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An Enzymatic Activity in Uninfected Cells That Cleaves the Linkage between Poliovirion RNA and the 5′ Terminal Protein

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Summary

The 5′ terminal protein (VPg) on poliovirion RNA can be removed by cell-free extracts from a variety of uninfected cells. This soluble enzymatic activity is found in both nuclear and cytoplasmic extracts of HeLa cells and is activated by Mg^{++}. The enzyme activity cleaves the tyrosine-phosphate bond that links the protein to the RNA. In a partially purified form it has insufficient nonspecific protease or nuclease activity to account for its action. The existence of this enzyme implies that poliovirus RNA is translated in cell-free extracts in a form that lacks the 5′ terminal protein. The role of this enzyme in the uninfected cell is not known.

Introduction

The single-stranded RNA genome of poliovirus contains a protein (VPg) covalently bound to its 5′ terminal phosphate (Flanegan et al., 1977; Nomoto et al., 1977a). VPg has a molecular weight of approximately 12,000 daltons and contains one tyrosine residue that is linked by a phosphodiester bond to the RNA molecule (Ambros and Baltimore, 1978). VPg is found on negative-strand viral RNA and on nascent poliovirus RNA molecules (Pettersson, Ambros and Baltimore, 1978). Poliovirus messenger RNA, however, lacks the 5′ terminal protein and instead terminates with a 5′ phosphate followed by the same nonanucleotide sequence that is 5′ terminal in virion RNA (UUAAAACAG) (Nomoto et al., 1977b; Pettersson et al., 1977). Since VPg is the only 5′ terminal structure detectable on nascent poliovirus RNA, it appears that the protein-RNA bond has been cleaved in RNA destined to act as viral messenger RNA. To investigate this cleavage reaction, we have assayed several cell-free extracts for an activity that breaks the tyrosine-phosphate bond between VPg and poliovirus RNA. Such an enzymatic activity exists in extracts of both poliovirus-infected and uninfected HeLa cells, as well as in cell-free extracts of rabbit reticulocytes, mouse L cells and wheat germ. The activity specifically cleaves the tyrosine-phosphate bond. A partially purified preparation obtained from washed HeLa cell nuclei removes VPg, leaving the protein intact.

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Results

To assay the removal of VPg from poliovirion RNA, gel exclusion chromatography was used. ^{3}H-tyrosine-labeled VPg in poliovirion RNA was excluded from a Biogel A1.5 M column in sodium dodecylsulfate, as was the ^{32}P-labeled poliovirus RNA marker (Figure 1A). Extensive ribonuclease digestion of the RNA caused the ^{3}H-tyrosine label to elute coincidentally with cytochrome c in agreement with the previous demonstration that free VPg has a molecular weight of 12,000 daltons (Ambros and Baltimore, 1978); the ^{32}P mononucleotides were totally included in the column (Figure 1B). When ^{3}H-tyrosine-labeled VPg was deliberately degraded with proteinase K while still attached to the RNA, ^{3}H-tyrosine radioactivity remained associated with the RNA (Figure 1C). This result is in agreement with the previous finding that a tyrosine-phosphate bond links VPg to poliovirus RNA (Ambros and Baltimore, 1978).

When ^{3}H-tyrosine-labeled poliovirion RNA was incubated in a cytoplasmic extract from HeLa cells, after 15 min almost 90% of the ^{3}H radioactivity was in very low molecular weight material, eluting with the included fractions of the Biogel A1.5 M column (Figure 1D). The ^{32}P marker RNA was degraded somewhat, but not sufficiently to account for the small size of the ^{3}H-tyrosine-containing material. Because the bond between the tyrosine residue in VPg and the poliovirus RNA 5′ terminal phosphate is protease-resistant (Ambros and Baltimore, 1978), the result shown in Figure 1D implies that HeLa cell cytoplasm contains an enzyme able to cleave the VPg-RNA linkage. VPg appears to be degraded by this extract because the ^{3}H radioactivity is in material smaller than the 12,000 molecular weight size of intact VPg. Incubation of ^{3}H-tyrosine-labeled poliovirus RNA in a nuclear wash from HeLa cells also removed the ^{3}H from the RNA but left most of the ^{3}H chromatographing at the position of cytochrome c (Figure 1E). As was the case with the cytoplasmic extract, the nuclear wash did not degrade the ^{32}P marker RNA sufficiently to account for the size of the ^{3}H-tyrosine-containing material.

The HeLa nuclear wash appears to contain an activity able to unlink VPg from poliovirus RNA while leaving both the RNA and VPg relatively intact. To confirm that the ^{3}H-tyrosine-containing material that was released was actually VPg free of RNA, a limit protease digestion was performed. For marker, a control sample of ^{3}H-tyrosine-labeled VPg, freed of covalently bound RNA by venom

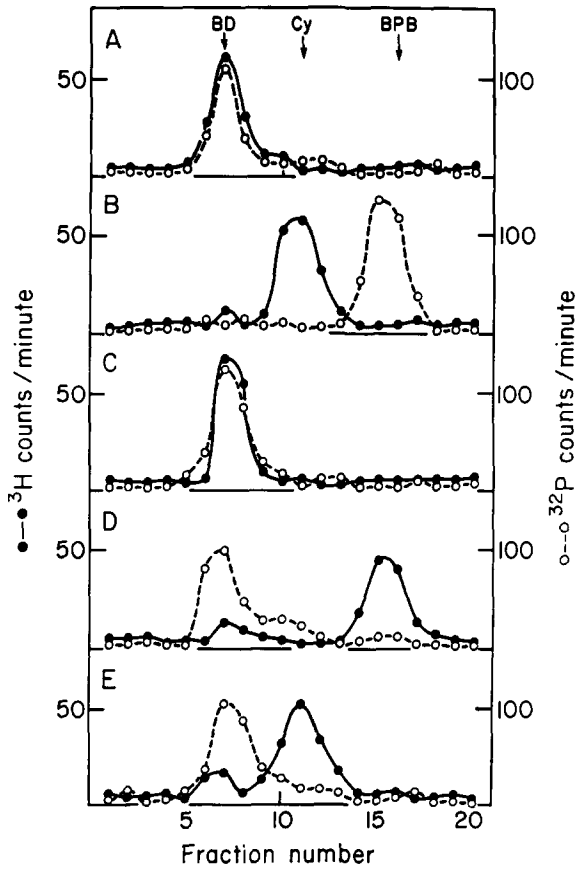

Figure 1. Biogel A1.5 M Column Chromatography of Poliovirus RNA after Incubation in Extracts of HeLa Cells

Samples containing a mixture of approximately 200 cpm each of ^{32}P-and ^{3}H-tyrosine-labeled 35S poliovirion RNA were incubated under each of the conditions described below, followed by gel filtration chromatography as described in Experimental Procedures. Fractions were collected and ^{32}P (O---O) and ^{3}H (●——●) radioactivity was measured. Incubation conditions were (A) RSB for 15 min at 37°C; (B) a mixture of ribonucleases T1, T2, and A for 15 min at 37°C; (C) 500 μg/ml proteinase K for 1 hr at 37°C; (D) HeLa cytoplasmic extract for 15 min at 37°C; (E) HeLa nuclear wash for 15 min at 37°C. Preparation of extracts is described in Experimental Procedures. The column was calibrated by chromatographing a sample containing blue dextran 2000 (BD), cytochrome (Cy) and bromophenol blue (BPB).

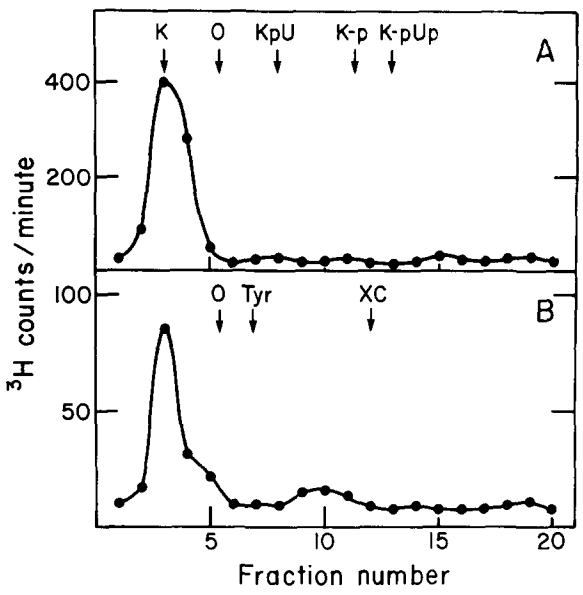

Figure 2. Ionophoretic Separation at pH 3.5 of ^{3}H-Tyrosine-Labeled Products of Proteinase K Digestion of VPg Unlinked from RNA

(A) ^{3}H-tyrosine-containing proteinase K-resistant oligopeptide (K) was prepared by proteinase K digestion of ^{3}H-tyrosine-labeled VPg, derived from VPg-pUp by venom phosphodiesterase digestion. The proteinase K digest was separated by pH 3.5 3MM paper ionophoresis, and the paper was fractionated and analyzed for ^{3}H radioactivity. K peptide covalently bound to phosphate, pU or pUp (K-p, K-pU, K-pUp) was made by proteinase K digestion of VPg-p, VPg-pU or VPg-pUp (Ambros and Baltimore, 1978), respectively, and the products were analyzed in identical ionophoretic analyses.

(B) ^{3}H-tyrosine-labeled 35S RNA was incubated with HeLa nuclear wash under the same conditions described in Figure 1D, followed by proteinase K digestion. The products were analyzed as described above. XC dye and tyrosine markers were included in a parallel lane. Tyrosine was detected by ninhydrin stain. (O) indicates the position of sample application. Anode is on the left.

phosphodiesterase digestion of VPg-pUp (Flanegan et al., 1977), was digested with proteinase K, and the products were analyzed by pH 3.5 3MM paper ionophoresis. All the ^{3}H radioactivity in the control sample was found in a peak which we have designated oligopeptide K (Figure 2A; see also Ambros and Baltimore, 1978). The mobility, relative to the XC dye of oligopeptide K covalently bound to either phosphate (K-p), uridine 5′-monophosphate (K-pU) or pUp (K-pUp), was established in parallel ionophoretic analyses. ^{3}H-tyrosine-containing material with mobility identical to oligopeptide K was generated by proteinase K digestion of the sample of 35S virion RNA that had been incubated in a HeLa nuclear wash (Figure 2B). This result further demonstrates that HeLa cells contain an activity capable of breaking the bond between the tyrosine in VPg and the 5′ terminal phosphate of poliovirus RNA.

To confirm the observation that removal of VPg from poliovirus RNA does not involve cleavage of any phosphodiester bonds between ribonucleotides near the 5′ end of the RNA, the 5′ terminal nonanucleotide was purified from ^{32}P-labeled poliovirion RNA and analyzed by two-dimensional electrophoresis and homochromatography. The nonanucleotide with VPg attached migrated slowly during cellulose acetate electrophoresis (first dimension) but rapidly in the homochromatography dimension (Figure 3A). After incubation of the nonanucleotide in a HeLa cell extract to remove VPg, all of the ^{32}P radioactivity migrated as a discrete species found at a position on the fingerprint markedly different from that of the nonanu-

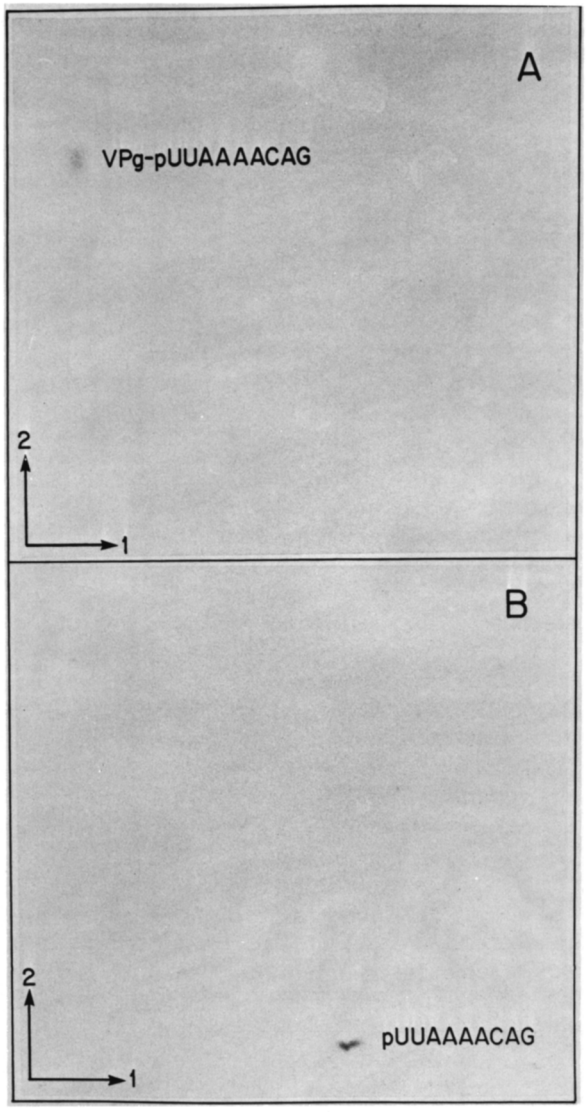

Figure 3. Two-Dimensional Analysis by Electrophoresis and Thin-Layer Chromatography of the 5′ Terminal RNAase T1-Resistant Nonanucleotide of Poliovirus Virion RNA

^{32}P-labeled 5′ terminal nonanucleotide was purified as described by Flanegan et al. (1977) and incubated for 15 min at 37°C in a HeLa cytoplasmic extract. A control sample was incubated in RSB. Samples were immediately phenol-extracted as described in Experimental Procedures. The phenol phase of the control sample and the aqueous phase of the extract treated sample were precipitated at −20°C with 10 vol of 95% ethanol in the presence of 0.4 M NaCl and 20 μg yeast-carrier tRNA. The precipitates were recovered by centrifugation and redissolved in H_2O, and a portion of each was analyzed by two-dimensional electrophoresis (dimension 1) and homochromatography (dimension 2) as described in Experimental Procedures. The remaining portion of each sample was analyzed by ribonuclease digestion as described in Figure 4 and Table 1. The sequences indicated are consistent with the data in Table 1 and with the known structure of the 5′ end of poliovirus RNAs (Flanegan et al., 1977; Pettersson et al., 1977).

cleotide with VPg attached (Figure 3B). The migration of this oligonucleotide was similar to that of the 5′ terminal nonanucleotide of poliovirus messenger RNA (pUUAAAACAG) (Pettersson et al., 1977). To analyze the composition of the species contained in the major spots in Figures 3A and 3B, a portion of each was digested with a mixture of ribonucleases T1, T2 and A. This treatment generates 3′ monophosphates and releases any 5′ terminal structure. The products were analyzed by pH 3.5 paper ionophoresis. Nuclease digestion of the nonanucleotide with VPg attached (control) released Up plus VPg-pUp but not pUp (Figure 4A); digestion of the nonanucleotide unlinked from VPg released pUp as well as Up (Figure 4B). The compositions of the two oligonucleotides were otherwise identical (Table 1) and are consistent with the published sequence of the 5′ end of poliovirus RNA (Flanegan et al., 1977; Pettersson et al., 1977). The identity of the spot labeled pUp in Figure 4B was confirmed after elution of the material from the paper: electrophoresis at pH 3.5 following P1 nuclease digestion yielded material migrating as uridine-5′-monophosphate plus inorganic phosphate. The lower than expected yield of VPg-pUp from the control nonanucleotide was probably due to the omission of sufficient bovine serum albumin carrier protein to prevent selective loss of VPg (Flanegan et al., 1977). (Yields were calculated without correcting for unequal labeling of the four nucleoside monophosphates.) Similar results were obtained when ^{32}P-labeled 5′ terminal nonanucleotide was incubated in a HeLa nuclear wash and subsequently analyzed as described above; pUp was found to be the 5′ terminal structure. These results confirm that VPg is removed by cleavage of the bond between the tyrosine in VPg and the 5′ terminal phosphate of RNA.

The solubility of the VPg-linked 5′ terminal nonanucleotide in phenol and its partition to the aqueous phase after removal of VPg was used as a rapid assay for VPg removal. Samples of purified ^{32}P-labeled 5′ terminal nonanucleotide were added to HeLa cell extracts as described in Figure 3. At various times of incubation, samples of each digest were removed and phenol-extracted, and the portion of radioactivity in the aqueous phase was calculated. The kinetics of removal of VPg from the ^{32}P-labeled 5′ terminal nonanucleotide were compared in HeLa cytoplasm (Figure 5A, open circles) and HeLa nuclear wash (Figure 5B, open circles). The amount of extract used for each time point was equivalent to the cytoplasm from about 1.8×10^5 cells and the nuclear wash from about 2.5×10^5 cells. The kinetics were slightly faster in the cytoplasmic extract than in the nuclear wash. The above dilutions of each extract were then assayed

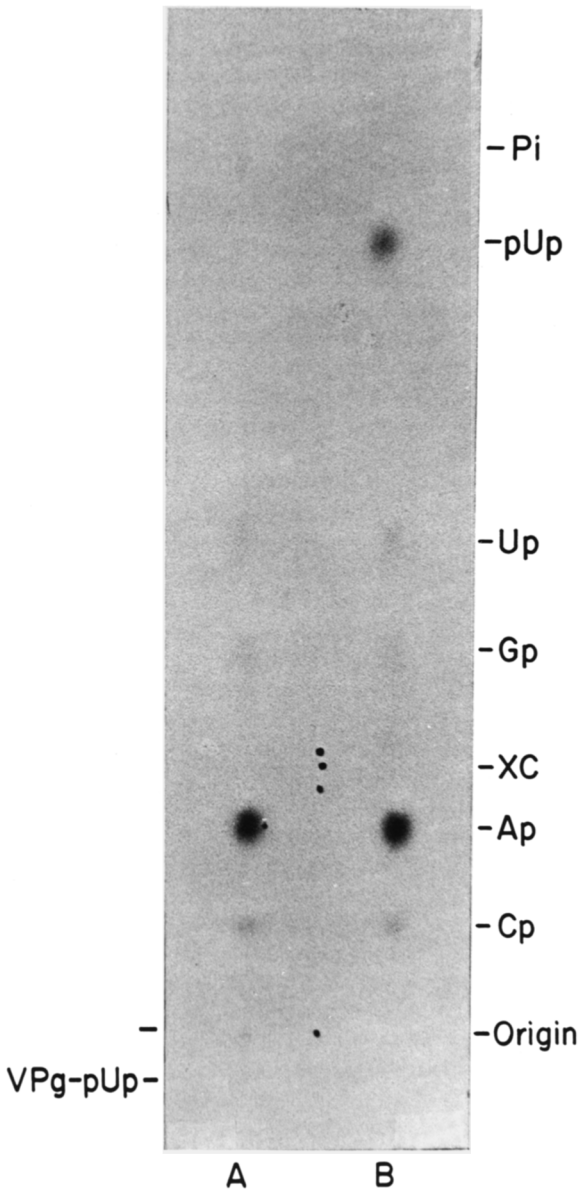

Figure 4. Ionophoretic Separation at pH 3.5 of Digestion Products Derived from the 5′ Terminal Nonanucleotide of Poliovirus RNA

A portion of the ^{32}P-labeled 5′ terminal nonanucleotide samples analyzed in Figure 3 was digested with a mixture of ribonucleases T1, T2, and A, and the products were separated by pH 3.5 ionophoresis on 3MM paper. The paper was dried and autoradiographed using Kodak XR5 film. (A) digest of untreated nonanucleotide (see Figure 3A). (B) digest of HeLa extract-treated nonanucleotide (see Figure 3B). The positions of XC dye, mononucleotides Cp, Ap, Gp, Up and free phosphate, Pi, were determined in a parallel lane. Samples were applied at origin and anode was below. The positions of pUp and VPg-pUp are indicated.

for VPg removal using ^{3}H-tyrosine-labeled 35S poliovirion RNA as a substrate. Samples were taken at various times of incubation and chromatographed on Biogel A1.5 M, and the percentage of

Table 1. Digestion Products Derived from the 5′ Terminal Nonanucleotide of Poliovirus RNA

Treatment of the Nonanucleotide	Relative Yield of Products					
	VPg-pUp	pUp	Cp	Ap	Gp	Up
None (Figure 3A)	0.8	0	1.2	5.1	1.0	1.0
HeLa Cell Extract (Figure 3B)	0	2.6	1.1	5.3	1.0	1.1

A portion of the ^{32}P-labeled nonanucleotide samples analyzed in Figure 3 was digested with a mixture of ribonucleases T1, T2 and A, and the products were separated by pH 3.5 3MM paper ionophoresis (Figure 4). The yield of each product is expressed as the ratio of radioactivity in that product to radioactivity in the Gp residue.

total radioactivity unlinked from RNA was calculated. ^{32}P-labeled marker 35S RNA was included in each sample to monitor for ribonuclease. In no case was ribonuclease activity sufficient to account for the appearance of significant ^{3}H radioactivity at the position in the Biogel column of free VPg or smaller. For each time of incubation in the cytoplasmic extract, all ^{3}H radioactivity unlinked from RNA appeared as a peak of small molecular weight material at the position of the peak in Figure 1D. The kinetics of removal of VPg as measured by this assay (Figure 5A, closed circles) are slower than those measured by the phenol partition assay (Figure 5A, open circles). This is probably due to protease activity present in the cytoplasmic extract capable of degrading large portions of the VPg attached to the nonanucleotide. Reconstruction experiments (data not shown) have determined that treatment with proteinase K or Pronase renders the 5′ terminal nonanucleotide aqueous-soluble. As shown earlier (Figure 1C), the ^{3}H-tyrosine assay for VPg removal is insensitive to contaminating protease, but measures breakage of the tyrosine-phosphate linkage (Figure 2). This assay is therefore a more accurate representation of the kinetics of breakage of the VPg-RNA bond. At 15 min of incubation in the cytoplasmic extracts, both assays indicate about 90% removal of VPg. This is consistent with the data presented in Table 1, which show that pUp is the predominant 5′ terminal structure of the nonanucleotide after 15 min of incubation in a HeLa cytoplasmic extract.

The kinetics of VPg removal in a HeLa nuclear wash are very similar when assayed with the nonanucleotide phenol extraction assay (Figure 5B, open circles) or with the column assay (Figure 5B, closed circles). Furthermore, at each time point, that ^{3}H radioactivity which was unlinked from RNA was found in a peak at the position of intact VPg (as in Figure 1E). Apparently the HeLa nuclear wash used in these experiments contains the activity able to unlink VPg from poliovirus RNA with

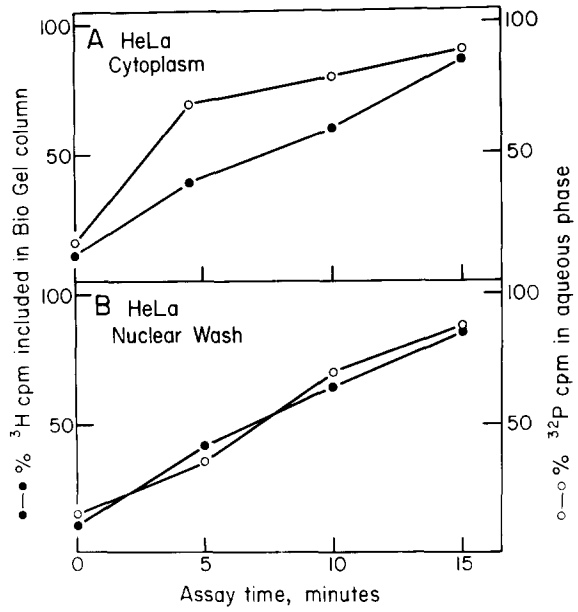

Figure 5. Kinetics of Removal of VPg from Poliovirus RNA in Extracts of HeLa Cells

Either the ^{32}P-labeled 5′ terminal nonanucleotide of poliovirus RNA or ^{3}H-tyrosine-labeled poliovirus 35S RNA was incubated at 37°C for the indicated times in a sample of HeLa cytoplasmic extract from approximately 1.8 × 10^6 cells (A) or HeLa nuclear wash equivalent to approximately 2.5 × 10^5 cells (B). VPg removal from RNA was assayed for each time point by phenol extraction of the nonanucleotide or Biogel column chromatography of the ^{3}H-tyrosine RNA as described in Experimental Procedures.

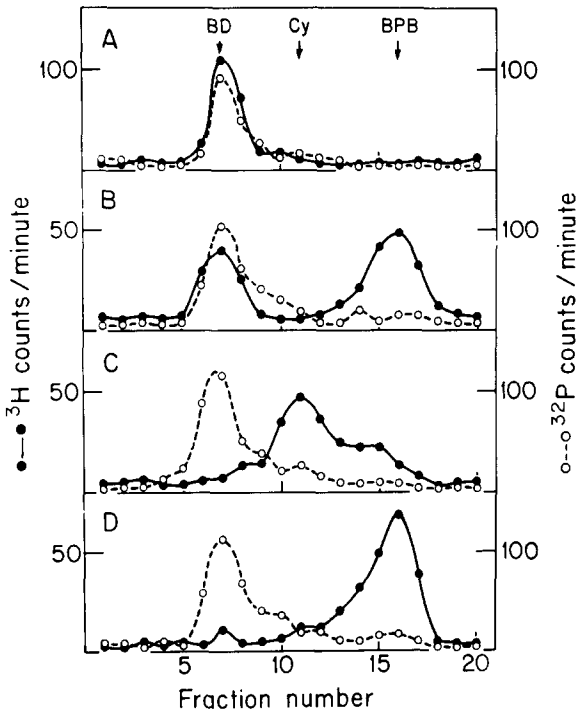

Figure 6. Biogel Column Chromatography of Poliovirus RNA after Incubation in Eucaryotic Cell-Free Extracts

The experiment is identical to the one described in Figure 1, except that RNA was incubated in (A) RSB, (B) a reticulocyte lysate, (C) a cell-free extract of mouse L cells or (D) a wheat germ cell-free extract. Sources of extracts and the method of their preparation are given in Experimental Procedures.

much less contaminating protease than is found in cytoplasmic extracts.

Removal of VPg from poliovirus RNA was found to follow the same kinetics (as measured by both assays described above) in extracts of poliovirus-infected HeLa cells as in uninfected HeLa cells (data not shown).

Cell-free extracts of three other cell types were assayed using a protocol identical to that used in the experiment described in Figure 1. Figure 6A shows that 35S virion RNA labeled with ^{32}P (open circles) and ^{3}H-tyrosine (closed circles) eluted at the excluded volume of a Biogel A1.5 M column. ^{3}H-tyrosine-labeled 35S RNA was incubated in a rabbit reticulocyte lysate, and after 15 min, >60% of the ^{3}H radioactivity was found in small molecular weight material unlinked from the ^{32}P-labeled 35S poliovirus RNA marker (Figure 6B). The same experiment performed with a mouse L cell cell-free extract (Figure 6C) and a wheat germ extract (Figure 6D) had similar results, except that the L cell extract left a large portion of the unlinked ^{3}H-tyrosine in material the size of VPg, while the wheat germ extracts appeared to degrade VPg. No ability to separate ^{3}H-tyrosine from viral RNA was detected in the soluble proteins released from E. coli by sonication (data not shown).

The phenol partition assay was used to determine some properties of unlinking activity in the HeLa nuclear wash. A 200 μl sample of the same nuclear wash assayed as shown in Figure 1E was sedimented through a 10–30% glycerol gradient as described in Experimental Procedures. Unlinking activity sedimented as a peak at approximately 3S (data not shown).

To determine whether divalent cations were required for unlinking activity, samples of a HeLa nuclear wash were assayed in the presence of 1 mM $MgCl_2$. Addition of 2 M EDTA completely inhibited unlinking activity, and $MgCl_2$ in 1 mM excess over EDTA reactivated the activity (Table 2). These results indicate that a divalent cation is necessary for unlinking activity.

To measure the heat stability of unlinking activity, samples of HeLa nuclear wash were incubated at 50 and 37°C for various times, and immediately thereafter assayed at 30°C for the amount of unlinking activity remaining. The half-life of unlinking activity at 50°C was found to be approximately 2 min, and the half-life at 37°C was found to be about 10 min (Table 3).

Discussion

Our finding that VPg is removed from poliovirus

Table 2. Divalent Cation Requirement of HeLa Cell Nuclear Wash Activity That Unlinks VPg from Poliovirus 35S RNA

Additions	% ^{3}H Radioactivity Phenol-Soluble
None	10
Nuclear Wash, 1 mM $MgCl_2$	79
Nuclear Wash, 1 mM $MgCl_2$, 2 mM EDTA	13
Nuclear Wash, 3 mM $MgCl_2$, 2 mM EDTA	96

Samples of a HeLa nuclear wash were assayed for ability to remove ^{3}H-tyrosine-labeled VPg from poliovirus 35S RNA under the indicated reaction conditions. Removal of VPg was measured by the phenol extraction method as described in Experimental Procedures.

Table 3. Heat Sensitivity of the HeLa Cell Nuclear Wash Activity That Unlinks VPg from Poliovirus 35S RNA

Temperature and Time of Heating	% Unlinking Activity Remaining
50°C	
0 min	100
1 min	75
2 min	40
5 min	25
10 min	15
37°C	
2 min	80
6 min	63
10 min	51

Samples of a HeLa nuclear wash were heated at the indicated temperatures for various times, and immediately thereafter incubated in the presence of ^{3}H-tyrosine-labeled poliovirus 35S RNA and 1 mM $MgCl_2$ for 10 min at 30°C. Removal of VPg was measured as in Table 2.

RNA by an activity in extracts of HeLa cells supports the hypothesis that the maturation of poliovirus messenger RNA during poliovirus replication involves the removal of VPg from the 5′ end of intracellular RNA molecules. The fact that the activity is able to remove VPg from exogenously added poliovirus RNA and leave the same 5′ end structure (pUUAAAACAG) as found on messenger RNA further supports the model that VPg is unlinked from poliovirus RNA by the specific cleavage of the bond between the tyrosine residue in VPg and the 5′ terminal phosphate of poliovirus RNA (Flanegan et al., 1977; Pettersson et al., 1977). Further evidence for the specificity of this unlinking activity is the fact that a preparation from HeLa cell nuclei cleaves VPg from the RNA, leaving VPg intact with the tyrosine of VPg completely free of covalently bound RNA.

We have described here three assays for activity able to unlink VPg from poliovirus RNA. One involves phenol extraction of the 5′ terminal nonanucleotide labeled with ^{32}P, and the second utilizes Biogel column chromatography of 35S RNA with ^{3}H-tyrosine label in VPg. The latter assay is not prone to artifactual results due to contaminating proteases, and in the absence of appreciable nuclease, measures only breakage of the tyrosine-phosphate linkage between VPg and the RNA. The 5′ terminal nonanucleotide assay is sensitive to proteolytic removal of VPg. When assaying preparations of unlinking activity which are relatively free of protease, we find that the phenol extraction assay is more rapid and convenient to use. A third assay utilizes the same phenol extraction procedure, but the substrate is ^{3}H-tyrosine-labeled 35S RNA. Unlinking activity is detected by the shift of ^{3}H radioactivity from the aqueous phase to the phenol phase. This assay is rapid but can be used only under conditions where ribonuclease activity does not interfere.

A partially purified preparation of unlinking activity can be obtained by a 0.2 M KCl wash of uninfected HeLa cell nuclei. Our finding that this activity sediments as a 3S peak on a glycerol gradient, is heat-labile and is found in uninfected cells is consistent with a single cellular enzyme being responsible. We have no evidence that the enzyme responsible for the activity studied in this paper is involved in normal nuclear processes. The cell fractionation procedure used in these experiments is relatively crude, and there is undoubtedly significant cross-contamination between our nuclear and cytoplasmic preparations. We do not know the normal function of this enzyme in uninfected cells, nor do we know whether the unlinking activities measured in extracts of rabbit reticulocytes, L cells or wheat germ are due to an enzyme functionally related to the one in HeLa cells. Answers to these questions will have to await further purification of this activity and detailed characterization of its substrate specificity. It seems reasonable at this point to postulate that protein-nucleic acid linkages via phosphodiester bonds to tyrosine may be found in normal cells, and that poliovirus uses for maturation of poliovirus messenger RNA a cellular enzyme normally involved in the cleavage of such linkages. One such linkage is known in bacteria—the joining of a uridylate residue to the regulatory protein (PII) of glutamine synthetase (Adler, Purich and Stadtman, 1975)—but to our knowledge, no equivalent bond in mammalian cells has been described.

Experimental Procedures

HeLa cells were infected with type I poliovirus and virions were purified as described elsewhere (Baltimore, Girard and Darnell, 1966). Purification of poliovirions RNA labeled with ^{32}P-or ^{3}H-tyrosine was as previously described (Hewlett, Rose and Balti-

more, 1976; Ambros and Baltimore, 1978). Isolation and analysis of isotopically labeled VPg by Biogel A1.5 M column chromatography in the presence of sodium dodecylsulfate and analysis of protease digestion products by 3MM paper ionophoresis are described elsewhere (Ambros and Baltimore, 1978). The conditions for ribonuclease T1, T2 and A digestion, proteinase K digestion and the method for purifying the 5′ terminal T1 RNAase-resistant nonanucleotide of poliovirion RNA are described elsewhere (Flanegan et al., 1977).

Cell Extracts

Reticulocyte lysates, L cell and wheat germ cell-free extracts were gifts from John K. Rose. Wheat germ extracts were prepared according to a method described elsewhere (Roberts and Paterson, 1976). Reticulocyte lysates, HeLa and L cell cytoplasmic extracts were prepared as described elsewhere (Villa-Komaroff et al., 1974), with some modifications (Rose et al., 1978).

Nuclear Wash

An extract of HeLa cell nuclei was made as follows: approximately 4×10^8 cells were washed twice with Earle's saline and resuspended in a volume of 10 mM HEPES (pH 7.5), 15 mM KCl, 1.5 mM Mg acetate, 6 mM 2-mercaptoethanol (hypotonic buffer) equal to about twice the packed cell volume. After 5 min at 0°C, the cells were broken with 15 strokes of a Dounce homogenizer. Nuclei plus larger cell debris were recovered by centrifugation at 5000 rpm for 2 min at 0°C in a Sorvall type SS-34 rotor. The sediment was washed twice by resuspension in hypotonic buffer followed by centrifugation for 2 min at 5000 rpm. It was then resuspended in 10 mM HEPES (pH 7.5), 1.5 mM Mg acetate, 6 mM 2-mercaptoethanol, 200 mM KCl, and allowed to stand on ice for 45 min with occasional gentle mixing. The supernatant was then recovered by centrifugation at 20,000 × g for 30 min. This nuclear wash was stored at −70°C in small portions.

Assay for Removal of VPg from Poliovirus RNA

All incubations were performed in 1.5 ml polypropylene Eppendorf microcentrifuge vials. To each vial was added a mixture of 15 μl RSB [10 mM Tris (pH 7.5), 10 mM NaCl, 1.5 mM $MgCl_2$], 2 μl of 20 mg/ml yeast tRNA carrier and 4 μl of poliovirus RNA substrate dissolved in RSB (approximately 200 cpm in 0.1 pmole of RNA). 5 μl of extract were then added, and the mixture was mixed briefly and left to incubate at 37°C. At the end of the assay time, 1 μl of 20 mg/ml bovine serum albumin was added, and the degree of removal of VPg from the RNA was immediately measured.

When the ^{32}P-labeled 5′ terminal nonanucleotide of poliovirus was used as a substrate, removal of the VPg attached to it was detected by phenol extraction. The nonanucleotide with VPg attached is phenol-soluble, whereas with VPg removed, it is partitioned to the aqueous phase. After incubation, the sample volume was adjusted to 80 μl with RSB and extracted with 80 μl of redistilled phenol. The aqueous phase was removed and the radioactivity in each phase was measured by liquid scintillation counting in Aquasol or by Cerenkov radiation. The degree of removal of the protein from the nonanucleotide was expressed as the ratio of radioactivity in the aqueous phase to the total radioactivity in both phases.

When ^{3}H-tyrosine-labeled virion RNA was used as a substrate, removal of the protein was detected by Biogel A1.5 M column chromatography. In the presence of sodium dodecylsulfate, VPg free of RNA elutes from this column at the position of a protein of about 12,000 molecular weight (Ambros and Baltimore, 1978). VPg attached to RNA elutes with the excluded volume of the column. ^{32}P-labeled 35S poliovirion RNA was included in each sample to monitor ribonuclease activity. After incubation in the cell extract, 20 μl of 30% sucrose, 0.5% sodium dodecylsulfate, 50 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA were added, and the sample was mixed thoroughly and layered onto a 1 × 10 cm Biogel A1.5 M column. The column was equilibrated with 0.5% sodium dodecylsulfate, 50 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and eluted in 15 drop fractions with the same buffer. Radioactivity was measured by liquid scintillation counting in Aquasol. Counter settings were adjusted so that ^{32}P and ^{3}H radioactivity was measured with no significant spillover. The degree of removal of VPg from RNA was expressed as the ratio of ^{3}H radioactivity included in the column fractions containing proteins of 12,000 molecular weight or less to the total ^{3}H radioactivity eluted from the column.

Removal of ^{3}H-tyrosine-labeled VPg from poliovirus 35S RNA was also assayed by phenol extraction exactly as described above for the ^{32}P-labeled 5′ terminal nonanucleotide substrate. When attached to 35S RNA, VPg remains in the aqueous phase after phenol extraction; freed from RNA, it partitions to the phenol phase. The portion of VPg removed was expressed as percentage of ^{3}H radioactivity which was phenol-soluble. We found this assay to be free from artifact only in preparations free of large amounts of contaminating ribonuclease (see Results).

Glycerol Gradient Centrifugation

A 200 μl sample of a HeLa cell nuclear wash was sedimented through a 10–30% glycerol gradient containing 10 mM HEPES (pH 7.5), 15 mM KCl, 1.5 mM Mg acetate, 6 mM 2-mercaptoethanol in a Beckman SW41 rotor at 40,000 rpm for 21 hr at 4°C. Samples were collected and a 50 μl portion of each was assayed for unlinking activity using ^{3}H-tyrosine-labeled 35S poliovirion RNA as substrate. Unlinking of VPg from RNA was measured by phenol extraction. Bovine serum albumin (4.4S) and cytochrome c (1.7S) markers were sedimented in a parallel gradient.

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Note Added in Proof

Rothberg et al. have recently published evidence for the tyrosine-phosphate linkage between VPg and poliovirus RNA [Rothberg, P. G., Harris, T. J. R., Nomoto, A. and Wimmer, E. (1978). O^4-(5′-uridylyl)tyrosine is the bond between the genome-linked protein and the RNA of poliovirus. Proc. Nat. Acad. Sci. USA *75*, 4868–4872].