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"Methylation-coupled" transcription by virus-associated transcriptase of cytoplasmic polyhedrosis virus containing double-stranded RNA

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ABSTRACT

S-adenosyl-L-methionine(SAM) activated the virus-associated RNA polymerase of cytoplasmic polyhedrosis virus in vitro. Synthesis of single-stranded viral RNA (mRNA) proceeded depending on the presence of SAM.

A methyl residue of SAM was incorporated into an RNA molecule. A ribose moiety of adenylic acid in the 5'-terminal region of the nascent RNA was methylated in the very early stage of the transcription. The dependence of the viral transcription on the presence of SAM and the methylation of terminal nucleotide suggests that the transcription of CPV is a "methylation-coupled" reaction.

INTRODUCTION

Cytoplasmic polyhedrosis virus(CPV) of silkworm contains segmented double-stranded RNA genome as does reovirus, blue tongue virus, rice dwarf virus and so on.[1,2] These viruses contain a virion-associated transcriptase which, in vitro, can copy one of the two chains of the double-stranded RNA segments.[3-11] It has been noted that the RNA-polymerizing activity of CPV preparations was relatively low, taking into consideration its infectivity, in comparison with the polymerase activity of reovirus. Most of the virus preparations have shown little polymerase activity in the standard reaction conditions. This was thought to be due to either to the failure to activate the masked enzyme or to the denaturation of the enzyme during the preparation of virus. However, attempts to obtain virus preparations with higher transcriptase activity have been unsuccessful.

Recently, Miura and co-workers found that the 5'-terminal nucleotide of one chain of double-stranded RNA of CPV is modified at the ribose moiety[12] with a methyl group(Watanabe,Furuichi and Miura, unpublished result). We thought that methylation

may also take place in the in vivo transcription system and tried adding a donor of methyl groups for nucleic acids into the reaction mixture for the in vitro RNA synthesis of CPV. As described in the present paper, the addition of S-adenosyl-L-methionine(SAM) to the reaction mixture activated the RNA polymerase in virus particle and the methyl residue of SAM was incorporated into the synthesized single-stranded RNA.

RESULTS

Enhancement of RNA synthesis by SAM

When SAM(0.4mM) was added to the in vitro RNA polymerase assay system, which consists of Tris buffer, Mg^{++}, the four ribonucleotide triphosphates(XTP), bentonite and purified CPV treated with an organic solvent[4], the incorporation of ^{3}H-UTP into acid-insoluble and RNase sensitive material was greatly enhanced. The reaction continued for at least 8 hours almost

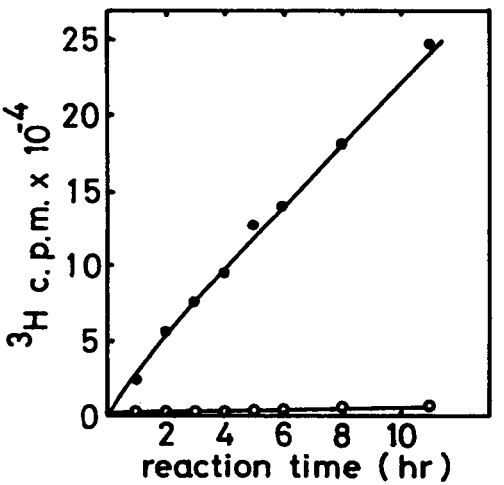

Fig.1. SAM-dependent RNA synthesis by the RNA polymerase associated with CPV. CPV was prepared from the midguts of infected silkworm larvae and purified as described previously (ref. 4,13). Virus particles which sedimented at ρ=1.4200g/cc in CsCl buoyant density gradients centrifugation was pooled and dialyzed against 5mM Tris-HCl(pH 8.0). The reaction mixture(0.25ml)consisted of 60mM Tris-HCl(pH 8.0); 12mM $MgCl_2$; 2mM each of ATP, CTP, GTP and 0.4mM of UTP; 0.5μCi ^{3}H-UTP (14 Ci/mmole); 60μg bentonite and 34μg CPV. 0.5mM SAM (Boehringer Mannheim) was added to the reaction mixture (+SAM). Incubation was carried out at 31°C. The reaction was stopped by the addition of 10% cold TCA and assayed for acid-precipitable radioactivity.
●——● +SAM ; o——o -SAM

linearly, although ^{3}H-UTP was scarcely incorporated into RNA in the absence of SAM. Fig.1 shows that RNA synthesis (incorporation of ^{3}H-UTP) was markedly enhanced by the addition of SAM, suggesting that the reaction depends on SAM.

Optimal condition for SAM-activated RNA synthesis were investigated. As shown in Fig.2, when 0.4mM of each XTP was used as a substrate in the reaction mixture, the optimal concentration of SAM was 0.1mM. However, it was rather variable, depending on the concentration of XTP's: for example, when 2mM of each XTP was used, the optimal concentration of SAM increased to 0.5mM. However, the concentration of SAM actually optimal may be lower than the value estimated here, since bentonite, which was added to the reaction mixture as an RNase inhibitor, has a tendency to adsorb SAM. Fig.2, also, shows that the RNA synthesis is dependent on the presence of SAM. The optimal temperature for SAM-dependent RNA synthesis was 31°C. It is a little higher than in the case where SAM is not added (27°C)[5].

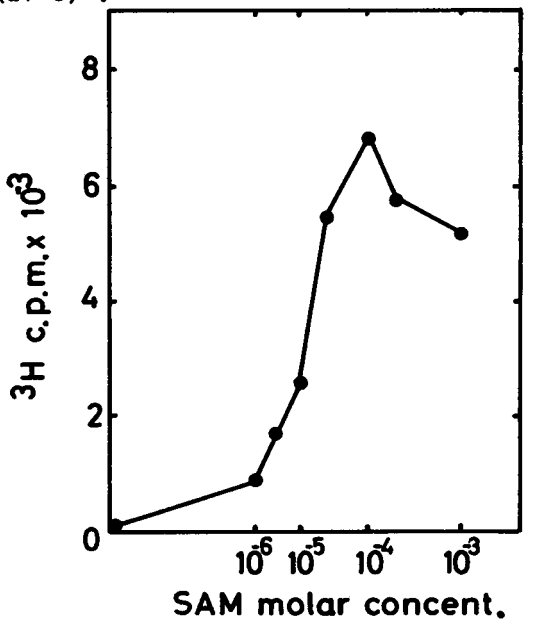

Fig.2 Optimal concentration of SAM for viral transcription. The reaction mixture consisted of 40mM Tris-HCl(pH 8.0); 4mM each of ATP, CTP, GTP and UTP; 0.5μCi ^{3}H-UTP (14Ci/mmole); 60μg bentonite; 10μg CPV; and variable amount of SAM. Incubation was carried out at 31°C for 1 hour.

Characterization of the synthesized RNA

After 8 hours incubation, the synthesized RNA was isolated by ultracentrifugation and gel filtration using Sephadex G-150. All the instruments and buffer-solutions used in these processes had been either heated at 170°C or autoclaved to prevent the possibility of contamination by ribonuclease and micro-organisms. ^{3}H-labeled RNA was eluted in the excluded fraction in column chromatography. The RNA was precipitated by the addition of 2.5 vol. of cold ethanol. The precipitate was dissolved in small volume of 5mM Tris-HCl(pH 8.0) for further characterization. When the RNA was incubated with 2μg/ml of pancreatic ribonuclease for 15min at 37°C, conditions under which single-stranded RNA is completely hydrolyzed, all label was found in acid-soluble material. When the RNA was dissolved in 4ml 2M LiCl-50mM Tris-HCl(pH 8.0) with 5 O.D.$_{260nm}$ units of E.coli ribosomal RNA as carrier and kept at 0°C overnight without ribonuclease, label was recovered quantitatively in the RNA precipitate upon centrifugation at 13,000 r.p.m. for 20min at 4°C. These results indicated that the RNA synthesized in the presence of SAM was single-stranded.

In order to determine whether the synthesized RNA was virus-specific, it was hybridized with double-stranded CPV RNA according to the procedure of Watanabe and Graham[13]. The RNA synthesized by CPV in the presence of SAM was mixed with a 7-fold excess of double-stranded CPV RNA and denatured in 90% DMSO. The denatured RNA was reannealed by heating at 72.5-73.0°C for 18 hr followed by cooling to room temperature. The annealed RNA was electrophoresed in 3% polyacrylamide gel and analyzed as described previously[14,15]. The results are shown in Fig.3. The ^{3}H count in the synthesized RNA was found in every band of double-stranded RNA segments in a given gel. This indicates that all ten segments were transcribed in the presence of SAM. In this respect, the RNA synthesized in the presence of SAM was similar to the single-stranded RNA transcribed from CPV in the absence of SAM, as described by Shimotohno and Miura[4]. However, the rate of SAM-dependent synthesis of RNA was 60 or more times larger than in the case of single-stranded RNA synthesis in the absence of SAM. Under optimal conditions,

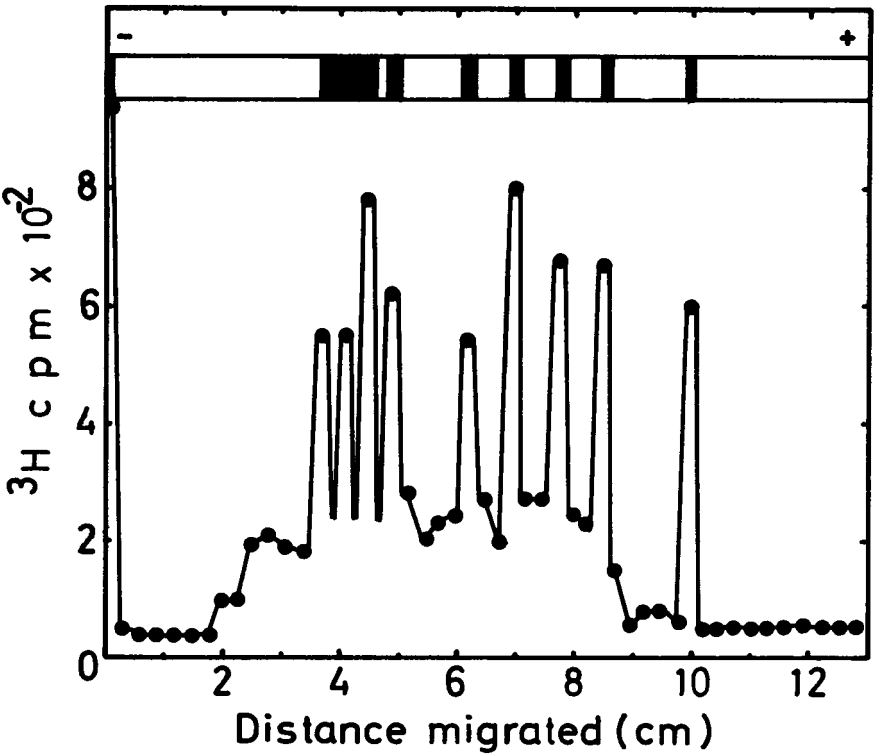

Fig. 3. Electrophoretic pattern of the in vitro RNA products hybridized with viral double-stranded RNA.
^{3}H-labeled single-stranded RNA was synthesized with 35µg CPV in the presence of 0.5mM SAM. After 11 hours at 31°C under standard conditions (See legend to Fig. 1), the reaction mixture was centrifuged at 80,000 x g for 60min, and the supernatant was passed through a Sephadex G-150 column.
Excluded fractions were pooled and RNA was isolated by alcohol precipitation. Single-stranded RNA (0.5 O.D.$_{260nm}$ units, 34,000 c.p.m.) was mixed with 3.7 O.D.$_{260nm}$ units of CPV double-stranded RNA in 0.1ml H_2O (final).
DMSO (1ml) was added to the solution and RNA was denatured by incubation at 40°C. After 30min incubation, RNA was precipitated by the addition of 2.5 vol. cold ethanol.
Precipitated RNA was dissolved in 0.4ml of a buffer solution consisting of 0.3M NaCl, 0.01M Tris-HCl(pH 8.0) and 1mM EDTA. Mixed RNAs were annealed by heating at 72.5-73.0°C for 18 hours and cooling to room temperature. RNAs were then precipitated by the addition of 2 vol. of cold ethanol. The jelly-like precipitate was spooled up with a glass rod, and dissolved in 0.1ml of the buffer solution used for gel electrophoresis.
The RNA solution was heated for 2min at 78°C to destroy aggregation, cooled in ice, and placed on a 3% polyacrylamide gel (0.7cm diameter) buffered with 0.036M Tris-0.032M KH_2PO_4-1mM-EDTA (pH 7.8)[27]. Electrophoresis was carried out at room temperature for 20 hours at 8 ma/tube. The gel was cut out into slices

after staining, and counted for ^{3}H-radioactivity as described previously (ref. 15). The stained band of double-stranded CPV RNA segments is shown in the upper part of the figure.

RNA polymerase in CPV polymerized 7 nucleotides per second at 31°C in the presence of SAM. This rate was comparable with the rate of RNA synthesis under optimal conditions for reovirus core RNA polymerase[16] and E.coli DNA-dependent RNA polymerase.[17] Since the optimal conditions (pH, temperature and Mg^{++}-concentration) for RNA synthesis in the presence of SAM were almost the same as those in its absence and the synthesized RNAs in both cases corresponded to all nucleotide sequence of the genome, as shown by hybridization experiments, RNA synthesis appeared to be carried out by the same RNA polymerase in CPV regardless of the presence or absence of SAM.

Incorporation of methyl groups into messenger RNA

For methylation of DNA and RNA, SAM acts as a donor of methyl groups[18,19]. It was investigated whether the methyl group in SAM is introduced into the RNA. SAM carrying a ^{3}H-labeled methyl group was added to the reaction mixture with ^{14}C-UTP, instead of ^{3}H-UTP, to monitor the RNA synthesis. After 2 hours incubation, the reaction mixture was centrifuged at 84,000 x g for 60min at 4°C, and the supernatant was passed through a Sephadex G-150 column. ^{3}H-Radioactivity was observed in the excluded fractions of the column together with ^{14}C-radioactivity. Radioactive fractions were pooled, precipitated with ethanol and dissolved in a small volume of 5mM sodium acetate buffer(pH 5.5). By centrifuging the sample in a glycerol density gradient (5-30%), it was shown that the ^{3}H-label sedimented with the ^{14}C-labeled single-stranded RNA products (Fig. 4). This indicates that ^{3}H-methyl group was incorporated into newly synthesized RNA. When the molar ratio of methyl groups to the number of nucleotides in a newly synthesized RNA molecule was calculated from the ^{3}H-SAM and ^{14}C-UTP radioactivities, assuming that the uracil content in an RNA molecule 25%, on average, of the total bases, it was found that one mole of methyl residue incorporated into one RNA molecule, since one methyl residue incorporated per 1,900 nucleotides in the total mRNAs, a value which is comparable to the average chain length of CPV mRNA

molecules (2,300 nucleotides).

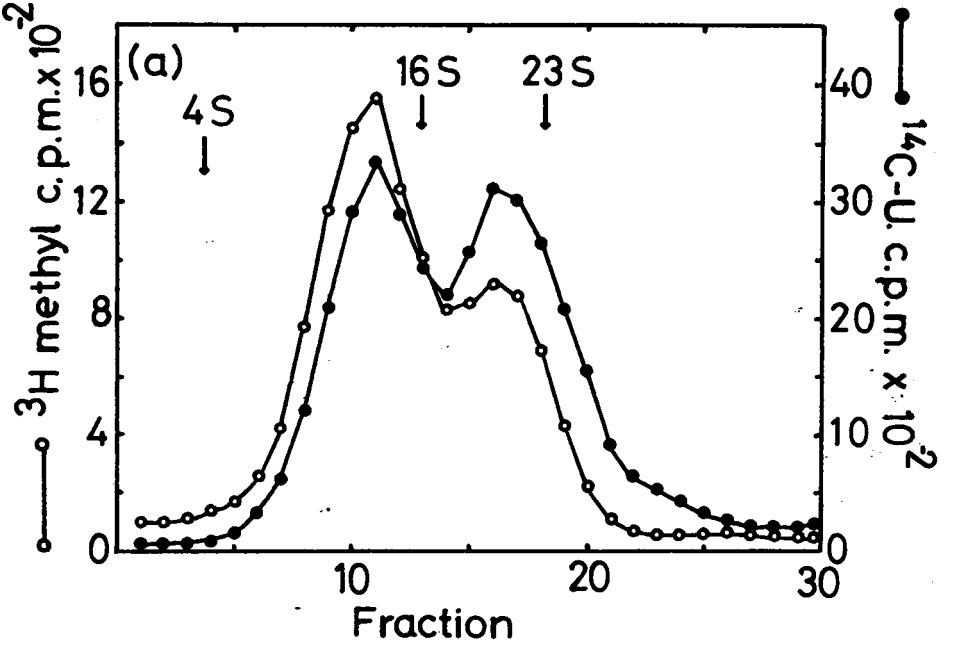

Fig. 4. Sedimentation of RNA products in glycerol gradients. RNA synthesis was carried out in the presence of ^{3}H-methyl SAM and ^{14}C-UTP in a reaction mixture (0.5ml) consisting of 60mM Tris-HCl(pH 8.0); 12mM $MgCl_2$; 2mM each of ATP,CTP and GTP, and 1mM UTP; 1.25µCi ^{14}C-UTP(475mCi/mmole, The Radiochemical Center, Amersham); 120µg bentonite; and 70µg virus. RNA synthesis was continued at 31°C for 2 hr and the reaction mixture was centrifuged and the supernatant was passed through a Sephadex G-150 column previously equilibrated with 0.01M acetate buffer(pH 5.5) containing 0.2% SDS and 0.1M NaCl. Labeled RNA was excluded in the void volume of the gel column and precipitated with ethanol using E.coli ribosomal RNA as carrier. The RNA was dissolved in a small volume of sterilized water, layered on 12ml of a linear gradient of 5-30% glycerol, containing 0.02M Tris buffer(pH 8.0) 0.1M NaCl and 5mM EDTA, and centrifuged at 4°C in the Spinco SW-41 rotor at 28,000 r.p.m. for 16 hr. Fractions (0.4ml) were collected from the top with an ISCO density gradient fractionator and mixed with 10ml of Kinards scintillator and the radioactivity counted.[28] ●——● ^{14}C-radioactivity; o——o ^{3}H-radioactivity.

In order to determine where the methylation occurred in the RNA molecule, ^{3}H-methyl-RNA was hydrolyzed with alkali or nucleases. Hydrolysis with alkali (0.3M NaOH, 37°C, 18 hr) did not produce methylated mononucleotides, indicating that the 2'-OH moiety of ribose was methylated. Successive treatment with Penicillium nuclease and venom phosphodiesterase, which cleave

phosphodiester linkage nonspecifically, produced ^{3}H-methylated mononucleotides. On treatment of the latter hydrolysate with E.coli alkaline phosphatase, ^{3}H-methyl nucleoside was obtained. This nucleoside was analyzed by paper electrophoresis (pH 3.5) and two dimensional paper chromatography using n-butanol-H_2O-conc. NH_4OH (86:14:5 v/v) and iso-butyric acid-0.5N NH_4OH (10:6 v/v) as solvents. The radioactivity migrated with authentic 2'-O-methyl-adenosine, prepared according to Broom and Robins[21]. Therefore, the methyl group is incorporated into one of the adenylic acid residues in CPV mRNA at the ribose moiety.

Since the reaction mixture for in vitro transcription of CPV contains only virus particles as a source of enzyme activity, the virus particle must contain a methylase which catalyzes the methylation of virus transcript using SAM as the methyl donor, and which is specific for the ribose moiety of adenosine.

When does methylation occur during transcription ?

It is well-known that ribosomal RNA and transfer RNA contain small amounts of methylated nucleotides[22,23]. Many studies on the methylation of these nucleic acids have shown that the methylation occurs after transcription is completed[24,25].
In order to determine when methylation takes place in relation to the transcription of CPV, the rate of methylation of nascent RNA was investigated. It is known that mRNA synthesis of CPV is carried out in the virus particle as does reovirus, and the mRNAs (13-20S) come out from the particle after completion of synthesis[5]. In order to detect the nascent RNA in the virus particle, the newly synthesized RNA in the whole reaction mixture was studied in due course of synthesis. As shown in Fig.5, the completed mRNA (13-20S) are found after 5-15min incubation, whereas nascent RNAs (3-4S), which are still in the process of chain elongation in the virus particles, are observed after 2min incubation. In the absence of SAM, the nascent RNA was not observed. This suggests that the effect of the addition of SAM was deeply correlated to the start of RNA synthesis.
The RNA synthesis was carried out adding ^{3}H-methyl-labeled SAM and ^{14}C-UTP to monitor methylation and RNA synthesis respectively. To use ^{14}C-UTP with high specific activity in this experiment, the addition of non-radioactive UTP was limited to 1/10

of the standard amount, which lowered the rate of RNA synthesis to about 1/5 of the case under the standard condition.

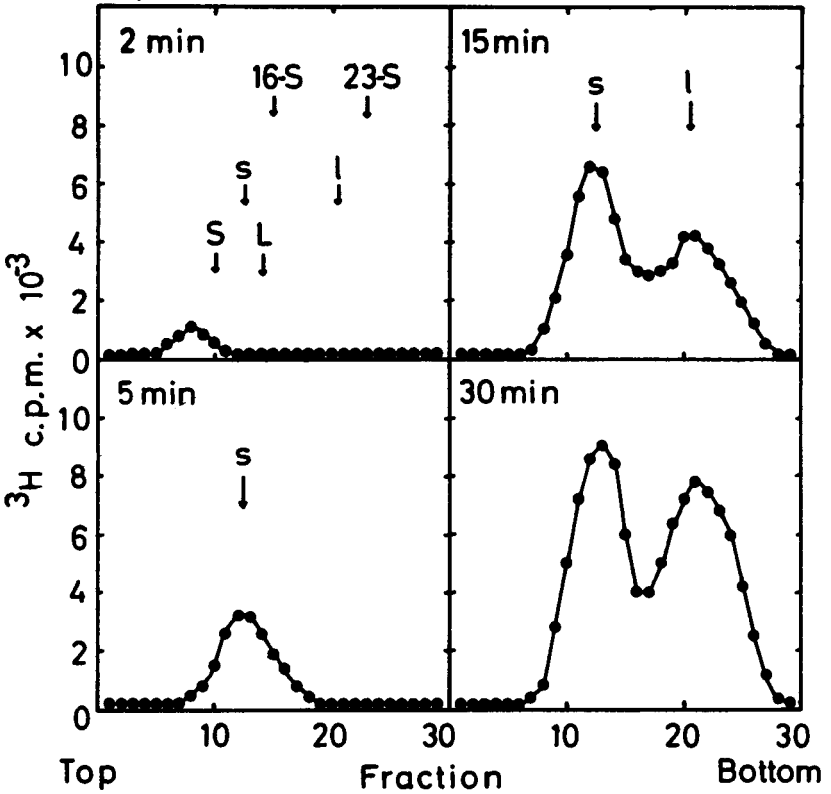

Fig. 5. Time course of the synthesis of RNA. RNA synthesis was carried out with ^{3}H-UTP in the presence of 0.5mM SAM for the time designated in the figure. After incubation, equal volume of 0.5% SDS-90% phenol was added to the reaction mixture and the RNA was extracted. An aliquot of the RNA solution was layered on 12ml of a linear density gradient of 5-30% glycerol, containing 0.02M Tris buffer(pH 8.0), 0.1M NaCl and 5mM EDTA, and centrifuged at 4°C in the Spinco SW-41 rotor at 28,000 r.p.m. for 16 hr. Fractions (0.4ml) were collected from the top with an ISCO density gradient fractionator and assayed for 5% TCA-insoluble radioactivity. S and L (or s and l) designated in the figure show the locations where double-stranded RNA segments (or single-stranded RNAs) having small molecular sizes (S, s) and large molecular sizes (L, l) sediment,respectively.

Synthesis of RNA was stopped by the addition of a large amount of EDTA at an early stage (2min) after the start of transcription. The virus particles were isolated from the reaction mixture by centrifugation. The virus precipitate was suspended in 0.5% SDS, and the total RNA was isolated by phenol treatment,

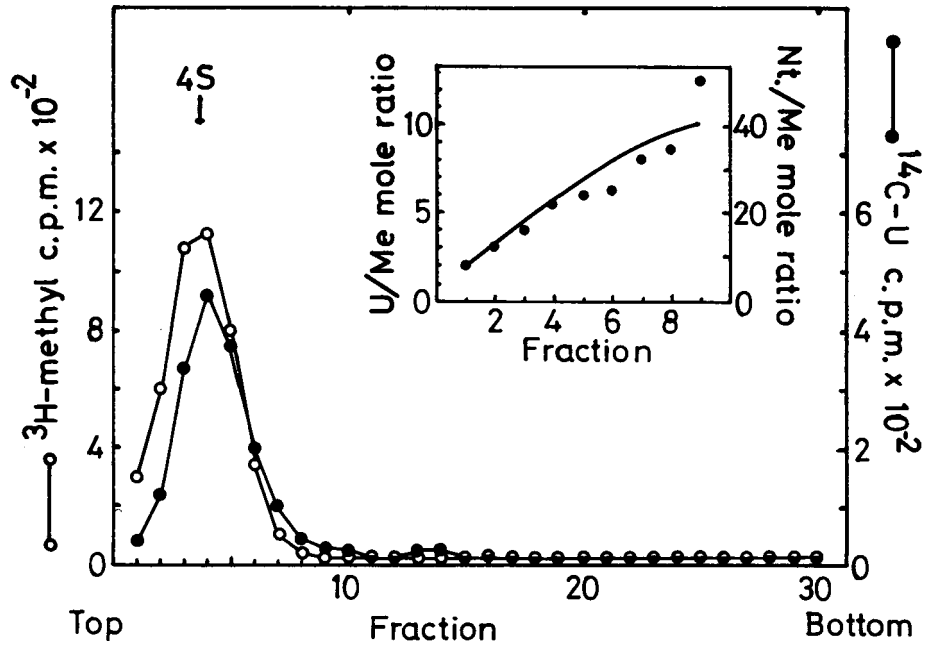

Fig. 6. Characterization of nascent RNA by glycerol gradient centrifugation. Reaction conditions were the same as those described for Fig. 4 except for the concentration of UTP, which was 0.1mM instead of 1mM. RNA synthesis was stopped after 2min incubation by the addition of 80mM(final) EDTA. The mixture was centrifuged at 80,000 x g for 60min at 4°C to isolate the virus particles. The virus pellet were disrupted in 0.01M acetate buffer(pH 5.5) containing 0.5% SDS and the RNA was extracted by shaking with 90% phenol. In order to remove contaminating ^{3}H-SAM and ^{14}C-UTP, RNA fraction was filtered through a Sephadex G-50 column equilibrated with 0.2% SDS-0.1M NaCl-0.01M acetate buffer(pH 5.5). Labeled RNA which eluted after the void volume fraction was analyzed by glycerol density gradient centrifugation as described in the legend to Fig. 4, using 2 O.D.$_{260nm}$ units of E.coli tRNA as a marker. The molar ratio of ^{14}C-uridine and ^{3}H-methyl incorporated in the RNA was calculated based on the specific activities of ^{14}C-UTP and ^{3}H-SAM used in the reaction, and is shown in the inset curve.
o——o ^{3}H-radioactivity; ●——● ^{14}C-radioactivity.

followed by Sephadex gel-filtration in the presence of 0.2% SDS. Then the RNA was characterized by centrifugation in a glycerol density gradient, as shown in Fig. 6. ^{14}C-labeled RNA sedimented at 3-4S, which is a little smaller than tRNA added as a marker. The radioactivity of ^{3}H-methyl group was also found in this nascent RNA fraction. The larger the molecular size of the RNA, the larger the ratio of the incorporated uridylate to

the methyl group (^{14}C-U/^{3}H-Me) becomes. For example, assuming the uridylate content in RNA is 25%, in the second fraction in Fig. 6, one methyl residue was incorporated per twelve nucleotides, whereas in the seventh fraction one methyl per thirty nucleotides was incorporated. These data suggested that the incorporation of methyl groups took place at a very early stage of the transcription, that is, the methylation took place at the 5'-terminal region of nascent RNA.

The RNA synthesis in the presence of SAM was slightly inhibited by the addition of actinomycin D (10μg/ml; 10% inhibition) or rifampicin (20μg/ml; 20% inhibition). In contrast, α-amanitin (40μg/ml) did not inhibit the SAM-dependent transcription; and S-adenosyl-homocystein(SAH), which is known to be a potent inhibitor of methylation[26], did not inhibit the reaction, but rather showed a stimulating effect (20-30%) on RNA synthesis when an amount of SAH(Boehringer Mannheim) corresponding to that of SAM was added to the standard reaction mixture as described in the legend to Fig. 1. This suggests that the methylation of RNA observed in this system is different from that so far reported[30].

DISCUSSION

Messenger RNA of the viruses containing double-stranded RNA such as CPV and reovirus is synthesized by the virus-associated RNA polymerase using double-stranded RNA as a template[4]. Miura and coworkers recently found that the 5'-terminal nucleotide of one chain of the double-stranded genome RNA is modified with methyl group at the 2' position of the ribose moiety (ref. 12 and the unpublished results by Watanabe, Furuichi and Miura). Based on these works, the addition of methyl donor SAM to the transcription system was tried. Here, I found a great stimulation of the mRNA synthesis by the addition of SAM, and the following facts:

(1) RNA synthesis is dependent on the addition of methyl donor SAM; The synthesized RNA is single-stranded and has a specific structure to the virus genome. All the segmented genome RNA are transcribed;

(2) RNA thus synthesized is methylated, the rate of methylation is calculated as one methyl group per one RNA molecule;

(3) The methyl residue incorporated into the RNA was detected in 2'-O-methyl adenylic acid which is found at the 5'-terminus of the double-stranded genome RNA;
(4) The methylation took place at very early stage of transcription.
Such an unique transcription system has not been known so far.

The methylation of transfer RNA and ribosomal RNA is known to be post-transcriptional. However, the methylation of mRNA of CPV occurs during transcription, moreover it does at very early stage, possibly at the initiation of transcription.
The incorporated methyl group was found in an adenylic acid residue in the 5'-terminal region. The methylation would correlate with the initiation of the transcription of CPV. A methyl donor SAM must play some important role there. Perry and Kelley, recently, reported that a very low level of methylation occurs in messenger RNA of mouse L-cell[20]. However, it was not clarified whether such methylation occurs post-transcriptionally or in the process of transcription.

As regards double-stranded RNA viruses other than CPV, the transcription of core particles of avian and human reovirus was studied in the presence or in the absence of SAM.
Although the dependence of transcription on SAM was remarkable for CPV, it was not so notable for reoviruses. This may be due to the states of enzymes in the core particle, which in turn depends on the pretreatment of the virus particles.
Although the presence of methylase in a virion has not been reported for double-stranded RNA viruses previously, it is known that a methylase is associated with tumor viruses, such as avian myeloblastosis virus (AMV) which transfers a methyl group from SAM to certain guanine bases in RNA [29]. Further, many studies have shown that the amount of methylated nucleotides increases in tRNA in a virus-induced tumor cell or a transformed cell[30]. However, the biological significance of these methylations has not been clarified. The methylation or the effect of SAM observed here for double-stranded RNA containing virus, CPV, seems to correlate with the initiation of transcription.

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