Biochemical and Genetic Studies of the Initiation of Human Rhinovirus 2 RNA Replication: Purification and Enzymatic Analysis of the RNA-Dependent RNA Polymerase 3D^{pol}

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The replication of human rhinovirus 2 (HRV2), a positive-stranded RNA virus belonging to the *Picornaviridae*, requires a virus-encoded RNA polymerase. We have expressed in *Escherichia coli* and purified both a glutathione *S*-transferase fusion polypeptide and an untagged form of the HRV2 RNA polymerase 3D^{pol}. Using in vitro assay systems previously described for poliovirus RNA polymerase 3D^{pol} (J. B. Flanagan and D. Baltimore, *Proc. Natl. Acad. Sci. USA* 74:3677–3680, 1977; A. V. Paul, J. H. van Boom, D. Filippov, and E. Wimmer, *Nature* 393:280–284, 1998), we have analyzed the biochemical properties of the two different enzyme preparations. HRV2 3D^{pol} is both template and primer dependent, and it catalyzes two types of synthetic reactions in the presence of UTP, Mn²⁺, and a poly(A) template. The first consists of an elongation reaction of an oligo(dT)₁₅ primer into poly(U). The second is a protein-priming reaction in which the enzyme covalently links UMP to the hydroxyl group of tyrosine in the terminal protein VPg, yielding VPgU. This precursor is elongated first into VPgUpU and then into VPg-linked poly(U), which is identical to the 5' end of picornavirus minus strands. The two forms of the enzyme are about equally active both in the oligonucleotide elongation and in the VPg-primed reaction. Various synthetic mutant VPgs were tested as substrates in the VPg uridylation reaction.

Rhinoviruses belong to the family of *Picornaviridae*, a large group of plus-strand RNA viruses with different host ranges and disease symptoms but similar genetic organizations and replication strategies (50, 60). This virus family has been subdivided into nine genera: *Enterovirus*, *Rhinovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Teschovirus*, and *Kobuvirus* (55). Rhinoviruses, with more than a hundred serotypes (23), are considered to be the most important causative agents of the common cold, for which no effective treatment or prevention has yet been developed (6, 22, 49). They can be classified into two groups based on their use of cellular receptors. The majority of human rhinoviruses (HRVs) bind to human intercellular adhesion molecule 1 (ICAM-1) (21, 57), while those belonging to the minor group, including HRV2, bind to various members of the low-density lipoprotein receptor gene family (27, 29).

HRV2, like other picornaviruses, is a small icosahedral particle composed of 60 copies each of the four capsid proteins VP1 to VP4, which enclose a plus-strand RNA genome covalently linked at the 5' end to the terminal protein VPg (33, 34, 50, 63). Replication of the viral RNA takes place in membranous vesicles in the cytoplasm of the infected host cell (31, 32, 68). Three RNA species have been recognized in cells infected with HRV2 (68). These include a single-stranded RNA of a length similar to that of genomic RNA; a double-stranded RNA, the replicative form (RF); and a multistranded

structure, called the replicative intermediate (RI) (68). It is generally accepted that picornavirus RNA replication is a two-step process carried out primarily by the viral RNA polymerase in conjunction with other viral and possibly also cellular proteins (reviewed in references 1 and 68). First the parental RNA is transcribed into a complementary minus strand, which is then used as the template for the synthesis of the progeny plus strands. Although the basic steps of RNA replication have been well defined, very little is known about the details of these processes. Most of the information accumulated thus far has been derived from studies of poliovirus, whose properties and replication are believed to parallel those of the other members of the entire picornavirus family.

The RNA genome of HRV2 (7,151 nucleotides [nt]) contains a long 5' nontranslated region (5' NTR), a single large open reading frame, and a short 3' NTR terminating with a poly(A) tail (52) (Fig. 1). The 5' phosphate of the terminal UMP in picornaviral RNA is linked in a phosphodiester bond to the hydroxyl group of a tyrosine in VPg (2, 48) (Fig. 1). The first segment of the 5' NTR forms a cloverleaf-like structure (47) that is required for plus-strand RNA synthesis (3, 4, 67) and possibly for progression from translation to replication (16). The remainder of the 5' NTR consists of a large and highly structured segment, the internal ribosomal entry site (IRES) (30), whose primary function is to direct translation (7). The polyprotein, containing one structural (P1) and two nonstructural (P2 and P3) domains, is processed to mature viral proteins by viral proteinases 2A^{pro}, 3C^{pro}, and 3D^{pro} (reviewed in references 25; 52, and 54). All of the nonstructural proteins and some of their precursors are required for RNA replication, with proteins of the P3 domain most directly

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FIG. 1. Structure of HRV2 genomic RNA. The single-stranded RNA genome of HRV2 is shown with the terminal protein VPg (3B) at the 5' end of the 5' NTR. The attachment site of the 5'-terminal UMP of the RNA to the tyrosine of VPg is shown enlarged. The polyprotein contains structural (P1) and nonstructural (P2 and P3) domains. Vertical lines within the polyprotein box represent proteinase cleavage sites. Processing of the P3 domain by proteinase 3CD^{pro} is shown enlarged. The location of the *cre* in the 2A^{pro} coding region is indicated (17).

involved in the process of RNA synthesis. These include a small hydrophobic and membrane-binding protein, 3A, with its precursor, 3AB; the terminal protein VPg (3B); proteinase 3CD^{pro}; and the RNA polymerase 3D^{pol} (reviewed in references 1 and 65). The two important precursors, 3AB and 3CD^{pro}, possess RNA binding activities that are essential for viral replication (3, 4, 26, 40, 67).

It was nearly 30 years ago that an RNA polymerase activity was first detected in membrane-bound complexes of HRV2-infected HeLa cells (68). Subsequently, this activity was partially purified, and the enzyme was shown to be template and primer dependent *in vitro* (37). In these studies either homopolymeric or viral RNA sequences were used as templates with oligonucleotide primers that were elongated into complementary strands. At about the same time, other *in vivo* and *in vitro* studies with poliovirus suggested that VPg might serve as a primer in the initiation of picornaviral RNA replication (64). This possibility was supported by the observation that virus-infected HeLa cells contain VPgpUpU, in which VPg is covalently linked to UMP (10). In addition, the synthesis of VPgpU and VPgpUpU could be achieved *in vitro* by using membranous replication complexes isolated from virus-infected cells (58, 59, 62). Recently it was directly shown that the RNA polymerase of poliovirus possesses a synthetic activity *in vitro* that catalyzes the linkage of UMP to the hydroxyl group of tyrosine in VPg, yielding VPgpU and VPgpUpU (41). The final product in the uridylation of VPg on a poly(A) template is VPg-linked poly(U) (41), the 5' end of viral minus strands (38).

In addition to the nonstructural proteins, RNA replication also requires important *cis*-replicating elements located in the 5' NTR, the 3' NTR, possibly in the IRES, and in the coding sequences of picornaviral RNAs (reviewed in references 1 and 66). The exact functions of the 5' cloverleaf structure, the IRES, and the 3' NTR poly(A) in picornaviral RNA replication are not yet known. The internal replication signals (*cre*) of different picornaviruses all consist of a relatively simple hairpin

structure with a conserved AAACA sequence in the loop (20, 35, 36, 42, 46), which is essential for initiation of minus-strand synthesis (20, 36). We have recently identified the function of this motif in the *cre*(2C) of poliovirus type 1 Mahoney

FIG. 2. Purification of GST-HRV2 3D^{pol} and HRV2 3D^{pol}. (A) GST-HRV2 3D^{pol} was expressed in *E. coli* and purified to near-homogeneity as described in Materials and Methods. The purity of the enzymes was tested by SDS-PAGE followed by Coomassie-staining. Protein sizes (in kilodaltons) are given on the left. Shown are bacterial lysates before (lane 1) and after (lane 2) induction of GST-HRV2 3D^{pol} expression, the insoluble (lane 3) and soluble (lane 4) fractions of lysed bacteria expressing GST-HRV2 3D^{pol}, the soluble fraction after absorption to glutathione-Sepharose (lane 5), and the eluate containing GST-HRV2 3D^{pol} (lane 6). The major fraction corresponds to the 76.5-kDa GST-HRV2 3D^{pol} fusion protein. (B) HRV2 3D^{pol} was expressed in *E. coli* and purified as described in Materials and Methods. Shown are bacterial lysates before (lane 1) and after (lane 2) induction of HRV2 3D^{pol} expression, proteins that did not precipitate with 50% ammonium sulfate (lane 3), the flowthrough fraction of the sulphopropyl (SP)-Sepharose column (lane 4), 50 mM NaCl eluate of the SP-Sepharose column (lane 5), and 200 mM NaCl eluate of the SP-Sepharose column (lane 6). The major fraction corresponds to the 50.5-kDa HRV2 3D^{pol} protein.

FIG. 3. Comparison of the synthetic activities of GST-HRV2 3D^{pol}, HRV2 3D^{pol}, and PV1(M) 3D^{pol}. (A) Oligo(dT)₁₅-primed poly(U) synthesis by GST-HRV2 3D^{pol} (lanes 1 to 4) and HRV2 3D^{pol} (lanes 6 to 9). Standard assays were used as described in Materials and Methods. Lanes 1 and 6, no enzyme; lanes 2 and 7, no poly(A) template; lanes 3 and 8, no oligo(dT)₁₅; lanes 4 and 9, complete reaction mixtures. As a control for the enzymatic activity of HRV2 3D^{pol}, the empty pT5T vector was carried through the same expression and purification process as the pT5T vector containing the HRV2 3D^{pol} sequence (see Materials and Methods). Purified bacterial proteins were tested for polymerase activity (lane 5). (B) Synthesis of HRV2 VPgU(pU) and VPg-poly(U) by GST-HRV2 3D^{pol} and HRV2 3D^{pol}. Standard assays for VPg-poly(U) synthesis were used as described in Materials and Methods. (Panel 1) VPgU(pU) synthesis by GST-HRV2 3D^{pol} (lane 1) and HRV2 3D^{pol} (lane 2). (Panel 2) VPg-poly(U) synthesis by GST-HRV2 3D^{pol} (lane 1) and HRV2 3D^{pol} (lane 2). (C) Synthesis of (dT)₁₅-poly(U) and VPgU(pU) by PV1(M) 3D^{pol} and HRV2 3D^{pol}. The oligonucleotide-elongating and VPg-uridylylating activities of HRV2 3D^{pol} were measured as described in Materials and Methods, and that of PV1(M) 3D^{pol} was measured as described previously (41) except that the reaction mixture contained 0.5 mM Mn²⁺. Each enzyme was tested with its cognate VPg as a substrate. (Panel 1) (dT)₁₅-poly(U) synthesis by PV1(M) 3D^{pol} (lane 1) and HRV2 3D^{pol} (lane 2). (Panel 2) VPgU(pU) synthesis by PV1(M) 3D^{pol} (lane 1) and HRV2 3D^{pol} (lane 2). Quantified data are shown at the top, and autoradiographs are shown at the bottom.

[PV1(M)] RNA as the primary template for the synthesis of VPgU and VPgUpU by 3D^{pol} (42, 46).

In order to gain more insight into the properties of the enzyme responsible for RNA synthesis by HRV2, a virus belonging to a genus of *Picornaviridae* different from *Enterovirus*, we have expressed in *Escherichia coli* and purified two different forms of HRV2 3D^{pol}, one with a glutathione *S*-transferase (GST) tag and one with only an extra N-terminal methionine. In this study we used both forms of purified HRV2 3D^{pol} to test their activities on a poly(A) template in the oligonucleotide- and VPg-primed reactions. Our accompanying paper (17) describes the synthetic activity of HRV2 3D^{pol} on its cognate *cis*-replicating element that is located in the coding sequence of protein 2A^{pro}.

MATERIALS AND METHODS

Construction of plasmids. HRV2 cDNA (pT7HRV2) (12) nucleotide sequences given for plasmids or oligonucleotides refer to the full-length (nt 1 to 7151) plus-strand HRV2 cDNA sequence (52). All PCR fragments used for cloning were sequenced to ensure their accuracy.

(i) **pGEX-HRV2 3D.** The sequence for 3D^{pol} was amplified from pT7HRV2 by PCR with oligonucleotide primers 5'-CCCGGATCCGGCCAAATAACGTTA TCA-3' (plus-strand sequence) and 5'-GGGGAATTCTTAAATTTTTCATACCACTC-3' (minus-strand sequence). The amplified product was cut with *Bam*HI and *Eco*RI and was cloned into the same sites of pGEX-2T (Pharmacia Biotech). The resulting plasmid (pGEX-HRV2 3D) contained the HRV2 3D^{pol}

coding sequence fused at its N terminus to the 26-kDa GST from *Schistosoma japonicum*.

(ii) **pT5T-HRV2 3D.** The coding sequence for 3D^{pol} was amplified from pT7HRV2 by PCR using *Pfu* DNA polymerase and the oligonucleotide primers 5'-CCCATATGATGGGCCAAATAACGTTATCAAG-3' (plus-strand sequence) and 5'-CCGAATTCTTAAATTTTTCATACCACTC-3' (minus-strand sequence). The amplified product was cut with *Nde*I and *Eco*RI and was cloned into the same sites of pT5T (39). The resulting plasmid (pT5T-HRV2 3D) contained the HRV2 3D^{pol} sequence preceded by a methionine.

Peptides and proteins. (i) **VPg peptides.** Wild-type (wt) and mutant synthetic VPg peptides were a generous gift of J. H. van Boom (41, 42, 46).

(ii) **GST-HRV2 3D^{pol}.** GST-HRV2 3D^{pol} was purified as described elsewhere for PV1(M) GST-2C (43), except for the following modifications. *E. coli* strain BL21(DE3)pLysS was transformed with plasmid pGEX-HRV2 3D, and the protein was expressed at 16°C for 14 h. The yield of pure 3D^{pol} protein was about 3.5 mg/liter of bacterial culture.

(iii) **HRV2 3D^{pol} and PV1(M) 3D^{pol}.** HRV2 3D^{pol} and PV1(M) 3D^{pol} were expressed in *E. coli* strain BL21(DE3)pLysS from pT5T-HRV2 3D and pT5T-PV1(M) 3D (39), respectively, and were purified as described elsewhere for PV1(M) 3D^{pol} by Pata et al. (39). The yield of pure HRV2 3D^{pol} was essentially the same as that of GST-HRV2 3D^{pol} (about 3.5 mg/liter of culture). Control proteins for HRV2 3D^{pol} were purified like HRV2 3D^{pol}, with the exception that the expression vector pT5T (39) did not contain the 3D^{pol} coding sequences.

RNA-dependent RNA polymerase assays. (i) **VPgU(pU) and VPg-poly(U) synthesis on a poly(A) template by HRV2 3D^{pol} and GST-HRV2 3D^{pol}.** The enzyme assay was the same as that previously described for PV1(M) 3D^{pol} (41) except for the pH of the buffer and the divalent cation. The standard reaction mixture (20 μ l) for VPg uridylylation by HRV2 3D^{pol} contained 50 mM HEPES (pH 7.6), 0.2 mM manganese(II) acetate (MnAc₂; Aldrich), 8% glycerol, 0.5 μ g (0.35 μ M) of poly(A) RNA template (~200-nt - fragments; Pharmacia), 2 μ g of

FIG. 4. Requirements of HRV2 VPg uridylation and VPg-poly(U) synthesis by HRV2 3D^{pol}. The standard assay for VPg-poly(U) synthesis was used, as described in Materials and Methods, except that various ingredients were omitted or added, as shown. Lane 1, complete reaction mixture; lane 2, no enzyme; lane 3, no VPg; lane 4, no poly(A); lane 5, no Mn²⁺; lane 6, complete mixture plus 0.1% NP-40; lane 7, complete mixture plus 5 mM DTT. (Panel 1) Autoradiography of the gel; (panel 2) quantified data.

synthetic HRV2 VPg (50 μ M), 1 μ g (1 μ M) of purified 3D^{pol}, and 1 μ Ci of [α -³²P]UTP (0.017 μ M) (3,000 Ci/mmol; Dupont, NEN). The standard reaction mixture for VPg-poly(U) synthesis contained, in addition, 10 μ M unlabeled UTP. Samples were incubated for 1 h at 30°C, and reactions were stopped by addition of 5 μ l of gel-loading buffer (Bio-Rad) and analyzed on Tris-Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad) with 13.5% polyacrylamide. Gels were dried at 68°C for 2 h without fixing and autoradiographed for 1 h (Kodak Biomax MS film). Reaction products were quantitated by measuring the amount of [α -³²P]UMP incorporated into VPgpU(pU) and VPg-poly(U) products (Molecular Dynamics PhosphorImager, Storm 860), and the data were translated into counts per minute with the help of a radioactive marker. VPgpU(pU) refers to the sum of VPgpU and VPgpUpU.

The assay with GST-HRV2 3D^{pol} was similar to that described above except that the standard reaction mixture for VPgpU(pU) synthesis contained 0.05 mM MnAc₂ and that for VPg-poly(U) synthesis contained 0.5 mM MnAc₂.

(ii) **Oligo(dT)₁₅-primed poly(U) synthesis on a poly(A) template.** The assay for oligo(dT)₁₅-primed poly(U) synthesis was the same as that described above except that 50 ng of oligo(dT)₁₅ primer (Boehringer Mannheim) replaced VPg.

RESULTS

Expression and purification of GST-HRV2 3D^{pol} and HRV2 3D^{pol}. We have initially undertaken the expression in *E. coli* and purification of a GST-fusion polypeptide of HRV2 3D^{pol} (GST-HRV2 3D^{pol}). The protein, expressed in good yields, can be solubilized in 1% Triton X-100 and batch purified on glutathione-Sepharose beads to near-homogeneity. The fusion polypeptide has the expected molecular size of 76.5 kDa (Fig. 2A, lane 6). In order to rule out the possibility that an N-terminal GST tag interferes with the activity of the enzyme, we have subsequently also expressed in *E. coli* and purified HRV2 3D^{pol} without a tag. Previous studies have indicated that an extra methionine linked to the N-terminal glycine of PV1(M) 3D^{pol} is to a large extent processed off in the bacterial cell and

that the activity of the resulting enzyme preparation is essentially the same as that of the wt protein (19). Therefore, we have proceeded to express in *E. coli* and purify HRV2 3D^{pol} with only an extra methionine linked to the N-terminal glycine of the 460-amino-acid 3D^{pol} polypeptide (Fig. 2B, lane 6). To distinguish this form from the GST fusion protein, we will refer to it as HRV2 3D^{pol}. The protein, with a molecular size of 50.5 kDa, was purified by ion-exchange chromatography to approximately 80% purity (Fig. 2B). Whether or not the minor fast-migrating bands of protein include degradation products of HRV2 3D^{pol} or contaminating bacterial proteins could not be determined, since no antibodies to this protein are available yet.

Oligo(dT)₁₅-primed poly(U) synthesis by GST-HRV2 3D^{pol} and HRV2 3D^{pol}. It has been previously shown by Morrow et al. (37) that HRV2 3D^{pol} isolated from virus-infected HeLa cells is active in an elongation reaction of an oligo(U) primer on a poly(A) template yielding poly(U). We have confirmed this observation with our enzyme preparations of HRV2 3D^{pol}, which were expressed in *E. coli* and purified. The two forms of 3D^{pol} were found to be about equally active in the synthesis of poly(U) on a poly(A) template (Fig. 3A; compare lane 4 with lane 9) in a reaction using UTP, Mn²⁺ as a cofactor, and oligo(dT)₁₅, instead of oligo(U), as a primer. Both preparations of polymerases are template (Fig. 3A; compare lane 4 with lane 2 and lane 9 with lane 7) and primer (Fig. 3A, compare lane 4 with lane 3 and lane 9 with lane 8) dependent. We used a control plasmid, with no 3D^{pol} insert, to rule out the possibility that the RNA polymerase activity of the enzyme preparations is due to a contaminating bacterial protein. Purified proteins isolated from extracts of cells that were produced after the induction of the control plasmid had no detectable activity (Fig. 3A; compare lane 5 with lanes 4 and 9).

VPgpU(pU) and VPg-poly(U) synthesis by HRV2 3D^{pol} and GST-HRV2 3D^{pol}. We have previously shown that under certain in vitro reaction conditions 3D^{pol} of PV1(M) is able to catalyze the covalent linkage of UMP to VPg, yielding VPgpU, a precursor that is elongated into VPgpUpU and then into VPg-poly(U) (41). The fact that the RNA polymerases of poliovirus and HRV2 are related (53) suggested the possibility that HRV2 3D^{pol} might also catalyze the same type of protein-priming reaction. As expected, HRV2 3D^{pol} is highly active in the synthesis of HRV2 VPgpU(pU) and VPg-poly(U) on a poly(A) template (Fig. 3B1 and 2). The in vitro reaction requires purified enzyme (Fig. 4, compare lanes 1 and 2), synthetic VPg (Fig. 4; compare lanes 1 and 3), UTP, and Mn²⁺ (Fig. 4; compare lanes 1 and 5). There appears to be no absolute requirement for poly(A) (Fig. 4; compare lanes 1 and 4), most likely since Mn²⁺ relaxes the specificity of the enzyme toward its template (5; A. V. Paul, J. Peters, and E. Wimmer, unpublished data). The yield of products is enhanced by the addition of a nonionic detergent (NP-40) (Fig. 4; compare lanes 1 and 6) but is not affected by the presence of dithiothreitol (DTT) (Fig. 4; compare lanes 1 and 7). The synthesis of VPg-poly(U) (Fig. 5B; compare lanes 1 and 7) is dependent on the presence of VPg, indicating that transcription of the poly(A) template is VPg primed. The small amount of polymeric product made in the absence of VPg (Fig. 4, lane 3, and Fig. 5B, lane 1) is most likely due to a terminal uridylyltransferase activity of the enzyme similar to that of poliovirus 3D^{pol}

FIG. 5. Effects of varying HRV2 3D^{pol}, HRV2 VPg, and UTP concentrations on the synthesis of VPgpU(pU) and VPg-poly(U). The standard assay was used for VPg-poly(U) synthesis as described in Materials and Methods, except that the 3D^{pol}, VPg, or UTP concentration was varied as indicated. Effects of concentrations of 3D^{pol} (A), VPg (B), and UTP (C) are shown as autoradiographs (panels 1) and quantified data (panels 2).

(44). Relatively high concentrations of 3D^{pol} (>3 μM [Fig. 5A]) and of VPg (50 μM [Fig. 5B]) are required for optimal synthesis of both the VPg-linked precursors and polymer. Under conditions of optimal VPg uridylylation (0.02 μM UTP) (Fig. 5C, lane 1), very little, if any, elongation of the precursors can be detected; however, polymers are synthesized when the UTP concentration is increased to >0.5 μM (Fig. 5C, lane 2). The optimum pH for the HRV2 3D^{pol} reaction is 7.5 to 8.0 (Fig. 6A), and the optimal incubation temperature is 33°C (Fig. 7A). The enzyme has a strong preference for Mn²⁺ over Mg²⁺ or Co²⁺ both in the VPg uridylylation reaction (Fig. 8A) and in the synthesis of VPg-poly(U) (data not shown). The optimal concentration of Mn²⁺ for both VPg uridylylation (Fig. 8A1) and VPg-poly(U) synthesis is 0.2 mM (data not shown).

The activity of the GST-tagged enzyme in the synthesis of HRV2 VPgpU(pU) and VPg-poly(U) is comparable to that of HRV2 3D^{pol} (Fig. 3B). The optimal reaction conditions are also similar for the two versions of the enzyme. The optimal pH of GST-HRV2 3D^{pol} for the synthesis of both precursors

and polymer is 7.6 (Fig. 6B). Interestingly, the size of the polymeric product decreases as the pH of the reaction is raised over pH 7.6 (Fig. 6B; compare lane 6 with lanes 7 to 9). The most favorable incubation temperature for the reactions is 30°C (Fig. 7B). Mn²⁺ is far superior to Mg²⁺ or Co²⁺ as a cofactor for GST-HRV2 3D^{pol} in both VPg uridylylation (Fig. 8B) and VPg-primed poly(U) synthesis (data not shown) under the conditions of the experiments. The optimal Mn²⁺ concentration for synthesis of the precursors is 0.05 mM (Fig. 8B), and that for synthesis of the polymer is 0.5 mM (data not shown).

Comparison of the RNA polymerase activities of HRV2 3D^{pol} and PV1(M) 3D^{pol}. Using equal amounts (1 μM) of purified HRV2 3D^{pol} or PV1(M) 3D^{pol} (41) in the reaction mixtures, we have compared the synthetic activities of these two viral RNA polymerases. Both of these polypeptides were expressed in *Escherichia coli* with only an N-terminal methionine preceding the 3D^{pol} amino acid sequences. As shown on Fig. 3C, the two enzymes are about equally active in the elongation of a (dT)₁₅ primer (panel 1; compare lanes 1 and 2). However, HRV2 3D^{pol} has two- to threefold-lower VPg-uridylylating activity than PV1(M) 3D^{pol} (panel 2; compare lanes 1 and 2) when each enzyme is tested with its cognate VPg as the substrate.

It has been reported previously that the uridylylated form of PV1(M) VPg, a 2-kDa peptide, migrates with an apparent molecular size of >7 kDa in SDS-PAGE gels (10, 34, 58). As can be seen from Fig. 3C, the apparent molecular size of HRV2 VPg is about the same. However, although HRV2 VPg is only 21 amino acids long, it migrates distinctly more slowly in the gel than the larger VPg of PV1(M) (22 amino acids) (Fig. 3C and 9C). The reason for the aberrant migration of the VPg peptides in these gels is not known.

FIG. 6. Effects of pH on HRV2 VPgU(pU) and VPg-poly(U) synthesis by HRV2 3D^{pol} (A) and GST-HRV2 3D^{pol} (B). Standard assays were used for VPg-poly(U) synthesis (see Materials and Methods) except that the pH of the reactions was varied as indicated. (Panels 1) Autoradiographs; (panels 2) quantified data, measured in counts per minute, are plotted as relative activity.

Comparison of wt and mutant VPg peptides as substrates for uridylylation by HRV2 3D^{pol} and GST-HRV2 3D^{pol}. Both the tagged- and untagged versions of HRV2 3D^{pol} are able to use PV1(M) VPg as a substrate in the in vitro reaction instead of HRV2 VPg (Fig. 9A and B; compare lanes 2 and 5). This observation has enabled us to assess the effects of amino acid substitutions in PV1(M) VPg, and presumably also in HRV2 VPg, on the in vitro activities of the peptides. Figure 9C shows a comparison of the wt and mutant PV1(M) VPg peptides as substrates for HRV2 3D^{pol} and GST-HRV2 3D^{pol} in the VPg uridylylation reaction. As expected, the tyrosine at position 3 in VPg, the attachment site to the viral RNA (2, 48), is essential for activity (Fig. 9A and B; compare lanes 5 and 13). The Y3T T4Y mutant VPg peptide is also totally inactive (Fig. 9A and B; compare lanes 5 and 14), confirming the importance of the tyrosine at the third position. Of the other mutant peptides, the R17E, K9A K10A, and G5P peptides had less than 10% of wt activity (Fig. 9A and B; compare lanes 5 with lanes 6, 8, and 11, respectively). Two mutations, P7A and N8A (Fig. 9A and B; compare lanes 5 with lanes 7 and 10, respectively), have no inhibitory effect, while others (K10A and T4A) give intermediate reductions in activity (Fig. 9A and B; compare lanes 5

with lanes 9 and 12, respectively). All of the mutant PV1(M) VPg peptides gave comparable results with the two different enzyme preparations. However, in contrast to HRV2 3D^{pol} (Fig. 9A; compare lane 5 with lane 3), the GST-tagged enzyme shows much-reduced activity with the VPg of HRV14 (56) compared with that of PV1(M) (Fig. 9B; compare lanes 5 and 3). The VPg of HRV89, a rhinovirus closely related to HRV2 (13), functions just as well as the VPg of HRV2 with both versions of the enzyme (Fig. 9A and B; compare lanes 2 and 4).

DISCUSSION

The RNA polymerase of poliovirus, a member of the genus *Enterovirus*, has been relatively well characterized. In contrast very little is known about the RNA polymerases of the other genera of *Picornaviridae*, such as HRVs. The synthetic activities of PV1(M) 3D^{pol} (15, 41) in vitro resemble those of the DNA polymerases of some double-stranded linear DNA viruses with genome-linked proteins such as phages ϕ 29 and PRD1 and adenovirus (reviewed in reference 51). The DNA or RNA polymerases of all these viruses are unique because they are able to catalyze two different types of synthetic reactions.

A. HRV2 3D^{pol} **B. GST-HRV2 3D^{pol}**

FIG. 7. Effects of temperature on HRV2 VPgU(pU) and VPg-poly(U) synthesis by HRV2 3D^{pol} (A) and GST-HRV2 3D^{pol} (B). Standard assays were used for VPg-poly(U) synthesis (see Materials and Methods) except that the incubation temperatures were varied as indicated. (Panels 1) Autoradiographs; (panels 2) quantified data, measured in counts per minute, are plotted as relative activity.

The first of these reactions is the elongation of an oligonucleotide primer on a DNA or RNA template, yielding a complementary strand. The second type of reaction consists of the covalent linkage of a nucleotide to the hydroxyl group of an amino acid in the viral terminal or preterminal protein (41, 51). The nucleotidylated proteins then serve as primers for elongation of the new strands.

More than 15 years ago, HRV2 3D^{pol} was partially purified from virus-infected HeLa cells, and the enzyme was shown to be active in the elongation of oligonucleotide primers on both homopolymeric and heteropolymeric RNA templates (37). The experiments reported in this paper were undertaken with the aim of gaining more information about the synthetic activities of HRV2 3D^{pol} and the replication of HRVs. We expressed two different forms of the protein in *E. coli*, one as a GST fusion polypeptide and one with only an extra N-terminal methionine. The enzymes were purified, and their activities were first compared in vitro in the oligonucleotide elongation reaction with poly(A) as a template. Our results clearly show that these two preparations of HRV 3D^{pol} are about equally active in the elongation of a (dT)₁₅ primer into (dT)₁₅-poly(U). The same is true when one compares the elongation activity of HRV2 3D^{pol} with that of PV1(M) 3D^{pol}. This is not surprising in view of the fact that there is a 56% homology in the amino

acid sequences of 3D^{pol} of these two viruses (53). However, the observation that GST-HRV2 3D^{pol} is highly active came as a big surprise (see below).

The picornaviral VPg is a peptide of about 20 to 25 amino acids whose single tyrosine residue provides the link to the 5'-terminal uridylic acid of the viral genome (48, 50, 66). In this paper we provide evidence that HRV2 VPg can be uridylylated about equally well by both the GST-tagged and untagged forms of the polymerase of HRV2, yielding VPgpU and VPgpUpU. The optimal assay conditions that are used by the two enzyme preparations are similar. The uridylylation reaction requires only purified HRV2 3D^{pol}, synthetic HRV2 VPg, UTP, a poly(A) template, and Mn²⁺ as a cofactor. These precursors can then prime the transcription of poly(A) by 3D^{pol} to produce VPg-poly(U). The formation of polymeric products requires the presence of VPg, confirming the role of VPg as a primer for VPg-linked poly(U) synthesis. Although we have not directly shown that the bond between VPg and nucleotide in VPgpU is O⁴-(5'-uridylyl)tyrosine (2, 48), our experiments carried out with sequence variants of VPg fully support this proposal. As expected, no VPg-linked products were formed with a Y3F mutant VPg peptide, and this in vitro defect correlates with our genetic studies that showed this mutation to be lethal to HRV2 (17). The inability of the hydroxyl group of Y4 or T3 in a mutant T3Y4 VPg peptide to function as an acceptor of UMP demonstrates the very strict specificity of the enzyme both for the location and for the identity of the amino acid involved in the linkage. Surprisingly, relatively few other amino acids are essential (R17) or very important (G5, K9 with K10) for VPg's function in the in vitro reaction. This finding is further supported by the results of in vitro assays in which the VPg of HRV2 was replaced by that of PV1(M) or HRV14 (56). All of these viral VPgs were active as substrates for HRV2 3D^{pol}, although these peptides not only have different lengths but also contain only a few conserved residues (Fig. 9). These conserved amino acids (Y3, G5, K10, and R17) are identical with those shown to be required for VPg's function from studies of the mutant VPg peptides (see above). We have previously shown that these same amino acids in PV1(M) VPg are also required for the protein-priming reaction catalyzed by PV1(M) 3D^{pol} in vitro and for viral viability (41; A. V. Paul, J. Peters, and E. Wimmer, unpublished data). It is interesting that the VPg's of the two closely related viruses HRV2 and HRV89 (13) contain two negatively charged glutamate residues, which are absent in the VPgs of the other two viruses (Fig. 9). Apparently the absence of these amino acids from the VPgs of PV1(M) and HRV14 does not interfere with their ability to function as substrates for HRV2 3D^{pol}. The only noticeable difference between the substrate specificities of the two preparations of HRV2 3D^{pol} is the inability of GST-HRV2 3D^{pol} to efficiently uridylylate HRV14 VPg.

A partial structure of the poliovirus RNA polymerase as a "right hand" with "fingers," "thumb," and "palm" domains is already known from X-ray crystallographic studies by Hansen et al. (24). From the structural data it was predicted that the N-terminal polypeptide segment of 3D^{pol} might be derived from another polymerase molecule such that the N terminus of one contributes to the active-site cleft of the second molecule (24, 28). The importance of this segment was strongly supported by the finding that the N-terminal amino acids of 3D^{pol}

A. HRV2 3D^{pol} **B. GST-HRV2 3D^{pol}**

FIG. 8. Effects of divalent metal concentrations on HRV2 VPgUpU(pU) synthesis by HRV2 3D^{pol} and GST-HRV2 3D^{pol}. Uridylation of VPg by HRV2 3D^{pol} (A) and by GST-HRV2 3D^{pol} (B) was measured by standard assays (see Materials and Methods) using either MnAc₂ (panels 1), MgAc₂ (panels 2), or CoAc₂ (panels 3) at the indicated concentrations. (Panels 4) Quantified data. The length of exposure of the gel to Kodak film is given above each autoradiograph.

are essential for its *in vitro* oligonucleotide elongation activity (24, 28, 44). In view of the fact that the enzymatic properties of HRV2 3D^{pol} are nearly identical to those of PV1(M) 3D^{pol}, it is remarkable that the bulky N-terminal GST tag on HRV2 3D^{pol} does not significantly interfere with its activity on a poly(A) template in either the oligonucleotide- or VPg-primed reactions. In contrast, a preparation of N-terminally fused GST-3D^{pol} of CAV21, an enterovirus closely related to PV1(M), is totally devoid of enzymatic activity in both of our assays, while the untagged form of the enzyme is fully active (E. Rieder and E. Wimmer, unpublished data).

It has been previously shown for PV1(M) 3D^{pol} that its RNA binding and polymerization activity is highly cooperative with respect to polymerase concentration, an observation suggest-

ing that protein-protein interactions are important for its function (24, 39, 41). We have found that both VPg uridylation and VPg-poly(U) synthesis by HRV2 3D^{pol}, just as by PV1(M) 3D^{pol} (41), are optimal at >1 μ M 3D^{pol}, a concentration that has been shown to be similar to that required for oligomerization of the poliovirus enzyme (39). Whether HRV2 3D^{pol} must form dimers to interact with its template or primer (VPg or oligonucleotide) remains to be determined.

All known DNA and RNA polymerases require a divalent cation cofactor for optimal activity. Most *in vitro* polymerase assays that measure elongation use Mg²⁺, which is not only an effective activator but is also considered to be the metal most likely used *in vivo*. The activity of poliovirus 3D^{pol} in the oligonucleotide elongation assay is 10 times higher with Mn²⁺

FIG. 9. Comparison of wt and mutant (mut) PV1(M) VPgs and rhinovirus VPgs as substrates for uridylylation by HRV2 3D^{pol} and GST-HRV2 3D^{pol}. Standard assays for VPgUpU(pU) synthesis were used (see Materials and Methods), except that HRV2 VPg was replaced by other VPgs, as indicated. (A) HRV2 3D^{pol}. (B) GST-HRV2 3D^{pol}. (C) Quantified data, measured in counts per minute, are shown as relative activity. Amino acid sequences of wt and mutant VPgs are given on the left. The mutant VPgs (with mutations shown in boldface) are derived from wt poliovirus VPg.

than with Mg²⁺, and this has been attributed to a reduction in the *K_m* value for 3D^{pol} binding to a primer and template (5). The protein-priming reactions catalyzed by DNA polymerases (8, 14, 45), the reverse transcriptase of hepatitis B virus (61), or PV1(M) 3D^{pol} (42) have an even more striking preference for Mn²⁺ as a metal cofactor. In this study we have confirmed this observation with both preparations of HRV2 3D^{pol}. In the presence of Mn²⁺, both VPg uridylylation and VPg-poly(U) synthesis on a poly(A) template by HRV2 3D^{pol} were increased about 100-fold over those in the presence of Mg²⁺. Whether or not the stimulatory activity of Mn²⁺ is due to an enhanced binding of the enzyme to the poly(A)-VPg complex is not yet known.

The facts that both minus and plus picornaviral RNA strands are VPg linked (38) and that the poly(A) tail of picornaviruses is genetically encoded (11) suggest that minus-strand synthesis commences with VPg-linked poly(U) synthesis. Therefore, one might expect that the uridylylation of VPg by 3D^{pol} on the

poly(A) tail represents the very first step in that process. Our simple in vitro uridylylation assay was designed to represent exactly that type of reaction (41). Recently, however, we have made the surprising observation that the primary template in PV1(M) RNA for the synthesis of VPgUpU(pU) in vitro is not the poly(A) tail but the *cre*(2C) hairpin (42), an RNA structure residing in the open reading frame of the poliovirus polyprotein (20). In our accompanying paper (17) we describe the VPg-uridylylating activity of HRV2 3D^{pol} using as a template its cognate *cis*-replicating element, which we have identified in the coding sequence of HRV2 2A^{pro}.

The common cold probably represents the most frequent type of human illness against which no effective vaccine or chemotherapy has yet been developed. Although HRV infections do not cause severe disease, they can lead to secondary infections (9, 22) and can trigger acute asthma symptoms (18). The RNA polymerase of rhinoviruses is an ideal target for the development of antiviral drugs not only because it is essential for viral growth but also because it catalyzes a unique type of enzymatic reaction in the infected human cell.

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