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Allosteric Effects of Ligands and Mutations on Poliovirus RNA-Dependent RNA Polymerase

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Protein priming of viral RNA synthesis plays an essential role in the replication of picornavirus RNA. Both poliovirus and coxsackievirus encode a small polypeptide, VPg, which serves as a primer for addition of the first nucleotide during synthesis of both positive and negative strands. This study examined the effects on the VPg uridylylation reaction of the RNA template sequence, the origin of VPg (coxsackievirus or poliovirus), the origin of 3D polymerase (coxsackievirus or poliovirus), the presence and origin of interacting protein 3CD, and the introduction of mutations at specific regions in the poliovirus 3D polymerase. Substantial effects associated with VPg origin were traced to differences in VPg-polymerase interactions. The effects of 3CD proteins and mutations at polymerase-polymerase intermolecular Interface I were most consistent with allosteric effects on the catalytic 3D polymerase molecule. In conclusion, the efficiency and specificity of VPg uridylylation by picornavirus polymerases is greatly influenced by allosteric effects of ligand binding that are likely to be relevant during the viral replicative cycle.

Picornaviruses have a single copy, positive-strand RNA genome with a small peptide, VPg (3B), covalently attached to the 5′-terminal nucleotide and a poly(A) segment at the 3′ end. RNA replication occurs in the cytoplasm of infected cells, proceeding through negative-strand intermediates which, in turn, serve as templates for production of progeny positive strands. A long-standing question has been how both positive- and negative-strand syntheses are initiated. Characterization of a reaction catalyzed by poliovirus 3D polymerase, in which the tyrosine of the VPg peptide was trans-esterified (uridylylated) in the presence of a poly(A) template to form VPg-pU (pU), provided insight into the mechanism of strand initiation (34): given the ability of poliovirus 3D polymerase to uridylylate VPg, the peptide-nucleotide conjugate could serve as the protein primer for progeny RNA strand synthesis. VPg must bind directly to poliovirus polymerase, because it can serve as an enzymatic substrate; furthermore, VPg-polymerase interactions have been observed in two-hybrid experiments (42). While the binding site on the poliovirus 3D polymerase for the VPg substrate of the uridylylation reaction has not yet been characterized, the binding site for its likely proteolytic precursor, 3AB, has been identified as a distinct site on the back of the palm of the polymerase molecule via extensive alanine-scanning mutagenesis (22).

Although VPg uridylylation could provide a protein primer for use in either positive- or negative-strand synthesis, this reaction is not sufficient to describe the mechanism of initiation for viral RNA synthesis in infected cells. For example, the use of a poly(A) template for VPg uridylylation does not provide specificity for a particular virus. This specificity could be provided if an RNA sequence or structure within the picornavirus genome were the authentic RNA template for VPg uridylylation. Such RNA sequences, such as in the capsid-coding region of rhinovirus 14 (24), the 2C-coding region of poliovirus RNA (9, 10, 33, 35), the VP2-coding region of Theiler's and Mengo viruses (20), the 2A-coding region of rhinovirus 2 (7), and the 5′ noncoding region (NCR) of foot-and-mouth-disease virus (23), have now been described for several picornaviruses.

When poliovirus protein 3CD, engineered to be proteinase-deficient and thus to remain in precursor form, is added to reactions that contain the 2C RNA, poliovirus 3D polymerase and VPg, VPg uridylylation is stimulated substantially above the rate observed in the absence of 3CD (32, 33, 35). Specific binding of 3CD to the $2C^{CRE}$ RNA sequence (2C RNA), which contains the stem-loop structure, has been demonstrated (44). Genetic data are consistent with 3CD binding contacts in both the stem and the loop of this RNA structure (43). Furthermore, 3CD has been shown to interact in two-hybrid experiments with 3D polymerase (42). Protein-protein interactions between poliovirus polymerase 3D molecules have been extensively documented (21, 30, 42). These data provide a picture in which 2C RNA-mediated VPg uridylylation involves a complex of 3D polymerase, VPg and 2C RNA-associated 3CD. Poly(A)-mediated VPg uridylylation, however, may involve only the polymerase and VPg, at least in vitro.

Recent reports (10, 25, 26) provide convincing evidence that 2C RNA is required for poliovirus positive-strand, but not negative-strand, synthesis. Specifically, mutations that disrupt the 2C RNA stem-loop structure were shown to block both synthesis of positive strands and accumulation of VPg-pU(pU) in an in vitro translation-replication system (10, 25). However, these mutations did not abrogate synthesis of negative strands, which were observed to have VPg at their 5′ termini. Therefore, negative-strand synthesis can occur in the absence of the

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2C RNA sequences and without measurable accumulation of VPg-pU(pU). However, when the linkage tyrosine residue in VPg was mutated to phenylalanine, synthesis of both positive and negative strands was inhibited (26). Thus, VPg must be uridylylated for detectable negative-strand synthesis by a mechanism that does not require 2C RNA as a template (11). It was therefore suggested that a template RNA that possesses an essential sequence element in the 3′ NCR followed by an extended stretch of adenylates could provide a suitable template for initiation of negative-strand synthesis, and thus it is likely that the mechanisms of initiation of positive- and negative-strand synthesis differ in detail. The existence of different mechanisms is consistent with the observation of disparate amounts of positive and negative strands within infected cells (8, 28) and the existence of mutations that preferentially affect either negative- or positive-strand synthesis (3, 8, 39).

In this study, we have examined the effects of two templates, poly(A) RNA and 2C RNA, and the presence of 3CD protein from either poliovirus or the closely related coxsackievirus B3, on uridylylation of both poliovirus and coxsackievirus VPg proteins by both poliovirus type 1 and coxsackie B3 RNA-dependent RNA polymerases. We observed significant variances in the efficiency of VPg uridylylation reactions depending on the VPg used and the presence or absence of 3CD. We began with an assumption that, in the in vitro conditions used, recognition specificity would be observed between the VPg/polymerase pairs from poliovirus and coxsackievirus that would be augmented by 3CD interactions. Instead, the data were consistent with a model in which both poliovirus and coxsackievirus polymerases recognize poliovirus VPg preferentially, and both 3CD proteins stimulate VPg uridylylation by binding to the polymerase, not to VPg, and affecting polymerase activity, VPg affinity, or both. Similarly, mutations of residues in Interface I, a site of interaction between adjacent monomer units in the three-dimensional structure of wild-type poliovirus 3D polymerase determined by x-ray crystallography (12, 14), were shown to have highly variable effects on the rate of VPg uridylylation. The effects of thse mutations did not correlate with predicted effects on the stability of Interface I. However, these effects were observed regardless of RNA template used and in the absence of 3CD protein, and therefore cannot be ascribed to 3CD–3D interactions as has been interpreted previously (31). The term "allostery" describes effects on proteins at sites distant from the binding or mutagenic event. We conclude that the effects on the rate of VPg uridylylation by the $2C^{CRE}$ RNA/3CD pair and the effects of mutations at two different polymerase surfaces arise from allosteric effects on polymerase activity.

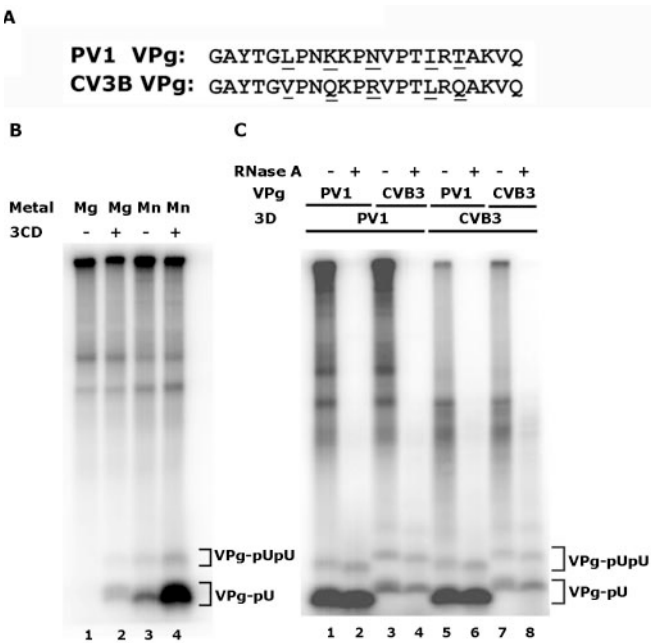

FIG. 1. VPg uridylylation catalyzed by poliovirus (PV-1) or coxsackievirus B3 (CVB3) 3D polymerase using a poliovirus 2C RNA template in the presence of poliovirus 3CD. (A) The sequences of PV-1 and CVB3 VPg proteins are shown. Differences in sequence are underlined. (B) PV-1 VPg uridylylation by PV-1 3D polymerase using a 2C RNA template in the presence of magnesium acetate (lanes 1 and 2) or manganese chloride (lanes 3 and 4). Poliovirus 3CD was absent in lanes 1 and 3 and present in lanes 2 and 4. (C) VPg uridylylation by PV 3D polymerase (lanes 1 to 4) and CVB3 3D polymerase (lanes 5 to 8) using a 2C RNA template; all reactions contained PV 3CD. The identity of the VPg substrate, from PV or CVB3, is indicated. Products were digested with RNase A (100 μg/ml, 30°C, 60 min) in even-numbered lanes as indicated. Products were resolved in 12% polyacrylamide-Tris-Tricine gels. The positions of migration of VPg-pUpU and VPg-pU are indicated in both (B) and (C). The identity of VPg-pU and VPg-pUpU were confirmed by RNase and snake venom phosphodiesterase treatment; slightly greater differences in mobility are seen in this gel than in subsequent separations of VPgpU and VPgpUpU.

MATERIALS AND METHODS

Protein purification. Wild-type Mahoney type 1 poliovirus polymerase and mutant polymerases (F377A, R379E, L342A, D349R, L446A, R455D, and R456D) were expressed from modified pT5T-3D (30) in *Escherichia coli* BL-21 (DE3)pLysS cells. Previously, it was shown that poliovirus 3D polymerase purified from *E. coli* displayed an identical specific activity in a poly(A)/oligo(U)-containing RNA-dependent RNA polymerase assay as polymerase purified from infected cells (27). Coxsackievirus B3 3D polymerase was expressed from a modified pT5T-3D plasmid in *E. coli* BL21(DE3) as described (1). Polymerase expression and purification were as described (14, 22). Poliovirus 3CD that contained mutation C147A in the active site of the 3C proteinase domain was expressed from a mutagenized pT5T-3CD plasmid and purified similarly to 3D polymerase except that the IPTG induction period was 60 min. Histidine-tagged 3CD proteins (both poliovirus and coxsackievirus) (29) were used in the experiments for Fig. 3. These proteins also contained mutations C147A in the proteinase active site and were expressed and purified as described (1, 30). The tagged poliovirus 3CD had no observable modified activity compared to untagged poliovirus 3CD. Poliovirus 3AB was expressed from plasmid pT7lac3AB (kindly supplied by Aniko Paul and Eckard Wimmer, Stony Brook, NY) in *E. coli* BL21(DE3) and was purified according to published procedures (18, 19). Poliovirus VPg (3B) was synthesized by Lofstrand Labs Limited (Gaithersburg, MD), and coxsackievirus VPg was synthesized by Chiron Technologies (Clayton, Victoria, Australia).

RNA preparations. Poly(A) RNA was obtained from Amersham/Pharmacia Biotech (Piscataway, NJ) and was purified by phenol-choroform extraction and isopropanol precipitation. Concentrations were based on total nucleotide content, assuming an average size of 300 nucleotides (nt). 2C RNA was expressed by T7 RNA transcription from pTM-2C plasmid (a gift from Natalya Teterina, NIH). The plasmid (pTM-2C) contains a T7 promoter from which a 1,091-nt RNA can be transcribed after BamH1 digestion of the plasmid DNA. The 1,091-nt transcripts contained the EMCV- IRES and nt 4124 to 4600 of poliovirus RNA, including the amino-terminal 477 nt of the 2C sequence and the 2C RNA stem-loop structure (9, 33). RNA transcripts were treated with DNase I, phenol-chloroform extracted, and precipitated with isopropanol; concentrations are reported, as indicated, either in strands or in nucleotides.

VPg uridylylation and analysis of products. VPg uridylylation was monitored in 20-μl or 30-μl reactions that contained 50 mM HEPES (pH 7.2), 2 mM dithiothreitol, 0.5 mM $MnCl_2$ (or 3.5 mM magnesium acetate, Fig. 1B), 8% glycerol, 50 μM VPg (either poliovirus or coxsackievirus), 10 μM UTP, 3-4.5 μCi

[α-^{32}P]UTP, template RNA [0.65 μM strands poly(A) for Fig. 3B, 15 μM strands 2C RNA for all other figures], 0.5 μM 3CD where indicated, 3AB as indicated, and 1 μM 3D polymerase (wild-type or mutant poliovirus or wild-type coxsackievirus). Reactions were incubated at 30°C for 2 h. Reactions were stopped with 2 mM EDTA on ice and then treated at 90°C for 4 min in sample buffer (5% glycerol, 2.5% β-mercaptoethanol, 1.5% sodium dodecyl sulfate, 0.06 M Tris-HCl [pH 6.8], 0.05% bromphenol blue; final concentrations). Uridylylation products were resolved in 12% polyacrylamide-Tris-Tricine gels (5, 37), after which the gels were transferred to 3MM filter paper, dried at 80°C under vacuum and exposed to Kodak BioMax MR film for autoradiography. Radioactive species in the gel were quantified by phosphorimager analysis (Typhoon 9400; Amersham Biosciences) and ImageQuant (Molecular Dynamics). To ensure that low uridylylation efficiency of coxsackievirus VPg did not reflect the presence of an inhibitor, mixing experiments were performed in which CV VPg was shown not to inhibit PV VPg uridylylation (data not shown). The 2-h time points used reflected the initial rates of the uridylylation reactions, as shown by time courses (data not shown).

RESULTS

Substrate specificity of VPg uridylylation by 3D polymerases using a poliovirus 2C RNA template in the presence of poliovirus 3CD. To test the molecular specificity of VPg uridylylation, we tested the ability of the RNA-dependent RNA polymerases from both poliovirus 1 (PV1) and the closely related coxsackievirus B3 (CVB3) to uridylylate the VPg proteins from both viruses. The 3D polymerase proteins from these two viruses display 74% amino acid identity. The amino acid sequences of the two VPg molecules are shown in Fig. 1A, in which the five amino acid differences between these 22-amino acid sequences are underlined. Uridylylation reactions were performed as described by Paul et al. (33), in which a specific stem-loop sequence in the 2C-coding region of poliovirus RNA, termed 2C^{CRE} or 2C RNA, acts as a template for VPg uridylylation and, in the presence of poliovirus 3CD and viral polymerase 3D, supports efficient VPg uridylylation.

The dependence of the 2C RNA-templated uridylylation reaction of poliovirus 3D polymerase on divalent cations is shown in Fig. 1B. Both singly uridylylated (VPg-pU) and doubly uridylylated (VPg-pUpU) species were produced; at the high concentrations of VPg used, the singly uridylylated species predominates. Both in the presence and absence of proteinase-deficient 3CD, this reaction required a divalent cation as reported previously (32, 33). In our hands, however, Mn^{2+} greatly stimulated the rate and extent of reaction using either poly(A) (33) (data not shown) or 2C RNA (Fig. 1B) as a template; the rate of VPg uridylylation in the presence of 3CD was about 25-fold higher with Mn^{2+} (lane 4) than with Mg^{2+} (lane 2). For this reason, all subsequent experiments were performed in the presence of Mn^{2+}.

The rate of uridylylation of these two VPg molecules was monitored by incorporation of [^{32}P]UMP (uridine monophosphate) in the presence of either poliovirus (PV1) or coxsackievirus (CVB3) polymerase. As shown in Fig. 1C, singly uridylylated poliovirus VPg (VPg-pU; lanes 1, 2, 5, and 6) migrated slightly faster than the VPg-pU of coxsackievirus (lanes 3, 4, 7, and 8). Both poliovirus and coxsackievirus B3 VPg molecules were uridylylated by both enzymes, but poliovirus VPg uridylylation was strongly favored using either 3D polymerase.

Possible mechanisms for the specificity of VPg uridylylation are shown in Fig. 2. The 2C RNA is pictured as an RNA hairpin because a specific stem-loop structure has been shown to be required both for viral viability (9, 33) and for 3CD-stimulated VPg uridylylation (33). In the presence of 3CD, which has been shown to bind specifically to the 2C stem-loop (43), uridylylation of poliovirus VPg, but not coxsackievirus VPg, was highly preferred (Fig. 1C). The "3CD as VPg binding platform" hypothesis (Fig. 2A) predicts that, in the presence of poliovirus 3CD, the uridylyation reaction is specific for poliovirus VPg because poliovirus VPg binds to poliovirus 3CD with higher affinity than does coxsackievirus VPg. The 3CD-bound VPg would then serve as the substrate for uridylylation by the 3D polymerases. Thus, in Fig. 2A, the binding site on poliovirus 3CD is predicted as showing specificity for poliovirus VPg.

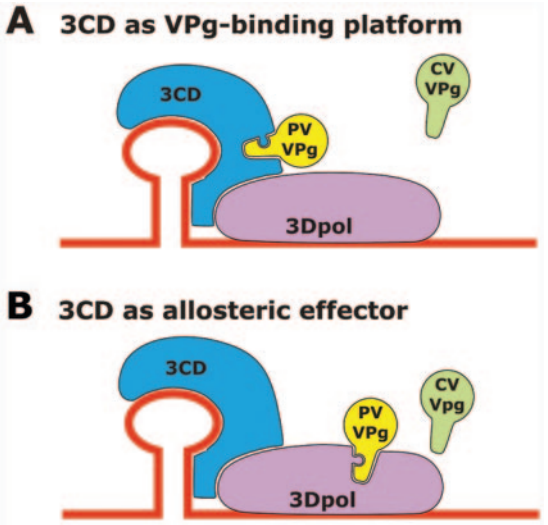

FIG. 2. Models for the mechanism by which 3CD stimulates VPg uridylylation by 3D polymerase. (A) In the "3CD as VPg binding platform" model, PV VPg preferentially binds to a similar binding site on both PV and CV 3CD. (B) In the "3CD as allosteric effector" model, 3CD binding to 3D causes a conformational change in either PV or CV 3D polymerase, modifying its uridylylation activity, VPg binding affinity, or both. During the catalysis of VPg uridylyation, the scenarios in both (A) and (B) must include additional contacts from those pictured, including the proximity of the active site of the 3D polymerase, the templating A residues in the 2C RNA, and the uridylylated tyrosine of the VPg molecule.

The "3CD as allosteric effector" model (Fig. 2B) suggests that, upon interaction with RNA-bound 3CD, both poliovirus and coxsackievirus 3D polymerases undergo conformational changes that increase their ability to uridylylate both VPg molecules. The increase in uridylylation rate for poliovirus VPg would then reflect either increased binding affinity of both polymerases for poliovirus VPg or an allosteric change in both polymerases that favored poliovirus VPg binding or reactivity. One way to distinguish between these two hypotheses is to determine whether the specificity for poliovirus VPg over coxsackievirus VPg is observed using coxsackievirus 3CD or in the absence of 3CD from any source.

Effect of 3CD on specificity of VPg uridylylation. To determine whether the presence and source of 3CD altered VPg substrate specificity, we tested the substrate specificity of 2C RNA-templated VPg uridylylation in the absence of 3CD and in the presence of either poliovirus and coxsackievirus 3CD. As shown in Fig. 3A (lanes 1 through 4), uridylylation of poliovirus VPg was more efficient than that of coxsackievirus VPg

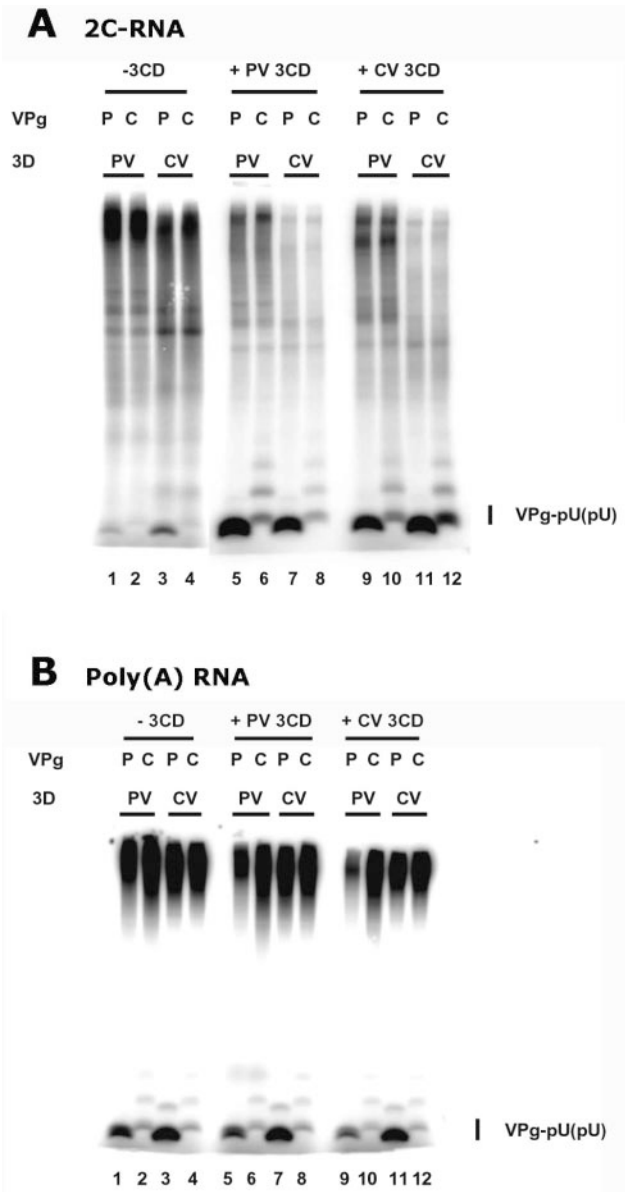

FIG. 3. VPg uridylylation by PV and CVB3 3D polymerase using two different RNA templates in the presence and absence of poliovirus or coxsackievirus 3CD. The relative rates of uridylylation of poliovirus (P) or coxsackievirus (C) VPg by poliovirus (PV) or coxsackievirus (CV) polymerase are reflected by the intensity of the uridylylated VPg bands. Lanes 1 to 4 show the results of reactions performed in the absence of 3CD; results shown in lanes 5 to 8 were performed in the presence of PV 3CD, and results shown in lanes 9 to 12 were performed in the presence of CV 3CD. (A) 2C RNA template and (B) Poly(A) template. Products were resolved in a 12% polyacrylamide-Tris-Tricine gel and analyzed as in Fig. 1.

even in the absence of 3CD from any source. Furthermore, the presence of either poliovirus 3CD (lanes 5 through 8) or coxsackievirus 3CD (lanes 9 through 12) stimulated uridylylation of both VPg molecules by either polymerase, with a preference for poliovirus VPg still observed. Furthermore, only when all three components (VPg, polymerase, 3CD) were derived from coxsackievirus (Fig. 3A, lane 12) did the extent of uridylylation of coxsackievirus VPg increase substantially. Thus, the coxsackievirus VPg peptide was not intrinsically unreactive. Perhaps the presence of a specific RNA sequence from coxsackievirus, which has yet to be described, would further increase the utilization of coxsackievirus VPg as a substrate.

When poly(A) was used as a template instead of 2C RNA, no 3CD stimulation was observed for any uridlylylation reaction, as expected (Fig. 3B). However, the preference for uridylylation of poliovirus VPg (lanes 5, 7, 9, and 11) over coxsackievirus VPg (lanes 6, 8, 10, and 12) remained. This is most consistent with the possibility (Fig. 2B) that the specificity of VPg uridylylation derives from increased affinity or reactivity of poliovirus VPg toward both poliovirus and coxsackievirus polymerases.

Identification of the VPg binding site for uridylylation on poliovirus polymerase. Although VPg is likely to bind directly to 3D polymerase (42), its binding site on 3D polymerase has not yet been characterized. On the other hand, poliovirus 3AB, which contains the VPg sequence at its C-terminus, has been shown to bind directly to poliovirus 3D polymerase by enzymatic stimulation, two-hybrid assays, and direct binding experiments (15, 19, 22, 42). The site of contact with 3D polymerase has been shown to involve amino acid residues F377, R379, E382, and V391 (15, 22) on the "back" of the viral polymerase structure, between the "thumb" and "fingers" domains (12) (Fig. 4A). Mutations in these residues in 3D polymerase also reduced the extent of VPg uridylylation in poly(A)-templated reactions in the absence of 3CD (22). Less is known about the points of contact with 3D polymerase on the 3AB molecule. Mutations in 3AB that interfere with its interaction with 3D polymerase in the yeast two-hybrid system were mapped predominately to the 3B (VPg) domain, and VPg alone was shown to interact with 3D polymerase by two-hybrid analysis (42). Therefore, it is likely that the amino acid residues that are responsible for VPg binding are similar to, or a subset of, those that are involved in 3AB binding.

To test the hypothesis that the VPg and 3AB binding sites on 3C polymerase are similar, we analyzed the concentration dependence of 3AB on the inhibition of VPg uridylylation. Figure 4B shows the effect of increasing 3AB concentration on the extent of VPg uridylylation by 3D polymerase in 2C RNA-templated reactions. The incubation time of two hours is shown, which under these conditions reflects the initial rate of the uridylylation reaction. Higher concentrations of 3AB were required to inhibit VPg uridylylation at 20 μM VPg than at 2 μM VPg, consistent with competitive inhibition of VPg uridylylation by 3AB. A plot of 1/v as a function of 3AB concentration reveals the mode of inhibition of VPg uridylylation by 3AB (38). The intersection of the two curves to the left of the y-axis, but above the *x* axis as shown in Fig. 4C, argues that 3AB is a competitive inhibitor of VPg uridylylation; noncompetitive inhibition would result in these two curves intersecting at the *x* axis. The intersection point at the y-axis yielded an approximate value of 100 nM for the K_i of 3AB for VPg uridylylation. Similar studies with fixed 3AB concentrations as a function of VPg concentration provided data for analysis by double reciprocal plots of 1/v versus 1/S and confirmed these results (data not shown). Our conclusions from these data are that 3AB is a competitive inhibitor of VPg uridylylation and displays a K_i for 3AB of 100 to 300 nM. Competitive inhibition of VPg uridylylation by 3AB is consistent with the hypothesis

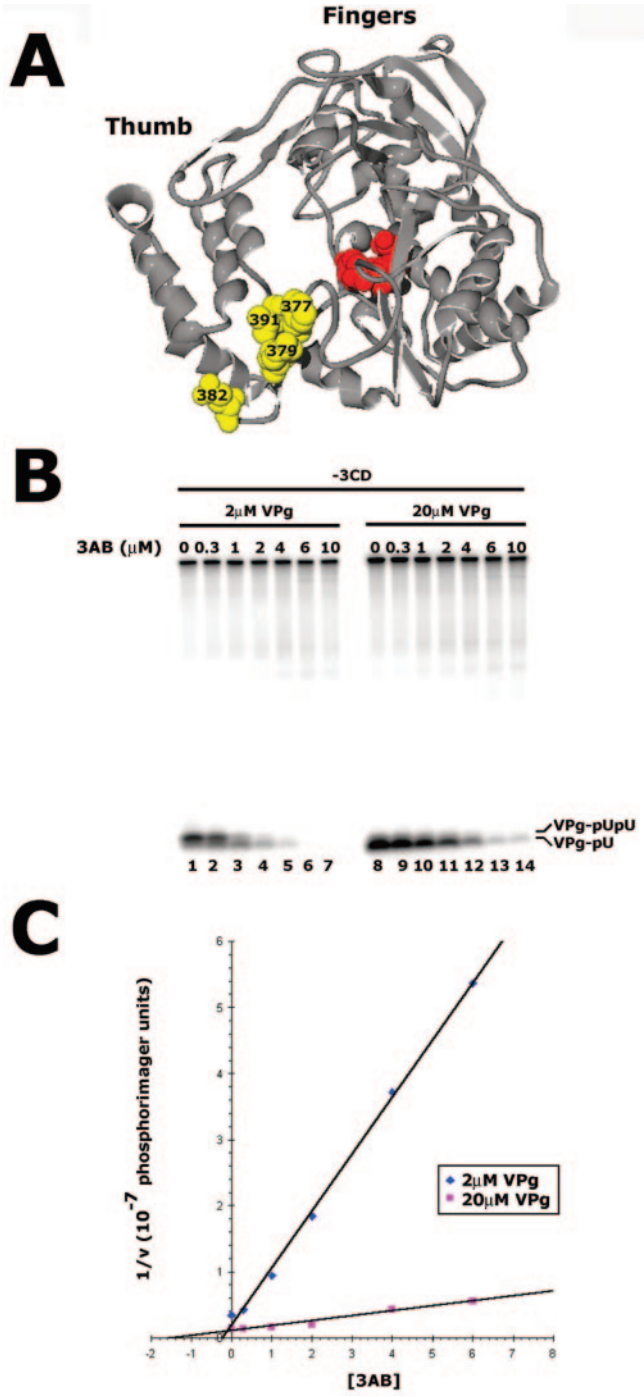

FIG. 4. Inhibition of PV VPg uridylylation by poliovirus 3AB. (A) A representation of the "back of the palm" region of the full-length poliovirus polymerase structure (40) is shown. Residues F377, R379, E382, and V391, known to be involved in 3AB binding to 3D polymerase, are identified in yellow. The active site is shown in red. (B) The effects on VPg uridylylation of increasing concentrations of 3AB at two VPg concentrations (2 μM, lanes 1 to 7; 20 μM VPg, lanes 8 to 14) with a 2C RNA template are shown. Products were resolved in a 12% polyacrylamide-Tris-Tricine gel as previously. (C) Graphical analysis of the data from panel B. A plot of 1/V as a function of inhibitor (3AB) concentration shows a y-intercept above the x-axis, arguing that the inhibition of VPg uridylylation by 3AB is competitive (30). An apparent K_i of approximately 100 nM was determined by extrapolation of the intercept of the two lines to the x-axis. Velocity, V, is in phosphorimager units per unit time.

that the 3AB and VPg binding sites on 3D polymerase are shared or overlapping.

Effect of mutations in the putative VPg binding site of 3D polymerase on 2C RNA-templated VPg uridylylation in the absence and presence of 3CD. The "3CD as binding scaffold" and "3CD as allosteric effector" models (Fig. 2) for 3CD stimulation of VPg uridylylation differ as to whether the 3CD protein or 3D polymerase is the binding site for VPg during the uridylylation reaction. To determine whether mutations in the 3AB and VPg binding sites on 3D polymerase affected the rates of VPg uridylylation in the presence or absence of 3CD, the rates of VPg uridylylation by F377A and R379E mutant polymerases using 2C RNA as a template were determined. Phe377 and Arg379 lie in a surface of the polymerase that has been shown to be the 3AB-binding site by alanine-scanning mutagenesis (22); the F377A and R379E mutant polymerases both showed reduced affinity for 3AB in two-hybrid and in direct binding assays, as well as decreased efficiency of VPg uridylylation. In the absence of 3CD (Fig. 5A), both F377A and R379E mutant polymerases displayed reduced VPg uridylylation activity, as shown previously (22). At all VPg concentrations, the R379E mutant polymerase displayed greater reduction of activity than the F377A mutant enzyme, consistent with its more severe defect in 3AB binding (22). For both mutant polymerases, the mutant phenotypes increased in severity as VPg concentrations decreased (Fig. 5A), consistent with the expectation that the reduction in VPg uridylylation observed is due to a defect of both polymerases in VPg binding, not intrinsic nucleotidyl transfer activity.

The effects of the F377A and R379E mutations on the extent of VPg uridylylation in the presence of 3CD are shown in Fig. 5B. The presence of 3CD greatly stimulated VPg uridylylation, and the radiolabeled products shown are displayed at a 10-fold lower exposure than those shown in Fig. 5A. In this experiment, the presence of the F377A mutation had no effect on the extent of VPg uridylylation at VPg concentrations down to 2 μM. However, the more severe mutation, R379E, consistently reduced VPg uridylylation in the presence of 3CD (Fig. 5B, lanes 3, 6, 9, and 12), as it had in its absence (Fig. 5A). This reduction in VPg uridylylation rate by the R379E mutation supports model B (Fig. 2), in which VPg binds directly to 3D polymerase even during the 3CD-stimulated reaction.

Allosteric effects of mutations at Interface I of 3D polymerase on VPg uridylylation. The experiments in Fig. 5 are consistent with the hypothesis that binding of 3CD protein to 3D polymerase has an allosteric effect on VPg uridylylation rate. We were curious whether mutations known to affect polymerase activity that were distant from the active site might also be due to allosteric effects on polymerase activity. For example, it was recently published that mutations at "Interface I", a binding interface observed in the crystal lattice of wild-type 3D polymerase (12), have disparate effects on the rate of VPg uridylylation (31). Specifically, Pathak et al. showed that mutation of Interface I residues L446, R455, and R456, on the "thumb" side of the putative polymerase-polymerase interface, significantly impaired 3CD-stimulated VPg uridylylation: a 100-fold reduction for R455, 456D polymerase was observed (31). On the other hand, mutations on the "palm" side of the interface had little effect (31). The authors concluded that a physical interaction between the "thumb" side of 3D polymer-

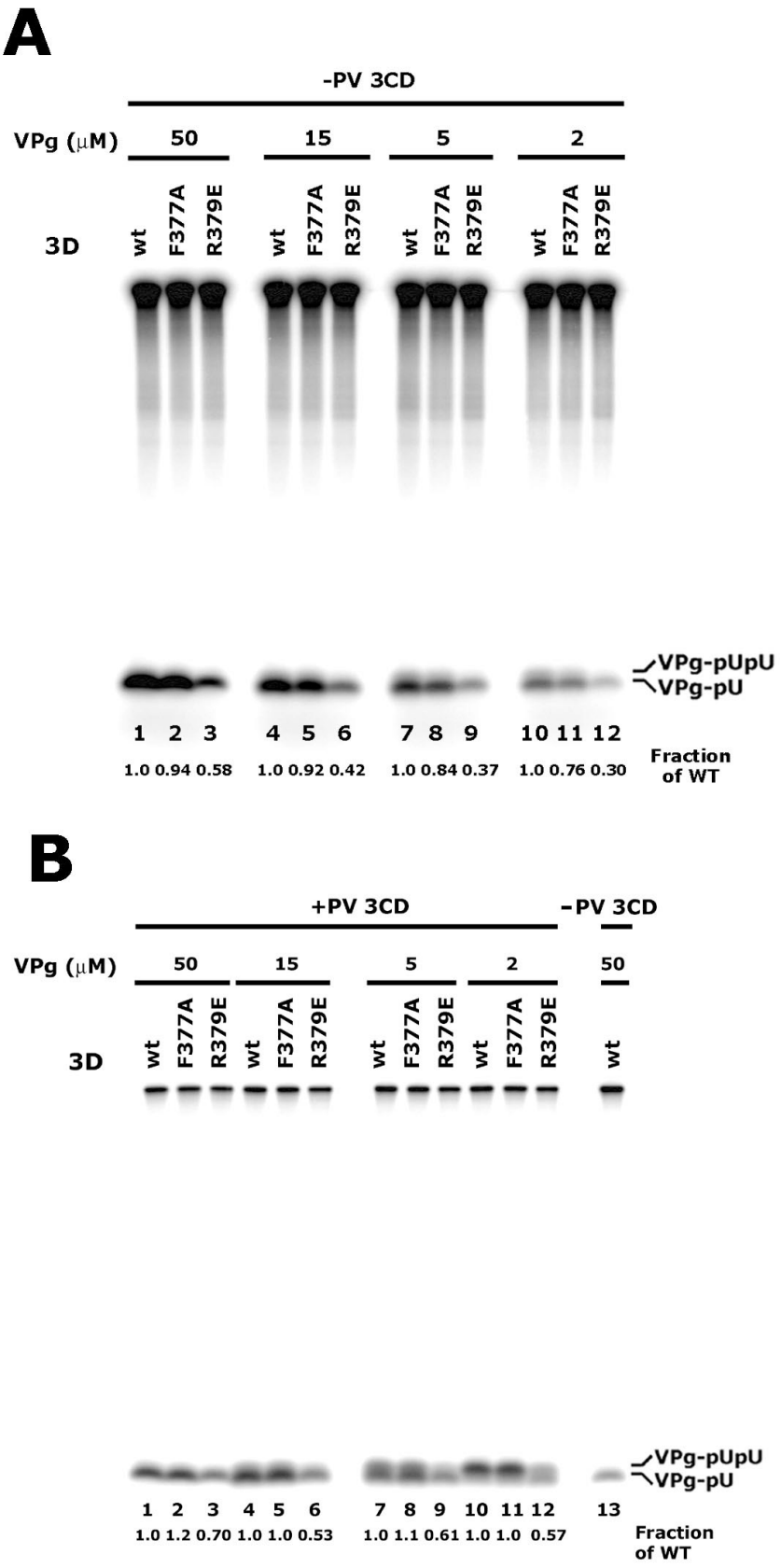

FIG. 5. The effect of mutations in the 3AB binding cluster on the rate of VPg uridylylation in the absence and presence of 3CD. (A). The rates of poliovirus VPg uridylyation by the wild type, F377A and R379E poliovirus 3D polymerases were measured at four different VPg concentrations in the absence of poliovirus 3CD as indicated. The fraction of wild-type activity displayed by the mutant polymerases is shown. (B). The extent of uridylylation in the presence of PV 3CD was assessed as described for panel A; the exposure shown, however, is one-tenth that shown in (A). For both panels, products were analyzed in 12% polyacrylamide-Tris-Tricine gels, as described previously.

ase with 3CD facilitates VPg uridylylation, and that this interaction is specifically affected by the combined mutations R455, R456D. However, we wondered whether the effects of these mutations were independent of the recruitment of ligands such as 3CD, and instead reflected allosteric effects on enzymatic activity.

To investigate whether mutations in residues L446, R455, and R456, on the "thumb" surface of putative polymerase-polymerase Interface I and residues L342 and D349 on the "palm" surface of this putative interface (Fig. 6A) affected the intrinsic ability of 3D polymerase to catalyze VPg uridylylation, we measured the extent of both poly(A) and 2C RNA-templated VPg uridylylation in the absence of 3CD. As can be seen in Fig. 6B, most of these mutations, on either the thumb or the palm side of Interface I, stimulated VPg uridylylation relative to wild-type polymerase in the presence of a poly(A) template (lanes 2, 3, 5, and 6). Only the R455D mutation, on the thumb side of Interface I, caused a reduction of VPg uridylylation (lane 4). In the absence of 3CD protein, mutations at Interface I of 3D polymerase could have affected polymerase homo-oligomerization, conformation, or both. Although not tested explicitly, it is unlikely that the stimulatory and inhibitory effects observed in Fig. 6B resulted from direct effects on oligomerization, because all five mutations would have been expected to reduce the affinity of polymerase-polymerase interactions. Affinity for 3CD was, of course, not a factor here as these reactions were performed in its absence.

When 2C RNA was used as a template instead of poly(A), slightly different results were obtained (Fig. 6B, lanes 7 through 12). Only the L342A mutation, which showed the greatest stimulation of VPg uridylylation in the poly(A)-templated reaction, stimulated the 2C RNA-templated reaction (lane 12). The other mutation on the palm side of Interface I, D349R, had no effect on the extent of VPg uridylylation, whereas all three mutations on the thumb side of the interface, L446A, R455D, and R456D, reduced the reaction rate (lanes 8 through 11). Again, the predicted effects of these mutations on the stability of Interface I did not correlate with their effects on VPg uridylylation. Instead, these results argue that mutations in Interface I residues lead to allosteric effects on the 3D polymerase structure which alter VPg uridylylation activity.

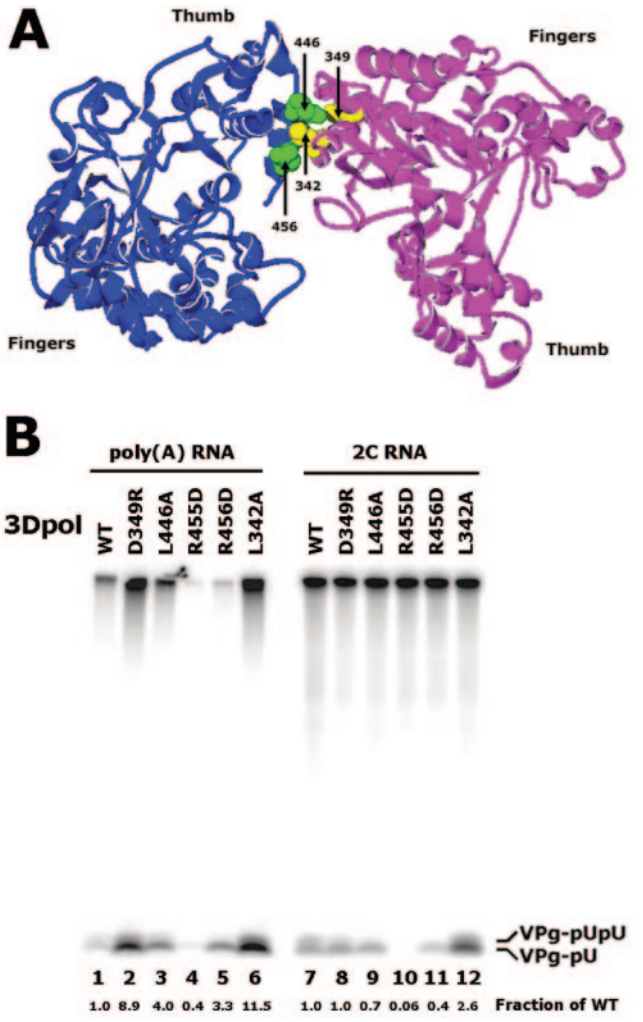

FIG. 6. The effect of mutations at Interface I in 3D polymerase on VPg uridylylation. (A) Two full-length polymerase monomers (40), interacting at Interface I identified from the wild-type polymerase structure (12) are shown. The "thumb" and "fingers" regions of each polymerase are marked to provide orientation, and the residues that were mutated individually in various polymerases are labeled. Residues L446, D455, and D456 on the "thumb" of the polymerase on the left are shown in green; residues L342 and D349 on the back of the "palm" region of the polymerase on the right are shown in yellow. (B) The extent of VPg uridylylation by wild-type and various 3D polymerases with mutations in residues at Interface I was measured in the presence of either poly(A) templates (1 μM strands) or 2C RNA templates (0.15 μM strands), as indicated, in the absence of poliovirus 3CD protein. The fraction of VPg uridylylation activity for each mutant polymerase, compared to the wild type, is shown. Products were resolved in 12% polyacrylamide-Tris-Tricine gels and analyzed as described in Materials and Methods. The radioactivity often present in the wells represents, for the most part, 3′-labeled template RNA, due to the terminal transferase activity of polymerase, as evidenced by the reduction of this signal when template RNA with a blocked 3′-hydroxyl was used (data not shown). The positions of migration of VPg-pUpU and VPg-pU are indicated.

DISCUSSION

Uridylylation of VPg by poliovirus 3D polymerase is a crucial step in initiation of viral RNA synthesis. This reaction requires an RNA template, substrate VPg and UTP, a divalent cation, and a viral RNA-dependent RNA polymerase (3D). In this study, insight into both the poly(A)-primed and the 2C RNA-primed reactions has been gained using a variety of substrates (poliovirus VPg and coxsackievirus VPg), allosteric effectors (poliovirus 3CD and coxsackievirus 3CD) and enzymes (coxsackievirus 3D polymerase, poliovirus 3D polymerase, and mutant poliovirus polymerases).

Where does VPg, the uridylylation substrate, bind to the catalyst? In the process of these studies we observed that poliovirus 3AB is a competitive inhibitor of VPg uridylylation. Therefore, we suggest that the VPg binding site on 3D polymerase involves similar residues as the binding of 3AB, including F377, R379, E382 and V391 (15, 22). At first glance, this finding is unusual as this binding site is on the side of the polymerase opposite to that expected for a nucleic acid template (2, 4, 22). Indeed, the recently determined structure of foot-and-mouth disease virus 3D polymerase, complexed with an RNA template-primer bound to the "front" of the polymer-

ase palm, has recently been published (6). In the case of poliovirus polymerase, it is therefore of interest to determine how the VPg protein primer can be uridylylated on the "back" of the palm and whether large conformational changes due to binary or ternary complexes are involved in this catalytic event.

Multiple ligands may be the agents responsible for the alteration of the 3D structure. We provide evidence that the nature of the RNA template affects the rate of VPg uridylylation even in the absence of 3CD (Fig. 6B), presumably by protein-RNA interactions. For example, with a poly(A) RNA template, significant differences in VPg uridylylation rate were observed with different mutant 3D polymerases, whereas with 2C RNA, the differences were moderate. Thus, the sequence of the RNA or its structure affects the uridylylation rate by various 3D polymerase mutants. This result may be a consequence of the contiguous runs of adenylates present in poly(A), as opposed to the heteropolymeric nucleotide sequence in 2C RNA, as the templates for addition of UMPs to VPg.

Another argument that ligand binding to 3D polymerase has pronounced effects on VPg uridylylation rate can be made from the influence of 3CD on 3D uridylylation activity (Fig. 1B and 3). As shown previously, the addition of 3CD to a reaction templated by 2C RNA substantially stimulated 3D polymerase activity (32, 33). Presumably, this stimulation is due to 3CD-3D interaction, although at present we do not know the interaction surfaces between these proteins. The presence of 3CD had an equivalent effect on VPg uridylylation whether poliovirus or coxsackievirus polymerase was used. However, added specificity was demonstrated by the selective use of one substrate, poliovirus VPg. Interestingly, preference for poliovirus VPg over coxsackievirus VPg was observed in the presence or absence of 3CD (Fig. 3), consistent with the idea that the same binding site was used in both reactions. In support of this hypothesis, mutations in the VPg binding site on the poliovirus polymerase reduced the efficiency of VPg uridylylation in both the presence and the absence of 3CD (Fig. 5). Therefore, we argue that, during 3CD-stimulated VPg uridylylation, VPg remains bound to the polymerase itself and the stimulation by 3CD is an allosteric effect.

Finally, mutations designed to disrupt Interface I (Fig. 6A), an interface observed in the three-dimensional crystal structure of wild-type poliovirus polymerase (12, 14), displayed a variety of disparate effects on VPg uridylylation rate (Fig. 6B). These differences, which did not correlate with predicted effects on Interface I formation, suggest that it was not disruption of the interface per se, but allosteric effects of changes at these molecular surfaces distant from the active site that affected VPg uridylylation rate. Oligomerization of proteins is often accompanied by allosteric effects that complicate the analysis of the resulting cooperativity (13, 41). For example, a mutation at a protein-protein interface could positively or negatively affect the protein-protein interaction, an allosteric change the interaction promotes, or both. As has proved to be the case in other higher-order assemblages, it will be interesting and challenging to deconvolute the effects of mutations designed to disrupt poliovirus polymerase-polymerase and polymerase-3CD interactions on oligomerization and allostery. In any case, it is difficult to rationalize the effects of activating mutations such as L342A (Fig. 6) without invoking an allosteric effect on polymerase activity.

A study by Pathak et al. (31) has also examined the effects of mutations at Interface I on VPg uridylylation. They found that VPg uridylylation was reduced substantially for mutant polymerase 3D-R455,456D (Fig. 6A) in the presence of 3CD, but observed much less effect of these mutations in the absence of 3CD. For another set of mutations (3D-D339, S341, D349A) (Fig. 6A), on the "palm" side of Interface I, little effect of 3CD on the rate of VPg uridylylation was observed. These results were interpreted as signifying that the polymerase surface that contains residues R455 and R456 interacts directly with 3CD during the uridylylation reaction. While this may be the case, we show here that observed differences in uridylylation activity using Interface I mutations in 3D polymerase, particularly mutant polymerases that alter residues 455, 456, and 349, are highly allele-specific even in reactions that do not involve 3CD (Fig. 6B). Therefore, the effects of these mutations on protein-protein interactions are difficult to ascertain due to the apparent sensitivity of VPg uridylylation efficiency to allosteric changes in the polymerase.

Profound conformational changes are known to be involved in the activity of many polymerases. For example, HIV reverse transcriptase, trapped by cross-linking in the act of polymerization, was shown to have a "closed" conformation in which the angle formed between the centers of mass in the thumb and fingers was approximately 50° (16), as measured from the base of the polymerase "hand". In an apoenzyme structure, on the other hand, the analogous angle was approximately 70° (36), and it was almost 90° in the presence of the non-nucleoside inhibitor nevirapine (17). These results suggest that the relative position of the thumb and fingers is likely to affect polymerase activity. For poliovirus polymerase, the putative VPg binding site lies on the back of the polymerase, at the fulcrum of the thumb and fingers. We show here that VPg uridylylation is exquisitely sensitive to polymerase alterations induced by binding of distant ligands and in response to distant mutations. We suggest that such allosteric interactions reflect the known dependence of the priming, elongation and regulation of viral RNA replication on a large multi-component complex whose composition, stoichiometry and intracellular architecture are just beginning to be elucidated.

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REFERENCES

1. **Bell, Y. C., B. L. Semler, and E. Ehrenfeld.** 1999. Requirement for RNA replication of a poliovirus replicon by coxsackievirus B3 RNA polymerase. J. Virol. **73:**9413–9421.
2. **Cheetham, G. M. T., and T. A. Steitz.** 1999. Structure of a transcribing T7 RNA polymerase initiation complex. Science **286:**2305–2309.
3. **Cornell, C. T., R. Perera, J. E. Brunner, and B. L. Semler.** 2004. Strand-specific RNA synthesis determinants in the RNA-dependent RNA polymerase of poliovirus. J. Virol. **78:**4397–4407.

4. **Crowder, S., and K. Kirkegaard.** 2004. Complete three-dimensional structures of picornaviral RNA-dependent RNA polymerases. Structure **12:**1336–1339.
5. **Dayhuff, T. J., R. F. Gesteland, and J. F. Atkins.** 1992. Electrophoresis, autoradiography and electroblotting of peptides: T4 gene 60 hopping. BioTechniques **13:**500–503.
6. **Ferrer-Orta, C., A. Arias, R. Perez-Luque, C. Escarmis, E. Domingo, and N. Verdaguer.** 2004. Structure of foot-and-mouth disease virus RNA-dependent RNA polymerase and its complex with a template-primer RNA. J. Biol. Chem. **279:**47212–47221.
7. **Gerber, K., E. Wimmer, and A. V. Paul.** 2001. Biochemical and genetic studies of the initiation of human rhinovirus 2 RNA replication: identification of a *cis*-replicating element in the coding sequence of $2A^{pro}$. J. Virol. **75:**10979–10990.
8. **Giachetti, C., and B. L. Semler.** 1991. Role of a viral membrane polypeptide in strand-specific initiation of poliovirus RNA synthesis. J. Virol. **65:**2647–2654.
9. **Goodfellow, I., Y. Chaudhry, A. Richardson, J. Meredith, J. W. Almond, W. Barclay, and D. J. Evans.** 2000. Identification of a *cis*-acting replication element within the poliovirus coding region. J. Virol. **74:**4590–4600.
10. **Goodfellow, I., C. Polacek, R. Andino, and D. J. Evans.** 2003. The poliovirus 2C cis-acting replication element-mediated uridylylation of VPg is not required for synthesis of negative-sense genomes. J. Gen. Virol. **84:**2359–2363.
11. **Goodfellow, I. G., D. Kerrigan, and D. J. Evans.** 2003. Structure and function analysis of the poliovirus cis-acting replication element (CRE). RNA **9:**124–137.
12. **Hansen, J. L., A. M. Long, and S. C. Schultz.** 1997. Structure of the RNA-dependent RNA polymerase of poliovirus. Structure **5:**1109–1122.
13. **Hattendorf, D., and S. L. Lindquist.** 2002. Cooperative kinetics of both Hsp104 ATPase domains and interdomain communication revealed by AAA sensor-1 mutants. EMBO J. **21:**12–21.
14. **Hobson, S. D., E. S. Rosenblum, O. C. Richards, K. Richmond, K. Kirkegaard, and S. C. Schultz.** 2001. Oligomeric structures of poliovirus polymerase are important for function. EMBO J. **20:**1153–1163.
15. **Hope, D. A., S. E. Diamond, and K. Kirkegaard.** 1997. Genetic dissection of interaction between poliovirus 3D polymerase and viral protein 3AB. J. Virol. **71:**9490–9498.
16. **Huang, H., R. Chopra, G. L. Verdine, and S. C. Harrison.** 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science **282:**1669–1675.
17. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz.** 1992. Crystal structure at 3.5 A of HIV-1 reverse transcriptase complexes with an inhibitor. Science **256:**1783–1790.
18. **Lama, J., and L. Carrasco.** 1992. Inducible expression of a toxic poliovirus membrane protein in Escherichia coli: Comparative studies using different expression systems based on T7 promoters. Biochem. Biophys. Res. Commun. **188:**972–981.
19. **Lama, J., A. V. Paul, K. S. Harris, and E. Wimmer.** 1994. Properties of purified recombinatnt poliovirus protein 3AB as substrate for viral proteinases and as cofactor for RNA polymerase 3Dpol. J. Biol. Chem. **269:**66–70.
20. **Lobert, P.-E, N. Escriou, J. Ruelle, and T. Michiels.** 1999. A coding RNA sequence acts as a replication signal in cardioviruses. Proc. Natl. Acad. Sci. USA **96:**11560–11565.
21. **Lyle, J. M., E. Bullitt, K. Bienz, and K. Kirkegaard.** 2002. Visualization and functional analysis of RNA-dependent RNA polymerase lattices. Science **296:**2218–2222.
22. **Lyle, J. M., A. Clewell, K. Richmond, O. C. Richards, D. A. Hope, S. C. Schultz, and K. Kirkegaard.** 2002. Similar structural basis for membrane localization and protein priming by an RNA-dependent RNA polymerase. J. Biol. Chem. **277:**16324–16331.
23. **Mason, P. W., S. V. Bezboradova, and T. M. Henry.** 2002. Identification and characterization of a *cis*-acting replication element (*cre*) adjacent to the internal ribosome entry site of foot-and-mouth disease virus. J. Virol. **76:**9686–9694.
24. **McKnight, K. L., and S. M. Lemon.** 1998. The rhinovirus type 14 genome contains an internally located RNA structure that is required for viral replication. RNA **4:**1569–1584.
25. **Morasco, B. J., N. Sharma, J. Parilla, and J. B. Flanegan.** 2003. Poliovirus *cre*(2C)-dependent syntheiss of VPgpUpU is required for positive- but not negative-strand RNA synthesis. J. Virol. **77:**5136–5144.
26. **Murray, K. E., and D. J. Barton.** 2003. Poliovirus CRE-dependent VPg uridylyation is required for positive-strand RNA synthesis but not for negative-strand RNA synthesis. J. Virol. **77:**4739–4750.
27. **Neufeld, K. L., O. C. Richards, and E. Ehrenfeld.** 1991. Purification, characterization and comparison of poliovirus RNA polymerase from native and recombinant sources. J. Biol. Chem. **266:**24212–24219.
28. **Novak, J. E., and K. Kirkegaard.** 1991. Improved method for detecting poliovirus minus strands used to demonstrate specificity of positive-strand encapsidation and the ratio of positive to minus strands in infected cells. J. Virol. **65:**3384–3387.
29. **Parsley, T. B., J. S. Towner, L. B. Blyn, E. Ehrenfeld, and B. L. Semler.** 1997. Poly(rC) binding protein 2 forms a ternary complex with the 5′ -terminal sequences of poliovirus RNA and the viral proteinase. RNA **3:**1124–1134.
30. **Pata, J. D., S. C. Schultz, and K. Kirkegaard.** 1995. Functional oligomerization of poliovirus RNA-dependent RNA polymerase. RNA **1:**466–477.
31. **Pathak, H. B., S. Kumar, B. Ghosh, A. W. Roberts, S. D. Sharma, J. D. Yoder, J. J. Arnold, D. W. Gohara, D. J. Barton, A. V. Paul, and C. E. Cameron.** 2002. Structure-function relationships of the RNA-dependent RNA polymerase from poliovirus (3Dpol): a surface of the primary oligomerization domain functions in capsid precursor processing and VPg uridylylation. J. Biol. Chem. **277:**31551–31562.
32. **Paul, A. V., J. Peters, J. Mugavero, J. Yin, J. H. van Boom, and E. Wimmer.** 2003. Biochemical and genetic studies of the VPg uridylylation reaction catalyzed by the RNA polymerase of poliovirus. J. Virol. **77:**891–904.
33. **Paul, A. V., E. Rieder, D. W. Kim, J. H. van Boom, and E. Wimmer.** 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. J. Virol. **74:**10359–10370.
34. **Paul, A. V., J. H. van Boom, D. Fillippov, and E. Wimmer.** 1998. Protein-primed RNA synthesis by purified poliovirus RNA polymerase. Nature **393:**280–284.
35. **Rieder, E., A. V. Paul, D. W. Kim, J. H. van Boom, and E. Wimmer.** 2000. Genetic and biochemical studies of poliovirus cis-acting replication element cre in relation to VPg uridylylation. J. Virol. **74:**10371–10380.
36. **Rodgers, D. W., S. J. Gamblin, B. A. Harris, S. Ray, J. S. Culp, B. Hellmig, D. J. Woolf, C. Debouck, and S. C. Harrison.** 1995. The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **92:**1222–1226.
37. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analyt. Biochem. **166:**368–379.
38. **Segel, I. H.** 1975. Enzyme kinetics. John Wiley & Sons, New York, N.Y.
39. **Teterina, N. L., M. S. Rinaudo, and E. Ehrenfeld.** 2003. Strand-specific RNA synthesis defects in a poliovirus with a mutation in protein 3A. J. Virol. **77:**12679–12691.
40. **Thompson, A. A., and O. B. Peersen.** 2004. Structural basis for proteolysis-dependent activation of the poliovirus RNA-dependent RNA polymerase. EMBO J. **23:**3462–3471.
41. **van Holde, K. E., K. I. Miller, and E. van Olden.** 2000. Allostery in very large molecular assemblies. Biophys. Chem. **86:**165–172.
42. **Xiang, W., A. Cuconati, D. A. Hope, K. Kirkegaard, and E. Wimmer.** 1998. Complete protein linkage map of poliovirus P3 proteins: interaction of polymerase $3D^{pol}$ with VPg and with genetic variants of 3AB. J. Virol. **72:**6732–6741.
43. **Yang, Y., R. Rijnbrand, S. Watowich, and S. M. Lemon.** 2004. Genetic evidence for an interaction between a picornaviral cis-acting RNA replication element and 3CD protein. J. Biol. Chem. **279:**12659–12667.
44. **Yin, J., A. V. Paul, E. Wimmer, and E. Rieder.** 2003. Functional dissection of a poliovirus *cis*-acting replication element [PV-*cre*(2C)]: analysis of single- and dual-*cre* viral genomes and proteins that bind specifically to PV-*cre* RNA. J. Virol. **77:**5152–5166.