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Influenza Virion RNA-Dependent RNA Polymerase: Stimulation by Guanosine and Related Compounds

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The activity of RNA-dependent RNA polymerase of several influenza viruses is stimulated by guanosine. Depending upon the virus strain used, the stimulation of initial reaction rate is up to 10-fold. 5'-GMP, 3',5'-cyclic GMP, and 5'-GDP show lesser stimulation effects. No other nucleosides or 5'-NMPs stimulate, but the dinucleoside monophosphates GpG and GpC show large stimulations. We present evidence that the stimulation represents preferential initiation of genome complementary RNA chains with guanosine: (i) [^{3}H]guanosine is incorporated specifically at the 5' terminus of RNA in polymerase reaction mixes in vitro. (ii) This incorporation reaction has several properties similar to those of the virion polymerase elongation reaction. (iii) RNA made in the stimulated reaction behaves as complementary RNA in annealing kinetic studies, as does RNA labeled with [^{3}H]guanosine.

The influenza virus particle consists of a protein-lipid membrane enclosing ribonucleoprotein structures which contain the genome RNA (1). These complexes contain an RNA-dependent RNA polymerase activity which can transcribe the virion RNA (3, 4, 5). So far the protein responsible for the polymerase activity has not been positively identified. This polymerase activity is thought to be responsible for the primary transcription of virion RNA (vRNA) to give complementary RNA (cRNA) in the newly infected cell (2). Some part of the transcriptive process is obscure, since primary transcription in vivo is sensitive to inhibitors of cellular DNA-dependent RNA polymerase, whereas in vitro no such inhibition is observed (2).

This paper describes the phenomenon of stimulation of the virion polymerase by guanosine (Guo) and related compounds, and examines the mechanism of this stimulation.

MATERIALS AND METHODS

Materials. ^{3}H-labeled guanosine, GTP, and UTP were purchased from the Radiochemical Centre, Amersham. Nucleoside triphosphates were purchased from Boehringer. Other nucleosides, nucleotides, etc. were from Sigma. Snake venom phosphodiesterase and RNase A were from Worthington. RNase T_1 was from Sankyo.

Purification of influenza viruses. Fowl plague virus (FPV), Rostock strain (an influenza A virus), was grown in the allantoic sacs of fertile hen eggs. Allantoic fluid was collected and debris removed by centrifuging for 10 min at 10,000 × *g* at 4 C. Virus was pelleted by centrifuging for 90 min at 40,000 × *g* at 4 C. The pellet was resuspended in about 5 ml of 0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA (NTE), layered on a 25-ml 15 to 60% (wt/vol) sucrose gradient in NTE and centrifuged for 90 min at 60,000 × *g* at 4 C. The visible virus band was collected and diluted with at least 2 volumes of NTE, and the virus was pelleted by centrifuging for 30 min at 140,000 × *g* at 4 C. The virus was resuspended in 2 ml of NTE, layered on a 10-ml 20 to 45% (wt/vol) gradient of potassium tartrate containing 10 mM Tris-hydrochloride (pH 7.4), and centrifuged for 5 to 16 h at 100,000 × *g* at 4 C. The virus band was collected and desalted by gel filtration on an agarose column (Biogel A5M) with NTE. Virus was stored at 0 C. Virus protein was measured by the method of Lowry et al. (9) with serum albumin as standard. Except where stated, FPV was used in all the experiments described here.

Influenza A viruses BEL and WS and influenza B LEE virus were purified in the same way. Purified Sendai virus was a gift from R. A. Lamb.

Assay of influenza virus RNA-dependent RNA polymerase. The polymerase assay procedure was based on those of Bishop et al. (3) and Mahy et al. (10). The reaction mixture contained 50 mM Tris-hydrochloride (pH 8.2), 150 mM KCl, 8 mM $MgCl_2$, 0.2 mM $MnCl_2$, 5 mM dithiothreitol, 0.5% Nonidet P-40, 2 mM ATP, 0.4 mM CTP, 0.4 mM [^{3}H]UTP (specific activity 30 μCi/μmol), 0.2 mM GTP, and virus to a concentration of 0.5 to 2 mg of protein/ml. Reaction mixes were incubated at 31 C. Samples (50 μl) were withdrawn and assayed for trichloroacetic acid-insoluble ^{3}H by spotting on glass fiber disks and washing the disks in two changes of 8% (wt/vol) trichloroacetic acid, 0.1% sodium pyrophosphate, fol-

lowed by three changes of 8% trichloroacetic acid and two rinses in EtOH. Disks were then dried and counted in toluene-based scintillation fluid in a scintillation spectrometer. Variations from these procedures are noted for individual experiments. Freshly prepared FPV incorporated 4 to 6 nmol of [^{3}H]UTP into trichloroacetic acid-insoluble material/h per mg of virus protein in this assay. All FPV preparations used in these experiments incorporated at least 1.2 nmol of UTP/h per mg of virus protein.

Purification of GTP and GMP. GTP was purified from Guo, GMP, and GDP by anion exchange column chromatography on DEAE-cellulose. Whatman DE52 DEAE-cellulose was packed in a column (1 by 10 cm) and washed with 0.5 M NaCl and then with 10 mM Tris-hydrochloride, pH 8.2. All column operations were carried out at 4 C with a column flow rate of 30 ml/h. GTP (20 μmol) was loaded on the column in 5 ml of 10 mM Tris-hydrochloride, pH 8.2. The column was developed with a 200-ml linear gradient of 50 to 150 mM KCl in 10 mM Tris-hydrochloride, pH 8.2. UV absorption of the effluent was monitored on a Uvicord. Trace amounts of Guo, GMP, and GDP were eluted and discarded. GTP was eluted at 120 mM KCl. Peak fractions were collected for immediate use in the polymerase assay.

5'-GMP was purified from Guo on a similar column at 20 C. GMP (15 μmol) was loaded in 5 ml of 10 mM Tris-hydrochloride, pH 8.2. The column was then washed with 40 mM KCl and 10 mM Tris-hydrochloride (pH 8.2) to remove Guo. 5'-GMP was eluted with 100 mM KCl and 10 mM Tris-hydrochloride (pH 8.2).

Preparation of RNA labeled in vitro. RNA for annealing and nuclease digestion analyses was prepared from polymerase reaction mixtures by two methods.

Method 1. After incubation at 31 C, a 0.3-ml polymerase reaction mix was treated with 20 μl of 0.5 M EDTA and chilled. RNA was then separated from [^{3}H]Guo or [^{3}H]GTP by gel filtration with NTE on a 12-ml column of Sephadex G25 (fine grade). Fractions (0.4 ml) were collected and the front peak was identified by scintillation counting of samples. The peak fractions were pooled, and 1 mg of yeast RNA and 20 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS) were added. The mixture was extracted three times with phenol saturated with NTE, then precipitated with 2.5 volumes of EtOH. The RNA was dissolved in 50 μl of 5 mM Tris-hydrochloride pH 7.4, and 1 mM EDTA (TE), 1 ml of Me_2SO was added, and the mixture was incubated for 20 min at 37 C to destroy any double-stranded structure (16) and then chilled. One-tenth milliliter of 4 M sodium acetate was added and the RNA was precipitated with 3 ml of EtOH, then dissolved in 50 μl of TE. This method was used to prepare RNAs for the first nuclease digestion experiment described below.

Method 2. Reaction mixture samples were made up to 0.5 ml with NTE and 0.2% SDS. Yeast RNA (0.5 mg) was added and the mixture was phenol extracted three times, precipitated twice with EtOH, and finally dissolved in 50 μl of TE. When it was necessary to remove all [^{3}H]Guo for nuclease digestion analysis, six cycles of EtOH precipitation were used. RNA for nuclease digestion was then treated with Me_2SO as in method 1.

RNA digestion and fractionation conditions. RNA prepared as above was digested with snake venom phosphodiesterase, RNase T_1, or RNase A using the conditions of Sanger and Brownlee (13). RNase T_1 and venom phosphodiesterase digests were fractionated by chromatography on DEAE-cellulose paper (Whatman DE81). The paper was prewashed with 6% (vol/vol) acetic acid and then water, and dried. (Paper pretreated in this way develops faster and without the secondary front found with untreated paper.) The sample was applied over a 1-cm line together with 0.1 μmol each of desired UV marker nucleotides and nucleosides, and the chromatogram developed descending for 20 cm with 7 M urea and 0.12 M ammonium acetate, pH 7.8 (8). The chromatogram was dried and cut into 1-cm strips for scintillation counting. R_f values in this system, referred to the solvent front, are: Guo, 0.95; 2',3'-cyclic GMP, 0.80; GMP, 0.45, oligonucleotides, <0.40.

RNase A digests were fractionated by chromatography on DEAE-paper with 7 M urea and 0.2 M ammonium acetate, pH 7.8. Yeast RNA (250 μg) digested with RNase A was used as UV marker. The chromatogram was developed descending for 35 cm. This procedure resolves oligonucleotides into length classes up to pentanucleotide (8). RNase A digests were also fractionated by electrophoresis at pH 1.9 on DEAE-paper (13). A 40-cm length of paper was run for 5 h at 1.8 kV. ^{32}P-labeled GpCp and GpUp were run in parallel and detected by autoradiography.

Measurement of guanosine incorporation into RNA. Virion polymerase reaction mixtures were labeled with 0.1 mM [^{3}H]Guo (specific activity 3 to 15.8 Ci/mmol in different experiments). Reactions were terminated by adding 0.25 volumes of 2.5 mM Guo and 0.25 M EDTA and chilling. It was found that trichloroacetic acid precipitation, even with extensive washing, yielded blank values too high to be of use as an assay method. ^{3}H incorporation into RNA was therefore measured as follows. The sample volume was adjusted to 0.5 ml with NTE and 0.2% SDS. Yeast RNA (0.5 mg) was added, the mixture was extracted with phenol, and the RNA was precipitated with 2.5 ml of EtOH. The RNA was dissolved in 50 μl of TE, 1 ml of Me_2SO was added, and the mixture was incubated for 20 min at 37 C. The RNA was then precipitated with 2 ml of 8% trichloroacetic acid, collected on a glass fiber disk, washed with six 10-ml batches of 8% trichloroacetic acid, and rinsed with EtOH. The filter was dried and counted in toluene scintillation fluid. This procedure, when applied to [^{3}H]GTP-labeled samples, yielded results which were very similar to those obtained by direct trichloroacetic acid precipitation, but less precise.

When studying dependence of Guo incorporation on the presence of NTPs, the following procedure was used in an attempt to remove any contained NTPs from the virus. FPV at 5 mg of protein/ml was treated with 0.5% Nonidet P-40 in 50 mM Tris-hydrochloride, pH 8.2, 0.15 M KCl, 1 mM dithiothreitol, and 1 mM EDTA for 15 min at 31 C, and then dialyzed against 50 mM Tris-hydrochloride, pH 8.2, 0.4 M KCl, 1 mM

dithiothreitol, and 1 mM EDTA for 3 h at 0 C, then against two changes of 70 mM Tris-hydrochloride, pH 8.2, 0.22 M KCl, 11 mM $MgCl_2$, 0.3 mM $MnCl_2$, and 1 mM dithiothreitol for direct use in a polymerase reaction mix.

Purification of FPV RNA. Purified FPV containing 15 mg of protein was suspended in 1 ml of NTE. One-tenth milliliter of 10% SDS was added, and the virus was incubated at 31 C for 10 min. The mixture was extracted with 1 ml of buffer-saturated phenol. The phenol phase was re-extracted with 1 ml of NTE and the pooled aqueous phases were given five more phenol extractions. The RNA was precipitated with 2.5 ml of EtOH, then dissolved in 0.5 ml of NTE, precipitated twice more with EtOH, and finally dissolved in 0.1 ml of TE. This procedure yielded 4.2 E_{260}-ml units with an E_{260}/E_{280} ratio of 2.1, at pH 7.4.

RNA-RNA annealing methods. We wished to use RNA labeled with [8-^{3}H]Guo for annealing studies. This label is rapidly exchanged at high temperatures (18), so it was desirable to use low temperature conditions for denaturation and annealing. RNA was labeled in vitro with [^{3}H]UTP, [^{3}H]GTP, or [^{3}H]Guo and purified as above. A 5-μl sample of RNA was diluted with 5 μl of TE, containing vRNA where indicated, and denatured by mixing with 0.39 ml of Me_2SO and incubating at 37 C for 20 min. A 0.59-ml amount of 3.4 × SSC (0.15 M NaCl plus 0.15 M sodium citrate), pH 7.0, with 0.5% SDS (at 37 C) was then added. The mixture now contained 40% Me_2SO, 2 × SSC, and 0.3% SDS: these constitute conditions suitable for formation of double-stranded RNA at 37 C (14). The mixture was incubated at 37 C and 50-μl samples were taken at intervals up to 100 h. Each sample was diluted into 2 ml of 2 × SSC (pH 7.4) containing 100 μg of RNase A per ml (except for RNA labeled with [^{3}H]Guo, where 3 μg of RNase per ml was used). RNase treatment was for 30 min at 37 C. The sample was then chilled, 0.3 ml of 50% (wt/vol) trichloroacetic acid was added, and the remaining RNA was collected on a glass fiber disk. The disk was washed with six 10-ml batches of 8% trichloroacetic acid, rinsed with EtOH, dried, and counted in toluene-based scintillation fluid.

RESULTS

Molecular specificity of the polymerase activators. We were interested in determining whether the virion RNA-dependent RNA polymerase is affected in vitro by intracellular metabolic regulators, specifically 3',5'-cyclic AMP and 3',5'-cyclic GMP. We found that cyclic AMP had no effect, but polymerase activity was stimulated 1.5 times by cyclic GMP. We tested related compounds and found that the largest stimulations were shown by guanosine (up to fourfold) and 5'-GMP (up to threefold). The polymerase assay system used at this time contained 2 mM GTP. We found that the optimum GTP concentration, for 30-min incubation periods, was 0.2 to 0.4 mM. In further experiments, GTP was used at 0.2 mM to minimize presence of guanosine or 5'-GMP from GTP breakdown.

Table 1 shows that stimulatory guanosine compounds, in order of decreasing effectiveness, were: Guo > 5'-GMP > 3',5'-cyclic GMP > GDP. 3'-GMP inhibited the polymerase. Table 1 also demonstrates that the guanosine structure, rather than a component part of it, constitutes the active agent. 5'-GMP was the only common 5'-NMP giving this effect, and guanosine was the only ribonucleoside: AMP, IMP, XMP, CMP, and UMP, and adenosine, inosine, xanthosine, cytidine, and uridine had no effect when added singly to the polymerase reaction at 0.5 mM.

The dependence of the activation effect on concentration of Guo and 5'-GMP was determined (Fig. 1). For a given stimulation, 10 to 30 times more GMP than Guo is required. Thus, a 3% contamination of the GMP with Guo could give the observed activation. However, we consider that 5'-GMP per se does give the activation effect, since activation was also observed with a 5'-GMP preparation from which any Guo had been specifically removed, but an activation effect caused by rapid conversion of GMP to Guo cannot be excluded.

We tested a number of Guo analogues to determine the parts of the molecule necessary for activation (Table 2). We conclude that the

TABLE 1. *Effects of guanosine-related compounds on polymerase activity*[a]

Addition	^{3}H counts/min	Stimulation
A		
None	723	1.0
Guo	2,889	4.0
2'-GMP	761	1.1
3'-GMP	314	0.4
5'-GMP	1,992	2.8
2',3'-cyclic GMP	711	1.0
3',5'-cyclic GMP	1,597	2.2
5'-GDP	1,350	1.9
B		
None	637	1.0
Guo	1,591	2.5
Guanine	613	1.0
Ribose	612	1.0

[a] Various compounds were added to 0.5 mM in the standard polymerase reaction mix. After 30 min incubation at 31 C, the trichloroacetic acid-insoluble ^{3}H was determined with 50-μl samples. The right hand column presents the activities relative to the activity obtained with no test compounds added. Parts A and B represent experiments performed on separate occasions.

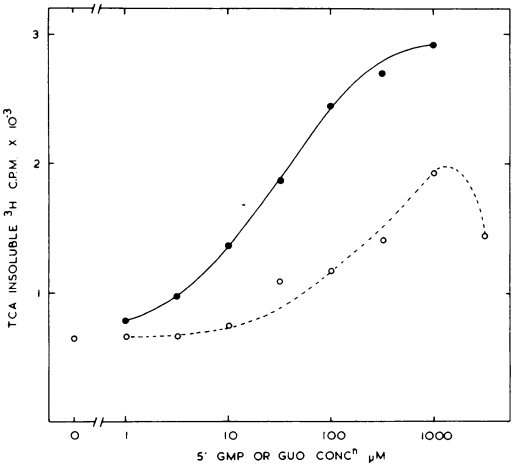

FIG. 1. *Dependence of polymerase stimulation on Guo and 5'-GMP concentration. Guo and 5'-GMP were added to standard polymerase reaction mixes (75 μl) at various concentrations. Incubation was for 30 min at 31 C, then 50 μl was taken for determination of trichloroacetic acid-insoluble ^{3}H. Symbols: O, Plus GMP; ●, plus Guo.*

TABLE 2. *Effects of guanosine analogues on polymerase activity*[a]

Addition	^{3}H counts/min	Stimulation
A		
None	637	1.0
Guanosine	1,591	2.5
N^2-Methylguanosine	1,269	2.0
7-Methylguanosine	556	0.9
1-Methylguanosine	808	1.3
2'-Deoxyguanosine	625	1.0
B		
None	660	1.0
Guanosine	1,715	2.6
6-Mercaptoguanosine	1,159	1.8
8-Mercaptoguanosine	638	1.0
6-Mercaptopurine riboside	730	1.1
N^2,N^2-Dimethylguanosine	581	0.9
5'-GMP	1,517	2.3
8-Azaguanosine 5'-phosphate	731	1.1

[a] Assays were performed as described for Table 1. Parts A and B represent experiments performed on separate occasions.

activation effect involves a number of features of Guo. (i) N-1 or its surroundings are involved since 1-methylguanosine has little activating effect. (ii) The 2-NH_2 group is necessary since inosine (which lacks this group) does not activate. N^2-methylguanosine activates but N^2,N^2-dimethylguanosine does not. We conclude that one of the amine H atoms is necessary (or that the two methyl groups cause inactivity by steric hindrance, rather than by substitution). (iii) The 6-OH group may be involved since substitution by 6-SH gives reduced activity. (iv) The imidazole ring is required intact and unmodified since 7-methylguanosine, 8-mercaptoguanosine, and 8-azaguanosine 5'-phosphate show no activity. (v) The 2'-OH group is required for activation, since 2'-deoxyguanosine and 2'-GMP show no effects. (vi) A free 3'-OH may be necessary, since 3'-GMP actually inhibits the polymerase. 3',5'-cyclic GMP does show a small activation effect, but this may result from hydrolysis to 5'-GMP.

Other aspects of polymerase activation. The time course of activation was studied (Fig. 2). For both Guo and 5'-GMP the stimulation was most pronounced at early times after initiation of RNA synthesis.

The influenza virion RNA polymerase is active in the absence of added Guo or 5'-GMP. However, it is apparent from Fig. 1 that very small amounts of contamination of GTP with 5'-GMP or Guo could give the observed basal activity. We therefore purified GTP to remove Guo, GMP, and GDP, and tested it immediately in the polymerase reaction. This preparation gave the same basal activity and activation effects as standard GTP. We consider, there-

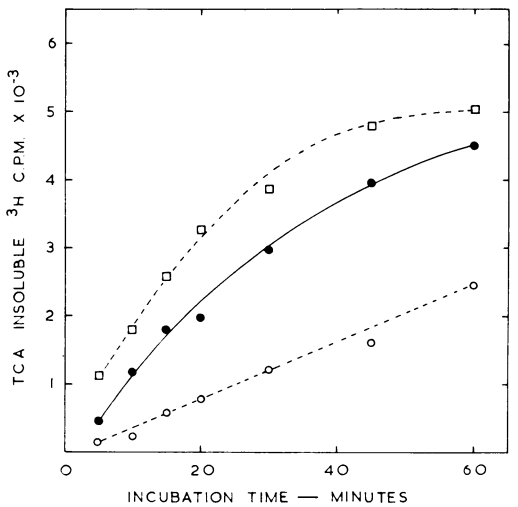

FIG. 2. *Time course of stimulation effects. Guo or 5'-GMP was added to 0.5 mM in standard polymerase reaction mixes, which were incubated at 31 C, and 50-μl samples were withdrawn up to 60 min for determination of trichloroacetic acid-insoluble ^{3}H. Symbols: O, no addition; ●, plus 5'-GMP; □, plus Guo.*

fore, that the enzyme does have activity in the absence of Guo or 5'-GMP.

We studied the activation effect with other myxoviruses: Table 3 shows that guanosine activates the virion polymerases of two other influenza A viruses (BEL and WS) and one influenza B virus (LEE), but not the virion polymerase of Sendai virus (a member of the paramyxo group). The polymerase of influenza A BEL virus showed much larger effects than FPV polymerase: the activation by Guo was about 10-fold up to 30 min.

Incorporation of guanosine into RNA. One possibility for the mechanism of activation is that Guo is preferentially incorporated as the first residue in cRNA chains synthesised in vitro. We examined this by setting up a virion polymerase reaction mix containing high specific activity [^{3}H]Guo. As a control, a separate mixture was labeled with [^{3}H]GTP. RNA was purified from both after incubation at 31 C for 15 min. We found that some [^{3}H]Guo was incorporated into RNA (quantitative aspects of this incorporation are dealt with later).

Incorporation of [^{3}H]Guo into RNA in vitro might occur by two routes, either directly at the 5'-terminus or via conversion to GTP by endogenous kinases. These two mechanisms can be distinguished by specific nuclease digestion and fractionation. Treatment of RNA with venom phosphodiesterase produces 5'-NMPs from residues internal in the RNA chain (12). If the [^{3}H]Guo is situated at the 5'-terminus of the RNA, it will appear as Guo. Digests were fractionated by chromatography on DEAE-paper with 7 M urea and 0.12 M ammonium acetate. When RNA was labeled with [^{3}H]GTP, 94% of the ^{3}H appeared as GMP after phosphodiesterase digestion, whereas RNA labeled with [^{3}H]Guo gave 92% of the ^{3}H in Guo (Table 4). For both preparations, RNA samples were incubated without enzyme and then chromatographed, and in both cases all the ^{3}H remained at the origin, as expected for RNA.

TABLE 3. *Guanosine activation of other virion polymerases*[a]

Virus	Activity minus Guo	Activity plus Guo	Stimulation
Influenza A BEL	1.77	18.70	10.6
Influenza A WS	1.26	3.98	3.2
Influenza B LEE	0.81	2.04	2.5
Sendai	0.018	0.018	1.0

[a] Polymerase activities were assayed ±0.5 mM Guo. Activities are expressed as nanomoles of [^{3}H]UTP incorporated/hour per milligram of virus protein into trichloroacetic acid-insoluble material. The estimates are based on 30-min incubations at 31 C except for Sendai virus, where incubation was for 60 min.

TABLE 4. *Nuclease digestion analysis of RNAs*[a]

RNA	Oligonucleotides	5'-GMP	3'-GMP	Guo
Venom phosphodiesterase				
[^{3}H]GTP label	4	94		2
[^{3}H]Guo label	6	2		92
RNase T_1				
[^{3}H]GTP label	76		24	0
[^{3}H]Guo label	4		94	2

[a] Nuclease digests were fractionated by chromatography on DEAE-paper. The ^{3}H detected in each fraction is shown as a percentage of the total detected (600 to 1,000 counts/min in different assays). In no case was any ^{3}H found in cyclic GMP fractions.

The RNA preparations were also digested with RNase T_1. In the case of internally labeled RNA, we expect that [^{3}H]GMP will only be produced from the sequence ---GpĜp---; otherwise the label should be in oligonucleotides (17). We therefore expect about one quarter of the label to appear as 3'-GMP. Table 4 shows that when RNA labeled with [^{3}H]GTP was digested with RNase T_1, 24% of the label was found in GMP and the rest in oligonucleotides. This pattern was stable to further digestion. With RNA labeled by [^{3}H]Guo, 94% of the label in an RNase T_1 digest chromatographed as 3'-GMP (Table 4). This is expected if the label is at the 5' terminus of the RNA (but note that [^{3}H]Guo from the sequence ---GpĜp--- would also appear as 3'-GMP, so this digestion is not so definitive as that with venom phosphodiesterase). The results of both enzyme digestions thus demonstrate that the [^{3}H]Guo was incorporated directly as the 5' terminus of some RNA species, whereas the [^{3}H]GTP was incorporated, as expected, into heteropolymeric RNA.

Samples of [^{3}H]Guo-labeled RNA were digested with RNase A and fractionated according to chain length by chromatography on DEAE-paper with 7M urea and 0.2 M ammonium acetate. All the ^{3}H was detected in the dinucleotide fraction (Fig. 3). Electrophoresis of the digest on DEAE-paper at pH 1.9 indicated that the ^{3}H was present as GpCp (Fig. 3).

Characteristics of the guanosine incorporation reaction. We analyzed several aspects of the Guo incorporation reaction and compared its properties with those of the virion RNA polymerase elongation reaction.

The time course of [^{3}H]Guo incorporation

into RNA was measured and compared with [^{3}H]GTP incorporation (Fig. 4). Guo incorporation continues at least until 60 min after starting incubation, when net incorporation of GTP has ceased. During the first 45 min of incubation, about one Guo molecule was incorporated for each 100 molecules of GTP incorporated. RNA was purified from samples labeled with [^{3}H]Guo for 30, 60, and 90 min, and digested with venom phosphodiesterase. As with a 15-min incubation sample (above), $\geq$88% of the ^{3}H was then found as Guo, i.e., had come from the 5′ terminus of an RNA chain.

The dependence of Guo incorporation on Guo concentration was determined (Fig. 5). Figure 5 also compares Guo incorporation with the stimulation of virion polymerase by Guo (data replotted from Fig. 1).

Incorporation of Guo into RNA was dependent on ATP and CTP, but omission of GTP or

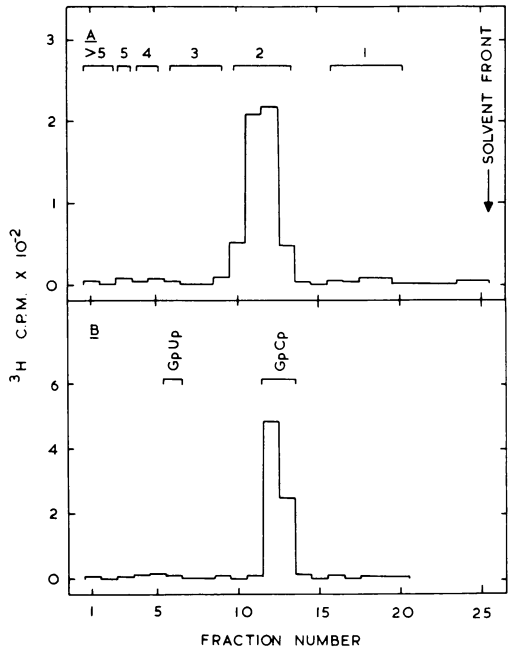

FIG. 3. *RNase A digestion of [^{3}H]Guo-labeled RNA. (A) A sample of an RNase A digest was chromatographed on DEAE-paper as described. The bars and numerals above represent the positions of marker oligonucleotides and their chain lengths. The paper was cut into 1.5-cm strips for scintillation counting, with the origin in fraction 1. (B) A sample was fractionated by electrophoresis on DEAE-paper at pH 1.9. The positions of GpUp and GpCp were determined by running authentic ^{32}P-labeled GpUp and GpCp in parallel and detecting them by autoradiography. The paper was cut into 1.5-cm strips for counting, with the origin in fraction 1.*

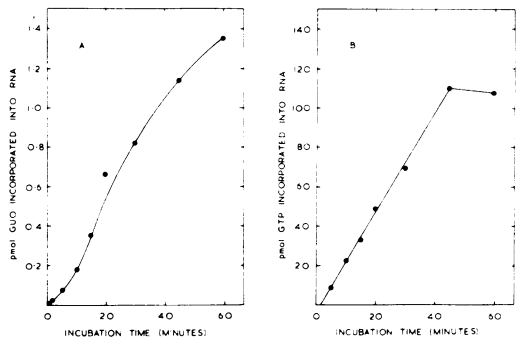

FIG. 4. *Time courses of incorporation of Guo and GTP into RNA. Two polymerase reaction mixes, each with a total volume of 0.66 ml, were set up. (A) Contained 0.1 mM [^{3}H]Guo, specific activity 15.8 Ci/mmol. (B) Contained 0.2 mM [^{3}H]GTP, specific activity 0.84 Ci/mmol, plus 0.1 mM unlabeled Guo. Each contained 930 μg of FPV protein, previously treated with 0.5% Nonidet P-40. The mixes were incubated at 31 C and 60-μl samples were withdrawn at intervals, chilled, and mixed with 20 μl of 0.25 mMGuo and 0.25 M EDTA. 20 μl of each sample was then used for determination of ^{3}H in RNA. The ordinates represent the incorporation of Guo or GTP into RNA in each 20-μl sample processed.*

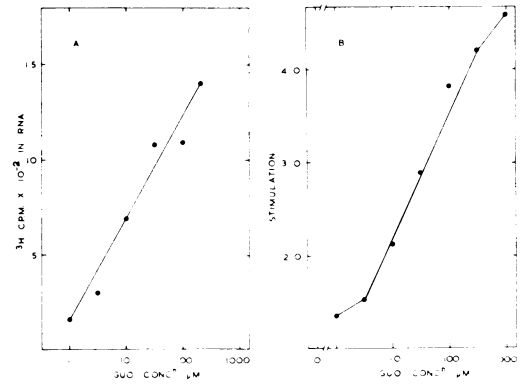

FIG. 5. *Dependence of Guo incorporation into RNA on concentration of Guo. (A) Polymerase reaction mixtures (75 μl) containing 31 μg of FPV protein and [^{3}H]Guo (3 Ci/mmol) at the concentrations shown, were incubated at 31 C for 60 min, then processed to determine ^{3}H in RNA as described. (B) For comparison, data from Fig. 1 are replotted to show the dependence of polymerase stimulation on Guo concentration. The ordinate scales have been selected so that each plotted line has the same ordinate displacement at 100 μM Guo.*

UTP did not reduce Guo incorporation (Table 5). We attempted to remove NTPs from a virus preparation by detergent treatment followed by dialysis. This preparation gave the same pattern of NTP dependence (Table 5). RNA was

purified from the minus GTP and minus UTP mixes, and analyzed by venom phosphodiesterase digestion. Again, at least 90% of the label was found as Guo.

FPV RNA polymerase is inhibited by Ca^{2+} (Fig. 6A). [^{3}H]Guo incorporation also shows inhibition, with a similar response to variation in Ca^{2+} concentration (Fig. 6B).

RNA-RNA annealing studies. We wished to demonstrate by the criteria of RNA annealing kinetics that the [^{3}H]UTP-labeled material synthesized in the stimulated reaction was in fact cRNA, and to study the nature of the [^{3}H]Guo-labeled RNA. RNA was purified from reaction mixes containing [^{3}H]UTP, [^{3}H]GTP, or [^{3}H]Guo and used for RNA-RNA annealing experiments with or without addition of vRNA. Figure 7 shows that RNA made in the standard polymerase reaction (no Guo stimulation) is partially and slowly converted to a double-stranded, RNase-resistant form in the absence of added vRNA. Addition of vRNA to the annealing mixture gives more rapid annealing to about 90% RNase resistance. The labeled RNA thus behaves as complementary to vRNA. A qualitatively similar result is obtained with RNA made in the stimulated polymerase reaction, demonstrating that in this case also the synthesised RNA is complementary to vRNA.

RNA labeled with [^{3}H]Guo also behaves as cRNA in its annealing kinetics, and behaves similarly to RNA labeled under the same conditions with [^{3}H]GTP (Fig. 8). The [^{3}H]Guo-labeled RNA is converted to an RNase-resistant form about twice as fast as the [^{3}H]GTP-labeled RNA, in the presence of added vRNA.

TABLE 5. *Dependence of [^{3}H]Guo incorporation into RNA on NTPs*[a]

Reaction mixture	^{3}H counts/min[b]	^{3}H counts/min[c]
Complete	1,803	2,464
No incubation at 31 C	29	42
No NTPs	36	58
No ATP	50	70
No GTP	1,036	4,918
No UTP	2,629	2,552
No CTP	113	203

[a] Polymerase incubation mixtures of 50 μl containing 0.1 mM [^{3}H]Guo (specific acitivity 15.8 Ci/mmol) and 4 mg of virus protein per ml were incubated for 30 min at 31 C, then chilled. 15 μl of 2.5 mM Guo and 250 mM EDTA was added. 10-μl samples were processed to determine incorporation of ^{3}H into RNA.

[b] An experiment with standard FPV.

[c] Virus was detergent treated and dialyzed before use.

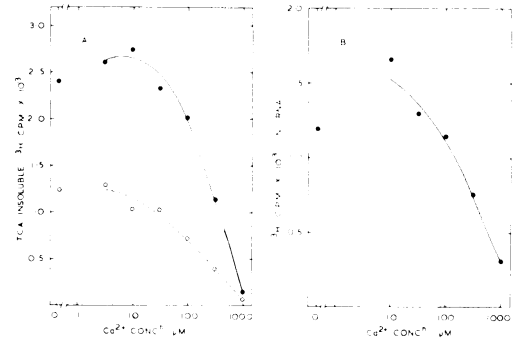

FIG. 6. *Inhibition by Ca^{2+} of [^{3}H]UTP and [^{3}H]Guo incorporation into RNA. (A) Polymerase reaction mixtures (75 μl), containing 31 μg of FPV protein and various concentrations of $CaCl_2$, were incubated for 60 min at 31 C (±0.1 mM Guo) then 50-μl samples were taken for determination of trichloroacetic acid-insoluble ^{3}H. Symbols: ○, No addition; ●, plus Guo. (B) Reaction mixes (50 μl) containing 0.1 mM [^{3}H]Guo (3 Ci/mmol) and various $CaCl_2$ concentrations were set up and incubated as for Fig. 7A and processed to determine ^{3}H in RNA.*

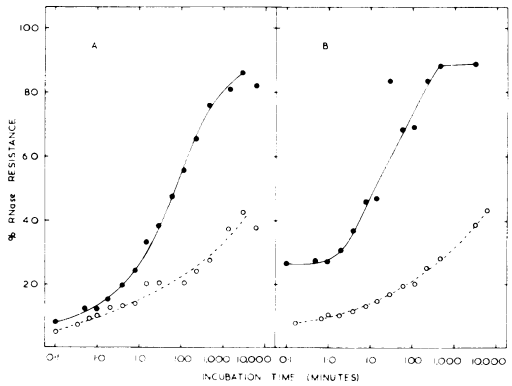

FIG. 7. *RNA-RNA annealing kinetics of [^{3}H]UTP-labeled RNA. Polymerase reaction mixtures (0.1 ml) containing [^{3}H]UTP (5 Ci/mmol) and 130 μg of FPV protein were incubated for 60 min at 31 C (±0.5 mM Guo). RNA was extracted and samples were incubated in annealing conditions as described. (A) RNA made without Guo; 563 counts/min per assay point. (B) RNA made in the presence of 0.5 mM Guo; 1,708 counts/min per assay point. Symbols: ●, RNA incubated in the presence of added 3 μg of vRNA; ○, RNA incubated without additions.*

However, digestion of samples of [^{3}H]Guo RNA was performed with 3 μg of RNase per ml instead of 100 μg of RNase per ml, since we had found the [^{3}H]Guo in RNA to be more sensitive to RNase digestion than ^{3}H internal in RNA. We consider, therefore, that the small difference in annealing rate cannot be given weight. We conclude that the [^{3}H]Guo is present as the 5′ terminus of cRNA.

Stimulation of polymerase by dinucleoside monophosphates. We tested GpN species for stimulation effects. GpG and GpC give very large stimulations, GpA gives slight stimulation and GpU has no effect (Table 6).

DISCUSSION

We have shown that influenza RNA polymerase is stimulated by Guo or 5'-phosphorylated Guo. A survey of compounds related to Guo showed that a number of features of the molecule are required for stimulation. We considered the interactions of various parts of the Guo molecule under three headings: (i) H bonding to cytosine in RNA (20); (ii) binding to protein; (iii) involvement in the catalytic mechanism of stimulation. If a Guo analogue is deficient in (i) or (ii) we expect the stimulation to be reduced or abolished. A deficiency in (iii) should either abolish activation or lead to inhibition via competition with the normal mechanism. Groups on the guanine structure necessary for H bonding to cytosine (1-OH, 2-N, 3-NH_2) are necessary for stimulation, but there is nothing in our results to demonstrate more directly that H bonding to RNA is in fact involved. Several other features of Guo (in the imidazole and ribose rings) are necessary, so some other interactions, presumably with protein, are involved. Finally, substitution of the 3'-OH by phosphate results in inhibition of the polymerase, so this group may be involved closely with the mechanism of stimulation.

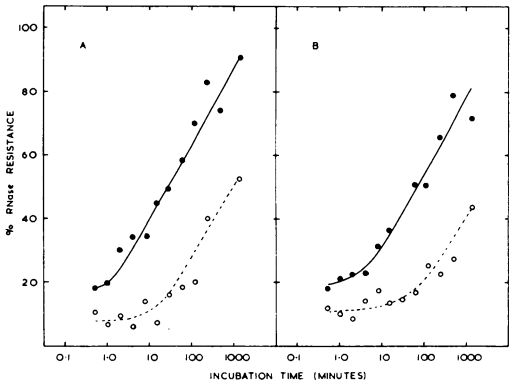

FIG. 8. *Annealing kinetics of RNA labeled with [^{3}H]Guo and [^{3}H]GTP. Two polymerase reaction mixtures of 0.2 ml each containing 400 μg of FPV protein were incubated for 60 min at 31 C. RNA was extracted and samples were incubated in annealing conditions except that total reaction volumes, for (A) and (B) respectively, were 4 and 2 times those given. (A) RNA from reaction mix containing 0.1 mM [^{3}H]Guo, 15.8 Ci/mmol; 200-μl samples were taken for each assay point, containing 166 counts/min in RNA. (B) RNA from reaction mix containing 0.1 mM unlabeled Guo and 0.2 mM [^{3}H]GTP, 1.2 Ci/mmol; 100-μl samples were taken for each assay point, containing 305 counts/min in RNA. Symbols: ●, RNA incubated in the presence of added vRNA [(A) 24 μg; (B) 12 μg]; ○, RNA incubated without additions.*

TABLE 6. *Stimulation of influenza virus RNA polymerases by dinucleoside monophosphates*[a]

Addition	FPV		Influenza A BEL virus	
	^{3}H counts/min	Stimulation	^{3}H counts/min	Stimulation
None	706	1.0	663	1.0
GpA	1,193	1.7	1,128	1.7
GpG	5,102	7.2	7,146	10.8
GpU	888	1.3	540	0.8
GpC	3,420	4.3	6,314	9.5

[a] GpN species were added to 0.5 mM in the standard polymerase assay. After incubation for 30 min at 31 C, trichloroacetic acid-insoluble ^{3}H in 50-μl samples was determined. Stimulation presents the activities relative to the basal activity.

We divided possible mechanisms of stimulation into three classes: class 1, the Guo is directly involved in the polymerase reaction and is incorporated into the cRNA; class 2, the guanosine activates the polymerase protein in a specific binding reaction but is not involved in covalent linkage in cRNA; class 3, the activation is indirect—for example, the guanosine inactivates a protein inhibiting the polymerase. The indication that the 3'-OH group of Guo was involved in the stimulation reaction suggested the following, class 1 mechanism: Guo, or 5'-GMP, is preferentially incorporated as the 5' terminus of the nascent cRNA chain synthesised by the virion polymerase. Most of our further work on Guo stimulation was aimed at studying this hypothesis.

We showed, using high specific activity [^{3}H]Guo, that Guo is indeed incorporated in vitro at the 5' end of some RNA species, in the sequence GpCp---. We wished to demonstrate that this incorporation reaction represented an aspect of the virion polymerase activity. The Guo incorporation reaction does behave similarly to the virion polymerase chain elongation reaction in several ways: (i) the stimulation of the virion polymerase by Guo shows the same dependence on Guo concentration as the Guo incorporation reaction does. (ii) The two reactions are similarly inhibited by Ca^{2+}. (iii) Guo incorporation is dependent on ATP and CTP. The lack of dependence on UTP and GTP could

occur if these molecules were incorporated into the growing RNA chain only after it was long enough to register as positive in our assay system. GTP could not have been formed in the minus GTP reaction by endogenous kinases from Guo, since 95% of the [^{3}H]Guo incorporated was present at the 5′ terminus. (iv) Guo is incorporated in the sequence GpCp---. In the absence of Guo, cRNA is initiated with pppGpCp--- (7).

We showed by the criteria of RNA-RNA annealing kinetics that the [^{3}H]UTP-labeled material, produced with or without Guo stimulation, behaves as cRNA, and that [^{3}H]Guo-labeled material also behaves as cRNA. If the distribution of chain lengths is the same for chains initiated with Guo and those not, and if Guo does not inhibit chain initiation by GTP, then, at the Guo concentration used for Fig. 4 (0.1 mM), about 75% of cRNA chains are initiated with Guo (Fig. 1) and each chain contains (weight average) about 75 GTP residues, i.e., is about 300 residues long. We conclude that the level of Guo incorporation observed is of an order consistent with the mechanism proposed.

We have shown that influenza polymerase can initiate cRNA synthesis with Guo and, probably, with 5′-GMP and 5′-GDP. Hefti et al. (7) have shown that GTP can also act as initiating nucleotide. The order of effectiveness for initiation is then Guo > GMP > GDP $\simeq$ GTP (data of Table 1). This preferential initiation is adequate to account for the activation effects described, although we have not formally excluded other possible mechanisms or effects. We believe this to be the first demonstration of initiation of synthesis of a nucleic acid chain with a nucleoside. The activation effects with GpG and GpC suggest that they too can act as chain initiators. The DNA-dependent RNA polymerase of *Escherichia coli* can also initiate chains with dinucleoside monophosphates in vitro (6).

It has been shown that influenza virus preparations contain a number of enzyme activities which can be removed with the surface components to leave internal ribonucleoprotein particles with active polymerase (7). We have not yet tested such isolated cores for guanosine stimulation or incorporation. Given the magnitude of the effects on RNA synthesis in vitro, it seems unlikely that they should be due to adventitiously associated enzymes.

This work has been concerned wholly with properties of the polymerase in vitro and may not relate directly to influenza RNA synthesis in vivo. However, two points emerge clearly. First, the chain initiation process is specific in that it always involves guanosine-containing molecules (NTPs other than GTP do not initiate [7]). Second, GTP is a relatively poor initiator. It is possible that in vivo some other molecule acts as initiator, for instance an RNA species.

There is evidence that some virus mRNAs are subjected to a modification of the 5′ terminus after transcription, by methylation and other substitutions (11, 15, 19). Do such modifications bear any functional relations to the phenomena described here? We have postulated that Guo can be incorporated as the first residue of the growing cRNA chain. An alternative (and more complex) schema is that Guo is added to a pre-existing partial cRNA chain by a novel enzyme activity, and that this addition then allows more rapid synthesis of the rest of the RNA.

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