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A Template RNA Entry Channel in the Fingers Domain of the Poliovirus Polymerase

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Abstract

Positive-strand RNA viruses within the *Picornaviridae* family express an RNA-dependent RNA polymerase (3D^{pol}) that is required for viral RNA replication. Structures of 3D^{pol} from poliovirus, coxsackievirus, human rhinoviruses, and other picornaviruses reveal a putative template RNA entry channel on the surface of the enzyme fingers domain. Basic amino acids and tyrosine residues along this entry channel are predicted to form ionic and base stacking interactions with the viral RNA template as it enters the polymerase active site. We generated a series of alanine substitution mutations at these residues in the poliovirus polymerase and assayed their effects on template RNA binding, RNA synthesis initiation, rates of RNA elongation, elongation complex stability, and virus growth. The results show that basic residues K125, R128, and R188 are important for template RNA binding while tyrosines Y118 and Y148 are required for efficient initiation of RNA synthesis and for elongation complex stability. Alanine substitutions of tyrosines 118 and 148 at the tip of the 3D^{pol} pinky finger drastically decreased the rate of initiation as well as elongation complex stability, but without affecting template RNA binding or RNA elongation rates. Viable poliovirus was recovered from HeLa cells transfected with mutant RNAs; however, mutations that dramatically inhibited template RNA binding (K125A-K126A and R188A), RNA synthesis initiation (Y118A, Y148A), or elongation complex stability (Y118A, Y148A) were not stably maintained in progeny virus. These data identify key residues within the template RNA entry channel and begin to define their distinct mechanistic roles within RNA elongation complexes.

Introduction

A common feature of all positive-strand RNA viruses is a virally encoded RNA-dependent RNA polymerase (RdRP). The RdRPs of picornaviruses, caliciviruses, flaviviruses, and other positive-strand RNA viruses share a core ~55 kDa structural fold composed of fingers, palm, and thumb domains arranged in a shape similar to a cupped right hand. A conserved feature of RdRP structures is an encircled active site where the fingers reach across the palm

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to interact with the top of the thumb. In this respect, RdRP structures differ from those of other single subunit DNA- and RNA-dependent polymerases whose fingers domains do not directly contact their thumbs. The encircled active site arrangement acts to tether the RdRP fingers domain, essentially fixing its position relative to the palm and thumb domains.

Replicative polymerase active sites are generally built on a central RRM-fold in the palm domain wherein aspartate residues in conserved motifs A and C coordinate the magnesium ions needed for catalysis and above which the NTP is positioned for catalysis. In most single-subunit polymerases, the initial selection for an incoming NTP occurs through base-pairing in a pre-insertion site and the nascent base pair is then transferred into the active site via a swinging motion of the fingers domain. The palm domain is fairly rigid and effectively pre-structured for catalysis prior to the arrival of the NTP. In contrast, viral RdRPs utilize a different method for closing their active sites wherein a subtle rearrangement within the palm domain acts to fully structure the active site for catalysis only upon correct NTP binding⁴. The incoming nucleotide binds directly into the active site via stacking and base-pairing to a fully pre-positioned templating nucleotide, and ribose hydroxyl recognition then drives the conformational changes necessary to structure the catalytic center. Importantly, this mode of active site closure via structural changes in the palm domain allows these viral RdRPs to function with an encircled active site and a fixed fingers domain.

While the poliovirus polymerase fingers domain does not play a direct role in NTP repositioning for catalysis, it is involved in binding the incoming template RNA⁴. These polymerase-template RNA interactions contribute to the stability of the elongation complex, allowing for processive elongation across the ≈ 7.5 Kb viral RNA genome⁵. The fingers domain exhibits characteristics consistent with a dynamic structure: a relatively loose packing density, high crystallographic B-factors, and structural fluctuations observed in molecular dynamics studies. Furthermore, this entire domain became disordered in the initial 3D^{pol} structure due to crystal packing clashes of the fingers domains in the lattice. The connection between the fingers and thumb domains is important for the structural integrity of 3D^{pol} as mutations that disrupt this interaction drastically reduce the thermal stability of the protein to below physiological levels¹⁰.

The 3D^{pol} fingers domain is important for maintaining a stable and processive elongation complex via a mechanism that is not yet fully understood at a structural level. For example, poliovirus 3D^{pol} Trp5 and the homologous Phe5 of coxsackievirus 3D^{pol} are found in an unusual solvent exposed conformation that is conserved among all picornaviral polymerases whose structures have been solved thus far. Mutating this residue in either polymerase significantly impairs polymerase activity, primarily through effects on elongation complex stability. However, the structure in this region does not differ between the apo polymerase and the RNA bound replicative polymerase elongation complex⁴ and similarly the fingers domains of other picornaviral and noroviral polymerases are highly homologous to each other and structurally indistinguishable in the absence and presence of bound template-primer RNA. These biochemical and structural data suggest the fingers domain may be interacting with the incoming template RNA in ways that have not yet been observed in crystal structures, perhaps by refolding to form transient interactions with the template RNA, or as part of larger assemblies in the viral replication complex¹⁵.

Atomic structures of 3D^{pol} from poliovirus, coxsackievirus, human rhinoviruses, and other picornaviruses reveal a putative template RNA entry channel on the surface of the fingers domain (Fig. 1). Basic amino acids and tyrosine residues along the template RNA entry channel are predicted to form transient ionic and base stacking interactions with the single-stranded viral RNA template as it moves into the active site of the polymerase. We engineered three sets of mutations into the poliovirus polymerase to investigate the roles of

amino acids along this channel. The first set of mutations was designed to disrupt potential charge interactions between basic residues of the polymerase and phosphate groups of the incoming template RNA (Fig. 1C). The second set of mutations was designed to disrupt potential base stacking interactions between tyrosine residues of the polymerase and nucleotides of the template RNA (Fig. 1D). A third set of control mutations were engineered at sites located far away from the RNA binding surface of the polymerase (Fig. 1A,B). The data indicate that basic residues K125, R128, and R188 are important for template RNA binding while tyrosines Y118 and Y148, in particular, are important for the initiation of RNA synthesis and the stability of the resulting elongation complex. Infectious virus studies show that several of these residues are essential for viral growth while other mutations result in the emergence of viable viruses containing pseudo-revertants that restore wildtype virus growth rates. Our data identify key residues within the template RNA entry channel and begin to define their mechanistic roles within RNA elongation complexes.

Results

All of the *in vitro* 3D^{pol} assays discussed below were carried out using polymerase with a L446D mutation located on the outside surface of the thumb domain (see Fig. 1A,B) while the infectious virus studies were done using the Type 1 Mahoney strain of poliovirus with a wildtype 3D^{pol}. Leucine 446 plays a key role in a crystal packing interaction that leads to the formation of extended polymerase fibers⁹ and mutating it to aspartate resulted in a new crystal lattice that allowed the complete structure to be solved⁸. The L446D mutation significantly increases the purification yield and solubility of 3D^{pol} from bacterial cell expression without significant effects on its structure or polymerase activity, allowing us to carry out the *in vitro* binding and activity studies requiring high protein concentrations. However, the L446D mutation does abolish growth of the virus in host cells¹⁶. The impact of the L446D mutation on virus growth might be due to the disruption of 3D^{pol}-3D^{pol} or 3D^{pol}-3C^{pro} domain protein-protein interactions implicated in RNA replication.

In this work the RNA nomenclature is referenced to the templating base being at the +1 position, the priming base pair being at the -1 position, and added superscripts ^P and ^T referring to product and template strands. Positive numbers are used for the bases of the incoming RNA template and negative numbers are used for the bases of the dsRNA product exiting the active site, with larger numbers indicating increased distance from the active site (see Fig. 1B).

RNA Binding

RNA binding was measured by a fluorescence polarization assay using a synthetic RNA hairpin template labeled with a fluorescein at its 5' end²¹. The RNA consisted of an 8-basepair duplex region followed by a 10-base long 5' single stranded template region (see Inset in Fig. 4A) and polymerase binding immobilizes the molecule and results in an increase in the fluorescence polarization (FP) value of the fluorescein label. The analyses of the various 3D^{pol} mutants showed RNA binding affinities ranging from 4.9 μM for the wildtype polymerase up to 170 μM for the mutants (Table 1). Alanine substitution mutations of charged residues consistently decreased RNA binding affinity (Fig. 2A) while mutations of tyrosine residues had no significant impact on RNA binding (Fig. 2B). The greatest effects were observed for the R188A mutation (170 μM) and the K125A-K126A double mutation (130 μM). Control mutations at charged residues located far from the putative RNA entry channel had no effect on RNA binding (Figs. 1A & 1B, Table 1). These data indicate that charged residues along the RNA entry channel mediate initial template RNA binding even if they do not directly contact the RNA in the current structure (e.g. K125 and K126), while nearby tyrosine residues have no significant impact on RNA binding.

To estimate the number of salt bridges involved in the 3D^{pol}-RNA template interaction we performed binding experiments at a number of salt concentrations ranging from 40 to 200 mM NaCl (Fig. 2C). The number of ionic contacts made with the RNA backbone can then be determined by plotting the logarithm of the observed binding constant as a function of the negative logarithm of the salt concentration (Fig. 2D). The $\approx 25 \mu\text{M}$ solubility limit of L446D 3D^{pol} in these conditions prevented us from obtaining clear maximal binding plateaus above $\approx 100 \text{ mM}$ NaCl for many of the mutant polymerases with reduced affinity (Fig. 2C). To obtain RNA dissociation constants from curve fitting of these incomplete datasets we fixed the maximal FP signal to the stable plateau value observed at lower salt concentrations and allowed the fitting algorithm to vary only the starting FP value and the midpoint of the binding curve. As an alternative method, we also carried out curve fitting where the amplitude of change in FP was fixed to the value observed for the wildtype protein; a direct comparison of the results from these two approaches showed at most a 2-fold difference in the fitted dissociation constants.

The analysis of the salt dependence of RNA binding is based on the slope of a double logarithmic plot that reflects how many monovalent ions are displaced from the RNA phosphate groups upon binding and the formation of ionic contacts with protein arginine or lysine residues²². As is often observed in such data, the resulting plots have an inflection point in the vicinity of 100 mM NaCl and non-ideal behavior in the salt dependence data was often observed below this salt concentration (Fig. 2D). Consequently, only data obtained at salt concentrations above $\approx 100 \text{ mM}$ NaCl value (i.e. <1 on a $-\log[\text{NaCl}]$ axis) were used to determine the slopes of the ionic strength dependence curves. The curve-fitted slopes were divided by 0.77 to account for the electrostatic shielding factor of the monovalent sodium ions used in the experiment, resulting in an estimate of the number of ionic contacts made between the polymerase and the RNA (Table 2). Note that our RNA binding assay buffer also contains 1.5 mM magnesium whose shielding constant is 0.88, potentially complicating the calculation of the number of ionic contacts.

The ionic contacts analysis indicates wildtype 3D^{pol} interacts with 7–8 RNA phosphate groups (Table 2), which is consistent with the eight interactions present in the crystal structure of the elongation complex (PDB code 3OL6⁴). The arginine and lysine mutations all show a reduction in the number of contacts, but in general the data do not accurately correlate with the loss of individual ionic contacts as predicted from the elongation complex structure. For example, neither K125 nor K126 interact with the RNA in the published atomic structures, but these residues have quite different effects on the salt dependence, with K125A showing the loss of only one contact while K126A reduces the number of ionic contacts by four. The R128A single mutation also results in an apparent loss of four contacts, but aK127A-R128A double mutant shows a loss of only two. The greater number of contacts lost as a result of single point mutations suggests the mutant proteins may not bind the RNA in the native conformation and perhaps instead form some alternate complex where the full set of native RNA contacts is not established. However, the low RNA affinities of the mutants and the required assumptions about the FP binding curve plateau values may also adversely affect the quality of the binding constants determined at $>100 \text{ mM}$ NaCl. In particular, a loss of incoming template RNA contacts could result in increased mobility of the 5' fluorescein label, which could in turn decrease the FP signal associated with saturating the binding equilibrium to a value that is lower than the value we are imposing when fitting the data.

Activity Assays

To further assess how the mutations affect 3D^{pol} function we performed three sets of assays to examine the rate of elongation complex formation, the rate of processive elongation, and the temporal stability of the elongation complexes. The full set of assays was done for all the

tyrosine mutants (Fig. 1D) and a set of control mutations of basic residues located away from the RNA entry channel (Fig. 1A,B). The elongation assays were not performed on many of the charge mutations because their weakened RNA binding efficiency reduced elongation complex yield to the point where reliable data could not be obtained.

Elongation Complex Formation

The rate-limiting step in poliovirus 3D^{pol} initiation is the addition of the first one or two nucleotides onto a primer, resulting in the formation of a stable and highly processive elongation complex (EC). We tested EC formation using two different RNAs simultaneously in the presence of ATP and GTP⁵, where the shorter RNA allows for incorporation of only guanosine to yield a +1 product and the longer RNA allows incorporation of both adenine and guanosine to yield a +2 product (Fig. 3A). Note that product formation in this reaction combines RNA binding and subsequent nucleotide incorporation steps. All of the mutant polymerases exhibited single exponential EC formation kinetics with a wide range of formation rates (Fig. 3, Table 1). 3D^{pol} with K125A, K126A, and K133A charge mutations exhibited reduced EC formation rates, but the effects were relatively small at 3–8-fold and correlated with the reduced RNA binding affinities of these mutants, suggesting the mutations do not significantly affect the catalytic steps of initiation. 3D^{pol} with a R188A mutation was incapable of forming detectable amounts of either +1 or +2 RNA products in the assay, presumably due to its profound defect in template RNA binding. 3D^{pol} with tyrosine mutations in the pinky finger domain, on the other hand, showed a much wider range of EC formation rates with the Y14A and Y157A mutations having only ~3-fold effects while Y118A and Y148A reduced EC formation rates by 10- and 30-fold, respectively.

Elongation Rates

To further examine how the tyrosine mutations affected 3D^{pol} function we measured maximal elongation rates and apparent NTP K_m values with a stopped-flow fluorescence assay²⁴ that uses a self-priming hairpin RNA with a fluorescein label at the very 5' end of a 26-nucleotide long template sequence (Fig. 4A). The data show an initial exponential decrease and lag phase that corresponds to the incorporation of the first 20 nucleotides followed by a large increase in fluorescence polarization corresponding to the incorporation of the last five nucleotides. The duration of the lag phase provides a measure of the time it takes for a pre-initiated polymerase to elongate through the 20-nucleotide single-stranded G-less cassette and is used to calculate an average elongation rate in nucleotides/second. Rates were measured at various NTP concentrations and V_{max} and K_m^{app} values were obtained by fitting the resulting data to a Michaelis-Menten style curve (Fig. 4B).

3D^{pol} with tyrosine mutations retained essentially wildtype elongation rates of ~3 nt/sec and nucleotide K_m^{app} values of ~20 μ M and similar data were obtained from 3D^{pol} with control mutations of lysine residues located away from the active site (Table 1). These rates were obtained at 22.5°C and pH 6.5 to maintain the stability of the stalled complexes, but note that more biologically relevant rates of 70–90 nt/sec are seen at 37°C and pH 7.5²⁴. Notably, the stopped-flow traces of 3D^{pol} with the tyrosine mutations showed reduced absolute fluorescence anisotropy values as compared to the wildtype polymerase (Fig. 4C) and significant decreases in the total fluorescence signal after ~30 seconds when elongation was complete (Fig. 4D). This behavior differs from that of the wildtype and control polymerases that display little or no drop in endpoint total fluorescence and these effects were most pronounced for the Y118A and Y148A polymerases, the two polymerases that exhibited highly reduced EC formation rates. These observations are similar to those previously seen for a series of mutations at the nearby Trp5 residue⁵ (shown in Fig. 1D) and indicate that while the tyrosine mutant polymerases are capable of elongation at wildtype rates, their

elongation complexes are unstable and prone to dissociate from the RNA template/product complexes. This will reduce the observed fluorescence anisotropy and decrease the total fluorescence signal from the bulk sample because less of the RNA is being actively elongated in the stopped-flow experiment⁵.

Elongation Complex Stability

To more specifically examine EC stability we performed an experiment to test how efficiently pre-initiated ECs can resume elongation after being stalled by nucleotide deprivation⁵. We first generated stalled EC's on the 9+2₂₄ RNA template by adding only ATP and GTP, and then diluted the resulting +2 product complexes 10-fold while increasing the monovalent salt concentration to 300 mM NaCl so as to prevent rebinding and reinitiation of any dissociated RNA. The diluted complex was incubated at room temperature for various amounts of time before being provided with the remaining nucleotides needed to elongate to a +20 product within a three minute time window that is too short for any new initiation events to take place.

The results indicate that the tyrosine mutations significantly destabilized stalled 3D^{pol} elongation complexes. The elongation competency of the wildtype EC decays with a ≈ 150 minute time constant, and this is reduced 6- and 9-fold for 3D^{pol} with the Y157A and Y14A mutations, respectively (Fig. 5, Table 1). Greater effects were observed for 3D^{pol} with the Y118A and Y148A mutations, whose EC stability was reduced by at least 30-fold. We can only place an upper limit on the decay of these complexes because we could no longer detect significant +20 elongation product after as little as 5 minutes of incubation in the high-salt buffer; the combination of slow formation rates and fast inactivation rates for these two mutant polymerases meant that only a small fraction of complex was active at any point in time. In contrast to the drastic stability defects observed with the tyrosine mutations, alanine substitution of charged residues within the template RNA entry channel reduced EC stability only ≈ 2.5 -fold and the lysine control mutations located far from the RNA entry channel did not significantly affect EC stability.

Virus viability and stability of engineered mutations

To assess the importance of the entry channel residues for 3D^{pol} function during initial virus replication we engineered the mutations into an infectious cDNA clone of Type 1 Mahoney poliovirus. Viral RNA transcripts were generated and transfected into HeLa cells to measure the impact of the mutations on virus viability, resulting in passage zero (P₍₀₎) virus that was recovered from transfected cells at 48 hours post-transfection (hpt) and quantified by plaque assay. The P₍₀₎ stocks were then used to infect naïve HeLa cells, yielding P₍₁₎ virus stocks from which cDNA was prepared and sequenced to determine the genetic stability of engineered mutations. Transfection of wildtype poliovirus RNA yielded 6.0×10^7 PFU per ml at 48 hpt and resulted in P₍₁₎ progeny virus stocks with a titer of 2.0×10^9 PFU per ml. All of the mutants produced P₍₁₎ progeny with similar titers, but about half of them showed slow initial growth rates after transfection and the emergence of fully revertant viruses or pseudo-revertant viruses with wildtype growth characteristics in the P₍₁₎ progeny population (Table 3). Among the basic residue mutations, the individual K125A, K126A, R128A, and K133A mutations were stably maintained in progeny virus and produced near wildtype viral titers, although the R128A mutant consistently produced small plaques. In contrast, the alanine mutations at K127 and R188, two residues that make direct contacts with the -1^T and -2^T RNA phosphates (Fig. 1C), were genetically unstable – the initial growth of virus following RNA transfection was down by three orders of magnitude and the isolation of progeny virus showed the emergence of fully revertant virus with wildtype growth rates. A similar growth pattern was seen when K127A was introduced in the context of K127A-R128A, a double mutant that also reverted to wildtype residues at both sites. Finally, there

appears to be less selective pressure on the adjacent K125 and K126 residues where growth of the double alanine mutation was consistently debilitated ≈ 10 -fold and the mutations were unstable in the context of virus, but without the emergence of a dominant single species; sequencing of 25 separate clones from the mixed $P_{(1)}$ population showed six clones (24%) had fully reverted to wildtype lysines, six (24%) had acquired a valine at 125 while retaining the alanine at 126, and thirteen (52%) maintained both alanine mutations.

Among the tyrosine mutations, alanine substitutions at Y118, Y148, and Y157 were not stable in the context of infectious virus, but the corresponding phenylalanine mutations were (Table 3). This suggests the hydrophobicity and/or aromatic ring of the side chain, rather than the tyrosine hydroxyl, is important for maintaining a functional polymerase elongation complex. Virus containing the Y157A mutation initially grew slowly and titered 2–3 logs lower than the wildtype virus, but the plaques that did form were phenotypically similar to wildtype plaques and suggested a reversion mutation had emerged. This was confirmed by sequencing of the Y157A progeny virus. The sequence analysis of the progeny virus from the Y118A and Y148A mutations showed that smaller hydrophobic residues could be accommodated at both sites provided the other remained as a large hydrophobic. For example, although the Y118A mutation was not stable and initially produced a slow growing $P_{(0)}$ virus with a 48-hour titer reduced by five logs compared to wildtype, the resulting progeny virus contained a mutation to methionine and the resulting $P_{(1)}$ population titer was down only 3-fold from that of wildtype. Similarly, Y148A resulted in the emergence of a pseudo-revertant valine mutation with a titer reduced only 5-fold from wildtype. Mutating both residues simultaneously via double mutations to all four possible combinations of alanine and phenylalanine also showed that neither alanine mutant was stable, but both phenylalanines were maintained in the progeny virus. Together, these virus growth data indicate there is selective pressure for having at least one, and preferably two, large planar and hydrophobic residues at positions 118 and 148 on the pinky finger side of the entry channel (Fig. 1D). However, this requirement does not extend across the channel to tyrosine 14 where an alanine is accommodated with essentially wildtype virus growth.

Discussion

The structures of poliovirus 3D^{pol} alone and bound to RNA in an active elongation complex show indistinguishable conformations for the fingers domain, yet mutations of several residues in this region of the protein have drastic effects on polymerase function. These include several mutations of the surface exposed tryptophan or phenylalanine at residues 5 of poliovirus and coxsackievirus polymerases and the *cis* proline 119 in the pinky finger of poliovirus polymerase^{5,6,8}. A systematic dissection of Trp5 mutations in poliovirus 3D^{pol} showed the most prominent effect on the temporal stability of the elongation complex via some allosteric interaction that is not yet understood at a structural level and this effect translated into reduced processivity of the enzyme when replicating kilobase-length template⁵. In the work presented here we carried out a similar systematic analysis on a series of positively charged residues in the polymerase fingers domain that may stabilize RNA interactions and on a set of four tyrosine residues that could interact with the bases of single-stranded RNA to tether the incoming template strand to the polymerase.

To assess the importance of these basic residues and the tyrosine lined groove for 3D^{pol} activity, we addressed multiple aspects of 3D^{pol} function *in vitro* and assessed the extent to which the mutant polymerases could support the growth of infectious virus in cell culture. The results show that both groups of residues are essential for optimal RNA synthesis and virus replication, but they affect different aspects of polymerase function. The charge mutations are primarily involved in RNA binding and have fairly minor effects on initiation and elongation rates and on elongation complex stability. In contrast, the tyrosines in the

groove have little effect on RNA binding or elongation rates but greatly destabilize the elongation complex. Incorporation of the polymerase mutations into the poliovirus genome and subsequent transfection of transcribed viral RNA into HeLa cells showed that many of the mutations could be tolerated in the context of infectious virus. However, some mutations significantly reduced viral growth and resulted in the rapid emergence of either revertant or pseudo-revertant viruses carrying informative compensatory mutations.

Ionic interactions between 3D^{pol} and RNA were probed by mutating six phylogenetically conserved lysine and arginine residues on the surface of the pinky finger that are well positioned to form salt bridges with the RNA phosphate backbone (Fig. 1C). Lysine and arginine residues are universally conserved at amino acid residues 126, 127, 128 and 188 in human enterovirus and rhinovirus polymerases (see Supplemental Information). Limited conservation of basic amino acids is also seen at residue 125 of group C and D enteroviruses and at residue 133 of group B, C, and D enteroviruses and group B and C rhinoviruses. The recently solved structure of the poliovirus polymerase elongation complex shows that four of these residues directly contact phosphate groups (Fig. 1); K127 forms a buried interaction with the template strand phosphate of the priming -1 base pair (i.e. -1^T, see Fig. 1C for nomenclature), R188 contact the -2^T phosphate, R128 contacts both -6^P and -7^P phosphates, and K133 contacts the -7^P phosphate. Lysines 125 and 126 lie downstream of the active site at the edge of the putative template binding groove on the pinky finger, but neither makes a definitive interaction with the incoming template RNA; K126 points in the general direction of the +3^T phosphate but is too far away to be considered a direct contact and K125 points away from the RNA altogether (Fig. 1C).

The major observations from this mutational study are summarized in Table 4 where the effects of the mutations on *in vitro* 3D^{pol} biochemistry and their ability to support infectious virus growth are compared with those of wildtype polymerase. Among the mutations of charged lysine and arginine residues on the pinky finger, the critical residues are K127 and R188 that form key salt bridges with the phosphates of template strand bases -1^T and -2^T in the heart of the active site. Alanine substitution of R188 reduces RNA affinity 35-fold and both mutations exhibit very slow initial virus growth rates prior to the emergence of revertants that rapidly become dominant in the progeny virus population. Reversion to the wildtype lysine is also observed for K127A and these data are consistent with results from a previous study showing that charge cluster triple mutations of K125A-K126A-K127A and K127A-R128A-D129A did not support virus growth when RNA was transfected into HeLa cells²⁵. Outside of these two key RNA interacting residues, the data from the remaining charged residue mutations show that the RNA affinity and initiation kinetics of 3D^{pol} can be reduced up to ~10-fold without significant effects on elongation complex stability or virus growth rates. For example, alanine substitution of R128 that interacts with both -6^P and -7^P phosphates reduces RNA affinity 7-fold and results in virus with wildtype infectivity titers despite a small plaque phenotype. Interestingly, the K125A and K126A mutants have comparable effects on RNA binding, but unlike R128 they do not directly contact the RNA backbone in the current atomic structures. The K125A-K126A double mutant was genetically unstable and sequence analysis of clones from the mixed population of P₍₁₎ virus did not demonstrate a clear population bias toward a specific sequence or the presence of at least one basic residue. This suggests that lysines at residues 125 and 126 improve polymerase function, but not enough to confer a particularly strong replicative growth advantage on the virus. The effects of these two mutations thus likely reflect either the loss of a general electrostatic contribution to the RNA association event or perhaps transient phosphate backbone interactions that have not yet been captured in the current crystal structures.

The second region targeted in our mutagenesis study was a tyrosine rich groove located between the pinky and index fingers (Fig. 1B,D). Phylogenetically conserved tyrosine residues in this region are part of a putative interaction surface for the RNA template as it enters the polymerase active site and this region may also serve as the site for template RNA duplex strand separation, although no specific tyrosine-RNA interactions were captured in the published atomic structures of elongation complexes. The groove starts near the active site where the ribose of the +2^T nucleotide packs against Y157 and continues across the surface of the fingers to end in the cluster of Y14, Y118, and Y148 residues. Tyrosines are conserved at amino acid positions 118, 148 and 157 in all human enteroviruses while human rhinoviruses have either tyrosine or phenylalanine at these positions. Y14 is less conserved with a variety of amino acids at this position of human enterovirus and rhinovirus polymerases. The edge of this groove includes the phylogenetically conserved G-motif²⁶, which contains a *cis* conformation proline 119 and a short alpha helix flanked by a pair of flexible glycine residues (G117, G124) that are essential for biochemical activity⁸. Together, these elements comprises a protein fold that is poised for a refolding event driven by proline *cis-trans* isomerization²⁷ that may play a role in the formation of template RNA interactions that stabilize the processive elongation complex.

The RNA binding assays in the absence of any initiation or elongation show only minor effects from the tyrosine to alanine mutations, with the greatest effect being only ≈ 2 -fold with Y157A that we attribute to the loss of interaction between the Y157 aromatic ring and the ribose of the +2^T nucleotide. Greater effects are seen in the biochemical assays that assess initiation efficiency as measured by the incorporation rate for the first one or two nucleotides onto a primer. In this assay, Y118A and Y148A mutations slowed initiation rates 15 to 40-fold, from five to ≈ 100 and ≈ 200 minutes, respectively, while the Y14A and Y157A mutations result in only 2 to 3-fold reductions. Despite this, all the tyrosine mutations exhibit essentially wildtype elongation rates in our stopped flow assay that utilizes pre-initiated stalled elongation complexes as a substrate, showing that the complexes that did form were fully capable of efficient elongation. However, the raw data from these assays showed reduced amplitudes for the signal change associated with the polymerase approaching the 5' end of the template (Fig. 4C) and clear instability of the endpoint fluorescence signals (Fig. 4D). Further examination of EC stability showed that all the tyrosine mutants destabilized the complex at least 5-fold and the Y118A and Y148A mutants had greater than 30-fold effects. We have previously observed such behavior from a series mutations at the nearby Trp5 residue and showed that reduced stability of the elongation complex correlated with reduced processivity when replicating kilobase-length RNA templates⁵.

To further explore the effects of these mutations on virus growth we transfected HeLa cells with poliovirus RNAs and monitored the amount of virus produced in the transfected cells, the stability of mutations within progeny virus, and the emergence of revertants or pseudo-revertants. The virus viability studies indicate there is a strong preference for aromatic residues at these sites (Table 3), consistent with the universal conservation of tyrosine or phenylalanine at residues 118, 148, and 157. Y14A was the only stable alanine substitution, but mutations to phenylalanine that preserve the planar ring structure of the side chain could be tolerated and were genetically stable at the other three sites. The Y118A-Y148A double mutant rapidly reverted to a pair of tyrosines, the Y118A-Y148F double mutant resulted in a pair of phenylalanines, and the double phenylalanine mutant Y118F-Y148F grew at wildtype levels and was genetically stable. The individual Y118A and Y148A mutations resulted in slow growing viruses and led to the emergence of pseudo-revertants with methionine and valine, respectively, implying that having at least one tyrosine residue at either position can support virus growth provided the residue at the other position is somewhat hydrophobic.

Taken together, the data suggest the tyrosine residues are involved in some interactions that occur after initial RNA binding but before elongation, and that 3D^{pol} requires this interaction to maintain the stability, and by inference from prior work on Trp5 mutations, also the processivity of the elongation complex. The residues certainly reside close enough to each other that a refolding event could be dependent on a synergistic re-packing interaction between Y118 and Y148 that could in the process flip K125 into position for template RNA interactions. A *cis-trans* isomerization of the *cis* Pro119 and unfolding of the adjacent short α -helix could potentially trigger this refolding, resulting in a RNA interacting structure protruding from the polymerase surface. Careful kinetic studies of the 3D^{pol} initiation pathway have implicated a conformational change after RNA binding and before nucleotide incorporation that results in a significant increase in the half-life of the 3D^{pol}-RNA complex. This conformational change has not been definitively identified in any of the poliovirus 3D^{pol} atomic structures solved thus far, nor in any of the atomic structures of highly homologous picornaviral polymerases from coxsackie, foot-and-mouth disease, or rhinoviruses. Methyl-¹³C methionine NMR studies of 3D^{pol}-RNA complexes have shown the existence of two different conformational states within palm domain residues just below the pinky finger region we mutated here³¹. However, it was not possible to make insightful T₂ relaxation time measurements of Met 101, 123, or 141, three methionines whose chemical environments are likely directly affected by a conformational change in the pinky finger; the M123 methyl is fully solvent exposed and the M101 and M141 methyl resonances were too broad for accurate intensity measurements.

In summary, we generated a set of mutations in a putative template RNA entry channel of poliovirus 3D^{pol} and analyzed their effects on polymerase function with *in vitro* biochemical experiments and cell based infectious virus studies. Data from mutations of basic residues are largely consistent with the contacts seen in the structure of the 3D^{pol}-RNA complex, but also show unexpectedly strong ionic interactions involving lysines 125 and 126. The mutation of tyrosine residues in a putative RNA template entry channel reveals that tyrosines 118 and 148 play a major role in the formation and stability of the elongation complex despite no observed structural changes upon RNA binding in this region of the 3D^{pol} structure. Together, these findings suggest that the current poliovirus polymerase elongation complex structure may not represent the full set of structural interactions necessary to maintain the complex in a processive conformation. These new interactions may involve direct RNA contacts with the tyrosine residues in the putative template entry groove, a refolding of the fingers domain to stabilize a processive enzyme conformation, or quite possibly a combination of the two. Given the significant effects of some of the mutations on the temporal stability of elongation complexes, the refolded conformation may be short lived compared to the days needed to grow crystals or hours needed to collect NMR data, and consequently may be difficult to directly visualize by traditional structural methods.

Materials and Methods

Protein Expression

Mutations were engineered into 3D^{pol} cDNAs using one of two different T7 promoter based poliovirus 3D^{pol} expression plasmids in the background of a L446D mutation to increase solubility⁸ and C-terminal GSSS-Hexahistidine purification tag (Table 1). 3D^{pol} with charged residues to alanine mutations were expressed using the pKK vector encoding full-length 3D^{pol} with an added initiator methionine residue that is cleaved off by bacterial host proteins⁸. 3D^{pol} with tyrosine and control site mutations were expressed using the pET26-Ub vector encoding for 3D^{pol} with a N-terminal ubiquitin domain that is cleaved off within the expression strain to generate a native glycine residue at the 3D^{pol} N-terminus³². Plasmids encoding wildtype and mutant polymerases were subsequently transformed into

Escherichia coli BL-21 (DE3) pLysS (for pKK vector) or pCGI (for ubiquitin fusion constructs) cells for protein expression as previously described.

Protein Purification

Bacteria were harvested by centrifugation, resuspended in lysis buffer [50 mM Tris (pH 8.0), 300 mM NaCl, 20% (vol/vol) glycerol, and 0.02% (wt/vol) NaN₃] and lysed with 18,000 lbs/cm² pressure in a model M-110L microfluidizer (Microfluidics Corp, Newton, MA). NP-40 was added to the lysate in a drop-wise manner to a final concentration of 0.1% (vol/vol) followed by DNA precipitation through the addition of PEI to a final concentration of 0.25% (vol/vol). The solution was centrifuged at 17,000 rpm for 45 minutes in a Sorvall RC2-B centrifuge. The supernatant was filtered through a 0.2 micron cellulose acetate filter, loaded onto a HisTrap HP nickel column (GE Healthcare), and eluted with 350 mM imidazole in buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 20% (vol/vol) glycerol, and 0.02% (wt/vol) NaN₃. Fractions containing the polymerase were pooled and diluted three-fold in low salt buffer consisting of 50 mM NaCl, 25 mM Tris (pH 8.5), 20% (vol/vol) glycerol, 0.02 (wt/vol) NaN₃ to allow for loading onto a HiTrap Q HP column (GE Healthcare). Polymerase was eluted via a linear gradient of 50 mM to 1 M NaCl, pooled and concentrated to a volume ~0.8 mL using 30K MWCO centrifugal filter and a model 5810R centrifuge (Eppendorf Inc.). The concentrated sample was loaded onto a MonoQ column (GE Healthcare) and eluted with a linear gradient using the same buffer conditions as for the Q-column. Fractions containing the polymerase were pooled, concentrated as before to a volume of ~0.8 mL, and loaded onto a Superdex 200 gel filtration column (GE Healthcare) equilibrated in 200 mM NaCl, 5 mM Tris (pH 7.5), 20% glycerol (vol/vol), and 0.02% (wt/vol) NaN₃. Fractions containing the polymerase were pooled, provided 5 mM tris (2-carboxy-ethyl) phosphine (TCEP) reducing agent, concentrated to a final [3D^{pol}] of 200 uM, flash frozen with liquid nitrogen, and stored at -80°C. Purification efficiency and sample purity were assessed by 12% polyacrylamide gel electrophoresis and Coomassie blue staining.

RNA Binding Assay

RNA binding assays were performed using an “8.10” polymerase elongation template element (PETE) RNA consisting of an eight base pair (bp) hairpin followed by a ten-nucleotide (nt) template with a fluorescein labeled to the 5' end via a 6-carbon linker²¹. 10 nM RNA was mixed with 50 mM HEPES (pH 6.5), 75 mM NaCl, 1.5 mM MgAc₂, 60 uM ZnCl₂, 4 mM dithiothreitol (DTT), and 0.1% (vol/vol) NP-40. 3D^{pol} was titrated into the RNA mix giving a range of protein concentrations from 10 nM to 25 uM and incubated at 4°C for 30 minutes, followed by an additional 30 minutes of incubation at room temperature. Fluorescence polarization (FP) was measured in a black 384-well polystyrene plate using a Perkin-Elmer Victor V multimode microplate reader. Kaleidagraph (Synergy Software) was used to determine RNA dissociation constants by fitting the data to single-site binding isotherm²¹.

Elongation Complex Formation

To determine the rate of elongation complex formation we employed two IRdye 800RS (Li-Cor Biosciences) labeled RNA constructs that allow for formation of either a +1 or +2 product in the same reaction when only ATP and GTP are added⁵ (Fig. 3A). The 10+1.12 PETE consists of a 10-bp hairpin primer and allows for incorporation of one nucleotide leading to a “locked” elongation complex, and leaves a 12-nt single-stranded template. The 9+2.24 PETE consists of a 9-bp hairpin primer, allows for incorporation of two nucleotides leading to a “locked” elongation complex, and leaves a 24-nt downstream template. In addition, the RNA templates do not contain guanosine until the sixth nucleotide from the 5' end, allowing us to avoid end-effects by omitting CTP from the elongation reactions. The

RNA constructs were mixed to a final concentration of 0.5 μM each with 15 μM 3D^{pol}, 40 μM of each ATP and GTP in a reaction buffer consisting of 50 mM HEPES (pH 6.5), 75 mM NaCl, 1.5 mM MgCl₂, and 5 mM TCEP. Reactions were quenched through equal volume mixing with quench buffer comprised of 50 mM HEPES, 75 mM NaCl, and 10 mM EDTA after various amounts of time. Reaction products were separated by electrophoresis in 7M Urea 15% polyacrylamide gels, analyzed with a Li-Cor Odyssey 9120 Infrared Imager and quantified using Odyssey software. Plots of percent of total RNA elongated versus time were constructed using Kaleidagraph and fit to a single exponential curve to determine the formation rate.

Elongation Rate Assays

RNA elongation assays utilized the 10.26 PETE RNA, consisting of a 10-bp hairpin and a 26-nt template, with a fluorescein attached to the 5' end of the construct²⁴. 1 μM RNA was premixed with 15 μM 3D^{pol}, 40 μM ATP and incubated at room temperature for 30 minutes to allow formation of the locked +1 elongation complex in the same buffer conditions used for the elongation complex formation assay. The resulting solution was diluted 10-fold in reaction buffer and stored on ice until ten minutes prior to use, at which point the reaction was placed in a water bath set to 22.5°C. Using an Applied Photophysics SX-20 stopped-flow instrument, the preformed elongation complex was rapidly mixed with an equal volume of the remaining NTPs to allow for full elongation of the 10.26 RNA. The final solution measured in the instrument contains 50 nM RNA, 750 nM 3D^{pol}, and a range of equimolar nucleotide concentrations from 5 μM to 120 μM each NTP. Fluorescence excitation was at 492 nm from a monochromator source and emission was detected using 515 nm high pass filters. From collecting the parallel and perpendicular polarization signals, we calculated total fluorescence (TF) and fluorescence polarization (FP) as previously described²⁴. Using Kaleidagraph, the lag time was calculated by independently fitting an initial decrease in signal and the final increase in signal, the latter being associated with terminal nucleotide addition. The intersection between these curves provides a measure of the amount of time needed to elongate through 20 nucleotides that was then used to calculate a rate in nt/second. The rates were plotted against substrate concentration to construct a Michaelis-Menten curve enabling determination of V_{max} and $K_{\text{m}}^{\text{app}}$.

Elongation Complex Stability Assays

Stability studies followed the protocol for elongation complex formation with the 9+2.24 RNA in 75 mM NaCl. Incubation times for complex assembly varied from 15 minutes to 90 minutes to provide for >90% product formation based on results from formation rate studies (Table 1). To prevent RNA degradation during the room temperature incubation, RiboLock RNase Inhibitor (Fermentas) was added to 3 units/ μL . The reaction was then diluted ten-fold in complex formation buffer containing 300 mM NaCl and equivalent concentration of 10+1.12 RNA was added to serve as a degradation and initiation control. After various amounts of time, samples were mixed with an equal volume of buffer with 80 μM each ATP, GTP, and UTP and incubated for an additional three minutes to allow for elongation of the 9+2.24 RNA to a +20 product. Quench buffer was added to stop the reaction, samples were separated using PAGE, and the Li-Cor Odyssey software was employed to image the gel and quantitate the bands. The efficiency of elongation as a function of incubation time in high salt buffer was calculated as the intensity of the +20 band divided by the sum of the +2 and +20 band intensities, and Kaleidagraph was used to fit the resulting decay curves to a single exponential function.

Transfection of HeLa cells and virus quantification

PV RNAs were produced by T7 transcription of MluI linearized cDNAs (type 1 Mahoney) and transfected in HeLa cells as previously described⁵. 2 μg of PV RNA was transfected in

duplicate into $\sim 10^6$ HeLa cells in each 35 mm well of 6-well plates using Transmessenger transfection reagent (Qiagen) according to manufacturer's instructions. Transfected HeLa cells were fed with 2 ml per well of cell culture media (DMEM containing 10% fetal bovine serum, 100 U per ml penicillin, and 100 μ g per ml streptomycin) and incubated at 37°C. Cells were examined for cytopathic effect (CPE) at 24, 48 and 72 hours post-transfection. P₀ virus was harvested from each 6-well plate at 48 and 72 hours post-transfection following three rounds of freeze-thaw, pooled (12 mls) and quantified by plaque assay. P₀ virus diluted in PBS (5 ml final volume) was used to infect $\sim 10^7$ HeLa cells in T₇₅ flasks at a multiplicity of infection (MOI) of 10 PFU per cell (or less when the titer of P₀ virus precluded an MOI of 10). P₁ virus was harvested after full CPE was observed at 24–72 hpi using three rounds of freeze-thaw and quantified by plaque assay.

Poliovirus sequencing

Poliovirus RNAs were isolated from purified virus particles and converted into cDNA for sequencing as previously described⁵. 8 mls of virus was layered onto 3 mls of 30% (w/v) sucrose in PBS followed by centrifugation at 36,000 rpm for 4 hours at 4°C using a Beckman SW41 rotor. Pelleted material containing virus particles was resuspended in 400 μ l 0.5% SDS buffer (0.5% SDS, 10 mM Tris-HCl, pH7.5, 100 mM NaCl). PV RNAs were isolated by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation.

Poliovirus RNAs were converted into cDNAs using Superscript III (Invitrogen) and a primer complementary to nucleotides 7400 – 7418 of the poliovirus 3' NTR (5' ₇₄₁₈CCCTACAACA GTATGACC₇₄₀₀ 3'). cDNA corresponding to the 3D^{pol} region of poliovirus RNA was amplified by 35 PCR cycles (2X) with high-fidelity Phusion DNA polymerase (New England Bio labs) using the reverse primer above and one of the following forward primers:

5' ₅₂₈₄GGCAATGACAATTCTACAAGC₅₃₀₄ 3'
 5' ₅₈₁₆CTCGGTGGGCGCGAAACTGCTCGTA₅₈₃₈ 3'
 5' ₆₂₇₈GAGGATGCCATGTCTGGC₆₂₉₅ 3'
 5' ₆₆₉₃GGTATGATGCATCTCTCAGCC₆₇₁₃ 3'
 5' ₇₁₉₄GGACTAAAGATCCTAGG₇₂₁₀ 3'

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 the forward primers above.

Viral sequences used for the residue conservation analysis were obtained from the rhinovirus sequencing study by Palmenberg et al. (33) and the Picornaviruses Project home page at <http://www.picornaviridae.com>. (34)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Template entry channel and mutation sites on the poliovirus 3D^{pol} elongation complex (A,B) Views from the front and top of the 3D^{pol}-RNA complex (PDB code 3OL6) showing exiting RNA duplex (green product strand, cyan template strand) and the putative RNA template entry channel (wide arrow). Structural elements of the polymerase are colored as in previous publications (palm domain in grey, thumb in blue, and individual fingers in green for index, orange for middle, yellow for ring, and red for pinky), the location of the 3D^{pol}-3D^{pol} interface disrupting L446D mutation on the thumb is shown in orange, and the sites of control mutations located away from RNA binding interface are indicated (K66, K314, K431). The RNA bases are numbered relative to the active site with positive numbers for downstream template, negative numbers for the upstream duplex product, and P and T denoting product and template strands. (C) Detailed view of the targeted basic residue (sticks) on the pinky finger, only some of which are directly interacting with the RNA in the

currently available elongation complex structure. RNA phosphate groups are shown as spheres. (D) Top view of the complex showing the putative RNA entry channel lined by tyrosines (CPK spheres) Y14 from the index finger, Y118 and Y148 from the pinky finger, and Y157 from the ring finger.

Figure 2. Effects of mutations and monovalent salt on RNA binding by 3D^{pol}

RNA binding curves for (A) charged residue mutations and (B) tyrosine mutations obtained in 75 mM NaCl. Data are fluorescence polarization measurements of a 5'-fluorescein labeled "8-10" RNA consisting of a 8 base pair stem with a tertraloop and a 10-base 5' single-stranded extension that serves as a template region²¹. Dissociation constants determined by curve fitting the data are listed in Table 1. (C) Data from wild-type polymerase showing the salt-dependence of the RNA binding equilibrium. Dissociation constants were obtained by curve fitting and for those datasets that did not reach a stable plateau value the maximal FP signal was constrained to equal that observed at lower salt concentrations. (D) Log-log plot of the RNA association constant as a function of salt concentration for wild type and R128A 3D^{pol}. The data reflect the commonly observed difference in behavior nucleic acid binding above (partially filled symbols) and below (open symbols) ≈ 100 mM salt (1.0 on $-\log[\text{NaCl}]$ axis), and only the data from above 100 mM NaCl were used to determine the slope and calculate the number of phosphate contacts made in the complex (see Table 2).

Figure 3. Elongation Complex Formation Rates

(A) Cartoon of the polymerase elongation template element (PETE) RNA constructs used to determine elongation complex formation rates. (B) Denaturing PAGE showing the formation of both the +1 and +2 products from the respective starting RNAs (S) by wildtype and Y148A polymerases in the presence of only ATP and GTP. (C) Quantitation of the +2 product formation data from the 9+2-24 RNA and fitting to a single exponential curve to determine formation rates. Data are shown for the wildtype and three control site mutations, three of the pinky finger charge mutations, and the four tyrosine mutations. Resulting elongation complex formation rates are listed in Table 1.

Figure 4. Elongation Rate Analysis

(A) Y14A 3D^{pol} elongation data illustrating both the lag phase and the subsequent increase in total fluorescence associated with the incorporation of the first twenty and last five nucleotides, respectively²⁴. The length of the lag phase decreases as the [NTP] increases, reflecting an increase in elongation rate. (B) Michaelis-Menten plots showing the maximum elongation rate and apparent K_m values of wild-type 3D^{pol} and the tyrosine and control mutants. The fitted values for these enzymatic parameters are listed in Table 1. (C) Fluorescence anisotropy based elongation data from wildtype and the four tyrosine mutants at 25 mM NTP showing the differences in the starting values and elongation dependent amplitude changes. The curves starting with a lower initial fluorescence anisotropy values also exhibit a smaller overall change in anisotropy, consistent with reduced RNA binding and fewer active complexes in solution. (D) Normalized total fluorescence of wildtype, Y14A, and Y118A at 25 mM NTP showing the differences in signal decay after reaching maximum total fluorescence that indicate the mutations destabilize the elongation complex.

Figure 5. Elongation Complex Stability

Comparison of the stability of elongation complexes as a function of time spent in a stalled state after initiation with ATP and GTP to form the +2 product on the 9+2₂₄ RNA. Data points show the fraction of the +2 material that could be efficiently elongated to full-length +20 product upon addition of the remaining NTPs. Data are shown for (A) control mutations, (B) basic residue mutations, and (C) tyrosine mutations and the time constants resulting from the single exponential curve fits are listed in Table 1.

Table 1

Effects of mutations on 3D^{pol} function *in vitro*

Mutation	RNA K_d (μ M) ^{<i>I</i>}	Initiation +1 (min) +2	V_{max} (nt/s)	K_m (μ M)	Stability (min)
Wildtype	4.9 $\pm$ 0.1	4.8 $\pm$ 0.3	3.0 $\pm$ 0.2	3.1 $\pm$ 0.1	23 $\pm$ 2
150 $\pm$ 9					
Pinky Finger Charged Residues					
K125A	38 $\pm$ 1	38 $\pm$ 5	16 $\pm$ 1	—	59 $\pm$ 5
K126A	23 $\pm$ 1	20 $\pm$ 2	13 $\pm$ 1	—	58 $\pm$ 4
K125A-K126A	130 $\pm$ 30	*	*	*	*
K127A-R128A	12 $\pm$ 1	—	—	—	—
R128A	35 $\pm$ 2	—	—	—	—
K133A	14 $\pm$ 1	17 $\pm$ 2	8 $\pm$ 1	—	65 $\pm$ 2
R188A	170 $\pm$ 20	*	*	*	*
Entry Channel Tyrosine Residues					
Y14A	5.6 $\pm$ 0.1	8.3 $\pm$ 0.4	8.7 $\pm$ 0.6	3.2 $\pm$ 0.1	17 $\pm$ 2
Y118A	4.5 $\pm$ 0.1	199 $\pm$ 3	30 $\pm$ 1	3.3 $\pm$ 0.1	20 $\pm$ 1
Y148A	6.7 $\pm$ 0.1	230 $\pm$ 70	90 $\pm$ 6	3.2 $\pm$ 0.1	19 $\pm$ 2
Y157A	9.8 $\pm$ 0.3	10.2 $\pm$ 0.4	10 $\pm$ 1	2.9 $\pm$ 0.1	16 $\pm$ 2
Control Residues					
K66A	4.2 $\pm$ 0.2	7 $\pm$ 2	3.5 $\pm$ 0.6	3.1 $\pm$ 0.1	22 $\pm$ 3
K314A	5.1 $\pm$ 0.3	7 $\pm$ 1	3.5 $\pm$ 0.6	3.1 $\pm$ 0.1	22 $\pm$ 3
K431A	—	8 $\pm$ 1	4.0 $\pm$ 0.6	3.1 $\pm$ 0.2	20 $\pm$ 4
120 $\pm$ 10					

^{*I*} Affinity as measured in 75–80 mM NaCl

— Not determined

* Low RNA affinity precluded further analysis of these 3D^{pol}-RNA complexes

Table 2

Phosphate contacts based on monovalent salt concentration dependence of RNA binding

Mutation	Interpolated K_d in 100 mM NaCl ¹ (μ M)	Slope of [NaCl] dependence ¹	Calculated number of contacts ²
Wildtype	7.1 ± 0.3	5.7 ± 0.4	7.4 ± 0.6
K125A	14.9 ± 0.6	4.6 ± 0.8	6.0 ± 1.1
K126A	19.9 ± 1.2	2.8 ± 0.2	3.6 ± 0.3
R128A	31.7 ± 1.5	2.8 ± 0.4	3.6 ± 0.5
K133A	11.8 ± 0.6	3.9 ± 0.4	5.1 ± 0.6
K127A-R128A	18.3 ± 0.6	4.1 ± 0.5	5.3 ± 0.7

¹Based on linear fit to log-log plots, as per Figure 2C,D²From slope of salt dependence divided by 0.77 shielding constant for sodium

Table 3

Virus viability and genetic stability of engineered polymerase mutations

Mutation	P ₍₀₎ titer ^a (PFU/mL)	P ₍₀₎ plaque phenotype	P ₍₁₎ titer (PFU/mL)	P ₍₁₎ plaque phenotype	Wildtype codon	Mutant codon	P ₍₁₎ virus codon	P ₍₁₎ Amino Acid
Wildtype	6.0×10 ⁷	wt	2.0×10 ⁹	wt				
Charged Residues								
K125A [*]	5.0×10 ⁷	wt	1.0×10 ⁹	wt	aag	gcc	gcc	ala
K126A [*]	3.0×10 ⁷	wt	2.0×10 ⁹	wt	aag	gcc	gcc	ala
K127A	1.0×10 ⁴	wt	1.0×10 ⁹	wt	aag	gcc	aag	lys
R128A [*]	2.0×10 ⁷	small	8.0×10 ⁸	small	aga	gcc	gcc	ala
K133A [*]	1.0×10 ⁸	wt	1.1×10 ⁹	wt	aaa	gcc	gcc	ala
R188A	6.0×10 ⁴	wt	9.0×10 ⁸	wt	aga	gcc	aga	arg
K125A-K126A	3.0×10 ⁶	mixed	3.0×10 ⁸	mixed	aag - aag	gcc - gcc	mixed	mixed
K127A-R128A	2.0×10 ⁴	wt	9.0×10 ⁸	wt	aag - aga	gcc - gcc	aag - aga	lys - arg
Tyrosine Residues								
Y14A [*]	8.0×10 ⁷	wt	9.0×10 ⁸	wt	uau	gcc	gcc	ala
Y118A	2.0×10 ²	small	8.0×10 ⁸	mixed	uac	gcg	aug	met
Y118F [*]	1.1×10 ⁸	wt	8.0×10 ⁸	wt	uac	uuu	uuu	phe
Y148A	9.0×10 ⁵	mixed	4.0×10 ⁸	wt	uau	gcc	guc	val
Y148F [*]	8.0×10 ⁷	wt	5.0×10 ⁸	wt	uau	uuu	uuu	phe
Y157A	5.0×10 ⁵	wt	2.0×10 ⁹	wt	uau	gcc	uau	tyr
Y157F [*]	3.0×10 ⁶	wt	1.3×10 ⁹	wt	uau	uuu	uuu	phe
Y118A-Y148A	6.0×10 ³	wt	7.0×10 ⁸	wt	uac - uau	gcg - gcc	uac - uac	tyr - tyr
Y118A-Y148F	1.0×10 ⁴	wt	4.0×10 ⁸	wt	uac - uau	gcg-uuu	uuu - uuu	phe - phe
Y118F-Y148A	1.8×10 ⁵	wt	7.0×10 ⁸	wt	uac - uau	uuu - gcc	uuu - guc	phe - val
Y118F-Y148F [*]	4.0×10 ⁷	wt	3.0×10 ⁸	wt	uac - uau	uuu - uuu	uuu - uuu	phe - phe

^a P₍₀₎ titers from 48 hours post transfection (hpt)

^{*} Mutations stably maintained in P₍₁₎ progeny

Table 4

Summary of effects on 3D^{pol} function and poliovirus growth.

Mutation	K _d RNA Binding (all expressed as fold-reduction from wildtype 3D ^{pol})	Initiation Rate (avg) ¹	Efficiency (V _{max} /K _m)	EC Stability	Virus Growth Remark ²
Wildtype	1.0	1.0	1.0	1.0	wildtype
Charged Residues					
K125A [*]	• 7.8	• 6.6		2.5	≈wildtype
K126A [*]	• 4.7	• 4.3		2.6	≈wildtype
K127A					reverted
R128A [*]	• 7.1				small plaque
K133A [*]	2.9	3.1		2.3	≈wildtype
R188A	• 35				reverted
K125A-K126A	• 27				mixed plaques & codons
K127A-R128A	2.4				doubly reverted
Tyrosine Residues					
Y118F [*]					≈wildtype
Y148F [*]					≈wildtype
Y157F [*]					≈wildtype
Y14A [*]	1.1	2.3	1.4	• 9.4	≈wildtype
Y118A	0.9	• 15	1.2	• >30	pseudo-reverted (Met)
Y148A	1.4	• 39	1.2	• >30	pseudo-reverted (Val)
Y157A	2.0	2.7	1.3	• 5.8	reverted
Control Residues					
K66A	0.9	1.3	1.0	1.3	
K314A	1.0	1.3	1.0	1.4	
K431A		1.5	1.2	1.3	

* Denotes mutations that were stably maintained in P₁(1) progeny virus

• Denotes significant differences from wildtype behavior

¹ Average of rates for +1 and +2 product formation from Table 1

² Progeny P(1) virus all tiered within one log of wildtype