THE FATE OF VPg DURING IN VITRO TRANSLATION OF POLIOVIRUS RNA

Andrew J. DORNER, Paul G. ROTHBERG and Eckard WIMMER
Department of Microbiology, State University of New York, Stony Brook, NY 11794, USA

Received 19 June 1981; revision received 3 August 1981

1. Introduction

Polio virion RNA (vRNA) is covalently linked at its 5′-end to a small protein, VPg, via an O^4-(5′-uridylyl) tyrosine bond [1,2]. The viral-specific RNA found associated with polyribosomes (mRNA) in infected cells, however, lacks VPg and terminates with a 5′-uridylate (pU) [3–5]. Biochemical analysis has shown that vRNA and mRNA differ only in the presence or absence of VPg [3,6]. Both types of RNA are reported to be infectious ([7], L. Fedus, unpublished).

Uninfected HeLa cells contain an activity, known as unlinking enzyme, which removes VPg from RNA, leaving the 5′-end of the RNA intact [8]. Cleavage of the bond between protein and RNA may serve a regulatory function: RNA lacking VPg may be directed to the ribosomes while the presence of VPg on the RNA may serve as a signal for encapsidation [9]. VPg itself has been postulated to be involved in the initiation of RNA synthesis perhaps through the cleavage of a precursor protein [10]. At present, however, any function for VPg remains obscure.

While the functional significance of the removal of VPg from the RNA associated with polyribosomes in infected cells is unknown, we have shown [17] that VPg is not removed during the formation of the 80 S initiation complex in a rabbit reticulocyte lysate. This indicates that the presence of VPg does not interfere with the initiation of protein synthesis in vitro. That work, however, did not detect any unlinking activity in this system under any conditions, contrary to [8,18].

Here we have explored the fate of VPg in both the rabbit reticulocyte lysate and the poliovirus-infected HeLa cell extract under conditions of ongoing protein synthesis and also in the presence of protein synthesis inhibitors. We have re-examined the unlinking activity under the ribosome binding conditions in [17] using a different assay for VPg removal [8]. We have also analyzed a cellular proteinase activity that appears to degrade free VPg while leaving intact VPg linked to RNA as well as newly synthesized viral-specific proteins.

Abbreviations: VPg, genome-linked viral protein; SDS, sodium dodecyl sulfate; VRC, vanadium ribonucleoside complex; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; M_r, relative molecular mass

2. Materials and methods

Poliovirus was grown in the presence of [^{3}H]tyrosine or [^{3}H]lysine in HeLa cell suspension culture as in [2]. The preparation of ^{32}P-labeled virus and the purification of ^{32}P-labeled vRNA have been described [6]. VPg-pU and the 5′-terminal nonanucleotide linked to VPg were prepared by digestion of vRNA with nuclease P1 and ribonuclease T1, respectively, as in [1].

A messenger-dependent reticulocyte lysate was prepared as in [11]. The conditions of the unlinking activity assay were as follows: 10 μl reticulocyte lysate in a 25 μl reaction contained 80 mM K(OAc), 0.1 mM $Mg(OAc)_2$, 4.5 mM DTT, 20 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase and 0.05 mg/ml tRNA. [^{3}H]Tyrosine- or [^{3}H]lysine-labeled vRNA was added to 40 μg/ml final conc. Occasionally ^{32}P-labeled vRNA was added to monitor ribonuclease levels. Incubation was usually for 30 min at 30°C. Inhibition of the unlinking activity was accomplished by addition of 8 mM vanadium ribonucleoside complex (VRC). The polio-infected HeLa cell extract was prepared essentially as in [12]. The HeLa cell extract was made messenger-dependent by treatment with micrococcal nuclease as described for the reticulocyte lysate [11]. Translation conditions in the HeLa cell extract were the same as for the reticulocyte lysate except that 100 mM K(OAc) and 0.4 mM $Mg(OAc)_2$

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50 © 1981 Federation of European Biochemical Societies

were used and 1 mM ATP and 0.2 mM GTP were added. To examine the level of endogenous mRNA activity in the HeLa cell extract nuclease treatment was omitted.

For analysis by SDS–polyacrylamide gel electrophoresis, in vitro translation reactions were incubated at 30°C for 2 h in the presence of [^{35}S]methionine. Aliquots were precipitated in 10% trichloroacetic acid, washed twice with acetone, dissolved in sample buffer, and run on 12.5% polyacrylamide gels containing 0.1% SDS according to [13]. The gel was dried and autoradiographed.

The level of covalent attachment of VPg to RNA was analyzed by a modification of the gel filtration column assay in [8]. To 25 μl unlinking reaction mixture, 75 μl column buffer (0.1 M NaCl, 50 mM Tris–HCl (pH 7.1), 1 mM EDTA, 0.5% SDS) was added then applied to a 1 cm × 10 cm Biogel A1.5 M column. Fractions (20 drop) were collected and the radioactivity measured by liquid scintillation counting. Full length RNA elutes from this column at fraction 10, intact VPg elutes at fractions 17–18 and low M_r material elutes at fractions 24–25. Background cpm were subtracted before plotting data.

3. Results and discussion

3.1. *In vitro translation systems contain an unlinking activity*

The gel filtration assay was used to detect an unlinking activity in the reticulocyte lysate. An elution profile of a mixture of [^{32}P]RNA and v[*tyrosyl*-^{3}H]-RNA is shown in fig.1a. The single peak of ^{32}P- and *tyrosyl*-^{3}H-radioactivity co-elutes with blue dextran, a marker of the excluded volume. Degradation of the RNA would result in elution of ^{32}P-labeled material after the void volume. The [^{3}H]tyrosine label monitors the single tyrosine residue of VPg which is involved in the linkage to RNA. Removal of VPg from the RNA

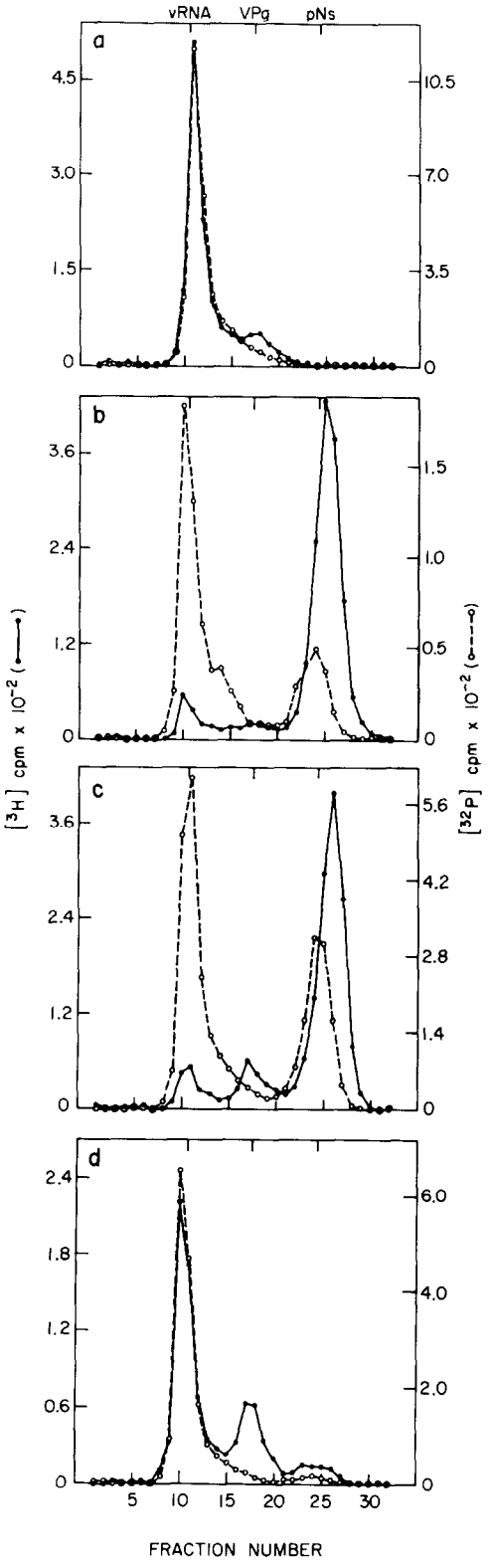


Fig.1. Gel filtration assay of unlinking activity. Biogel A1.5 M column profiles of polio RNA incubated in: (a,c) a HeLa cell extract, 0 min (a) or 30 min (c); (b,d) a reticulocyte lysate, 30 min (b) or 4 min under ribosome binding conditons (d). [*tyrosyl*-^{3}H]RNA was used to monitor the status of the nucleic acid/protein linkage by virtue of the involvement of VPg's single tyrosine in the covalent bond. [^{32}P]RNA was used to monitor the integrity of the RNA. Incubation conditions were as in section 2.

is indicated by elution of the *tyrosyl*-^{3}H-radioactivity after the void volume. Fig.1b shows the elution profile of a mixture of v[*tyrosyl*-^{3}H]RNA and v[^{32}P]RNA afer 30 min incubation in a reticulocyte lysate. The elution of *tyrosyl*-^{3}H-radioactivity after the void volume indicates the presence of an unlinking activity. As seen from the ^{32}P radioactivity profile the level of ribonuclease is not sufficient to account for this result. This result alone does not directly demonstrate the cleavage of the O^4-(5′-uridylyl) tyrosine bond. A nuclease which preferentially attacks the 5′-end of polio vRNA could remove VPg still covalently attached to a short stretch of nucleotides. In this situation the VPg would still be effectively removed from the RNA.

When [^{3}H]tyrosine labeled vRNA was added to a poliovirus-infected HeLa cell extract a similar column profile indicative of unlinking activity can be seen (fig.1c). Unlinking activity has been reported in both systems [8].

Because the unlinking activity assay was performed under conditions of on-going protein synthesis, the state of the RNA/protein bond was determined under conditions in which the RNA is functional. Both the micrococcal nuclease-treated reticulocyte lysate (fig.2d) and the polio-infected HeLa cell extract (fig.2e) faithfully translate added polio vRNA. Without the addition of polio vRNA no discrete protein products can be observed in either the micrococcal nuclease treated reticulocyte lysate (fig.2a) or the micrococcal nuclease treated polio-infected HeLa cell extract (fig.2b). Non-micrococcal nuclease treated polio-infected HeLa cell extract, translating endogenous vRNA (fig.2f), exhibits a range of proteins similar to the in vivo cell extract (fig.2c). The polio-infected HeLa cell extract synthesizes only polio-specific proteins due to the shutoff of host cell protein synthesis during viral infection [19].

3.2. *Proteinase activity*

In both systems that we examined, the [^{3}H]tyrosine-labeled product of the unlinking activity eluted from the Biogel A1.5 M column in the low M_r region, co-eluting with bromophenol blue. This indicates that VPg is being degraded under the assay conditions, as in [8]. Similar analysis of the fate of [^{3}H]lysine labeled RNA reinforced this conclusion. [^{3}H]Lysine labeled material also eluted in the low M_r region of the column after incubation in these systems (not shown). Lysine occurs in VPg in positions 9, 10 and 20, and tyrosine at position 3 from the amino-terminus of the 22 amino

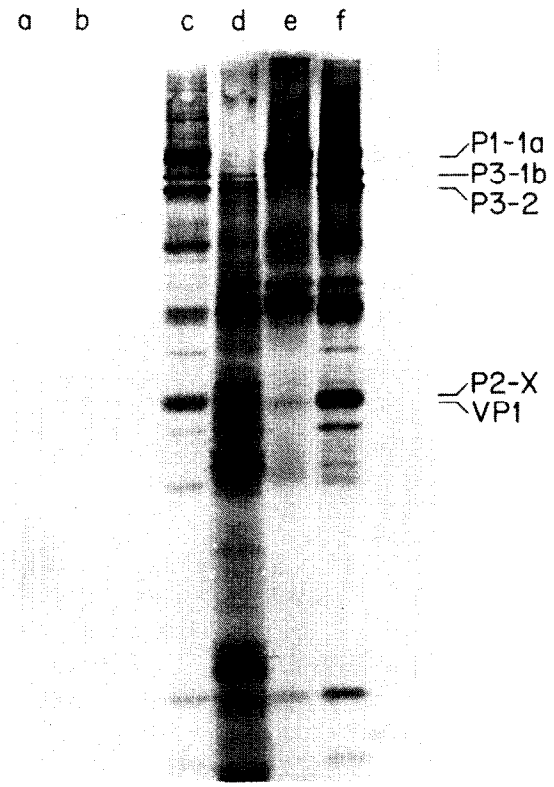
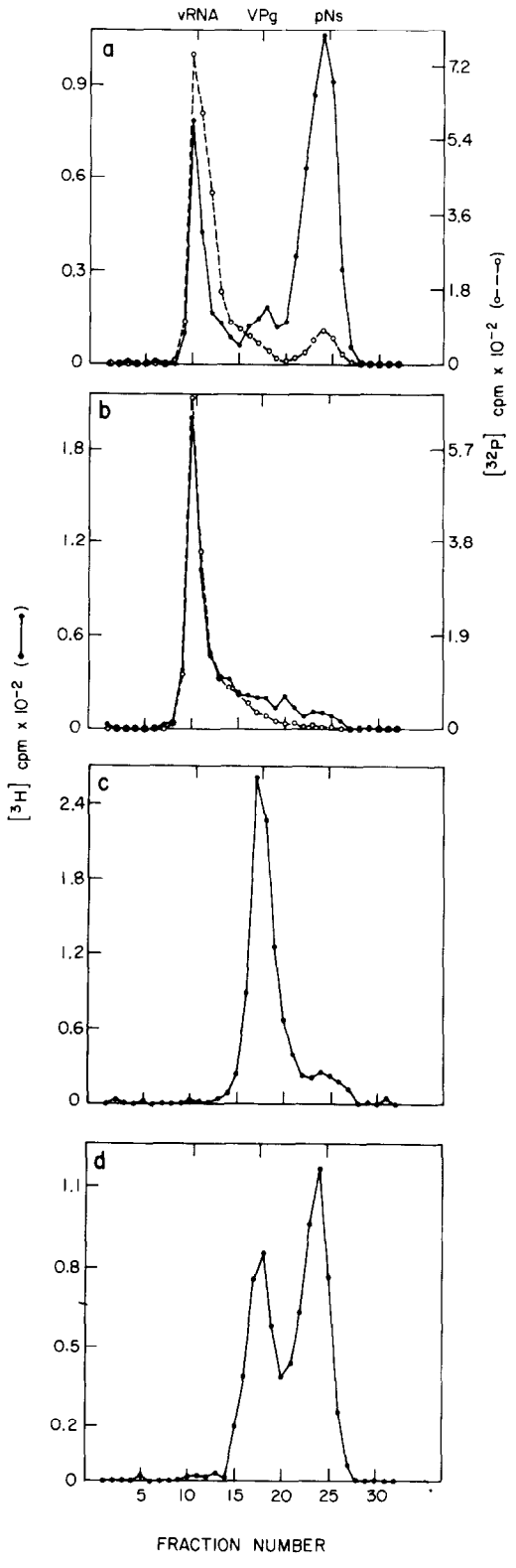


Fig.2. SDS–PAGE analysis of in vitro synthesized polio proteins. Autoradiogram of a 12.5% polyacrylamide gel of [^{35}S]-methionine-labeled polio-specific proteins synthesized in the messenger-dependent reticulocyte lysate and the poliovirus-infected HeLa cell extract: (a) micrococcal nuclease-treated reticulocyte lysate without added RNA; (b) micrococcal nuclease-treated poliovirus-infected HeLa cell extract without added RNA; (c) in vivo lysate from polio-infected HeLa cells labeled with [^{35}S]methionine; (d) micrococcal nuclease-treated reticulocyte lysate supplemented with polio vRNA; (e) micrococcal nuclease-treated poliovirus-infected HeLa cell extract supplemented with polio vRNA; (f) poliovirus-infected HeLa cell extract translating endogenous polio vRNA. P1-1a, P3-1b, P3-2, and P2-X are polio-specific non-capsid proteins. VP1 is a capsid protein.

acid protein [14,15]. Several peptide bonds would have to be cut to result in the elution of [^{3}H]lysine and [^{3}H]tyrosine in the low M_r region of the column.

To determine the integrity of VPg while covalently linked to RNA, VRC was used to inhibit both the unlinking activity and ribonuclease. VRC, a ribonuclease inhibitor [16], also inhibits a partially purified preparation of unlinking enzyme (not shown). Fig.3b shows that [^{3}H]lysine-labeled RNA was not degraded after a 30 min incubation in a VRC-inhibited reticulo-



cyte lysate reaction. Degradation of covalently linked [^{3}H]lysine VPg with pronase exhibits an elution profile with the ^{3}H counts eluting at fraction 25 (not shown). These data suggest that in the reticulocyte lysate, VPg that is covalently linked to intact viral RNA is protected from proteolytic digestion.

We then determined whether the presence of one nucleotide linked to VPg was sufficient to protect VPg from proteolytic digestion in the reticulocyte lysate. As can be seen in fig.3d, [^{3}H]lysine VPg-pU, prepared by nuclease P1 digestion of vRNA, is degraded in the VRC-inhibited reticulocyte lysate. Fig.3c is the elution profile of VPg-pU prior to incubation in the lysate. Complete degradation of the VPg-pU into low M_r products does not occur in this set of reactions presumably because a low concentration of SDS was used to prepare the hydrophobic VPg-pU. We assume that the low concentration of SDS also partially inhibits the unlinking activity as measured in fig.3a. We have also observed proteolytic degradation of VPg-pU in the HeLa cell extract (not shown). This proteinase activity offers a possible explanation for the observation that free VPg cannot be found in the infected cell [10].

RNase T1 digestion of vRNA produces a VPg-linked 5′-terminal nonanucleotide. This VPg is also degraded in the reticulocyte lysate (not shown). Work is currently in progress to determine the minimum length of RNA needed to protect VPg from proteinase digestion.

Another assay has been described [8] that detects unlinking activity by the partitioning of free VPg to the phenol phase during phenol extraction. VPg linked to RNA partitions to the aqueous phase. This assay had been used to measure unlinking activity in the reticulocyte lysate [17]. Based on these results, the phenol extraction assay is an unreliable measure of unlinking activity because the low M_r products of proteolytic degradation of VPg also partition to the

Fig.3. Gel filtration analysis of proteolysis of VPg. Biogel A1.5 M column profiles of: (a,b) [^{3}H]lysine and ^{32}P-labeled polio RNA incubated in a reticulocyte lysate in the absence (a) and presence (b) of 8 mM VRC; (c,d) [*lysyl*-^{3}H]VPg-pU before (c) and after (d) a 30 min incubation in a VRC-inhibited reticulocyte lysate. All incubations contained 0.02% SDS which has needed in the case of (d) to prevent adsorption of VPg to the tube before incubation in the reticulocyte lysate. Preparation of VPg-pU and column chromatography conditions were described in section 2.

aqueous phase. Thus the effect of unlinking activity is masked by proteolytic degradation. We conclude that the phenol extraction assay [8] is only useful for measuring relatively pure preparations of unlinking enzyme.

3.3. *VPg unlinking activity and inhibition of protein synthesis*

Inhibition of initiation of protein synthesis by pactamycin and inhibition of elongation by sparsomycin has no effect on VPg removal in the reticulocyte lysate (not shown). Thus viral protein synthesis is not required for this unlinking activity. The degradation of free VPg is also unaffected indicating that these activities are not viral encoded. However, we have not succeeded in inhibiting the unlinking activity without concomitant inhibition of protein synthesis. This may be due to the fact that in vitro protein synthesis is very sensitive to a variety of perturbations.

3.4. *VPg removal and ribosome binding*

Under the conditions of ribosome binding in [17] we find little VPg is removed from the RNA and that the released VPg is not degraded to smaller fragments (fig.1d). Our work substantiates the data in [17] that during the short incubation times used for in vitro initiation complex formation little VPg is removed from the RNA. However the reticulocyte lysate does contain an unlinking activity manifest at longer incubation times. This activity was not detected using the phenol extraction assay [17] due to the presence of a proteinase as we have shown here.

The proteinase activity may prove to be of interest due to its apparent preference for VPg release from RNA. The resistance of VPg covalently attached to RNA from proteolytic degradation implies some protective interaction between the RNA and VPg. We believe the proteinase activity could be useful in defining non-covalent interactions between VPg and polio RNA.

Acknowledgements

We thank B. Semler for incisive criticism, L. Fedus for excellent technical assistance, M. A. Huntington and J. Demian for preparation of artwork, and S. Studier for typing the manuscript. A. J. D. and P. G. R. are members of the Molecular Biology Program at Stony Brook. E. W. is a Josiah Macy Scholar.

References

[1] Nomoto, A., Detjen, B., Pozzatti, R. and Wimmer, E. (1977) Nature 268, 208–213.
[2] Rothberg, P. G., Harris, T. J. R., Nomoto, A. and Wimmer, E. (1978) Proc. Natl. Acad. Sci. USA 75, 4868–4872.
[3] Nomoto, A., Lee, Y. F. and Wimmer, E. (1976) Proc. Natl. Acad. Sci. USA 73, 375–380.
[4] Hewlett, M. J., Rose, J. K. and Baltimore, D. (1976) Proc. Natl. Acad. Sci. USA 73, 327–330.
[5] Fernandez-Munoz, R. and Darnell, J. E. (1976) J. Virol. 18, 719–726.
[6] Nomoto, A., Kitamura, N., Golini, F. and Wimmer, E. (1977) Proc. Natl. Acad. Sci. USA 74, 5345–5349.
[7] Flanegan, J. B., Pettersson, R. F., Ambros, V., Hewlett, M. J. and Baltimore, D. (1977) Proc. Natl. Acad. Sci. USA 74, 961–965.
[8] Ambros, V., Pettersson, R. F. and Baltimore, D. (1978) Cell 15, 1439–1446.
[9] Rothberg, P. G., Adler, C. J., Kitamura, N. and Wimmer, E. (1980) in: Biosynthesis, Modification, and Processing of Cellular and Viral Protein (Koch, G. and Ricter, D. eds) pp. 309–319, Academic Press, New York.
[10] Wimmer, E. (1979) in: The Molecular Biology of Picornaviruses (Perez-Bercoff, R. ed) pp. 175–190, Plenum, London, New York.
[11] Pelham, H. R. B. and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247–256.
[12] Celma, M. L. and Ehrenfeld, E. (1974) Proc. Natl. Acad. Sci. USA 71, 2440–2444.
[13] Laemmli, U. K. (1970) Nature 227, 680–685.
[14] Kitamura, N., Adler, C. J., Rothberg, P. G., Martinko, J., Nathenson, S. G. and Wimmer, E. (1980) Cell 21, 295–302.
[15] Semler, B. L., Anderson, C. W., Kitamura, N., Rothberg, P. G., Wishart, W. L. and Wimmer, E. (1981) Proc. Natl. Acad. Sci. USA in press.
[16] Berger, S. L. and Birkenmeier, C. S. (1979) Biochemistry 18, 5143–5149.
[17] Golini, F., Semler, B. L., Dorner, A. J. and Wimmer, E. (1980) Nature 287, 600–603.
[18] Sanger, D. V., Bryant, J., Harris, T. J. R., Brown, F. and Rowlands, D. J. (1981) J. Virol. 39, 67–74.
[19] Baltimore, D. (1969) in: The Biochemistry of Viruses (Levy, H. B. ed) pp. 101–176, Dekker, New York.