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5'-Terminus of Moloney Murine Leukemia Virus 35S RNA Is $m^7G^{5'}ppp^{5'}GmpCp$

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The 5'-terminal sequence $m^7G^{5'}ppp^{5'}GmpCp$ was isolated from Moloney murine leukemia virus 35S RNA after digestion of ^{32}P-labeled RNA with RNases T1, T2, and A followed by pH 3.5 ionophoresis on DEAE paper.

RNAs from a wide variety of eukaryotic cells and their viruses have an unusual 5'-terminal structure with a 7-methylguanosine linked 5'-5' via a triphosphate to a second 2'-*O*-methylated nucleotide (review in A. J. Shatkin, New Sci., in press). Such "capped" structures are represented $m^7G^{5'}ppp^{5'}NmpNp$; in some cases they have a greater extent of methylation (12; Shatkin, in press).

It has recently been found that poliovirus mRNA and virion RNA, which are identical nucleic acid sequences, are neither capped nor methylated (9, 10). Like poliovirus RNA, the genome of RNA tumor viruses can also act as mRNA for the synthesis of viral proteins in vitro (8). We therefore have examined the 5'-terminus of the RNA from Moloney murine leukemia virus (M-MuLV). We show here that the 5'-termini of the 35S subunits of M-MuLV RNA are $m^7G^{5'}ppp^{5'}GmpCp$.

While this work was in progress, two groups have reported that the 5'-termini of the genome RNA of two avian RNA tumor viruses, Rous sarcoma virus (6) and avian sarcoma virus (7), have a structure identical to that which we find on the M-MuLV genome.

Preparation of viral RNA. For this analysis, uniformly ^{32}P-labeled M-MuLV viral RNA was prepared. The virions were disrupted by treatment with sodium dodecyl sulfate, and the 70S RNA complex was separated from the low-molecular-weight virion RNAs and then deproteinized by phenol-chloroform extraction. The 35S genomic RNA was prepared by heating the 70S complex to 95°C for 2 min followed by sucrose gradient sedimentation. The fraction of the RNA sedimenting as a sharp 35S peak (45% of the total radioactivity) was used in subsequent analysis.

To isolate the 5'-terminus of M-MuLV 35S RNA, the ^{32}P-labeled RNA was digested with RNases T1, T2, and A. This combination of enzymes will degrade RNA to 3'-mononucleotides, but does not digest 5'-terminal structures of the form $m^7G^{5'}ppp^{5'}NmpNp$ because pyrophosphate linkages and nucleotides with 2'-*O*-methylations are resistant. To separate the mononucleotides from the 5'-terminus, the digested RNA was subjected to pH 3.5 ionophoresis on DEAE paper as described previously (12). The autoradiogram of the separation is shown in Fig. 1. Lane 1 shows the multiple 5'-termini obtained from vesicular stomatitis virus (VSV) mRNA, with the location of the major terminus $m^7G^{5'}ppp^{5'}(m)AmpAp$ (12) indicated in the figure. From M-MuLV 35S RNA, only one potential 5'-terminus migrating just ahead of the major VSV terminus was found (Fig. 1, lane 2). The traces of other spots seen between the origin and the mononucleotides were more intense in similar analyses of the low-molecular-weight RNA derived from the 70S RNA complex, and thus are presumably derived from termini or nuclease-resistant dinucleotides in low-molecular-weight RNAs contaminating the 35S RNA.

Penicillium nuclease. To determine the structure of this potential 5'-terminus, it was eluted from the paper and digested with penicillium nuclease (P1), an enzyme that degrades RNA to 5'-mononucleotides regardless of base or ribose modifications and also has a 3'-phosphatase activity (5). The products of this digestion were separated by pH 3.5 ionophoresis on Whatman 3MM paper, and the autoradiogram is shown in Fig. 2A. Spots migrating with markers of pC and P_i and an additional spot migrating just slower than pG were obtained in the approximate ratios (counts per minute) of 0.8:1.0:2.7. The mobility of the spot migrating just slower than pG was unchanged after phosphatase digestion, indicating that it contained phosphate in internucleotide linkage, whereas the radioactivity in the spot migrating with pC

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was converted to ^{32}P after phosphatase digestion. The phosphatase-resistant structure was found to be sensitive to digestion by venom phosphodiesterase, an enzyme that digests most readily from an unphosphorylated 3'-terminus to give 5'-mononucleotides (1). This enzyme will also digest from both 3'-termini of a structure $N^{5'}ppp^{5'}N$ to yield pN and P_i (12). The products of venom phosphodiesterase digestion were separated by two-dimensional thin-layer chromatography, using a solvent system that fractionates methylated and unmethylated nucleotides (12, 14). The three products obtained co-migrated with markers of pm^7G, pGm, and P_i (Fig. 2B). These identities were also verified by one-dimensional pH 3.5 ionophoresis on Whatman 3MM paper, where pGm migrated with pG and pm^7G moved just to the negative side of the origin (data not shown). The initial structure of this dinucleotide must have been $m^7G^{5'}p(p)p^{5'}Gm$ because it did not contain phosphate available to phosphatase digestion. The triphosphate nature of the linkage was uncertain because of possible phosphatase activity in the phosphodiesterase and was demonstrated as described below. The identity of the product migrating with pC in the initial P1 nuclease digestion of the potential 5'-terminus was confirmed by thin-layer chromatography (Fig. 2C), establishing the complete P1 digestion products as $m^7G^{5'}p(p)p^{5'}Gm$, pC, and P_i, in approximately equimolar ratios. Given the specificity of P1 for cleaving to give 5'-mononucleotides as well as having a 3'-phosphatase activity, initial structures of $m^7G^{5'}p(p)p^{5'}GmpCp$ or $mG^{5'}p(p)p^{5'}m^7GpCp$ are possible with the latter structure unlikely because RNases T1 and T2 presumably would have cleaved between m^7G and C in the initial digestion, which generated the terminus.

Periodate oxidation and β-elimination. To determine the order of the constituents and the number of phosphates in the 5'-5' linkage, a second approach was taken. A sample of the potential 5'-terminus isolated after RNase T1, T2, and A digestion was oxidized with periodate followed by β-elimination with aniline. This procedure removes a nucleoside containing free 2'-3'-hydroxyls from an oligonucleotide (13). To free the ^{32}P-labeled product of this reaction from periodate and aniline, it was subjected to pH 3.5 ionophoresis on 3MM paper, located by autoradiography, eluted with water, and lyophilized as described previously (12). Subsequent digestion with P1 nuclease gave products that migrated with markers of pC and P_i and just ahead of GTP when subjected to pH 3.5 ionophoresis on DEAE paper (Fig. 3A, lane 1). All radioactivity in the P1 digestion products was converted to $^{32}P_i$ after phosphatase digestion (Fig. 3A, lane 2), showing that the β-elimination was complete. Digestion of the material in the spot migrating just ahead of GTP with venom phosphodiesterase gave products migrating with markers of PP_i and pGm (Fig. 3B, lane 1). Because venom phosphodiesterase cleaves a nucleoside triphosphate to give PP_i and pN (11), the initial structure must have been pppGm, indicating a triphosphate linkage before β-elimination. The identity of pGm was

FIG. 1. *Autoradiogram of a DEAE paper ionophoretic purification of M-MuLV and VSV 5'-termini. ^{32}P-labeled M-MuLV virions were prepared from a strain of NIH/3T3 cells that produces a cloned line of M-MuLV (4). Three roller bottles were incubated for 4 h in phosphate-free Dulbecco minimal medium containing 10% dialyzed calf serum (DME/CS). The medium was removed, and 30 ml of the same medium containing 1 mCi of carrier-free [^{32}P]phosphoric acid (New England Nuclear Corp.) per ml was added to each bottle. After 12 h the medium was collected, and 25 ml of fresh phosphate-free DME/CS was added and collected again after 12 h. Medium from the three harvests was pooled, and cell debris was removed by centrifugation for 5 min at 2,000 × g. Virus was pelleted from the medium by centrifugation at 19,000 rpm in a Beckman L19 rotor for 2.5 h. The virus was resuspended in 0.01 M Tris (pH 7.5), 0.1 M NaCl, and 0.1 mM EDTA. The virus was banded, and the 70S RNA was prepared as described by Fan and Baltimore (3). The 70S RNA was precipitated by addition of 2 volumes of ethanol and after centrifugation was resuspended in 0.2 M sodium acetate, 0.01 M Tris (pH 7.5), and 1 mM EDTA. The RNA was deproteinized by addition of an equal volume of a solution containing equal volumes of phenol and chloroform. The nonaqueous phase was reextracted with the resuspension buffer. The combined aqueous phases were pooled, and the RNA was precipitated by addition of 2 volumes of ethanol. After centrifugation, the RNA was resuspended in 0.01 M Tris (pH 7.5) and 1 mM EDTA and was denatured by heating to 95°C for 2 min. The RNA was loaded onto a 12-ml 15 to 30% sucrose gradient containing 0.1 M NaCl, 0.05 M Tris (pH 7.5), 1 mM EDTA, and 0.5% sodium dodecyl sulfate. The gradient was centrifuged at 35,000 rpm in a Beckman SW41 rotor for 5.5 h. The region of the gradient containing 35S RNA was pooled, and the RNA was precipitated by addition of 2 volumes of ethanol. Limit digests of M-MuLV 35S RNA (3 × 10^6 cpm) and VSV mRNA (5 × 10^5 cpm) with RNases T1, T2, and A were prepared as described previously (12). The digested samples were spotted on a sheet of Whatman DEAE 81 paper (100 by 30 cm) and subjected to ionophoresis at pH 3.5 for 6 h at 30 V/cm (12). Autoradiography was for 14 h on Kodak RPR-14 film. Quantitation and elution of the 5'-terminal structures has been described previously (12). Lane 1, VSV mRNA; lane 2, M-MuLV RNA.*

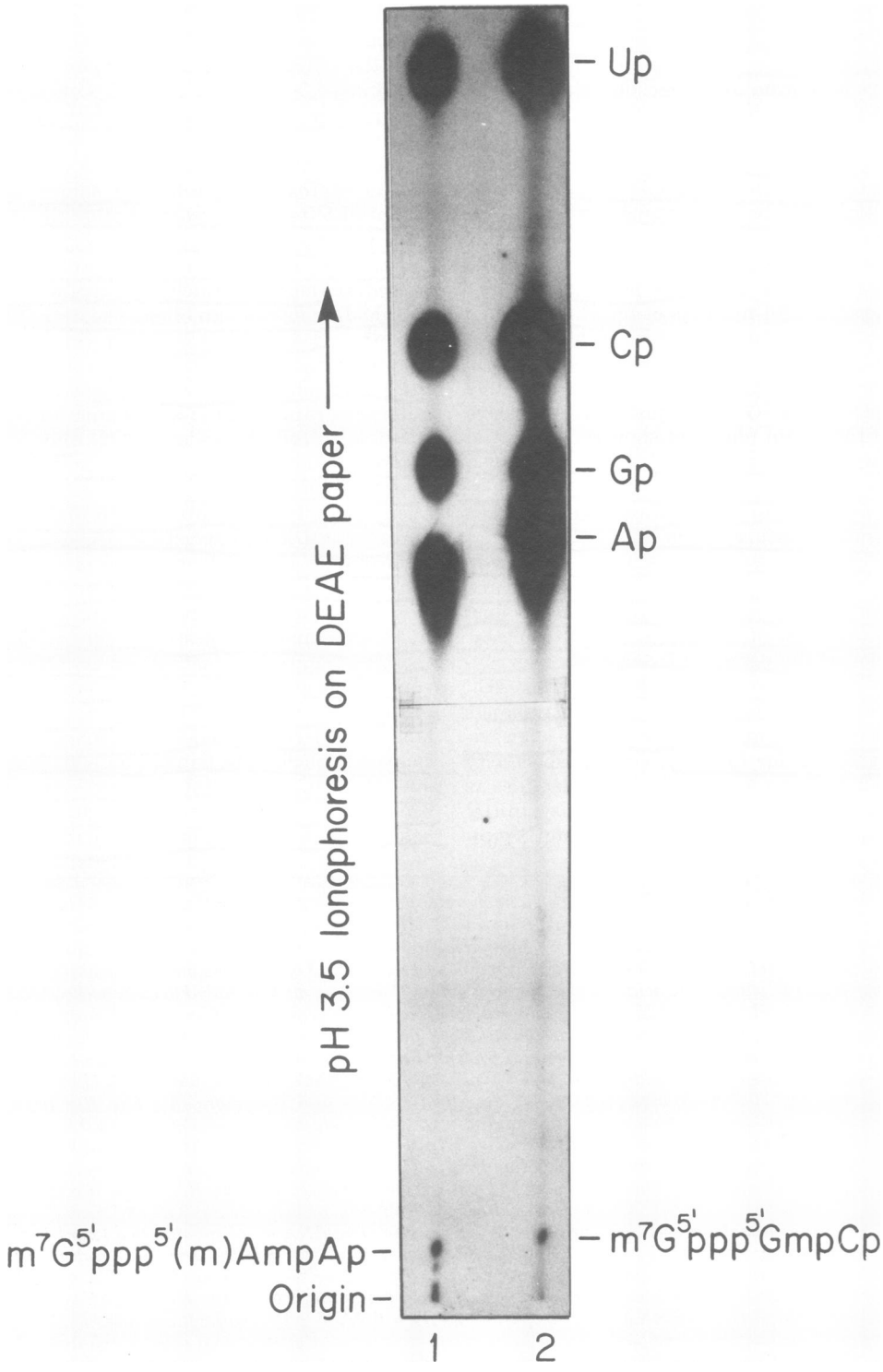
– Up
– Cp
– Gp
– Ap
pH 3.5 Ionophoresis on DEAE paper
m7G5'ppp5'(m)AmpAp –
Origin –
– m7G5'ppp5'GmpCp
1
2

FIG. 1

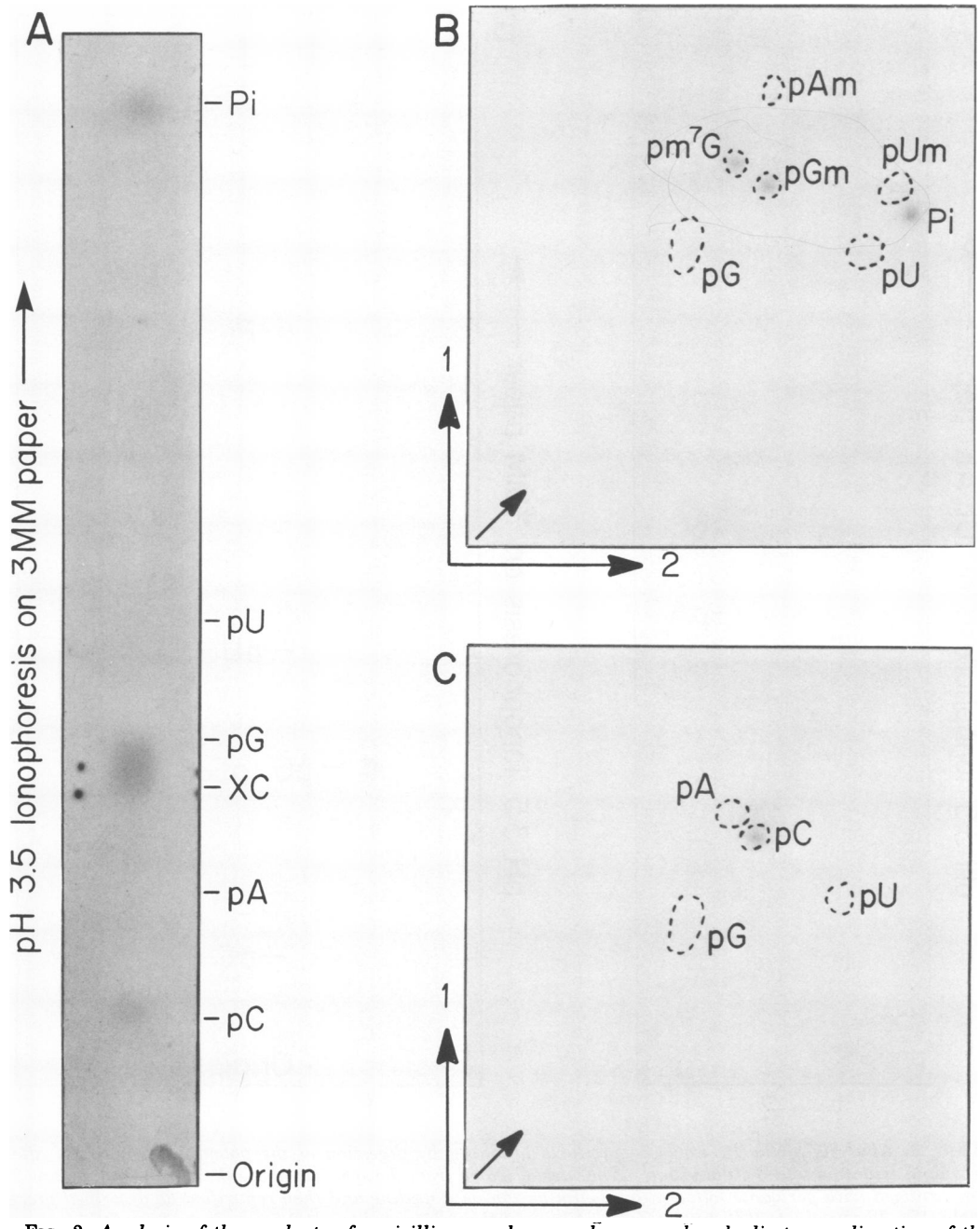

FIG. 2. *Analysis of the products of penicillium nuclease and venom phosphodiesterase digestion of the potential 5'-terminus from MuLV RNA. (A) A portion of the material eluted from the spot just above the origin (Fig. 1, lane 2) was digested with penicillium nuclease, and the products were separated by pH 3.5 ionophoresis on 3MM paper. (B) The material migrating with the blue dye xylene cyanol FF (XC) (A) was eluted from the paper with water, lyophilized, and digested with venom phosphodiesterase followed by separation of the products by two-dimensional thin-layer chromatography using a modification of the system of Nishimura (12, 14). First dimension: isobutyric acid-NH_3-water (152:10:100); 2nd dimension: t-butanol-HCl-water (70:15:15). (C) The material migrating with pC (A) was eluted and analyzed by chromatography as above. Exposures of the autoradiograms were from 1 week (A) to 4 weeks (B and C) on Kodak NS film. Enzyme digestion conditions were described elsewhere (12). Arrows indicate origins on the thin-layer plates. Dotted circles indicate positions of nucleotides that were located by UV illumination. Methylated nucleotides were purchased from P-L Biochemicals.*

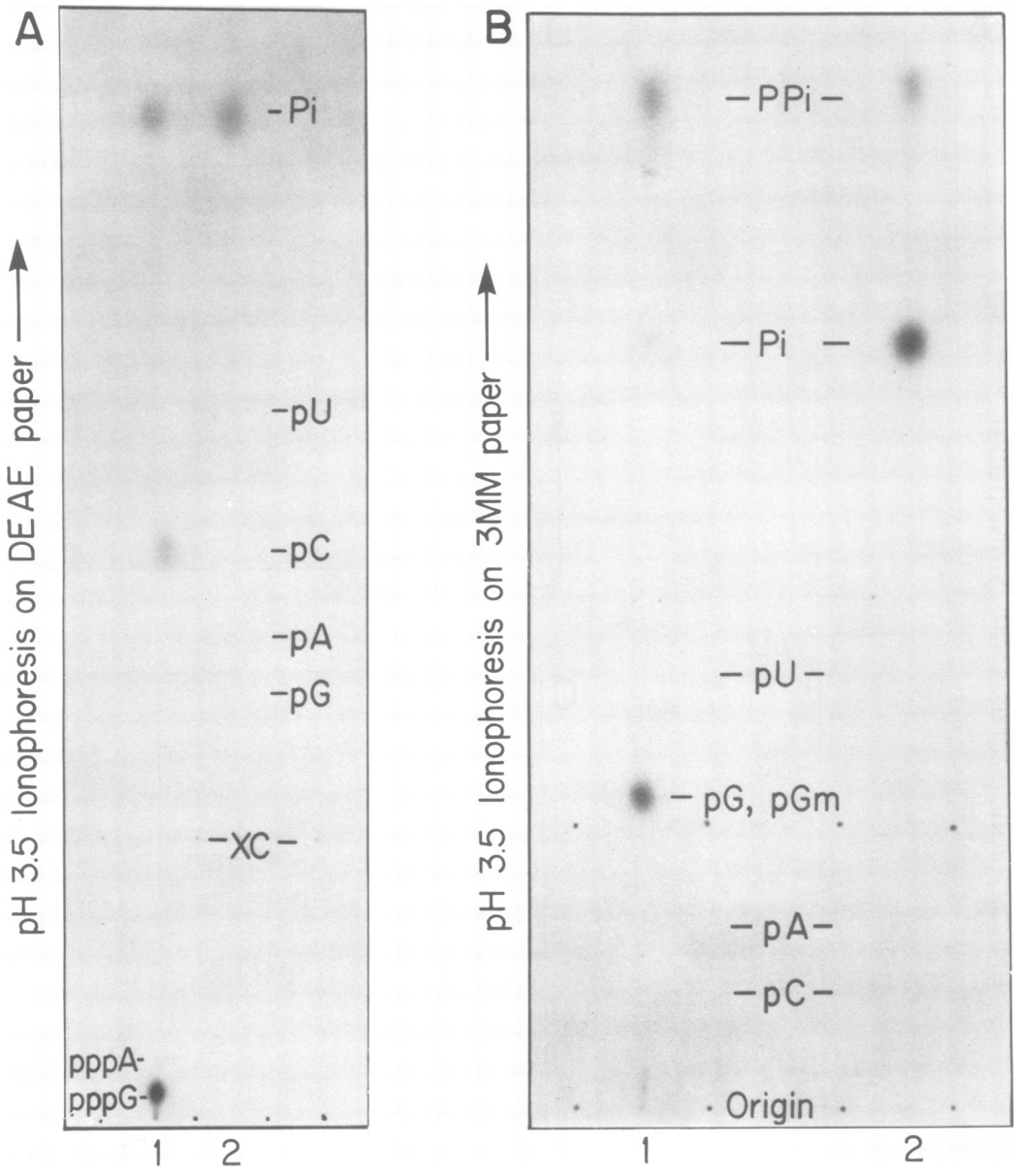

FIG. 3. *Analysis of the P1 nuclease products produced by digestion of the potential 5'-terminus after periodate oxidation and β-elimination. (A) Material eluted from the spot just above the origin (Fig. 1, lane 2) was oxidized with periodate followed by β-elimination with aniline and then repurified as described previously (12). The products of P1 nuclease digestion were then separated by pH 3.5 ionophoresis on DEAE paper (lane 1). The same material spotted in lane 1 was digested with alkaline phosphatase before pH 3.5 ionophoresis (lane 2). (B) Autoradiogram of the products produced by venom phosphodiesterase digestion of the GTP-like material from panel A, lane 1. The products were separated by pH 3.5 ionophoresis on Whatman 3MM paper (lane 1). ^{32}P-labeled markers of PP_i and P_i (lane 2). Autoradiography (15 days to 4 weeks) and location of markers was as in Fig. 2. Enzymatic digestions were performed as described previously (12).*

also confirmed by thin-layer chromatography as in Fig. 2B. Thus, the products of P1 nuclease digestion of the ^{32}P-labeled dinucleotide obtained after periodate oxidation and β-elimination are pppGm, pC, and P_i. Since P1 nuclease removes 3'-phosphates and cleaves only 5'-3'-

phosphodiester bonds, the only structure consistent with the enzyme specificity is ppp$^{5'}$GmpCp. Furthermore, because m^{7}G was not present after periodate oxidation and β-elimination, it must have been terminal with free 2'-3'-hydroxyls and therefore must have been linked by its 5'-phosphate as follows: m^{7}G$^{5'}$ppp$^{5'}$GmpCp.

The RNases used to isolate this trinucleotide from the 35S RNA leave 3'-phosphorylated termini. Since m^{7}G lacks a 3'-phosphate, it must have been located at one end of the RNA. The 5'-3'-linkages in the adjacent nucleotides indicate a 5'-terminal origin.

The yield of 0.062% for the radioactivity in this terminus relative to the total radioactivity in the RNA indicates the presence of one such terminus (five phosphates) per 8,065 nucleotides (5/8,065 = 0.062%). Since the 35S RNA subunits are approximately 9,000 nucleotides long (2), presumably all 35S RNA subunits terminate with the cap structure.

In our initial analysis of M-MuLV 35S RNA 5'-termini, we found that it was essential to avoid contamination of the 35S RNA with lower-molecular-weight RNAs derived from the 70S complex. These RNAs contribute multiple 5'-termini, including a phosphatase-sensitive terminus (presumably ppNp) that co-migrates with m^{7}G$^{5'}$ppp$^{5'}$GmpCp on pH 3.5 DEAE paper ionophoresis (Fig. 1). Thus, careful purification of 35S RNA is essential if this system is used to analyze for the 5'-terminus.

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LITERATURE CITED

1. **Barrell, B. G.** 1971. Fractionation and sequence analysis of radioactive nucleotides, p. 751-828. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research, vol. II. Harper and Row, New York.
2. **Beemon, K. L., A. J. Faras, A. T. Haase, P. H. Duesberg, and J. E. Maisel.** 1976. Genomic complexities of murine leukemia and sarcoma, reticuloendotheliosis, and visna viruses. J. Virol. 17:525-537.
3. **Fan, H., and D. Baltimore.** 1973. RNA metabolism of murine leukemia virus: detection of virus-specific RNA sequences in infected and uninfected cells and identification of virus-specific messenger RNA. J. Mol. Biol. 80:93-117.
4. **Fan, H., and M. Paskind.** 1974. Measurement of the complexity of cloned Moloney murine leukemia virus 60 to 70S RNA: evidence for a haploid genome. J. Virol. 14:421-429.
5. **Fujimoto, M., A. Kuniaka, and H. Yoshino.** 1969. Specificity of a nuclease from penicillium citrinum. Agric. Biol. Chem. 33:1517-1518.
6. **Furuichi, Y., A. J. Shatkin, E. Stavnezer, and J. M. Bishop.** 1975. Blocked, methylated 5'-terminal sequence in avian sarcoma virus RNA. Nature (London) 257:618-620.
7. **Keith, J., and H. Fraenkel-Conrat.** 1975. Identification of the 5'-end of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. 9:3347-3350.
8. **Kerr, I., U. Olshevsky, H. F. Lodish, and D. Baltimore.** 1976. Translation of murine leukemia virus RNA acid in cell-free systems from animal cells. J. Virol. 18:627-635.
9. **Hewlett, M. J., J. K. Rose, and D. Baltimore.** 1976. 5'-Terminal structure of poliovirus polyribosomal RNA is pUp. Proc. Natl. Acad. Sci. U.S.A. 73:327-330.
10. **Nomoto, A., Y. F. Lee, and E. Wimmer.** 1976. The 5'-end of poliovirus mRNA is not capped with m^{7}G$^{5'}$ppp$^{5'}$Np. Proc. Natl. Acad. Sci. U.S.A. 73:375-380.
11. **Roblin, R.** 1968. The 5'-terminus of bacteriophage R17 RNA: pppGp. J. Mol. Biol. 31:51-61.
12. **Rose, J. K.** 1975. Heterogeneous 5'-terminal structures occur on vesicular stomatitis virus mRNAs. J. Biol. Chem. 250:8098-8104.
13. **Steinschneider, A., and H. Fraenkel-Conrat.** 1966. Studies of nucleotide sequence in tobacco mosaic virus nucleic acid. IV. Use of aniline in stepwise degradation. Biochemistry 5:2735-2743.
14. **Walker, R. T., and U. L. RajBhandary.** 1975. Formylatable methionine transfer RNA from mycoplasma: purification and comparison of partial nucleotide sequences with those of other procaryotic initiator tRNAs. Nucleic Acids Res. 2:61-78.