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Dinucleotide Sequences at the 5′ Ends of Vaccinia Virus mRNA's Synthesized In Vitro

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The diversity of dinucleotide sequences at the 5′ ends of vaccinia virus mRNA's was determined by a two-dimensional electrophoresis procedure. RNA labeled with *S*-adenosyl[*methyl*-^{3}H]methionine was synthesized in vitro by enzymes present in vaccinia virus cores. The RNA, ending in m^{7}G(5′)pppN^{m}pN–, was β-eliminated and treated with alkaline phosphatase. After digestion with RNases T_2, T_1, and A, all eight possible dinucleotides containing G^m and A^m were identified. They are, in decreasing order of abundance: G^{m}pUp (22%), A^{m}pCp (18%), G^{m}pAp (16%), G^{m}pCp (15%), A^{m}pAp (11%), A^{m}pUp (10%), A^{m}pGp (7%), and G^{m}pGp (2%).

Enzymes necessary for synthesis of mRNA are present within infectious vaccinia virus particles, making it possible to study transcription in vitro (10, 16). The total sequence complexity of mRNA synthesized by virus cores is about 150 kilobases (2, 18), and the abundant mRNA's have been shown to code for authentic early viral proteins (4, 7, 9, 19). It is not yet known whether the mRNA's have similar or diverse leader sequences. The presence of at least two 5′-terminal sequences was indicated by the finding of m^{7}G(5′)pppA^m and m^{7}G(5′)pppG^m ends (23). In the present communication, we show that all eight possible m^{7}G(5′)pppA^{m}pNp and m^{7}G(5′)pppG^{m}pNp nucleotide sequences are present, indicating considerable 5′-terminal sequence diversity.

In previous experiments, the ratios of m^{7}G(5′)pppA^m to m^{7}G(5′)pppG^m ends varied considerably, leading us to suspect that this might be related to the relative concentration of ribonucleoside triphosphates used for transcription (14). Such a result might be expected if initiated ends are capped and the concentrations of ATP and GTP needed for initiation are greater than those needed for elongation. To evaluate this relationship, RNA was synthesized in reaction mixtures containing 2 mM GTP and varied ATP concentrations. *S*-Adenosyl[*methyl*-^{3}H]methionine was used to specifically label the 5′ ends of the RNA. After purification, the RNA was digested with nuclease P1, and the cap structures, m^{7}G(5′)pppA^m and m^{7}G(5′)pppG^m, were separated by paper chromatography as shown in Fig. 1. With ATP concentrations of 2 mM, 1 mM, 0.5 mM, and 0.2 mM, m^{7}G(5′)pppA^m comprised 36, 32, 25, and 17% of the total caps formed, respectively. In a separate experiment, the concentration of ATP was fixed at 2.5 mM and the GTP concentration was varied. At 2.5 mM GTP, 40% of the caps were m^{7}G(5′)pppA^m, whereas at 0.1 mM, the percentage was 54%. Thus, the relative amount of m^{7}G(5′)pppA^m varied from 17 to 54%, depending on the ATP and GTP concentrations used for transcription. In subsequent experiments, the concentrations of ATP and GTP were each 2.5 mM to provide high levels of both A^m and G^m caps.

Two approaches were used to identify the nucleotides following m^{7}G(5′)pppA^m and m^{7}G(5′)pppG^m. Both depend on the fact that 2′-*O*-methylation occurs exclusively on the penultimate nucleotide of RNA synthesized in vitro by vaccinia virus (23). There are no sites of internal methylation, and cap II structures [m^{7}G(5′)pppN^{m}pN^m] are found only in RNA synthesized in vivo (1). In the first approach, methyl-labeled RNA was digested with either RNase T_1 (which cleaves after unmethylated guanosine residues) or RNase A (which cleaves after unmethylated pyrimidine residues) or with both enzymes, and then treated with alkaline phosphatase. The methyl-labeled oligonucleotides were resolved by DEAE-cellulose column chromatography under conditions in which m^{7}G(5′)pppN^{m}pN elutes with a net charge between −3 and −4 and each additional nucleotide adds one extra negative charge. After RNase T_1 digestion, less than 8% of the labeled oligonucleotides eluted with a charge of −3, indicating that guanosine is relatively uncommon immediately after A^m or G^m (Fig. 2A). In contrast, 61% of the labeled material eluted with a −3 charge after

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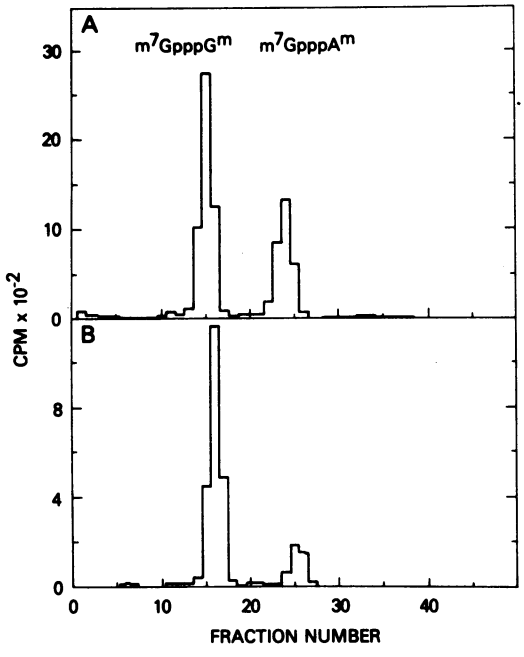

FIG. 1. *Paper chromatography of methylated ends isolated from RNA synthesized in the presence of different ATP concentrations. RNA was synthesized for 30 min at 37°C in reaction mixtures containing 50 mM Tris-hydrochloride (pH 8.5), 10 mM dithiothreitol, 0.05% Nonidet P-40 detergent, 10 mM $MgCl_2$, 2 mM GTP, 1 mM CTP, 1 mM UTP, either 2 mM, 1 mM, 0.5 mM, or 0.2 mM ATP, 3 μM S-adenosyl[methyl-^{3}H]methionine (3.4 Ci/mmol), and 1.3 units of absorbancy at 260 nm of purified vaccinia virus per ml. After sodium dodecyl sulfate-phenol-chloroform extraction, ethanol precipitation, and gel filtration, the RNA was digested with nuclease P1, applied to Whatman no. 1 paper, and chromatographed in isobutyric acid–0.5 M NH_4OH (5:3). Strips, 1 cm wide, were counted. Only results obtained with 2 mM ATP (A) and 0.2 mM ATP (B) are shown.*

RNase A digestion, indicating that pyrimidines are common in this position (Fig. 2B). Since approximately 70% of the labeled oligonucleotides eluted with a charge of −3 after combined digestion with RNase T_1 and RNase A, 30% of the nucleotides following A^m and G^m must be adenosine (Fig. 2C). Further analysis of the −3, −4, and −5 peaks obtained after RNase T_1 digestion indicated that all contain more than one sequence (unpublished data).

A "fingerprinting" procedure was used as the second approach to determine the dinucleotide sequence at the ends of vaccinia virus mRNA's. Previously, Cory and Adams (5) separated capped oligonucleotides obtained by RNase T_2 digestion of ^{32}P-labeled myeloma cell mRNA by two-dimensional electrophoresis. Resolution was limited, however, by the low mobility of capped oligonucleotides on DEAE-paper. To eliminate this problem, first the m^7G residue was removed from methyl-labeled RNA by periodate oxidation and β-elimination (15). After treatment with alkaline phosphatase to remove the terminal phosphates, the RNA now ending in A^m or G^m was treated with a mixture of RNases T_2, T_1, and A to liberate labeled RNase-resistant dinucleotides of the types A^mpNp and G^mpNp. In initial experiments, the dinucleotides were purified by chromatography on a DEAE-cellulose column in 7 M urea. However, since all of the radioactive material eluted as a peak with the expected charge of −3, this step was eliminated in subsequent experiments. The *methyl*-^{3}H-labeled dinucleotides were then mixed with all 16 possible N^mpNp's, obtained by RNase digestion of *methyl*-^{14}C-labeled HeLa cell rRNA, and subjected to two-dimensional electrophoresis on cellulose acetate (Cellogel) and DEAE-paper. The positions of the ^{14}C-labeled dinucleotides determined by autoradiography (Fig. 3) were similar to those previously described and served as markers. The X-ray film was then aligned with the DEAE-paper, and appropriate sections of the latter were counted either directly or after the dinucleotides were eluted with 2 M triethylamine bicarbonate. As anticipated, ^{3}H-labeled material was found exclusively in areas containing dinucleotides of the type A^mpNp and G^mpNp but not in areas containing exclusively U^mpNp or C^mpNp. The identities of the methylated nucleosides in all spots were confirmed by paper chromatography after digestion with snake venom phosphodiesterase and alkaline phosphatase (not shown). The latter analysis also allowed us to correct for some cross-contamination of A^mpGp and G^mpAp. The results of an experiment of this type are presented in Table 1. All eight possible dinucleotide sequences were found. Their frequency in descending order was:

TABLE 1. *5′-Terminal dinucleotide sequences*

Dinucleotide[a]	^{3}H cpm	% of total
G^mpUp	1,680	22
A^mpCp	1,430	18
G^mpAp	1,277	16
G^mpCp	1,150	15
A^mpAp	820	11
A^mpUp	780	10
A^mpGp	520	7
G^mpGp	116	2

[a] Dinucleotides were derived from 5′-terminal $m^7G(5')pppN^mpN$- sequences and isolated as described in the text.

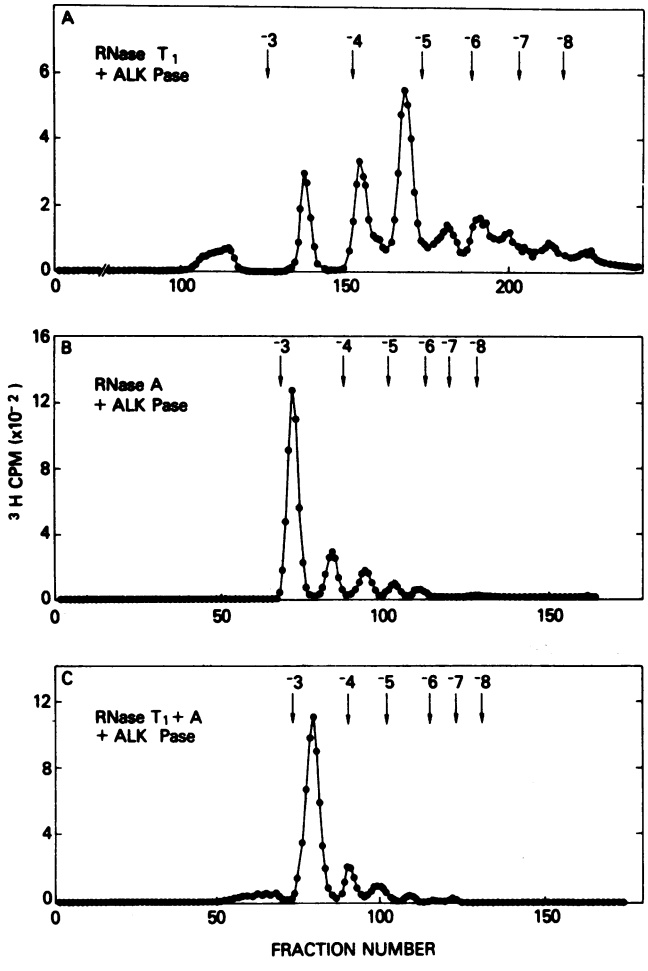

FIG. 2. *DEAE-cellulose column chromatography of RNase digests of vaccinia virus RNA which was synthesized as described in the legend to Fig. 1, except that all ribonucleoside triphosphates were present at 2.5 mM. Enzyme digestions with (A) RNase T_1 and alkaline phosphatase, (B) RNase A and alkaline phosphatase, and (C) RNase T_1, RNase A, and alkaline phosphatase were as described (3). DEAE-cellulose chromatography was in 7 M urea at pH 7.6 (23). The negative charges of uncapped marker oligonucleotides detected by continuous UV monitoring are indicated.*

G^mpUp, A^mpCp, G^mpAp, G^mpCp, A^mpAp, A^mpUp, A^mpGp, and G^mpGp. Of note was the diversity of 5'-terminal sequences and the relatively low amount of guanosine in the second position.

In regard to the ability to synthesize RNA with diverse 5' sequences, the transcriptase activity packaged in vaccinia virus appears to resemble that of procaryotic and eucaryotic DNA-dependent RNA polymerases. By contrast, the transcriptase activities packaged in RNA viruses, such as reovirus (6, 8) or vesicular stomatitis virus (20), produce multiple RNAs with identical 5'-nucleotide sequences (6, 8, 20). We have assumed, from studies showing the incorporation of the β-phosphate from GTP and ATP into cap structures during transcription (14) and from the specificity of the purified mRNA guanylyltransferase for RNA with di- or triphosphate ends (12, 13, 22), that the ribonucleoside triphosphate used to initiate RNA synthesis is the one that becomes 2'-*O*-methylated after capping. However, evidence for endonucleolytic processing has been obtained (17), and the isolation of a 5'-polynucleotide kinase that could add phosphates at a site of RNA cleavage to make it suitable for capping has led to alternative proposals (21). The recent cloning of vaccinia virus

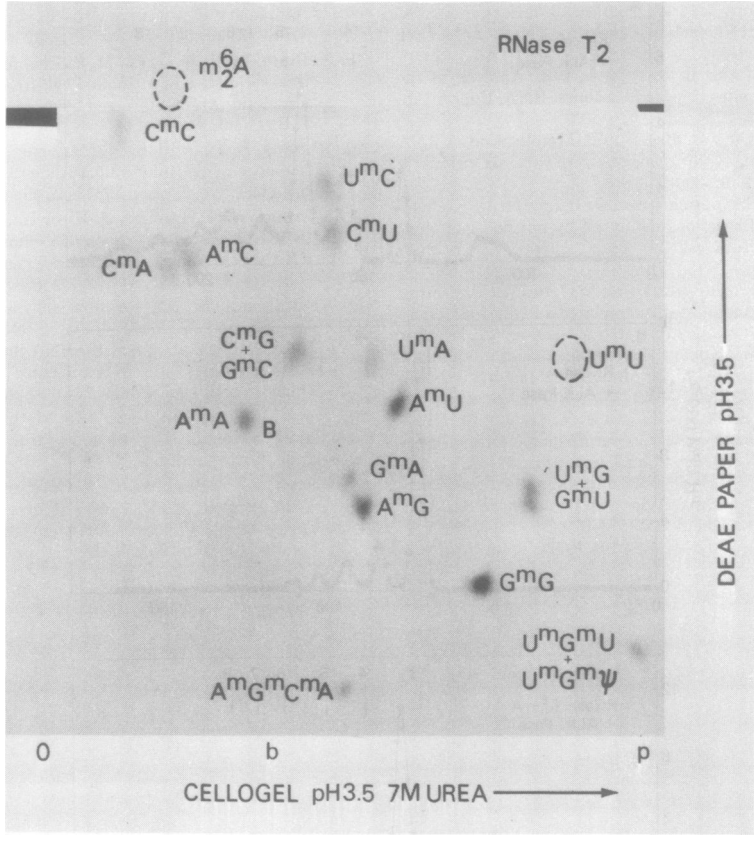

FIG. 3. *Autoradiograph of two-dimensional electropherogram of methylated dinucleotides. Vaccinia virus RNA labeled with S-adenosyl[methyl-^{3}H]methionine was periodate oxidized and β-eliminated as previously described (15) and treated with alkaline phosphatase. The RNA now ending in A^mpN- and G^mpN- was digested with a mixture of RNases T_1, T_2, and A and then mixed with marker methyl-^{14}C-labeled dinucleotides obtained from HeLa cell rRNA. The latter was prepared by incubating 2 × 10^7 HeLa cells with 250 μCi of [methyl-^{14}C]methionine (50.6 mCi/mmol) at 37°C for 42 h in methionine-free medium supplemented with 10 μM adenosine, 10 μM guanosine, and 20 mM sodium formate. Two-dimensional electrophoresis was carried out as described (11) except for the use of Cellogel in place of cellulose acetate strips. ^{14}C-labeled dinucleotides were located by autoradiography and eluted with 2 M triethylamine bicarbonate.*

DNA sequences (24), however, should facilitate the examination of individual mRNA's and the determination of their mode of formation.

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