ELSEVIER

Antiviral Research 42 (1999) 35–46

Antiviral Research

Guanosine nucleotide analogs as inhibitors of alphavirus mRNA capping enzyme

Anja Lampio [a,*], Tero Ahola [a,1], Edward Darzynkiewicz [b], Janusz Stepinski [c], Marzena Jankowska-Anyszka [c], Leevi Kääriäinen [a]

[a] *Institute of Biotechnology, Viikki Biocenter, PO Box 56, (Viikinkaari 9), FIN-00014 University of Helsinki, Helsinki, Finland*
[b] *Department of Biophysics, University of Warsaw, 02-089 Warsaw, Poland*
[c] *Department of Chemistry, University of Warsaw, 02-089 Warsaw, Poland*

Received 15 September 1998; accepted 12 January 1999

Abstract

The two virus-specific reactions in the capping of alphavirus RNAs, catalyzed by the replicase protein nsP1, are promising targets for developing virus-specific inhibitors. In this report, we have studied the effect of over 50 cap analogs on the guanine-7-methyltransferase and guanylyltransferase activities of Semliki Forest virus nsP1. Recombinant nsP1 was expressed in *Escherichia coli* and partially purified by flotation in a discontinuous sucrose gradient. The methyltransferase activity had a pH optimum between pH 6.5 and 7.1, and the apparent K_m values were 1.9 mM for GTP, 6.0 μM for *S*-adenosyl-L-methionine and 170 μM for Mg^{2+}. NsP1 methyltransferase was able to methylate efficiently GTP (relative activity 100%), GDP (16%), GpppG (35%), GppppG (50%) and less efficiently GpppA (12%), m^2GTP (9%), and $m^{2,2}$GTP (25%), but not m^7GppG. The most potent inhibitors for nsP1 methyltransferase were et^2m^7GMP (K_i value 42 μM), $m^{2,7}$GMP, (64 μM), $m^{2,7}$GpppG (82 μM), m2et7GMP (105 μM), m^2(2-phet)^{7}GMP (194 μM) and m^2GMP (386 μM). Of these compounds, m^2GMP, m^2et^7GMP and m^2(2-phet)^{7}GMP showed competitive inhibition, whereas the others showed mixed type inhibition. All compounds that inhibited the methyltransferase activity inhibited also the guanylyltransferase activity of nsP1. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Alphaviruses; Antiviral compounds; Nucleotide analogs; Methyltransferase; Guanylyltransferase

1. Introduction

Alphaviruses are enveloped positive-strand RNA viruses. They replicate in the cytoplasm of the infected cells and do not need nuclear functions (Kääriäinen and Ranki, 1984; Strauss and

* Corresponding author. Fax: +358-9-708-59560.
E-mail address: anja.lampio@helsinki.fi (A. Lampio)
[1] Present address: Institute for Molecular Biology, University of Winsconsin, 1525 Linden Drive, Madison, WI 53706, USA.

PII: S0166-3542(99)00011-X

Strauss, 1994). The genomic 42S RNA (ca. 11.5 kb) and the subgenomic 26S mRNA have a m^7GpppA cap-structure at their 5′ end (Dubin et al., 1977; Pettersson et al., 1980). They are synthesized in association with membrane-bound replication complexes, which contain the four virus-specific nonstructural proteins nsP1-4 (Kääriäinen and Söderlund, 1978; Froshauer et al., 1988; Barton et al., 1991).

Recent findings have shown that nsP1 is involved in the capping of the viral RNAs. Semliki Forest (SFV) and Sindbis virus nsP1 catalyze the guanine-7-methyltransferase (MT) reaction, in which *S*-adenosyl-methionine (AdoMet) reacts with GTP to yield 7-methyl-GTP (m^7GTP) and *S*-adenosyl-homocysteine (AdoHcy) (Mi and Stollar, 1991; Laakkonen et al., 1994). The reaction is specific for GTP and dGTP, while capped RNA is not a substrate (Laakkonen et al., 1994). In the presence of AdoMet, GTP also reacts with nsP1 to form a unique covalent complex m^7GMP-nsP1 (Ahola and Kääriäinen, 1995), similar to the covalent complex formed by cellular guanylyltransferases with GMP (Shuman and Hurwitz, 1981; Venkatesan and Moss, 1982; Mizumoto and Kaziro, 1987). The MT-reaction can take place in the presence of EDTA, while the guanylyltransferase reaction (GT) requires divalent cations (Mg^{2+} or Mn^{2+}). The novel specificities of nsP1-catalyzed reactions have led us to propose that RNA capping of alphaviruses consists of the following reactions:

$$GTP + AdoMet \Rightarrow m^7GTP + AdoHcy$$

$$m^7GTP + nsP1 \Rightarrow m^7GMP - nsP1 + PP_i$$

The 7-methyl-GMP would then be transferred to the mRNA to create the cap 0-structure (Ahola and Kääriäinen, 1995). Mutations of nsP1 amino acids conserved in the alphavirus-like superfamily revealed that His 38 is essential for the formation of the covalent guanylate complex but not for the MT-reaction, whereas Asp 64 and Asp 90 are involved in the binding of AdoMet to nsP1 (Ahola et al., 1997). The conserved residues, and hence the capping activities, are essential for alphavirus RNA replication (Wang et al., 1996).

Since both MT and GT reactions are unique for alphaviruses they should offer a possibility for development of virus-specific inhibitors. To this end we have studied the effect of over 50 synthetic cap analogs on MT and GT activities of nsP1 in vitro.

2. Materials and methods

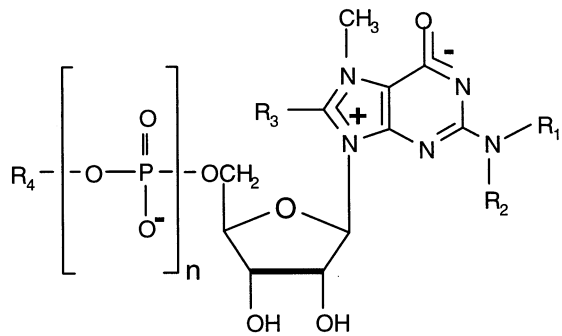
2.1. Chemicals

The chemical formulas of the cap analogs are shown in Figs. 1–3 and references for their synthesis are given in Table 1. Guanosine (G), GMP, GDP, GTP, m^7G, m^7GMP, m^7GDP and m^7GTP were from Sigma-Aldrich.

2.2. Expression and partial purification of recombinant nsP1

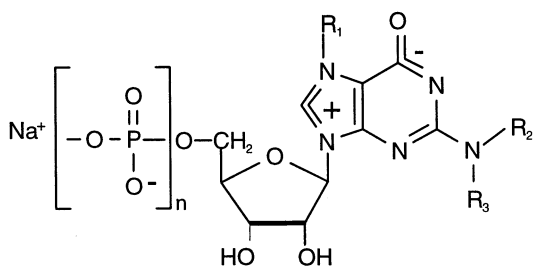
Plasmid pBAT-nsP1, in which the coding sequence of nsP1 is under T7 promoter inducible with isopropyl 1-thio-β-galactopyranosidase (IPTG) (Peränen et al., 1996), was transformed into *Escherichia coli* BL21 (DE3) (Novagen), and the protein was expressed at 15°C after induction with 500 μM IPTG as described (Laakkonen et al., 1994). Pelleted cells were resuspended in lysis buffer (50 mM Tris–HCI pH 8.0, 50 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10% glycerol) and disrupted with a French press (Laakkonen et al., 1994). The lysate was centrifuged for 15 min at 15 000 × g, and the resulting supernatant was used for further analysis. The flotation was carried out as described previously for HeLa cell membranes (Laakkonen et al., 1994). Briefly, the bacterial supernatant S15 was mixed with 67% sucrose in 50 mM Tris, pH 8.0, to give a final concentration of 60%. Discontinuous flotation gradients were prepared in SW50.1 (Beckman) ultracentrifuge tubes by layering first 0.5 ml 67% (w/w) sucrose, then 0.5 ml of the sample, followed by 3 ml of 50% (w/w) sucrose and 1 ml of 10% (w/w) sucrose. The samples were centrifuged overnight (>16 h) at 35,000 rpm in SW50.1 rotor at 4°C. Fractions of 0.55 ml were collected and analyzed by SDS-PAGE and for methyltransferase activity.

A



2.	$m^{2,2,7}$Gppp:	$R_1 = R_2 = CH_3$,	$R_3 = H$,	$R_4 = *$,	n = 3
3.	$m^{2,2,7}$Gp:	$R_1 = R_2 = CH_3$,	$R_3 = H$,	$R_4 = *$,	n = 1
4.	$m^{2,2,7}$G:	$R_1 = R_2 = CH_3$,	$R_3 = H$,	$R_4 = H$,	n = 0
7.	$m^{2,7}$Gppp:	$R_1 = CH_3$,	$R_2 = R_3 = H$	$R_4 = *$,	n = 3
8.	$m^{2,7}$Gp:	$R_1 = CH_3$,	$R_2 = R_3 = H$	$R_4 = *$,	n = 1
10.	$m^{2,7}$G:	$R_1 = CH_3$,	$R_2 = R_3 = H$	$R_4 = H$,	n = 0
23.	$m^7NH_2{}^8$Gp:	$R_1 = R_2 = H$,	$R_3 = NH_2$,	$R_4 = *$,	n = 1
24.	$m^{7,8}$Gp:	$R_1 = R_2 = H$,	$R_3 = CH_3$.	$R_4 = *$,	n = 1
43.	et^2m^7Gp:	$R_1 = C_2H_5$,	$R_2 = R_3 = H$	$R_4 = *$,	n = 1
55.	m^7G:	$R_1 = R_2 = R_3 = R_4 = H$,			n = 0
56.	m^7Gp:	$R_1 = R_2 = R_3 = H$,		$R_4 = *$,	n = 1
56.	m^7Gpp:	$R_1 = R_2 = R_3 = H$,		$R_4 = *$,	n = 2
57.	m^7Gppp:	$R_1 = R_2 = R_3 = H$,		$R_4 = *$,	n = 3

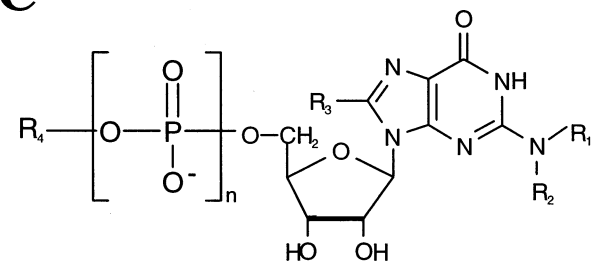
B



26.	allyl^{7}Gp:	$R_1 = CH_2\text{-}CH{=}CH_2$,	$R_2 = R_3 = H$,		n = 1
27.	butyl^{7}Gp:	$R_1 = CH_2CH_2CH_2CH_3$,	$R_2 = R_3 = H$,		n = 1
28.	cm^{7}Gp:	$R_1 = CH_2COOH$,	$R_2 = R_3 = H$,		n = 1
29	bn^{7}Gp:	$R_1 = CH_2C_6H_5$,	$R_2 = R_3 = H$,		n = 1
30.	(1-phet)^{7}Gp:	$R_1 = CH(CH_3)C_6H_5$,	$R_2 = R_3 = H$,		n = 1
31.	(2-phet)^{7}Gp:	$R_1 = CH_2CH_2C_6H_5$,	$R_2 = R_3 = H$,		n = 1
37.	m^2(pFbn)^{7}Gppp:	$R_1 = CH_2C_6H_4pF$,	$R_2 = CH_3$,	$R_3 = H$,	n = 3
38.	$m^{2,2}$(pFbn)^{7}Gppp:	$R_1 = CH_2C_6H_4pF$,	$R_2 = R_3 = CH_3$,		n = 3
39.	m^2(pClbn)^{7}Gpp:	$R_1 = CH_2C_6H_4pCl$,	$R_2 = CH_3$,	$R_3 = H$,	n = 2
40.	m^2(pFbn)^{7}Gp:	$R_1 = CH_2C_6H_4pF$,	$R_2 = CH_3$,	$R_3 = H$,	n = 1
41.	m^2bn^{7}Gp:	$R_1 = CH_2C_6H_5$,	$R_2 = CH_3$,	$R_3 = H$,	n = 1
42.	m^2(2-phet)^{7}Gp:	$R_1 = CH_2CH_2C_6H_5$,	$R_2 = CH_3$,	$R_3 = H$,	n = 1
44.	m^2et^7Gp:	$R_1 = C_2H_5$,	$R_2 = CH_3$,	$R_3 = H$,	n = 1

Fig. 1. Guanosine analogs with a methyl group at position 7 (A), with substituents other than methyl at position 7 (B), and without a substitution at position 7 (C). * Anionic form of the terminal phosphate group in mono-, di- or triphosphates.

C



32.	m^2G:	$R_1 = CH_3$,	$R_2 = R_3 = R_4 = H$,		n = 0
33.	m^2Gp:	$R_1 = CH_3$,	$R_2 = R_3 = H$,	$R_4 = *$,	n = 1
34.	m^2Gppp:	$R_1 = CH_3$,	$R_2 = R_3 = H$,	$R_4 = *$,	n = 3
35.	$m^{2,2}G$:	$R_1 = R_2 = CH_3$,	$R_2 = R_3 = H$,		n = 0
36.	$m^{2,2}Gppp$:	$R_1 = R_2 = CH_3$,	$R_3 = H$,	$R_4 = *$,	n = 3
45.	m^8G:	$R_1 = R_2 = R_4 = H$,	$R_3 = CH_3$,		n = 0
46.	m^8Gp:	$R_1 = R_2 = H$,	$R_3 = CH_3$	$R_4 = *$,	n = 1
48.	Gp:	$R_1 = R_2 = R_3 = H$,		$R_4 = *$,	n = 1
50.	Gpp:	$R_1 = R_2 = R_3 = H$,		$R_4 = *$,	n = 2
51.	Gppp:	$R_1 = R_2 = R_3 = H$,		$R_4 = *$,	n = 3

Fig. 1. (*Continued*)

2.3. Enzyme assays

The reaction mixture (25 μl) for methyltransferase assays contained 10 mM GTP, 2 mM $MgCl_2$, 2 mM dithiothreitol,20 μM AdoMet, 0.5 μCi S-adenosyl-L-[methyl-^{3}H]methionine (60 to 85 Ci/mmol), 100 mM HEPES (pH 6.95), and 1–2 μl of the enzyme source, representing methyltransferase activity of 3.8 pmol/min/μl in S15 and 0.44 pmol/min/2 μl in flotated membrane fraction. After incubation for 30 min at 30°C, the labeled product was purified using 1-ml DEAE-Sephadex columns and quantified by liquid scintillation (Laakkonen et al., 1994).

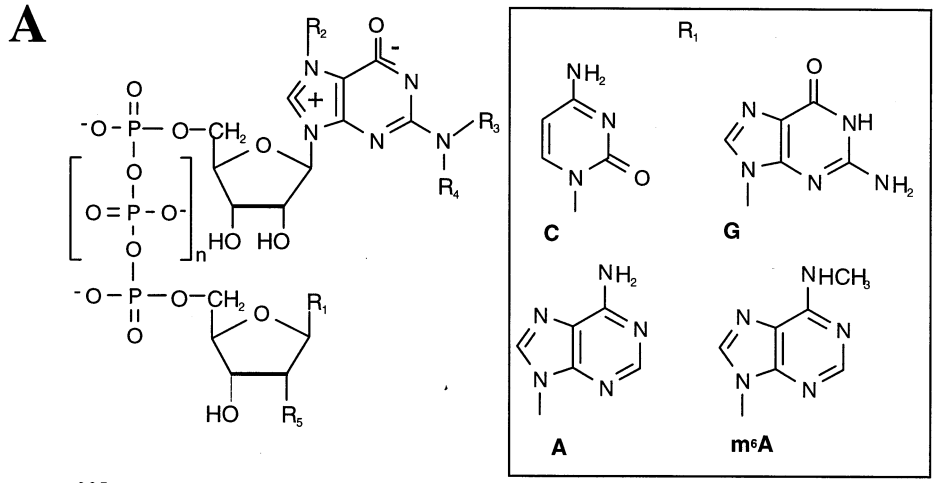
Reaction mixtures for covalent guanylate complex formation were incubated in a 30 μl volume containing 5 μCi of [α-^{32}P]GTP (> 400 Ci/mmol) and 100 μM GTP in 50 mM Tris–HCl (pH 7.5), 10 mM KCl, 2 mM $MgCl_2$, 5 mM dithiotreitol, 100 μM AdoMet, and 100 μM cap analog for 20 min at 30°C and 1–2 μl of S15. The reactions were stopped by boiling in the presence of Laemmli sample buffer. The proteins were separated by SDS-PAGE in 10% gels, and the radioactive bands were visualized by autoradiography (Ahola and Kääriäinen, 1995).

3. Results

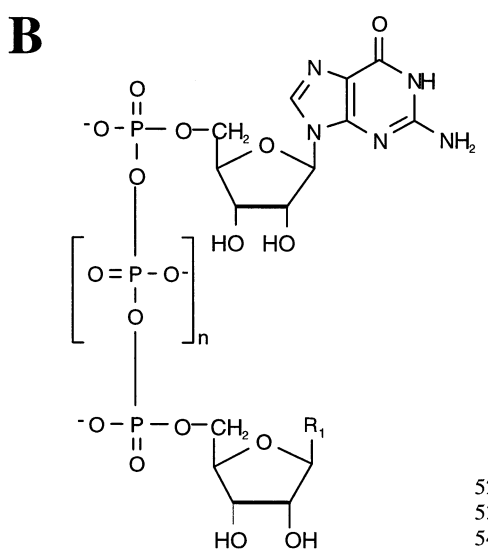
3.1. Partial purification and characterization of nsP1

Initial studies of the inhibitory effect of cap analogs on recombinant nsP1 were performed using S15 supernatant from lysed *E. coli* cells. No guanine-7-methyltransferase activity capable of methylating GTP was detectable in untransformed *E. coli* cells or in cells transformed with control plasmids expressing other SFV nonstructural proteins (Laakkonen et al., 1994; Ahola et al., 1997). Since bacterial lysates contain numerous enzymes which might affect enzymatic assays, the S15 preparation was partially purified by flotation in a discontinuous sucrose gradient. A substantial portion of nsP1 floated to the 50% and 10% sucrose interface, and most of the contaminating proteins remained in the 60–67% sucrose layer (Fig. 4). Flotated nsP1 was purified 16-fold as compared to the S15 supernatant.

The methyltransferase activity of flotated nsP1 was characterized in detail. The pH optimum was determined using several buffers. In HEPES,

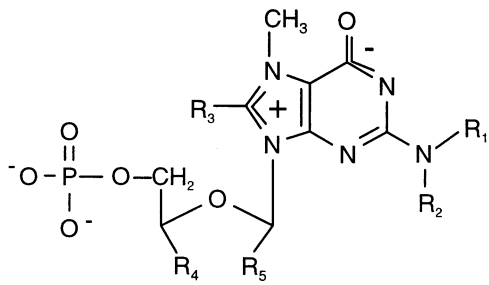


1.	$m^{2,2,7}$GpppG:	R_1 = **G**,	$R_2 = R_3 = R_4 = CH_3$,	R_5 = OH,	n = 1
5.	$m^{2,7}$GppppG:	R_1 = **G**,	$R_2 = R_3 = CH_3$, R_4 = H,	R_5 = OH,	n = 2
6.	$m^{2,7}$GpppG:	R_1 = **G**,	$R_2 = R_3 = CH_3$, R_4 = H,	R_5 = OH,	n = 1
11.	m^7Gppppm^{7}G:	R_1 = **m^7G**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 2
12.	m^7Gpppm^{7}G:	R_1 = **m^7G**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 1
13.	m^7GppppG:	R_1 = **G**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 2
14.	m^7GpppG:	R_1 = **G**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 1
15.	m^7GppG:	R_1 = **G**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 0
16.	m^7GpppA:	R_1 = **A**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 1
17.	et^7GpppG:	R_1 = **G**,	$R_2 = C_2H_5$ $R_3 = R_4$ = H,	R_5 = OH,	n = 1
18.	m^7GpppC:	R_1 = **C**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 1
19.	m^7Gppp2'dG:	R_1 = **G**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = H,	n = 1
20.	m^7Gpppm$^{2'O}$G:	R_1 = **G**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	$R_5 = OCH_3$,	n = 1
21.	m^7Gpppm^{6}A:	R_1 = **m^6A**,	$R_2 = CH_3$, $R_3 = R_4$ = H,	R_5 = OH,	n = 1

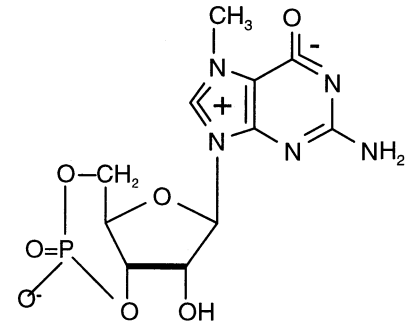


52.	GpppG:	R_1 = G,	n = 1
53.	GppppG:	R_1 = G,	n = 2
54.	GpppA:	R_1 = A	n = 1

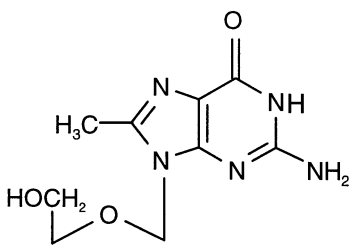
Fig. 2. Dinucleotides with an N-7 substituted guanosine moiety (A) and with an unsubstituted guanosine moiety (B).



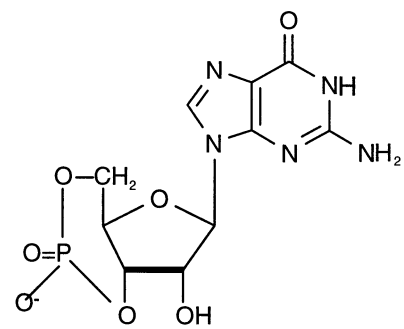
9. $m^{2,7}$secoGp: $R_1 = CH_3$, $R_2 = R_3 = H$, $R_4 = R_5 = CH_2OH$;
25. $m^{7,8}$acycloGp: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = CH_3$.



22. m^7Gcp.



47. m^8acycloG.



49. Gcp.

Fig. 3. Guanosine nucleotide analogs with a modified sugar moiety.

PIPES, MOPS, and MES buffers optimal activity was found between pH 6.5 and 7.1, whereas no activity was detected in 100 mM Na-cacodylate buffers, pH 6–7.5. In further studies, HEPES buffer at pH 6.95 was used. The nsP1 associated methyltransferase activity was enhanced about two-fold by 2 mM Mg^{2+}, Ca^{2+}, and Mn^{2+} ions as compared with reaction in the presence of EDTA. Ni^{2+}, Fe^{2+}, and Co^{2+} had little or no effect on the enzyme activity, whereas Cu^{2+} inhibited the reaction completely and Zn^{2+} to a lesser extent. At pH 6.95, the apparent K_m values for GTP, AdoMet, and Mg^{2+} were 1.9 mM, 6.0 μM, and 170 μM, respectively. When the MT-reaction was carried out at 25, 30, 37 and 42°C, the highest activity was observed at 30°C. At this temperature the incorporation of tritiated methyl groups from AdoMet into m7GTP was linear at least for 120 min.

3.2. Inhibition of methyltransferase activity by cap analogs

For screening of potential inhibitors, altogether 58 cap analogs were tested in a methyltransferase assay at concentrations of 3 and 0.3 mM. In these experiments, the concentration of GTP was 3.0 mM, and the enzyme source was the S15 supernatant (Table 1). The most effective inhibitors included those containing a methyl group at the position 7 of the guanine ring and one methyl or ethyl group at the position 2 (compounds No.

Table 1
Cap analogs, translation, and methyltransferase activity of SFV nsP1

No.	Cap analog	References [a]	Translation inhibition [b]	MT activity(%) remaining [c] 3 mM	0.3 mM	AdoMet acceptor [d] (%GTP)	K_i [e] (μM)
1	$m^{2,2,7}$GpppG	1, 2	+	58	96		
2	$m^{2,2,7}$Gppp	3	++	250	400		
3	$m^{2,2,7}$Gp	1	+	61	89		
4	$m^{2,2,7}$G	4	NT	69	82		
5	$m^{2,7}$GppppG	5	NT	NT	53		
6	$m^{2,7}$GpppG	1	+++	24	36		82
7	$m^{2,7}$Gppp	3	++++	60	68		
8	$m^{2,7}$Gp	1	++	30	64		64
9	$m^{2,7}$secoGp	6	+	61	86		
10	$m^{2,7}$G	4	NT	47	82		
11	m^7Gppppm^{7}G	5	NT	86	107		
12	m^7Gpppm^{7}G	5	NT	89	111		
13	m^7GppppG	5	NT	77	93		
14	m^7GpppG	1	+++	75	98		
15	m^7GppG	5	NT	76	88	<1	
16	m^7GpppA	7	NT	87	97	4	
17	et^{7}GpppG	8	++	56	80	5	
18	m^7GpppC	7	NT	82	94		
19	m^7Gppp2′dG	7	NT	70	93		
20	m^7Gpppm$^{2'O}$G	7	NT	88	89		
21	m^7Gpppm^{6}A	7	NT	75	91		
22	m^7Gcp	10	NT	95	NT		
23	$m^7NH_2^8$Gp	9	++	70	95		
24	$m^{7,8}$Gp	9	+	94	NT		
25	$m^{7,8}$acycloGp	9	+	94	99		
26	allyl^{7}Gp	8	+	91	NT		
27	butyl^{7}Gp	8	−	93	NT		
28	cm^{7}Gp	8	−	99	NT		
29	bn^{7}Gp	8	++	102	NT		
30	(1-phet)^{7}Gp	8	+	115	NT		
31	(2-phet)^{7}Gp	8	++	100	NT		
32	m^2G	1	NT	NT	90		
33	m^2Gp	1	NT	38	70		386
34	m^2Gppp	3	++	39	69	9	
35	$m^{2,2}$G	1	NT	78	97		
36	$m^{2,2}$Gppp	3	++	270	130	25	
37	m^2(pFbn)^{7}Gppp	3	NT	87	79		
38	$m^{2,2}$(pFbn)^7 Gppp	3	NT	206	114		
39	m^2(pClbn)^{7}Gpp	3	NT	58	73		
40	m^2(pFlbn)^{7}Gp	3	NT	NT	54		
41	m^2bn^{7}Gp	3	++++	NT	57		
42	m^2(2-phet)^{7}Gp	3	++	35	64		194
43	et^{2}m^{7}Gp	3	++	30	41		42
44	m^2et^{7}Gp	3	++	32	57		105
45	m^8G	11	NT	98	NT		
46	m^8Gp	11	NT	99	NT		
47	m^8acycloG	9	NT	101	NT		
48	Gp		NT	81	99		
49	Gcp	10	NT	100	NT		

Table 1 *(Continued)*

No.	Cap analog	References [a]	Translation inhibition [b]	MT activity(%) remaining [c] 3 mM	0.3 mM	AdoMet acceptor [d] (%GTP)	K_i [e] (μM)
50	Gpp		NT	118	104	16	
51	Gppp		NT			100	
52	GpppG	5	++			35	
53	GppppG	5	++	110	111	50	
54	GpppA		NT	86	89	12	
55	m^7G	12	++	76	93		
56	m^7Gp	13	++	84	98		
57	m^7Gpp	14	++++	43	82		
58	m^7Gppp	13	++++	58	86		

[a] 1, Darzynkiewicz et al., 1988a; 2, Darzynkiewicz et al., 1990; 3, Jankowska et al., 1993; 4, Wieczorek et al., 1995; 5, Stepinski et al., 1995; 6, Stepinski et al., 1990; 7, Jankowska et al., 1996; 8, Darzynkiewicz et al., 1989; 9, Darzynkiewicz et al., 1987; 10, Darzynkiewicz et al., 1988b; 11, Lassota et al., 1984; 12, Jones and Robins, 1963; 13, Darzynkiewicz et al., 1985; 14, Adams et al., 1978.

[b] Inhibition of in vitro translation detected previously in the respective reference. NT, not tested.

[c] Methyltransferase activity in the presence of the cap analog as % of the MT-activity in the absence of the analog.

[d] Percentage of incorporation of tritiated methyl group from AdoMet into cap analog as percentage of incorporation into GTP.

[e] Inhibition constants (K_i) determined for cap analogs as described in Section 2.

6–10 and 43 in Table 1). The nucleoside was the minimal unit recognized by methyltransferase, since even $m^{2,7}G$ (No.10, Fig. 1A) was inhibitory to the enzyme. However, addition of at least one phosphate group to the 5′ position of ribose enhanced the inhibition. In contrast, opening of the ribose ring ($m^{2,7}$secoGMP, No.9, Fig. 3) reduced the inhibition.

Among the best inhibitors were m^2et^7GMP (No. 44 in Table 1 and Fig. 1B), $m^2(2phet)^7GMP$ (No. 42, Fig. 1B) and et^2m^7GMP (No. 43, Fig. 1A). Single methyl substitutions in position 2 only were also somewhat inhibitory (compounds No. 33 and 34 in Table 1, Fig. 1C), whereas two methyl groups alone (No. 36) or in combination with a substitution at position 7 (compounds 2, Fig. 1A and 38, Fig. 1B) of phosphorylated guanosine seemed to stimulate the methyltransferase reaction (see later).

Bulky substitutions only at the position 7 of the guanine ring such as $allyl^7GMP$, $butyl^7GMP$, cm^7GMP, $benzyl^7GMP$,1-$phet^7GMP$, and 2-$phet^7GMP$ showed little or no inhibitory activity (compounds 26–31 in Table 1, Fig. 1B). All the dinucleotide cap analogs with substitutions only at the position 7 (No. 11–21 in Table 1 and Fig. 2A) were also poor inhibitors, whereas an additional substitution at the position 2 produced inhibitory compounds (No.1, 5, 6 in Table 1 and Fig. 2A). Single substition by a methyl group at position 8 had no effect on the methyltransferase reaction of nsP1 (No.45–47, Table 1 and Fig. 1 C). Double substitutions at positions 7 and 8 were ineffective as well (No. 23–25; Fig. 1A).

Interestingly, $m^{2,2,7}GTP$ (No.2 in Table 1), $m^{2,2}GTP$ (No. 36), and $m^{2,2}(pFbzl)^7GTP$ (No. 38)

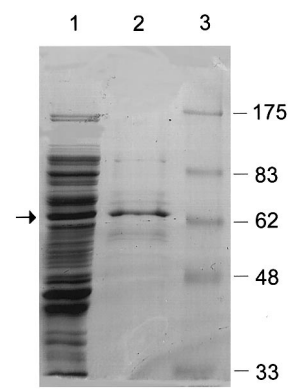


Fig. 4. Coomassie Blue staining of proteins from *E. coli* expressing Semliki Forest virus nsP1, separated by SDS-PAGE in a 10% gel. Lane 1, S15 fraction; lane 2, flotated membrane fraction from 50/10% sucrose interface; lane 3, molecular weight markers in kDa. The arrow indicates the position of nsP1.

showed an apparent stimulatory effect on nsP1 methyltransferase, although $m^{2,2,7}G$ (No. 4), $m^{2,2,7}GMP$ (No. 3), and $m^{2,2,7}GpppG$ (No. 1) were inhibitory at 3.0 mM concentration. However, when $m^{2,2,7}GTP$ was tested using a flotated nsP1 preparation, there was very little stimulatory effect on the methyltransferase activity. Further experiments indicated that millimolar concentrations of $m^{2,2,7}GTP$ protected the product of the methyltransferase reaction, tritium-labeled m^7GTP, from being degraded by contaminating enzymes present in the S15 supernatant. The contaminating activity was reduced to an insignificant level when flotated enzyme preparations were used (data not shown).

3.3. Inhibition constants of selected analogs

The inhibition constants for the most effective analogs were determined by using the flotated nsP1 preparation as the enzyme source. The reaction velocity of the enzyme was measured at different concentrations of GTP in the presence and absence of 0.3 mM inhibitor. The K_i values were calculated from Lineweaver–Burk reciprocal plots. The inhibition of nsP1 by $m^2(2\text{-phet})^7GMP$ (No. 42, Table 1, Fig. 1B), m^2et^7GMP (No. 44), and m^2GMP (No. 33, Fig. 1C) was of the competitive type. A mixed competitive non-competitive type inhibition was observed with $m^2\ ^7GMP$ (No. 8, a Lineweaver-Burk plot is shown as an example, Fig. 5) and et^2m^7GMP (No. 43) which were the most potent inhibitors of the MT reaction in this study with a K_i of 64 and 42 μM, respectively (Table 1).

3.4. Inhibition of guanylyltransferase reaction

Covalent complex formation between nsP1 and ^{32}P-labeled m^7GMP was used as an indicator of the guanylyltransferase (GT) reaction (Ahola and Kääriäinen, 1995). Normal GT activity was found with all compounds which were not inhibitory for methyltransferase. As expected, the GT activity was partially inhibited by the same analogs that inhibited the MT reaction (especially No. 6, 8, 33, 34, 39, 41–44), since methylation is a prerequisite for covalent complex formation. Examples of the GT reaction and its inhibition are shown in Fig. 6.

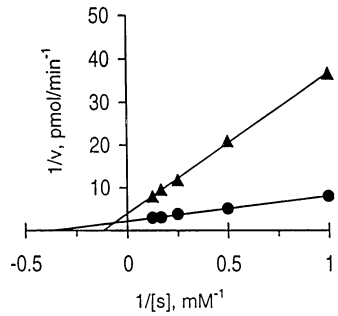


Fig. 5. Lineweaver-Burk plot analysis of inhibition of nsP1 methyltransferase by $m^{2,7}GMP$. Reciprocals of reaction velocities in the presence (triangles) and absence (circles) of the inhibitor are plotted versus the reciprocals of different substrate concentrations.

3.5. Cap analogs as substrates for nsP1-methyltransferase

Some analogs were tested as acceptors of the methyl group from AdoMet by using flotated nsP1 preparations as the enzyme source. Dinucleotides substituted at the position 7 of one guanine ring (compounds 15–17 in Table 1) were poor acceptors. The methyl group was incorporated

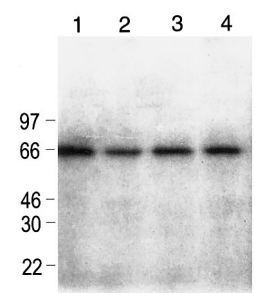


Fig. 6. Inhibition of the covalent complex formation (GT-reaction) of nsP1 with [α-^{32}P]m7GMP by cap analogs as analyzed by SDS-PAGE and autoradiography. Lane 1, nsP1 control (100%); lane 2, $m^{2,7}GMP$ (compound No. 8 in Table 1, 20% activity remaining); lane 3, $m^{2,2,7}GMP$ (No. 3, 65%); lane 4, $m^{2,7}GTP$ (No. 7, 83%). Molecular weight markers on the left in kDa. Percentage values in parentheses were determined by densitometry using the control band as 100%.

more efficiently into m^2GTP (No. 34) and $m^{2,2}$GTP (No. 36) indicating that single or double methylations at position 2 do not completely interfere with the transfer of the methyl group. The nonsubstituted guanosine mono- and dinucleotides were much better substrates (No. 50–52). Interestingly, GppppG (No. 53) was a better substrate than GpppG (No. 52). The dinucleotide GpppA (No. 54), which also corresponds to the 5′ end of Semliki Forest virus 42S and 26S RNA, was a relatively poor substrate for the enzyme.

4. Discussion

The properties of alphavirus RNA capping enzyme nsP1 differ in many respects from the cellular mRNA capping enzymes. It only forms a covalent complex nsP1-m^7GMP, unlike the cellular guanylyltransferase, which forms an enzyme -GMP complex (Ahola and Kääriäinen, 1995). As there is no m^7GTP pool present in animal cells, nsP1 catalyzes first the synthesis of this compound in a guanine-7-methyltransferase reaction (Mi and Stollar, 1991; Laakkonen et al., 1994). Both these reactions are specific for alphaviruses, and possibly for the large alphavirus-like superfamily of plant and animal viruses (Rozanov et al., 1992; Ahola et al., 1997). In this communication, we have tested a set of cap analogs, which have been shown to inhibit in vitro translation of capped mRNAs by competing with the cap binding protein at the initiation step (Darzynkiewicz et al., 1985, 1987, 1988a, 1989) or to interfere with the transport of small nuclear RNAs with an hypermethylated 5′ cap-structure (Hamm et al., 1990; V Izaurralde et al., 1992, 1994).

Interestingly, m^7G, m^7GMP, m^7GDP, m^7GTP (Table 1, compounds No. 55–58), which are powerful inhibitors of translation, were poor inhibitors of the nsP1 enzyme. The best inhibitors of the MT-reaction had a substitution of one of the hydrogens of the amino group at the position 2 of the guanine ring (compounds No. 6, 8, 42, 43, 44, Table 1). Although they were inhibitory at micromolar concentrations, they attest that a single substitution gives better inhibition than substitution of both hydrogens (compare compounds No. 1 and 6; 3 and 8). Inhibitory activity was also seen with an analog having a rather bulky substitution at position 7 of the guanine ring, m^2(2-phet)^{7}GMP (No. 42 in Table 1).

The substrate specificity of the MT reaction was also tested for some analogs. Interestingly, $m^{2,2}$GTP and m^2GTP (No.34 and 36 in Table 1) could serve as substrates for the alphavirus enzyme, whereas 7-subsituted analogs were poor substrates (No.15–17, 57–58, Table 1). It has been reported previously that 26S mRNA molecules in both SFV- and Sindbis virus- infected cells contain subpopulations (20–30%) with hypermethylated 5′ cap-structures $m^{2,2,7}$GpppN and $m^{2,7}$GpppN (HsuChen and Dubin, 1976; Dubin et al., 1977; van Duijn et al., 1986). Furthermore, we have shown that nsP1 methyltransferase is unable to methylate unmethylated capped RNA (Laakkonen et al., 1994). Our present finding that m^7GTP was not able to serve as methyl group acceptor for nsP1 suggests that m^7GTP cannot be hypermethylated in position 2 by nsP1. Thus, we assume that another methyltransferase must be responsible for the hypermethylation of 5′ cap of the alphavirus 26S RNA subpopulation. A good candidate would be an enzyme methylating U snRNAs in the cytoplasm. These RNAs are co-transcriptionally capped (m^7GpppN) in the nucleus and transported to the cytoplasm, where they are hypermethylated to yield trimethylguanosine ($m^{2,2,7}$GpppN) cap which serves as a signal for back import to the nucleus (Mattaj, 1986; Zieve et al., 1988; Hamm et al., 1990; Fischer et al., 1991).

When looking for an inhibitor of virus replication, several aspects have to be considered. One of them is its inhibitory activity towards cellular functions. Many cap analogs tested in this study have been shown to be powerful inhibitors of translation. They compete with the 5′ cap of mRNAs for the binding to initiation factor elF4E or cap binding protein (Carberry et al., 1990; Matsuo et al., 1997). One of the best inhibitors of SFV nsP1 methyltransferase, $m^{2,7}$GpppG (No.6, Table 1), was an efficient translational inhibitor, whereas $m^{2,7}$GMP (No. 8), m^2et^{7}GMP (No.44), et$^2m^7$GMP (No. 43), m^2 (2-phet)^{7}GMP (No. 42) inhibited translation only moderately.

We are currently purifying nsP1 for structural analysis which together with the present data should help to design more powerful inhibitors of the alphavirus RNA capping enzyme.

Acknowledgements

We thank Tarja Välimäki and Airi Sinkko for technical assistance and Hannu Väänänen for preparation of the Figures. This work has been supported by Finnish Technology Development Center (TEKES), and grants from the Polish Committee for Scientific Research (Grants No. KBN 6 P04A034 09 and BST-592/14/98) and the Academy of Finland. LK is a Biocentrum Helsinki fellow.

References

Adams, B.L., Morgan, M., Muthukrishnan, S., Hecht, S.M., Shatkin, A.J., 1978. The effect of 'cap' analogs on rheovirus binding to wheat germ ribosomes. Evidence for enhancement of ribosomal binding via a preferred cap conformation. J. Biol. Chem. 253, 2589–2595.

Ahola, T., Kääriäinen, L., 1995. Reaction in alphavirus mRNA capping: Formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP. Proc. Natl. Acad. Sci. USA 92, 507–511.

Ahola, T., Laakkonen, P., Vihinen, H., Kääriäinen, L., 1997. Critical residues of Semliki Forest virus RNA capping enzyme involved in methyltransferase and guanylyltransferase-like activities. J. Virol. 71, 392–397.

Barton, D.J., Sawicki, S.G., Sawicki, D.L., 1991. Solubilization and immunoprecipitation of alphavirus replication complexes. J. Virol. 65, 1496–1506.

Carberry, S.E., Darzynkiewicz, E., Stepinski, J., Tahara, S.M., Rhoads, R.E., Goss, D.J., 1990. A spectroscopic study of the binding of N-7-substituted cap analogues to human protein synthesis initiation factor 4E. Biochemistry 29, 3337–3341.

Darzynkiewicz, E., Ekiel, I., Lassota, P., Tahara, S.M., 1987. Inhibition of eukaryotic translation by analogues of messenger RNA 5′-cap: chemical and biological consequences of 5′-phosphate modifications of 7-methylguanosine 5′-monophosphate. Biochemistry 26, 4372–4380.

Darzynkiewicz, E., Ekiel, I., Tahara, S.M., Seliger, L.S., Shatkin, A.J., 1985. Chemical synthesis and characterization of 7-methylguanosine cap analogues. Biochemistry 24, 1701–1707.

Darzynkiewicz, E., Labaldi, I., Haber, D., Burger, K., Lönnberg, H., 1988b. 7-methylguanine nucleotides and their structural analogues: protolytic equilibria, complexing with magnesium(II) ion, and kinetics for alkaline opening of the imidazole ring. Acta Chem. Scand. B. 42, 86–92.

Darzynkiewicz, E., Stepinski, J., Ekiel, I., Goyer, C., et al., 1989. Inhibition of eukaryotic translation by nucleoside 5′-monophosphate analogues of mRNA 5′-cap: changes in N7 substituent affect analogue activity. Biochemistry 28, 4771–4778.

Darzynkiewicz, E., Stepinski, J., Ekiel, I., Jin, Y., Haber, D., Sijuwade, T., Tahara, S. M., 1988a. β-globin mRNAs capped with m7G, m2,2,7G or m3,2,2,7G differ in intrinsic translation efficiency. Nucleic Acids Res. 16, 8953–8962.

Darzynkiewicz, E., Stepinski, J., Tahara, S. M., Stolarski, R., Ekiel, I., Haber, D., Neuvonen, K., Lehikoinen, P., Labaldi, I., Lönnberg, H., 1990. Synthesis, conformation, and hydrolytic stability of P-1, 3-dinucleoside phosphates related to messenger RNA 5'-cap and comparative kinetic studies on their nucleoside and nucleoside monophosphate analogs.

Dubin, D.T., Stollar, V., HsuChen, C.-C., Timko, K., Guild, G.M., 1977. Sindbis virus messenger RNA: The 5′-termini and methylated residues of 26 and 42 S RNA. Virology 77, 457–470.

van Duijn, L.P., Kasperaitis, M., Ameling, C., Voorma, H.O., 1986. Additional methylation at the N(2)-position of the cap of 26S Semliki Forest virus late mRNA and initiation of translation. Virus Res. 5, 61–66.

Fischer, U., Darzynkiewicz, E., Tahara, S.M., Dathan, N.A., Luhrmann, R., Mattaj, I. W., 1991. Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. J. Cell Biol. 113, 705–714.

Froshauer, S., Kartenbeck, J., Helenius, A., 1988. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. J. Cell Biol. 107, 2075–2086.

Hamm, J., Darzynkiewicz, E., Tahara, S.M., Mattaj, I.W., 1990. The trimethylguanosine cap structure of U1 snRNA is a component of bipartite nuclear targeting signal. Cell 62, 569–577.

HsuChen, C.-C., Dubin, D.T., 1976. Di- and tri-methylated congeners of 7-methylguanine in Sindbis virus messenger RNA. Nature 264, 190–191.

Izaurralde, E., Lewis, J., McGuigan, C., Jankowska, M., Darzynkiewicz, E., Mattaj, I.W., 1994. A nuclear cap binding protein complex involved in pre-mRNA splicing. Cell 78, 657–668.

Izaurralde, E., Stepinski, J., Darzynkiewicz, E., Mattaj, I.W., 1992. A cap binding protein that may mediate nuclear export of RNA polymerase II-trascribed RNAs. J. Cell Biol. 118, 1287–1295.

Jankowska, M., Stepinski, J., Stolarski, R., Temeriusz, A., Darzynkiewicz, E., 1993. Synthesis and properties of new NH2 and N7 substituted GMP and GTP 5′- mRNA cap analogues. Collect. Czech. Chem. Commun. 58, 138–141.

Jankowska, M., Stepinski, J., Stolarski, R., Wieczorek, Z., Temeriusz, A., Haber, D., Darzynkiewicz, E., 1996. 1H

NMR and fluorescence studies of new mRNA 5′-cap analogues. Collect. Czech. Chem. Commun. 61, S197–S202.

Jones, J.W., Robins, R.K., 1963. Purine nucleosides. III Methylation studies of certain naturally occuring purine nucleosides. J. Am. Chem. Soc. 85, 193–201.

Kääriäinen, L., Ranki, M., 1984. Inhibition of cell functions by RNA-virus infections. Annu. Rev. Microbiol. 38, 91–109.

Kääriäinen, L., Söderlund, H., 1978. Structure and replication of alphaviruses. Curr. Top. Microbiol. Immunol. 82, 15–69.

Laakkonen, P., Hyvönen, M., Peränen, J., Kääriäinen, L., 1994. Expression of Semliki Forest virus nsP1-specific methyltransferase in insect cells and in Escherichia coli. J. Virol. 68, 7418–7425.

Lassota, P., Stolarski, R., Shugar, D., 1984. Conformation about the glycosidic bond and susceptibility to 5′-nucleotidase of 8-substituted analogues of 5'-GMP. Z. Naturforsch. 38C, 55–63.

Matsuo, H., Li, H., McGuire, A.M., Fletcher, C. M., Gingras, A.-C., Sonenberg, N., Wagner, G., 1997. Structure of translation factor elF4E bound to m7GDP and interaction with 4E-binding protein. Nature Struct. Biol. 4, 717–724.

Mattaj, I.W., 1986. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. Cell 46, 905–911.

Mi, S., Stollar, V., 1991. Expression of Sindbis virus nsP1 and methyltransferase activity in *Escherchia coli*. Virology 184, 427–433.

Mizumoto, K., Kaziro, Y., 1987. Messenger RNA capping enzymes from eukaryotic cells. Prog. Nucleic Acids Res. Mol. Biol. 34, 1–28.

Peränen, J., Rikkonen, M., Hyvönen, M., Kääriäinen, L., 1996. T7 vectors with a modified T7*lac* promoter for expression of proteins in *Escherichia coli*. Anal. Biochem. 236, 371–373.

Pettersson, R. F., Söderlund, H., Kääriäinen, L., 1980. The nucleotide sequences of 5′-terminal T1 oligonucleotides of Semlilin Forest virus 42-S and 26-S RNAs are different. Eur. J. Biochem. 105, 435–443.

Rozanov, M. N., Koonin, E. V., Gorbalenya, A. E., 1992. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. J. Gen. Virol. 73, 2129–2134.

Shuman, S., Hurwitz, J., 1981. Mechanism of mRNA capping by vaccinia virus guanylyltransferase: Characterization of an enzyme-guanylate intermediate. Proc. Natl. Acad. Sci. USA 78, 187–191.

Stepinski, J., Bretner, M., Jankowska, M., Felczak, K., et al., 1995. Synthesis and properties of P1,P2-, P1,P3- and P1,P4-dinucleoside di-, tri-, and tetraphosphate mRNA 5′-cap analogues. Nucleosides Nucleotides 14, 717–721.

Stepinski, J., Grabowska, L., Darzynkiewicz, E., Temeriusz, A., Stolarski, R., Tahara, S.M., Shugar, D., Järvinen, P., Lönnberg, H., 1990. Synthesis, conformation, and hydrolytic stability of modified mRNA 5′-cap structures: P1,P3-dinucleoside triphosphates derived from guanosine and acyclic analogues of 7-methyl-, N2,7-dimethyl and N2,N2,7-trimethylguanosines. Collection Czech. Chem. Commun. 55, 117–120.

Strauss, J.H., Strauss, E.G., 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. 58, 491–562.

Venkatesan, S., Moss, B., 1982. Eukaryotic mRNA capping enzyme-guanylate covalent intermediate. Proc. Natl. Acad. Sci. USA 79, 340–344.

Wang, H.-L., O'Rear, J., Stollar, V., 1996. Mutagenesis of the Sindbis virus nsP1 protein: Effects on methyltransferase activity and viral infectivity. Virology 217, 527–531.

Wieczorek, Z., Stepinski, J., Jankowska, M., Lönnberg, H., 1995. Fluorescence and absorption spectroscopic properties of RNA 5′-cap analogues derived from 7-methyl-, N2, 7-dimethyl, and N2,N2,7-trimethyl guanosines. J. Photochem. Photo Biol. B: Biol. 28, 57–63.

Zieve, G.W., Sauterer, R.A., Feeney, R.J., 1988. Newly synthesized small nuclear RNAs appear transiently in the cytoplasm. J. Mol. Biol. 199, 259–267.