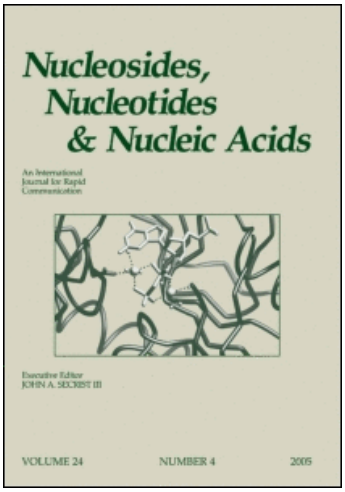

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Synthetic Templates and the RNA Polymerase of Influenza a Virus

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SYNTHETIC TEMPLATES AND THE RNA POLYMERASE OF INFLUENZA A VIRUS

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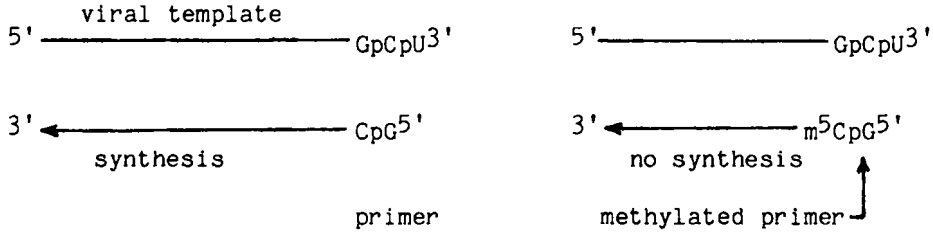
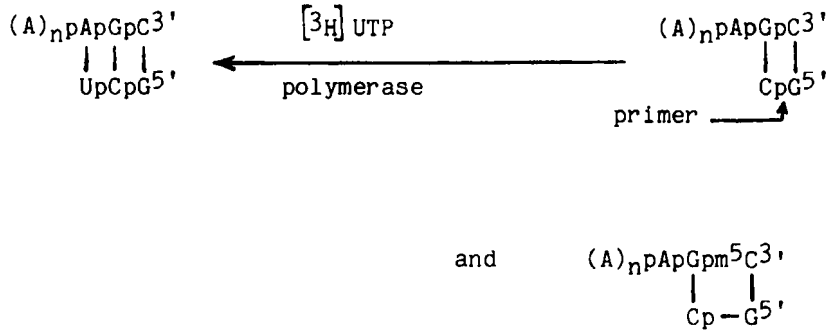
Abstract - Polyadenylic acid (poly A) and polyguanylic acid (poly G) have been modified to give polymers containing GpC and Gpm^5C termini. Polymers containing methylated (Gpm^5C) termini are inactive as templates for the RNA - dependent RNA polymerase of Influenza A virus.

In a previous communication[1], it was shown that the methylated dinucleoside monophosphate (Gpm^5C) did not function as a primer for the initiation of RNA synthesis using the Influenza A viral RNA polymerase with viral RNA templates. All eight RNA molecules comprising the Influenza virus genome end in a -GpCpU terminus (3')[2,3,4], thus allowing one to use an <u>in vitro</u> assay system for studies on the initiation of RNA synthesis as depicted in Scheme 1.



SCHEME 1



SCHEME 2

In the present work it was considered of interest to ascertain whether (i) the RNA template component of the system (Scheme 1) could be modified to contain a 5-methylcytidine residue at its 3' terminus and (ii) whether such a modified RNA would function as a template with the viral polymerase. For this purpose we made use of poly(A) and poly(G) which were modified through enzymatic and chemical methods to give $(A)_npGpC$, $(A)_npGpm^5C$, $(G)_npC$ and $(G)_npm^5C$. With poly(A)-containing templates, the assay system contained detergent disrupted virus (RNA polymerase), GpC primer and [^{3}H] UTP. This assay system is shown in Scheme 2.

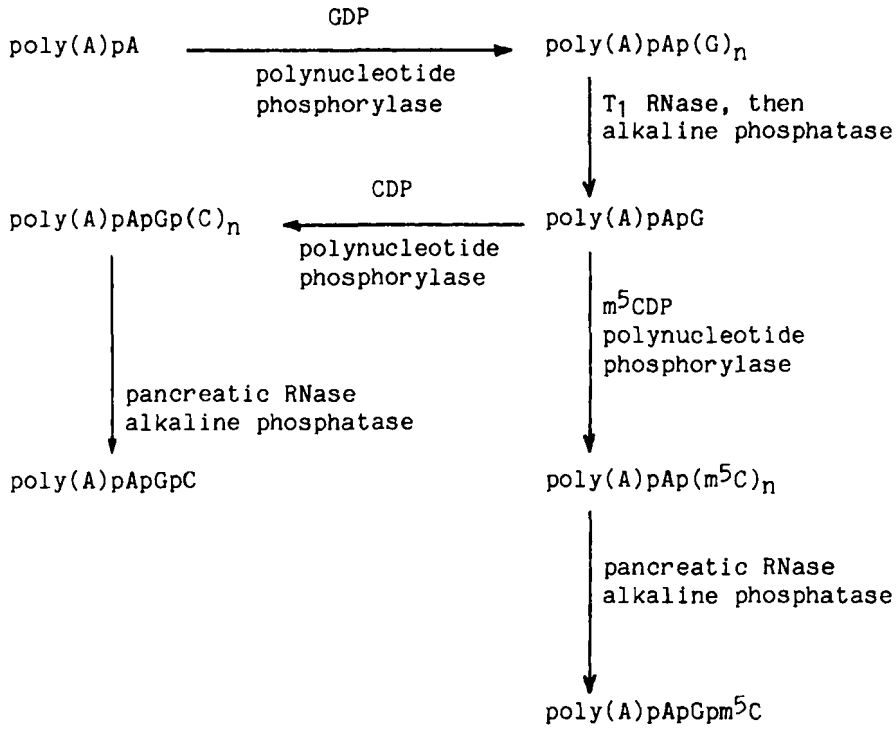
With $(A)_npApGpm^5C$ as the template, reaction conditions were as above. In the case of poly(G)-modified templates, on the other hand, the assay was similar but with [^{3}H] CTP as the labelled precursor. The preparation of modified templates (Scheme 3) and their use with influenza A viral RNA polymerase are reported in this communication.

MATERIALS AND METHODS

All enzymes were obtained from P-L Biochemicals.

Synthesis of poly(A)pGpC

The synthesis of poly(A) containing a GpC group at its 3' end was carried out using enzymatic methods (Scheme 3).



SCHEME 3

Poly(A)$(G)_n$: Each reaction mixture contained 10mM $Mg(OAc)_2$, 0.1M $CuSO_4$, 200 mM tris-HCl (pH 8.0), 0.12 M NaCl, 100 µg (1 nmole) poly A, 300 µg (670 nmole) GDP, 5µCi [^{3}H] GDP (specific activity, 15.4 Ci/mmole) and 10 µg polynucleotide phosphorylase (Micrococcus lysodeicticus) in a total volume of 0.5 ml. Incubation was carried out at 37°C for 3.5 hours. Aliquots (25 µl) were taken at various times to monitor the reaction. To each aliquot was added 100 µg of tRNA (carrier) and the mixture precipitated with cold 10% TCA (tricholoroacetic acid). Precipitates were collected on Whatman GF/C filters and washed with 25 ml of cold 5% TCA followed by 10 ml of 96% ethanol. Filters were dried and radioactivity determined by scintillation counting using a toluene-based counting fluid. To the remaining reaction mixture (0.35 ml) was added 0.18 ml water followed by 0.6 ml of 90% phenol. The aqueous phase from the phenol

extraction was recovered and to it was added 0.1 volume of 20% KOAc and 2.5 volumes of cold ethanol. The precipated polymer was collected by centrifugation, dissolved in distilled water and exhaustively dialysed against distilled water. RNA polymers were stored in solution at -15°C or as lyophilized powders. Further purification of modified RNA polymers was also carried out by gel filtration on columns of Sephadex G25 or G200 (1 x 45 cm) using 0.75 M NH_4HCO_3 (pH 7.5) as eluent.

Poly(A)pG: To 300 µg of [^{3}H] poly(A)$(G)_n$ in 0.5 ml of 0.05M tris-HCl (pH7.6) containing 1mM $MgCl_2$ was added 20 units of T_1 RNase and the resulting mixture incubated at 37°C for 3 hours. Loss of [^{3}H] guanosine residues was monitored by acid-precipitable radioactivity as described above. After the T_1 RNase treatment, alkaline phosphatase (E. coli) was added and the incubation continued at 37°C for 1 hour. The modified polynucleotide was then extracted with phenol and ethanol precipitated. The precipitated RNA was taken up in a small volume of distilled water, heated at 60°C for 5-7 minutes to destroy any traces of alkaline phosphatase and then dialysed against water. The final solution was stored at -15°C.

Poly(A)pGpC: Cytidine residues were incorporated into poly(A)pG using polynucleotide phosphorylase (Micrococcus) and a mixture of CDP and [^{3}H] CDP under the same reaction conditions as used for the addition of guanosine residues to poly(A). The final product was phenol extracted, ethanol precipitated and dialysed against water. Pancreatic RNase treatment of the polymer was carried out as follows: the reaction mixture in a final volume of 1.5 ml contained 170mM tris-HCl (pH 8.0), 66mM $CuSO_4$, 70 mM $MgCl_2$, 90mM NaCl, poly(A)pGp$(C)_n$, and bovine pancreatic RNase (20 units). Incubation was at 37°C for 45 minutes, the course of the reaction being monitored by loss of TCA-precipitable radioactivity. Following pancreatic RNase treatment, the mixture was treated with alkaline phosphatase (3 units) at 37°C for 45 minutes. Extraction with

phenol, ethanol precipitation and dialysis against water was as described previously.

Synthesis of Poly (A)pGpm^{5}C

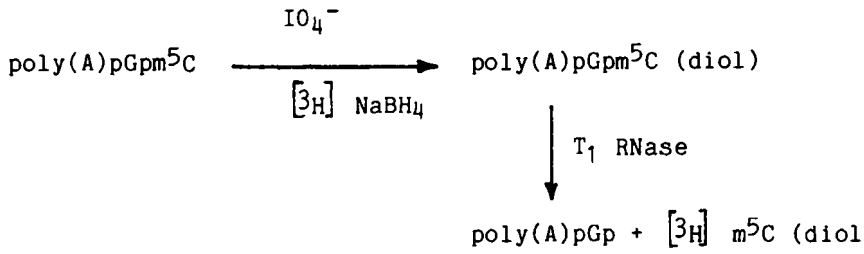
5-Methylcytidine-5'-diphosphate (m^5CDP): A suspension of 5-methylcytidine (91 mg, 0.31 mmole) in 1.0 ml of triethylphosphate was cooled in ice and to it was added 0.6 ml of $POCl_3$ (0.4 mmole). The resulting mixture was allowed to stand at 4°C for 3 hours. A slurry of 20 ml of ice-water-pyridine (1:1;1, v/v/v) was then added and the resulting solution concentrated to approximately 5 ml by evaporation under reduced pressure. Water (10 ml) was added and the evaporation repeated. The resulting concentrated solution was extracted three times with $CHCl_3$ and the aqueous phase then adjusted to pH 10 with 1M LiOH. A precipitate was removed by centrifugation and the supernatant taken to dryness to give a glassy residue. This was taken up in 1.0 ml of water and applied to a DEAE- 52 cellulose column (1 x 45 cm). Elution was carried out with a linear gradient of 0-0.35M triethylamine bicarbonate (pH 7.5). Four main fractions were eluted from the column in the order, 1 and 2 (unreacted m^5C), fraction 3 (m^5CMP) and fraction 4 (m^5CDP). The method of preparation used usually gives the nucleoside-5'-monophosphate in good yield[5]. In our hands, we also observed formation of the 5'-diphosphate (m^5CDP) in a yield (10%) which was satisfactory for our purpose. m^5CDP was identified by (a) TLC chromography on silica gel $60F_{254}$ plates using solvent A (isopropanol-ammonia-water, 7:1:2 v/v/v), solvent B (96% ethanol-1M ammonium acetate, 5:2, v/v) and solvent D (96% ethanol-1M triethylamine HCl, 5:2, v/v), and chromatography on polyethyleneimine (PEI) cellulose with solvent C (1.0M LiCl)[6]; (b) u/v spectra and (c) determinationn of acid-labile to total phosphate which gave a ratio of 1:1.9.

Poly(A)pGpm^{5}C: This was prepared from poly(A)pG and 5-methylcytidine-5'-diphosphate (m^5CDP) in the presence of polynucleotide phosphorylase and subsequent treatment with

pancreatic RNase and alkaline phosphatase. A reaction mixture containing poly(A)pG (100 μg), 0.6 mM m^5CDP (sodium salt), 0.2M tris-HCl (pH 8.0), 10 mM $Mg(OAc)_2$, 100 mM $CuSO_4$, 120 mM NaCl and 0.1 units *E. coli* polynucleotide phosphorylase in a final volume of 80μl was incubated at 37°C for 4.5 hours. The mixture was extracted with phenol, ethanol precipitated and then dialysed exhaustively against distilled water. Treatment with pancreatic RNase and alkaline phosphatase was carried out as described above for the preparation of poly(A)pG. Poly(A)pGpm^5C after dialysis was stored at -15°C.

Determination of 5-methylcytidine in RNA polymers:

As radioactive m^5CDP was not available, an indirect method was used to determine 5-methylcytidine at the 3' terminus of modified RNA polymers (Scheme 4). Briefly, polymers such as poly(A)pGpm^5C and poly(G)pGpm^5C were treated with periodate and then reduced with $[^3H]$ $NaBH_4$. The labelled polymer was finally digested with T_1 RNase releasing m^5C as a labelled 2'3'-dialcohol which could be identified by TLC and autoradiography using standards[7]. Experimentally this was carried out as follows: Poly(A)pGpm^5C or poly(G)pGpm^5C (28 μg) in 50 μl of water was added to 15 μg of 9mM $NaIO_4$ and the reaction allowed to proceed in the dark at 25°C for 2 hours. The reaction mixture was then cooled briefly on ice and to it was added 1μl of 1M K_2HPO_4 followed immediately by 10 μl of $[^3H]$ $NaBH_4$ (0.1M). After standing in the dark at 25°C for 2 hours, 200 μl of 1M acetic acid was added to destroy excess unreacted borohydride. The reaction mixture was finally evaporated to dryness under a stream of nitrogen at room temperature. The resulting $[^3H]$- labelled RNA-diol was dissolved in 50 μl of distilled water to which was added 50 μl of 50 mM sodium bicine buffer (pH 8.0). T_1 RNase (2-4 units) was added and the mixture incubated at 37°C for 2 hours. It was then heated at 100°C for two minutes and placed on ice. Thin layer chromatography of T_1 RNase digests was carried out on (i) cellulose plates (20 x 20 cm or 10 x 10 cm) using in the first dimension 1% formic acid and in the second dimension, t-butanol-1% formic acid



SCHEME 4

(1:1, v/v), and (ii) polyethyleneimine (PEI) cellulose plates using ascending chromatography with 1.0 M acetic acid followed by 0.3M LiCl. Unlabelled 5- methylcytidine diol (for use as markers) was prepared from 5-methylcytidine through periodate oxidation and $NaBH_4$ reduction. Autoradiography was carried out with Kodak X-Omat AR medical X-ray film.

Influenza A virus:

Influenza A virus (Brazil/78/HINI) was grown in embryonated eggs. Isolation of the virus from allantoic fluid was carried out as described[8].

System for RNA Synthesis by Influenza A virus:

In studies concerning the use of different RNA templates the incubation mixtures contained 50mM tris-HCl (pH 8.2), 150 mM KCl, 7mM $MnCl_2$, 2mM $MgCl_2$, 15 mM mercaptoethanol, 0.5% triton X-100, 0.10 mM UTP or 0.10 mM CTP (depending on whether the template contained poly(A) or poly(G)), 10.0 μCi [^{3}H] UTP (46 Ci/nmole) or 10.0 μCi [^{3}H] CTP (20 Ci/mmole), 0.5 mM synthetic template, 5.0 mM primer (GpC) and Influenza A virus (18 μg protein) in a final volume of 200 μl. Incubations were at 30°C. Aliquots (20 μl) were taken at various times, and carrier tRNA (100 μg) added, followed by cold 10%

TCA. Samples were collected on Whatman GF/C filters, washed thoroughly with cold 5% TCA followed by ethanol, dried and then counted in scintillation fluid with appropriate blanks and corrections.

RESULTS AND DISCUSSION

Synthesis of RNA complementary copies of all eight RNA genome molecules of the Influenza A virus can be investigated in vitro through use of detergent disrupted virus preparations. The viral associated RNA-dependent RNA polymerase is able to function under these conditions and synthesis of RNA is stimulated by the addition of the dinucleoside monophosphates ApG, GpG and GpC which appear to act as primers (Scheme I). Previously we have shown that the methylated dinucleoside monophosphate (Gpm^5C) is not able to function as a primer for RNA synthesis in this system[1]. The results with modified primers encouraged us to investigate the effects of methylation on the RNA template component of the replication system. Reactions depicted in Scheme 2 outline the use of synthetic RNA templates containing 3'- GpC and Gpm^5C termini (3' end) in the Influenza A virus system. We prepared poly(A)pApGpC, poly(A)pApGpm^5C and the corresponding poly(G) derivatives, and used these templates to ascertain whether: (i) the Influenza A virus polymerase can use a synthetic RNA template, (ii) whether a 3'-terminal GpC group is necessary to initiate synthesis and, (iii) if the templates are functional, would a synthetic template ending in 5-methylcytidine (Scheme 2) still prime synthesis of new RNA chains?

Synthesis of modified poly(A) and poly(G) templates was accomplished through enzymatic methods. It should be mentioned that the starting materials were heterogeneous with regard to chain length and therefore the modified RNA templates obtained were also heterogeneous with regard to chain length.

Studies on the incorporation of [^{3}H] UTP into RNA using a system of triton X-100 disrupted viral preparations and synthetic RNA templates were carried out in the presence or absence of a dinucleoside monophosphate (GpC) primer. Results of these experiments are shown in Fig. 1 (c) which indicate that the Influenza A virus polymerase can use poly(A)pApGpC as a template with GpC as a primer. In the absence of added primer, synthesis was considerably slower and with poly(A), synthesis was not observed. On the other hand when poly(A)pApGpm^{5}C was used as a template with or without GpC primer, synthesis of RNA was very much slower (Fig. 1d) thus indicating that the 3' -terminal 5-methylcytidine residue was interfering with the initiation of RNA synthesis in this system. A similar series of results was obtained with poly(G)pGpC and poly(G)pGpm^{5}C templates with or without GpC primer (Fig. 1, a and b).

The results of the present experiments show that: (i) synthesis of RNA through complementary copying of a synthetic poly(A) or poly(G) RNA template occurs if the template has attached a terminus ending in GpC and a GpC primer is present, (ii) if the synthetic template ends in Gpm^{5}C, synthesis of complementary RNA is strongly inhibited with or without a GpC primer and (iii) a certain amount of RNA synthesis occurs with GpC-terminated synthetic RNA templates in the absence of a GpC primer. No synthesis is found with unmodified poly(A) and poly(G) templates. It is of interest here to note that our previous work showed that the dinucleoside monophosphate Gpm^{5}C was unable to function as a primer for viral RNA replication. At present, we are not able to offer a reasonable explanation as to why the 5-methylcytidine residue at the 3' -terminus of a synthetic RNA template prevents initiation of synthesis of new RNA chains. It is possible that Gpm^{5}C interferes with the interaction and movement of the polymerase complex along the template.

Our observations are nevertheless interesting and suggest that modified dinucleoside monophosphates and short modified

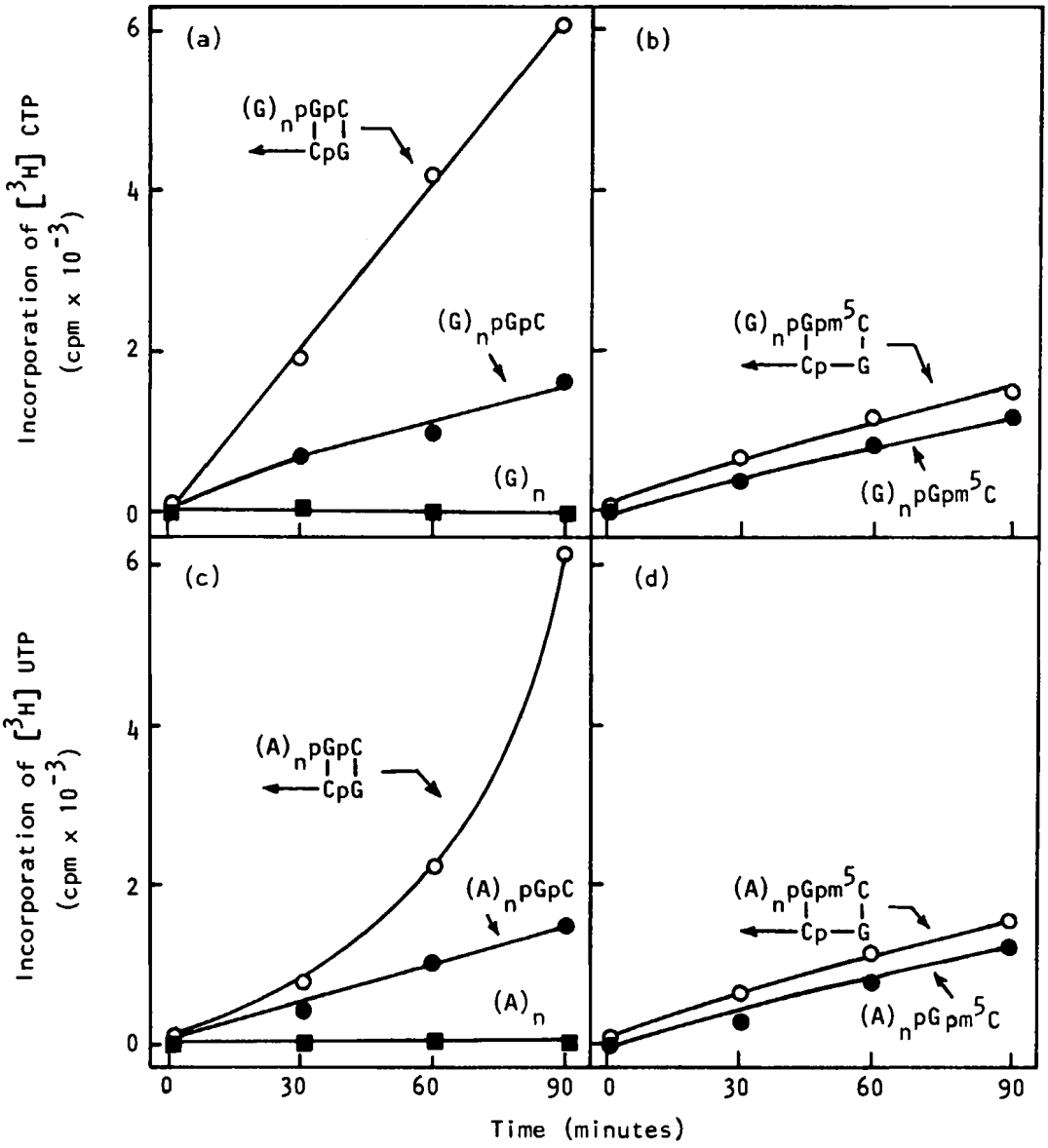
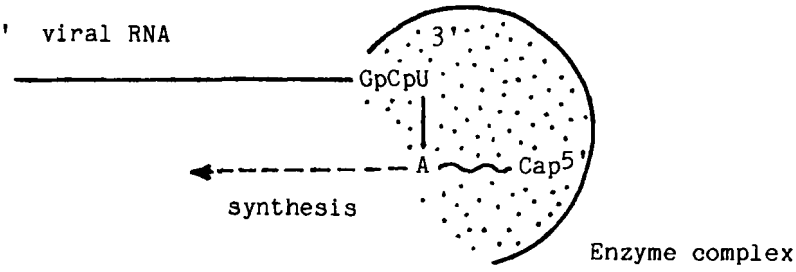


Fig. 1. Synthesis of RNA with synthetic RNA templates.
(a) $(G)_n pGpC$, (b) $(G)_n pGpm^5C$, (c) $(A)_n pGpC$, (d) $(A)_n pGpm^5C$ with (○) and without (●) a GpC primer.



SCHEME 5

oligonucleotides are worthy of further study as possible inhibitors of RNA synthesis in the Influenza A virus system. It is of interest to note that synthesis of RNA by the Influenza A viral RNA polymerase can be initiated in one of two ways. The system used in the work presented here and which involves dinucleoside monophosphate primers (Schemes 1 and 2) is the non-physiological one [8,9]. Interestingly, a highly purified ribonucleoprotein complex consisting of three protein subunits associated with viral RNA has been recently obtained from Influenza A virus which is capable of replicating viral RNA in the presence of complementary dinucleoside monophosphates [10]. The alternative and apparently physiological mechanism for initiation of RNA synthesis is complicated and consists of a number of steps. Cellular capped mRNA molecules are cleaved by the RNA polymerase-associated endonuclease at a purine residue 10-13 nucleotides from the cap to give fragments which serve as primers for viral RNA synthesis [11,12,13,14]. This mode of initiation is shown in Scheme 5.

At present we do not know what effect synthetic RNA molecules with either GpC or Gpm^5C groupings at thier 3' termini have on this particular mode of initiation. Further experimental work should yield information on these problems.

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