THE PROCESS OF FORMATION OF THE 5′-TERMINAL MODIFIED STRUCTURE IN MESSENGER RNA OF CYTOPLASMIC POLYHEDROSIS VIRUS

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1. Introduction

The modified structure including the 7-methylguanosine blocking through 5′-5′-pyrophosphate linkage and the ribose-methylation at 2′-position of the first nucleotide was found at the 5′-terminus of mRNA of cytoplasmic polyhedrosis virus (CP virus) [1]. The same modified structure has been detected generally in mRNA of cells as well as of viruses in an eukaryote system (for example, [2–6]). CP virus, which contains double-stranded RNA as a genome and enzymes including RNA polymerase [7–8], phosphohydrolase [9] and methylases, is able to synthesize mRNA carrying the 5′-terminal modification in vitro in the presence of a methyl-donor *S*-adenosylmethionine (SAM) [1,10]. In order to know the mechanism of the formation of the modified structure at the 5′-terminus of this mRNA, the process of methylation was studied by the limiting synthesis of the terminal part of the RNA. Methylation occurred firstly at the 7-position of base in the blocking guanylic acid residue after the confront structure ($G^{5'}ppp^{5'}A$) had been made. The 2′-position of the 5′-terminal nucleotide of RNA was methylated after chain elongation had proceeded a few nucleotides length.

2. Materials and methods

CP virus was purified from a diseased silkworm as described previously [11]. Further purification of the virus particle was performed by CsCl density equilibrium centrifugation [12].

The in vitro RNA synthesis of CP virus was carried out under the condition reported previously [1,7–8, 10], adding [methyl-^{3}H]SAM to label the blocked 5′-terminal nucleotide components [1,10]. A condition for RNA synthesis with the addition of the limited substrate was described in the legend of fig.1. Since the RNA synthesis proceeds from the 5′-terminus [10,12], the limited synthesis yields the oligonucleotides that correspond to the 5′-terminal parts of this mRNA. Analysis of the primary structure of the product oligonucleotide was carried out by the radioactivity of [methyl-^{3}H] by column chromatographies. To separate oligonucleotides according to their chain length, DEAE-cellulose or DEAE-Sephadex was used in the 7 M urea solution. To separate the blocked nucleotides and the mononucleotides, anion exchange resin Bio-Rad AG-1 was used. The conditions for chromatographies and the enzymatic digestions were also described in the legends of figures. These are essentially the same to those reported previously [1,4,12,14].

[Methyl-^{3}H]*S*-adenosylmethionine (SAM) was purchased from Radiochemical Center. Ribonucleoside triphosphates were purchased from Boeringer-Yamanouchi Co. The confront nucleotides, GpppA and GpppG, were synthesized chemically.

3. Results and discussion

The 5′-terminal structure of the mRNA of CP virus is $m^7G^{5'}ppp^{5'}$Am-G-Y- [1,12]. The last pyrimidine nucleoside (Y) must be uridine judging from the genome nucleotide sequence [14]. If CTP is omitted from the reaction mixture of the in vitro RNA synthesis, the 5′-terminal oligonucleotide which precedes to the first cytidylate should be produced.

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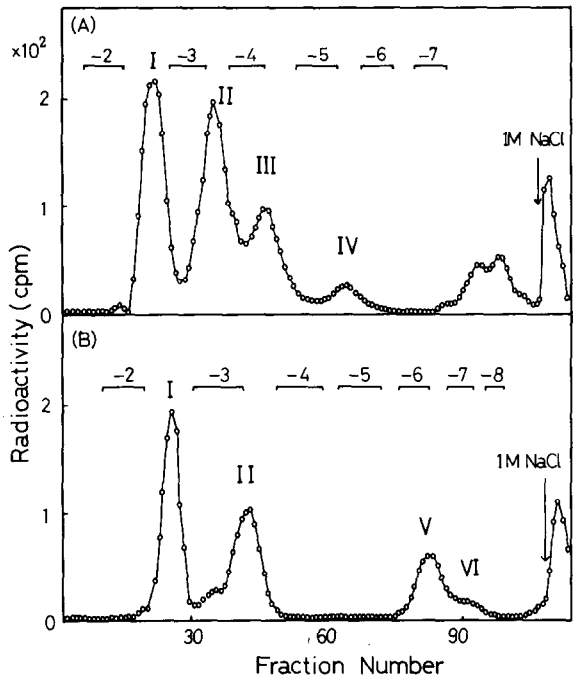

Fig.1. DEAE cellulose and DEAE Sephadex A-25 column chromatography of the nascent oligonucleotides synthesized in vitro by CP virus under the limited condition. The reaction mixture consists of 120 mM of Tris-HCl (pH 8.0), 12 mM of $MgCl_2$, 0.5 μmol of ATP, GTP and UTP, 0.1 μmol of cold SAM. 50 μCi of [^{3}H]SAM (75 Ci/mmole) and 4 A_{260} units of CP virus in 250 μl. After the reaction mixture was incubated at 34°C for 3 h, temperature was shifted down to 27°C. Then 0.25 μmol of CTP was added to it, and incubation was continued another 15 (A) and 30 (B) sec. After the reaction was stopped by adding sodium dodecyl sulfate and urea to final concentration of 0.1% and 7 M respectively, the reaction mixture was applied to DEAE-cellulose (A) or DEAE-Sephadex (B). The column was washed with 7 M urea sufficiently to remove remaining [^{3}H]SAM and eluted with a linear concentration gradient of NaCl from 0 M (100 ml) to 0.3 M (100 ml) (for A) or from 0.1 M (150 ml) to 0.4 M (150 ml) (for B) in 7 M urea. The 30-drop fractions were collected, and optical density at 260 nm was measured. The [methyl-^{3}H] radioactivity in 0.3 ml sample was measured with the addition of 2 ml ethylene glycol monomethyl ether and 5 ml Kinard's scintillation fluid [13].

Analysis showed that the oligonucleotides synthesized without CTP have 1 to 3 chain length and contain the 5′-blocked structure. However, the oligonucleotides did not contain 2′-*O*-methylation of the first adenylate residue. The methylation of the ribose moiety of this nucleotide would occur after elongation of the RNA chain has proceeded to some extent. In order to clarify the sequence of the 5′-terminal modification of CP virus mRNA, the nascent oligonucleotides having various chain length were prepared by limiting substrates and reaction speed.

CP virus was incubated with ATP, GTP, UTP and [^{3}H]SAM in the presence of Mg^{++} at 34°C for 3 h. Then the temperature of the reaction mixture was shifted down to 27°C. Here, CTP in a half amount of the usual reaction condition was added to the reaction mixture and the reaction was continued another 15 and 30 sec to allow production of short RNA chains. Sodium dodesyl sulfate (final 1%) and urea (final 7 M) were added to stop the reaction.

The reaction mixtures were applied to DEAE-urea system and washed with an excess volume of 7 M urea (pH 7.8) to remove remaining [^{3}H]SAM. Then the column was eluted with a linear gradient of NaCl in 7 M urea. As shown in fig.1 the [methyl-^{3}H]-labeled oligonucleotide fractions I–VI, which carry 1 to 6 chain length respectively and the blocked structure, were separated. To remove salt, each fraction was adsorbed on a DEAE-Sephadex column (A 25, 0.6 × 2 cm), washed with 0.05 M triethylamine bicarbonate (TBC), and eluted with 2 M TBC solution. The eluate was evaporated in vacuum. These nascent oligonucleotide preparations were digested with venom phosphodiesterase. The digests were applied to the Bio-Rad AG-1 columns and eluted with a linear gradient of NaCl in 0.01 N HCl as described in the legend of fig.2. Thus the rate of methylation in the blocked 5′-terminal part of the mRNA was elucidated. The [methyl-^{3}H] radioactivity was not detected in 2′-*O*-methyl adenylate in the oligonucleotides I and II, although the radioactivity was detected in 7-methyl guanylate. But in oligonucleotide III–VI the [^{3}H] radioactivity was observed both in 2′-*O*-methyl adenylate and in 7-methyl guanylate. In the case of oligonucleotides of III and IV, lesser radioactivity was found in 2′-*O*-methyl adenylate than in 7-methyl guanylate. The [^{3}H] radioactivity ratios of 7-methyl guanylate to 2′-*O*-methyl adenylate in these oligonucleotides are listed in Table 1. The ratio reached to 1 at the pentanucleotide and it was kept in constant for further polymerization of nucleotides. This result means that methylation at the 5′-terminus of the mRNA occurs at first on the blocking guanylate

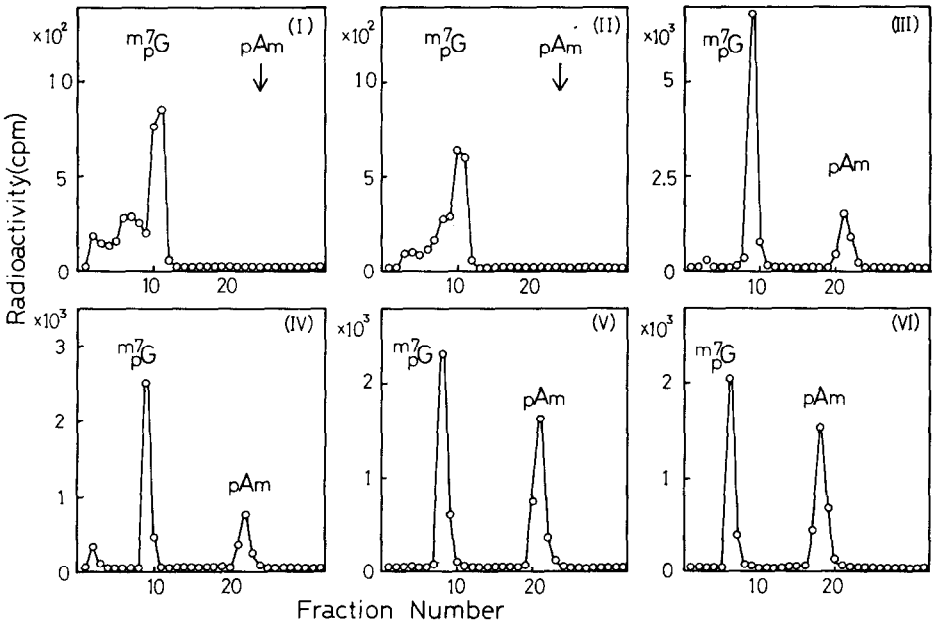

Fig.2. Chromatography on a Bio-Rad AG 1 column of the venom phosphodiesterase digest of oligonucleotide (I–VI) in fig.1. Oligonucleotides from I to VI in fig.1 were collected and digested with venom phosphodiesterase. The digests were applied to the Bio-Rad AG-1 column (0.6 × 20 cm) and eluted with a linear concentration gradient of NaCl from 0 M (50 ml) to 0.1 M (50 ml) in 0.01 N HCl. The 50-drop fractions were collected, and absorbance at 260 nm of marker nucleotides, pm^7G and pAm, and radioactivity were measured as in fig.1.

Table 1
Correlation between methylation of ribose moiety of adenylate at the 5′-terminus and nucleotide length of the growing chain of CP virus mRNA

Number of nascent oligonucleotides (see fig.1)	Chain length of oligonucleotide	[methyl-^{3}H] ratio $\frac{pAm}{pm^7G}$
I	1	< 0.03
II	2	< 0.04
III	3	0.36
IV	4	0.46
V	5	0.95
VI	6	1.16
An mRNA molecule carrying a 'complete' chain length		1.08

The ratios of the [methyl-^{3}H] radioactivity in 7-methylguanylate to that in 2′-*O*-methyladenylate were calculated from fig.2. The mRNA listed as a molecule carrying a 'complete' chain length was synthesized under usual reaction condition for 7 h.

at 7-position of the base residue, and the methylation at the 2′-position of the ribose moiety of the first adenylate starts only after RNA chain has reached three nucleotide length.

In order to confirm the steps of methylation the blocked nucleotides without methyl groups, GpppA and GpppG, were prepared by chemical synthesis and added to the reaction mixture. The mixture was incubated at 34°C for 3 h in the presence of [^{3}H]SAM as a methyl donor, but without nucleoside triphosphates. The reaction was stopped by solubilizing a virion protein by sodium dodecyl sulphate. The mixture was chromatographed by the DEAE-urea system. The fractions including GpppR and its methylated derivatives were then chromatographed on the AG-1 anion exchange column (fig.3). When GpppA was used as a substrate, the [methyl-^{3}H] radioactivity was detected at the position of m^7GpppAm or m^7GpppA. But in the case of GpppG, no radioactivity was detected at the position of

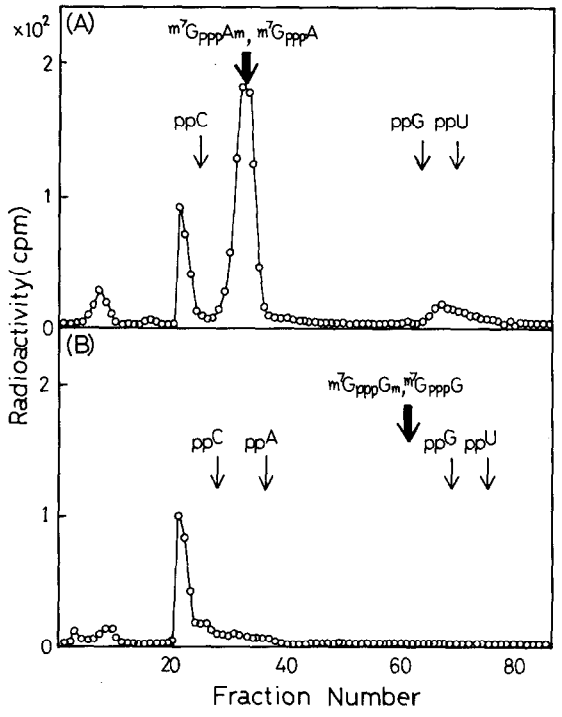

Fig.3. Methylation of the confront nucleotides GpppA and GpppG. The reaction mixture for methylation consisted of the following materials in 250 μl; 120 mM of Tris-HCl (pH 8.0), 12 mM of $MgCl_2$, 0.1 μmol of cold SAM, 50 μCi of [^{3}H]SAM, 5 A_{260} units of GpppA (a) or GpppG (b), and 4 A_{260} units of CP virus. This reaction mixture was incubated at 34°C for 3 h. After the reaction was stopped by adding sodium dodecyl sulfate and urea to final concentrations of 1% and 7 M respectively. The solution was applied to the DEAE-cellulose column (0.6 × 40 cm) and washed with an excess volume of 7 M urea (pH 7.8) to remove [^{3}H]SAM. Elution was carried out with the linear concentration gradient of NaCl in 7 M urea (pH 7.8). The fractions, which correspond to the −2- to −3-charged marker nucleotides, were collected and diluted 100 times with water. This solution was applied on Bio-Rad AG-1 column (0.6 × 20 cm) and eluted with a linear gradient of NaCl from 0 M (50 ml) to 0.3 M (50 ml) in 0.01 N HCl. The 50-drop fractions were collected. Their absorbance at 260 nm and radioactivity were measured as in fig.1.

m^{7}GpppGm or m^{7}GpppG. The radioactivity eluted earlier than these confront nucleotides would be residual [^{3}H]SAM or its degradation products. This was confirmed by the chromatography of [^{3}H]SAM under the same condition. The result tells us that GpppA can be a substrate for the methylase associated to CP virus, but GpppG can not. This is not conflict with the 5′-terminal structure of CP virus mRNA, m^{7}GpppAm. The methylation enzyme in CP virus seems to recognize the substrate structure strictly and to methylate after the blocked structure is constructed. In fact, GTP in the reaction mixture was not methylated. The methylated nucleotide fractions which correspond to m^{7}GpppAm or m^{7}GpppA was then digested by venom phosphodiesterase and analysed by the AG-1 column chromatography. The [methyl-^{3}H] radioactivity was eluted only at the position of 7-methylguanylic acid. No radioactivity was eluted in the fraction of 2′-*O*-methyladenylic acid. Therefore, the added GpppA was converted to m^{7}GpppA, not to m^{7}GpppAm, by CP virus particle. Here again it is clear that methylation at the 7 position of the blocking guanosine residue is carried out at first, and the ribose moiety in the A residue is not methylated before a few nucleotides linked to it.

Based on these results, the process of the 5′-terminal modification during the synthesis of CP virus mRNA are written as follows:

GTP + ATP → GpppA
(or GTP + ADP → GpppA)
GpppA + SAM → m^{7}GpppA
m^{7}GpppA + GTP →
m^{7}GpppApG (with or without SAM)
m^{7}GpppApG + UTP + CTP + SAM →
m^{7}GpppAmpGpUpC - - -

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