The EMBO Journal vol.3 no.11 pp.2593-2598, 1984

# Encephalomyocarditis virus RNA synthesis *in vitro* is protein-primed

A.B.Vartapetian[1], E.V.Koonin[2], V.I.Agol[1,2] and A.A.Bogdanov[1]

[1]A.N.Belozersky Laboratory of Bioorganic Chemistry and Molecular Biology, Moscow State University, Moscow 119899, and [2]Institute of Poliomyelitis and Viral Encephalitides of the USSR Academy of Medical Sciences, Moscow Region, 142782, USSR

Communicated by L.Kääriäinen

**A crude membrane-bound replication complex isolated from encephalomyocarditis virus-infected cells is able to initiate the synthesis of viral RNA. Both the formation of the primer, VPg-pU, and its utilization for the initiation of RNA chains take place in this system. A significant amount of the synthesized VPg-pU is found in the free form. The predominant product of the *de novo* initiation is represented by short phenol-soluble VPg oligonucleotide species, and only a small percentage of the latter appear to be elongated into longer RNA chains.**

*Key words:* picornaviruses/protein priming/RNA synthesis/VPg

## Introduction

The RNA genome of a variety of animal and plant viruses can be isolated from the virions as a covalent complex with a virus-specific polypeptide attached to the 5'-terminal nucleotide; this polypeptide is usually referred to as VPg (for viral protein genome-linked) (for reviews, see Wimmer, 1979, 1982; Vartapetian, 1982). Clues as to the possible VPg function(s) came largely from studies on picornaviruses. The finding of VPg association with nascent strands of intracellular replicative intermediate (RI) RNA of poliovirus led to the suggestion that the polypeptide is involved in the initiation of virus-specific RNA synthesis, probably as a primer (Flanegan *et al.*, 1977; Nomoto *et al.*, 1977; Pettersson *et al.*, 1978).

To elucidate the actual role and the mechanism of the VPg participation in the replication of the viral genome, cell-free systems are evidently required. Two kinds of such systems are currently available. The first is exemplified by purified preparations of the poliovirus replicase supplemented with the viral RNA template and a host cell-derived protein ('host factor'). The synthesis of complementary RNA species by such preparations was shown to be severely inhibited by antibodies raised against poliovirus VPg or synthetic VPg oligopeptide fragments; moreover, a part of the product synthesized by the uninhibited system could be immunoprecipitated by these antibodies. The antibodies exert, however, no appreciable effect on RNA synthesis if the system contained exogenously added oligo(U) primer, indicating that the elongation of polynucleotide chains was not impaired (Baron and Baltimore, 1982; Morrow and Dasgupta, 1983). These findings may be interpreted to support the notion that VPg is an essential component of the initiation machinery. Nevertheless, the purified system has not yet provided any direct information on this point, since (i) VPg or VPg-containing polypeptides were present in the system only as unidentified contaminants, and (ii) the synthesized RNA product appeared to be attached to unidentified, relatively large proteins antigenically related to VPg rather than to *bona fide* VPg.

The other cell-free system suitable for investigating VPg functions is a crude, membrane-associated preparation of virus-specific replication complexes (RC), which can be isolated from virus-infected cells. These RC contain an apparently complete set of enzymes, 'factors' and templates required for efficient RNA synthesis. Recently, we described the formation, by encephalomyocarditis (EMC) virus RC, of short oligonucleotides covalently linked to VPg (Vartapetian *et al.*, 1982). Similar results were also obtained with the poliovirus RC (Takegami *et al.*, 1983). Such VPg-oligonucleotide complexes may be regarded as very early products of elongation of RNA strands initiated with the participation of VPg. These findings encouraged us to undertake a more detailed study of the initiation of EMC virus RNA synthesis in this cell-free system.

In the case of picornaviruses, the initiation reactions may be assumed to include two major steps. The first consists of the formation of a primer, VPg-mononucleotide complex. The second step, priming or the initiation proper, is the formation of a phosphodiester bond between the nucleotide moiety of the primer and the nucleotide destined to be the 5' penultimate nucleotide of the RNA chain. Here we show that both these postulated steps, the formation of the primer, VPg-pU, and its utilization for the initiation of RNA chains, are accomplished *in vitro* by the EMC virus RC preparations.

## Results

### *General properties of the cell-free system*

A crude membrane fraction from EMC virus-infected Krebs-2 cells obtained by sedimenting the post-nuclear supernatant at 30 000 *g* for 30 min contained the bulk of the virus-specific RNA-synthesizing activity. Using endogenous RNA as a template, such membrane-bound RC preparations promote extensive synthesis of heteropolymeric RNA species so that the incorporation of label into RNA in the presence of [$^3$H]UTP and the three other unlabelled nucleoside triphosphates (NTP) exceeded that in the presence of [$^3$H]UTP alone at least 10-fold. Sucrose gradient sedimentation of the synthesized products revealed the RNA species typical for picornaviruses, i.e., single-stranded genome-length 35 S RNA and smaller amounts of fully double-stranded replicative form (RF) 20 S RNA and heterogeneous partially double-stranded RI RNA (cf. Dmitrieva and Agol, 1974). The major denatured product of the RC was represented by RNA species with an electrophoretic mobility identical to that of the genome-sized single-stranded EMC virus RNA; in addition a set of incomplete chains was generated (Figure 1A). Most of the newly synthesized RNA hybridized to an excess of EMC virus double-stranded RF RNA, while hybridization with the virion RNA was negligible (Figure 1B). Thus, the major

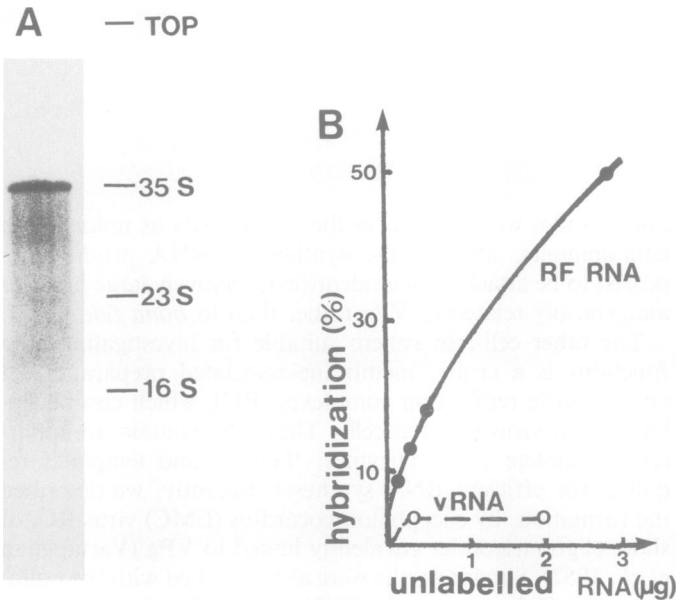


**Fig. 1.** Genome-length EMC virus RNA of positive polarity is synthesized *in vitro*. **(A)** Agarose gel electrophoresis under completely denaturing conditions of the [α-$^{32}$P]UTP-labelled products synthesized by the EMC virus RC. The positions of genome-length virion RNA of EMC virus (35 S) and *E. coli* rRNAs (23 S and 16 S) are indicated; **(B)** hybridization of high mol. wt. [$^{32}$P]RNA products with the increasing amounts of unlabelled virion RNA (plus strand) and RF RNA (double-stranded) of EMC virus.

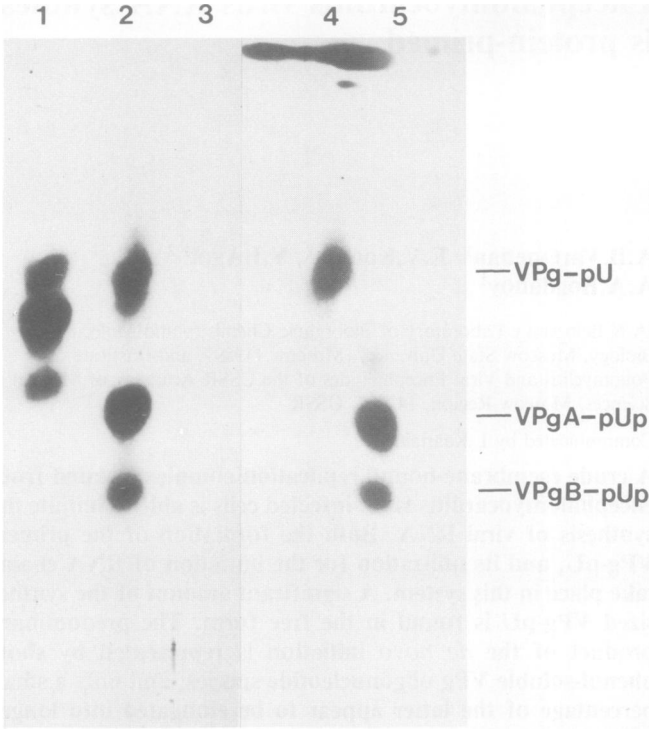


**Fig. 2.** Nucleotidyl proteins synthesized by the EMC virus RC in the presence of [α-$^{32}$P]UTP. Phenol-soluble products of the *in vitro* system were treated as indicated below and analyzed by electrophoresis in 12.5% gels in the presence of SDS. **1**, untreated RC products; **2**, RNase-treated RC products; **3**, RNase + pronase treated RC products; **4**, $^{32}$P-labelled EMC virion RNA treated with RNases and phosphatase to produce VPg-pU; **5**, $^{32}$P-labelled EMC virion RNA treated with RNases only to produce VPgA-pUp and VPgB-pUp.

product synthesized was viral RNA species of positive polarity.

*Evidence for the VPg-mediated initiation of RNA chains in vitro*

The 5′-terminal nucleotide of the EMC virus RNA, the uridylic acid residue, is known to be covalently linked with proteins VPgA and VPgB, which could be detected as two distinct VPg-pUp species upon electrophoresis of the $^{32}$P-labelled virion RNA treated with a mixture of RNases A, T1 and T2 (Vartapetian *et al.*, 1980).

To study the initiation of the EMC virus RNA synthesis *in vitro*, the membrane-bound RC was incubated with [α-$^{32}$P]-UTP and the three other unlabelled NTP, the sample was extracted with phenol and both the phenol- and water-soluble fractions were analyzed. The rationale of this procedure was that VPg bound to short oligonucleotides is known to partition into phenol phase upon extraction with phenol (Ambros *et al.*, 1978; Vartapetian *et al.*, 1982). Such a method appeared to be especially suitable for the search for early products of initiation.

When the phenol-soluble products were subjected to polyacrylamide gel electrophoresis (PAGE), several protease-sensitive bands could be revealed (Figure 2, lanes 1 and 3). Neither of these bands could be seen when [α-$^{32}$P]ATP was used as the source of the label instead of [α-$^{32}$P]UTP; nor were they found upon incubation of [α-$^{32}$P]UTP with membrane preparations from mock-infected cells or with detergent-solubilized preparations of EMC virus RC (not shown). The slowest of these bands co-migrated with the VPg-pU marker (see below). The other bands were identified as VPg oligonucleotides. The presence of an oligonucleotide moiety was indicated by their sensitivity to treatment with RNases (Figure 2, lane 2). The formation, after this treatment, of the VPgA-pUp and VPgB-pUp bands demonstrated that the protein moiety of these entities was VPg. The validity of the identification of the RNase-generated bands as VPg-pUp's was based not only on their co-migration with the appropriate markers, but also on their conversion, after treatment with phosphatase or P1 nuclease, into a labelled material with the electrophoretic mobility of VPg-pU (not shown), which retained half of the $^{32}$P radioactivity originally found in VPg-pUp. This result indicated also that both the VPg-linked and the penultimate nucleotides of the VPg-bound oligonucleotides were represented by the uridylic acid residues. Finally, when the material from the putative VPgA-pUp and VPgB-pUp bands was individually subjected to acid hydrolysis, [$^{32}$P] phosphotyrosine was the only labelled phosphoamino acid found (Figure 3). The structure of the VPg-nucleotide linkage in these complexes, thus identified, was VPg(Tyr-O$^4$)-pUpU . . ., as expected of the genuine VPg oligonucleotides (Vartapetian *et al.*, 1980, 1983).

Thus, the VPg-adjacent, that is the 5′-terminal nucleotide, and the penultimate nucleotide of the newly synthesized VPg oligonucleotides contained the $^{32}$P label, unequivocally demonstrating that they were derived from the exogenous substrate. It can therefore be concluded that the VPg-mediated *de novo* initiation (the formation of the VPg-pU linkage and of the first internucleotide bond) does take place *in vitro* and that the elongation of newly initiated chains occurs to at least some extent.

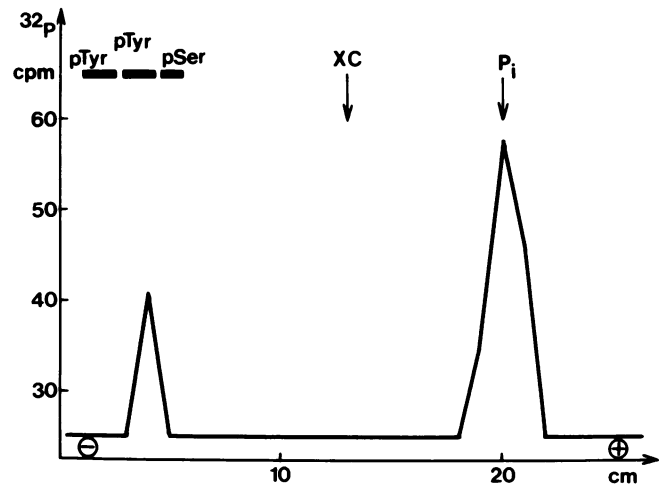


**Fig. 3.** VPg is bound to the 5′-uridylic acid of the newly synthesized oligonucleotides via the hydroxy group of its tyrosine residue. [$^{32}$P]VPgA-pUp and [$^{32}$P]VPgB-pUp derived upon RNase treatment of phenol-soluble VPg oligonucleotides synthesized by the RC (Figure 2, lane 2) were subjected to acid hydrolysis. The products were separated by paper electrophoresis at pH 1.7. The experiment shown is for VPgA-pUp; a similar result was obtained with VPgB-pUp.

*Identification of the free VPg-pU among the reaction products*

To obtain a marker VPg-pU, the RNase hydrolysate of the virion RNA (which contained VPgA-pUp and VPgB-pUp) was treated with phosphatase and subjected to PAGE. This treatment led to the disappearance of the VPg-pUp bands and emergence of the VPg-pU band with a considerably lower mobility (Figure 2, lanes 4 and 5). VPg-pU derived from the $^{32}$P-labelled EMC virion RNA by treatment with nuclease P1 (an enzyme known to generate 3′-unphosphorylated nucleotides) exhibited an identical mobility (not shown). VPgA-pU and VPgB-pU could not be resolved under the conditions employed. Thus, the EMC virus VPg-pU demonstrated peculiar electrophoretic behaviour in that the removal of only one phosphate group of VPg-pUp resulted in a remarkable mobility shift. In this respect it is different from the poliovirus VPg-pU which migrated slightly ahead of the respective VPg-pUp (Crawford and Baltimore, 1983; our unpublished observations).

Inspection of the phenol-soluble products of the RC electrophoresed without RNase digestion (Figure 2, lane 1) revealed some labelled material in the position expected of VPg-pU. Treatment with the mixture of RNases did not appear to change the mobility of this material (Figure 2, lane 2), but it disappeared after pronase digestion (Figure 2, lane 3). These observations allowed us to suggest that the slowly migrating RNase-resistant labelled material was indeed VPg-pU. To verify this suggestion, the putative VPg-pU was excised from the gel, subjected to digestion with pronase and the digestion products were electrophoresed in a concentrated polyacrylamide slab. This procedure was known to generate, from VPg-pUp, a unique set of nucleotidyl peptides, which appeared to differ from each other by the length and/or the sequence of their peptidyl moiety (Vartapetian *et al.*, 1980). As shown in Figure 4, the nucleotidyl peptides derived from the putative free VPg-pU were identical, in terms of their electrophoretic mobility, to nucleotidyl peptides derived from the marker VPg-pU (lanes 1, 3 and 5). This was true irrespective of whether this material was pre-treated with the mixture of RNases or not (compare lanes 1 and 2 in Figure 4). The absence of the 3′ phosphate on the U residue of the VPg-pU-derived nucleotidyl peptides appeared to slow down their electrophoretic mobility, since the mobilities of their VPg-pUp-derived counterparts were markedly greater (Figure 4, lanes 3, 4 and 5, 6). Thus, the newly synthesized free VPg-pU is accumulated in the cell-free system.

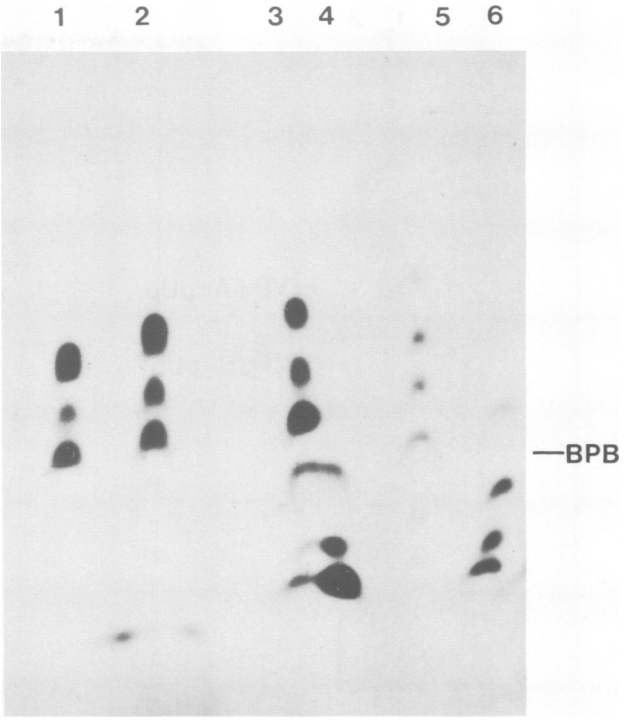


**Fig. 4.** Polyacrylamide gel electrophoresis of nucleotidyl peptides derived from [$^{32}$P]VPg-pUp and [$^{32}$P]VPg-pU. Individual VPg-pUp and VPg-pU species isolated by electrophoresis were digested with pronase and analyzed by electrophoresis in 20% polyacrylamide gels. **1**, VPg-pU (untreated with RNases, from lane 1 in Figure 2); **2**, VPg-pU (RNase-treated, from lane 2 in Figure 2); **3**, VPg-pU marker obtained from VPgA-pUp (lane 2 in Figure 2) by treatment with phosphatase prior to pronase; **4**, VPgA-pUp, the same as in **3** but without phosphatase treatment; **5**, VPg-pU marker obtained from VPgB-pUp (lane 2 in Figure 2) by treatment with phosphatase prior to pronase; **6**, VPgB-pUp, the same as in **5** but without phosphatase treatment.

*Relative proportions of VPg-pU, VPg oligonucleotides and VPg polynucleotides accumulated in the cell-free system*

The water-soluble fraction of the products synthesized on incubation of [$\alpha$-$^{32}$P]UTP with membrane-bound RC preparations was also analyzed. As already mentioned, this fraction contained long newly synthesized RNA species. When the total water-soluble product was digested with the RNase mixture and was then subjected to PAGE, two distinct labelled bands with the mobilities of VPgA-pUp and VPgB-pUp were generated (Figure 5), implying that at least some polynucleotide chains were initiated during incubation of the cell-free system.

Since VPg-pU was detected among the RC products alongside *in vitro* initiated VPg oligonucleotides and VPg polynucleotides, the latter were regarded as the products of elongation of the VPg-pU primer. Then, the efficiency of VPg-pU utilization for priming RNA chains and for further elongation of these chains could be deduced from the relative molar proportions of VPg-pU, VPg oligonucleotides and

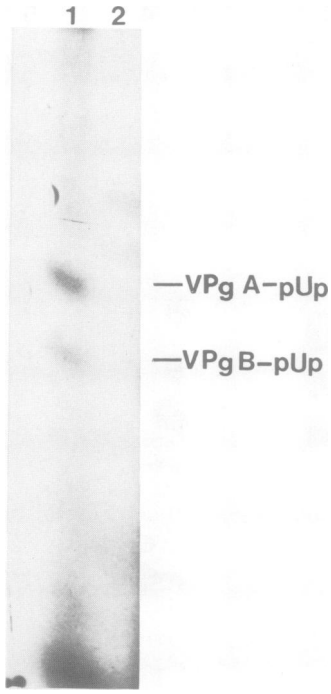


**Fig. 5.** Water-soluble [$\alpha$-$^{32}$P]UTP-labelled products of the EMC virus RC. The products were separated by PAGE after (1) or before (2) the RNase treatment. Shown is the radioautograph of the 12.5% separating gel into which the high mol. wt. labelled RNA species did not enter.

VPg polynucleotides accumulated *in vitro*. The phenol-soluble products were treated with the RNase mixture, and radioactivity in the (VPgA-pUp + VPgB-pUp) and VPg-pU bands was determined. The results allowed us to estimate that 25–70% of VPg-pU were elongated to VPg oligonucleotides (Table I). A similar analysis using both phenol- and water-soluble products showed that < 5% of the VPg oligonucleotides were further transformed into the water-soluble VPg polynucleotides (Table II).

For these estimates to be valid, it was important to be sure that VPg oligonucleotides were actually produced by a kind of abortive elongation rather than by degradation of longer RNA species. Although unambiguous proof of this notion is lacking, the VPg oligonucleotides appeared to possess free 3′-OH termini as evidenced by their sensitivity to snake venom phosphodiesterase under the conditions where only 3′ unphosphorylated oligonucleotides could be digested (not shown). On the contrary, degradation products are more likely to have the 3′ phosphorylated termini. Another assumption implicit in the above calculations is that no significant cleavage of VPg from the newly synthesized polynucleotide chains has occurred in our system.

## Discussion

This study demonstrates that crude RC preparations from picornavirus-infected cells accomplish initiation of viral RNA chains. Both the formation of the primer, VPg-pU, and its utilization for priming could be reproduced *in vitro*. A significant part of this work relied on the discovery of anomalous electrophoretic behaviour of the EMC virus-specific VPg-pU complex. Initially, we were surprised by this anomaly, but perhaps we should not have been, because aberrant electrophoretic mobilities of picornaviral VPg's and VPg-related polypeptides have been described repeatedly (Adler *et al.*, 1983; Morrow and Dasgupta, 1983).

**Table I.** Relative proportions of VPg-pU and VPg oligonucleotides accumulated in the cell-free system

| Exp. no | Radioactivity in | | % of VPg-pU elongated |
|---|---|---|---|
| | free VPg-pU (c.p.m.) a | VPg-pUp's (c.p.m./2)[a] b | b/(a+b) x 100 |
| 1 | 280 | 600 | 68 |
| 2 | 350 | 180 | 34 |
| 3 | 7630 | 2620 | 26 |
| 4 | 7040 | 7150 | 50 |
| 5 | 550 | 610 | 53 |

[a]The values of the radioactivity in VPg-pUp's were divided by two because they contain two labelled phosphates as compared with one in VPg-pU.

**Table II.** Relative proportions of VPg oligonucleotides and VPg polynucleotides accumulated in the cell-free system

| Exp. no | Radioactivity in VPg-pUp's (c.p.m.) derived from | | % of VPg oligonucleotides elongated |
|---|---|---|---|
| | VPg oligonucleotides a | VPg polynucleotides b | b/(a+b) x 100 |
| 1 | 25 360 | 1170 | 4.6 |
| 2 | 24 500 | 60 | 0.25 |

Intracellular membranes appear to play an important, though not necessarily direct, part in the VPg uridylation reaction, since detergent destruction of the membranes abolished accumulation of detectable amounts of VPg oligonucleotides (Vartapetian *et al.*, 1982; Takegami *et al.*, 1983) and of VPg-pU. Yet, the molecular mechanism of this reaction remains obscure. What is its actual substrate, VPg itself or some of its precursor polypeptides? Is this reaction catalyzed by the viral RNA polymerase, or are other proteins of viral or host origin involved? Is VPg-pU formation a template-dependent reaction? Analogous questions have been answered in other systems where initiation of polynucleotide chains also involves a virus-specific terminal protein primer; e.g., adenovirus and bacteriophage $\phi$29 DNA replication (Stillman, 1983; Matsumoto *et al.*, 1983). It would be interesting to know whether or not similar problems are solved in a similar way in viruses which are so divergent.

The fact that free VPg-pU was accumulated in amounts comparable to those of VPg-pU that has been utilized in the priming reaction, deserves some comment. It cannot, of course, be ruled out that the accumulation of VPg-pU reflected merely some deficiency in the cell-free initiation reaction. It is, however, more interesting to consider the possibility that a certain surplus of the primer is normally needed to achieve an adequate rate of RNA initiation. It may be noted that recent results of Takegami *et al.* (1983) suggested that small amounts of poliovirus VPg-pU were formed by membrane-bound RC preparations *in vitro*, though no direct identification of this substance was presented. Determination of relative VPg-pU concentrations in the virus-infected cells may help to elucidate the actual function of the free VPg-pU.

Another intriguing observation concerns the abundance of newly synthesized short VPg oligonucleotides. Our preliminary experiments indicate that one of the major VPg oligonucleotide products had the structure VPg-pUpUpG, which is

identical to the 5′ terminal trinucleotide of the EMC virion RNA (Vartapetian *et al.*, 1983). Thus, it looked as if 'strong stop' signals were present several nucleotides from the 3′ end of the template. Such abortive elongation may be regarded as another *in vitro* artifact. However, selective accumulation of VPg-pUpU also occurred in poliovirus-infected cells (Crawford and Baltimore, 1983), mimicking the situation observed with poliovirus RC *in vitro* (Takegami *et al.*, 1983). The reason for accumulation of short VPg oligonucleotides is unknown. Perhaps their association with the template is too weak to retain them in the RC, especially when adding subsequent nucleotides proceeds relatively slowly. The abortive elongation is known to occur in a variety of other RNA-synthesizing systems (Carpousis and Gralla, 1980; Yamakawa *et al.*, 1981). Again, search for the unity or diversity of the relevant mechanisms appears to be of general importance.

Our study evidently poses more questions than it gives answers. We believe, however, that it also provides a tool for finding the answers, since investigation of the mode of formation of the protein-nucleotide primer and of its utilization for viral RNA synthesis now becomes feasible in a cell-free system.

## Materials and methods

*Infection of the cells*

For preparation of $^{32}$P-labelled EMC virus virion RNA, Krebs-2 ascites carcinoma cells were infected with the virus at a low m.o.i. (0.01 – 0.1 p.f.u./cell). [$^{32}$P]orthophosphoric acid (5 – 20 mCi per 100 ml of the infected cells suspension) was added simultaneously with the virus and the cells were incubated for 15 h. For RC isolation, the cells were infected at a m.o.i. of 10 – 100 p.f.u./cell.

*Preparation of $^{32}$P-labelled EMC virion RNA, VPg-pUp and VPg-pU*

EMC virion RNA was isolated from partially purified virions by the procedure of Kerr and Martin (1972) modified as described previously (Drygin *et al.*, 1979). For preparation of [$^{32}$P]VPg-pUp, ~200 μg of $^{32}$P-labelled EMC virion RNA was dissolved in 50 μl of AE buffer (50 mM ammonium acetate, pH 4.5, 1 mM EDTA) and digested with RNases A (Worthington, 25 μg), T1 (Sigma, 30 U) and T2 (Calbiochem, 7.5 U) at 37°C for 4 h. For preparation of VPg-pU, the RNase treated samples were made 100 mM in Tris-HCl, pH 8.0, and 5 mM in $MgCl_2$ and treated with 18 μg of *Escherichia coli* alkaline phosphatase (Sigma) for 2 h at 37°C. The nucleotidyl proteins were precipitated with 4 vol of acetone and processed for electrophoresis.

*Fractionation of the cells and isolation of the membrane-bound RC*

This was done as described (Koonin and Agol, 1983). Briefly, the cells were harvested at 5 h p.i., disrupted in a Dounce homogenizer and the cytoplasmic fraction sedimentable at 30 000 *g* for 30 min was recovered and used for further experimentation.

*Conditions of the RNA-synthesizing reaction*

A standard sample contained, in 250 μl, 50 mM Hepes/NaOH pH 8.0, 20 mM KCl, 8 mM $MgCl_2$, 4 mM dithiothreitol, 0.4 mM EGTA, 10 μg/ml Actinomycine D, 1 mM ATP, GTP and CTP, [α-$^{32}$P]UTP (10 μM, 40 – 80 μCi/nmol) and 100 – 150 μl of RC preparation (from 1 – 1.5 x $10^8$ cells). The samples were incubated at 37°C for 30 min and the reaction was terminated by rapid freezing.

*Analysis of the products synthesized by the RC*

After thawing, the samples were made 1% in SDS, boiled for 2 min and extracted with 10 volumes of chloroform/methanol (2:1, v/v). The aqueous phase was recovered and extracted twice with water-saturated phenol-chloroform (1:1, v/v). VPg-linked RNA species were precipitated with 4 vol of acetone from the phenol phase and with 2.5 vol of ethanol from the aqueous phase.

The precipitates were collected by centrifugation, dissolved in 10 μl of AE buffer and treated with the mixture of RNases as described above. If indicated, the samples, after addition of Tris-HCl pH 7.5 to 50 mM, were subsequently treated with pronase (Calbiochem) at 1 mg/ml for 2 h at 37°C.

Nucleotidyl proteins were separated by PAGE in a modified system of Swank and Munkres (1971). The stacking gel was 3.5% acrylamide/bis-acrylamide (10:1) containing 100 mM Tris-phosphate, pH 6.9, 0.1% SDS and 8 M urea. The separating gel was 12.5% acrylamide/bis-acrylamide (10:1) containing 100 mM Tris-phosphate, pH 7.2, 0.1% SDS and 8 M urea. The gels were run at 10 V/cm for ~20 h and the labelled bands were visualized by autoradiography at −70°C with intensifying screens.

Electrophoresis of $^{32}$P-labelled RNA products of the RC was performed in 1% agarose prepared in a buffer containing 40 mM Tris-acetate, pH 7.3, 1 mM EDTA, 25 mM LiCl and 75% DMSO; the electrode buffer was the same, with the omission of DMSO. Under these conditions RNA molecules are completely denatured (Chumakov, 1979) and migrate inversely proportional to their mol. wts (our unpublished observations). After electrophoresis, the gel was stained with ethidium bromide and autoradiographed. High mol. wt. [$^{32}$P]RNA was then eluted from the gel and hybridized with unlabelled virion RNA and RF RNA of EMC virus as described (Chumakov, 1979).

*Analysis of nucleotidyl-peptides*

For preparation of nucleotidyl peptides, gel slices containing VPg nucleotides were cut out and the radioactive material was eluted by shaking in 150 μl of 10 mM Tris HCl, pH 7.5, 0.1% SDS for 4 h. The nucleotidyl proteins were precipitated from the eluate with 4 vol of acetone and digested with pronase (5 mg/ml) for 4 h at 37°C. If indicated, the sample, prior to pronase digestion, was treated with phosphatase as described above. Nucleotidyl peptides were separated in 20% polyacrylamide gels (Maxam and Gilbert, 1980).

*Identification of $^{32}$P-labelled phosphoamino acids*

$^{32}$P-labelled VPgA-pUp and VPgB-pUp were eluted from the gel and digested with pronase as described above. The resulting nucleotidyl peptides were treated with a mixture of concentrated HCl and $CF_3COOH$ as described (Tsugita and Scheffler, 1982). The hydrolysate was subjected to electrophoresis on Whatman 3MM paper at pH 1.7 as described (Vartapetian *et al.*, 1980). After electrophoresis, the paper was cut into 1 cm strips and the amount of radioactivity in each strip was determined by liquid scintillation counting. The positions of phosphoserine, phosphothreonine and phosphotyrosine markers was determined by ninhydrine staining.

*Chemicals and isotopes*

Unlabelled NTP were from Calbiochem or Sigma. All other chemicals were reagent grade. In some experiments [α-$^{32}$P]UTP (400 Ci/mM) was from Amersham. Alternatively, [α-$^{32}$P]UTP was synthesized according to Johnson and Walseth (1979) using [γ-$^{32}$P]ATP (Isotop, USSR) as the source of the label.

## Acknowledgements

We are grateful to Dr A.Gorbalenya for helpful suggestions and to Drs E. Wimmer and N.Crawford for communicating their data prior to publication.

## References

Adler,C.J., Elzinga,M. and Wimmer,E. (1983) *J. Gen. Virol.*, **64**, 349-355.
Ambros,V., Pettersson,R.F. and Baltimore,D. (1978) *Cell*, **15**, 1439-1446.
Baron,M. and Baltimore,D. (1982) *Cell*, **28**, 395-404.
Carpousis,A.J. and Gralla,J.D. (1980) *Biochemistry (Wash.)*, **19**, 3245-3253.
Chumakov,K.M. (1979) *Biokhimiya*, **44**, 57-65.
Crawford,N.M. and Baltimore,D. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7452-7455.
Dmitrieva,T.M. and Agol,V.I. (1974) *Arch. Ges. Virusforsch.*, **45**, 17-26.
Drygin,Yu.F., Vartapetian,A.B. and Chumakov,K.M. (1979) *Mol. Biol.*, **13**, 777-787.
Flanegan,J.B., Pettersson,R.F., Ambros,V., Hewlett,M.J. and Baltimore,D. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 961-965.
Johnson,R.A. and Walseth,T.F. (1979) *Adv. Cyclic Nucleotide Res.*, **10**, 135-167.
Kerr,I.M. and Martin,E.M. (1972) *J. Virol.*, **9**, 559-561.
Koonin,E.V. and Agol,V.I. (1983) *Virology*, **129**, 309-318.
Matsumoto,K., Saito,T. and Hirokawa,H. (1983) *Mol. Gen. Genet.*, **191**, 26-30.
Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.
Morrow,C.D. and Dasgupta,A. (1983) *J. Virol.*, **42**, 429-439.
Nomoto,A., Detjen,B., Pozzatti,R. and Wimmer,E. (1977) *Nature*, **268**, 208-213.
Pettersson,R.F., Ambros,V. and Baltimore,D. (1978) *J. Virol.*, **27**, 357-365.
Stillman,B.W. (1983) *Cell*, **35**, 7-9.
Swank,R.T. and Munkres,K.D. (1971) *Anal. Biochem.*, **39**, 478-485.
Takegami,T., Kuhn,R.J., Anderson,C.W. and Wimmer,E. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7447-7451.
Tsugita,A. and Scheffler,J.-J. (1982) *Eur. J. Biochem.*, **124**, 585-588.
Vartapetian,A.B. (1982) *Bioorg. Khim.*, **8**, 1581-1606.
Vartapetian,A.B., Drygin,Yu.F., Chumakov,K.M. and Bogdanov,A.A. (1980) *Nucleic Acids Res.*, **8**, 3729-3742.

Vartapetian,A.B., Koonin,E.V., Chumakov,K.M., Bogdanov,A.A. and Agol,V.I. (1982) *Dokl. Akad. Nauk SSSR,* **267**, 963-965.
Vartapetian,A.B., Mankin,A.S., Skripkin,E.A., Chumakov,K.M., Smirnov, V.D. and Bogdanov,A.A. (1983) *Gene,* **26**, 189-195.
Wimmer,E. (1979) in Perez-Bercoff,R. (ed.), *The Molecular Biology of Picornaviruses,* Plenum Publ. Corp., NY, pp. 175-190.
Wimmer,E. (1982) *Cell,* **28**, 199-201.
Yamakawa,M., Furuichi,Y., Nakashima,K., La Fiandra,A.J. and Shatkin, A.J. (1981) *J. Biol. Chem.,* **256**, 6507-6514.

*Received on 5 July 1984*