A MODIFIED STRUCTURE AT THE 5′-TERMINUS OF mRNA OF VACCINIA VIRUS

Toshiyuki URUSHIBARA*, Yasuhiro FURUICHI, Chiaki NISHIMURA** and Kin-ichiro MIURA
*National Institute of Genetics, Mishima, 411 Japan; and **Kitasato University, Department of Pharmaceutical Sciences, Shirokane, Tokyo, 108 Japan*

Received 13 November 1974

1. Introduction

When transcription of cytoplasmic polyhedrosis virus (CPV), containing double-stranded RNA and its transcriptase, is carried out in vitro in the presence of a methyl-donor S-adenosylmethionine, the synthesized messenger RNA carries methylated nucleotides at the 5′-terminus in an unusual form [1,2]. As such an odd structure seems to be related to the function of mRNA, we decided to investigate whether other viral mRNA also contains an unusual terminal structure. Thus, the in vitro transcription of vaccinia virus, which contains double-stranded DNA and transcriptase [3,4], was studied. Addition of *S*-adenosylmethionine to the reaction mixture caused synthesis of the methylated mRNA. The methylation was restricted to the 5′-terminal part, where an unusual structure like that in CPV was detected, namely the 5′-terminal nucleotide is methylated at the 2′-position of ribose and its 5′-phosphate is blocked by 7-methylguanylic acid with a pyrophosphate linkage.

2. Materials and methods

Vaccinia virus cultured in baby hamster kidney cells was collected as described in ref. [5]. Crude virus preparation suspended in 0.02 M Tris–HCl buffer (pH 7.8) containing 0.1 M NaCl and 5 mM EDTA was homogenized with the same volume of difluorodichloromethane (Daiflon, Daikin Co.) [6]. The emulsion was centrifuged to separate the organic solvent. These treatments were effective in extracting the activities of RNA polymerase and methylase contained in the virion. The virus was then purified by banding in a density gradient of CsCl. The details will be published elsewhere.

The in vitro RNA synthesis was carried out with the Daiflon-treated vaccinia virus under the conditions employed by Kates and Beeson [3] with the addition of SAM in the final concentration of 40 μmol/ml (for labelling, [methyl-^{3}H] SAM (125 μCi/0.0184 μM) was added).

Methods for analysis of the terminal oligonucleotides were published previously [2,7,8].

3. Results and discussion

The RNA synthesis by vaccinia virions was performed in the reaction mixture according to Kates and Beeson [3]. If we added 0.3 M SAM to the reaction mixture, RNA synthesis was barely stimulated. It is similar to the situation with in vitro synthesis of reovirus mRNA in the presence of SAM, although synthesis of CPV mRNA was greatly stimulated by SAM [1]. However, a small amount of methyl-^{3}H in SAM was incorporated into the synthesized mRNA in every case. Vaccinia virus mRNA was synthesized for 12 hr in a reaction mixture containing [methyl-^{3}H] SAM and [^{14}C] UTP. It was purified by passing through a Sephadex G-100 column to separate the mRNA from substrates, and centrifuged in a density gradient of 5 to 30% glycerol. The size of the synthesized RNA ranged from 7S to 14S (peak is about 11S). This is the same as the mRNA synthesized in the absence of SAM by Kates and Beeson [3]. Vaccinia virus mRNA consists of various

* on leave from Kitasato University

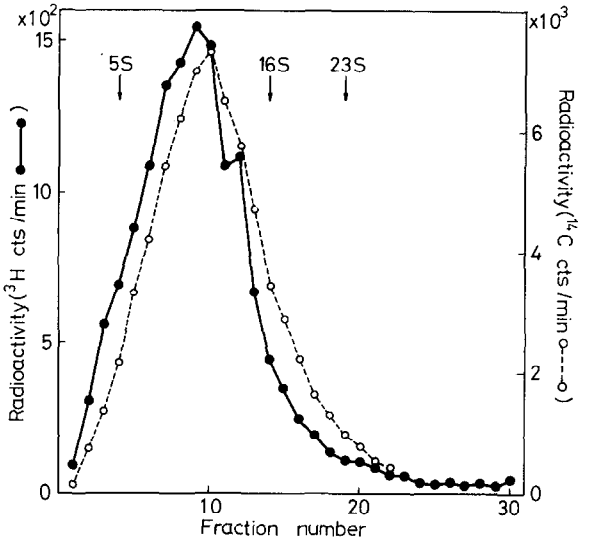

Fig.1. Sedimentation pattern of vaccinia virus mRNA. mRNA was synthesized with [^{14}C]UTP (10 μCi, 0.168 μmol/ml) and [methyl-^{3}H]SAM (125 μCi, 0.00184 μmol/ml) in the medium for in vitro transcription at 32°C for 12 hr. After virus particles were centrifuged off, mRNA in the supernatant was recovered by Sephadex (G-100) column chromatography and by precipitation with ethanol adding carrier ribosomal RNA. mRNA was then centrifuged in the linear gradient of 5–30% glycerol in 0.02 M Tris–HCl buffer (pH 7.8) containing 0.1 M NaCl and 0.005 M EDTA using a Beckman-Spinco ultracentrifuge L2 and rotor SW 41 at 28000 rev/min for 16 hr at 5°C.

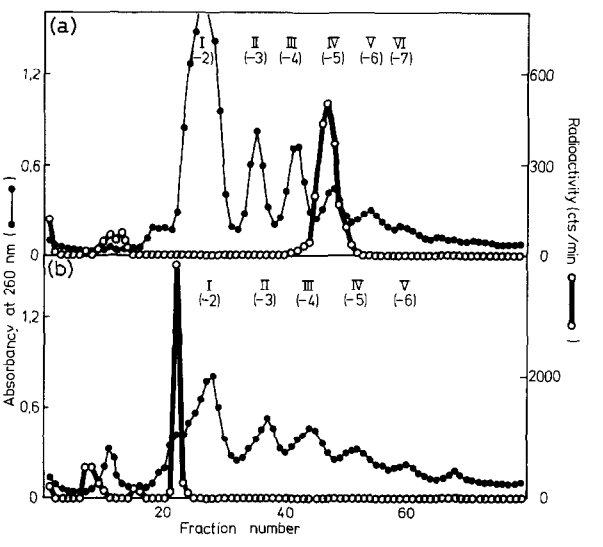

Fig.2. DEAE-urea column chromatography of hydrolysed [methyl-^{3}H] labeled vaccinia virus mRNA. (a) Alkaline digest. (b) *Penicillium* nuclease digest. Digests were chromatographed on a DEAE cellulose (DE-11) column (0.6 cm × 30 cm) with the pancreatic ribonuclease digest of ribosomal RNA included as a marker. Elution was carried out with a linear gradient of NaCl from 0.05 M (50 ml) to 0.4 M (50 ml) in 7 M urea–0.02 M Tris–HCl (pH 8.2). Roman numerals over peaks indicate the chain length of nucleotides, and numerals in parentheses indicate net charge. Details are the same as in ref. [8].

molecular species having different sizes, which were transcribed from different cistrons. As shown in fig.1, the radioactivity of [methyl-^{3}H] is distributed in the same area as the [^{14}C]uridine, which shows a synthesized RNA. The ratio of ^{3}H/^{14}C, however, decreased with increase of the size of RNA. In the fractions around the peak the incorporated [methyl-^{3}H] was calculated as about 1.3 mol per mRNA molecule, and this value was almost constant throughout the size range of the synthesized RNA. This strongly suggests that the same number of methyl residues is incorporated at definite sites in every mRNA molecule.

The [methyl-^{3}H] labeled RNA was then hydrolysed by alkali, and the digest was applied on a DEAE-urea column. Only one major radioactive peak appeared at the position of −5 charge (or tetranucleoside tetraphosphates (fig.2a)). If it is a tetranucleotide, the preceeding three nucleosides should be methylated at the 2′-position of the ribose moiety. When the [methyl-^{3}H] labeled RNA was hydrolysed by *Penicillium* nuclease, which splits polynucleotide into 5′-nucleotides including a 2′-*O*-methyl nucleotide, the radioactivity in the digest behaved as one major peak at −2 charge (or mononucleoside monophosphate) (fig.2b). This corresponds to the mixture of pNm (2′-*O*-methyl nucleoside-5′-phosphate). However, if the 5′-terminal is blocked as shown in CPV mRNA [2], XppNm will also be contained in this fraction.

Paper electrophoresis of the *Penicillium* nuclease digest showed one radioactive peak near pG (fig.3a). Even after this radioactive component was treated with phosphomonoesterase, the electrophoretic pattern did not change. The phosphate should be blocked as XppNm; this component, therefore, is not pG or pGm.

The radioactive nucleotide in the *Penicillium* nuclease digest was then hydrolysed with venom phosphodiesterase. The electrophoretic pattern of the hydrolysate revealed three components as shown in fig. 3b.

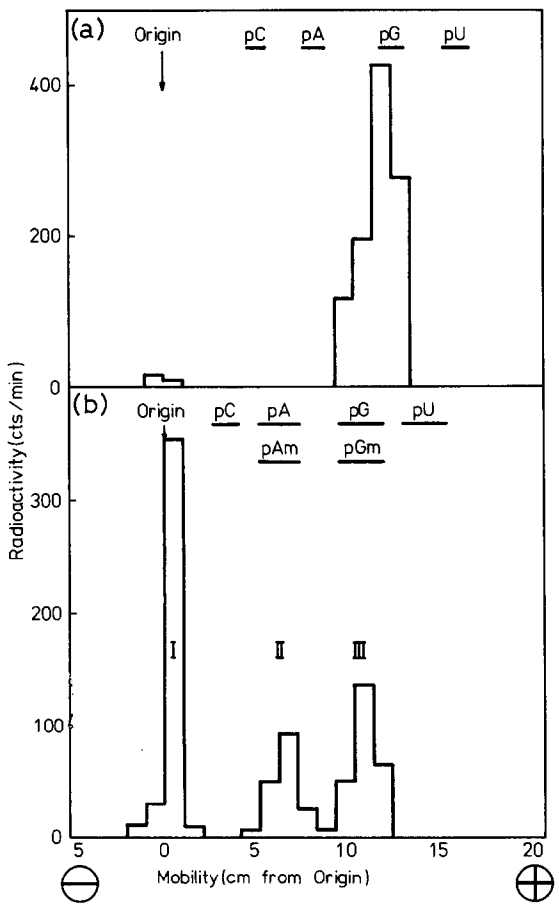

Fig.3. Paper electrophoresis of the terminal oligonucleotide and its phosphodiesterase digest. (a) [methyl-^{3}H] labeled vaccinia virus mRNA was digested by *Penicillium* nuclease, in which the conditions were the same as in ref. [8]. The digest was paper electrophoresed on Whatman 3MM paper in 5% acetic acid–morpholine buffer (pH 3.5) at 35 V/cm for 70 min. The radioactivity in each 1cm-piece was counted in 5 ml of PPO- and POPOP-containing toluene. When the sample was digested further with phosphomonoesterase (see [8]), a quite similar electrophoregram was obtained. This means that the phosphate groups in the sample oligonucleotide are protected from phosphomonoesterase. (b) Phosphodiesterase digest of the oligonucleotide recovered from (a). The [^{3}H] labeled oligonucleotide in (a) was digested further by venom phosphodiesterase (conditions were the same to ref. [8]). Paper electrophoresis was carried out as in (a).

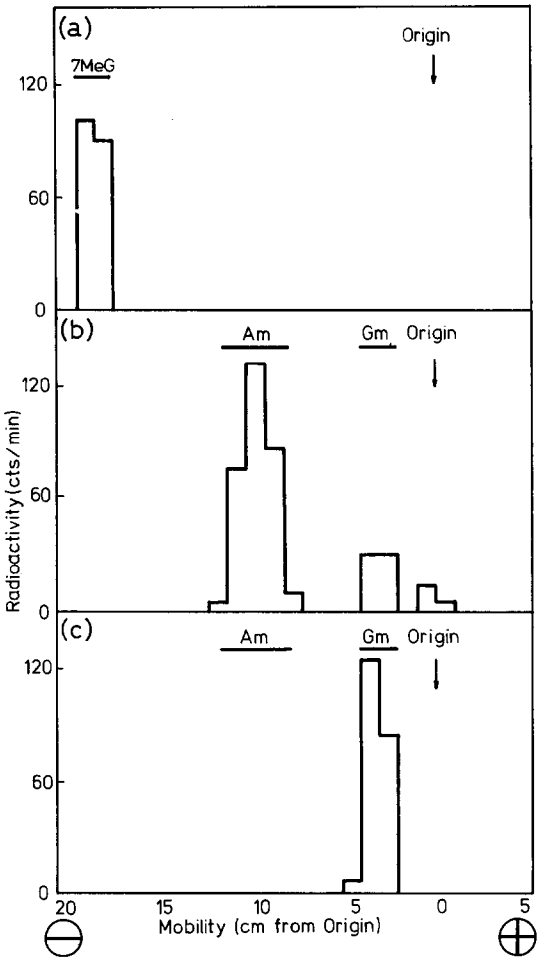

Fig.4. Paper electrophoresis of the phosphomonoesterase digest of three components in fig. 3b. (a) (b) and (c) are the phosphomonoesterase digest (conditions are written in ref. [8] of the components I, II and III respectively. Paper electrophoresis was carried out as in fig. 3a 42 V/cm for 70 min, (b) 35 V/cm for 140 min, (c) 35 V/cm for 140 min.

The ratio of ^{3}H in components I, II and III was approx. 5/2/3 in the mean of two experiments. The component I is hydrolysed further with phosphomonoesterase and electrophoresed again (fig.4a). The [methyl-^{3}H] count was found to overlap perfectly the ultraviolet absorption of the added authentic 7-methylguanosine. This was confirmed also by two-dimensional paper chromatography as shown in fig. 5. Since the component I has a cis-diol (at the 2′- and 3′-positions–details will be published elsewhere), it is considered to be 5′-phosphorylated 7-methylguanosine (pm^{7}G) as found in the similar digest of CPV mRNA (Furuichi and Miura [2]). The component II was identified as 2′-

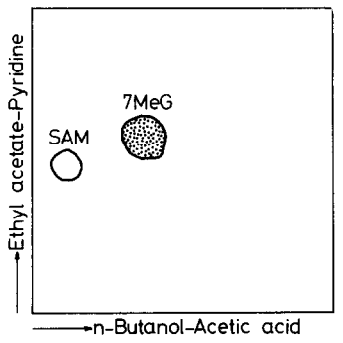

Fig.5. Identification of the component I in fig. 4a with 7-methylguanosine by two-dimensional paper chromatography. First dimension: *n*-butanol/acetic acid/H_2O, 5/1/4. Second dimension: ethylacetate/pyridine/H_2O, 75/25/165. Ultraviole spots of the added standards m^7G and SAM and other parts were cut out, added to 5 ml of PPO- and POPOP-containing toluene and counted in a scintillation spectrometer. A radioactive spot is represented by dots.

O-methyladenosine-5′-phosphate (pAm) by co-running with the authentic pAm in paper electrophoresis and two-dimensional paper chromatography. It was confirmed by phosphomonoesterase treatment, which yielded Am (Am was compared with the authentic material as shown in fig. 4b). The component III was identified as 2′-*O*-methylguanosine-5′-phosphate, pGm, by methods similar to those used for pAm (fig. 4c).

As written above, the phosphates of the 5′-terminal nucleotide in vaccinia virus mRNA are blocked by some material, and three components, that is 7-methyl-5′-guanylic acid, 2′-*O*-methyl-5′-guanylic acid, and 2′-*O*-methyl-5′-adenylic acid, are obtained from the 5′-terminal part. Since the 5′-terminal nucleotide of CPV mRNA is a 2′-*O*-methylated adenylic acid, and its 5′-phosphates are blocked by 7-methyl-5′-guanylic acid with pyrophosphate linkage [2], the vaccinia virus mRNA would be blocked at the 5′-terminal in a similar manner. If components I, II and III were derived from a continuing sequence as $m^7G^{5'}pp^{5'}$AmpGmpNp ——— (or $m^7G^{5'}pp^{5'}$GmpAmpNp———) at the 5′-terminal, *Penicillium* nuclease digestion must yield $m^7G^{5'}pp^{5'}$Am and pGm (or $m^7G^{5'}pp^{5'}$Gm and pAm) as the [methyl-^{3}H] labeled components. $m^7G^{5'}pp^{5'}$Am and pGm (or $m^7G^{5'}pp^{5'}$Gm and pAm) carry −2 charge in DEAE-urea column chromatography (fig.2b), and have similar mobilities near pG in paper electrophoresis (fig.3a). However, this is not likely the case, since no pAm and pGm were found in the *Penicillium* nuclease digest. And the molar ratio of pm^7G/pAm/pGm in the phosphodiesterase digest was not 1/1/1, but 5/2/3; in any case the radioactivity of component I corresponds to the sum of components II and III. If there are two kinds of mRNA for vaccinia virus, whose 5′-terminal structures are $m^7G^{5'}pp^{5'}$AmpNp——— and $m^7G^{5'}pp^{5'}$GmpNp———, alkali digestion must yield $m^7G'^{5'}pp^{5'}$AmpNp and $m^7G'^{5'}pp^{5'}$GmpNp as the [methyl-^{3}H] labeled components (m^7G' means a change of m^7G caused by alkali). Since the imidazole ring of 7 methylguanosine decomposes by alkali treatment to lose a plus charge, $m^7G'^{5'}pp^{5'}$AmpNp and $m^7G'^{5'}pp^{5'}$GmpNp would have -5 charge. Here, if ratio of the amounts of $m^7G^{5'}pp^{5'}$Am and $m^7G^{5'}pp^{5'}$Gm is 2:3, all the experimental results can be explained without inconsistency. In fact the ^{3}H labeled oligonucleotides in the *Penicillium* nuclease digest were separated into two components by the Dowex-1 column chromatography.

The transcript from polycistronic gene may have at least two kinds of starting as shown already for phage transcription [9]. Kates and Beeson [3] stated that γ-^{32}P-ATP and very little amount or γ-^{32}P-CTP are incorporated into vaccinia mRNA as its 5′-terminal nucleotide. However, this is the in vitro transcription without SAM. Even in the in vitro transcription, the presence of SAM may change the situation.

These results lead us to the conclusion that : (1) the RNAs transcribed from vaccinia virus in the presence of SAM are methylated at the 2′-position of the 5′-terminal nucleotide; (2) their 5′-terminal phosphates are blocked by 7-methylguanosine-5′-phosphate through a pyrophosphate linkage; (3) 3/5 of the mRNA molecules carry 2′-*O*-methyl guanylic acid as the 5′-terminal nucleotide and the rest 2′-*O*-methyl adenylic acid. Thus the 5′-terminal structures of the mRNA transcribed from vaccinia virus in the presence of SAM in vitro are summarized in the following two formulas: $m^7G^{5'}pp^{5'}$Gm–and $m^7G^{5'}pp^{5'}$Am–. These modified structures at the 5′-terminal of vaccinia virus mRNA are similar to the mRNA of CPV [2], except for the number of phosphates. That the blocking at the 5′-terminal phosphates by 7-methylguanosine as well as the methylation at the 2′-position of the terminal nucleotide in a mRNA molecule are common for these

viral mRNA's, suggests that these abnormal structures are necessary for the function and/or the control of metabolism of mRNA.

Acknowledgements

We thank Professor James F. Crow for correcting the English, and Dr Akira Kuninaka for the supply of *Penicillium* nuclease. This work was partially supported by grants from the Ministry of Education of Japan and Japan Society for the Promotion of Science.

References

[1] Furuichi, Y. (1974) Nucleic Acid Res. 1, 809–822.
[2] Furuichi, Y. and Miura, K submitted to Nature.
[3] Kates, J. and Beeson, J. (1970) J. Mol. Biol. 50, 1–18.
[4] Kates, J. and Beeson, J. (1970) J. Mol. Biol. 50, 19–33.
[5] Planterose, K. N., Nishimura, C. and Salzman, N. P. (1962) Virology 18, 294–301.
[6] Shimotohno, K. and Miura, K. (1974) J. Biochem. 74, 117–125.
[7] Shimotohno, K. and Miura, K. (1974) J. Mol. Biol. 86, 21–30.
[8] Miura, K., Watanabe, K. and Sugiura, M. (1974) J. Mol. Biol. 86, 31–48.
[9] Sugiura, M., Okamoto, T. and Takanami, M. (1969) J. Mol. Biol. 43, 299–315.