BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 103–106 (1998)
ARTICLE NO. RC989085

Inhibition of Influenza Virus RNA Polymerase by 5′-Capped Short RNA Fragments

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Received June 26, 1998

We have demonstrated that 5′-capped short RNA fragments inhibit the expression of chloramphenicol acetyltransferase (CAT) in the murine 76 cell line, derived which expresses the genes for the RNA polymerases (PB1, PB2, and PA) and the nucleoprotein (NP) of influenza virus in response to treatment with dexamethasone. We have synthesized 5′-capped short RNA fragments (8-13 ntds long) with a 5′-capped structure (m7GpppGm) using T7 RNA polymerase. The 5′-capped short RNA fragments (8-13 ntds long) were encapsulated in liposomes and were tested for their inhibitory effect by a CAT-ELISA assay using the clone 76 cells. The RNA fragments that were 9-12 ntds long showed inhibitory effects. In particular, the 9 ntds long RNA fragment, was highly inhibitory. On the other hand, the inhibitory effect of the 13 ntds long RNA fragment was considerably decreased in comparison with the other short RNA fragments. The minimal RNA chain length required for priming activity was found to be 12 ntds long. Furthermore, the 5′-capped RNA fragments exhibited higher inhibitory activities than the antisense phosphorothioate oligonucleotide (PB2-AUG-as, 20 ntds long) complementary to the site of the PB2-AUG initiation codon. Liposome encapsulation protected the RNA fragments in serum-containing medium and substantially improved their cellular accumulation.

Influenza A virus is a segmented, negative-strand RNA virus that encodes its own RNA-dependent RNA polymerase. The polymerase exists as a complex of three proteins (PB1, PB2, and PA) at the termini of nucleoprotein (NP)-encapsulated viral genome segments. The mRNA synthesis is initiated at the negative-strand RNA segments by endonucleolytic cleavage of the host precursor mRNA in the nucleous of infected cells, by a specific endonuclease activity of the influenza virus RNA polymerase. These cellular transcripts possess a "cap-1" structure (m7GpppXm) at their 5′ ends and are bound and then cleaved by the influenza virus RNA polymerase at positions 9-15 ntds from their 5′ ends, preferentially after purine residues, to yield primers for viral transcription (1,2). This overall process produces chimeric viral mRNAs that contain host-derived heterogeneous sequences at their 5′ ends (3,4). These capped short RNA fragments are potential inhibitors of cap-dependent transcription in vitro. A recent study has confirmed earlier data (5-9) that priming by capped oligonucleotides can be uncoupled from the endonuclease activity of the influenza RNA polymerase, and has suggested the use of inhibitors such as a decoys of cap-dependent transcription in vitro (10). On the other hand, we recently demonstrated that liposomally encapsulated antisense phosphorothioate oligonucleotides against the PB2-AUG mRNA sequence exhibited the highest inhibitory activity toward influenza virus RNA polymerase expression (11,12). However, among the various antisense DNA or RNA based strategies against influenza virus infection, the decoy strategy has a potential advantage over the use of other inhibitors, such as antisense DNA or RNA and ribozymes, because the generation of escape mutants might be less frequent: the alteration of an influenza virus RNA polymerase that prevents binding to a decoy would also prevent binding to a native element [cap of primer RNA] (11-13).

In this report, we present a detailed analysis of the inhibition of influenza virus RNA polymerase expres-

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0006-291X/98 $25.00

sion by the 5′-capped short RNA fragments (8-13 ntds) as determined by CAT protein expression (CAT-activity) in the clone 76 cell line (14,15). Furthermore, we describe the CAT-activities of the 5′-capped short RNA fragments containing the different cap-1 structures, m7GpppGm- and m7GpppGOH-.

MATERIALS AND METHODS

Oligonucleotide synthesis. The template oligonucleotides containing the T7 RNA polymerase promoter were synthesized by the phosphoramidite method, using an Applied Biosystems Model 392 DNA/RNA synthesizer on the 1 μM scale, with controlled pore glass supports. The oligonucleotides were purified by reverse phase HPLC chromatography. The extinction coefficients of the oligonucleotides were determined by calculating the theoretical extinction coefficients as the sum of the nucleosides and multiplying with the experimentally determined enzymatic hypochromicity (16).

5′-Capped short RNA strand synthesis. The DNA template containing the T7 RNA polymerase promoter was annealed in a one to one molar ratio in 0.8 mM HEPES-KOH (pH 7.5) by heating at 90 °C for 3 min, followed by snap-cooling on ice. The T7 RNA transcription reactions contained 0.6 μg DNA template, 80 mM HEPES-KOH (pH 7.5)/12 mM $MgCl_2$, 2 mM spermidine, 20 mM DTT, 30 μg of either m7GpppGm (Pharmacia Biotech) or m7GpppGOH, 4 mM of each NTP, 10 mM KCl, 10 mM $MgCl_2$, 20 μg/μl BSA, 40 U/μl RNasin, and 7 mg/ml T7 RNA polymerase (10 μl). The reaction mixture was incubated for 2 h at 37°C. Full-length transcripts (8-13 nucleotides) were purified by 7 M urea/20% polyacrylamide gel electrophoresis, elution, and ethanol precipitation in the presence of 3 M sodium acetate.

Enzymatic assay of 5′-capped RNA fragments (m7GpppGmAAUACUC and m7GpppGOHA-AUACUC). The capped oligoribonucleotide (0.2 A260), which was freeze-dried, was incubated with snake venom phosphodiesterase (0.2 μg) in the presence of 1 M $MgCl_2$ in Tris-HCl buffer (pH 8.0, 50 μl) at 37°C for 5 h. Aliquots were removed and extracted with phenol-chloroform. Samples were analyzed by HPLC (buffer A: 0.005 M KH_2PO_4, pH 4.1; buffer B: 0.1 M KH_2PO_4, 1 M KCl, pH4.5).

Cells. The murine C127 derivative cell line, clone 76 (14,15), was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

In vitro RNA synthesis. The plasmid pOUMS101 contains the CAT gene sandwiched between the 5′- and 3′-terminal sequences of the cDNA encoding RNA segment 8 of influenza virus A/PR/8/34, in the antisense orientation of the T7 RNA polymerase (17). Transcription of Mbo II-digested pOUMS101 by T7 RNA polymerase results in the synthesis of the antisense CAT RNA. The template DNA was removed by treatment with DNase I. The RNA transcript was purified by TBE / 7 M urea, 5 % polyacrylamide gel electrophoresis, elution, and ethanol precipitation in the presence of 3 M sodium acetate.

RNA transcription in clone 76 cells. Clone 76 cells, grown in 60 mm dishes and approximately 50 % confluent, were treated with 10^{-6} M dexamethasone in DMEM containing 10 % FBS at 37°C. After 24 h, the cells were washed with PBS, a mixture of 5′-capped RNA fragments and 5 μg of lipofection reagent (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, DOTAP, Boehringer Mannheim) in DMEM was added, and the cells were inoculated for 4 h at 37°C. The cells were washed with PBS, 100 ng of RNA was mixed with 5 μg of DOTAP in DMEM, and the mixture was incubated for 4 h at 37 °C. The medium was changed to fresh DMEM containing 10 % FBS and the cells were incubated for a further 20 h. The amount of CAT protein in the cells was monitored by the CAT-ELISA kit (anti-CAT-DIG, Boehringer Mannheim).

TABLE 1

The 5′-Capped Short RNA Sequences Used in This Study

Abbreviation	5′-capped RNA sequence
Gm8	m7GpppGmAAUACUC
Gm9	m7GpppGmAAUACUCA
Gm10	m7GpppGmAAUACUCAA
Gm11	m7GpppGmAAUACUCAAA
Gm12	m7GpppGmAAUACUCAAAC
Gm13	m7GpppGmAAUACUCAAACU
GOH8	m7GpppGOHAAUACUC
S-ODN-PB2-AUG-as	5′-TsTsTsCsTsTsTsCsCsAsTsAsTsTs-GsAsAsTsAsTsA-3′ [a]

18 37

[a] Targeted mRNA:5′UAUAUUCAAUAUGGAAAGAA-3′. The phosphorothioate derivative is denoted by "S".

RESULTS AND DISCUSSION

The synthesis of influenza viral mRNA requires initiation by host cell primers, which are specifically capped (m7GpppNm-containing) RNA fragments derived from host cell RNA polymerase II transcripts (18-20). This occurs in the nucleus of the infected cell. As a consequence, viral mRNA synthesis requires the continuous functioning of the cellular RNA polymerase II, and is inhibited by α-amanitin. The host cell primers are generated by a viral cap-dependent endonuclease that cleaves the capped cellular RNAs 10 to 13 nucleotides from their 5′ ends, preferentially at a purine residues. Transcription is initiated by the incorporation of a G residue onto the 3′ end of the resulting fragments, directed by the penultimate C residue of the vRNAs. Viral mRNA chains are then elongated until a stretch of 4 to 7 uridine residues is reached 17 to 22 nucleotides before the 5′ ends of the vRNAs, where transcription terminates and polyadenylate (poly[A]) is added to the mRNAs.

First, we tried to synthesize the 5′-capped oligonucleotides (see Table 1) by in vitro transcription from the DNA template containing the T7 RNA polymerase promoter using T7 polymerase, NTPs, and either m7GppGm or m7GpppGOH as primers (described in the Materials and Methods). The T7 RNA polymerase method produced these 5′-capped oligonucleotides in good yields (60-70%). Furthermore, we detected the 5′-capped short RNA fragments (Gm8 and GOH8) by enzyme assay with snake venom phosphodiesterase. The degraded products of Gm8 and GOH8 were analyzed by HPLC. The enzyme assays of m7GPPPGm and Gm8, and m7GpppGOH and GOH8, with snake venom phosphodiesterase exhibited peaks corresponding to pm7G, pGm, pG, pC, pU, and pA, respectively, as shown in Fig. 1A and B. Therefore, we could monitor the addition of the 5′-end cap structure.

The 5′-capped short RNA fragments are potential targets for a decoy (cap of the primer RNA). Next,

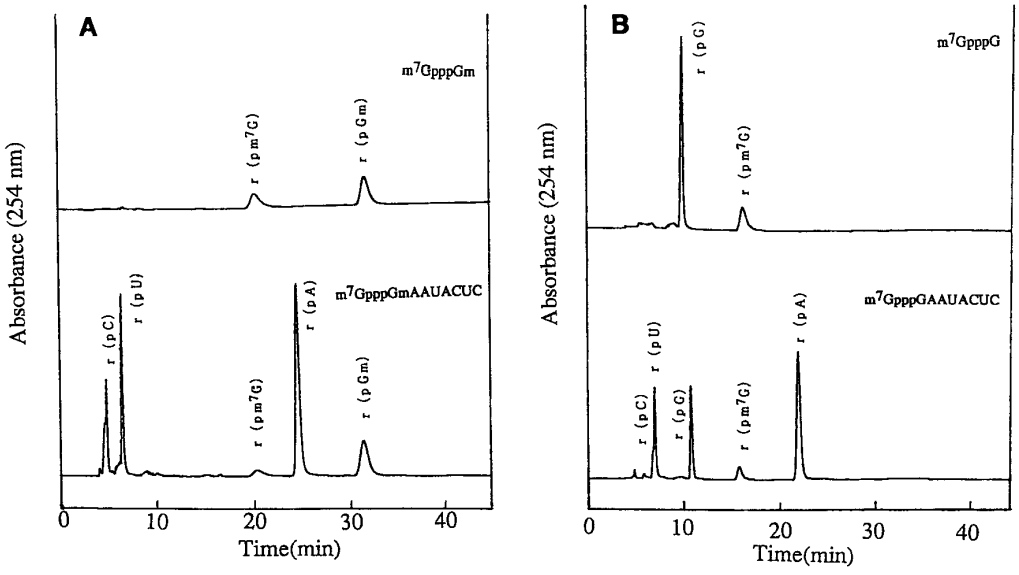

FIG. 1. Analysis of products obtained after hydrolysis of 5′-capped RNA fragments (Gm8 (A) and GOH8 (B)) with snake venom phosphodiesterase. Separation on a TSK-gel ODS-80TM column in 0.005 M KH_2PO_4 (pH4.1, buffer A) and 0.6% 0.1 M KH_2PO_4 -1 M KCl (pH4.5, buffer B) (0-5 min) followed by a gradient to 100% buffer B (60 min).

to clarify the cap-1 chain length and its structural specificity as an inhibitor of the RNA polymerase, we tested the 5′-capped short RNA fragments containing either cap-1 (m7GpppGm-) or a cap-1 analogue (m7GpppGOH-) for their effects on the expression of the RNA polymerase in clone 76 cells (Table 1). Recently, Nakada *et al.* reported an *in vitro* replication system for influenza virus using the clone 76 cell line, in which the viral RNA polymerase (PB1, PB2, PA) and nucleoprotein (NP) genes of influenza virus A can be expressed in response to dexamethasone (14,15). A chimeric NS-chloramphenicol acetyltransferase (CAT) RNA, consisting of the full-length negative-strand RNA of the CAT gene positioned between the 5′-and 3′-terminal sequences of the influenza virus RNA segment 8, can be transcribed in the absence of RNA polymerase in this cell line following transfection (17). The 5′-capped short RNA fragments were introduced into the clone 76 cells by liposome-mediated transfection (21-23). The *in vitro* activities of these 5′-capped short RNA fragments on the expression of the influenza virus RNA polymerase were assessed on the basis of their inhibition of CAT protein expression with the CAT-ELISA method.

When we analyzed the inhibitory effect of the liposomally endocapsulated 5′-capped short RNA fragments containing the 5′-cap-1 structure (m7GpppGm-), the 9-12 ntds long 5′-capped RNA fragments (Gm9, Gm10, Gm11, and Gm12) had highly inhibitory effects, causing more than 61% inhibition at 0.03 μM concentration (Table 2). At a 0.3 μM concentration, they showed the highest inhibitory effects, causing more than 72% inhibition. In particular, at the 5′-capped RNA fragment (9 ntds long) concentrations of 0.03 μM and 0.3 μM, the percentages of inhibition were 82% and 88%, respectively. This capped RNA had higher inhibitory effects than the 5′-capped RNA fragments that were 10-12ntds long. However, the 8 ntds long 5′-capped RNA fragment (Gm8), containing the 5′-cap-1 structure (m7GpppGm-), had lower inhibitory effects as compared with the 5′-capped RNA fragments, Gm9-Gm12.

TABLE 2

Inhibition of Influenza Virus RNA Polymerase by Encapsulated 5′-Capped Short RNA Fragments

	Inhibitory effect (%)[b,c]	
	0.03	0.3
5′-Capped RNAs[a]	μM	
Gm8	55	77
Gm9	82	88
Gm10	69	83
Gm11	66	72
Gm12	61	82
Gm12[d]	<5	10
Gm13	23	<5
GOH8	29	51
S-ODN-PB2-as	45	53

[a] The 5′-capped short RNA strands are described in Table 1.

[b] The inhibitory effects are given as the percentage of inhibition of CAT protein expression, and are compared and normalized to 100% CAT protein expression.

[c] Data represent average values for at least three different experiments.

[d] This reaction was carried out in the absence of DOTAP.

This results suggest that the oligomer chain length has a great effect on the anti-CAT activity. The 9-12 ntds long 5′-capped RNA fragments containing the 5′-end cap-1 structure showed increased inhibition efficiency at higher concentrations. On the other hand, the 13 ntds long 5′-capped RNA fragment (Gm13), containing the 5′-cap-1 structure (m7GpppGm-), showed lower anti-CAT activity at 0.03-0.3 μM concentrations. We suggest that the PB2 protein recognized and bound to the Gm13 as a positive strand RNA primer. The viral polymerase can efficiently bind the short capped RNAs that are less than 12 nucleotides in length, but the viral enzymes cannot utilize those RNAs as primers.

Furthermore, we evaluated the anti-CAT activity of the 8 ntds long 5′-capped RNA fragment (GOH8), containing the cap-1 analogue (m7GpppGOH-). The GOH8 RNA showed less anti-CAT activity as compared with the Gm8 RNA (Table 2). These results suggest that the influenza virus RNA polymerase (PB2 protein) recognizes and binds to the cap-1 (m7GpppGm-) structure of the primer RNA. The antisense phosphorothioate oligonucleotide (S-ODN-PB2-as, 20 mer), containing an AUG initiation codon as the target of PB2, was chosen for comparison with the 5′-capped short RNA fragments. The short capped oligonucleotides showed higher inhibitory effects than the liposomally encapsulated S-ODN-PB2-as (Table 2).

Finally, we investigated the inhibition of CAT protein expression by the 12 ntds long free 5′-capped RNA fragment (Gm12) in clone 76 cells. This RNA fragment had no detectable inhibitory effect in its free form, because it was apparently degraded in the culture medium (Table 2). These results suggest that the increased cell association of the 5′-capped short RNA fragments can explain the enhanced decoy effect of the liposomally encapsulated oligonucleotides. Thus, in the presence of DOTAP, the oligonucleotide first enters the cytoplasm and then quickly accumulates in the nucleus (24). Moreover, the effectiveness of the liposomally encapsulated 5′-capped short RNA fragments was probably due mainly to their relative nuclease resistance in the culture medium (Table 2).

In conclusion, the 9-12 ntds long 5′-capped RNA fragments, containing the 5′-end cap-1 structure (m7GpppGm-), showed the greatest inhibition of influenza virus RNA polymerase expression in clone 76 cells. In particular, at the 9 ntds long 5′-capped RNA fragment, showed higher inhibitory effects than the 10-12 ntds long 5′-capped RNA fragments. In contrast, the modified 5′-capped short RNA fragment containing the 5′-end cap-1 analogue (m7GpppGOH-) showed decreased inhibition. Furthermore, the use of cationic lipids (DOTAP) provided the nuclease resistance and enhanced cellular uptake of the 5′-capped short RNA fragments. The 5′-capped short RNA fragments are potent inhibitors of cap-dependent transcription *in vitro.*

ACKNOWLEDGMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research (09278223) on Priority Areas from the Ministry of Education, Science and Culture of Japan and by a Grant of the "Research for Future" Program of the Japan Society for the Promotion of Science, Ministry of Education, Science and Culture of Japan.

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