nucleotide of CPV mRNA must be cleaved at the β-γ position during cap formation, the results suggest that, in this viral transcription system, cap formation is a prerequisite to mRNA synthesis—i.e., a "pretranscriptional" event.**

Most eukaryotic cells and viruses have been found to contain a 5′-terminal "cap" structure, m^{7}GpppN(m), in their mRNAs (1). Studies of cytoplasmic polyhedrosis virus (CPV) of the silkworm *Bombyx mori* opened the way to the discovery of cap structures (2). Like that of human reovirus, the genome of CPV consists of 10 double-stranded RNA segments, and the virus particle contains an RNA polymerase activity that transcribes the duplex RNA to form mRNAs either in the infected animal or *in vitro* under the appropriate conditions. Synthesis of mRNA *in vitro* by purified CPV was found to be dependent upon the presence of a methyl donor, *S*-adenosylmethionine (AdoMet), and the resulting viral mRNAs contained methylated 5′ termini, m^{7}GpppAm (3). Because methylation of the 5′terminus of nascent mRNA was detected at the initiation of mRNA synthesis and synthesis itself showed strong dependency on the presence of AdoMet, this peculiar system of viral mRNA formation was reported as "methylation-coupled transcription" (2).

It was also reported previously that low levels of a potent methylation inhibitor, *S*-adenosylhomocysteine (AdoHcy), an analogue of AdoMet, did not prevent the stimulation of CPV mRNA synthesis by AdoMet. Rather, the addition of equimolar amounts of AdoMet and AdoHcy to a reaction mixture increased mRNA synthesis by 20%–30% more than with AdoMet alone (2). The effect of AdoHcy on CPV mRNA synthesis and methylation has now been investigated in detail. AdoHcy stimulated CPV mRNA synthesis as effectively as AdoMet but also inhibited methylation. Other experiments designed to assess the relationship between mRNA synthesis and cap formation suggested that capping (or some cap-related reaction) is tightly coupled with and probably a prerequisite for CPV mRNA synthesis. A similar order of reactions could explain certain aspects of capping of cellular nuclear RNAs.

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MATERIALS AND METHODS

Preparation of Purified CPV and CPV mRNA Synthesis *In Vitro*. CPV was prepared by essentially the same procedure as before (4). Reaction conditions for mRNA synthesis and analysis were as described (5), with slight modifications depending upon the purpose of the experiments. Details of the conditions are shown in the legends to the tables and figures.

Materials. [^{3}H-*methyl*]AdoMet (specific activity, 12.2 Ci/mmol) and [^{3}H]UTP (specific activity, 16.4 Ci/mmol) were purchased from Amersham/Searle. Nonradioactive AdoMet, AdoHcy, adenosine, and ribonucleoside triphosphates were from Calbiochem and Boehringer/Mannheim (West Germany). *S*-Adenosylethiomine (AdoEt) was purchased from Sigma. [α-^{32}P]GTP (specific activity, 11.8 Ci/mmol) and [α-^{32}P]CTP (specific activity, 41.6 Ci/mmol) were obtained from New England Nuclear; β,γ-imidoribonucleoside triphosphates (AMP-pNHp, GMP-pNHp, and UMP-pNHp) were from ICN. P1 was purchased from Yamasa Shoyu; bacterial alkaline phosphatase and pyruvate kinase were from Worthington Biochemicals.

RESULTS

Stimulation of CPV mRNA Synthesis *In Vitro* by Both AdoMet and AdoHcy. In the presence of AdoMet, purified CPV synthesizes viral-specific mRNA *in vitro* (2). In the absence of AdoMet, little (<3% of maximum) RNA synthesis takes place. In the previous study, when AdoMet and AdoHcy (i.e., methyl donor and methylation inhibitor, respectively), each at 0.1 mM, were added to the reaction mixture, the extent of mRNA synthesis was stimulated by 20%–30% compared to reactions in the presence of 0.1 mM AdoMet alone. In order to study this effect in detail, increasing amounts of AdoHcy were added to complete reaction mixtures that contained 0.2 mM AdoMet, a subsaturating concentration sufficient to stimulate transcription by 330-fold over the control level without AdoMet. As shown in Fig. 1*A*, the addition of AdoHcy stimulated further the incorporation of [^{3}H]UMP into virus mRNA. At 2.5 mM AdoHcy, RNA synthesis was promoted by about 2-fold (620-

Abbreviations: CPV, cytoplasmic polyhedrosis virus; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; AdoEt, *S*-adenosylethionine; AMP-pNHp, 5′-adenylyl-imidodiphosphate; GMP-pNHp, 5′-guanylyl-imidodiphosphate; UMP-pNHp, 5′-uridylyl-imidodiphosphate; m^{7}G, 7-methylguanosine; G^m, 2′-*O*-methylguanosine; A^m, 2′-*O*-methyladenosine.

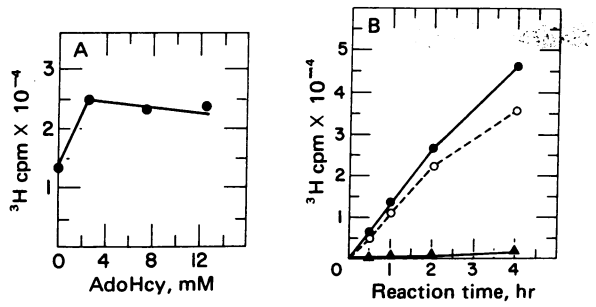

FIG. 1. Stimulation of CPV mRNA synthesis *in vitro* by AdoHcy. (*A*) CPV mRNA synthesis *in vitro* in the presence of AdoMet with increasing amounts of AdoHcy. Reaction mixtures (0.25 ml) contained 60 mM Tris·HCl (pH 8.0), 12 mM $MgCl_2$, 4 mM ATP, 2 mM CTP, 2 mM GTP, 1 mM [^{3}H]UTP (2 μCi), 0.2 mM AdoMet, 60 μg of bentonite, 5 mM phospho*enol*pyruvate, 3 units of pyruvate kinase, 30 μg of purified CPV, and different amounts of AdoHcy (0–12.5 mM). Incubation was carried out at 31° for 1 hr. The reaction was stopped by the addition of 5% cold trichloroacetic acid, and acid-precipitable radioactivity was assayed. (*B*) Time course of CPV mRNA synthesis in the presence of AdoMet (●) or AdoHcy (○). Reaction mixtures (0.1 ml) contained 50 mM Tris·HCl, 4 mM $MgCl_2$, 0.8 mM ATP, 0.4 mM CTP, 0.4 mM GTP, 0.4 mM [^{3}H]UTP, 10 μg of bentonite, 2 mM phospho*enol*pyruvate, 1.5 units of pyruvate kinase, 0.1 mM AdoMet (or 0.1 mM AdoHcy, or neither), and 1 μg of purified CPV. Incubation and assay for viral mRNA synthesis was performed as in *A*. Control (no additions).

Table 1. Effect of AdoHcy on CPV mRNA synthesis: Stimulation of RNA synthesis and inhibition of methylation

Conditions	Radioactivity incorporated in RNA: [^{3}H]-Methyl, cpm	Radioactivity incorporated in RNA: [^{32}P] GMP, cpm	Inhibition of methylation, %
Control	—	3,345	—
With 75 μM AdoMet*	4486	162,255	0†
With 750 μM AdoHcy	—	184,206	—
With 75 μM AdoMet* and 750 μM AdoHcy	513	152,444	89*

In each experiment, the reaction mixture (0.1 ml) contained 50 mM Tris·HCl (pH 8.0), 4 mM $MgCl_2$, 0.8 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 0.4 mM GTP containing 0.6 μCi of [α-^{32}P]GTP, 10 μg of bentonite, 2 mM phospho*enol*pyruvate, 1.5 units of pyruvate kinase, and 2 μg of CPV. Incubation was carried out at 31° for 6 hr. In an uninbated aliquot of reaction mixture, background levels of trichloroacetic acid-insoluble radioactivity were 203 and 319 cpm for ^{3}H and ^{32}P, respectively. These values were subtracted.

* The AdoMet included 10 μCi of [^{3}H-*methyl*]AdoMet.

† [^{3}H]Methyl radioactivity incorporated into CPV mRNA in the presence of 75 μM AdoMet was assumed as 100% methylation.

fold total stimulation), indicating that the effect of these compounds on RNA synthesis is additive. This result confirmed the previous observation that AdoHcy does not suppress the stimulatory effect of AdoMet on CPV mRNA synthesis. When AdoHcy was added to the CPV RNA synthesis reaction mixture in the absence of AdoMet, it stimulated CPV mRNA synthesis to almost the same extent as did AdoMet (Fig. 1*B*).

Inhibition of Methylation of CPV mRNA by AdoHcy. mRNA synthesis *in vitro* by cores of reovirus, another double-stranded RNA-containing virus, was not stimulated or inhibited by either AdoMet or AdoHcy (6). However, methylation of the RNA was prevented by the addition of an excess of AdoHcy to reaction mixtures containing AdoMet (6, 7). In order to determine if AdoHcy also inhibits CPV mRNA methylation, a 10-fold excess of AdoHcy over AdoMet was added to the mRNA synthesis incubation mixture. The experiment was carried out with [α-^{32}P]CTP to check RNA synthesis and [^{3}H-*methyl*]AdoMet for assaying methylation. In the presence of [^{3}H-*methyl*]AdoMet, RNA synthesis was stimulated about 50-fold over the control (Table 1). On the basis of specific activity of the precursors, it was calculated that the RNA was methylated to the extent of 2 methyl groups per 4000 nucleotides. When AdoMet and a 10-fold molar excess of AdoHcy were included in the reaction mixture, methylation was inhibited by about 90%, whereas RNA synthesis was not significantly inhibited (<7%). Because AdoHcy stimulated RNA synthesis quite well (55-fold) at the same concentration that blocked methylation, these results indicate that AdoHcy functions in two different ways: as a strong stimulator of RNA synthesis and as a potent methylation inhibitor.

It has previously been shown that CPV mRNAs synthesized in the presence of AdoMet contain 5′-terminal m^{7}GpppAmpG- (3) whereas the small amount of mRNA made in the absence of AdoMet contains the unblocked structure ppAp- (8). It was also found that the β-phosphate of the 5′-terminal residue of CPV mRNA is incorporated into the middle position in the 5′-cap structure m^{7}Gp*ppAm (3) and into the β-position in the unblocked structure p*pAp- (8). In order to analyze the 5′-terminal structure of CPV mRNA synthesized in the presence of AdoHcy, [β-^{32}P]ATP was prepared according to the method of Furuichi and Shatkin (9) and used to label mRNAs specifically at the 5′ termini. [^{3}H]UTP was included in the incubation mixture in order to label the RNAs internally and to check the precision of the subsequent enzymatic analyses. The RNA was digested to the constituent nucleosides, inorganic phosphate, and 5′-blocked structures (m^{7}GpppAm or GpppA) by treatment with P1 nuclease followed by bacterial alkaline phosphatase. The digests were analyzed by paper electrophoresis, in parallel with appropriate marker compounds.

As shown in Fig. 2*C*, ^{32}P radioactivity from the 5′ terminus of CPV mRNA split into two fractions, inorganic phosphate (63%) and a radioactive compound (37%) migrating with marker GpppA. Internal [^{3}H]pU was converted to uridine and stayed at the origin. The ^{32}P-labeled compound that migrated with marker GpppA was further identified by comigration with standard marker GpppA in high-voltage electrophoresis on DEAE-cellulose (data not shown). Therefore, CPV mRNAs synthesized in the presence of AdoHcy contained 5′-Gp*ppA (37%) and 5′-p*pA (63%). These results indicated that mRNAs synthesized with AdoHcy were not methylated and, more interestingly, were only partially blocked at the 5′ termini, which is in contrast to the mRNA synthesized in the presence of AdoMet (the 5′ terminus was almost completely blocked and methylated). The effect of AdoHcy—an increase in the proportion of RNA molecules with unblocked 5′ termini—will be discussed later.

Effect of AdoMet Analogues on CPV mRNA Synthesis *In Vitro*. As described in the previous sections, both methyl donor AdoMet and methylation inhibitor AdoHcy showed similar striking stimulation of CPV mRNA synthesis. Both compounds contain adenosine and an amino acid residue as fundamental structural units, and AdoMet also has a methyl group and a positive charge. Apparently, the absence of both a methyl group and a positive charge from AdoHcy did not prevent its stimulatory effect on CPV mRNA synthesis. Consequently, it was of interest to study which structural unit, adenosine or the amino acid residue, is more important for this unique effect on CPV

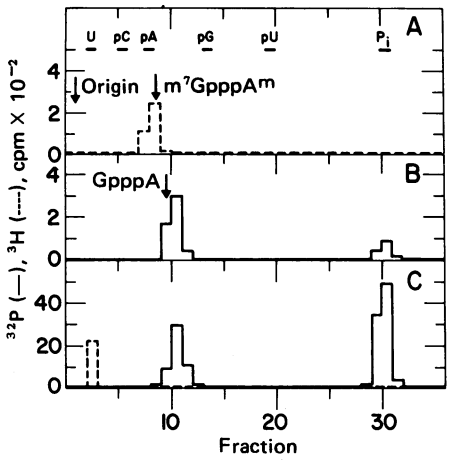

FIG. 2. Analysis of 5′ terminus of CPV mRNA synthesized in the presence of [β-^{32}P]ATP and AdoHcy. Reaction mixture (200 μl) contained: 75 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 0.75 mM ATP containing 45 μCi of [β-^{32}P]ATP, 2 mM CTP, 2 mM GTP, 2 mM UTP containing 0.1 μCi of [^{3}H]UTP, 40 μg of bentonite, 2.5 mM phospho*enol*pyruvate, 1.5 units of pyruvate kinase, 0.5 mM AdoHcy, and 40 μg of CPV. After 6-hr incubation at 31°, newly synthesized CPV mRNA was purified by passage through Sephadex G-100 and alcohol precipitated. The mRNA was digested completely by incubation with P1 nuclease followed by bacterial alkaline phosphatase (5). The digests were applied to Whatman 3MM paper, and, after electrophoresis for 60 min at 60 V/cm, 1-cm strips were cut and assayed for radioactivity in toluene-based scintillant. As standard marker, [^{3}H]methyl-labeled $m^7Gp\overset{*}{p}pA^m$ prepared from CPV mRNA synthesized in the presence of [^{3}H-*methyl*]AdoMet and ^{32}P-labeled $Gp\overset{*}{p}pA$ prepared from unmethylated vesicular stomatitis virus mRNA (10) were included. (*A*) m^7GpppA^m/ (*B*) GpppA; (*C*) CPV mRNA digested by P1 nuclease and phosphatase.

transcription. CPV mRNA synthesis was carried out in the presence of AdoMet, AdoHcy, AdoEt, adenosine, or methionine. As shown in Table 2, either AdoMet or AdoHcy (0.1 mM) stimulated transcription ~70-fold. AdoEt, another structurally similar analogue of AdoMet, was half as effective as AdoMet at its optimal concentration (0.5 mM). Replacement of the methyl group by an ethyl group apparently had an inhibitory effect on CPV transcription. The ethyl group may be too bulky to function in a key-and-keyhole model. Adenosine, which is common to AdoMet, AdoHcy, and AdoEt, also stimulated RNA synthesis 4- to 5-fold but at a higher concentration (0.5–1.0 mM). On the other hand, another constituent group, L-methionine, gave a 1.5-fold stimulation at a concentration of 0.5 mM. These results clearly indicate that the adenosine moiety in AdoMet (and its analogues) is more important than the methionine portion of the molecule and, moreover, the structural integrity of the adenosine–amino acid complex is required for an efficient stimulatory effect on RNA synthesis.

Mechanism of Stimulation of CPV mRNA Synthesis by AdoMet. There are present in CPV particles at least four enzyme activities that are involved in capped mRNA synthesis, including RNA polymerase, guanylyl transferase, methyl transferase, and nucleotide phosphohydrolase (2, 3, 11). In order to get some information about the possible relationship of capping to transcription, experiments were carried out with β,γ-imido analogues of ribonucleoside triphosphates. These analogues are utilized for chain elongation by the RNA polymerases associated with vesicular stomatitis virus and reovirus because they possess a normal α-β pyrophosphate linkage (12, 13). On the other hand, the imido group between the β- and γ-phosphates renders them inactive as substrates for nucleotide phosphohydrolase, an enzyme that cleaves the γ-phosphate of nucleoside triphosphates as part of the mechanism of cap formation (14). For example, reovirus cores can synthesize mRNA *in vitro* in the presence of one imido nucleotide and three normal ribonucleoside triphosphates. With AMP-pNHp or UMP-pNHp, reovirus cores produced mRNA containing 5′-terminal $m^7GpppGm$. In the presence of GMP-pNHp, the RNA products contained uncapped pNHp-pG 5′ termini due to the inability of nucleotide phosphohydrolase to yield a 5′-ppG terminus, the substrate for subsequent blocking catalyzed by guanylyl transferase (14).

Table 2. Effect of analogues of AdoMet and other compounds on CPV mRNA synthesis

Addition	mM	[^{3}H]UTP incorp., cpm	RNA synthesis, -fold incr.
Control		1,218	1.0
AdoMet	0.1	92,429	75.9
AdoHy	0.1	83,115	68.2
AdoEt	0.1	21,034	17.3
AdoEt	0.5*	40,226	33.0
Methyl-5′-thioadenosine	0.1	3,462	2.8
Methyl-5′-thioadenosine	0.5*	4,410	3.6
Adenosine	0.1	2,492	2.1
Adenosine	0.5*	6,505	5.3
L-Methionine	0.1	1,597	1.3
L-Methionine	0.5*	1,659	1.4
Guanosine	0.1	1,272	<1.0

Reaction conditions were essentially the same as in Table 1 except that 80 μCi of [α-^{32}P]CTP instead of [α-^{32}P]GTP and 0.01 μg of CPV instead of 2 μg were used. Besides the basic components for RNA synthesis, the reaction mixture in each experiment contained AdoMet or its analogue at the concentration indicated. Incubation was performed for 3 hr at 31°.

* Experiments that resulted in maximal stimulation at the concentrations (0.05–2 mM) tested.

In the following experiments with CPV, one species of each ribonucleoside triphosphate was replaced by the corresponding imido analogue, and mRNA synthesis as well as methylation were investigated by including [^{3}H-*methyl*]AdoMet and [α-^{32}P]CTP in the reaction mixtures. When GTP or UTP was replaced by GMP-pNHp or UMP-pNHp, respectively, both mRNA synthesis and methylation proceeded at reduced rates (Fig. 3 *C* and *D* compared to the control (Fig. 3*A*). However, when ATP was replaced by AMP-pNHp, little or no mRNA synthesis or methylation was observed (Fig. 3*B*). That this was not due to the presence of a nonspecific inhibitor contained in the AMP-pNHp preparation or to the inability of CPV RNA polymerase to utilize the analogue was clearly shown in the following experiment. *In vitro* synthesis of CPV mRNA was driven for a short time (2 min) in the presence of normal ribonucleoside triphosphates and AdoMet. Then, the reaction was stopped by chilling the reaction mixture on ice followed by dilution with 26 volumes of cold 10 mM Tris·HCl buffer (pH 8.0). This short-time reaction allows CPV to accumulate template-bound nascent small mRNAs (~4S), whereas completed, released mRNA molecules are 16–23 S (2). CPV containing the nascent RNAs was collected from the reaction mixture by centrifugation and reincubated in a second RNA-synthesizing reaction mixture in which ATP was replaced by AMP-pNHp. Fig. 4 demonstrates that only the CPV that had been incubated with normal ribonucleoside triphosphates and AdoMet in the first reaction was able to continue RNA synthesis in the second incubation. When ATP was replaced by AMP-pNHp or Ado-

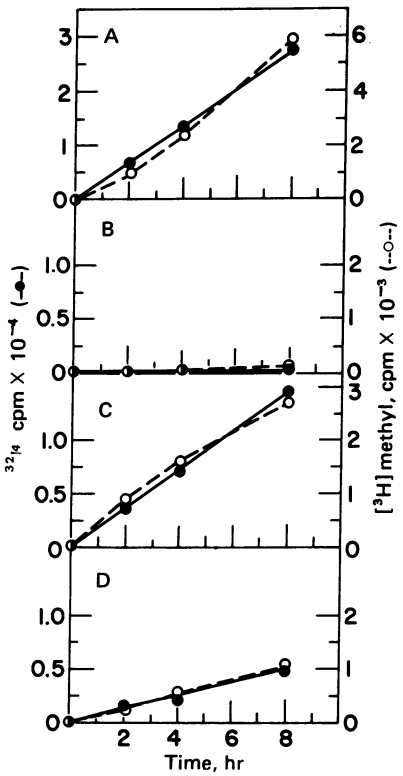

FIG. 3. CPV RNA synthesis with β,γ-imido analogues of ribonucleoside triphosphates. (*A*) Reaction mixture (0.1 ml) contained 50 mM Tris·HCl (pH 8.0), 4 mM $MgCl_2$, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM UTP, 0.4 mM CTP with 1.4 μCi of [α-^{32}P]CTP, 60 μM [^{3}H-*methyl*]AdoMet (20 μCi), 2 mM phospho*enol*pyruvate, 1.5 units of pyruvate kinase, 10 μg of bentonite, and 2 μg of CPV and was incubated at 32°. At the indicated times, acid-precipitable, labeled RNA was measured. For (*B*), (*C*), and (*D*), the same reaction condition was used except that the indicated ribonucleoside triphosphate was replaced by its imido analogue: *B*, AMP-pNHp; *C*, GMP-pNHp; *D*, UMP-pNHp (note change in scales on ordinates).

Met was absent in the first reaction, no mRNA was synthesized in the second reaction mixture with AMP-pNHp. These results suggest that CPV RNA polymerase has the potential to utilize AMP-pNHp for RNA chain elongation but cannot initiate RNA synthesis with the nonhydrolyzable analogue.

The results in Fig. 3 suggest that the synthesis of CPV mRNA requires, in addition to AdoMet, some reaction step that is involved in cap formation—for example, removal of the γ-phosphate from ATP by the virion-associated nucleotide phosphohydrolase to produce 5′-ppA (11), or a condensation of pG (from pppG) and 5′-ppA by guanylyl transferase. Replacement of ATP by AMP-pNHp at the 5′ terminus woud make removal of the γ-phosphate impossible and consequently prevent the capping reaction. More importantly, the inability to form caps apparently prevents the subsequent mRNA synthesis entirely, although CPV mRNA polymerase can utilize imido nucleotides. Thus, CPV mRNA synthesis appears to be tightly coupled with cap formation in a way that makes cap synthesis, or at least some step involved in cap formation, obligatory for the commencement of RNA synthesis.

DISCUSSION

Previously it was found that mRNA synthesis *in vitro* by CPV was dependent upon the presence of the methyl donor AdoMet (2). In this report, we describe additional interesting observations concerning this unique viral transcription system. The conclusions from each subsection are as follows.

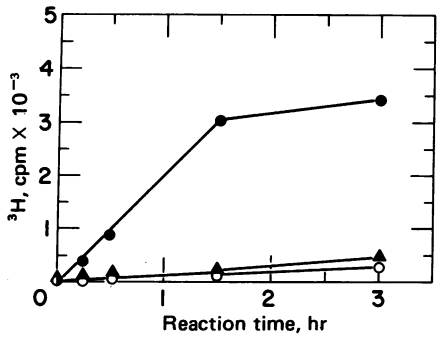

FIG. 4. CPV mRNA synthesis in the presence of ATP analogue AMP-pNHp. *In vitro* synthesis of CPV mRNA was carried out in two steps. After the first incubation at 31° for 2 min, the reaction mixture was chilled on ice and diluted with 26 volumes of cold 10 mM Tris·HCl (pH 8.0). The virus particles were isolated from the diluted reaction mixture by centrifugation (SW 50.1, 30,000 rpm, 90 min, 4°). The virus pellet was rinsed once with 10 mM Tris·HCl (pH 8.0) and was resuspended in the second reaction mixture for continuation of RNA synthesis. The first reaction mixture (25 μl) contained 50 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.2 mM UTP, 0.1 mM AdoMet, 6 μg of bentonite, and 0.1 μg of purified CPV (●). Two controls were included, one without AdoMet (▲), and one containing AMP-pNHp (0.4 mM) in place of ATP (○). The second reaction mixture (200 μl) contained 50 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 0.8 mM AMP-pNHp, 0.2 mM GTP, 0.2 mM CTP, 0.05 mM UTP, and 0.1 mM AdoMet. Incubation was carried out at 31°. A 40-μl aliquot was extracted and trichloroacetic acid-precipitable ^{3}H radioactivity was determined. [^{3}H]UTP was included only in the second reaction mixture.

(*i*) AdoHcy, which is known to be a competitive inhibitor of methylation by AdoMet, was found to stimulate CPV mRNA synthesis as efficiently as AdoMet.

(*ii*) Although AdoHcy stimulated CPV mRNA synthesis, it inhibited methylation of mRNA. The 5′ termini of the mRNA synthesized in the presence of AdoHcy were mixtures of GpppA- (37%) and ppA- (63%), whereas the mRNA synthesized in the presence of AdoMet contained exclusively m^7GpppAm-. An increase in the proportion of unblocked termini in unmethylated mRNA probably resulted from the reverse reaction upon blocked mRNA by viral guanylyl transferase—i.e., GpppAp- + PPi → GTP + ppAp—. Similar observations have been made for reovirus mRNA in which mRNA synthesized in the presence of AdoMet contained a higher ratio of blocked versus unblocked 5′ termini than RNAs produced in the presence of AdoHcy (7, 15). The addition of inorganic pyrophosphatase, which decreases the concentration of pyrophosphate in the reaction mixture, increased the ratio of blocked versus unblocked 5′ termini of reovirus mRNA (15). A similar observation was also obtained in CPV mRNA synthesis in the presence of AdoHcy. With addition of inorganic pyrophosphatase, the level of 5′-terminal blocked structure of CPV mRNAs was enhanced [86% blocked 5′ termini and 14% unblocked 5′ termini (unpublished data)].

(*iii*) AdoEt and adenosine also stimulated CPV mRNA synthesis. This is probably related to their structural similarity to the natural stimulator, AdoMet. How, then, can AdoMet and these AdoMet analogues that are not direct substrates for RNA synthesis cause stimulation of transcription? Although there is no clear answer available, these observations suggest a model in which the presence of the substrate AdoMet, or a similar molecule, at the active site of the methyl transferase induces a conformation change in the enzyme molecule. This change allows cap formation and subsequently RNA synthesis to proceed, possibly as a result of an interaction between cap-forming proteins(s) and RNA polymerase.

(*iv*) CPV, reovirus, and vaccinia virus (16) apparently share similar mechanisms of cap formation. However, CPV appears to have an additional regulatory system in which AdoMet "switches on" mRNA synthesis, probably as an allosteric effector that influences the RNA polymerase and capping enzymes. In the experiment designed to determine the relationship between cap formation and RNA synthesis, in which β,γ-imido nucleoside analogues were used together with AdoMet, only AMP-pNHp (the nucleotide species at the 5′ end of CPV mRNA) failed to support methylation and RNA synthesis. This probably derived from the inability of AMP-pNHp, as a replacement for 5′-ATP, to be processed by nucleotide phosphohydrolase, an enzyme involved in cap formation. Therefore, the results are interpreted as indicating that the presence of AdoMet is not adequate for the stimulation of CPV RNA synthesis. Some condition that allows cap formation—for instance, the production of 5′-ppA by nucleotide phosphohydrolase—is also required for stimulation of RNA synthesis.

In CPV transcription, AdoMet functions as a methyl donor for as well as an allosteric-like stimulator of mRNA synthesis. This effect may involve the release of viral RNA polymerase from a repressed state on the genome. Consistent with this idea are observations that the extent of appearance of AdoMet-insensitive CPV (i.e., virus that is able to synthesize mRNA in the absence of AdoMet) is variable from one preparation to another. In addition, repeated pelleting of viruses by high-speed centrifugation during purification increases the proportion of AdoMet-independent virions (unpublished data). This "constitutive type" of CPV might be due to structural alterations in the viral enzyme complexes due to physical damage occurring during pelleting.

With influenza virus *in vitro* transcription, McGeoch and Kitron (17) and Krug *et al.* (18) found that oligonucleotides such as ApG, GpC, and GpG efficiently stimulated mRNA synthesis *in vitro*. Apparently, these dinucleotides functioned as initiators in a manner similar to polynucleotide formation by primer-dependent polynucleotide phosphorylase of *Micrococcus luteus* (19). In preliminary experiments, the addition of cap precursors such as pppApG, ppApG, GpppA, GpppAm, and m^7GpppA to the CPV mRNA-synthesizing system did not stimulate mRNA synthesis (unpublished data). Shimotohno and Miura (20) have reported that 5′-GpppA was produced in the partial reaction and subsequently methylated to m^7GpppA(m); however, both compounds apparently could not precede CPV mRNA synthesis. Inability of these cap precursors to replace AdoMet in the stimulation of CPV mRNA synthesis may imply that CPV RNA polymerase, for its full function, is not missing an initiator but requires the removal of some barrier, that occurs by an unknown mechanism in the presence of AdoMet or AdoHcy.

Recently, we reported (21) that the 5′ cap (or 5′-blocked structure) affords reovirus mRNAs stability by protecting them from degradation by exonuclease(s) which cleaves uncapped RNA in a 5′-to-3′ direction. Consistent with this function of the cap, Perry and Kelley (22) also have observed that caps in L cell nuclear RNA are conserved during processing to form mRNA. Thus, addition of caps to nuclear transcripts at an early stage of cellular mRNA synthesis (as in CPV mRNA synthesis) and their retention during processing would result in protection of 5′-terminal sequences that are known to be important for subsequent formation of translational initiation complexes in the cytoplasm (23).

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