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Absence of 5' terminal capping in encephalomyocarditis virus RNA

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ABSTRACT

The nature of the 5' terminus of encephalomyocarditis (EMC) virion RNA has been investigated. We have failed to detect any capped products or nucleoside polyphosphates arising upon complete digestion of the RNA with T_1, T_2 and pancreatic ribonucleases, and it would therefore appear that the 5' terminus of EMC virus RNA is not phosphorylated and not capped with m^7G.

EMC virions do contain, however, large amounts of all four 5'-mono-substituted nucleoside triphosphates (4.2M pppG; 16.4M pppA; 3.0M pppU and 2.5M pppC), of which at least a proportion (about 15-20%) appear to remain bound to fully denatured RNA in the presence of divalent cations.

INTRODUCTION

Messenger RNA (mRNA) molecules of eukaryotic cells and their viruses have been shown to contain m^7G_5,ppp_5,NmpNp, or related structures at their 5' termini[1-14]. It has been shown that the introduction of the m^7G cap is associated with the initiation of transcription in two viruses containing double-stranded segmented RNA genomes, namely reovirus and cytoplasmic polyhedrosis virus (CPV)[4,5]. Furthermore, the presence of a 5' terminal m^7G is important for the efficient translation of mRNA [e.g. mRNAs from reovirus and vesicular stomatitis virus (VSV)] and is required for binding of reovirus mRNA to 40S or 80S ribosomes[6,15,16,17]. The finding that chemical removal of m^7G from globin mRNA prevented its translation led to the proposal that the presence of this nucleotide at the 5' terminus might be required universally for the efficient translation of mRNAs in eukaryotic cells[6].

We have examined the RNA of encephalomyocarditis (EMC) virus for the presence of a 5' terminal 'cap'. EMC virus is a member of the picornavirus group, and contains a single plus-stranded, polyadenylated genome, of molecular weight $2.5\text{-}2.7 \times 10^6$, which acts as mRNA during the initiation of infection[18]. We have failed to detect any cap, or any nucleoside di-, tri-, or tetra-phosphate arising upon complete nuclease digestion of EMC virus RNA.

We therefore infer that its 5' terminus is a non-capped, non-phosphorylated nucleoside. However a possible 5'-terminal linkage to material other than a nucleotide cannot be entirely excluded.

MATERIALS AND METHODS

Virus RNA

^{32}P-labelled EMC virus RNA was prepared from EMC virus grown in Krebs ascites tumour cells in the presence of ^{32}P-orthophosphate, according to Porter *et al.*[19].

[^{32}P-] EMC virus RNA, extracted by the SDS-phenol-chloroform procedure, was routinely purified by centrifugation on an 11 ml 10.2-22.4% (w/w) isokinetic sucrose gradient in PBS-A for 3.5 h at 36,000 rpm at 4°C in the SW41 rotor of the Beckman L265-B ultracentrifuge. In later experiments, to remove putative contaminating nucleotides either linked to the RNA via divalent cations or by hydrogen bonding, [^{32}P-] EMC virus RNA was centrifuged on a 5 ml 10-56.3% (w/w) isokinetic sucrose gradient prepared in 10mM EDTA/PBSA for 2 h at 65,000 rpm at 4°C in the SW65 Ti rotor of the Beckman L265-B ultracentrifuge or on an 11 ml 5-20% w/v linear sucrose gradient prepared in barbital buffered, 98% formamide pH 9.0[34]. Peak fractions were pooled, dialyzed against 20mM Tris pH 7.5 and the RNA finally precipitated with 2 volumes of ethanol at -20°C in the presence of 0.2M Na acetate.

Enzymatic digestions

10-30 x 10^6 cpm of [^{32}P-] EMC virus RNA (50-100 µg, including carrier tRNA) was digested for 6 h at 37°C with 20 units T_2 RNase (Sankyo), 7 µg T_1 RNase (Sankyo) and 7 µg RNase A (Worthington) in 30 µl of buffer (0.015M ammonium acetate, pH 4.5; 0.002M EDTA) according to Adams and Cory[3]. The digest was dried *in vacuo*, resuspended in 5 µl water and subjected to high voltage electrophoresis on cellulose acetate (Schleicher and Schüll Selectron) at pH 3.5 in 7M urea, 5% acetic acid, 0.5% pyridine and 2mM EDTA at 5Kv (Gilson Electrophorator) for ~4 h. The cellulose acetate strip was then removed and the region from the origin to beyond the orange G marker blotted through onto two sheets of DEAE paper (Whatman # DE81, both 46 x 90 cm) using 3MM paper wetted with distilled water and glass pressure plates. After the origin of the DEAE sheets had been washed with 70% ethanol (to remove the urea) and dried, the second dimension was electrophoresed at 1.1Kv in 5% acetic acid and 0.5% pyridine (pH 3.5) for ~15 h[3,11].

Products possibly arising from the 5' terminus of the RNA were further analysed under the following conditions:

(a) Bacterial alkaline phosphatase (BAPF, electrophoretically purified grade, Worthington) digestion was carried out for 30 min at 37°C, using 0.5 unit/ml enzyme 7-20 µl 0.01M Tris-HCl, pH 7.5 in the presence of 20 µg tRNA carrier.

(b) Nucleotide pyrophosphatase (Sigma) digestion was performed for 1 h at 37°C with 1 unit/ml enzyme in 20 µl 0.05M Tris-HCl pH 7.5, 0.01M $MgCl_2$ in the presence of 10 µg nicotinamide adenine dinucleotide (NAD) carrier.

(c) Snake venom phosphodiesterase (Worthington) digestion was carried out for various periods of time (up to 2 h) at 37°C in 20 µl of 0.01M Tris-HCl, pH 8.9, 0.01M $MgCl_2$ at an enzyme concentration of 0.1 mg/ml.

(d) Acid stability was determined by dissolving nucleotides (100,000 cpm) in 10 µl 1M HCl and heating for 8 min at 100°C.

(e) Base stability was examined by dissolving the nucleotides (100,000 cpm) in 20 µl of 0.3M NaOH and incubating overnight at 37°C.

In all cases the nucleotides under examination were previously eluted from DEAE paper with 30% triethylammonium bicarbonate, pH 10.0. The eluant was evaporated to dryness and excess triethylamine removed by evaporation of water; the product was stored at -20°C. It must be noted that a small amount of hydrolysis does occur on drying the electrophoresis paper and/or eluting the nucleotides. Products resulting from the above digestions were fractionated either on DEAE or Whatman # 52 paper at pH 3.5[20] as described in the Figure legends.

Comparison of nucleoside polyphosphates with standards

The unknown nucleotides were co-electrophoresed with authentic marker nucleoside tri- and tetra-phosphates on DEAE paper at pH 3.5 as described in the Figure legends.

Chromatography of mononucleotides with authentic markers was carried out on Whatman # 1 paper using isopropanol 70 : concentrated ammonia 1 : water 29 as the developing system.

The markers adenosine-5'-phosphate, guanosine-5'-phosphate, adenosine-5'-tetraphosphate and guanosine-5'-tetraphosphate were purchased from Sigma. The nucleoside triphosphates were purchased from P-L Biochemicals Inc.

RESULTS

The combined T_1, T_2 and pancreatic RNase digestion of EMC virus RNA would be expected to cleave it entirely into its constituent nucleoside-3'-monophosphates (Np), except for a product arising from its 5'-terminus, which might be a nucleoside di-, tri- or tetraphosphate (pNp, ppNp or

pppNp) or a capped and methylated structure of the type m^7G_5,ppp_5,NmpNp. Furthermore, 2' ribose methylations would be detected as di- or oligonucleotides [NmpNp or Nmp(Nmp)$_n$Np] in combined digests of the RNA since the phosphodiester bond adjacent to a 2'-O-methyl group is resistant to the RNases used[3]. Base methylations and other modified mononucleotides would be detected by their characteristic mobility in this two-dimensional electrophoresis system.

A fingerprint of such a combined RNase digestion of [^{32}P-] EMC virus RNA is shown in Figure 1. It may be seen that there are one major and four minor spots in addition to the four principal 3' mononucleotides and their 2',3'- cyclic intermediates. The position of these spots on the fingerprint suggests that all the products contain at least three phosphates. The mobilities of the spots in the first dimension would appear too great to correspond to any of the capped oligonucleotides described by Adams & Cory[3].

Initially, the identity of the four unknown nucleotides, obtained by combined RNase digestion of [^{32}P-] EMC virus RNA prepared using sucrose gradients in PBS-A alone, were investigated by the following analyses:

(a) Bacterial alkaline phosphatase treatment of products [designated spots 1-4 (Figure 1)] arising from four independent RNA preparations and subsequent fractionation on DEAE paper (pH 3.5) gave rise to free phosphate alone in all cases, indicating that all of the products are mononucleoside polyphosphates, and that there are no internal phosphates (Figure 2, BAPF, lanes 1,2,3 and 4). BAPF digestion of the material in spot 5 resulted in the material remaining on the origin of the paper after the electrophoresis at pH 3.5 (data not shown) indicating that the material was not nucleic acid or, alternatively, that it was protected in some fashion from ribonuclease action even though the RNA had originally been extracted three times with phenol-chloroform-SDS. The fact that this material was not present in combined RNase digests of EMC virus RNA that had been prepared in the presence of 10mM EDTA (vide infra) suggests that the material is a contaminant. Digestion of marker oligonucleotides under the same conditions released only the terminal phosphate.

(b) Treatment of spots 1 and 2 with nucleotide pyrophosphatase (nPPase) released pyrophosphate (PPi), phosphate (Pi) and pG or pA respectively, (Figure 2, nPPase lanes 2 and 3). Treatment of spot 3 released PPi, Pi and pU (data not shown). Spot 4 was not appreciably digested. The pA and pG released by digestion were co-chromatographed with authentic

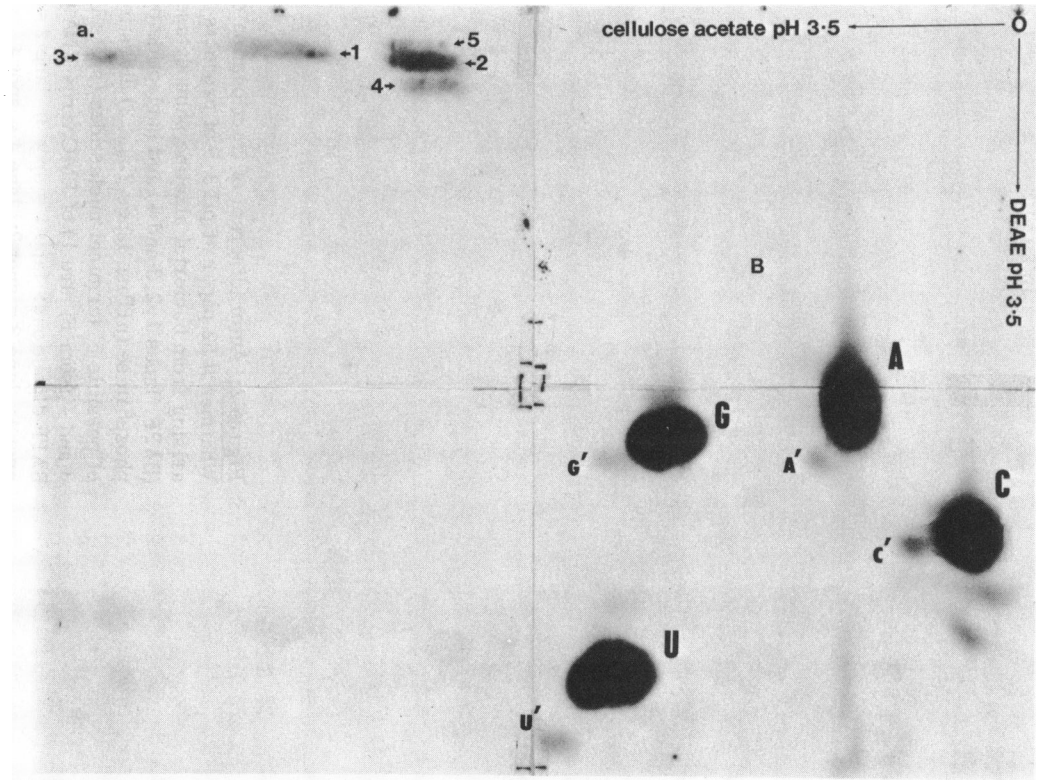

Figure 1 : Autoradiogram of two-dimensional fractionation of a total T_1, T_2 and pancreatic RNase digestion of PBS-A/ sucrose gradient purified EMC virus RNA. First dimension on cellulose acetate pH 3.5, second dimension DEAE paper pH 3.5. Further details as described in Materials and Methods.

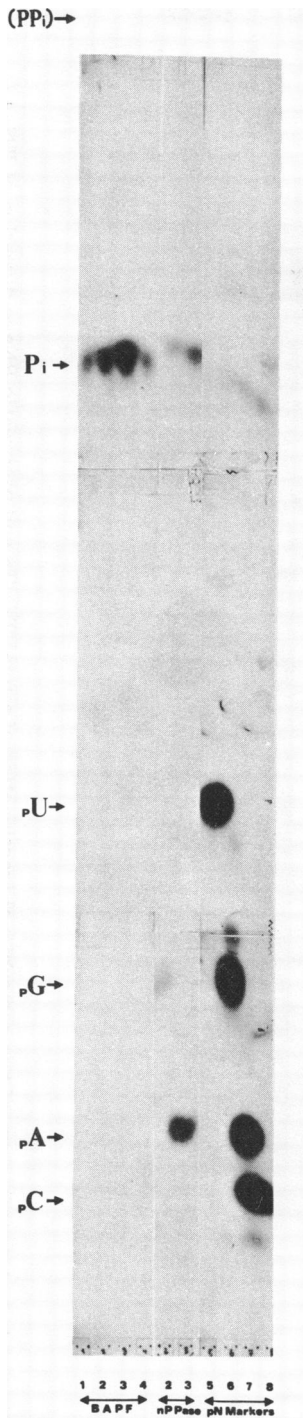

Figure 2: Autoradiogram of fractionation on Whatman #52 paper at pH 3·5 of products arising from bacterial alkaline phosphatase (BAPF, lanes 1,2,3 and 4) and nucleotide pyrophosphatase (nPPase lanes 2 and 3) digestion of possible 5' terminal nucleotides (spots 1,2, 3 and 4 from Figure 1) of EMC virus RNA. BAPF digestions: lane 1 = spot 3; lane 2 = spot 1; lane 3 = spot 2; lane 4 = spot 4. nPPase digestions lane 2 = spot 1; lane 3 = spot 2; lanes 5,6,7 and 8 are pU, pG, pA and pC markers respectively. In the above example, pyrophosphate (PPi) was electrophoresed off the paper, although its theoretical position is marked.

markers in the isopropanol-NH_3 system (see Methods) to determine whether they might be pm^6A or pm^2G, or other methylated derivatives. Their chromatographic properties, however, were identical with the marker spots (data not shown). This finding, together with their electrophoretic behaviour, indicates that they are unlikely to be methylated.

(c) Digestion with snake venom phosphodiesterase released some pyrophosphate, free phosphate and pG, pA and pU from spots 1,2 and 3 respectively, as indicated by electrophoresis of the products on Whatman # 52 paper (Figure 3) or DEAE paper (data not shown). The mononucleotides arose in lower yields than those from the nucleotide pyrophosphatase digestion, possibly due to the phosphatase activity certainly present in the venom phosphodiesterase. Spot 4 was slightly digested by the venom phosphodiesterase preparation yielding some pC and free phosphate.

The results of treatment of products from three independent preparations with nucleotide pyrophosphatase and venom phosphodiesterase are also consistent with them being 5'-mononucleotide polyphosphates, and exclude the presence of inverted m^7G caps in any of them. However, similar analyses on the product obtained after combined RNase digestion of Semliki Forest virus RNA revealed the presence of a capped structure containing m^7G (P.F. and S.I. Kennedy, unpublished results).

(d) All four nucleotides (spots 1-4) were found to be stable to digestion with alkali, eliminating structures like pNpp, Nppp and pNppp or 'magic-spot' like structures such as ppNpp; their positions on DEAE paper after electrophoresis at pH 3.5 remaining unchanged (Figure 4, lanes 1,2,3 and 4).

(e) When spots 1,2,3 and 4 were subjected to treatment with 1M HCl and subsequently analysed on DEAE paper at pH 3.5 (Figure 4, lanes 5,6,7 and 8), free phosphate and ribose phosphate, pU and pC respectively, were released; the ribose phosphate in lanes 5 and 6 is due to depurination of pA and pG by the acid. This confirms that the original nucleotides were all 5' mono-substituted nucleoside polyphosphates (pppN or possibly ppppN). If any of the nucleotides contained a 3' phosphate then upon either venom phosphodiesterase or acid treatment nucleoside diphosphates (pNp) would have been released which would have been easily identified.

The molar yields and subsequent identities (obtained by the analyses

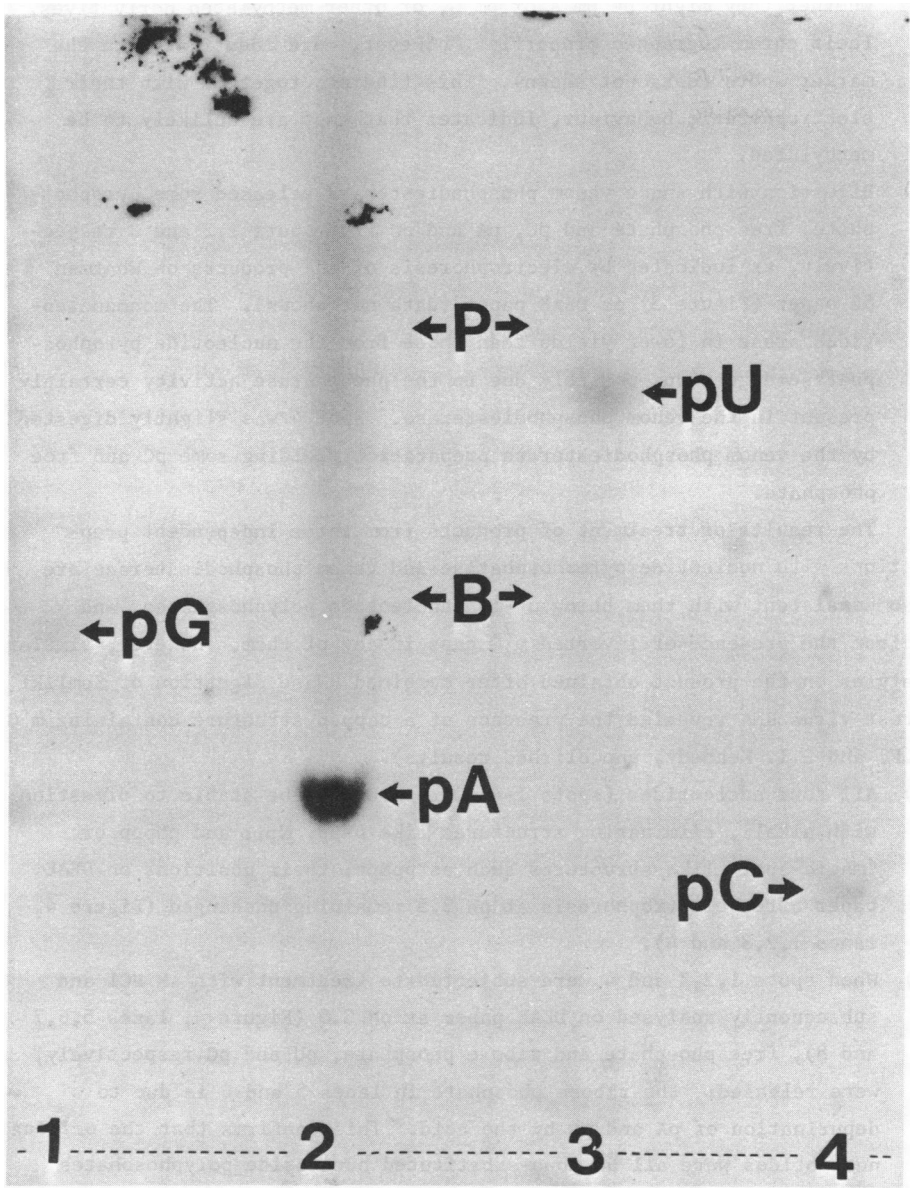

Figure 3: Autoradiogram of fractionation on Whatman # 52 paper at pH 3.5 of snake venom phosphodiesterase digestion of possible 5' terminal nucleotides (spots 1,2,3 and 4 from Figure 1 or Figure 6). The position of the bromophenol blue marker is indicated (B).

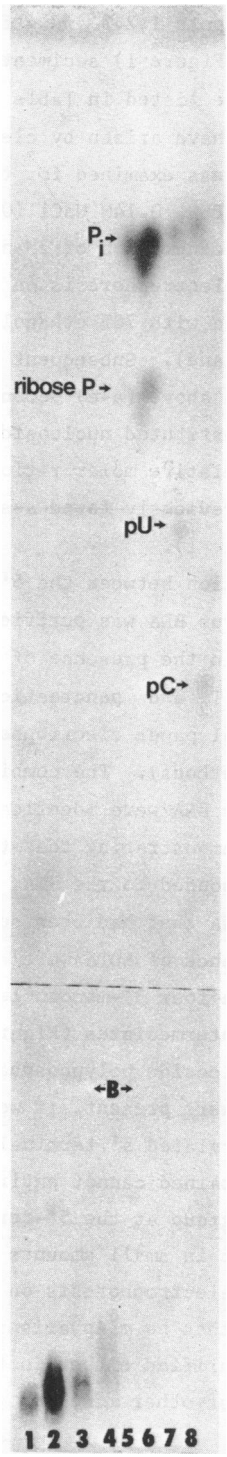

Figure 4: Autoradiogram of fractionation, on DEAE paper at pH 3·5, of products arising from alkali digestion (lanes 1,2,3 and 4) or acid treatment (lanes 5,6,7 and 8) of possible 5' terminal nucleotides (spots 1,2,3 and 4 from Figure 1 or Figure 6). (Spot 1, lanes 1 and 5; spot 2, lanes 2 and 6; spot 3, lanes 3 and 7; spot 4, lanes 4 and 8). The position of the bromophenol blue (B) is indicated.

referred to above) of the four unknown nucleotides (spots 1,2,3 and 4 from a two-dimensional fingerprint such as illustrated in Figure 1) sedimenting with PBS-A sucrose-gradient purified EMC virus RNA are listed in Table 1.

As these four 5'-nucleotides could not possibly have arisen by cleavage from intact EMC virus RNA, purified [^{32}P-] EMC virus was examined for their presence. [^{32}P-] EMC virus (15 x 10^6 cpm) in 10mM HEPES, 0.14M NaCl (0.66 ml) was adjusted to pH 6.2, urea added to a final concentration of 2M and the solution incubated for 20-30 min at 37°C. Upon electrophoresis on DEAE paper at pH 3.5 (after washing the origin free of urea with 70% ethanol) four major products were observed (Figure 7, centre lane). Subsequent analysis of these products under the conditions given above (a-e) demonstrated that these products were the four 5' mono-substituted nucleoside polyphosphates (pppN or ppppN) present in the same relative molar ratios, but in sixfold excess over the same 5'-nucleotides previously found associated with phenol-chloroform-SDS extracted RNA (Table 1).

In order to determine the nature of the association between the 5' nucleoside polyphosphates and the RNA, [^{32}P-] EMC virus RNA was purified on formamide-sucrose gradients or on sucrose gradients in the presence of 10mM EDTA, subjected to combined RNase digestion with T_1, T_2 and pancreatic nucleases and the products analysed by two-dimensional paper electrophoresis on cellulose acetate and DEAE paper at pH 3.5 (see Methods). The combined RNase digestion products of fully denatured EMC virus RNA were identical to those derived from native EMC virus RNA (Figure 1) demonstrating that the four 5' nucleoside polyphosphates were not hydrogen-bonded to the RNA. When the combined RNase digestion products of EMC virus RNA that had been subjected to sucrose gradient centrifugation in the presence of EDTA were examined, however, the only products present were the four 3'-mononucleotides Ap, Cp, Up and Gp and their 2' and 3' cyclic intermediates (Figure 5). As no other products (such as capped structures, nucleoside polyphosphates or 2'-O-methyl containing di- or oligo nucleotides) were present, it would appear that EMC virus RNA has an uncapped, unphosphorylated 5' terminal nucleoside. However, the results which have been obtained cannot entirely exclude the presence of some other type of blocking group at the 5'-terminus.

The methylated nucleotide m^6Ap, which is present in small amounts in many eukaryotic mRNAs, is well-separated from Ap by electrophoresis on DEAE-paper at pH 3.5[3]. It may be seen (Figures 1 and 5) that no m^6Ap arises from EMC virus RNA upon RNase digestion, using RNA purified either in the presence or absence of EDTA. However, in the cases of other modified

TABLE 1

nucleotide		molar abundance* isolated from RNA	isolated from virus
(1)	pppG	2.0	4.2
(2)	pppA	3.9	16.4
(3)	pppU	1.1	3.0
(4)	pppC	1.3	2.5

*expressed in moles/mole RNA, based on a chain length of 8000 nucleotides for EMC virus RNA; the values given are the mean of two determinations

nucleotides, where the modification has occurred on the base moiety, these might not have been fractionated from the four main nucleotides by the procedures used, and we therefore cannot exclude the possibility of their occurrence at low frequency in EMC virus RNA.

In later experiments, to determine the structure of the four 5' nucleoside polyphosphates, work was carried out on the ethanol supernatant obtained after phenol-chloroform-SDS extraction. The same nucleoside polyphosphates are isolated by this method with the advantage that it is less wasteful of viral RNA. The ethanol supernatants containing the nucleoside polyphosphates were filtered through DEAE paper discs and washed with water, ethanol and then air dried. The nucleotides were eluted with 30% triethylammonium bicarbonate pH 10.0 and washed as described in Methods. An autoradiograph of the four nucleotides isolated in this manner and fractionated in one dimension on DEAE paper at pH 3.5 is shown in Figure 6.

The electrophoretic mobilities of spots 1,2,3 and 4 on both the two-dimensional and one-dimensional fingerprints are consistent with them being 5' nucleoside tri- or tetra-phosphates. To distinguish between these two possibilities, UV-absorbing quantities of 5' tetraphosphate and 5' triphosphate markers were co-electrophoresed on DEAE paper, pH 3.5, with the unknown nucleotides. 'Magic-spot' (ppGpp), and its analogue ppApp, were also included as markers but neither co-migrated with spots 1 and 2 respectively (M.E. and P.F., unpublished results). It was found, however, that spots 1,2,3 and 4 co-migrated with authentic pppG, pppA, pppU and pppC markers respectively (Figure 7).

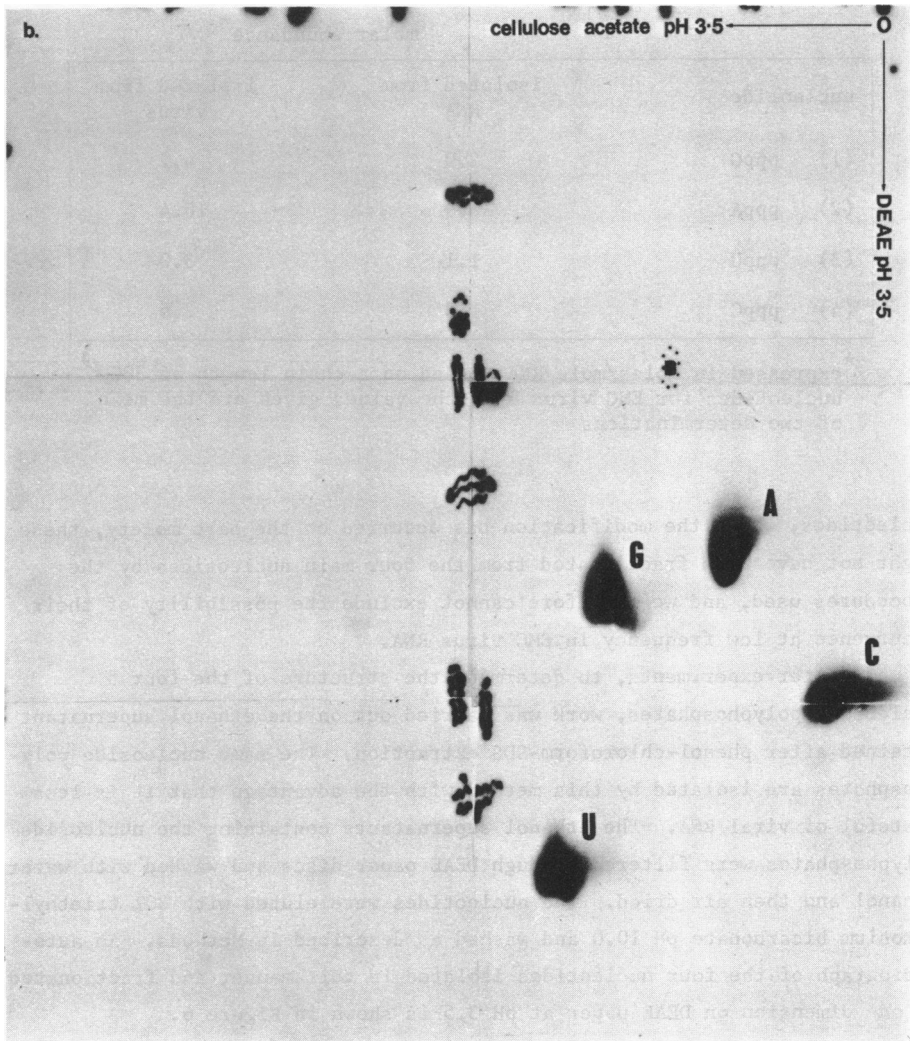

Figure 5: Autoradiogram of two-dimensional fractionation of a total T_1, T_2 and pancreatic RNase digestion of PBS-A/10mM EDTA-sucrose gradient purified EMC virus RNA. First dimension on cellulose acetate pH 3.5, second dimension DEAE paper, pH 3.5. Further details as described in Materials and Methods.

DISCUSSION

Our results indicate that EMC virus RNA, which acts as a plus-stranded messenger RNA in the infected cell, does not contain a blocked, methylated

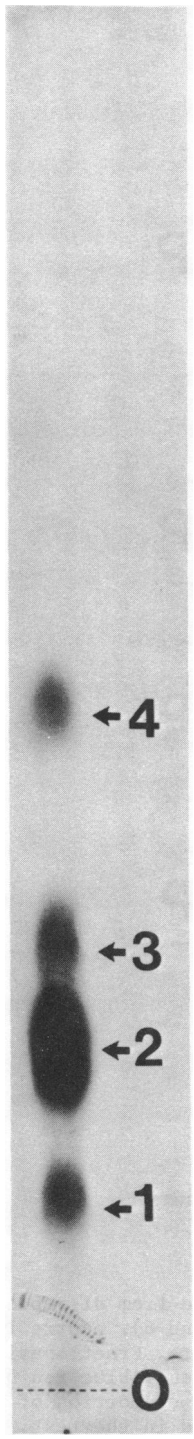

Figure 6: Autoradiogram of the fractionation on DEAE paper, pH 3·5, of the ethanol supernatant obtained after phenol-chloroform-SDS extraction of EMC virus RNA isolated from a PBS-A sucrose gradient. Further details are described in the text.

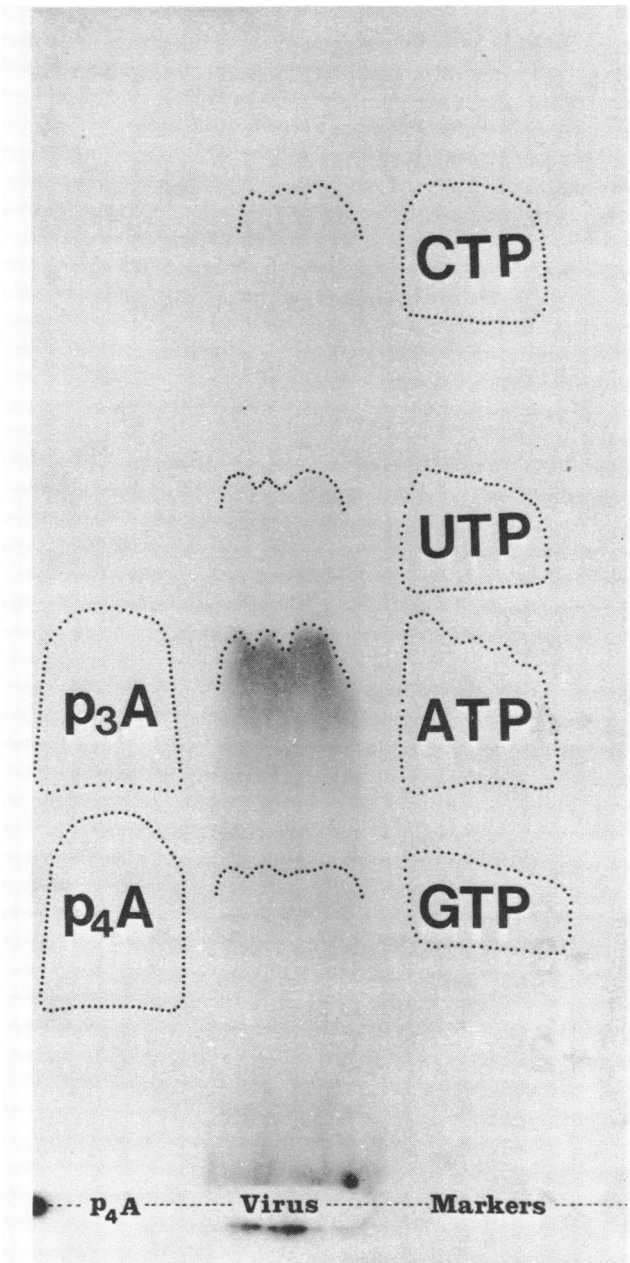

Figure 7: Co-migration of unknown nucleotides derived from disrupted EMC virions (equivalent to spots 1,2,3 and 4, Figures 1 and 6), centre lane, with authentic pppG, pppA, pppU, pppC and ppppA markers. Fractionation was on DEAE paper at pH 3.5. In this example the bromophenol blue ran 52 cm and the orange G 85.5 cm from the origin, only the lower portion of the autoradiogram is shown. Further details are described in the text.

5'- terminal structure of the type $m^7G_5,ppp_5,NmpNp$ as found at the 5' terminus of the mRNA of many eukaryotic cells and viruses[10]. It is also of interest to note that this picornavirus RNA contains no N^6-methyladenylic acid (m^6Ap), a modified nucleoside in poly(A)-containing RNA from eukaryotic cells[2,3,21,22,23,24] and in avian sarcoma virus genome RNA[25]. This has previously been suggested to occur in cellular mRNAs and mRNAs from viruses replicating in the nucleus, but not in cytoplasmic viral mRNAs[2], and our failure to detect it would be consistent with this. The four standard ribose methylated nucleotides were also absent.

The absence of a capping group from EMC virion RNA would suggest that capping and methylation might not be an obligate requirement for translation of eukaryotic mRNAs of either cellular or viral origin as suggested by various workers[6,15]. As emphasized by previous workers[26], however, lack of the capping group (7-methylguanosine in 5',5'-pyrophosphate linkage) from the 5' terminus of a picornavirus virion RNA does not exclude the necessity of $m^7G_5,ppp_5,NmpNp$ for picornavirus protein synthesis as viral mRNA in polyribosomes does not appear to become encapsidated[18], and since certain cell-free extracts have been found capable of re-capping non-methylated mRNAs[6,15]. Recently both Nomoto et al.[26] and Hewlett et al.[27] have reported that the capping group is absent from poliovirus mRNA isolated from infected HeLa cell polyribosomes in the absence of sparsomycin as well as absent from poliovirion RNA as previously reported[28]. Furthermore, it has been shown that poliovirion RNA is not labelled, in a wheat germ cell-free extract, with [^{3}H]-methyl in the presence of S-[methyl-^{3}H] adenosylmethionine and that the addition of S-adenosylmethionine or S-adenosylhomocysteine did not alter poliovirion RNA stimulated [^{3}H-] leucine incorporation in this system[26]. As found for poliovirion RNA[29], we have failed, thus far, to label EMC virus RNA (in Krebs ascites cells in vivo) with [^{3}H-methyl] methionine (D.F. and P.F unpublished).

Recently, it has been found, both in the wheat germ cell-free system[30,31] and in a fractionated, reticulocyte cell-free system[32], that 'cap' analogues such as 7-methylguanosine-5'-monophosphate (m^7G_5,p) and synthetic 'caps' like m^7G_5,ppp_5,Gm inhibit initiation of translation of several 'capped' eukaryotic mRNAs. Translation of non-capped messengers such as satellite necrosis virus (STNV) RNA[30], EMC virus RNA[31,32], SV40 cRNA[31] or coliphage T4 mRNA[31], however, was significantly less inhibited by 'cap' analogues or synthetic 'caps' in these cell-free systems. Interestingly, this lack of inhibition by small 'cap' analogues like

$m^7G_{5'}p$ and $m^7G_{5'}ppp$ was also demonstrated in L-cell extracts where globin mRNA translation was inhibited but Mengovirus (which is closely related to the cardiovirus EMC virus) RNA was even stimulated; a complete 'cap' ($m^7G_{5'}ppp_{5'}Gm$), however, inhibited the translation of both Mengovirus and globin RNA[31]. Furthermore, Filipowicz et al.[33] have isolated a cap binding protein (CBP) from ribosomal salt washes of Artemia salina embryos that binds m^7GpppGpC and m^7GpppGmpC in a nitrocellulose membrane filter assay. It was found that capped mRNAs (e.g. globin and reovirus mRNAs) were strong competitors of cap binding whereas ribosomal, STNV, unmethylated reovirus and EMC virus RNAs did not compete with synthetic caps for CBP. Taken together, these results would strongly suggest that a capped and methylated 5' terminal structure containing m^7G is not required for picornavirus mRNA translation. Although it is reasonable to suppose that lack of a 5' terminal cap in picornavirus mRNAs might, in some way, be connected with the ability of picornaviruses to inhibit host cell protein synthesis, this does not seem to be mediated by an alteration of the size or the 'cap' of host cell mRNA after infection[26]. However, it is possible that a virus-specific protein could induce changes in initiation factors, for example, IF-M3 which in the uninfected cell has a high affinity for 'capped' mRNAs[32], so that they have a higher affinity for uncapped than capped mRNAs. Alternatively, there might be some other interesting structural feature present in picornavirus RNA that is responsible for selective binding by ribosomes or initiation factors over host cell mRNAs.

It is clear that capping and methylation cannot be coupled to transcription of EMC virus genomic RNA from a hypothetical double-stranded replicative form, and this differs from the situation found with the double-stranded segmented RNA viruses.

The biological significance of the 5' nucleoside triphosphates found within EMC virions and associated with EMC virus RNA, if any, is unknown.

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