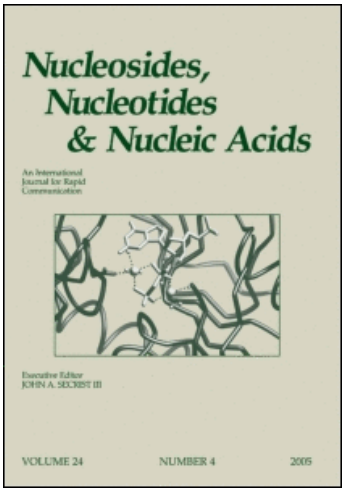

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Synthesis of Guanylyl (3'→5')-5-methylcytidine (Gpm⁵C), a Dinucleoside Monophosphate which is Unable to Function as a Primer in the Synthesis of RNA by the Influenza A Virus RNA Polymerase

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SYNTHESIS OF GUANYLYL (3'→5')-5-METHYLCYTIDINE (Gpm^5C), A DINUCLEOSIDE MONOPHOSPHATE WHICH IS UNABLE TO FUNCTION AS A PRIMER IN THE SYNTHESIS OF RNA BY THE INFLUENZA A VIRUS RNA POLYMERASE

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Abstract - Guanylyl (3'→5')-5-methylcytidine (Gpm^5C) has been synthesized enzymatically through the use of T_1RNAse at high enzyme dilution. In contrast with GpC, the methylated dinucleoside monophosphate is shown to be inactive as a primer for RNA synthesis by the RNA-dependent RNA polymerase of Influenza A virus.

Synthesis of RNA can be initiated in one of two ways by the RNA polymerase enzyme present in the Influenza A virus. The first, which appears to be the normal physiological mechanism involves the use of cell messenger RNA (mRNA) molecules capped at their 5' termini. The mRNA molecules are cleaved by a unique endonuclease activity of the Influenza A polymerase to give primer fragments containing 5'-terminal methylated caps ($m^7GpppNm$) which are 10-13 nucleotides long[1,2,3,4]. Synthesis of new individual RNA molecules from all eight viral RNA templates appear to commence at the penultimate residue (C) of the templates which all end in a -GpCpU terminus (3')[5,6,7,8]. The mechanism of this type of initiation is shown in Scheme 1.

An alternative mode of initiation which has been investigated under in-vitro conditions and which is well documented, involves the use of dinucleoside monophosphate primers ApG, GpG and GpC[9,10], which appear to initiate RNA synthesis at the -GpCpU(3') termini in all eight viral RNA templates and are incorporated into the newly synthesized RNA chains (Scheme 2).

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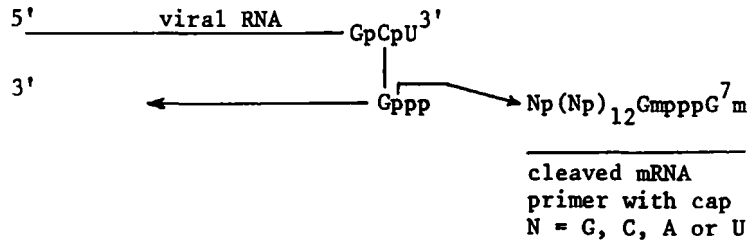
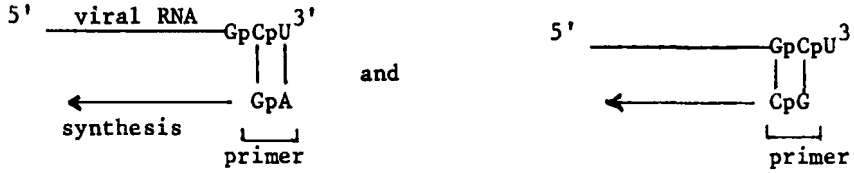


SCHEME 1



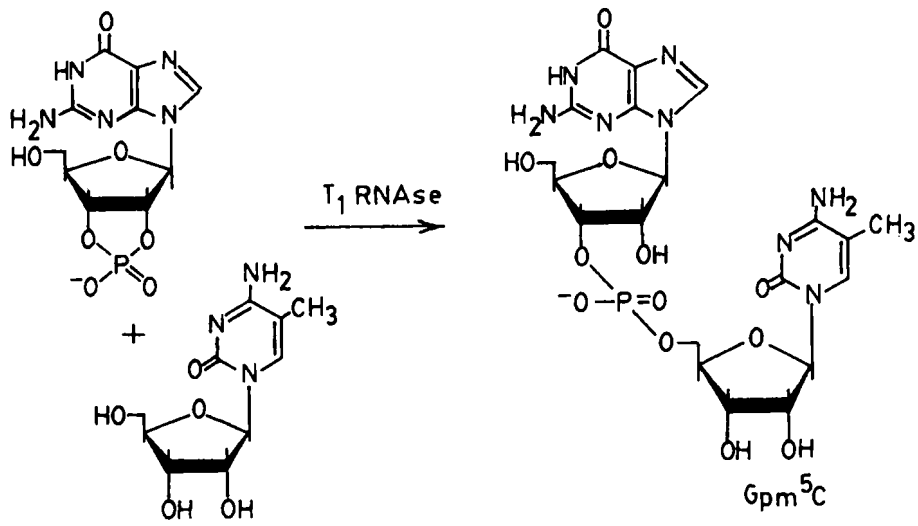
SCHEME 2

We are studying the priming/initiating action of these dinucleoside monophosphates with the object of furthering our understanding of the priming reaction in the Influenza A virus system and for the purpose of synthesizing modified dinucleoside monophosphates as possible inhibitors of the polymerase catalysed reactions. We are particularly interested in substitution at the 5 position of the cytosine ring in GpC, and therefore synthesized the dinucleoside monophosphate, Gpm^5C (Scheme 3) which has a 5-methylcytidine in place of cytidine and have studied the effects of this compound on the priming of RNA synthesis in the Influenza A virus system.

MATERIALS AND METHODS

Guanylyl (3'→5')-5-methylcytidine (Gpm^5C)

The synthesis of this dinucleoside monophosphate (Scheme 3) was carried out enzymatically with T_1 RNAse at high enzyme dilution[11]. Guanosine-2',3'-cyclic phosphate (16 mg, 48 μmole) was dissolved in distilled water, neturalized to pH 7.6 (dilute KOH) and lyophilized. To the lyophilized cyclic phosphate was added 90 mg (350 μmole) of 5-methyl-cytidine dissolved in 1.6 ml of 0.01 M sodium phosphate buffer (pH 7.6). The reaction mixture was cooled in ice, and to it was added a cold aqueous solution of T_1 RNAse (100 μl;2.5 μg T_1 RNAse). After careful mixing,



SCHEME 3

the reaction was allowed to proceed at 5°C for 48 hours. The reaction mixture was then diluted to 4.0 ml with water and fractionated on a DEAE-cellulose (52) column (1.8 x 40 cm) using a linear triethylamine-HCO_3^- gradient, pH 7.5 (0 - 0.3 M) and collecting 5 ml fractions. The peak eluting at 0.13 - 0.21 M was pooled and concentrated to dryness under vacuum at 37°C. The resulting solid was repeatedly treated with water and methanol, followed by evaporation as described. The final residue was dissolved in 4 ml water and fractionated on a DEAE-Sephacel column (1.8 x 30 cm) using a linear triethylamine-HCO_3^- gradient, pH 7.7 (0 - 0.25 M). The required Gpm^5C eluted in peak 1 (Fig. 1). This material was pooled and taken to dryness as described and stored at -15°C. Yield - 11 mg, 39%.

Treatment of the Gpm^5C with T_1 RNAse followed by TLC chromatography on silica gel $60F_{254}$ plates using ethanol-1M NH_4OAc, pH 7.5 (5:2 v/v) solvent gave two spots corresponding to guanosine-3'-phosphate and 5-methylcytidine. Analysis of the separated material by UV spectra gave a Gp to m^5C ratio of 1:1.07.

The ^1H-NMR spectrum at 80 MHz of Gpm^5C (0.014 M in D_2O with 0.013 M 3-(trimethylsilyl)-D_4-propionic acid sodium salt as internal reference) was recorded in the Fourier transform mode. Guanosine residue: H8, 8.02 (s, 1H); H1', 5.86 (s, 1H) ppm. 5-Methylcytidine residue: H6, 7.67 (s, 1H), H1', 5.76 (s, 1H);5-CH_3, 1.51 (s, 3H) ppm. The dinucleoside was present

as the mono-triethylammonium salt, ethyl CH_3-, 1.28 (t, 9H);ethyl -CH_2-, 3.2 (q, 6H) ppm.

Growth and purification of Influenza A virus

The Influenza A virus (Brazil/78/H1N1) was grown in embryonated eggs. Isolation and purification of the virus from the allantoic fluid was carried out by centrifugation of the allantoic fluid followed by sucrose-gradient centrifugation of the virus[9].

System for RNA synthesis by Influenza A virus

For studying the priming action of different dinucleoside monophosphates on RNA synthesis by the Influenza A polymerase, the incubation mixture contained - 66.6 mM Tris-HCl (pH 8.2), 200 mM KCl, 2 mM $MnCl_2$, 8 mM $MgCl_2$, 20 mM mercaptoethanol, 0.66% Nonidet P40, 2.7 mM ATP, 0.13 mM UTP, 0.26 mM GTP, 0.53 mM CTP, 12.5 μCi [^{3}H] CTP (20 Ci/mmole), 0.5 mM primer (ApG, GpC or Gpm^{5}C) and Influenza A virus (17 μg RNA) in a final volume of 180 μl. Incubations were at 30°C. Aliquots (30 μl) were taken at various times, and carrier t-RNA (100 μg) added, followed by cold 10% TCA. Samples were allowed to stand for 20 minutes at 5°C, washed thoroughly with cold 5% TCA and ethanol on GF/C filters, dried and counted.

RESULTS AND DISCUSSION

The Influenza A virus contains its own RNA-dependent RNA polymerase enzyme. On disruption of the virus particles with the non-ionic detergent Nonidet P40, the solubilized polymerase is able to transcribe the viral RNA templates and synthesize complementary copies of RNA. The synthesis of RNA in these preparations is strongly stimulated by the addition of the dinucleoside monophosphates ApG, GpG and GpC which appear to act as primers at the 3'-termini of the viral RNA molecules (Scheme 2). We are interested in synthesizing primers of the GpC variety in which the cytosine ring of the dinucleoside monophosphate is modified at its 5 position (Scheme 3). A dinucleoside monophosphate of this type should hydrogen bond normally with the viral RNA templates. However, it is possible that such a primer might interfere with RNA synthesis by

interaction with the polymerase or alternately inhibit its movement along the RNA template.

The synthesis of guanylyl(3'→5')-5-methylcytidine (Gpm^5C) is outlined in Scheme 3.

In order to test this idea, we synthesized Gpm^5C through the action of T_1 RNAse at high enzyme dilution, and carefully purified the compound by DEAE-52 and DEAE-Sephacel column chromatography. The fractionation on DEAE-Sephacel is shown in Fig. 1. The desired Gpm^5C eluted in peak 1. A sample of this material when subjected to HPLC (Zorbax-NH_2) gave a single peak (see inset of Fig. 1).

The HPLC was on a Zorbax-NH_2 column (4.6 mm x 25 cm) with 0.16 M triethylammonium acetate (pH 6.2) in 10% acetonitrile.

The results showing the incorporation of [^{3}H] CTP into RNA by a Nonidet P-40 disrupted viral preparation in the presence of different dinucleoside monophosphate primers is given in Fig. 2(a). Both GpC and ApG were active as primers, the latter showing the greater stimulation. In these experiments, the methylated dinucleoside monophosphate (Gpm^5C) showed no priming activity over a period of 40 minutes of incubation. Further, the very limited incorporation of [^{3}H] CTP observed in the presence of Gpm^5C was always lower than the endogenous incorporation (no added primer). Similar results were obtained with a number of different viral preparations.

In experiments where Gpm^5C was added with either GpC or ApG at the start of the reaction (Fig. 2b), the methylated dinucleoside monophosphate

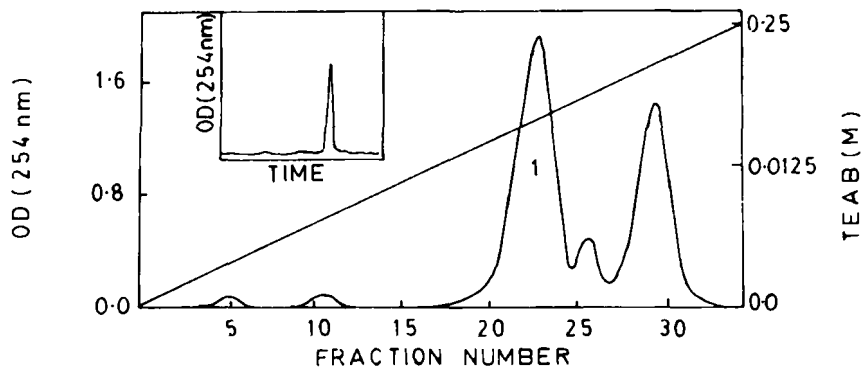


FIG. 1 DEAE-Sephacel chromatography of Gpm^5C

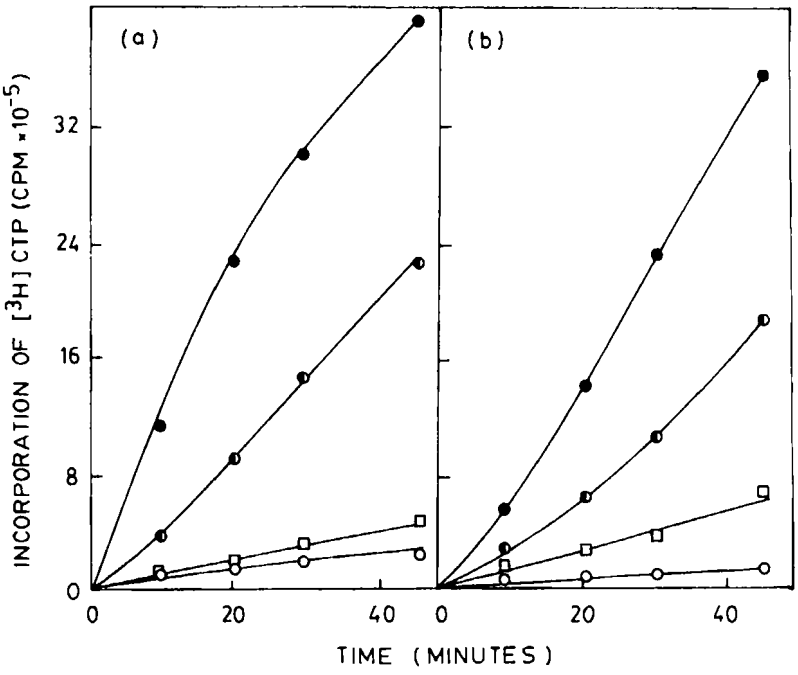


Fig. 2. Priming of RNA synthesis by dinucleoside monophosphates in the Influenza A virus system. (a) ApG,●; GpC,◐; Gpm^5C,○; and endogenous, □; (b) Gpm^5C added with other dinucleotides at zero time. ApG,●; ApG+Gpm^5C, □; GpC,◐; GpC+Gpm^5C,○.

inhibited the incorporation of $[^3H]$ CTP into RNA to a considerable extent. In the case of ApG plus Gpm^5C, the inhibition observed was approximately 85%, whereas with GpC plus Gpm^5C, the inhibition was 95%. The results clearly indicate that Gpm^5C is unable to prime or initiate the synthesis of RNA. The reason for this is not known. Molecular models show that Gpm^5C hydrogen-bonds normally with CpG of an RNA strand, and that the 5-methyl group of the C residue projects to the outside of the helix. We suggest that Gpm^5C possibly interferes with the interaction and movement of the polymerase enzyme along the RNA template.

The present findings on the inhibition of Influenza A virus RNA synthesis by Gpm^5C, a dinucleoside monophosphate modified in the 5 position of the cytosine ring, suggests the design and synthesis of useful drugs of this type as possible agents for interfering with the replication of Influenza A virus. The transport of such compounds into cells is

obviously a problem. This would have to be taken into account when planning the design of the drug.

It is of interest to note that 5-methylcytidine is found in the DNA of a number of eukaryotic cells where it occurs principally in the sequence $-m^5CpG-$ which is present as a palindrome in double-stranded DNA and occasionally in the alternative palindromic sequence $-GGm^5CC-$. There is considerable evidence to indicate and suggest that 5-methylcytidine serves as a regulatory signal in eukaryotic gene expression[12,13]. It would be premature at this stage of the experimental work to suggest a connection between our findings with the Influenza A virus system and the possible involvement of 5-methylcytidine in gene regulation of eukaryotic cells.

In a recent review on the structure and function of the Influenza virus genome, Mc Cauley and Mahy[14] discuss the work of Kawakami and Ishihama (unpublished) which is aimed at further purification of the Influenza viral genome ribonucleoprotein complex. The Japanese workers have managed to purify a ribonucleoprotein consisting of three polymerase protein subunits associated with the viral RNA. Of considerable interest is their finding that the complex can transcribe viral RNA in the presence of 3'-terminal complementary dinucleoside monophosphate primers. This mode of initiation of RNA synthesis is depicted in Scheme 2 of the present communication.

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