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NOTES

Lack of Evidence for VPg Priming of Poliovirus RNA Synthesis in the Host Factor-Dependent In Vitro Replicase Reaction

NANCY C. ANDREWS† AND DAVID BALTIMORE*

Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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Anti-VPg immunoprecipitable RNA labeled in vitro during a poliovirus RNA polymerase reaction was formed by the elongation of VPg-containing template fragments rather than by initiation with VPg. The reaction was dependent on a host factor (terminal uridylyl transferase). The incorporation of labeled UTP could be detected with only the host factor present.

Poliovirus is a single-stranded RNA virus of positive polarity with poly(A) at its 3′ end (7, 14, 18). It encodes a primer-dependent RNA polymerase, which has been purified and shown to utilize oligo(U) to copy virion RNA in vitro (3, 9, 16, 17). A protein from uninfected cells termed host factor can replace oligo(U) in the initiation of RNA synthesis in cell-free systems (2, 3, 5, 6). We have found that the host factor is a terminal uridylyl transferase which adds uridylic acid residues to the 3′ end of poly(A) on virion RNA (1; N. C. Andrews and D. Baltimore, Proc. Natl. Acad. Sci. USA, in press).

A 22-amino-acid peptide, VPg, is attached to the 5′ ends of viral RNAs in vivo (8, 12, 13). Earlier results suggested that VPg was involved in the initiation of poliovirus RNA synthesis in vitro. Anti-VPg antibodies immunoprecipitated 1% of the material labeled with [α-^{32}P]UTP in the host factor-stimulated replicase reaction, and these products yielded specific low-molecular-weight bands when analyzed by electrophoresis (4, 11). These small molecules were thought to be nascent transcripts primed by VPg. In this communication, we present evidence that they are, instead, aberrant products unrelated to the replication of intact molecules of RNA.

To find out which components of the replicase reaction were required for the synthesis of anti-VPg immunoprecipitable products, we incubated various combinations of viral polymerase, host factor, and viral RNA with [α-^{32}P]UTP in replicase reaction mixtures (1) and immunoprecipitated the products with anti-VPg antibody (4). As shown previously (4, 11), host factor plus highly purified viral polymerase and viral RNA incorporated labeled UTP into anti-VPg immunoprecipitable products (Fig. 1, lane 4), but in the absence of host factor no reaction was evident (lane 3). Surprisingly, however, host factor alone formed similar products (Fig. 1, lane 1) in a reaction which required viral RNA (lane 2). All immunoprecipitable material could be competed away by excess VPg peptide (data not shown). In previous work, immunoprecipitable products made in the absence of viral polymerase were not detected (4). Those experiments used a 10- to 25-fold-less-concentrated source of host factor. When we used less-concentrated host factor, we also did not see incorporation without viral polymerase (data not shown). Because host factor exhibits a strong terminal uridylyl transferase activity (1; Andrews and Baltimore, in press), this enzyme is presumably responsible for

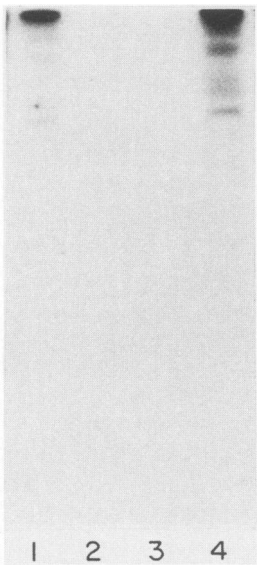

FIG. 1. Formation of anti-VPg immunoprecipitable products by host factor alone. Replicase reactions with various combinations of enzymes were immunoprecipitated with anti-VPg antibodies as described in the text. Immunoprecipitated material was fractionated on an 18.5% polyacrylamide–sodium dodecyl sulfate gel with BPB marker just run off the bottom. Previous work indicated that the RNA components of VPg-linked immunoprecipitable products were 50 to 150 nucleotides long (4). An autoradiogram of the gel is shown here. Lanes: 1, virion RNA plus host factor; 2, host factor alone; 3, viral polymerase plus virion RNA; 4, viral polymerase plus host factor and virion RNA.

* Corresponding author.
† Present address: Harvard Medical School, Boston, MA 02115.

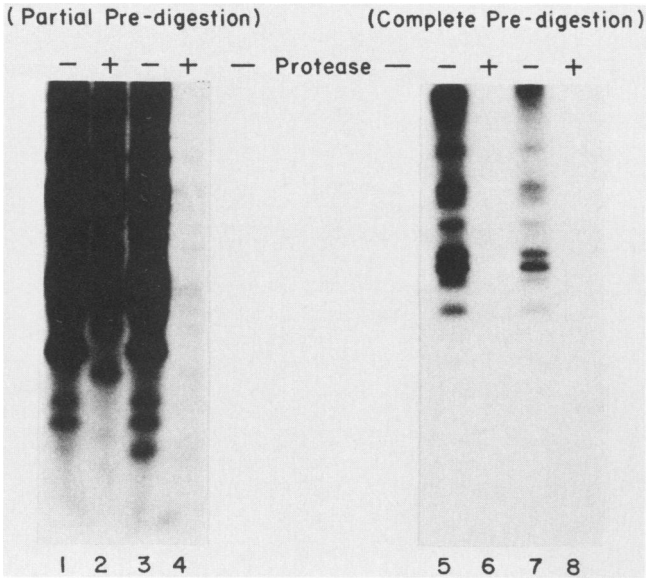

FIG. 2. Anti-VPg immunoprecipitation of replicase reaction products after predigestion of the template with proteinase K. Replicase reactions were carried out with virion RNA which had or had not been predigested with proteinase K. Products were immunoprecipitated with anti-VPg antibodies and electrophoresed through 12.5% polyacrylamide–sodium dodecyl sulfate gels. Autoradiograms of two gels are shown here. Lanes 2 and 4 show products of reactions in which the template had been partially predigested; lanes 6 and 8 show products of reactions in which the template had been completely predigested. Lanes 1, 2, 5, and 6, Crude viral polymerase plus host factor; lanes 3, 4, 7, and 8, highly purified viral polymerase plus host factor. Crude viral polymerase was severalfold more active than highly purified viral polymerase, although this is not evident from lanes 1 and 3 because of overexposure.

the incorporation of [α-^{32}P]UTP in the absence of viral polymerase.

In the absence of viral polymerase, no source of viral protein is added, except for the VPg present on input template RNA. Thus, it must provide the antigenicity that mediates immunoprecipitation. The incorporated radioactivity behaves as if it were covalently linked to VPg-containing RNA (4), implying that either short, 5′-terminal fragments of virion RNA are being elongated by host factor or VPg is being recycled from the template by cleavage and reutilization.

To examine whether the VPg from virion RNA was also responsible for the antigenicity of the [α-^{32}P]UTP-labeled products of the replicase reaction including viral polymerase, we digested virion RNA with proteinase K prior to incubation. RNA (3.3 μg) was treated with either 2 μg of proteinase K for 15 min at 37°C (partial digestion) or 56 μg of proteinase K for 60 min at 37°C (complete digestion by the criterion that VPg was no longer antigenically detectable). Proteinase K was removed by phenol extraction. Partial digestion prior to the replicase reaction shifted the mobilities of the products when two different preparations of viral polymerase were tested (Fig. 2, lanes 1 to 4). Complete digestion prior to the replicase reaction made the products totally lose antigenicity (Fig. 2, lanes 5 to 8). Predigestion did not interfere with the ability of RNA to stimulate the incorporation of [α-^{32}P]UTP into nonimmunoprecipitable products (data not shown). Thus, it appears that all immunoprecipitable products in these reactions gain their antigenicity from VPg on input RNA molecules.

In the presence of viral polymerase, in vitro synthesis of labeled, anti-VPg immunoprecipitable products was stimulated by the inclusion of ATP, GTP, and CTP (4). The dependence on all four nucleotides suggests that the products are heteropolymers, although nascent negative strands should contain predominantly poly(U). By contrast, in the absence of viral polymerase, synthesis was not impaired by the omission of any one of the three unlabeled nucleotides (Fig. 3). This result is consistent with evidence that the host factor is terminal uridylyl transferase and that this enzyme makes polymers consisting mainly of UMP moieties. There was a slight stimulation when CTP was omitted (Fig. 3, lane 4), presumably because CTP weakly competes with UTP as a substrate for terminal uridylyl transferase (1). Thus, the immunoprecipitable material made by terminal uridylyl transferase in the presence of viral RNA consists of short RNA molecules containing labeled stretches of UMP residues. These RNA molecules could either be elongated fragments of viral RNA or de novo-synthesized poly(U) primed by VPg removed from the input viral RNA.

Because free VPg is not a substrate for terminal uridylyl transferase (Andrews and Baltimore, in press), we examined whether the host factor-mediated incorporation might be due to the elongation of 5′-terminal fragments of viral RNA. To this end, we prepared a filter with bound, defined fragments of cloned poliovirus cDNA and hybridized the labeled RNA to it. If the incorporation occurred selectively on the ends of 5′-terminal RNA, the labeled RNA should hybridize to 5′ but not 3′ DNA fragments.

Immunoprecipitated RNA was treated with proteinase K as described above and added to hybridization buffer (50% formamide, 100 μg of tRNA per ml, 0.45 M NaCl, 45 mM sodium citrate). Filters were prepared by standard proce-

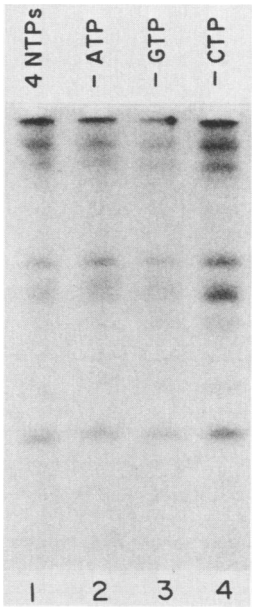

FIG. 3. Anti-VPg immunoprecipitation of products of host factor incubated with virion RNA. Host factor was incubated with virion RNA in the presence of [α-^{32}P]UTP and different combinations of nucleoside triphosphates. Products were immunoprecipitated with anti-VPg antibodies and fractionated on a 12.5% polyacrylamide–sodium dodecyl sulfate gel. An autoradiogram of the gel is shown here. Lanes: 1, all four nucleoside triphosphates; 2, minus ATP; 3, minus GTP; 4, minus CTP.

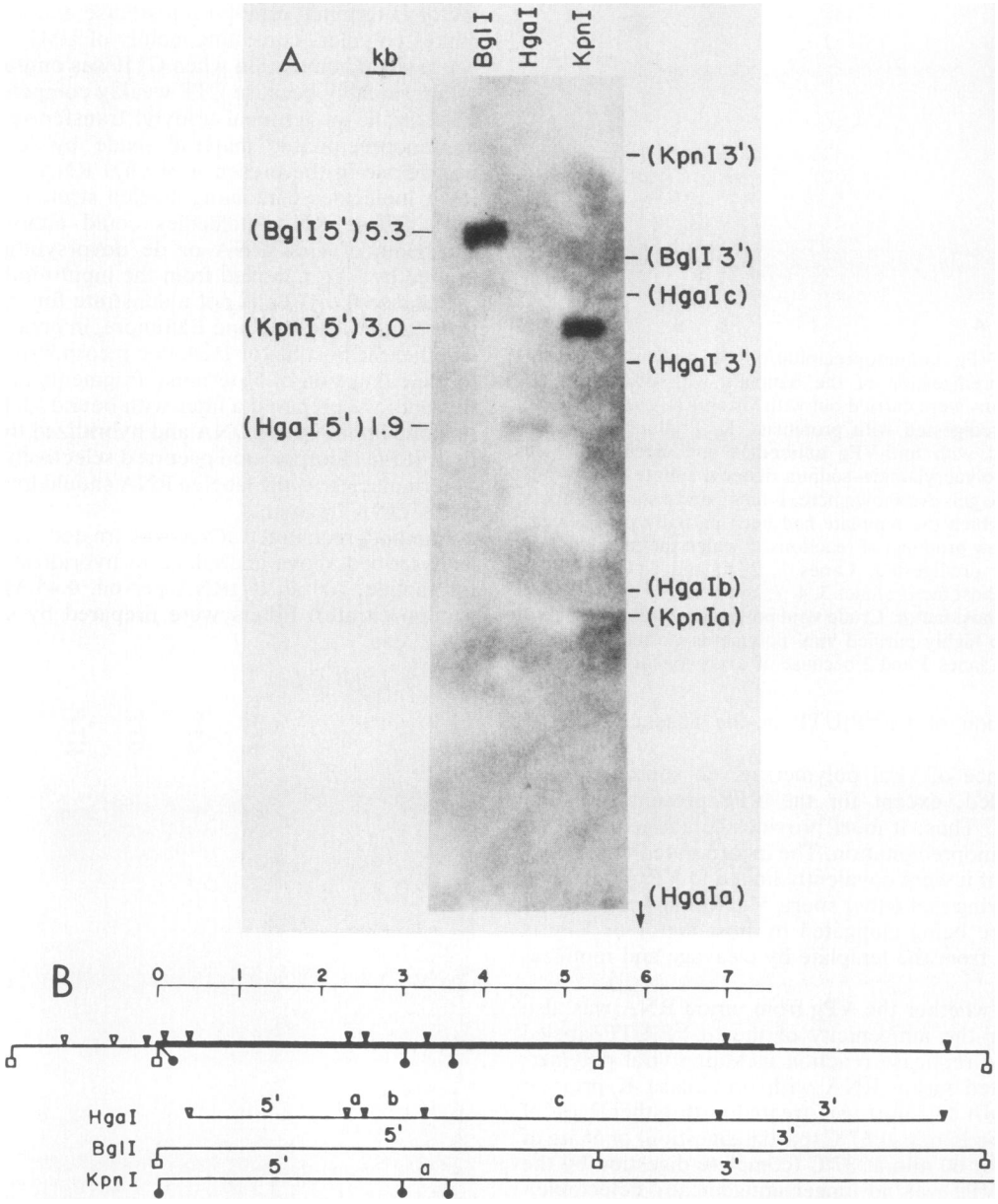

FIG. 4. Hybridization of anti-VPg immunoprecipitable products to poliovirus cDNA fragments. (A) Autoradiogram prepared from a filter hybridized to ^{32}P-labeled RNA from anti-VPg immunoprecipitable replicase reaction products. (B) Restriction sites. The dark bar represents poliovirus sequences; the plasmid sequences are represented in a linear fashion starting arbitrarily with one *BglI* site. The positions of fragments carrying poliovirus sequences are indicated beside the blot in panel A. kb, Kilobases.

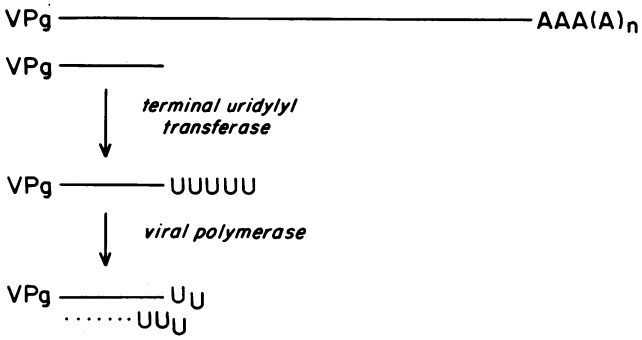

FIG. 5. Model of formation of anti-VPg immunoprecipitable products by terminal uridylyl transferase plus viral polymerase. Shown is a model to explain the formation of [α-^{32}P]UTP-labeled anti-VPg immunoprecipitable products in the in vitro replicase reaction. Terminal uridylyl transferase adds uridylic acid residues to the 3′ ends of short 5′-terminal fragments of virion RNA. The oligo(U) tract contributes to the formation of a hairpin primer by annealing to purine stretches within the heteropolymeric sequences. The hairpins are recognized and elongated by viral polymerase. In this way, label added to the reaction can be covalently linked to VPg through small fragments of the template.

dures (10). Poliovirus DNA from a plasmid clone was digested under standard conditions. Filters were prehybridized for 30 min and then hybridized overnight at 37°C. They were washed with 0.3 M NaCl–30 mM sodium citrate.

Restriction enzymes cleaved the cloned DNA as shown in the map in Fig. 4. The positions of fragments containing poliovirus sequences were visualized by ethidium bromide staining and are indicated by arrows. For each digest, only one of the several bands hybridized with the RNA made in vitro. Immunoprecipitated RNA always hybridized with fragments from the 5′ end of the viral genome and never with fragments from the 3′ end.

A model for the formation of anti-VPg immunoprecipitable [α-^{32}P]UTP-labeled products is easily formulated from these results (Fig. 5). The enzymatic activity of terminal uridylyl transferase apparently uridylylates 5′-terminal fragments that inevitably contaminate virion RNA preparations. The oligo(U) formed probably anneals to purine stretches within the fragments, creating a hairpin primer similar to that proposed for the copying of virion RNA (1). Viral polymerase elongates these molecules. This elongation of subgenomic molecules probably does not occur in vivo, where intact virion RNA molecules are assumed to act as templates for replication.

We presently believe that there is no conclusive evidence that VPg is involved in the initiation of RNA synthesis in vitro. Takegami et al. demonstrated the synthesis of VPgPUpU in a crude in vitro system but could not chase this material into long RNA strands (15). Either VPg does not prime negative-strand synthesis in vivo or the in vitro systems do not faithfully model the initiation of viral replication.

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