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FUNCTIONAL ANALYSIS OF INFLUENZA RNA POLYMERASE ACTIVITY BY THE USE OF CAPS, OLIGONUCLEOTIDES AND POLYNUCLEOTIDES

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The effects of caps, dinucleotides, oligonucleotides and polynucleotides on influenza virus RNA polymerase activity have been investigated. The results show that both methyl groups in a cap are necessary for optimal stimulation of polymerase activity. Both $m^7G(5')ppp(5')A^m$ and ApG stimulated the influenza RNA polymerase activity and seemed to interact at different sites. Out of the 16 homopolynucleotides tested, seven inhibited influenza RNA polymerase by 50% at 2–10 μg/ml. Poly(G) gave a 90% reduction of influenza virus plaque formation at 10 μg/ml. An oligodeoxyribonucleotide complementary to the 12 terminal nucleotides of the 3′ end of influenza virus RNA was synthesized. This oligonucleotide did not selectively inhibit influenza RNA polymerase.

influenza virus RNA polymerase inhibition antiviral activity caps oligonucleotides polynucleotides

INTRODUCTION

The molecular mechanism of influenza mRNA synthesis has recently been analyzed in great detail and found to be different from what is known for other mRNA's [3–7,9, 11–14,16–20]. The virion contains a RNA polymerase responsible for the synthesis of a polyadenylated influenza mRNA. In the infected cell, cap structures and 6–14 further nucleotides are cleaved off from cellular mRNA and incorporated as a primer at the 5′ end of the influenza mRNA [7,9,13]. Since this can also take place in a cell-free assay with purified virions [3–5,16,19], the nuclease activity found in the virions [1] could be responsible for the cleavage of the cap-containing primer from cellular mRNA. The sequence of some primers as well as the rest of the 5′ end of influenza mRNA have been determined [7–9,19]. It has also been shown that dinucleotides can initiate RNA synthesis by the virion polymerase [14,17,18]. This detailed knowledge of the influenza mRNA synthesis has made it possible to search for inhibitors of the different steps of initiation. In this paper, we describe attempts to interfere with influenza mRNA synthesis

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at the cap binding site, at the primer cleavage stage, and by using different initiating nucleotides and an oligonucleotide complementary to the 3′ end of the virion RNA.

MATERIALS AND METHODS

Virus

Influenza A Victoria 3/75 X-47 and influenza B Hongkong 8/73 were purchased from Orion OY Helsinki, Finland.

Polymerase assay

The cell-free influenza RNA polymerase assay was carried out as previously described [22]. The incorporations of guanosine monophosphate (GMP) are given for 60 min incorporation at 30°C if not otherwise indicated. The polymerase reactions were linear for at least 60 min. In all assays, 6 mM $MgCl_2$ was used. The values given in the tables are corrected for zero time incorporation.

Plaque assay

Plaque assay on MDCK cells using influenza A Victoria 3/75 X-47 has been described [22]. A clone of influenza A Victoria 3/75 X-47 was used which did not need trypsin in the overlay in order to form plaques.

Chemicals

[^{3}H]Guanosine triphosphate (GTP) (13.3 Ci/mmol) was from New England Nuclear. Poly(G), poly(A), poly(C) and poly(U) were obtained from Calbiochem, all other homopolynucleotides as well as cap structures were from P.-L. Biochemicals. The dinucleotides (3′→5′) ApG, (3′→5′) GpG, and (2′→5′) ApG were from Sigma, and the other dinucleotides from Collaborative Research.

Oligonucleotides

The dodecamer DNA segment, d(AGCAAAAGCAGG), complementary to the 3′ end of influenza virion RNA [15] was synthesized on a solid support, and the methodology of its synthesis has been reported elsewhere [10]. The tetradecamer, d(GCCCATTTTTGGAA), the nonamer, d(GCCACTTT), and the undecamer, d(GCTTCTTTCAT) were synthesized according to the phosphotriester approach [2], using 9-phenylxanthen-9-yl for 5′-hydroxy protection. The 3′ end was protected by a phosphotriester containing β,β,β-tribromoethyl group. The oligonucleotide T_{12-18} was obtained from P.-L. Biochemicals. The dinucleotides 2′-d-GpG, 2′-d-pGp, 2′-d-pGpG, 2-d-ApG, 2′-d-pApG, used for oligonucleotide synthesis, were chemically synthesized using the phosphotriester approach [2].

Globin mRNA

Globin mRNA was purified from rabbit reticulocytes [5].

RESULTS

Effects of cap structures on the influenza RNA polymerase activity

The effects of cap structures on the cell-free influenza RNA polymerase reaction is shown in Table 1. None of the structures inhibited the polymerase in the absence of globin mRNA. The stimulation observed was dependent on the degree of methylation of the cap. Caps containing two methyl groups were more stimulatory than those containing one and those lacking both methyl groups The most active caps were $m^7G(5')ppp(5')G^m$ and $m^7G(5')ppp(5')A^m$. In the presence of globin mRNA, added as a primer, an inhibition of influenza A RNA polymerase activity was observed for the caps, which gave the highest stimulation in the absence of globin mRNA.

TABLE 1

Effects of cap structures on influenza RNA polymerase activity

Compound	% Inhibition of RNA synthesis with (+) or without (-) globin mRNA in the assay			
	Influenza A		Influenza B	
	-	+	-	+
G(5')ppp(5')G	-18	13	-32	16
G(5')pppp(5')G	-56	-5	-9	20
$m^7G(5')ppp(5')G$	-102	28	-38	11
$G(5')ppp(5')G^m$	-77	6	-54	18
$m^7G(5')ppp(5')G^m$	-155	42	-54	-10
G(5')ppp(5')A	-7	13	-6	0
$m^7G(5')ppp(5')A$	-75	8	-4	0
$G(5')ppp(5')A^m$	-38	14	-12	9
$m^7G(5')ppp(5')A^m$	-159	37	-18	-4
$m^7G(5')ppp(5')N^6mA^m$	-95	0	-12	-2
A(5')pp(5')C	-58	-8	8	8
A(5')pp(5')G	-16	0	-12	-1
G(5')ppp(5')C	-9	17	-4	5
G(5')ppp(5')U	-7	12	-13	6
$m^7G(5')ppp(5')C^m$	-30	19	-6	4
$m^7G(5')ppp(5')U^m$	-70	19	-19	0

The compounds were tested at a concentration of 65 μM apart from G(5')pppp(5')G, which was used at 110 μM. Globin mRNA was added to 1 2 μg/100 μl for influenza A and 1 9 μg/100 μl for influenza B At a concentration of 32 μM these compounds did not cause any plaque reduction using influenza A Victoria The incorporation of GMP was, in the absence of mRNA, 37–64 pmol/50 μl and, in the presence of mRNA, 150–204 pmol/50 μl in the different influenza A assays. In the influenza B assays the incorporation of GMP was, in the absence of mRNA, 13–25 pmol/50 μl and, in the presence of mRNA, 39–144 pmol/50 μl

Inhibition of influenza RNA polymerase activity by polynucleotides

The effect of polynucleotides on influenza RNA polymerase activity was first determined in the absence of any primer. As shown in Table 2, poly(X) was an efficient inhibitor and a 50% inhibition was seen at a concentration of 2 μg/ml. A 50% inhibition was observed with poly(U), poly(s^4U), poly(dU), poly(dT), poly(dC) and poly(dI) at concentrations ranging from 5 to 10 μg/ml. Poly(G) was the only polynucleotide giving a plaque reduction in cell culture at 10 μg/ml, but poly(X) and poly(I) gave a reduction in the size of plaques.

The influence of different primers on the ability of polynucleotides to inhibit influenza RNA polymerase activity is shown in Table 3. Addition of $m^7G(5')ppp(5')N^6mA^m$ or ApG did not significantly alter the inhibition caused by poly(U), poly(dU), poly(s^4U) or poly(G). When globin mRNA was added as a primer, all the polynucleotides became less inhibitory to influenza RNA polymerase activity.

TABLE 2

Effects of polynucleotides on influenza A RNA polymerase activity in the absence of primer, and plaque formation

Compound	Concentration giving 50% inhibition of RNA synthesis (μg/ml)	% Inhibition of plaque formation at 10 μg/ml
Poly(A)	> 100	-6
Poly(dA)	> 100	-46
Poly(1-N^6-etheno A)	> 100	-16
Poly(2'-*O*-methyl A)	50	-4
Poly(G)	29	90
Poly(dG)	> 100	-14
Poly(I)	10	37[a]
Poly(dI)	> 100	-16
Poly(X)	2	4[a]
Poly(C)	> 100	-14
Poly(dC)	6	0
Poly(2'-*O*-methyl C)	> 100	-41
Poly(U)	5	-14
Poly(dU)	9	-27
Poly(dT)	8	2
Poly(s^4U)	5	-32

The uninhibited polymerase reactions incorporated GMP in the range from 38 pmol/50 μl to 71 pmol/50 μl

[a] Small plaques

TABLE 3

Effects of polynucleotides on influenza A RNA polymerase activity

Compound	Primer	Concentration giving 50% inhibition of RNA synthesis (μg/ml)	Incorporation of GMP (pmol/40 μl in the absence of homopolymer)
Poly(G)	None	29	50
Poly(G)	$m^7G(5')ppp(5')N^6mA^m$	20	117
Poly(G)	ApG	36	338
Poly(G)	Globin mRNA	65	192
Poly(U)	None	5	56
Poly(U)	$m^7G(5')ppp(5')N^6mA^m$	8	117
Poly(U)	ApG	8	405
Poly(U)	Globin mRNA	20	207
Poly(dU)	None	9	46
Poly(dU)	$m^7G(5')ppp(5')N^6mA^m$	9	118
Poly(dU)	ApG	9	360
Poly(dU)	Globin mRNA	> 50	187
Poly(s^4U)	None	5	43
Poly(s^4U)	$m^7G(5')ppp(5')N^6mA^m$	4	112
Poly(s^4U)	ApG	6	316
Poly(s^4U)	Globin mRNA	13	163
Poly(dC)	None	6	48
Poly(dC)	$m^7G(5')ppp(5')N^6mA^m$	7	113
Poly(dC)	ApG	13	331
Poly(dC)	Globin mRNA	40	218

The concentration of added ApG was 500 μM, of $m^7G(5')ppp(5')N^6mA^m$, 65 μM, and of globin mRNA, 1.2 μg/100 μl.

Effects of dinucleotides on influenza RNA polymerase activity

In Table 4 is shown that GpG and ApG stimulated the non-primed influenza A and B RNA polymerase activities. ApG(2′→5′) was also stimulatory but not 2′-d-ApG. Both 2′-d-GpG and 2′-d-GpG were found to inhibit influenza A RNA polymerase. None of the compounds at a concentration of 100 μM caused any reduction in the plaque formation by influenza A Victoria.

Combined effects of ApG and $m^7G(5')ppp(5')A^m$ on influenza RNA polymerase activity

The dose-response curves for the stimulation of influenza RNA polymerase activity by ApG and $m^7G(5')ppp(5')A^m$ are shown in Fig. 1. In the presence of both ApG and the cap, an additive effect was observed at a saturating concentration of ApG.

TABLE 4

Effects of dinucleotides on influenza RNA polymerase activity

Compound	% Inhibition of RNA synthesis at 500 μM	
	Influenza A	Influenza B
GpG	-724	-1194
2'-d-GpG	47	15
2'-d-pGpG	42	8
2'-d-pGpG]	56	7
ApG (3'→5')	-806	-2038
ApG (2'→5')	-630	-1074
2'-d-ApG	4	0
2'-d-pApG	26	3
2'-d-pApG]	40	-31

At a concentration of 100 μM these compounds did not cause any plaque reduction using influenza A Victoria Without any compound added an incorporation of 42 pmol/50 μl GMP was observed for influenza A and 10 pmol/50 μl for influenza B The incubation time was 45 min

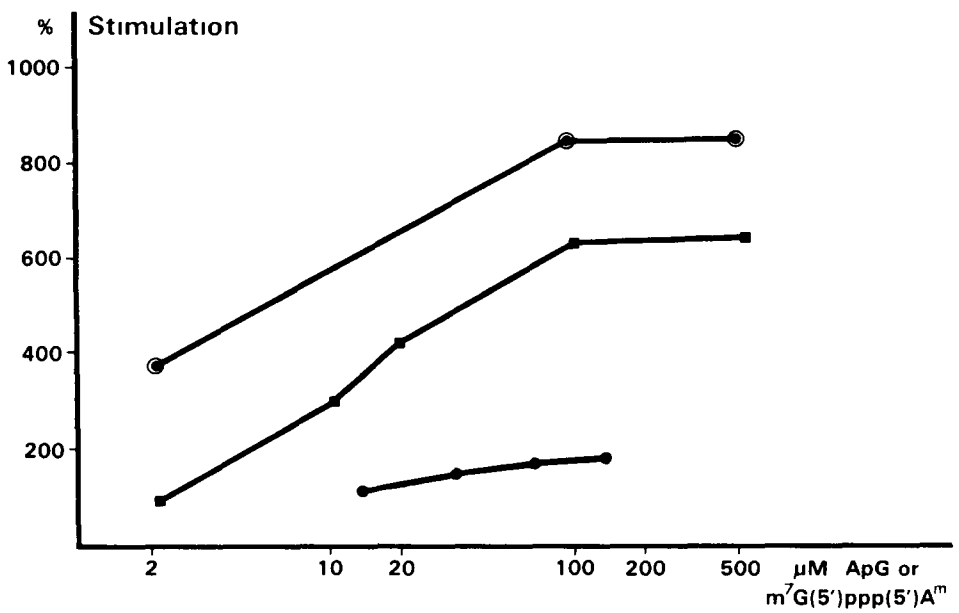

Fig 1 Stimulation of influenza A RNA polymerase activity by ApG and $m^7G(5')ppp(5')A^m$ The stimulation of the polymerase activity was determined at increasing concentrations of ApG and $m^7G(5')ppp(5')A^m$ and for various combinations of these compounds ○—○, 65μM $m^7G(5')ppp(5')A^m$ and increasing concentration of ApG ■—■, ApG ●—●, $m^7G(5')ppp(5')A^m$

Inhibition of influenza RNA polymerase activity by oligodeoxyribonucleotides

The possibility to inhibit influenza RNA polymerase activity by hybridization of a DNA sequence complementary to the 3′ end of influenza viral RNA was investigated by comparing the effects of complementary and non-complementary oligonucleotides. The results shown in Table 5 indicate that the complementary sequence d(AGCAAAAGCAGG) was not a better inhibitor than four other non-related sequences. The inhibition observed was reduced by adding globin mRNA to the assay.

DISCUSSION

The transfer of a capped oligonucleotide from a cellular mRNA to influenza mRNA offers several unique events suitable for inhibition. The importance of the methyl groups in the cap of an active primer has been reported earlier [4], and it was recently shown [6] that removal of either the 7-methyl or 2′-*O*-methyl groups decreased the priming ability of brome mosaic virus RNA in an influenza A RNA polymerase reaction. The present results (Table 1) confirm the observation that both methyl groups on the cap are necessary for optimal activity. It is thus likely that the polymerase can recognize both the 7-methyl and the 2′-*O*-methyl group. In the presence of globin mRNA, the dimethylated caps showed a slight inhibition of the polymerase activity, indicating a competition with globin mRNA. The influenza B polymerase activity was less stimulated by cap structures, but both the 7-methyl and the 2′-*O*-methyl groups seemed to be

TABLE 5

Inhibition of influenza A RNA polymerase activity by oligodeoxyribonucleotides

Compound	Concentration (μM)	Primer	% Inhibition of RNA synthesis
d(AGCAAAAGCAGG)	22	None	32
	22	Globin mRNA	8
d(GCCCATTTTTGGAA)	9	None	79
	22	Globin mRNA	12
d(GCCCACTTT)	26	None	-13
	26	Globin mRNA	-3
$d(T)_{12-18}$	20	None	22
	20	Globin mRNA	24
d(GCTTCTTTCAT)	22	None	-14
	22	Globin mRNA	-3

The concentration of globin mRNA in the assay was 0 6 μg/50 μl. The molar concentration of $d(T)_{12-18}$ was calculated assuming an average of 15 nucleotides. The control reaction without globin mRNA incorporated 76 pmol/40 μl GMP and the globin mRNA primed reaction 385 pmol/40 μl GMP

recognized. In contrast to the influenza A polymerase, influenza B polymerase seemed to prefer caps with two guanosines.

It is not clear if the cleavage of the primer from the cellular mRNA is mediated by a virus enzyme. However, a virion-associated ribonuclease has been reported [1] and the interaction of ribopolymers with this cleavage has been suggested [12]. Poly(X) seemed to be one of the most effective inhibitors of the non-primed influenza RNA polymerase activity (Table 2) and also reduced the size of influenza plaque in cell culture; this was also observed for poly(I). Poly(G) caused a 90% plaque reduction at a concentration of 10 μg/ml but, since it only inhibited the polymerase at a high concentration, it is doubtful that its effect in cell culture was primarily due to an inhibition of the viral polymerase. The ribopolymers, poly(I), poly(G), and poly(U) were more active as inhibitors than the corresponding 2′-deoxyribopolymers. The reverse was observed for poly(C). Inhibitions caused by homopolynucleotides were not affected by addition of caps or ApG but were reduced by globin mRNA (Table 3), indicating that this inhibition was not caused by interaction at the cap-binding site or at the initiating site where ApG binds [17, 18]. The inhibitions observed for poly(s^4U), poly(U), and poly(G) correspond well with the results from Smith et al. [21] and Krug et al. [13]. A correlation between polymerase inhibition and plaque inhibition might be affected by differences in degradation of the polynucleotides by cellular nucleases.

In contrast to the stimulation of influenza RNA polymerase activity by GpG and ApG, an inhibition was observed if the corresponding 2′-deoxydinucleotides were used (Table 4). A change from a (3′→5′) to a (2′→5′) phosphodiester bond in the dinucleotide ApG did not, however, suppress the priming ability. When the combined effects of m^7G(5′)ppp(5′)A^m and ApG were investigated as shown in Fig. 1, the results indicated an additive effect, which might mean that the cap and ApG interact with different sites on the polymerase.

A further possibility of inhibiting polymerase activity has been suggested by Zamecnik and Stephenson [23], who found that a deoxy-tridecamer complementary to the 3′ end of Rous sarcoma virus RNA inhibited Rous sarcoma virus replication. One explanation to this result could be that the oligonucleotide by hybridization to viral RNA prevented the polymerase from using its template. This approach was also used for influenza virus by synthesizing a deoxy-dodecamer [10] complementary to the 3′ end of influenza virus RNA. When tested for its ability to inhibit influenza RNA polymerase activity, it was not more active than a few of the non-complementary DNA sequences tested (Table 5). This indicates that the 3′ end of influenza virus RNA is shielded from base pairing, possibly by proteins not removed by the complementary oligonucleotide. This result supports an earlier observation that base pairing to virion RNA was not necessary for the primer sequence [12].

These results indicate that there are different functional sites, suitable as targets for inhibitors, on the influenza RNA polymerase complex; one which recognizes the two methyl groups in the cap, one for the cleavage of the primer and another for the initiation at the end of the primer. Our results also show that influenza RNA polymerase

can not be inhibited by an oligonucleotide complementary to the 3′ end of influenza virion RNA.

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