Structural features of a picornavirus polymerase involved in the polyadenylation of viral RNA

Brian J. Kempf¹, Michelle M. Kelly¹, Courtney Springer³, Olve B. Peersen³, and David J. Barton¹,² *

¹Department of Microbiology and ²Program in Molecular Biology, University of Colorado School of Medicine, Aurora, CO 80045
³Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO 80523

*Corresponding Author

David J. Barton

Department of Microbiology (MS8333)
University of Colorado School of Medicine
12800 East 19th Avenue
Aurora, CO 80045 Ph: (303) 724-4215
Fax: (303) 724-4226
E-mail: David.Barton@UCDenver.edu

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ABSTRACT

Picornaviruses have 3’ polyadenylated RNA genomes, but the mechanisms by which these genomes are polyadenylated during viral replication remain obscure. Based on prior studies, we proposed a model wherein the poliovirus RNA-dependent RNA polymerase (3D<sub>pol</sub>) uses a reiterative transcription mechanism while replicating the poly(A) and poly(U) portions of viral RNA templates. To further test this model, we examined whether mutations in 3D<sub>pol</sub> influenced the polyadenylation of virion RNA. We identified nine alanine substitution mutations in 3D<sub>pol</sub> that resulted in shorter or longer 3’ poly(A) tails in virion RNA. These mutations could disrupt structural features of 3D<sub>pol</sub> required for the recruitment of a cellular poly(A) polymerase; however, the structural orientation of these residues suggest a direct role of 3D<sub>pol</sub> in the polyadenylation of RNA genomes. Reactions containing purified 3D<sub>pol</sub> and a template RNA with a defined poly(U) sequence provided data consistent with a template-dependent reiterative transcription mechanism for polyadenylation. The phylogenetically conserved structural features of 3D<sub>pol</sub> involved in the polyadenylation of virion RNA include a thumb domain alpha-helix that is positioned in the minor groove of the dsRNA product and lysine and arginine residues that interact with the phosphates of both the RNA template and product strands.

(196 words)

Keywords: RNA-dependent RNA polymerase, polyadenylation, poly(A), RNA replication, picornavirus.
INTRODUCTION

Picornaviruses, like many other positive-strand RNA viruses, have RNA genomes with variable length 3’ poly(A) tails (1). The poly(A) tails of picornaviruses are important for viability (2), with the length of the poly(A) tails influencing the magnitudes of both viral mRNA translation and RNA replication (3, 4). RNA sequences and structures within the 3’ non-translated region are reported to influence both the length of poly(A) tails in picornaviral RNA (5) and the efficiency of virus replication (6, 7). Viral RNA-dependent RNA polymerases are predicted to catalyze the polyadenylation of picornaviral RNA in a template-dependent manner during viral RNA replication (8); however, the mechanisms by which RNA genomes are polyadenylated during viral RNA replication remain obscure. In particular, there is little understanding of the mechanisms regulating the length of poly(A) tails synthesized during RNA replication. The RNA-dependent RNA polymerases of negative-strand RNA viruses reiteratively transcribe a small poly(U) sequence within intergenic regions of the viral RNA genome to produce long poly(A) tails on viral mRNAs (9). In a similar manner, the poliovirus RNA-dependent RNA polymerase can reiteratively transcribe poly(A) and poly(U) sequences during viral RNA replication, producing poly(A) and poly(U) sequences longer than those in the respective template RNAs (8).

Viral RNA-dependent RNA polymerases are well studied at the structural level (10-12), yet there have been no reports describing structural features of viral polymerases involved in the polyadenylation of viral RNA. The RNA-dependent RNA polymerase of picornaviruses assume a “right hand” conformation with thumb, palm and fingers domains (Figure 1) (13, 14). Polymerases from phylogenetically distinct picornaviruses exhibit similarity at the amino acid sequence and structural levels (Figure 1 and Table S1). The fingers domain reaches across the top of the polymerase to interact with the thumb domain, effectively encircling the active site that is located at the top of the palm.
Atomic structures of the poliovirus polymerase elongation complex reveal features involved in interactions with viral RNA templates and dsRNA products (Figure 1D) (16).

In order to identify the structural features of picornavirus polymerases associated with the polyadenylation of viral RNA, we mutated specific amino acid residues in the 3D\textsuperscript{pol} thumb, palm, and fingers domains, recovered viable virus from HeLa cells, assessed the stability of the engineered mutations in virus populations, and determined whether stably-maintained mutations affected the length of poly(A) sequences at the 3’ end of virion RNA. We found that alanine substitution mutations within phylogenetically conserved regions of 3D\textsuperscript{pol} resulted in populations of virion RNA with altered poly(A) tail lengths, consistent with the predicted reiterative transcription mechanisms of viral RNA replication (8). Data from reactions containing purified 3D\textsuperscript{pol} confirmed that 3D\textsuperscript{pol} mutations influenced the amounts of reiterative transcription on a defined poly(U) template. We describe and discuss the conserved features of picornavirus RNA-dependent RNA polymerases involved in the synthesis of poly(A) tails at the 3’ end of viral RNA genomes.
MATERIALS AND METHODS

Viral RNA and cDNA clones.

Poliovirus type 1 (Mahoney) was used for this study. Poliovirus cDNA clones with genetically encoded poly(A) tails 32, 51, and 84 bases in length were previously described (8). 3Dpol mutations were introduced into a subgenomic cDNA clone [pRNA2 poly A(84)] by site-directed mutagenesis (Quick Change XL-II, Stratagene, Inc.) and verified by sequencing the 3CD genes (nucleotides 5438-7440). The parental codons, mutant codons and DNA oligonucleotides used for site-directed mutagenesis are listed in Table S1. Defined 3Dpol mutations in pRNA2 poly A(84) were subcloned into a full-length infectious cDNA via unique KasI and MluI restriction sites. Full-length infectious poliovirus RNAs were transcribed from MluI-linearized cDNA clones using T7 RNA polymerase (AmpliScribe T7 High Yield Transcription Kit, CellScript, Inc.).

Encephalomyocarditis virus (EMCV) was derived from an infectious cDNA clone (pEC9) kindly provided by Ann Palmenberg (University of Wisconsin, Institute of Molecular Virology, Madison, WI). T7 transcription of SalI-linearized pEC9 cDNA produces infectious EMCV RNA encoding 17 adenine residues at the 3' end (17).

Poliovirus RNA translation, polyprotein processing and RNA replication in cytoplasmic extracts from HeLa cells.

Poliovirus RNA translation, polyprotein processing and RNA replication were assayed in reactions containing cytoplasmic extracts from uninfected HeLa cells as previously described (18-20). Radiolabeled proteins from reactions containing [35S]methionine were fractionated by SDS-PAGE and detected by phosphorimaging. Preinitiation RNA replication complexes were used to assay viral RNA replication in reactions with and without 2 mM guanidine HCL, an inhibitor of RNA replication (21). Radiolabeled viral
RNAs from reactions containing [$\alpha$-$^{32}$P]UTP were fractionated by electrophoresis in non-denaturing agarose gels and detected by phosphorimaging.

**Viral RNA transfection, specific infectivity and infection of HeLa cells.**

Poliovirus RNAs containing 3D$^{\text{pol}}$ mutations were produced by T7 transcription and transfected into HeLa cells as previously described (22).

The specific infectivity of poliovirus RNAs was determined by transfecting serial 10-fold dilutions of viral RNA (10, 1.0, 0.1, 0.01, 0.001 ng) into ~$10^6$ HeLa cells per 35 mm well of 6-well plates. Transfected cells wereoverlayed with 1.2% methylcellulose in media (DMEM, 100 Units per ml of penicillin and 100 ug per ml of streptomycin, 10% fetal bovine serum, 10 mM MgCl₂) and incubated at 37°C in 5% CO₂ for 72 hours. Plates were then rinsed with PBS, fixed with 5% TCA, and stained with crystal violet to visualize plaques. The specific infectivity was reported as plaque forming units (PFU) per ug of viral RNA, with a lower limit of detection of $10^2$ pfu per ug.

P(0) virus was harvested at 72 hours post-transfection (hpt) and quantified by plaque assay. P(0) virus was used to infect ~$10^7$ HeLa cells in T75 flasks at an MOI of 10 PFU per cell. P(1) virus was harvested by freeze-thawing cells at 24-72 hours post-infection (hpi), based on the timing of cytopathic effects, and quantified by plaque assay.

EMCV RNA from an infectious cDNA clone was transfected into HeLa cells (17). EMCV was grown and titered by plaque assay as described above for poliovirus.

**Virus purification and isolation of virion RNA.**

Virus (8 ml) was layered onto 3 ml of 30% (w/v) sucrose in phosphate-buffered saline followed by centrifugation at 36,000 rpm for 4 h at 4°C using a Beckman SW41 rotor. Pelleted material containing virus particles was resuspended in 400 μl 0.5% SDS buffer...
[0.5% SDS, 10 mM Tris–HCl (pH 7.5), and 100 mM NaCl]. Virion RNAs were isolated by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation.

cDNA synthesis and sequencing of the 3CD region of virion RNA.

The stability of 3Dpol mutations engineered into poliovirus was determined by sequencing the 3CD region of cDNA. Poliovirus RNAs were converted into cDNAs using Superscript III (Invitrogen) and a primer complementary to nucleotides 7400–7418 of the poliovirus 3’ NTR (Table S2, Reverse Primer 1). cDNA corresponding to the 3CD region of poliovirus RNA was amplified by 35 PCR cycles with high-fidelity Phusion DNA polymerase (New England Biolabs, Ipswich, MA) using reverse primer 1 and forward primer 1 (Table S2). When low amounts of P(0) and P(1) virus limited the initial yield of cDNA, a second round of PCR was used to increase the yield of DNA for sequencing (35 cycles using a 1:50 dilution of DNA template from the first reaction). PCR products were analyzed by agarose gel electrophoresis, purified using Qiagen QiaQuick PCR Purification Kit, and sequenced in the University of Colorado Cancer Center DNA Sequencing Core using forward primers 1-5 (Table S2).

[^32P]-pCp end-labeling of virion RNA and urea-PAGE.

Poliovirus and EMCV virion RNAs were 3’ end-labeled in reactions containing T4 single-stranded RNA ligase (New England Biolabs) and [^32P]pCp (MP Biomedicals, Solon, OH). Poliovirus RNA transcripts with genetically encoded poly(A) tails 32, 51, and 84 bases in length were radiolabeled in T7 transcription reactions containing [α[^32P]]ATP as previously described (8). Radiolabeled RNAs were digested with RNase T1 and fractionated by electrophoresis in 7 M urea 18% polyacrylamide gels in TBE buffer (89 mM Tris base, 89 mM boric acid pH 8.3, 2 mM EDTA) for 6 hours at 25 W (8). Radiolabeled RNAs within the gels were detected and quantified by phosphorimaging (Bio-Rad).
TOPO-TA cloning and cDNA sequencing of poly(A) tails in virion RNA.

The 3’ end of poliovirus and EMCV virion RNAs were converted into cDNA, cloned and sequenced using methods previously described (8). An RNA linker (Table S2) was added to the 3’ ends of purified virion RNAs using T4 single-stranded RNA ligase. Virion RNAs with the 3’ linker RNA were concentrated by ethanol precipitation and transcribed into cDNA using a primer complementary to the 3’ end of the RNA linker (Table S2, Linker cDNA Primer) and Superscript III Reverse Transcriptase (Invitrogen). cDNA corresponding to the 3’ end of poliovirus and EMCV RNAs, including the poly(A) tail and RNA linker, was amplified in 30 PCR cycles with high fidelity Phusion DNA polymerase (New England Biolabs) using poliovirus forward primer 5 or EMCV forward primer 1 and the linker amplification primer (Table S2). PCR products were TOPO-TA cloned (Invitrogen) according to the manufacturer’s instructions. The cloning reactions were transformed into TOP10 chemically competent E. coli and plated onto LB plates containing 100 μg/ml ampicillin. Colonies were selected and PCR screened for insert. Plasmids containing cDNA from the 3’ end of poliovirus and EMCV virion RNA were extracted using a QIAPrep Spin Miniprep kit (Qiagen) and sequenced in the University of Colorado Cancer Center DNA Sequencing Core Laboratory. Plasmid DNA was sequenced using poliovirus forward primer 5 or EMCV forward primer 1 (Table S2). These primers provided sequence data corresponding to 235 bases of heteropolymeric sequence at the 3’ end of PV RNA as well as the size and sequence of the poly(A) tail or 526 bases of heteropolymeric sequence at the 3’ end of EMCV RNA as well as the size and sequence of the poly(A) tail. The reliability of these TOPO-TA cloning and sequencing methods was validated using T7 transcripts of poliovirus RNA with defined poly(A) tails 32, 51, and 84 bases in length (Figure S1).
Reactions containing purified 3D\textsuperscript{pol} and a defined RNA template.

Wildtype and mutant poliovirus 3D\textsuperscript{pol} were purified as previously described (23). A self-priming RNA template was synthesized by Integrated DNA Technologies (Figure 7A). An amino modifier deoxythymi dine at the 5'-end of the RNA template was labeled with IRdye 800RS NHS ester (Li-Cor Biosciences), as previously described (23).

Reactions containing 15 μM 3D\textsuperscript{pol}, 1 μM RNA template, 65 μM of the individual NTPs (as indicated in the figure), 25 mM HEPES, pH 6.5, 87.5 mM NaCl, 2 mM MgCl\textsubscript{2}, and 5 mM TCEP (a reducing agent) were mixed on ice for five minutes and then incubated at room temperature for the elongation reaction. Samples (1 μL) were taken from the reactions at various time points and quenched with 19 μL of reaction buffer containing 300 mM NaCl and 25 mM EDTA. Gel loading dye (10 μL of solution containing 95% formamide, 0.5% bromophenol blue, 0.5% xylene cyanol) was added to the quenched samples. RNAs in each sample were separated by electrophoresis in 15% polyacrylamide-7M urea gels and detected using a Li-Cor Odyssey 9120 infrared imager system.

TOPO-TA cloning and cDNA sequencing of virion RNA to determine the fidelity of RNA replication.

Poliovirus RNAs were converted into cDNAs using Superscript III (Invitrogen) and poliovirus reverse primer 2 (Table S2). cDNA corresponding to the 3D\textsuperscript{pol} region of poliovirus RNA was amplified by 35 PCR cycles with high-fidelity Phusion DNA polymerase (New England Biolabs) using poliovirus reverse primer 2 and forward primer 2 (Table S2). PCR products were analyzed by agarose gel electrophoresis and TOPO-TA cloned (Invitrogen) according to the manufacturer’s instructions. The cloning reactions were transformed into TOP10 chemically competent E. coli and plated onto LB plates containing 100 μg/ml ampicillin. Colonies were selected and PCR screened.
for insert. Plasmid DNA from 50 individual clones containing poliovirus cDNA were extracted using a QIAprep Spin Miniprep kit (Qiagen) and sequenced with poliovirus reverse primer 2 and forward primer 2 in the University of Colorado Cancer Center DNA Sequencing Core Laboratory.
RESULTS

3D\textsuperscript{pol} mutations and summary regarding polyadenylation of virion RNA.

Based on the structure of the poliovirus RNA-dependent RNA polymerase elongation complex (16), we mutated 17 amino acids that are intimately involved in interactions with viral RNA templates and dsRNA products, residues potentially involved in the polyadenylation of virion RNA (Table 1). Amino acid substitution mutations were engineered into an infectious cDNA clone of poliovirus and the effects of the mutations on various aspects of viral gene expression and replication were assayed.

The impact of 3D\textsuperscript{pol} mutations on the specific infectivity of viral RNA and virus growth was assayed in HeLa cells (Table 1) (22). Infectious virus produced in transfected HeLa cells was titered by plaque assay and amplified by passage ($P_{(0)}$ & $P_{(1)}$ titers in Table 1).

The genetic stability of mutations in virus populations was determined by sequencing the 3CD genes of virion RNA (Table 1 and Table S1). The sequence of parental codons, mutant codons, and reversion mutations for virus populations are described in Table S1. No compensatory mutations were found in the 3CD genes of virus with stably maintained mutations.

3D\textsuperscript{pol} mutations that dramatically reduced the specific infectivity of viral RNA were unstable in virus populations whereas 3D\textsuperscript{pol} mutations that had only minor impact on the specific infectivity of viral RNA were stable in virus populations (Table 1). Viral RNA with unstable G64D, Y118A, Y157A, Y118A-Y148A, Y118A-Y148F, K127A, K127A-R128A, and R188A mutations each had specific infectivities several orders of magnitude below that of wildtype viral RNA. Viral RNA with unstable G64T, Y148A, Y118F-Y148A, and K125A-K126A mutations had specific infectivities ranging from $7.0 \times 10^5$ to $1.2 \times 10^6$ PFU per ug of viral RNA as compared to $9.0 \times 10^6$ PFU per ug for wildtype RNA. Viral RNA with stable G64S, Y14A, Y118F, Y148F, Y157F, Y118F-Y148F, K125A, K126A,

Virus populations with unstable mutations precluded further analysis of their impact on polyadenylation of virion RNA (Table 1, Unstable 3D\text{pol} mutations). Seven mutations stably maintained in virus populations failed to impact the polyadenylation of virion RNA (Table 1, Stable 3D\text{pol} mutations with wildtype poly(A) tail phenotypes). Nine alanine substitution mutations stably maintained in virus populations affected the polyadenylation of virion RNA (Table 1, Stable 3D\text{pol} mutations that altered poly(A) tail phenotypes), as described in further detail below.

**Viral mRNA translation, polyprotein processing and RNA replication.**

The impact of 3D\text{pol} mutations on viral mRNA translation, polyprotein processing and RNA replication was assayed in HeLa cell-free reactions (Figure 2 and Figure S2) (19, 21, 24). Mutant viral RNAs were divided into four groups based on the structural and functional characteristics of particular 3D\text{pol} amino acids: mutations of glycine 64, an amino acid involved in the fidelity of viral RNA replication (Figures 2A and S2A) (25-29); mutations of tyrosine residues in the fingers domain located along the viral RNA template entry channel (Figures 2B and S2B) (22); mutations of charged lysine and arginine residues that can interact with phosphate residues of the viral RNA template and dsRNA products (Figures 2C and S2C) (16); and mutations of amino acids in a thumb domain alpha helix positioned in the minor groove of dsRNA products (Figures 2D and S2D) (16).

Glycine 64 mutations (G64S, G64T, and G64D) resulted in reduced amounts of viral RNA replication in cell-free replication reactions as compared to wildtype poliovirus (Figure 2A). The amounts of replicative form RNA (RF RNA) synthesized by G64 mutants were indistinguishable when compared to wildtype poliovirus; however, the
amounts of accumulating positive-strand RNA products were reduced as compared to wildtype poliovirus (Figure 2A), consistent with the slower rate of RNA catalysis associated with a G64S mutation (28). The G64S mutation was stably maintained in virus populations, consistent with previous studies (25-27); however, the G64S mutation had no effect on the lengths of poly(A) tails in virion RNA (Table 1), as described in further detail below. The G64T and G64D mutations, which were associated with defects in capsid polyprotein processing (Figure S2A, lanes 3 and 4), were not analyzed further.

Tyrosine residues in the fingers domain (Y14, Y118, Y148, Y157) are located near the template RNA entry channel (22) and may help denature dsRNA stem-loop structures as the template strand enters the polymerase. Alanine and phenylalanine substitution mutations of these residues resulted in notable effects on polyprotein processing (Figure S2B), RNA replication (Figure 2B), and virus replication (Table 1). Alanine substitutions at Y118, Y148, and Y157 were not stably maintained in virus populations, consistent with severe defects in viral RNA replication, while more conservative phenylalanine mutations at these positions were stably maintained, consistent with detectable levels of viral RNA replication. However, none of the tyrosine mutations affected the polyadenylation of virion RNA (Table 1), as described in further detail below.

Lysine and arginine residues have the capacity to interact with phosphates in viral RNA templates and dsRNA products (16, 22). Alanine substitutions of K125, K126, R128, K133, and K276 were stably maintained in virus populations (Table 1), showed normal polyprotein processing (Figure S2C) and detectable levels of viral RNA replication (Figure 2C). Notably, individual alanine substitutions at four of these residues affected the polyadenylation of virion RNA (Table 1, K126A, R128A, K133A, and K276A). K127A, R188A, K125A-K126A, and K127A-R128A mutations were not stably
maintained in virus populations (Table 1), consistent with undetectable levels of viral RNA replication in HeLa cell extracts (Figure 2C), and consistent with a previous report of alanine substitution mutations in poliovirus (30).

An alpha helix in the thumb domain was targeted for mutagenesis because it fits snugly into the minor groove of the dsRNA product as it exits the polymerase (16). Alanine substitution mutations of N409, D412, S416, L419, and L420 were stably maintained in virus populations (Table 1), exhibited normal polyprotein processing (Figure S2D) and detectable levels of viral RNA replication in cell-free reactions (Figure 2D). Alanine substitution mutations at each of the five sites also affected the polyadenylation of virion RNA (Table 1), as described in detail below.

Lengths of poly(A) tails in virion RNA measured by $^{32}$P-pCp end-labeling and urea-PAGE.

The variable lengths of poly(A) at the 3' end of radiolabeled poliovirus RNA are easily measured by RNase T1 digestion followed by urea-PAGE (1). RNase T1, which cleaves RNA at the 3' side of G bases, liberates the poly(A) tail from poliovirus RNA as illustrated in Figure 3A. Three other relatively large T1- liberated oligonucleotides from the viral genome serve as convenient size markers in urea-polyacrylamide gels (Figure 3A and 3B, note the mobilities of the 37, 36, and 31-mers in lanes 1-3). RNA transcripts from poliovirus cDNA clones with poly(A) tails of 84, 51, and 32 bases in length provide additional size markers in the urea-polyacrylamide gels (Figure 3B, note the mobilities of poly(A) in lanes 1-3). $^{32}$P-pCp end-labeling of poliovirus and EMCV virion RNA, in conjunction with RNase T1 digestion and urea-PAGE, reveals the distinct characteristics of poly(A) tails in these two viruses (Figure 3B, lanes 8 and 9). The poly(A) tails in poliovirus RNA genomes tend to be longer than the poly(A) tails of EMCV RNA genomes (Figure 3B, compare lanes 8 and 9), as previously reported (1).
Phosphorimaging allows for detailed quantitative analyses of the size distribution of poly(A) tails in populations of virion RNA (Figure 3C). Poliovirion RNA has poly(A) tails ranging in size from 20 to 120 bases long, with a classic bell curve distribution around a mean ~ 50 bases (Figure 3B, lane 8 and Figure 3C). EMCV virion RNA has poly(A) tails ranging in size from less than 20 bases long to greater than 70 bases long, with a mean distribution ~ 30 bases (Figure 3B, lane 9 and Figure 3C).

The impact of 3Dpol mutations on the polyadenylation of virion RNA was determined for G64S, K125A, K126A, and R128A (Figure 3); Y14A, Y118F, Y148F, Y157F, and Y118F-Y148F (Figure 4A); K133A and K276A (Figure 4B); N409A, D412A, S416A, L419A and L420A (Figure 5). The size distribution of poly(A) tails in virion RNA was indistinguishable from wildtype for G64S virus, the tyrosine mutant viruses (Y14A, Y118F, Y148F, Y157F, and Y118F-Y148F), and the K125A virus. The size distribution of poly(A) tails in virion RNA was modestly different from wildtype for K126A, R128A, K133A, and K276A viruses. The most striking differences in the size distribution of poly(A) tails were observed for N409A, D412A, S416A, L419A and L420A viruses (Figure 5). N409A, L419A and L420A viruses had poly(A) tails substantially longer than wildtype poliovirus (Figure 5A, lanes 4, 7 & 8), with a classic bell curve distribution (Figures 5B, 5E and 5F). D412A virus, on the other hand, had poly(A) tails substantially shorter than wildtype poliovirus (Figure 5A, lane 5), with a classic bell curve distribution (Figure 5C). S416A virus had poly(A) tails with a broader size distribution than wildtype (Figure 5A, lane 6 and 5D).

Advantages of [\(^{32}\text{P}\)]-pCp end-labeling of virion RNA, in conjunction with RNase T1 digestion and urea-PAGE, include the single base resolution of the urea-polyacrylamide gels, the equimolar distribution of radiolabel on each molecule of poly(A), and the comprehensive representation of virion RNA molecules in each sample. A disadvantage of this method is the inability to quantify poly(A) tails less than 20 bases long, due to an
abundance of background signal in this region of polyacrylamide gels (data not shown). This limitation is particularly problematic for EMCV RNA, which has an abundance of small poly(A) tails less than 20 bases long.

Lengths of poly(A) tails in virion RNA measured by TOPO-TA cloning and cDNA sequencing.

Due to the limitations noted above and our desire to confirm the results with an independent method, we re-analyzed the size of poly(A) tails in virion RNA using cDNA sequencing (8). This method reliably reveals the size of poly(A) sequences at the 3' end of viral RNA (Figure S1). The 3' end of virion RNA was converted into cDNA, TOPO-TA cloned and sequenced as diagrammed in Figure 6A. Fifty individual clones were sequenced from each mutant virus population. The size of poly(A) tail in each cDNA clone was plotted accordingly (Figure 6B) and the cumulative data were summarized in Table 2. Fifty individual EMCV poly(A) tails ranged from 10-58 bases long, with a mean of 28.2 bases and a median of 26.0 bases (Figure 6B and Table 2). Fifty individual wildtype poliovirion poly(A) tails ranged from 25 to 93 bases long, with a mean of 52.9 bases and a median of 51.0 bases (Figure 6B and Table 2). Overall, the size of EMCV poly(A) tails was statistically different from the size of poliovirus poly(A) tails (Table 2, P value of <0.0001).

The impact of 3Dpol mutations on the polyadenylation of virion RNA was analyzed using this TOPO-TA cloning and cDNA sequencing method. G64S virus poly(A) tails were not statistically different from wildtype (Figure 6B and Table 2). Likewise, K125A poly(A) tails were not statistically different from wildtype (Figure 6B and Table 2). In contrast, the size of K126A, R128A, K133A, K276A, N409A, D412A, L419A, and L420A virus poly(A) tails were statistically different from wildtype (Figure 6B and Table 2). D412A poliovirus poly(A) tails were the smallest, with a median size of 40.5 bases whereas
L420A poly(A) tails were the largest, with a median size of 59.0 bases (Table 2). These data, which are consistent with the size distribution of poly(A) tails measured using urea-polyacrylamide gels, confirm that 3D\textsuperscript{pol} mutations influence the polyadenylation of virion RNA.

**Impact of 3D\textsuperscript{pol} mutations on template-dependent reiterative transcription in vitro.**

We used reactions containing purified 3D\textsuperscript{pol} to assess the impact of N409A, D412A, S416A, L419A and L420A mutations on template-dependent reiterative transcription (Fig. 7). The RNA template, which was labeled at its 5' end with an infrared dye for detection purposes, had a self-priming hairpin at the 3' end followed by a poly(U) sequence 10 bases in length (Fig. 7A). Beyond the poly(U) sequence were heteropolymeric sequences, a poly(C) sequence, and additional heteropolymeric sequence (Fig. 7A). Wildtype 3D\textsuperscript{pol} converted this template into different products over time depending on the presence of one or more rNTPs (Fig. 7B). When all four rNTPs were present, 3D\textsuperscript{pol} produced defined length RNA products 39 bases longer than the template corresponding to continuous transcription over the full length of the template sequence (Fig. 7B, +39). When ATP and UTP were present in the reaction, 3D\textsuperscript{pol} produced RNA products 12 bases longer than the template, corresponding to templating by the U\textsubscript{10} sequence and the two adenosines immediately thereafter, resulting in a heteropolymeric end on the new transcript (Fig. 7B, +12). When ATP was the only NTP present in the reaction, 3D\textsuperscript{pol} initially produced RNA products 10 bases longer than the template during the first minute of the reaction, reflecting the initiation kinetics and subsequent elongation we typically observe in these reactions (22, 23), but this is then followed by a progressively longer smear of poly(A) products ranging in size from 11 to more than 40 bases in length (Fig. 7B, ATP only reaction). These poly(A) products, which are significantly longer than the 10 base long poly(U) sequence in the RNA template, are due to reiterative transcription of the poly(U) template by 3D\textsuperscript{pol} when the
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polymerase is stalled at the end of the homopolymer region because the next needed nucleotide is not available. We used this ATP only reaction condition to assess the impact of N409A, D412A, S416A, L419A and L420A mutations on template-dependent reiterative transcription (Fig. 7C).

N409A, D412A, S416A, L419A and L420A 3D<sup>pol</sup> mutations influenced the amounts of poly(U)-dependent reiterative transcription as compared to wildtype 3D<sup>pol</sup> (Fig. 7C). The N409A, S416A, L419A and L420A mutations resulted in poly(A) products longer than those synthesized by wildtype 3D<sup>pol</sup> whereas the D412A mutation resulted in shorter poly(A) products (Fig. 7C). These in vitro data show that 3D<sup>pol</sup> can reiteratively transcribe poly(U) sequences and that N409A, D412A, S416A, L419A and L420A mutations influence the magnitudes of reiterative transcription on poly(U) sequences.

Fidelity of polyadenylation and hyper-polymorphism associated with G7440 and A7441.

TOPO-TA cloning and cDNA sequencing of 600 poliovirus 3' terminal cDNA clones provided data regarding the fidelity of polyadenylation (Figure 6 and Table 3). There was only one polymorphism detected in the 600 poly(A) tails themselves (~30,000 cumulative bases of poly(A) sequence), a C four bases from the 3' end of heteropolymeric RNA in one K125A cDNA clone (Figure 6A and Table 3). In contrast, there were more frequent polymorphisms in the heteropolymeric regions of poliovirus cDNA (Table 3, 12 polymorphisms in the 126,600 cumulative heteropolymeric region between nucleotides 7230 and 7440). Finally, there were very frequent polymorphisms at G7740 and A7741, the junction between heteropolymeric RNA sequences and the poly(A) tail (Figure 6A and Table 3).

Among polioviruses and other group C enteroviruses, the reported RNA sequences adjacent to the poly(A) tail are GGAG<sub>7440</sub>-poly(A), GGAGG-poly(A), GG-poly(A) and GGGG<sub>7440</sub>-poly(A) (Figure 6A and Figure S3). Among the 600 polio virus cDNAs we
analyzed, there were 31 polymorphisms in the terminal G residues (Table 3). Thus, 5% (31/600 = 5%) of poliovirus RNA genomes had a polymorphism at this location. The GGAG sequences proximal to the poly(A) tails of viral RNA were invariant in 85 cDNA clones from T7 transcripts (30 listed in Figure S1 of this manuscript and 55 listed in Figure 8B of Steil et al., 2010), suggesting that enzymes other than 3D<sup>pol</sup> were not responsible for the GGAG polymorphisms in cDNA from virus populations. These enzymes include T7 RNA polymerase, Superscript III reverse transcriptase, Phusion DNA-dependent DNA polymerase, <i>E. coli</i> DNA polymerase, and the DNA polymerase used for sequencing. Notably, GGAG polymorphisms were not identified in virion RNA from wildtype poliovirus (Table 3). Rather, GGAG polymorphisms were identified in virus with 3D<sup>pol</sup> mutations (Table 3).

**Fidelity of RNA Replication**

The impact of 3D<sup>pol</sup> mutations on the fidelity of RNA replication was further analyzed using additional TOPO-TA cloning and cDNA sequencing (Table 4). The 3D<sup>pol</sup> region (nts 5860-7360) of virion RNA was sequenced in 30 clones from wildtype and mutant virus preparations, corresponding to 45,000 bases of sequence data for each virus (Table 4). Eight mutations were detected in 45,000 bases of sequence data from wildtype virion RNA, corresponding to an average of 1.33 mutations per genome. Only two mutations were detected in 45,000 bases of sequence data from G64S virion RNA, corresponding to 0.33 mutations per genome, consistent with the previously reported impact of the G64S mutation on the fidelity of RNA replication (31). R128A and L420A 3D<sup>pol</sup> mutations had no significant impact on the fidelity of RNA replication as compared to wildtype 3D<sup>pol</sup> (Table 4). In contrast, the K133A and S416A mutations increased the fidelity of RNA replication to levels comparable to the G64S mutation (Table 4). The remainder of 3D<sup>pol</sup> mutations (K126A, K276A, N409A, D412A, and L419A) had intermediate effects on the fidelity of RNA replication. These data indicate that features
of 3D\textsuperscript{pol} involved in the polyadenylation of virion RNA also influence the fidelity of RNA replication.

**Impact of 3D\textsuperscript{pol} mutations on poliovirus replication in HeLa cells.**

In order to begin to address the biological impact of 3D\textsuperscript{pol} mutations associated with longer or shorter poly(A) tails, we examined virus replication under one-step growth conditions in HeLa cells (Figure 8). The 3D\textsuperscript{pol} mutations that affected the size of poly(A) tails in virion RNA had no significant effects on the kinetics or magnitudes of poliovirus replication in HeLa cells.

HeLa cells do not reveal every important, biologically relevant phenotype associated with poliovirus infections. Nonetheless, the biochemical data in Figure 2 and Figure S2 correspond nicely with the data from HeLa cells. Stable 3D\textsuperscript{pol} mutations are associated with normal viral mRNA translation and polyprotein processing (Figure S2), detectable viral RNA replication in cell-free reactions (albeit at slightly reduced magnitudes as compared to wildtype, Figure 2), near normal specific infectivity of viral RNA (Table 1), and one-step virus growth comparable to wildtype (Figure 8). Unstable 3D\textsuperscript{pol} mutations were associated with defects in viral polyprotein processing, and/or undetectable viral RNA replication in cell-free reactions, decreased specific infectivity of viral RNA, and lower amounts of viable virus from transfected cells (Table 1).

A close examination of the data shows that the specific infectivities of viral RNAs with stable 3D\textsuperscript{pol} mutations were slightly lower than that for wildtype RNA (Table 1). In fact, the specific infectivities of viral RNA with 3D\textsuperscript{pol} mutations correspond well with the various magnitudes of viral RNA synthesis detected in Figure 2 (with two exceptions; Y14A and Y148A). Yet one must consider that a Y14A 3D\textsuperscript{pol} mutation decreases the stability of 3D\textsuperscript{pol} elongation complexes (22), consistent with undetectable RNA replication in cell-free reactions (Figure 2B, lanes 3 & 4). Furthermore, the unstable
Y148A 3D<sup>pol</sup> mutation results in a minor polyprotein processing defect analogous to that in G64T (Figure S2), perhaps explaining reduced RNA replication (Figure 2B, lanes 9 & 10). Thus, the biochemical data and the data from HeLa cells are largely congruent.
DISCUSSION

The polyadenylation of picornavirus RNA genomes is an important but poorly defined aspect of viral RNA replication. In theory, host poly(A) polymerases and/or viral RNA-dependent RNA polymerases could be involved in the polyadenylation of viral RNA. We used two independent assays to measure the lengths of poly(A) sequences at the 3’ end of poliovirion RNA; $[^{32}P]$-pCp end-labeling in conjunction with urea-PAGE (Figures 3-5) and TOPO-TA cloning in conjunction with cDNA sequencing (Figure 6 and Tables 2-3). Data from both methods were congruent and clearly indicate that alanine substitution mutations within the poliovirus 3Dpol influenced the lengths of poly(A) sequences at the 3’ end of poliovirion RNA. We also show that purified 3Dpol can reiteratively transcribe poly(U) sequences and that 3Dpol mutations which influence the lengths of poly(A) sequences in poliovirion RNA also influence the magnitudes of reiterative transcription on poly(U) sequences (Fig. 7). Our new experimental evidence, in conjunction with previously published data showing the reciprocal nature of poly(A) and poly(U) sequences during viral RNA replication (8), indicates that poliovirus RNA-dependent RNA polymerase (3Dpol) is responsible for the polyadenylation of virion RNA during viral RNA replication. Furthermore, because poly(A) and poly(U) products of viral RNA replication are frequently longer than the corresponding poly(A) and poly(U) sequences in defined viral RNA templates (8), we conclude that poly(A) and poly(U) sequences can be reiteratively transcribed by 3Dpol during viral RNA replication.

It is important to note that our data do not exclude the potential impact of cellular enzymes on viral RNA poly(A) tails. Cellular poly(A) polymerases and deadenylases could theoretically increase or decrease the size of poly(A) tails in viral RNA (32). We chose to measure the size of poly(A) tails in virion RNA because virion RNA is easily purified and newly synthesized virion RNA is most likely packaged into virus particles before coming into contact with cellular poly(A) polymerases and deadenylases. The
contribution of cellular poly(A) polymerases and deadenylases to poly(A) tails in poliovirion RNA, if any, must be relatively minor as compared to the contributions of 3D$^{\text{pol}}$ because 3D$^{\text{pol}}$ mutations influenced the overall size of poly(A) tails (see Table 2 and the discussion below).

RNA-dependent RNA polymerase structures involved in polyadenylation of virion RNA.

The 3D$^{\text{pol}}$ residues involved in the polyadenylation of poliovirus RNA are phylogenetically and structurally conserved among picornaviruses (Figure 9, Table 5 and Table S3). K126, R128, K133, K276, N409, D412, S416, L419, and L420 all influenced the polyadenylation of poliovirus RNA and are highly conserved among polioviruses, Coxsackieviruses, echoviruses, enteroviruses and rhinoviruses. Mapping the phenotypic effects of the various mutations onto the structure of the poliovirus polymerase elongation complex shows clear structural delineation: mutations at the periphery of the fingers domain had no effect on the size of poly(A) tails, mutations of finger residues in contact with the RNA resulted in modest effects on the size of poly(A) tails, and the set of mutations with the strongest effects on the size of poly(A) tails all map to the thumb domain helix that is in direct contact with the minor groove of the RNA duplex as it exits the polymerase (Figure 9A-9C). Furthermore, these amino acids occupy key positions within a clamping structure on 3D$^{\text{pol}}$ that grips the dsRNA product between the pinky finger and thumb domains (Figure 9). The hydrophobic leucines at residues 419 and 420 are packed up against the ribose groups of bases on both the product and template strands, cradling the RNA duplex in the correct position as it exits the active site. Within the fingers domain, K133 interacts with a phosphate residue of dsRNA across from the aforementioned alpha helix and R128 contacts the product RNA strand phosphate backbone one base pair closer to the active site than K133. K126 has a weaker interaction with the template strand RNA as it enters the polymerase active site. K276 is located at the top of the middle finger and is near the RNA template entry.
channel, but it does not directly interact with the RNA in any of the picornaviral 3D\textsuperscript{pol}-
RNA complexes whose structures have been solved thus far.

The structural orientation of the phylogenetically conserved amino acids implicated in
the polyadenylation of viral RNA are similar in the atomic structures of enterovirus 71
3D\textsuperscript{pol} (33), a group A enterovirus; Coxsackievirus B3 3D\textsuperscript{pol} (34, 35), a group B
enterovirus; poliovirus 3D\textsuperscript{pol} (13, 16), a group C enterovirus; rhinovirus 16 3D\textsuperscript{pol} (36, 37),
species A; rhinovirus 14 3D\textsuperscript{pol} (37), species B; and foot-and-mouth disease virus 3D\textsuperscript{pol}
(38, 39). These phylogenetically conserved sequences and structures likely contribute
to the reiterative transcription of poly(A) and poly(U) sequences during viral RNA
replication in these other picornaviruses as well, thereby regulating the lengths of
poly(A) at the 3’ end of viral RNA.

At present it is not clear how 3D\textsuperscript{pol} and the viral RNA template and product strands
move relative to one another to allow for reiterative transcription. Nonetheless, our data
clearly implicate 3D\textsuperscript{pol} thumb alpha helix residues N409, D412, S416, L419, and L420
in reiterative transcription mechanisms (Fig. 7). The dynamic movements seemingly
required for reiterative transcription would disrupt the complex and tight network of
interactions evident in the atomic resolution structures of the poliovirus elongation
complex (16). However, the architecture of the polymerase must accommodate
profound realignment of RNA templates and products during homologous recombination
(40, 41), an event wherein the structural rearrangements might mimic those needed for
reiterative transcription. In fact, homologous recombination between templates could
also give rise to poly(A) products longer than expected, much like reiterative
transcription mechanisms. Additional insight can be obtained from the slippage by DNA
polymerases, a common phenomenon whose frequency is often comparable to that of
nucleotide misincorporation, especially on homopolymer sequences (42, 43). While no
detailed studies of slippage rates in RNA-dependent RNA polymerases have been
carried out, there is precedent for active site slippage by poliovirus 3D<sup>pol</sup>. The double uridylylation reaction that generates the VPg-pUpU primer used for viral genome replication is templated by a pair of adenosines in the cre(2C) stem-loop RNA element, but the reaction itself uses a slide-back mechanism where the same adenosine templates both incorporation events (44). The molecular details of slippage across a longer 8-10 base pair homopolymer duplex in the polymerase during poly(A) tail synthesis are likely to be somewhat different than those needed for a simple single base pair slippage step during VPg uridylylation, but similar template shifting mechanisms in the active site are probably used by both events.

**Fidelity of polyadenylation**

The fidelity of polyadenylation by the viral RNA-dependent RNA polymerase appears to be quite high as we identified only one polymorphism in 600 poly(A) tails corresponding to ~30,000 cumulative bases of poly(A) sequence (Table 3). This corresponds to an error rate of 0.25 errors per 7,500 bases of viral poly(A) tail. In contrast, we found more abundant polymorphisms in the heteropolymeric regions of viral RNA (as discussed below). The low frequency of polymorphisms in the poly(A) tails of virion RNA is consistent with previous results in which substitution mutations throughout the poly(A) tail of poliovirus RNA transcripts reverted during replication in HeLa cells (8). Reiterative transcription mechanisms, which may resemble homologous recombination, could easily eliminate substitution mutations throughout the poly(A) tails, thereby maintaining the integrity of poly(A) tails.

The high frequency of polymorphisms observed at the junction between the poly(A) tail and heteropolymeric genomic sequence were unexpected (Figure 6A and Table 3). 31 out of 600 viral 3’ ends had a G deleted or inserted in this one location, corresponding to 5% of the genomes. The mechanism(s) responsible for the insertion and deletion of
single G residues in the GGAG sequence are undetermined; however, reiterative transcription mechanisms could involve RNA templates and products that melt, translocate, re-anneal and resume elongation. Because GGAG bases in positive-strand RNA at this location can base pair with the reciprocal poly(U) sequences at the 5’ end of negative-strand RNA replication intermediates (8), a reiterative transcription mechanism could eliminate or duplicate a G residue at this location, depending on the manner in which templates and products realign. Furthermore, the pseudoknot in the 3’ UTR of poliovirus RNA could perhaps forestall the elongation of 3D\textsuperscript{pol} at this location during negative-strand RNA synthesis, thereby increasing the frequency of template-product realignments specifically at this position.

Fidelity of RNA replication

The fidelity of RNA replication is reported to influence the quasispecies diversity and the pathogenesis of poliovirus infections (27). A G64S mutation in 3D\textsuperscript{pol} renders poliovirus resistant to ribavirin (25), increases the fidelity of RNA replication (26), and attenuates poliovirus replication in mice (26, 27, 31). Similar fidelity effects are also seen for a K359R mutation that subtly alters a key charged residue that contacts and helps to position the gamma phosphate of the incoming NTP (45). Conversely, mutations engineered into the palm domain of the coxsackievirus polymerase have been shown to decrease fidelity, resulting in mutator phenotype viruses that grow well in tissue culture but have reduced fitness and are thus attenuated for growth in vivo (46). Finally, a M296I mutation in the structurally homologous foot and mouth disease virus polymerase also decreases fidelity (47). These prior results have implicated residues in the vicinity of the active site and within the palm domain or palm-fingers junction region as fidelity control points. Our findings extend on these earlier results by implicating residues in both the fingers and thumb domains that are quite distant from the active site itself as fidelity modulators. In particular, the K133A and S416A mutations that together straddle
both sides of the exiting RNA duplex and affect poly(A) tail length also increase fidelity to levels comparable to those associated with the G64S mutation (Table 4). The location of these residues suggests that there might be an interplay between replication fidelity and how rigidly the product RNA duplex is being held by the polymerase in the replication complex.

**Size of poly(A) tails in poliovirus RNA**

Poliovirus RNA 3’ poly(A) tails ranged from 21 to 121 bases in length, with a mean of 52.9 bases (Figure 6 and Table 2). Notably, among the 600 individual poly(A) tails analyzed by cDNA sequencing, none were less than 21 bases in length. The minimum size of poliovirus poly(A) tails required for optimal negative-strand RNA synthesis in cell-free reactions is 20 bases in length (3), consistent with the minimum size of poly(A) tails in virion RNA (Figure 6 and Table 2).

**Biological significance of slightly longer or shorter poly(A) tails in viral RNA.**

The poly(A) tails of picornaviruses are important for viability (2), with the length of the poly(A) tails influencing the magnitudes of viral mRNA translation (4) and viral RNA replication (3, 48). Nonetheless, 3Dpoly mutations that altered the lengths of poly(A) in viral RNA had limited impact on the specific infectivity of viral RNA in HeLa cells (Table 1), plaque phenotypes in HeLa cells (Table 1), or the kinetics and magnitudes of virus replication during one-step growth conditions in HeLa cells (Figure 7). Consequently, the biological significance of slightly longer (60 bases) or shorter (40 bases) poly(A) tails in poliovirus RNA is uncertain. It is important to note, however, that HeLa cells are extremely permissive host cells that do not reveal every important, biologically relevant phenotype. For instance, a G64S 3Dpoly mutation has little impact on the specific infectivity of poliovirus RNA or the replication of poliovirus in HeLa cells (Table 1); however, this mutation has a dramatic impact on the replication and pathogenesis of...
Poliovirus in animal models (26, 27, 31). Thus, the biological significance of slightly longer or shorter poly(A) tails in poliovirus RNA is yet to be determined. Ongoing studies in tissue culture cells and future studies in animal models will be required to elucidate the biological significance of longer or shorter poly(A) tails in viral RNA.

An Evolutionary Perspective

The RNA-dependent RNA polymerases of modern picornaviruses and picorna-like viruses are predicted to have evolved from a common ancestral polymerase that existed before the origin of eukaryotic hosts (49). Furthermore, many contemporary positive-strand RNA viruses have 3' polyadenylated RNA genomes, including all of the viruses in the order Picornavirales (50). The viral RNA-dependent RNA polymerases of these other positive-strand RNA viruses are likely responsible for the polyadenylation of viral RNA during viral RNA replication; however, experimental evidence will be required to confirm this prediction. Additional experimental evidence will also be required to further define the constellation of amino acid residues and structures involved in regulating the size of poly(A) tails.

Intriguingly, the catalytic domain of telomerase reverse transcriptase (TERT) has structural and functional characteristics in common with picornaviral RNA-dependent RNA polymerases. The catalytic domain of TERT assumes a right hand conformation with thumb, palm and fingers domains encircling the telomerase RNA template and cDNA products (51-53). Furthermore, TERT uses a template-dependent reiterative transcription mechanism to synthesize repetitive DNA sequences of variable length at the 3' end of eukaryotic chromosomes (54). By analogy with TERT and eukaryotic chromosomes, we suggest that poly(A) tails are a telomere of picornavirus RNA genomes, that 3D\textsuperscript{pol} polyadenylates viral RNA during viral RNA replication using an evolutionarily ancient reiterative transcription mechanism (8), and that phylogenetically conserved architectural features of 3D\textsuperscript{pol} regulate the lengths of poly(A) tails.
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FIGURE LEGENDS

Figure 1. Picornavirus RNA-dependent RNA polymerases. A. Diagram of a poliovirus/rhinovirus RNA genome. B. Structure of the poliovirus RNA-dependent RNA polymerase (3Dpol) in the apo form (13). C. The structurally homologous rhinovirus RNA-dependent RNA polymerase (3Dpol) (37). D. The poliovirus 3Dpol elongation complex with RNA template and product (16). The structures are shown in similar orientations and with the core fingers, palm, and thumb domains color coded.

Figure 2. Impact of 3Dpol mutations on viral RNA replication. Poliovirus RNAs with the indicated 3Dpol mutations were incubated in cytoplasmic extracts from uninfected HeLa cells as previously described (18, 21, 24, 55). SDS-PAGE and phosphorimaging were used to detect [35S]-methionine labeled viral proteins (Figure S1). Viral RNA replication was assayed for 90 minutes in reactions containing [α-32P]UTP, in the presence and absence of 2 mM guanidine HCl, an inhibitor of viral RNA synthesis, as previously described (19, 21). Radiolabeled viral RNAs were fractionated by electrophoresis in 1% agarose gels and detected by phosphorimaging. A. G64 mutations in 3Dpol. B. Tyrosine mutations in the fingers domain of 3Dpol. C. Alanine substitution mutations of charged residues in 3Dpol. D. Alanine substitution mutations of thumb residues N409, D412, S416, L419, and L420 in 3Dpol. The mobilities of replicative intermediate (RI), replicative form (RF RNA), and single-stranded poliovirus RNA (PV RNA) are indicated.

Figure 3. Lengths of poly(A) tails in virion RNA measured by [32P]-pCp end-labeling and urea-PAGE. A. Diagram highlighting RNase T1 cleavage sites at G residues adjacent to poliovirus RNA poly(A) tail, along with large T1-resistant oligos from the heteropolymeric region of the viral RNA. B. Electrophoresis of radiolabeled RNAs in 7M urea-18% polyacrylamide gel reveals the size of poly(A) tails in viral RNAs.
Radiolabeled poliovirus RNA transcripts with poly(A) tails of 84, 51, and 32 bases were RNase T1-digested and used as size markers (Lanes 1-3). Wildtype poliovirus and EMCV virion RNAs were isolated from purified virus particles, end-labeled with $[^{32}\text{P}]$-pCp and RNA ligase, digested with RNase T1 and fractionated by electrophoresis (lanes 8 & 9). Virion RNA from 3D$^{\text{pol}}$ mutant virus particles was end-labeled with $[^{32}\text{P}]$-pCp, digested with RNase T1, and fractionated by electrophoresis (lanes 4-7) (1). C. Phosphorimaging was used to analyze the quantitative distribution of poly(A) tails in virion RNAs. Wildtype poliovirion RNA poly(A) tails (black line), EMCV virion RNA poly(A) tails (blue line), and poliovirus 3D$^{\text{pol}}$ mutant virion RNA poly(A) tails (red lines). Peaks of 84, 51, and 32 base long poly(A) tails (in grey).

**Figure 4. Impact of tyrosine mutations in the fingers domain of 3D$^{\text{pol}}$, K133A and K276A on the size of poly(A) tails in poliovirus RNA.** A. Tyrosine mutations in the fingers domain of 3D$^{\text{pol}}$. B. K133A and K276A 3D$^{\text{pol}}$ mutations. A & B. Virion RNA was isolated from wildtype and mutant virus particles, end-labeled with $[^{32}\text{P}]$-pCp, digested with RNase T1, fractionated by electrophoresis, and radiolabeled poly(A) tails were detected by phosphorimaging. Radiolabeled poliovirus RNA transcripts were used as size markers as described in Figure 3. C. Phosphorimaging was used to analyze the quantitative distribution of poly(A) tails in virion RNAs. Wildtype poliovirion RNA poly(A) tails (black line), EMCV virion RNA poly(A) tails (blue line), and poliovirus 3D$^{\text{pol}}$ mutant virion RNA poly(A) tails (red lines). Peaks of 84, 51, and 32 base long poly(A) tails (in grey).

**Figure 5. Impact of N409A, D412A, S416A, L419A and L420A 3D$^{\text{pol}}$ mutations on the size of poly(A) tails in poliovirus RNA.** A. Virion RNA was isolated from wildtype and mutant virus particles, end-labeled with $[^{32}\text{P}]$-pCp, digested with RNase T1, fractionated by electrophoresis, and radiolabeled poly(A) tails were detected by phosphorimaging. Radiolabeled poliovirus RNA transcripts were used as size markers.
as described in Figure 3. **B-E.** Phosphorimaging was used to analyze the quantitative distribution of poly(A) tails in virion RNAs. Wildtype poliovirion RNA poly(A) tails (black line), EMCV virion RNA poly(A) tails (blue line), and poliovirus 3D<sup>pol</sup> mutant virion RNA poly(A) tails (red lines). Peaks of 84, 51, and 32 base long poly(A) tails (in grey).

**Figure 6. Lengths of poly(A) tails in virion RNA measured by TOPO-TA cloning and cDNA sequencing. A.** Diagram illustrating 3' terminal region of poliovirus RNA targeted for cDNA synthesis, TOPO-TA cloning and sequencing. cDNA sequencing revealed the length of poly(A) sequence in each cDNA, as well as various polymorphisms, including frequent polymorphisms in the 3' terminal GGAG sequence. **B.** Dot plot indicating the length of poly(A) in 50 random TOPO-TA cDNA clones from each virus. Mean lengths indicated by horizontal lines.

**Figure 7. Reiterative transcription of a poly(U) sequence by purified 3D<sup>pol</sup>. A.** Diagram of the RNA template. The RNA is labeled at its 5' end with an infrared dye so that both starting material and products can be imaged on gels. A five base self-priming hairpin is present at the 3' end, followed by a 10 base long poly(U) sequence. Beyond the poly(U) sequence are 13 bases of heteropolymeric sequence, a 10 base long poly(C) sequence, and 6 bases of additional heteropolymeric sequence. **B.** RNA products synthesized by wildtype 3D<sup>pol</sup> in reactions containing ATP only, ATP and UTP, and all four NTPs. Reactions containing purified 3D<sup>pol</sup> (15 μM), RNA template (1 μM), and the indicated NTP(s) (65 μM each) were incubated for 0 to 60 minutes. RNA products from the reactions were quenched by addition of EDTA and high salt, fractionated by denaturing gel electrophoresis, and visualized by scanning of the infrared label on the RNA. **C.** RNA products synthesized by wildtype and mutant 3D<sup>pol</sup> in reactions containing ATP (65 μM) after 30 minutes of incubation.
Figure 8. Impact of $3D^{\text{pol}}$ mutations on the kinetics and magnitudes of poliovirus replication in HeLa cells. HeLa cells (~$10^6$ cells) were infected with wildtype or $3D^{\text{pol}}$ mutant viruses using an MOI of 10 PFU per cell. Virus was harvested at the indicated times in 2 ml of tissue culture media by freeze-thawing cells. Virus titers were determined by plaque assay and plotted versus hours post-adsorption. A. Lysine and arginine mutations in $3D^{\text{pol}}$. B. Thumb alpha-helix mutations in $3D^{\text{pol}}$.

Figure 9. Structural orientation of poliovirus RNA-dependent RNA polymerase residues involved in the polyadenylation of viral RNA. Structure of the elongation complex showing the RNA template strand (light blue), RNA product strand (gold), and the locations of the mutated residues with atom spheres and their effects on poly(A) tail length by color coding. Residues resulting in unchanged poly(A) tail lengths are shown in tan, those with modest effects are in light green, the thumb domain residues that resulted in longer poly(A) tails are in bright green, D412A that yielded shorter poly(A) tails is in red, and S416A that broadened the poly(A) tail length distribution is in orange. The three panels show front (A) and top (B) views of the $3D^{\text{pol}}$-RNA complex and a close up of the thumb domain helix (C) that inserts into the minor groove of the product RNA duplex.
Table 1. Impact of 3D<sup>pol</sup> mutations on virus growth and polyadenylation.

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</tr>
<tr>
<td>R188A</td>
<td>1.0 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>6.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>9.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>wt</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Stable 3D&lt;sup&gt;pol&lt;/sup&gt; mutations with wildtype poly(A) tails</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G64S</td>
<td>4.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.4 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>Y14A</td>
<td>3.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>9.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>Y118F</td>
<td>3.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.8 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>Y148F</td>
<td>2.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>5.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>Y157F</td>
<td>5.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>7.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>Y118F-Y148F</td>
<td>9.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>9.5 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.0 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>K125A</td>
<td>1.9 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.0 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td><strong>Stable 3D&lt;sup&gt;pol&lt;/sup&gt; mutations with altered poly(A) tails</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K126A</td>
<td>1.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.0 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>shorter</td>
</tr>
<tr>
<td>R128A</td>
<td>8.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>small</td>
<td>shorter</td>
</tr>
<tr>
<td>K133A</td>
<td>2.9 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>9.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.1 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>longer</td>
</tr>
<tr>
<td>K276A</td>
<td>2.4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>9.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>wt</td>
<td>shorter</td>
</tr>
<tr>
<td>N409A</td>
<td>2.6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>9.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.1 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>longer</td>
</tr>
<tr>
<td>D412A</td>
<td>2.2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.2 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>shorter</td>
</tr>
<tr>
<td>S416A</td>
<td>5.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>wt</td>
<td>broader</td>
</tr>
<tr>
<td>L419A</td>
<td>4.2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>7.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>wt</td>
<td>longer</td>
</tr>
<tr>
<td>L420A</td>
<td>8.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>8.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.1 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>wt</td>
<td>longer</td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific Infectivity of viral RNA (PFU per ug of viral RNA).
<sup>b</sup> Titers (PFU per ml) at 72 h.p.t. for P<sub>0</sub> virus, at 48 h.p.i. for P<sub>1</sub> virus.
<sup>c</sup> Plaque phenotypes of P<sub>1</sub> virus as compared to wildtype (wt).
<sup>d</sup> Poly(A) tail phenotypes as determined in Figures 3-6 and Table 2; not done (nd).
**Table 2. Poly(A) tails from wildtype and 3D<sup>PoI</sup> mutant polioviruses<sup>a</sup>.**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Range</th>
<th>Mean</th>
<th>Median</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV WT</td>
<td>25-93</td>
<td>52.9</td>
<td>51.0</td>
<td>NA</td>
</tr>
<tr>
<td>G64S</td>
<td>30-101</td>
<td>52.2</td>
<td>50.0</td>
<td>0.7940**</td>
</tr>
<tr>
<td>K125A</td>
<td>28-98</td>
<td>53.7</td>
<td>49.5</td>
<td>0.7923**</td>
</tr>
<tr>
<td>K126A</td>
<td>21-71</td>
<td>47.1</td>
<td>47.5</td>
<td>0.0224</td>
</tr>
<tr>
<td>R128A</td>
<td>23-61</td>
<td>43.8</td>
<td>43.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>K133A</td>
<td>29-96</td>
<td>58.6</td>
<td>58.0</td>
<td>0.0443</td>
</tr>
<tr>
<td>K276A</td>
<td>21-85</td>
<td>46.6</td>
<td>44.5</td>
<td>0.0145</td>
</tr>
<tr>
<td>N409A</td>
<td>25-110</td>
<td>60.2</td>
<td>59.0</td>
<td>0.0272</td>
</tr>
<tr>
<td>D412A</td>
<td>21-80</td>
<td>41.9</td>
<td>40.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>S416A</td>
<td>28-97</td>
<td>53.2</td>
<td>50.0</td>
<td>0.9251**</td>
</tr>
<tr>
<td>L419A</td>
<td>39-113</td>
<td>65.7</td>
<td>58.5</td>
<td>0.0004</td>
</tr>
<tr>
<td>L420A</td>
<td>28-121</td>
<td>61.7</td>
<td>59.0</td>
<td>0.0061</td>
</tr>
<tr>
<td>EMCV</td>
<td>10-58</td>
<td>28.2</td>
<td>26.0</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Data from cDNA clones described in Figure 6
* P values calculated compared to wildtype poliovirus using unpaired t-test.
** Not statistically different from wildtype poliovirus
Table 3. Fidelity of polyadenylation and polymorphisms in the 3' end of wildtype and 3D\textsuperscript{pol} mutant virion RNAs.

<table>
<thead>
<tr>
<th>Virus</th>
<th># of Mutations\textsuperscript{a} (in 50 cDNA clones)</th>
<th>Heteropolymeric</th>
<th>G\textsubscript{7440} Deletion\textsuperscript{b}</th>
<th>G\textsubscript{7441} Insertion\textsuperscript{c}</th>
<th>Poly(A) Mutations\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV WT</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>G64S</td>
<td>2</td>
<td>--</td>
<td>--</td>
<td>2</td>
<td>--</td>
</tr>
<tr>
<td>K125A</td>
<td>3</td>
<td>--</td>
<td>1</td>
<td>1</td>
<td>C4</td>
</tr>
<tr>
<td>K126A</td>
<td>5</td>
<td>A\textsubscript{7330}U,G\textsubscript{7239}A,T\textsubscript{7249}C,A\textsubscript{7380}G</td>
<td>3</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>R128A</td>
<td>6</td>
<td>--</td>
<td>3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>K133A</td>
<td>3</td>
<td>G\textsubscript{7310}U,A\textsubscript{7362}U</td>
<td>1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>K276A</td>
<td>3</td>
<td>--</td>
<td>2</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>N409A</td>
<td>3</td>
<td>--</td>
<td>3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>D412A</td>
<td>3</td>
<td>U\textsubscript{7334}C,U\textsubscript{7368}C</td>
<td>1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>S416A</td>
<td>4</td>
<td>A\textsubscript{7404}U</td>
<td>2</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>L419A</td>
<td>1</td>
<td>--</td>
<td>1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>L420A</td>
<td>5</td>
<td>--</td>
<td>5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>EMCV</td>
<td>6</td>
<td>G\textsubscript{7632}A,A\textsubscript{7635}G,extra U\textsubscript{7776}</td>
<td>1</td>
<td>2</td>
<td>--</td>
</tr>
</tbody>
</table>

\textsuperscript{a} # of mutations in 50 cDNA clones from 3' end of each virus as described in Figure 6.

\textsuperscript{b} 24 out of 600 cDNA clones missing G\textsubscript{7440} (see illustration in Figure 6A)

\textsuperscript{c} 8 out of 600 cDNA clones have G\textsubscript{7441}

\textsuperscript{d} 1 out of 600 clones had a C in the poly(A) sequence
Table 4. Fidelity of poliovirus RNA replication.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Bases</th>
<th>Mutations</th>
<th>Frequency</th>
<th>Transitions</th>
<th>Transversions</th>
<th>Insertions</th>
<th>Deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV WT</td>
<td>45,000</td>
<td>8</td>
<td>1.33</td>
<td>2</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G64S</td>
<td>45,000</td>
<td>2</td>
<td>0.33</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K126A</td>
<td>45,000</td>
<td>5</td>
<td>0.83</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>R128A</td>
<td>45,000</td>
<td>10</td>
<td>1.67</td>
<td>8</td>
<td>2</td>
<td></td>
<td></td>
</tr>
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<td>K133A</td>
<td>45,000</td>
<td>1</td>
<td>0.17</td>
<td>1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>K276A</td>
<td>45,000</td>
<td>4</td>
<td>0.67</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N409A</td>
<td>45,000</td>
<td>3</td>
<td>0.50</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D412A</td>
<td>45,000</td>
<td>4</td>
<td>0.67</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S416A</td>
<td>45,000</td>
<td>0</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L419A</td>
<td>45,000</td>
<td>3</td>
<td>0.50</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L420A</td>
<td>45,000</td>
<td>9</td>
<td>1.50</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

- The 3D<sup>pol</sup> region (nucleotides 5860-7360) of 30 TOPO-TA clones was sequenced for each virus.
- Number of mutations detected per 7,500 bases of sequence (mutations per genome equivalent).
- Details of each mutation.
Table 5. Picornavirus 3D\textsuperscript{pol} amino acids involved in the polyadenylation of viral RNA.

<table>
<thead>
<tr>
<th>HEV Species\textsuperscript{a}</th>
<th>126</th>
<th>128</th>
<th>133</th>
<th>276</th>
<th>409</th>
<th>412</th>
<th>416</th>
<th>419</th>
<th>420</th>
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</thead>
<tbody>
<tr>
<td>HEV A</td>
<td>K</td>
<td>R</td>
<td>P</td>
<td>R</td>
<td>N</td>
<td>D</td>
<td>S</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>HEV B</td>
<td>K(98.2)</td>
<td>R(1.8)</td>
<td>K(5.5)</td>
<td>R(94.5)</td>
<td>K</td>
<td>N</td>
<td>D</td>
<td>S</td>
<td>L</td>
</tr>
<tr>
<td>HEV C</td>
<td>K(95.0)</td>
<td>R(5.0)</td>
<td>R</td>
<td>K</td>
<td>K(75.0)</td>
<td>R(25.0)</td>
<td>N</td>
<td>D</td>
<td>S</td>
</tr>
<tr>
<td>HEV D</td>
<td>K</td>
<td>R</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>N</td>
<td>D</td>
<td>S</td>
<td>L(66.7)</td>
</tr>
<tr>
<td>Rhinovirus A</td>
<td>K(92.3)</td>
<td>R(7.7)</td>
<td>K(38.5)</td>
<td>K(60.3)</td>
<td>N(35.0)</td>
<td>T(1.3)</td>
<td>K(82.1)</td>
<td>R(16.7)</td>
<td>S(1.3)</td>
</tr>
<tr>
<td>Rhinovirus B</td>
<td>K(92.0)</td>
<td>R(4.0)</td>
<td>K(12.0)</td>
<td>K(68.0)</td>
<td>R(84.0)</td>
<td>K(16.0)</td>
<td>N(96.0)</td>
<td>S(4.0)</td>
<td>D</td>
</tr>
<tr>
<td>Rhinovirus C</td>
<td>K</td>
<td>K(33.3)</td>
<td>R(66.7)</td>
<td>K(91.7)</td>
<td>R(8.3)</td>
<td>N</td>
<td>D</td>
<td>S</td>
<td>L(83.3)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The enterovirus genus includes human enterovirus species A, B, C, and D, as well as rhinovirus species A, B, and C (56).

\textsuperscript{b} 3D\textsuperscript{pol} amino acids involved in the polyadenylation of viral RNA are phylogenetically conserved across human enterovirus and rhinovirus species. Residues at each location are absolutely conserved in reported 3D\textsuperscript{pol} sequences unless indicated otherwise. The frequency of polymorphisms, when present, are indicated (% of reported 3D\textsuperscript{pol} sequences). The data in Table 5 were derived from a comprehensive list of human enterovirus and rhinovirus 3D\textsuperscript{pol} amino acid sequences (see Table S3) (56, 57).
A. **Poliovirus / Rhinovirus RNA Genomes**

B. **Poliovirus 3D^pol**

C. **Rhinovirus 3D^pol**

D. **Template**

**Figure 1**
Kempf et al.
Figure 2
Kempf et al.
**Figure 3**

Kempf et al.

**A.**

```
<table>
<thead>
<tr>
<th>Lane</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<tbody>
<tr>
<td>Poly(A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

**B.**

Poly(A)

**C.**

Poly(A)
A. PV RNAs
Virion RNA

B. PV RNAs
Virion RNA

C. K133A
K276A

Figure 4
Kempf et al.
Figure 5
Kempf et al.
A.

Polymorphisms in 3' terminal GGAG_{7440}poly(A) sequences:

- 568 out of 600 cDNA clones have wildtype sequence
- 24 out of 600 cDNA clones missing G_{7440}
- 8 out of 600 cDNA clones have extra G_{7441}
- 1 out of 600 cDNA clones had a C in the poly(A) tail

B.

Figure 6
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7437GGAGAAAAAAAAA\_(N)
7437GGAGAAACAAAAAA\_(N)
Figure 7
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A

ATP only
ATP & UTP
All NTPs

BC

+39
+10
Initial RNA
+12
+39
Figure 8
Kempf et al.
Figure 9
Kempf et al.