POLIOVIRUS RNA-DEPENDENT RNA POLYMERASE (IN)FIDELITY:
MECHANISMS, CONSEQUENCES AND APPLICATIONS

A Thesis in
Biochemistry, Microbiology and Molecular Biology

by
Victoria S. Korneeva

© 2007 Victoria S. Korneeva

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2007
The thesis of Victoria S. Korneeva was reviewed and approved* by the following:

Craig E. Cameron  
Paul Berg Professor of Biochemistry and Molecular Biology  
Thesis Advisor  
Chair of Committee

Squire J. Booker  
Associate Professor of Biochemistry and Molecular Biology

Michael Teng  
Assistant Professor of Biochemistry and Molecular Biology

Ming Tien  
Professor of Biochemistry

Blake R. Peterson  
Associate Professor of Chemistry

Richard J. Frisque  
Professor of Molecular Virology  
Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School
ABSTRACT

RNA viruses are causative agents of numerous human and animal diseases, such as hepatitis (hepatitis C virus), common cold (rhinoviruses), influenza, foot-and-mouth disease, hemorrhagic fever (Ebola and Dengue viruses), encephalitis and meningitis (West Nile, coxsackievirus, echoviruses), and myocarditis (coxsackievirus). Although some progress in vaccine and antiviral drug development has been made in recent years, there is still a great demand in creating more powerful and versatile antiviral compounds. The viral RNA-dependent RNA polymerase (RdRp) is a key enzyme in viral genome replication and represents a unique target for antiviral drug development. Lethal mutagenesis of the RNA viruses has been recently explored as a promising antiviral strategy. Therefore, understanding structural determinants of the polymerase fidelity will strongly facilitate rational drug design.

In this thesis, studies on structure-function relationships of poliovirus (PV) polymerase, 3Dpol, and the mechanistic basis for RdRp fidelity are described, including biochemical and biological evaluation of three 3Dpol derivatives: Glu-297, Tyr-30 and Arg-273. Previous studies of PV RdRp have shown that Asn-297 permits the enzyme to distinguish ribose from 2’-deoxyribose. Mutation of this highly conserved amino acid residue to Glu, which is present in all phage RdRps, resulted in a 3Dpol derivative with decreased efficiency of deoxyribonucleotide incorporation. At the same time, the fidelity of the ribonucleotide incorporation displayed by Glu-297 3Dpol was substantially reduced, providing a mechanistic explanation for the elevated mutation frequency observed for RNA phages. In addition, evaluation of the protein-primed initiation
reaction catalyzed by 3Dpol led us to the conclusion that even though the same polymerase active site is employed in both reactions, substantial structural differences exist between initiation and elongation complexes. Moreover, Glu-297 3Dpol was able to excise deoxyribonucleotides, suggesting that RNA phage RdRps might have utilized this ability to proofread. We introduced Phe-30 to Tyr mutation in the PV 3Dpol in order to disrupt a strong interaction between the fingers and thumb subdomains of the polymerase, observed crystallographically. Contrary to our expectations, Tyr-30 mutation had no effect on the polymerase function. Unexpectedly, this mutation within 3D domain altered an unknown 3CD function after genome translation and replication. This discovery shed some light on a potential role of 3CD in virus maturation and/or viral RNA packaging; the mechanisms of both of these processes are still unknown. The last, but not least, 3Dpol derivative described in this thesis is the Arg-273 3Dpol. Although residue 273 is located about 20 Å from the active site of the polymerase, mutation changing wildtype amino acid residue His-273 to Arg resulted in both a PV and a polymerase with a mutator phenotype. Biochemical evaluation of the Arg-273 3Dpol showed that the conformational change preceding the chemistry step is accountable for the relaxed fidelity observed for this polymerase; X-ray analysis of the Arg-273 3Dpol did not reveal any substantial changes in the crystal structure when compared to the WT 3Dpol, suggesting that Arg-273 may alter conformational flexibility of the enzyme. Arg-273 PV, in combination with the WT and high fidelity Ser-64 PV represents a unique experimental system for exploring relationships between virus fidelity, fitness, and pathogenicity.
Together, these studies provide insight into yet unexplored properties of the 3Dpol and provide basis for further studies on the polymerase fidelity, virus maturation and virus-host interactions and towards designing novel strategies to treat RNA viruses.
# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... x

LIST OF TABLES ............................................................................................................. xiii

LIST OF ABBREVIATIONS ........................................................................................... xiv

ACKNOWLEDGEMENTS ............................................................................................... xvii

**Chapter 1**  Introduction ........................................................................................... 1
  Viruses ....................................................................................................................... 1
  Poliovirus as a model system for RNA viruses .................................................... 5
  The life cycle of a picornavirus ........................................................................... 8
  Poliovirus ................................................................................................................ 12
  Poliovirus 3Dpol .................................................................................................. 15
  Complete mechanism of nucleotide incorporation catalyzed by 3Dpol .......... 19
  Two-metal ion mechanism of catalysis .............................................................. 21
  Kinetic basis for 3Dpol fidelity ......................................................................... 23
  Conclusions ......................................................................................................... 25
  References ............................................................................................................. 26

**Chapter 2**  Experimental Procedures ..................................................................... 29
  Materials ................................................................................................................. 29
  Construction of expression plasmids for 3Dpol derivatives ................................ 30
  Construction, expression and purification of 3CD ............................................ 32
  Expression and purification of 3Dpol derivatives ............................................. 32
  Construction of mutated viral cDNA clones and mutated replicons ............... 34
  Cloning and transcription of 61-nt 2C-cre (cis-replicating element) ............... 35
  Purification of RNA substrate sym/sub ............................................................... 35
  5’-32P Labeling of sym/sub ................................................................................. 36
  Annealing of sym/sub ......................................................................................... 37
  Poly(rU) polymerase assay ............................................................................... 37
  3Dpol kinetic assays ........................................................................................... 37
  3Dpol active site titration .................................................................................. 38
  3Dpol-sym/sub complex assembly ................................................................... 38
  3Dpol-sym/sub complex dissociation .............................................................. 39
  Inactivation of 3Dpol ......................................................................................... 39
  Chemical-quench-flow assay .......................................................................... 39
  Product analysis ................................................................................................. 40
  Data analysis ....................................................................................................... 40
  Kinetic simulations ............................................................................................. 41
  VPg uridylylation assay ...................................................................................... 41
Chapter 3  Structure-Function Relationships of the Viral RNA-dependent RNA Polymerase: Fidelity, Replication Speed and Initiation Mechanism Determined by a Residue in the Ribose-Binding Pocket

Summary.................................................................54
Introduction........................................................................55
Results.................................................................................60
Rationale.................................................................................60
Glu-297 3Dpol Incorporates 2’-deoxyribonucleotides as Efficiently as WT 3Dpol............................60
Glu-297 PV is Quasi-Infectious ...........................................68
Glu-297 PV is Severely Impaired for RNA Synthesis in Cells.........70
The Product Yield and Processivity of VPg-primed RNA Synthesis by Glu-297 3Dpol is Severely Impaired ....................72
The Processivity of RNA-primed RNA Synthesis by Glu-297 3Dpol is Normal.......................................................76
Efficient and Processive Uridylylation of VPg by Gly-297 3Dpol......77
Attempts to Rescue Glu-297 3Dpol RNA-primed Elongation Rate by Rational Design...............................................79
Discussion..............................................................................81
Acknowledgements................................................................87
References..............................................................................87

Chapter 4  Proofreading by Glu-297 3Dpol: use of the First and Second Conformational-Change Steps in Nucleotide Selection

Summary.............................................................................90
Introduction.........................................................................91
Results..................................................................................94
Apparent Dissociation Constants for Glu-297 3Dpol are Elevated in the Case of Deoxyribonucleotide Incorporation..................94
Phosphorothioate Effect Suggests that Steps other than Chemistry are more Rate Limiting for Glu-297 3Dpol .................................................97
EDTA-HCl Quench Analysis of the Glu-297 3Dpol-Catalyzed Nucleotide Incorporation Supports the Hypothesis that the Conformational-change step Prior to the Chemistry Step Becomes more Rate Limiting .................................................98
The Rate Constant of the Dissociation of a Nucleotide Analog, AMPCP, from a Ternary Complex Suggests that Decrease in $K_2$ was due to Change in $k_{-2}$, not in $k_{-2}$ ..................................................103
Successive Incorporation of Two Nucleotides ......................................107
Evaluation of the Reverse Reaction Showed that Glu-297 3Dpol can Proofread after Deoxyribonucleotide Incorporation .....................112
Discussion .............................................................................................116
References.................................................................................................123

Chapter 5 Biological Evidence for Post-replication Function of Picornavirus Protein 3CD .........................................................................................................................125

Summary .....................................................................................................125
Introduction ..................................................................................................126
Results ..........................................................................................................130
Kinetic Properties of Tyr-30 3Dpol were Unaffected by Mutation ..........130
Biological Evaluation of the Tyr-30Allele ..................................................130
Tyr-30 PV Conferred a Small Plaque Phenotype in Cell Culture .............134
RNA Synthesis by Tyr-30 PV was Identical to WT PV .............................135
The Tyr-30 Mutation Caused Delay in Structural Proteins Processing During Viral RNA Translation in vitro ..................................................138
Tyr-30 3CD was Incompetent in in vitro Virus Maturation Stimulation Assay .................................................................143
Discussion ..................................................................................................145
Acknowledgements ....................................................................................152
References ..................................................................................................152

Chapter 6 Residue Arg-273 as a Modulator of the polymerase Fidelity ..........155

Summary .....................................................................................................155
Introduction ..................................................................................................156
Results ..........................................................................................................161
Biochemical Evaluation of Arg-273 3Dpol, a Low Fidelity Polymerase .........161
Arg-273 3Dpol is a Functional Polymerase with Decreased Fidelity ........161
Decreased Arg-273 3Dpol Fidelity Originates from Tighter Binding and Increased Rate of Nucleotide Incorporation ..........................164
Arg-273 3Dpol Exhibits an Increase in the Equilibrium Constant for the Conformational-Change Step Preceding Phosphoryl Transfer .........166
Relaxed Conformational-change Step Results in Altered 3Dpol-RNA Complex Assembly Rate and Complex Stability ..................173
X-ray Crystallographic Analysis of Arg-273 3Dpol Confirms only Minimal Changes to the Polymerase Structure .....................174
Biological Evaluation of the Arg-273 3Dpol Allele .........................................179
Poliovirus with Arg-273 3Dpol Resembles WT PV in Tissue Culture ..................179
Arg-273 PV Exhibits a Mutator Phenotype in Cell Culture ..........................183
Arg-273 PV was not Extinguished during Serial Passage ...............................186
Arg-273 PV Exhibited Reduced Fitness Relative to WT PV in the Coinfection Experiment ..................................................187
Arg-273 PV is greatly Attenuated in cPVR Mice .........................................192
Transmission Electron Microscopy (TEM) Revealed that Arg-273 PV produced more Empty Viral Particles than WT PV .......193
Discussion ...............................................................................................................199
References ............................................................................................................210

Chapter 7 General Discussion and Future Directions ........................................212
General conclusions ..........................................................................................212
Future directions ................................................................................................216
Additional studies of the conformational-change step preceding phosphoryl transfer ............................................................216
Further analysis of the structural basis for 3Dpol fidelity .................................218
Theory of lethal mutagenesis .......................................................................219
Biochemical and biological evaluation of the Ser-64/Arg-273 3Dpol ...222
References .............................................................................................................222

Appendix A Introduction of Additional Binding Steps and Stalling into Kinetic Mechanism for Successive 2 Nucleotides Incorporation Significantly Improves Fit of the Data .................................................................224

Appendix B Kinetic and Thermodynamic Constants for Arg-273/Glu-297 3Dpol-Catalyzed Nucleotide Incorporation .................................................................233

Appendix C Intracellular Concentrations of Nucleotides Differ between Mammals and Bacteria .................................................................234

Appendix D WT and Arg-273 PV: Serial Passage at Low MOIs ..........................235

Appendix E Pulse-Chase Pulse-Quench Analysis of Glu-297 3Dpol-Catalyzed Nucleotide Incorporation .................................................................236

Appendix F Supplemental Materials for Chapter 4 ................................................242
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Classification scheme for animal viruses.</td>
<td>3</td>
</tr>
<tr>
<td>1-2</td>
<td>Overview of a picornavirus replication cycle.</td>
<td>11</td>
</tr>
<tr>
<td>1-3</td>
<td>Structure of the poliovirus genome.</td>
<td>13</td>
</tr>
<tr>
<td>1-4</td>
<td>Crystal structure of the poliovirus RNA-dependent RNA polymerase 3Dpol.</td>
<td>18</td>
</tr>
<tr>
<td>1-5</td>
<td>Complete kinetic mechanism for 3Dpol-catalyzed nucleotide incorporation.</td>
<td>20</td>
</tr>
<tr>
<td>1-6</td>
<td>Two-metal ion mechanism for the catalysis.</td>
<td>22</td>
</tr>
<tr>
<td>1-7</td>
<td>Comparison of use of the conformational-change and phosphoryl transfer steps for WT 3Dpol-catalyzed correct and incorrect nucleotide incorporation in the presence of Mg\textsuperscript{2+} and Mn\textsuperscript{2+}.</td>
<td>24</td>
</tr>
<tr>
<td>3-1</td>
<td>Differences in ribose/2'-deoxyribose specificity for RNA phage polymerases relative to animal and plant RNA virus polymerases predicted from structure/sequence comparison.</td>
<td>59</td>
</tr>
<tr>
<td>3-2</td>
<td>Kinetic analysis of Glu-297 3Dpol.</td>
<td>65</td>
</tr>
<tr>
<td>3-3</td>
<td>Minimal kinetic mechanism for 3Dpol-catalyzed nucleotide incorporation.</td>
<td>67</td>
</tr>
<tr>
<td>3-4</td>
<td>Glu-297 PV is quasi-infectious.</td>
<td>69</td>
</tr>
<tr>
<td>3-5</td>
<td>Replication of Glu-297 PV in cells is as debilitated as a PV encoding a catalytically inactive polymerase.</td>
<td>71</td>
</tr>
<tr>
<td>3-6</td>
<td>VPg uridylylation reaction.</td>
<td>73</td>
</tr>
<tr>
<td>3-7</td>
<td>VPg-primed RNA synthesis by Glu-297 3Dpol is impaired.</td>
<td>76</td>
</tr>
<tr>
<td>3-8</td>
<td>Processive RNA-primed RNA synthesis by Glu-297 3Dpol is normal.</td>
<td>78</td>
</tr>
<tr>
<td>3-9</td>
<td>VPg-primed RNA synthesis by Gly-297 3Dpol is normal.</td>
<td>80</td>
</tr>
<tr>
<td>3-10</td>
<td>Asn-297 interacts with the 3'-OH of the VPg-pU product.</td>
<td>85</td>
</tr>
<tr>
<td>4-1</td>
<td>The amount of intermediate that accumulated prior to chemistry step for Glu-297 3Dpol was reduced relative to WT 3Dpol.</td>
<td>101</td>
</tr>
</tbody>
</table>
Fig. 4-2: Stability of the Glu-297 3Dpol ternary complex with AMPCPP was similar to WT.  ................................................................. 106

Fig. 4-3: Successive incorporation of two nucleotides suggested block in translocation for Glu-297 3Dpol after first dNMP incorporation. ....................... 111

Fig. 4-4: Pyrophosphate exchange. ................................................................. 115

Fig. 5-1: PV 3Dpol crystal structure revealed an interface between fingertips and thumb subdomains................................................................. 128

Fig. 5-2: Kinetic properties of Tyr-30 3Dpol were unaffected by the mutation........ 132

Fig. 5-3: Biological evaluation of the Tyr-30 allele. ........................................ 137

Fig. 5-4: Processing cascade of the poliovirus polyprotein. ............................ 139

Fig. 5-5: Delay in structural protein processing was observed during in vitro translation of the Tyr-30 viral RNA. ................................................. 142

Fig. 5-6: Tyr-30 3CD was incompetent in in vitro virus maturation stimulation assay................................................................. 144

Fig. 6-1: Location of Arg-273 and Gly-64 in the PV 3Dpol crystal structure. ....... 158

Fig. 6-2: Arg-273 3Dpol has reduced fidelity relative to WT 3Dpol in vitro........... 163

Fig. 6-3: The observed kinetics of Arg-273 3Dpol-catalyzed AMP incorporation in the presence of Mg$^{2+}$ is quench agent-dependent. ............................ 170

Fig. 6-4: The conformational-change step preceding phosphoryl transfer is relaxed for Arg-273 3Dpol. ................................................................. 172

Fig. 6-5: X-ray crystallographic analysis of Arg-273 3Dpol confirms subtle (minimal) effects on the polymerase structure. ........................................ 178

Fig. 6-6: Arg-273 PV kinetics of growth in cell culture is similar to WT PV. .......... 182

Fig. 6-7: Arg-273 has a mutator phenotype in cell culture. ............................. 185

Fig. 6-8: Arg-273 PV was not extinguished during serial passage. .................... 188

Fig. 6-9: Arg-273 PV exhibits reduced fitness relative to WT PV in a coinfection experiment. ................................................................. 191

Fig. 6-10: Arg-273 PV was attenuated in cPVR mice. ...................................... 195
Fig. 6-11: Arg-273 virus presents bottlenecks in RNA packaging. .........................198

Fig. 7-1: Determination of the lethal mutagenesis and virus extinction threshold. ....221

Fig. A-1: Introduction of additional binding steps into kinetic mechanism for successive 2 nucleotides incorporation significantly improves fit of the data. ............................226

Fig. A-2: Introduction of additional stalling step prior to translocation into kinetic mechanism for successive 2 nucleotides incorporation improves fit of the data. ........................................................................................................................228

Fig. A-3: Introduction of additional stalling step after translocation into kinetic mechanism for successive 2 nucleotides incorporation significantly improves fit of the data.  ..............................................................................................230

Fig. A-4: Introduction of additional binding steps and stalling into kinetic mechanism for successive 2 nucleotides incorporation significantly improves fit of the data. ...........................................................................................................232

Fig. D-1: Serial passage of the WT and Arg-273 PV at MOI of 0.1 and 0.01. .........235

Fig. E-1: The amount of the intermediate that accumulates prior to chemistry for the Glu-297 derivative is reduced relative to WT 3Dpol. .................................241

Fig. F-1: Substantial block is observed for Glu-297 3Dpol-catalyzed dNMP incorporation even in the presence of Mn^{2+}. .................................................................243

Fig. F-2: Deoxy- and ribonucleotide incorporation by Glu-297 and WT 3Dpol in the presence of Mg^{2+}, (B) and (C), respectively. .................................................245

Fig. F-3: dATP excision by Glu-297 3Dpol upon addition of exogenous pyrophosphate. ........................................................................................................247

Fig. F-4: dNTP incorporation in the presence of Mg^{2+} catalyzed by Glu-297 and WT 3Dpol, (A) and (B), respectively.................................................................248

Fig. F-5: GMP incorporation after 3’dUMP by Glu-297 and WT 3Dpol, (B) and (C), respectively. .............................................................................................250

Fig. F-6: dNTP incorporation in the presence of Mg^{2+} catalyzed by Glu-297 and WT 3Dpol.................................................................................................251
### LIST OF TABLES

Table 1-1: D. Baltimore’s Virus Classification ...........................................................4

Table 1-2: Picornaviridae Family .................................................................................7

Table 2-1: Oligonucleotides Used in this Study .........................................................31

Table 2-2: Extinction Coefficients at 260 nm for Nucleoside Monophosphates.........36

Table 3-1: Glu-297 3Dpol Incorporates 2’-deoxyribonucleotides as Efficiently as WT 3Dpol .............................................................................................................61

Table 3-2: Activity of Glu-297 3Dpol was not Enhanced by Introduction of Residues Conserved in Phage RdRps .....................................................................62

Table 4-1: Kinetic and Thermodynamic Constants for 3Dpol-Catalyzed Nucleotide Incorporation .................................................................................................96

Table 4-2: Observed Phosphorothioate Effect for 3Dpol-catalyzed Nucleotide Incorporation in the Presence of Mg$^{2+}$ and Mn$^{2+}$ ......................................................100

Table 6-2: Observed Phosphorothioate Effects for 3D$^\text{pol}$-catalyzed Nucleotide Incorporation Suggest that the Conformational-Change Step Remains Partially Rate Limiting for Arg-273 3Dpol in the Presence of Mg$^{2+}$ ..................168

Table 6-3: Rate of the Complex Assembly ($k_{\text{ass}}$) and Dissociation ($k_{\text{off}}$) and Enzyme Inactivation for WT and Arg-273 3Dpol in the Presence of Mg$^{2+}$ .....175

Table B-1: Kinetic and Thermodynamic Constants for Arg-273/Glu-297 3Dpol-Catalyzed Nucleotide Incorporation .................................................................................233

Table C-1: Intracellular Concentrations of Nucleotides Differ between Mammals and Bacteria ...........................................................................................................234
LIST OF ABREVIATIONS

2-AP   2-aminopurine
2C-cre  2C-cis-acting replication element
2'-dAMP 2'-deoxyadenosine 5'-monophosphate
2'-dATP 2'-deoxyadenosine 5'-triphosphate
2'-dNMP 2'-deoxynucleoside-5'-monophosphate
2'-dNTP 2'-deoxynucleoside-5'-triphosphate
AMP   adenosine 5'-monophosphate
AMPCPP α,β-methyleneadenosine 5'-triphosphate
AMPαS  adenosine 5'-O-(1-thiomonophosphate)
ATP   adenosine 5'-triphosphate
ATPαS  adenosine 5'-O-(1-thiotriphosphate)
BPB   bromophenol blue
cDNA  complementary deoxyribonucleic acid
CL    cloverleaf
CMP   cytidine 5'-monophosphate
CPE    cytopathic effect
cPVR   poliovirus receptor
dAMP   2'-deoxyadenosine 5'-monophosphate
dAMPαS 2'-deoxyadenosine 5'-O-(1-thiomonophosphate)
dATP   2'-deoxyadenosine 5'-triphosphate
dATPαS 2'-deoxyadenosine 5'-O-(1-thiotriphosphate)
DdDp   DNA-dependent DNA polymerase
DMEM/F-12 Dulbecco's modified Eagle medium:nutrient mix F-12
dNMP  2'-deoxynucleoside-5'-monophosphate
dNTP   2'-deoxynucleoside 5'-triphosphate
DTT   dithiothreitol
dUTP   2'-deoxyuridine 5'-triphosphate
EDTA  ethylenediaminetetraacetic acid
ER    endoplasmic reticulum
FBS   fetal bovine serum
FMDV   foot-and-mouth disease virus
GMP   guanosine 5'-monophosphate
GTP   guanosine 5'-triphosphate
HCV   hepatitis C virus
HIV   human immunodeficiency virus
ICTV  International Committee on Taxonomy of Viruses
IPTG  isopropyl-β-D-thiogalactopyranoside
IRES  internal ribosome entry site
KF    Klenow fragment
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD$_{50}$</td>
<td>lethal dose 50%</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cutoff</td>
</tr>
<tr>
<td>NMP</td>
<td>nucleoside-5’-monophosphate</td>
</tr>
<tr>
<td>NP40</td>
<td>nonidet P40</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside-5’-triphosphate</td>
</tr>
<tr>
<td>NTR</td>
<td>non-translated region</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>PABP</td>
<td>polyadenylate binding protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCBP</td>
<td>polycytidylate binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>polyadenylate</td>
</tr>
<tr>
<td>PP$_1$</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>PV</td>
<td>poliovirus</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RLU</td>
<td>relative light units</td>
</tr>
<tr>
<td>RMP</td>
<td>ribavirin monophosphate</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>rNMP</td>
<td>ribonucleoside monophosphate</td>
</tr>
<tr>
<td>rNMP</td>
<td>ribonucleoside-5’-monophosphate</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>rNTP</td>
<td>ribonucleoside triphosphate</td>
</tr>
<tr>
<td>rNTP</td>
<td>ribonucleoside-5’-triphosphate</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RTP</td>
<td>ribavirin 5’-triphosphate</td>
</tr>
<tr>
<td>S/S</td>
<td>symmetrical substrate</td>
</tr>
<tr>
<td>SARS</td>
<td>severe acute respiratory syndrome</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sym/sub</td>
<td>symmetrical substrate</td>
</tr>
<tr>
<td>T$<em>{10}$E$</em>{1}$</td>
<td>10 mM Tris, 1 mM ethylenediaminetetraacetic acid pH = 8.0</td>
</tr>
<tr>
<td>TBE (1X)</td>
<td>89 mM Tris base, 89 mM boric acid, 2 mM EDTA</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>UMP</td>
<td>uridine 5’-monophosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
<tr>
<td>VPg</td>
<td>genome-linked virion protein</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like first to acknowledge my family, my mother and my grandmother, for all the support they provided to me during these years. I would also like to thank all of my friends, who made all these years I spent in the graduate school an unforgettable experience.

I would like to thank my advisor, Dr. Craig Cameron, for giving me an opportunity to join his lab and to work on so many exciting and diverse projects, and for all of his help, advice and patience. I would also like to thank my committee members for their understanding and support during rough times. I would like to thank all previous and current members of the Cameron lab, for sharing their knowledge, for teaching me how to work in a biochemical lab, and for creating a friendly environment.
Chapter 1

Introduction

Viruses

Viruses are small infectious particles, ranging from 20 to 300 nm in diameter. They are obligatory parasites that can replicate themselves only upon the infection of their host organism; they cannot propagate on their own. Viruses were first distinguished from bacteria and other disease-causing organisms in the end of the 19th century. Viruses have most likely existed on the Earth for billions of years, but left no fossil record. Therefore virus origins can be predicted only based on the virus population that is present nowadays. There are three major theories on virus origins; the first theory considers viruses as the remnants of the pre-cellular world and that viruses coevolved with all the known forms of life. The regressive theory views viruses as descendants of the intracellular microorganisms-parasites. According to the progressive theory, viruses come from the cellular genetic elements that gained the ability to replicate autonomously and therefore were able to evolve.

Because viruses are neither living nor not-living organisms, their classification is based on their phenotypic characteristics, such as virus size, the organisms the viruses infect, virus morphology (capsid type), type of the nucleic acid that constitutes its genome, or mode of virus replication. In the 1960s, a comprehensive system for virus classification based on the common properties shared by viruses was established. Major
criteria for this classification included the nature of the nucleic acid in a viral genome, symmetry of the protein shell (capsid), presence or absence of a lipid envelope, and the dimensions of the virion and capsid (Fig. 1-1). Later, D. Baltimore introduced virus classification based entirely on the genomic material and the mechanisms of virus genome replication (Table 1-1).

In the early 1990s, an International Committee on Taxonomy of Viruses (ICTV) introduced a set of rules on the naming and classification of viruses, which combined both classical and Baltimore’s characteristics. According to ICTV viral classification starts at the level of order and follow as thus: order (-virales), family (-viridae), subfamily (-virinae), genus (-virus) and species (-virus). In a 2000 report by the ICTV approximately 30,000 to 40,000 virus isolates were assigned to 3 orders, 56 families, 9 subfamilies, 233 genera and 1550 species (1).
Fig. 1-1: Classification scheme for animal viruses. Summary of the major characteristics of 23 representative families of viruses that infect vertebrates. Taken from (1).
Table 1-1: D. Baltimore’s Virus Classification

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Double-stranded DNA viruses</td>
</tr>
<tr>
<td>II</td>
<td>Single-stranded DNA viruses</td>
</tr>
<tr>
<td>III</td>
<td>Double-stranded RNA viruses</td>
</tr>
<tr>
<td>IV</td>
<td>Positive-sense single-stranded RNA viruses</td>
</tr>
<tr>
<td>V</td>
<td>Negative-sense single-stranded RNA viruses</td>
</tr>
<tr>
<td>VI</td>
<td>Reverse-transcribing diploid single-stranded RNA viruses</td>
</tr>
<tr>
<td>VII</td>
<td>Reverse-transcribing circular double-stranded DNA viruses</td>
</tr>
</tbody>
</table>
Poliovirus as a model system for RNA viruses

Poliovirus (PV) was identified as a causative agent of poliomyelitis almost 100 years ago (2). Nearly fifty years later two effective vaccines for poliovirus were developed. This discovery opened the doors for a new era of exploration for poliovirus. Numerous advances were made in elucidating molecular biology, structure and genetics of PV. However, the investigation of the pathogenesis of the virus ceased after vaccine discovery. The identification of the cellular receptor CD155, involved in the virus attachment to the cell, led to the creation of the transgenic mice susceptible to poliovirus infection (3,4). The development of the transgenic mice model created a novel opportunity for studying pathogenesis of the disease and virus-host interaction. Thus, PV has proved to be an excellent experimental system, for which numerous biochemical and cell culture techniques had been established, producing a large body of biochemical and genetic information. As a result, poliovirus serves as a paradigm for positive-sense, single-stranded RNA viruses.

Although poliovirus is probably one of the most studied viruses nowadays, there still remain many unanswered questions regarding the virus life cycle, initiation of replication, spread of the virus within an infected host organism, virus maturation, RNA encapsidation, and last, but not least, the mechanism by which PV causes poliomyelitis.

Poliovirus belongs to the picornaviridae family, which encompasses small non-enveloped viruses with a single-stranded genome of the positive polarity. This family consists of nine genera and includes many important human and animal pathogens.
(Table 1-2) (5). The viruses of the picornairidae family cause numerous diseases that range from common cold to myocarditis, meningitis and hepatitis.
Table 1-2: Picornaviridae Family

<table>
<thead>
<tr>
<th>Genus</th>
<th>Type species</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aphthovirus</td>
<td>Foot-and-mouth disease virus</td>
<td>Foot-and-mouth disease viruses A, C, Asia 1, SAT 1, SAT 2, SAT 3</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td></td>
</tr>
<tr>
<td>Cardioviruses</td>
<td>Encephalomyocarditis virus</td>
<td>Theiler’s murine encephalomyelities virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mengovirus</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>Poliovirus 1</td>
<td>Polioviruses 1, 2, 3,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine enteroviruses 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human coxsakieviruses A1-A16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human coxsakieviruses B1-B5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human ecoviruses 1-33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human enteroviruses 68-71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simian enteroviruses 1-18</td>
</tr>
<tr>
<td>Hepatovirus</td>
<td>Human hepatitis A virus</td>
<td>Simian hepatitis A virus</td>
</tr>
<tr>
<td>Parechovirus</td>
<td>Human parechovirus 1</td>
<td>Human parechovirus 2</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>Human rhinovirus 1A</td>
<td>Human rhinoviruses 2-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine rhinoviruses 1-3</td>
</tr>
<tr>
<td>Erbovirus</td>
<td>Equine rhinitis B virus</td>
<td>Equine rhinitis B virus 2</td>
</tr>
<tr>
<td>Kobuvirus</td>
<td>Aichi virus</td>
<td>Aichi virus</td>
</tr>
<tr>
<td>Teschovirus</td>
<td>Porcine teschovirus 1</td>
<td>Porcine teschovirus 2-7, 11-13</td>
</tr>
</tbody>
</table>
The life cycle of a picornavirus

The replication cycle of a picornavirus starts when the virus encounters its receptor on the cell surface (Fig. 1-2, step 1). After the virion binds to its cognate receptor the capsid undergoes a series of transformations that lead to the release of the viral RNA into the cytoplasm (Fig. 1-2, step 2). The viral RNA is of a positive sense polarity (i.e. can serve as a mRNA), and its translation by the host cell translational machinery begins immediately (Fig. 1-2, step 3). A picornavirus RNA has only one open reading frame, and its translation yields one large polyprotein, which is cotranslationally processed by viral proteases (Fig. 1-2, steps 4, 5). Viral non-structural proteins from P2 and P3 region begin to modify cellular environment by forming vesicles from the membranes of the endoplasmic reticulum and Golgi complex (Fig. 1-2, step 6). At some point in the beginning of the infection switch from translation to replication occurs; translation of the viral genome is reduced at this point, and replication of the viral RNA begins (Fig. 1-2, step 7). The incoming genomic RNA first serves as a template for negative strand RNA synthesis (Fig. 1-2, step 8) and then the minus strand RNAs are amplified to produce numerous copies of the viral RNA of the positive sense polarity (Fig. 1-2, step 9); the RNA replication is carried out by the virally encoded RNA-dependent RNA polymerase. Some of the newly synthesized viral RNAs are shuffled back to the cytoplasm where they are translated to produce more viral proteins (Fig. 1-2, step 10). Structural proteins, formed by partial cleavage of P1 precursor, associate with the plus strand viral RNA to form progeny virions (Fig. 1-2, steps 11 and 12). These
newly formed viruses are released upon the cell lysis and ready to undergo next cycle of infection (Fig. 1-2, step 13).
Fig. 1-2: **Overview of a picornavirus replication cycle.** Virus binds to the cellular receptor (step 1), and the genome gets uncoated (step 2). VPg is removed from the viral RNA, which can serve as a mRNA. Viral RNA associates with the cellular translation machinery (step 3). Viral polypeptide is synthesized (step 4). The polyprotein is cotranslationally processed by viral proteases 2Apro and 3Cpro to yield partially and fully processed viral proteins (step 5). Non-structural proteins from P2 and P3 regions transform ER and Golgi membranes into membranous vesicles (step 6). Viral RNA associates with the newly formed membrane vesicles (step 7). Minus strand RNA is synthesized from viral RNA (step 8), these minus strand RNAs then serve as templates for the positive strand RNA synthesis (step 9). RNA synthesis is unsymmetrical; strands of the positive polarity accumulate to a much higher concentration. Some of the newly synthesized viral RNAs can relocate back to the cytoplasm and be translated to produce more viral proteins (step 10). Structural proteins of P1 region are cleaved, and begin to assemble into pentamers and/or into viral particles (step 11). Newly synthesized RNA is packaged into viral particles (step 12). Upon cell lysis the virus is released (step 13). The figure is taken from (1).
Poliovirus

Poliovirus is relatively small; its genomic RNA is only 7440 nucleotides long and contains one open reading frame (ORF) flanked by non-translated regions (NTRs) on both 5’- and 3’-ends (Fig. 1-3). Within the 5’-NTR cloverleaf structure and internal ribosome entry site (IRES) are found; 3’-NTR is composed of a pseudoknot and a poly(A) tail. Upon entry into the cell, genomic RNA serves as a messenger RNA and is translated by the host translation machinery to produce one 247 kDa polyprotein. This polyprotein is then processed in cis and trans by viral proteases. 2Apro releases P1 region from P2-P3 precursor, further polyprotein cleavage is then carried out by 3Cpro, either in the form of 3C or 3CD, to yield partially processed and mature viral proteins (Fig. 1-3). P1 region codes for structural proteins that form viral capsid. The poliovirus capsid consists of 60 copies of each of the four structural proteins; during the virus infection protomers are spontaneously formed from three single copies of each of the structural proteins – VP0, VP1 and VP3; VP0 is processed later inside the capsid to produce VP2 and VP4 by an unidentified mechanism. The protomers in turn assemble a pentamer unit; twelve pentamers form an icosahedral viral capsid. The mechanism by which viral RNA is encapsidated remains unknown, although only actively transcribed RNA molecules are subject to packaging (6). Proteins of the P1 region are not essential for PV replication; subgenomic PV replicons with a deletion of the entire P1 region is capable of replication. P2 and P3 regions of the poliovirus code for non-structural
Fig. 1-3: Structure of the poliovirus genome. PV genomic RNA is comprised of one open reading frame flanked by 3’- and 5’-non-traslational regions (NTRs). P1 region encompasses capsid proteins, whereas regions P2 and P3 code for non-structural proteins. 2A protease releases P2-P3 precursor from the polypeptide upon its translation (shown by curved arrow). Further processing of the remainder polypeptides is carried on by 3C protease in the form of either 3C (\(\vee\)) or 3CD (\(\triangledown\)). All structural and non-structural mature proteins are shown along with their corresponding molecular weights (kDa). Protein primer VPg (3B) is covalently attached to the 5’ end of the viral RNA and is shown as a black sphere. Location of an internal ribosome entry site (IRES) and cis-replicating elements is shown: IRES and the cloverleaf structure reside within the 5’-NTR; 2C-cre within 2C-coding region, and the pseudoknot and poly(A) tail comprise the 3’-NTR.
proteins that are essential for virus replication. 2A is a protease that releases P1 region from the remainder of the polyprotein. Besides polyprotein processing 2Apro cleaves eukaryotic translation initiation factor eIF4G; this event results in a shut off of the cellular host mRNA translation. Viral translation is not affected by eIF4G cleavage because viral RNA possesses an internal ribosome entry site (IRES) that does not require initiation factors. Other proteins of the P2 region, 2B, 2C and their common precursor 2BC, induce changes of the host cell environment within the cytoplasm by rearranging ER and Golgi membranes into membranous vesicles (7-9). Virus replication is thought to occur inside these rosette-like structures formed by the membranous vesicles (9-11).

3AB protein exhibits non-specific RNA binding activity; however together with 3CD it forms ribonucleoprotein complexes at the 5’ cloverleaf and 3’NTR (12-17). A transmembrane domain within the C-terminus of 3AB is responsible for anchoring 3AB to the membranes of the rosette-like membranous vesicles. 3CD and 3D proteins are thought to be recruited to these vesicles, where replication complexes form, via interactions with 3AB (14,18,19).

The second protein of the P3 region, 3B, is the smallest viral peptide and consists of only 22 amino acids in the case of PV. This peptide is commonly called VPg – viral protein genome linked. VPg serves as a primer for both positive and negative sense RNA synthesis. Newly synthesized RNAs and RNAs found inside the viral capsids all have VPg covalently attached to their 5’-end. Uridylylation of VPg (attachment of two uracil monophosphate units) at a completely conserved Tyr-3 amino acid residue comprises the first step in the initiation of the viral genome replication (20,21). The 2C-cre (cis-
replicating element) stem-loop RNA structure within the 2C-coding region serves as a template for VPg uridylylation by viral polymerase 3Dpol (Fig. 1-3) (22-25).

3C is a protease that cleaves non-structural proteins from their precursor proteins P2-P3. Cleavage of the TATA-binding protein (TBP) by 3C efficiently shuts off RNA polymerase II-mediated transcription (26,27). 3C cleaves host poly(A)-binding protein (PABP) as well; removal of the C-terminal domain of this protein results in severe inhibition of the host mRNA translation (21). 3CD, precursor of both 3C and 3D, functions as a protease; however, only the active site of the 3C domain is responsible for RNA binding and catalysis and 3D domain confers substrate specificity to the P1 region. 3CD is also an RNA-binding protein; formation of the ribonucleoprotein complexes with poly(rC)-binding protein (PCBP) at 5’-cloverleaf RNA structure and 3’-NTR is essential for RNA replication (12,13,15,17). The binding of the 3CD to the 5’-NTR is important for slowing down viral RNA translation and serves as a signal for switch to replication (16,28). 3CD also interacts with 2C-cre loop and significantly stimulates VPg uridylylation by the virally encoded RdRp. Recently, 3CD was shown to stimulate virus maturation by an unknown mechanism in *in vitro* translation/replication assay (29,30).

**Poliovirus 3Dpol**

The last and largest protein encoded by PV is the RNA-dependent RNA polymerase, 3Dpol, the enzyme that carries out viral RNA replication. PV 3Dpol was the first enzyme for which RNA-dependent RNA polymerase activity was shown (31). PV 3Dpol was also the first enzyme among RdRps for which the crystal structure was solved.
(32). The availability of the crystal structure for 3Dpol encouraged detailed kinetic and biochemical evaluation of 3Dpol to be performed. Although the first crystal structure of 3Dpol was missing part of the fingers subdomain, it proved that the overall shape of the enzyme resembled canonical fold of other classes of nucleic acid polymerases, commonly described as a cupped right hand with the fingers, thumb and palm subdomains. A few years later, Peersen’s group intentionally disrupted a known and persistent crystal packaging interaction between the thumb and back of the palm subdomains and solved a complete 3Dpol crystal structure (Fig. 1-4) (33). This structure revealed that PV 3Dpol adopts a “closed” conformation, where extensive interactions between the fingertips and thumb subdomains completely encircle the active site of the enzyme. A completely encircled active site is a characteristic feature of RdRps (33-43). Four conserved structural motifs A, B, C, and D make up the palm subdomain, motif E is positioned between the thumb and palm domains, and motif F is located at the tip of the fingers domain. Motifs A to D are conserved among all single subunit polymerases, whereas motif E is found only in RTs and RdRps, and motif F is a unique element of RdRps (Fig. 1-4) (32,33,44). Within motifs A and C reside three absolutely conserved aspartate residues (Asp-233, Asp-328 and Asp-329 in PV) that serve as ligands for the two metals essential for catalysis (Fig. 1-6). Asp-238 of motif A along with Asn-297 of motif B are implicated in the incoming nucleotide base selection. Asp-238 is thought to hydrogen-bond with both 2’- and 3’-hydroxyls of the ribose ring, whereas Asn-297 is hydrogen bonded to only the 2’-OH group (45,46). The function of motif D is poorly understood. It was shown to be involved in nucleotide selection in the RT and was proposed to stabilize structural integrity of the palm subdomain; although an essential role in catalysis
have been recently suggested for motif D (47,48). Motif E is termed “primer grip” in RTs, emphasizing its interactions with nucleic acid; in PV 3Dpol mutations within motif E affect 3AB protein binding and efficiency of the protein primer VPg uridylylation (14,49-53). Motif F is unique to RdRps, and is most likely involved in binding of the triphosphate moiety of the incoming nucleotide (39,54).
Fig. 1-4: Crystal structure of the poliovirus RNA-dependent RNA polymerase 3Dpol. The overall shape of the polymerase resembles cupped right hand. The conserved structural motifs in the palm subdomain are colored as follows: motif A - in red; motif B - in green; motif C - in yellow; motif D - in blue; motif E - in magenta, and motif F - in purple. The image was generated using the program WebLab Viewer (Molecular Simulations Inc., San Diego, CA). PDB access code is 1RA6.
Complete mechanism of nucleotide incorporation catalyzed by 3Dpol

The availability of a crystal structure for PV 3Dpol combined with the development of a symmetrical RNA primer/template substrate (sym/sub) that allows 3Dpol to establish stable elongation complexes sky-rocketed detailed kinetics and mechanistic studies of a single nucleotide incorporation catalyzed by 3Dpol (32,33,55-57). The outcome of these studies was a solution of a complete kinetic mechanism of single nucleotide incorporation in the presence of both metal cofactors, Mg$^{2+}$ and Mn$^{2+}$ (Fig. 1-5) (56,57). Mg$^{2+}$ is a biologically-relevant metal cofactor for 3Dpol, whereas Mn$^{2+}$ can be utilized by the polymerase in vitro; use of Mn$^{2+}$, however, decreases the polymerase efficiency and incorporation fidelity (56,57). In general, PV 3Dpol follows the same sequence of steps proposed for RTs and DNA polymerases (58-61) (Fig. 1-5).

First, 3Dpol-RNA binary complex (ER$_n$) binds an incoming nucleotide (NTP) to form ternary complex (ER$_n$NTP) (step 1). This ternary complex undergoes a conformational change to form an activated complex competent for phosphoryl transfer (*ER$_n$NTP) (step 2). Chemistry step occurs, resulting in the formation of an activated ternary product complex (*ER$_{n+1}$PP$_i$) (step 3), followed by isomerization of this complex to a ternary product complex (ER$_{n+1}$PP$_i$) (step 4), from which pyrophosphate (PP$_i$) can dissociate (step 5). After pyrophosphate dissociation, the remaining product complex (ER$_{n+1}$) is ready to undergo the next round of catalysis. For PV 3Dpol two steps, the first conformational change and chemistry, are partially rate-limiting in the presence of Mg$^{2+}$; in the presence of Mn$^{2+}$, however, only the chemistry step remains rate limiting (56,57).
Fig. 1-5: Complete kinetic mechanism for 3Dpol-catalyzed nucleotide incorporation. ERₙ (3Dpol – sym/sub complex); NTP (nucleotide); ERₙNTP (ternary complex); *ERₙNTP (activated elongation complex); *ERₙ₊₁PPᵢ (activated product complex); ERₙ₊₁PPᵢ (product complex); ERₙ₊₁ (3Dpol – sym/sub product complex); PPᵢ (pyrophosphate). Kinetic parameters for the WT 3Dpol in the presence of Mg²⁺ or Mn²⁺ as a metal cofactor are shown in red and blue color, respectively (56,57).

<table>
<thead>
<tr>
<th>Metal</th>
<th>k⁺₁</th>
<th>k⁺₂</th>
<th>k⁺₃</th>
<th>k⁺₄</th>
<th>k⁺₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn²⁺</td>
<td>10 μM⁻¹ s⁻¹</td>
<td>300 s⁻¹</td>
<td>30 s⁻¹</td>
<td>≥160 s⁻¹</td>
<td>2000 s⁻¹</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>10 μM⁻¹ s⁻¹</td>
<td>300 s⁻¹</td>
<td>520 s⁻¹</td>
<td>500 s⁻¹</td>
<td>8100 s⁻¹</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
ERₙ + NTP & \rightleftharpoons ERₙNTP \\
ERₙNTP & \rightleftharpoons *ERₙNTP \\
*ERₙNTP & \rightleftharpoons ERₙ₊₁PPᵢ \\
ERₙ₊₁PPᵢ & \rightleftharpoons ERₙ₊₁PPᵢ \\
ERₙ₊₁PPᵢ & \rightleftharpoons ERₙ₊₁PPᵢ
\end{align*}
\]
Two-metal ion mechanism of catalysis

PV polymerase 3Dpol is the enzyme that is solely responsible for replication of the viral RNA. The phosphoryl transfer reaction is thought to be the same for all polymerases and was previously described to utilize so-called two-metal-ion mechanism of catalysis (62). According to this mechanism, metal A in the active site of the polymerase lowers down the pKₐ of the 3’-OH of the primer and thus, facilitates the attack on the α-phosphate of the incoming nucleotide (Fig. 1-6). Metal B coordinates both β- and γ-phosphates of the incoming nucleotide and is thought to stabilize the incoming nucleotide in the conformation competent for catalysis. Furthermore, these two metals were implied to stabilize the structure and charge of the transition state during the phosphoryl transfer. Further elucidation of the phosphoryl transfer reaction mechanism performed in our laboratory showed the existence of two protons being transferred during polymerase-catalyzed nucleotide incorporation; based on these findings general base catalysis was proposed for 3’-OH deprotonation step and general acid for protonation of the pyrophosphate leaving group for the nucleotidyl-transfer reactions (48).
Fig. 1-6: **Two-metal ion mechanism for the catalysis.** Primer and incoming nucleotide are shown. Two metal cations are labeled A and B. Metal ion A is coordinated by conserved aspartates of the active site and thought to lower the pKₐ of the 3’-OH to facilitate nucleophilic attack on the α-phosphate of the incoming nucleotide. Metal ion B coordinates the β- and γ-phosphates and likely stabilizes the triphosphate in a conformation competent for phosphoryl transfer. Adapted from (62).
**Kinetic basis for 3Dpol fidelity**

When Mg\(^{2+}\) is used as a metal cofactor both the conformational-change and chemistry steps (steps 2 and 3 in Fig. 1-5) are rate limiting and play crucial role for incorrect nucleotide discrimination (Fig. 1-7) (56,63). The conformational change preceding chemistry step is thought to involve reorientation of the triphosphate moiety of the incoming nucleotide from the ground-state binding to the conformation proper for catalysis (Fig. 1-5, step 1 and 2). In the presence of Mg\(^{2+}\) only the triphosphate moiety of a correct nucleotide can adopt appropriate orientation for catalysis, for incorrect nucleotides the orientation will be suboptimal. Use of Mn\(^{2+}\) as a metal cofactor significantly slows down chemistry step for the correct nucleotide, making it equal for both correct and incorrect nucleotides; thus only the conformational-change step is involved in nucleotide selection (Fig. 1-7) (63). Mn\(^{2+}\) is thought to increase the stability of the activated elongation complex (*ER\(_{a}\)NTP) through additional advantageous interactions with the amino acid residues of the ribose-binding pocket, in addition to tighter binding to the β- and γ-phosphates of the bound nucleotide. However, these advantageous interactions are not dependent on the nature of the incoming nucleotide, i.e. both correct and incorrect nucleotides would be able to establish those interactions, and the efficiency of the phosphoryl transfer step becomes equal for correct and incorrect nucleotides. The inability to couple the nature of the bound nucleotide to the efficiency of phosphoryl transfer is proposed to be a basis for the loss of WT 3Dpol fidelity observed in the presence of Mn\(^{2+}\) (57).
Fig. 1-7: Comparison of use of the conformational-change and phosphoryl transfer steps for WT 3Dpol-catalyzed correct and incorrect nucleotide incorporation in the presence of Mg$^{2+}$ and Mn$^{2+}$. In the presence of Mg$^{2+}$, both conformational change and phosphoryl transfer modulate fidelity; in the case of incorrect nucleotide incorporation conformational change becomes much less favorable and the rate of the chemistry step is significantly decreased (green arrow in the reverse direction, and red arrow in the forward direction, respectively). Use of Mn$^{2+}$ as a divalent metal cofactor diminishes rate of phosphoryl transfer for correct nucleotide to the level of the rate of incorrect nucleotide incorporation (red arrows); and only conformational change defines fidelity (green arrows). Adapted from (63).
Conclusions

In spite of poliovirus being one of the most studied viruses, the detailed mechanism of its replication is still poorly understood. The work described in this thesis encompasses wide range of aspects of RNA virus replication. Further interrogation of the structure-function relationships of the amino acid residues within the nucleotide-binding pocket led us to the conclusion that protein-primed initiation complex and RNA-primed elongation complex exhibit substantial functional differences, in spite of employing the same polymerase active site (Chapter 3). Regardless of the availability of the 3Dpol polymerase crystal structure and solution of the complete kinetic mechanism for single nucleotide incorporation, the kinetic and structural determinants of the fidelity are still not completely understood. Detailed kinetic analysis of deoxyribonucleotides misincorporation suggested that a single amino acid substitution within the ribose-binding pocket can enforce use of the 1st conformational-change step for dNTP discrimination, enhance dNTP excision, but at the same time compromise enzyme’s selectivity for the correct base (Chapter 4). Preliminary analysis of the conserved structural interaction between the fingertips and thumb subdomains of viral polymerase led us to an intriguing observation of this interface (within 3CD protein) being essential for processes after translation and replication, such as virus maturation, and perhaps, virus spread (Chapter 5). Discovery of the first PV and 3Dpol with mutator phenotype and their biological and biochemical evaluation are presented in Chapter 6. Availability of the PV with mutator polymerase allowed us to begin addressing questions of connectivity between polymerase
fidelity and virus fitness and its pathogenicity within the host organism, and evolutionary aspects of RNA virus replication.

References

Chapter 2

Experimental Procedures

The purpose of this chapter is to introduce all of the experimental procedures and materials used throughout this thesis. Experimental procedures will be referenced to this chapter by the name of the appropriate protocol. If any modification to the following protocols was done, it would be stated further in the text or in the figure legends of the consecutive chapters.

Materials

$[\gamma^{32}P]ATP$ (>7,000 Ci/mmol) was from MP Biomedicals (former ICN); $[\alpha^{32}P]ATP$ (3,000 Ci/mmol), $[\alpha^{32}P]UTP$ (>6,000 Ci/mmol) Perkin-Elmer Life Sciences Inc.; DNA oligonucleotides were from Integrated DNA Technologies, Inc.; T4 polynucleotide kinase, Deep Vent DNA polymerase, and restriction enzymes were from New England Biolabs, Inc.; Shrimp Alkaline Phosphatase was from USB; T4 DNA ligase was from Invitrogen Life Technologies; Difco-NZCYM was from BD Biosciences; QIAEX was from QIAGEN; Sephadex G-25 was from Sigma; Phosphocellulose (P-11) and DE-81 filter paper were from Whatman; all nucleotides (ultrapure solutions), Q- and S-Sepharose fast flow were from GE Healthcare; polyethylenimine-cellulose TLC plates were from EM Science; RNA oligonucleotides were from Dharmacon Research, Inc. (Boulder, CO); synthetic VPg peptide was from Alpha Diagnostic International (San
Antonio, TX); all other reagents were of the highest grade available through Sigma or Fisher or VWR.

**Construction of expression plasmids for 3Dpol derivatives**

Mutations were introduced into a modified 3Dpol-coding sequence by using an overlap-extension PCR and expressed in *Escherichia coli* by using a ubiquitin fusion system. The ubiquitin fusion system, PCR conditions, and modified gene sequence were described previously (1). In brief, Glu-297 clone was produced by an overlap-extension PCR with oligonucleotides 1, 7 and 2, 8; Arg-273 clone – with oligonucleotides 1, 15 and 2, 16; Tyr-30 – with oligonucleotides 1, 17 and 5, 18 (Table 2-1) with pET26Ub-3D-BPKN-I92T as a template. For Glu-297, Arg-273 the final products of the second PCR step were purified and digested with PstI and NheI and ligated into pET26Ub-3D-BPKN-I92T that has been digested with the same enzymes. Double and triple mutants (S288M/N297E, C290N/N297E and S288M/C290N/N297E) were constructed in the same way using oligonucleotide primers 1, 2, 9-14 (Table 2-1), with pET26Ub-3D-BPKNP-I92T plasmid used as the template, this plasmid has silent PmlI site in the 3Dpol coding sequence. For the Tyr-30 derivative the second PCR product and the pET26Ub-3D-BPKNP-I92T vector were digested with AvrII and SacII restriction enzymes. Mutations were confirmed by DNA sequencing (Nucleic Acid Facility, Pennsylvania State University).
Table 2-1: Oligonucleotides Used in this Study\(^a,b\)

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 3D-PV-\textit{Avr}II-rev</td>
<td>5'- CCT GAG TGT TCC TAG GAT CTT TAG T - 3'</td>
</tr>
<tr>
<td>2 3D-PV-\textit{Pst}I-for</td>
<td>5'- GGA GTG ATA ACA GGT TCT GCA GTG GGG TGC GAT - 3'</td>
</tr>
<tr>
<td>3 3D-\textit{Avr}II-for</td>
<td>5'- AAC GAT CCC AGG \textit{Ctt} AAG ACA GAT TTT GAG - 3'</td>
</tr>
<tr>
<td>4 3D-\textit{Bam}HI-rev</td>
<td>5'- GCG GGA TCC TTA CTA AAA TGAGTC AAG CCA ACG GCG GTA - 3'</td>
</tr>
<tr>
<td>5 3D-\textit{Sac}II-for</td>
<td>5'- GCG \textit{Ccg} \textit{Cgg} TGG AGG TGA AAT CCA GTG \textit{Gat} GAG A - 3'</td>
</tr>
<tr>
<td>6 3Dseq500</td>
<td>5'- AGG TTG AGC AGG GGA AA - 3'</td>
</tr>
<tr>
<td>7 3D-PV-N297E-for</td>
<td>5'- GGT ACC TCA ATT TTT \textit{Gaa} TCA ATG ATT AAC - 3'</td>
</tr>
<tr>
<td>8 3D-PV-N297E-rev</td>
<td>5'- GTT AAT CAT TGA \textit{Ttc} AAA AAT TGA GGT ACC - 3'</td>
</tr>
<tr>
<td>9 3D-PV-S288M-N297E-for</td>
<td>5'- AAG GGC GGT \textit{ATg} CCA ATG GGC TGT TCA GGT ACC TCA ATT TTT \textit{Gaa} TCA ATG ATT AAC AAC - 3'</td>
</tr>
<tr>
<td>10 3D-PV-S288M-N297E-rev</td>
<td>5'- GTT GTT AAT CAT TGA TTC AAA AAT TGA GGT ACC TGA ACA GCC CAT TGG \textit{Gtt} GCC AGA TGG CAT ACC - 3'</td>
</tr>
<tr>
<td>11 3D-PV-C290N-N297E-for</td>
<td>5'- GGT ATG CCA TCT GGC AAC TCA GGT ACC TCA ATT TTT \textit{Gaa} TCA ATG ATT AAC AAC - 3'</td>
</tr>
<tr>
<td>12 3D-PV-C290N-N297E-rev</td>
<td>5'- GTT GTT AAT CAT TGA \textit{Ttc} AAA AAT TGA GGT ACC TGA ACA GCC CAT TGG \textit{Gtt} GCC AGA TGG CAT ACC - 3'</td>
</tr>
<tr>
<td>13 3D-PV-S288M-C290N-N297E-for</td>
<td>5'- AAG GGC GGT \textit{ATg} CCA ATG GGC \textit{Aac} TCA GGT ACC TCA ATT TTT \textit{Gaa} TCA ATG ATT AAC AAC - 3'</td>
</tr>
<tr>
<td>14 3D-PV-S288M-C290N-N297E-rev</td>
<td>5'- GTT GTT AAT CAT TGA \textit{Ttc} AAA AAT TGA GGT ACC TGA \textit{Gtt} GCC CAT TGG \textit{Cat} ACC GCC CTT - 3'</td>
</tr>
<tr>
<td>15 3D-PV-H273R-for</td>
<td>5'- CTA AAC \textit{Cac} TCA \textit{Cac} \textit{agg} CTG TAC AAG AAT AAA ACA - 3'</td>
</tr>
<tr>
<td>16 3D-PV-H273R-rev</td>
<td>5'- TGT TTT ATT CTT GTA CAG CCT GTG TGA GTG GTT TAG - 3'</td>
</tr>
<tr>
<td>17 PV-3D-F30Y-for</td>
<td>5'- CCT GGA CCC AGT GCT \textit{Tac} CAC TAT GTG TTT GAA - 3'</td>
</tr>
<tr>
<td>18 PV-3D-F30Y-rev</td>
<td>5'- TTC AAA CAC ATA GTG \textit{Gta} AGC ACT GGG TTT AAG - 3'</td>
</tr>
<tr>
<td>19 pET26Ub\textit{Eco}RI-rev</td>
<td>5'- \textit{Gag} CTC \textit{Gaa} TTC TTA CTA CTA - 3'</td>
</tr>
<tr>
<td>20 PV-3D-MfeI-L446D-for</td>
<td>5'- \textit{CAatt} GGT AGG GCT TTA \textit{Gat} CTC CCA GAG TAC TCA - 3'</td>
</tr>
</tbody>
</table>

\(^a\) Codons that introduce amino acid residue change in 3Dpol sequence are in bold.

\(^b\) Restriction enzyme sites are underlined.
Construction, expression and purification of 3CD

WT viral protein 3CD was expressed and purified as described previously (2). In order to create Tyr-30 3CD derivative the second PCR product obtained during construction of the 3D expression vector was digested with AvrII and BstBI restriction enzymes and ligated and pET26Ub-3CD-C147G-GSSG-6HIS plasmid. DNA sequencing was used to verify the integrity of the clone.

Expression and purification of 3Dpol derivatives

3Dpol derivatives were expressed and purified as described previously (1). In brief, pET-26Ub-3D-BPKN-I92T vectors were transformed into E.coli cells, strain BL21(DE3)pCG1. This E.coli strain carries pCG1 vector that encodes ubiquitin protease Ubp1. This enzyme efficiently cleaves ubiquitin from the ubiquitin-3Dpol fusion to yield 3Dpol with an authentic N-terminus. BL21(DE3)pCG1 cells transformed with pET-26Ub-3Dpol vectors were grown in 100 mL of NZYCM media supplemented with 25 µg/ml of kanamycin (K25), 20 µg/ml of chloramphenicol (C20) and 0.4% of dextrose overnight at 30 ºC. The overnight cultures were then used to inoculate 2.5 L of NZYCM media (K25, C20) to the OD$_{600}$ of 0.05. The cultures were then grown at 37 ºC till OD$_{600}$ reached 0.8 - 1. At this point, the cultures were cooled down to 30 ºC, and IPTG was added to final concentration of 500 µM. After 4 hours post induction cells were harvested by centrifugation at 6000 rpm in JLA-16.250 Beckman rotor for 15 minutes at 4 ºC, washed once with 200-300 mL of T$_{10}$E$_{1}$ (10 mM Tris, 1 mM ethylenediaminetetraacetic acid pH = 8.0) and stored at – 80 ºC. Typical yields from 2.5
L culture were 16-20 grams of the cell paste. Frozen cells were resuspended in the lysis buffer (100 mM potassium phosphate pH 8.0, 0.5 mM EDTA, 20% glycerol, 1 mM DTT, 60 µM ZnCl₂, 2.8 µg/mL Pepstatin A, 2.0 µg/mL Leupeptin), in the ratio 4 mL of the buffer per 1 gram of the cell paste. Cells were lysed by passing two times through a French Press; PMSF and NP-40 were added immediately to the cell extract to the final concentrations of 1mM and 0.1%, respectively. PEI was added slowly to the cell lysate to a final concentration of 0.25% (v/v) in order to precipitate nucleic acids. The lysate was allowed to equilibrate at 4 °C for additional 15-30 minutes, after which it was spun at 25000 rpm in Beckman JA-30.50 Ti rotor at 4 °C for 30 minutes. PEI supernatant was decanted and pulverized ammonium sulfate was added to it very slowly to 40% saturation. The solution was left stirring at 4 °C for 30 minutes, the precipitated protein was then pelleted at 25000 rpm at 4 °C for 30 minutes. The ammonium sulfate pellet was resuspended in buffer A (50 mM Tris pH 8.0, 20% glycerol, 1 mM DTT, 0.1% NP-40, 60 µM ZnCl₂) to a final salt concentration of 50 mM (measured by a conductivity meter). This solution was loaded onto pre-equilibrated in buffer A plus 50 mM NaCl phosphocellulose column at the flow rate of 1 mL per minute. Volume of the column was determined by total protein amount in the ammonium sulfate solution and the resin capacity – 1 mL of bed volume holds about 20 mg of protein. The column was washed with Buffer A plus 50 mM NaCl to the baseline and then eluted with a gradient from 50 to 350 mM NaCl in buffer A (6 bed volumes). Fractions, containing 3Dpol, were pooled together based on the purity and activity. Pooled fraction from the phosphocellulose column were then loaded onto S-sepharose fast flow column, pre-equilibrated in buffer A plus 50 mM NaCl, washed with 6-bed volumes of the buffer A plus 50 mM NaCl and
eluted with the salt gradient from 50 to 500 mM in buffer A. Pooled fractions, diluted to 50 mM salt (based on conductivity) were loaded onto Q-column, washed and eluted – essentially, in the same way as was described for S-sepharose column. Pooled fractions from Q-sepharose column were diluted to 50 mM salt and were loaded onto a final concentrating 1 mL Q-sepharose column. This column was washed with buffer B (50 mM HEPES pH 7.5, 50 mM NaCl, 20% glycerol, 1 mM DTT, 0.1% NP-40 and 60 µM ZnCl₂), and eluted by stepping of in the same buffer, but with 500 mM NaCl. Protein concentration was determined by measuring absorbance at 280 nm in 6M guanidine chloride solution and using calculated for WT extinction coefficient (71840 M⁻¹ cm⁻¹) (1). Purified enzyme was stored at – 80 ºC. For double and triple mutants (S288M/N297E, C290N/N297E and S288M/C290N/N297E) poly r(U) activity assay was performed with partially purified enzymes recovered from ammonium sulfate pellets.

**Construction of mutated viral cDNA clones and mutated replicons**

In order to introduce mutation into the 3Dpol coding sequence of viral cDNA, an overlap extension PCR was performed with oligonucleotides 1, 3 and the appropriate protein expression vector as a template. PCR products were purified and digested with *Avr*II and *Afl*III restriction enzymes. The digested PCR products were ligated into pUC18-*Bg*II-*Eco*RI-3CD vector, a subclone of the viral cDNA (2,3). From these vectors, the fragment between *Bg*II and *Eco*RI was cloned into the viral cDNA plasmid (pMoRA) (4). To introduce mutations into the replicon, the fragment between the *Bg*II and *Avr*II sites from the mutated pUC18-*Bg*II-*Eco*RI-3CD subclone vector was ligated
into pRLucRA (also known as pRLuc31-rib⁺polyAlong) (4,5). DNA sequencing was used to verify the integrity of all clones.

**Cloning and transcription of 61-nt 2C-cre (cis-replicating element)**

2C-cre RNA was cloned and transcribed and gel-purified by denaturing polyacrylamide gel electrophoresis as described previously (2).

**Purification of RNA substrate sym/sub**

Sym/subs, 10 nucleotide long RNA substrates were purchased from Dharmacon Research, Inc. (Boulder, CO). The oligonucleotides were purified by polyacrylamide denaturing gel electrophoresis on gels composed of 21.3% of polyacrylamide, 1.7% bis-acrylamide, 7 M urea and 1X TBE. RNA bands were visualized by UV shadowing, and only gel piece containing full-length RNA was excised. RNA was eluted from the gel in 1X TBE using Elutrap apparatus (Schleicher & Schuell). RNA solution was passed through Sep-Pak cartridges (Waters) and eluted in 6 mL of methanol. Methanol was evaporated in a Speed-Vac (Savant), and protection group was removed from 2’-OH of the RNA, essentially as recommended by the manufacturer. In brief, RNA pellet was dissolved in 100 µL of 500 mM acetic acid, heated for 15 minutes at 65 °C, and then neutralized with 100 µL 660 mM Tris base. Concentration of the RNA was determined by taking an absorbance reading at 260 nm in 50 mM K₂PO₄ pH 7.0, 6 M guanidine chloride and using calculated extinction coefficient (Table 2-2). Concentrated hydrolyzed RNA stocks were usually around 1 mM and contained 330 mM Tris-acetate,
pH 7.0. The stock RNA was diluted in T_{10}E_{0.1} (10mM Tris pH 8.0, 0.1 mM EDTA) to the desired concentration, aliquotted and stored at –20 °C.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>( \varepsilon, \text{ M}^{-1} \text{ cm}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>15,300</td>
</tr>
<tr>
<td>CMP</td>
<td>7,400</td>
</tr>
<tr>
<td>GMP</td>
<td>11,700</td>
</tr>
<tr>
<td>UMP</td>
<td>9,900</td>
</tr>
</tbody>
</table>

5'\text{-}^{32}\text{P Labeling of sym/sub}

RNA oligonucleotides were end-labeled by using \([\gamma-^{32}\text{P}]\text{ATP}\) and T4 polynucleotide kinase (New England Biolabs) essentially as recommended by the manufacturer. Labeling reactions, usually 20 \(\mu\text{L}\), contained 11 \(\mu\text{M}\) \([\gamma-^{32}\text{P}]\text{ATP}\), 10 \(\mu\text{M}\) sym/sub, 1X kinase buffer and 0.4 units/\(\mu\text{L}\) T4 polynucleotide kinase. Reactions were incubated at 37 °C for 45-60 min, and then T4 polynucleotide kinase was inactivated at 65 °C for 15 min. Unincorporated nucleotide was removed by passing the reaction mixture over two consecutive 1 mL Sephadex G-25 (Sigma) spun columns in T_{10}E_{0.1} (10 mM Tris pH 8.0, 0.1 mM EDTA). Concentration of the end-labeled RNA was assumed unchanged. The fraction of the end-labeled RNA in the polymerase reactions constituted only 1 to 2\%, therefore any changes in the end-labeled RNA concentration were insignificant.
Annealing of sym/sub

Typically, 1% of the end-labeled sym/sub was mixed with 99% of the unlabeled sym/sub in T<sub>10</sub>E<sub>0.1</sub> to desired concentration and heated to 90 °C for 1 min and then cooled down to 10 °C at a rate of 5 °C per min in a Progene Thermocycler.

Poly(rU) polymerase assay

Poly r(U) activity assay was performed as described previously with minor modifications (3). Briefly, reactions contained 50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl<sub>2</sub>, 60 µM ZnCl<sub>2</sub>, 500 µM UTP, 0.2 µCi/µl [α<sup>32</sup>-P]UTP, 2 µM dT<sub>15</sub>/0.2 µM poly rA<sub>467</sub> primer-template complex, and 500 ng of 3Dpol. Reactions were carried out in a total volume of 25 µl with 500 ng of enzyme at 30 °C for 5 min, and then quenched by mixing 5 µl with equal volume of 0.1 M EDTA. Five µl of the quenched reaction were spotted onto DE81 filter paper discs and dried completely. The discs were washed three times for 10 min in 250 ml of 5% dibasic sodium phosphate and rinsed in absolute ethanol. Bound radioactivity was quantitated by liquid scintillation counting in 5 ml of EcoScint scintillation fluid (National Diagnostics).

3Dpol kinetic assays

Reactions contained 50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl<sub>2</sub>, 60 µM ZnCl<sub>2</sub>, 500 µM NTPs (unless specified otherwise), 1 µM sym/sub (0.5 µM duplex) and 1 µM 3Dpol (unless specified otherwise). Reactions were quenched by
addition of EDTA to final concentration of 50 mM. 3Dpol was diluted immediately prior to use in 50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 60 µM ZnCl₂, and 20% glycerol. The volume of enzyme added to any reaction was always less than or equal to one-tenth of the total volume. All reactions were performed at 30 ºC.

3Dpol active site titration

Typically active site titration reaction contained 20 µM sym/sub (10 µM duplex), 500 µM ATP and 1 or 2.5 µM 3Dpol in the reaction buffer (50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 µM ZnCl₂). Reactions were initiated by the addition of 3Dpol and incubated at 30 ºC, at fixed time points the reactions were quenched by the addition of EDTA to a final concentration of 50 mM. The data best fit to a straight line with the y-intercept corresponding to the concentration of active 3Dpol.

3Dpol-sym/sub complex assembly

In order to determine the rate of the 3Dpol-sym/sub complex assembly 2 µM 3Dpol was rapidly mixed with 2 µM sym/sub RNA (1 µM duplex) in the reaction buffer (50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 µM ZnCl₂) containing 500 µM ATP. Reactions were quenched at indicated times by the addition of EDTA to a 50 mM final concentration. The data best fit to a single exponential.
3Dpol-sym/sub complex dissociation

Typically, 2 μM 3Dpol were incubated at 30 °C with 2 μM 5’-32P-labeled sym/sub RNA (1 μM duplex) for 90 sec in the reaction buffer (50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 μM ZnCl₂), at which point 100 μM unlabeled sym/sub RNA trap was added. At fixed timepoints after trap addition, 5μL reaction aliquots were taken out and rapidly mixed with 5 μL of 1mM ATP solution in the reaction buffer. Reactions were allowed to proceed for an additional 30 sec at 30 °C, and then were quenched by addition of EDTA to a final concentration of 50 mM. The data best fit to a single exponential.

Inactivation of 3Dpol

3Dpol (2 μM) was incubated at 30 °C in the reaction buffer (50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 μM ZnCl₂) in the presence of 500 μM ATP. At the indicated times the reaction was started by the addition of 2 μM sym/sub RNA (1 μM duplex) and the reactions were allowed to proceed for an additional 90 sec at which time the reaction was quenched by addition of EDTA to 50 mM final concentration. The data best fit to a single exponential.

Chemical-quench-flow assay

Rapid mixing/quenching experiments were performed by using a Model RQF-3 chemical-quench-flow apparatus (KinTek Corp, Austin, TX). All experiments were
performed at 30 °C, the temperature was controlled by using a circulating water bath. 3Dpol-sym/sub complex in 50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 60 µM ZnCl₂ and 5 mM MgCl₂ was rapidly mixed with the nucleotide substrate in the same buffer, and the reactions were either quenched by addition of 0.5 M EDTA to final concentration of 0.3 M or by addition of 2 N HCl to a final concentration of 1.2 N. Immediately after addition of HCl, the solution was neutralized by addition of 3 M KOH in 1 M Tris pH 8.0.

**Product analysis**

Products were analyzed on denaturing PAGE. An equal volume of loading buffer, 5 µL, (90% formamide, 0.025% bromophenol blue and 0.025% xylene cyanol, 1X TBE) was mixed with 5 µL of the quenched reaction mixtures and heated at 65 °C for 2-5 minutes prior to loading 5 µL on denaturing 32% polyacrylamide gel containing 1X TBE and 7 M urea. Electrophoresis was performed at 90 watts. Gels were visualized by using a PhosphorImager and quantified by using ImageQuant software (Molecular Dynamics).

**Data analysis**

Data was fit by non-linear regression using the program KaleidaGraph (Synergy Software, Reading, PA). Time courses at fixed nucleotide concentration were fit to equation (2-1)

\[
[\text{product}] = A * e^{-k_{obs} * t} + C
\]  

(2-1)
where $A$ is the maximal concentration of product formed, $k_{obs}$ is the observed first-order rate constant describing product formation, $t$ is the time, and $C$ is a constant. The apparent dissociation constant ($K_{d,app}$) and maximal rate for nucleotide incorporation ($k_{pol}$) were determined using equation (2-2)

$$\frac{k_{pol} \cdot [NTP]}{K_{d,app} + [NTP]} = k_{obs}$$

(2-2)

**Kinetic simulations**

Kinetic simulations were performed by using KinTekSim version 2.03 (KinTek Corp., Austin, TX). Agreement between the experimental data and kinetic simulations was determined by visual inspection.

**VPg uridylylation assay**

VPg uridylylation reaction mixtures contained 1 $\mu$M 3Dpol, 1 $\mu$M 61-nt 2C-cre, and 5 $\mu$M VPg in reaction buffer (50 mM HEPES pH 7.5, 5 mM magnesium acetate, 10 $\mu$M UTP, and 0.04 $\mu$M $[\alpha-^{32}P]$UTP (6000 Ci/mmol)). All reactions were adjusted to a final NaCl concentration of 20 mM. 3Dpol and 3CD were diluted immediately prior to use in buffer containing 50 mM HEPES, 10 mM 2-mercaptoethanol and 20% glycerol. First, VPg were mixed with 61-nt 2C-cre RNA in 10 $\mu$L of the reaction buffer described above. This mixture was incubated at 30 $^\circ$C for 5 min prior to addition of 3CD to a final concentration of 1 $\mu$M. After addition of 3CD the reaction mixture was incubated at 30 $^\circ$C for another 5 min prior to adding 3Dpol to final concentration of 1 $\mu$M. The
uridylylation reaction was allowed to proceed for 30 min, after which 5 µL of reaction were quenched by addition of equal volume of 100 mM EDTA in a loading dye (90% formamide, 1X TBE, 0.025% BPB). Products were separated by Tris-Tricine SDS-polyacrylamide gel electrophoresis. Gels contained 15% acrylamide and 0.4% bisacrylamide. The cathode buffer (upper chamber) contained 0.1 M Tris, 0.1 M Tricine, and 0.1% (w/v) SDS; the anode buffer contained 0.2 M Tris pH 8.9. Gels were run at 80 watts. Products were visualized by using a PhosphorImager and quantified by using ImageQuant software.

**Infectious center assay**

RNA transcripts were generated from the appropriate pMoRA plasmids after linearization with *Eco*RI. Transcription reactions, typically 20 µL, consisted of 350 mM HEPES pH 7.5, 32 mM Mg Acetate, 40 mM DTT, 2 mM Spermidine, 28 mM NTPs, 0.5 µg template, and 0.5 µg T7 RNA polymerase. Reactions were incubated at 37 ºC for 3 hr followed by removal of magnesium pyrophosphate. DNase treatment with RQ1DNase (Promega) was used to remove the template; lithium chloride precipitation was used to remove unincorporated nucleotides. RNA concentration was calculated by measuring absorbance at 260 nm, assuming an A$_{260}$ of 1 was equivalent to 40 µg/ml. HeLa cells were propagated in DMEM/F-12 (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen), and 1% (v/v) penicillin and streptomycin (P/S) (Invitrogen), always keeping the cultures between 20% and 80% confluency. Subconfluent monolayers of HeLa cells were detached from the culture flasks by trypsin treatment, washed with 1X phosphate-buffered saline (PBS), and cell number adjusted to $1.2 \times 10^6$
cells/0.4 mL in PBS. Cell suspension (400 µl) was mixed with 5 µg of RNA in a microcentrifuge tube, transferred to an electroporation cuvette (0.2-cm gap width; Bio-Rad) and subjected to an electric pulse at 500 microfarads and 0.13 V using a Gene Pulser system (Bio-Rad). 400 µl of electroporated cells were plated on to appropriate wells of a 6-well plate containing a monolayer of HeLa cells plated with 5 × 10^5 cells the day before; for WT control 1/100th, 1/1000th and 1/2000th dilutions were plated. Cells were allowed to adsorb to the plate for 1 h at 37 ºC, and then the medium/PBS was aspirated; the cells were then covered with 3 mL of a mixture of 1X DMEM/F-12 plus 10% FBS, 1% P/S and 1% low melting point agarose (American Bioanalytical). Plates were then incubated at either 32 ºC or 37 ºC for 2-3 days. The agarose overlay was removed by using a spatula. Wells were stained with crystal violet, and viral plaques were counted. Infectious center assay was also employed in order to determine titer of the virus. In this case the HeLa cells monolayers were infected with an appropriate dilution of virus stock in 1X PBS, allowed to absorb for 30 min at room temperature, and then the input virus solution was aspirated and the cell monolayers were covered with 3 mL of a mixture of 1X DMEM/F-12 plus 10% FBS, 1% P/S and 1% low melting point agarose. Plates were incubated at 37 ºC for 2-3 days.

Serial passage and RT-PCR

For serial passage of Glu-297 virus 1 x 10^6 HeLa cells were electroporated with 5 µg of RNA (no RNA as a negative control), essentially as was described above for the infectious center assay. The electroporated cells were then plated on to a monolayer of HeLa cells (plated with 5 x 10^5 cells the previous day onto 6-well plates). Plates were
incubated at 37 ºC. Within 2 days cells infected with WT RNA started showing signs of cytopathic effect (CPE). In contrast, Glu-297-infected cells started showing signs of CPE only on day 4. Cells in mock control looked healthy at that time. For the first passage, 100 µl media from the transfected cell were taken on day 2 and transferred onto a fresh monolayer of HeLa cells. Cells infected with the media from WT virus died within one day and for Glu-297 within 2 days. Mock control cells did not show any signs of CPE. One hundred microliters of media from day 2 (passage 1) was again transferred onto a fresh monolayer for passage 2. The infected cells from passage 2 showed the same kinetics for CPE. Passage 3 was done in the same manner as the first two. On the second day, when both WT and Glu-297 PV infected cells were dead, 1 mL of media was collected and viral RNA was extracted with a NucleoSpin nucleic acid purification kit (Machery-Nagel) according to the manufacturer’s manual. The 3Dpol cDNA was prepared from purified viral RNA by reverse transcription with Endo-Free RT (Ambion) with oligonucleotide 4 (Table 2-1). The resulting DNA product was then PCR amplified using SuperTaq DNA polymerase (Ambion) and oligonucleotides 4 and 5 as primers. The PCR product for the N297E 3Dpol gene was sequenced with oligonucleotide 6 as primer. Codon GAA that encodes Glu-297 was mutated to GGA, encoding Gly.

However, when the serial passage experiment was performed for Arg-273 virus, in RT-PCR step MMuLV-RT (New England Biolabs) in combination with its buffer was used, instead of discontinued Endo-Free RT from Ambion. No modifications were done to the second PCR step. Slow disappearance of the Arg-273 viral RNA was determined by sequencing of the nucleic acid obtained in second PCR step with oligonucleotide 2 (Table 2-1).
**Coinfection assay**

A monolayer of HeLa cells (0.6 x 10^6) was plated in a 6-well plate one day prior to infections. The cells were infected at a multiplicity of infection (MOI) of 10 with a virus mixture of the WT and Arg-273 at 1:10 ratio. Virus was allowed to absorb for 15 minutes at room temperature. After absorption, input virus was removed and cells were covered with the fresh media (DMEM/F12, 10% FBS, 1% PS). Infections were allowed to proceed to the CPE, at which point cells and media were collected, subjected to 3 freeze-thaw cycles, and then span down at 6000 rpm in order to get rid of the cell debris (when this experiment was performed at MOI of 1 and 0.1, the infections were not allowed to proceed to CPE but instead were terminated after 8 hrs). Clarified lysate was used to inoculate fresh HeLa cells monolayer for the consecutive passage. Total of 4 passages were performed. Viral RNA was isolated from every passage with QiaAmp viral RNA purification kit (Qiagen), as recommended by the manufacturer. Viral RNA was subjected to RT-PCR and then PCR amplified, essentially as described above in Serial passage and RT-PCR section.

**One-step growth curve in suspension**

For this experiment HeLa cells were split one day prior to the experiment. On the day of the experiment HeLa cells were treated with trypsin and 2.5 x 10^5 cells per timepoint were taken and centrifuged at room temperature at 4000 rpm for 4 min, and then washed with 1 mL of phosphate-buffered saline (PBS). Cells were then resuspended in 400 µL of 1X PBS buffer containing virus (at 10 MOI). Virus was allowed to absorb
at room temperature for 30 minutes, while constantly rotating. Cells were then centrifuged (4000 rpm for 4 min), washed with 1 mL 1X PBS and then resuspended in 400 µL of the prewarmed media (DMEM/F12, 10% FBS, 1% P/S). Cells were incubated at 37 ºC, and at each timepoint the appropriate tube was frozen using dry ice and virus was released from cells by three freeze-thaw cycles. Cellular debris was spun down and clarified virus was used to determine virus titer. Virus was titered by performing infectious center assay using serial dilutions of the clarified virus stocks in 1X PBS. In brief, fresh HeLa cells monolayers (plated one day prior to the experiment, 0.6 x 10^6 cells) were infected with 100 µl of the appropriate virus dilution, extra 200 µl of 1X PBS were added to keep cells moist. After virus was allowed to absorb for 15 minutes at room temperature, virus solution was removed and cell monolayers were covered with the mixture of 1X DMEM/F12 media, 10% FBS, 1% P/S, 1% low melting agarose (American Bioanalytical). Agarose was allowed to solidify at room temperature for about 10-15 minutes and the plates were transferred to a 37 ºC incubator for 48 hours. At that time agar layers were removed and cell monolayers were stained with crystal violet solution (0.1% in 20% ethanol) and number of plaques counted.

**Guanidine resistance assay**

For this experiment 4 10-mm plates were seeded with 4 x 10^6 HeLa cells one day prior to the experiment. On the day of the experiment cells in 3 plates were infected with 10^6 PFU of either WT or Arg-273 virus. For the control plates only 50 PFU of each virus were used. Virus was allowed to absorb for 15 min at room temperature. After absorption, input virus was removed and cells were covered with 20 mL of a mixture of
1X DMEM/F-12 plus 10% FBS, 1% P/S and 1% low melting point agarose, and 3mM guanidine chloride (Sigma). For the control plates guanidine chloride was omitted. Plates were then incubated at 37 °C 3 days. The agarose overlay was removed by using a spatula. Wells were stained with crystal violet, and viral plaques were counted. The apparent error frequencies of WT and Arg-273 PV were calculated as the titer (PFU/mL) obtained on the plates containing guanidine chloride divided by the titer (PFU/mL) on plates without guanidine chloride.

**Subgenomic replicon assay**

RNA transcripts were produced from the pRLucRA plasmids after linearization with *Apa*I. RNA concentration was calculated as described above. HeLa cells (1.2 × 10⁶) were electroporated with subgenomic replicon (5 µg) essentially as described above for infectious center assay. Immediately after electroporation 400 µL of cells were mixed with 6 mL of prewarmed media (DMEM/F-12, 10% FBS, 1% P/S); 0.5 mL of this mixture were aliquotted into 1.7 mL eppendorf tube per single timepoint and transferred into either 32 °C or 37 °C incubator. At fixed timepoints, cells were pelleted by centrifugation at 14,000 rpm for 2 min. Cells were lysed in 100 µL of 1X cell culture lysis reagent (CCLR) (Promega). Lysates were left on ice until luciferase and protein concentration assays were performed at which point the lysates were centrifuged for 2 min at 14,000 rpm to remove cellular debris and nuclei. Lysates were assayed for luciferase activity by mixing 10 µL of lysate supernatant with 10 µL of luciferase assay substrate (Promega) and quantifying light production for 10 seconds by using a Junior LB
9509 luminometer (Berthold). RLU were adjusted to total protein concentration in the samples. Protein concentration was measured at 595 nm using the Bradford reagent (Bio-Rad).

**In vitro translation reactions**

HeLa cell S10 extracts were prepared by Dr. Ian Goodfellow as described previously (6). Typically, 50 µL in vitro translation reaction contained 60% by volume of HeLa S10 extract, 10% by volume 10X nucleotide mix (10 mM ATP, 2.5 mM GTP, 2.5 mM UTP, 600 mM KCH₃CO₂, 300 mM creatine phosphate, 4 mg of creatine kinasae/mL, and 155 mM HEPES pH 7.0), 8% by volume of [³⁵S]methionine (1.2 mCi/mL, Amersham), and 8% by volume rabbit reticulocyte lysate (Promega), 2 mM guanidine chloride, and 1 µg of viral RNA. Reactions were incubated at 30 °C, and at fixed timepoints 5 µL of the reaction were quenched by mixing with 30 µL of 1X SDS-PAGE sample buffer (2.5% SDS (Sigma), 112.5 mM Tris pH 6.8, 2.5% 2-mercaptoethanol, 25% glycerol, 0.025% bromophenol blue). Samples were heated at 90 °C for 10 min prior to loading on sodium dodecyl sulfate – 12.5% polyacrylamide gel, the gels were fixed and dried, radiolabeled proteins were detected by phosphorimaging.

**Stimulation of poliovirus synthesis by purified 3CD**

Stimulation of poliovirus synthesis was performed by Dr. David Franco. Viral RNA (500 ng) was translated at 34 °C in the presence of unlabeled methionine, 200 µM each CTP, GTP, UTP, and 1 mM ATP in a total volume of 25 µL. Purified 3CD protein
was added to the translation-RNA replication reactions at 400 ng/ml. After incubation for 12 to 15 h, the samples were diluted with 1X PBS and were added to fresh HeLa cell monolayers in order to determine virus titer; virus titers were determined by infectious center assay, as described above.

**Virus purification and transmission electron microscopy**

In order to obtain highly purified and concentrated viral stocks three 10 mm plates of HeLa cells were infected with WT, Arg-273 or Tyr-30 virus at MOI of 1. Upon cell death, media and cells were collected and subjected to 3 freeze-thaw cycles. Cellular debris was removed by centrifugation in Beckman JLA-16.250 rotor at 6000 rpm for 15 min. at room temperature. The pH of the supernatant was adjusted to 7 – 8, and the virus was precipitated with 8% (w/v) of polyethylene glycol (PEG 8000) (American Bioanalytical) and 0.3 M of NaCl for 16 hours at 4 ºC while constantly rotating. Precipitated virus was centrifuged at 6000 rpm for 30 min. at room temperature. The pellet was resuspended in 0.5 mL of TN buffer (50 mM Tris pH 8.0, 10mM NaCl). Two hundred fifty microliters of this suspension were then overlayed onto a 1.5 mL 30% sucrose cushion (30% sucrose, 30 mM Tris pH 8.0, 0.1 mM NaCl) and centrifuged in a Sorvall S55S-582 swinging bucket rotor at 190,000 x g for 14 hours at 4 ºC. The pellet was resuspended in 100 µL of TN buffer with 2% deoxycholic acid. Virus solution was dialyzed overnight using a 50-100 kDa cut-off membrane against 1L of TN buffer at 4 ºC. After dialysis, virus solution was subjected to the second purification step through 30% sucrose cushion. The final pellet was resuspended in 100 µL of TN buffer and used to prepare samples for transmission electron microscopy (TEM). Titer of the purified
virus was determined essentially as described above for one-step growth curve assay. The purified virus was negatively stained on either 200- or 400-mesh formvar carbon coated copper grids with 2% uranium acetate aqueous solution following standard protocols by the Penn State Electron Microscopy (EM) facility. Transmission electron microscopy images were collected at the EM facility on a JEM 1200 EXII microscope. In order to determine the fraction of empty viral particles at least 10 images for each virus were analyzed.

**Infection of susceptible mice**

All animal studies were performed by Dr. Marco Vignuzzi. Protocols for animal studies shown here were approved by the UCSF Institutional Animal Care and Use Committee (IACUC). All experiments were performed in accordance to guidelines and regulations overseen by the IACUC.

For determination of the 50% lethal dose (LD$_{50}$), 8 week old cPVR transgenic mice expressing the poliovirus receptor were inoculated intramuscularly (50 µL in each hind leg) with 10-fold serial dilutions (10-20 mice per dilution) of each virus, with doses ranging from $10^6$ to $10^9$. Mice were monitored daily for onset of paralysis and were euthanized when total paralysis was imminent. LD$_{50}$ values were determined using the Reed and Muench method. Survival curves show percentage of surviving mice over time (days of infection).

For isolation of virus from brain and feces, mice were inoculated intravenously with $10^7$ PFU of each virus. Each day following infection, 5 mice from each group were sacrificed and tissues were removed, washed, and homogenized with an Ultraturrax T8
homogenizer (IKA Works Inc., Wilmington, NC). Homogenates were clarified of cell debris and stored at -80 ºC. Tissue homogenates were then titered for virus on HeLa cells by standard infectious center assay. The average titers and standard deviation of 5 individual mice are shown.

**Purification, crystallization and structure solution of the Arg-273 3Dpol**

It was shown before that disrupting the interface I, between the front of the thumb of one polymerase molecule and the back of the palm of the other, highly increases solubility of 3Dpol and produces crystals more suitable for crystallization (7). Mutating Lys-446 and Arg-455 to aspartic acid efficiently disrupts the interface without affecting 3Dpol polymerase activity (7). Therefore, for the crystallographic study of Arg-273 3Dpol the mutation was cloned into pET26Ub-3D-BPKN-I92T-L446D-R455D expression vector essentially as described above. First, pET26Ub-3D-BPKN-I92T-L446D-R455D vector was produced by an overlap-extension PCR using oligonucleoides 19, 20 (Table 2-1) and pET26Ub-3D-BPKN-I92T-R455D as a template (2). The PCR fragment was digested with EcoRI and MfeI restriction enzymes and ligated into pET26Ub-3D-BPKN-I92T vector that has been digested with the same enzymes. Arg-273 clone was then produced essentially as described above with oligonucleotides 1, 15 and 2, 16 (Table 2-1) with pET26Ub-3D-BPKN-I92T-L446D-R455D as a template. Otherwise, Arg-273/Asp-446/Asp-455 3Dpol was expressed and purified essentially as described above, with the following modifications. First, 60% ammonium sulfate salt cut was used instead of 40% to precipitate 3Dpol. Second, after Q-Sepharose column the protein fractions were pooled and loaded onto a HiLoad 26/60 Superdex 200 column
equilibrated and eluted with buffer containing 200 mM NaCl, 5 mM Tris pH 7.5, 0.1 mM EDTA, and 2 mM DTT. The purified protein was concentrated to 10 mg/mL. Crystals were grown using hanging drop vapor diffusion at 20°C, mixed 1:1 with well solution. Crystals of Arg-273 3Dpol grew overnight over a well solution containing 2 M sodium acetate and 0.1 M sodium cacodylate pH 6.8. These crystals were transferred to a cryoprotectant containing the crystallization solution with 20% glycerol and were flash frozen in a nitrogen stream. Diffraction data were collected at the 19-ID beamline at the Advanced Photon Source (Argonne, IL). The data were integrated, merged, and scaled using DENZO and SCALEPACK (8). The structures were solved by molecular replacement using the program PHASER (9). The search model was the structure of PV-3Dpol (PDB code 1RA6) with solvent and key residues omitted (7). The model was built using Coot and refined with REFMAC5 (10,11).
References

Chapter 3

Structure-Function Relationships of the Viral RNA-dependent RNA Polymerase: Fidelity, Replication Speed and Initiation Mechanism Determined by a Residue in the Ribose-Binding Pocket

Summary

Studies of the RNA-dependent RNA polymerase (RdRp) from poliovirus (PV), 3Dpol, have shown that Asn-297 permits this enzyme to distinguish ribose from 2’-deoxyribose. All animal RNA viruses have Asn at the structurally homologous position of their polymerases, suggesting a conserved function for this residue. However, all prokaryotic RNA viruses have Glu at this position. In the presence of Mg$^{2+}$, the apparent affinity of Glu-297 3Dpol for 2’-deoxyribonucleotides was decreased by six fold relative to wild type without a substantial difference in the fidelity of 2’-dNMP incorporation. The fidelity of ribonucleotide misincorporation for Glu-297 3Dpol was reduced by 14 fold relative to wild type. A 4- to 11-fold reduction in the rate of ribonucleotide incorporation was observed. Glu-297 PV was unable to grow in HeLa cells due to a replication defect equivalent to that observed for a mutant PV encoding an inactive polymerase. Evaluation of the protein-(VPg)-primed initiation reaction showed that only half of the Glu-297 3Dpol initiation complexes were capable of producing VPg-pUpU product and that the overall yield of uridylylated VPg products were reduced by 20-fold relative to wild-type enzyme, a circumstance attributable to a reduced affinity for UTP. These studies identify the first RdRp derivative with a mutator phenotype and provide a
mechanistic basis for the elevated mutation frequency of RNA phage relative to animal RNA viruses observed in culture. Although protein-primed initiation and RNA-primed elongation complexes employ the same polymerase active site, the functional differences reported here imply significant structural differences between these complexes.

Introduction

RNA viruses cause a variety of acute and chronic diseases in humans: common cold, summer flu, hepatitis, severe acute respiratory syndrome and liver cancer, to name a few (1-5). The genomes of these viruses are transcribed and replicated by a virus-encoded RNA-dependent RNA polymerase (RdRp) (4,6,7). Like other viral polymerases, the RdRp represents an important target for antiviral drug development (8-11). The RdRp from poliovirus (PV), 3Dpol, has emerged as an important model for understanding the structure, function and mechanism of this class of nucleic acid polymerases (12-15).

PV 3Dpol will incorporate a ribonucleotide (rNMP) with an incorrect base at a frequency of $\sim 10^{-7}$ to $10^{-4}$ (16,17). However, this enzyme is much more tolerant of nucleotides with an incorrect sugar configuration. Both 2’- and 3’-deoxynucleotides (dNMPs) are incorporated at a frequency of $\sim 10^{-2}$ (15). It is known that incorporation of more than one incorrect ribonucleotide per PV genome decreases the specific infectivity of the RNA (16,18). Whether or not 2’-dNMP incorporation has an effect on viral RNA infectivity is not known.
Several factors likely limit 2’-dNMP incorporation into the genomes of RNA viruses of eukaryotes. First, dNTP pools in cells are thought to be low, in the 5-30 µM range (19). Low dNTP levels are maintained by regulating the activity and localization of ribonucleotide reductase (RNR) (20,21). In the G1-phase of the cell cycle, very little RNR holoenzyme exists due to the localization of one subunit in the nucleus and the other in the cytoplasm. During the S-phase of the cell cycle, the RNR holoenzyme is distributed in both cytoplasm and nucleus; allosteric control mechanisms would likely permit only the nuclear enzyme to function catalytically (20-22). If nuclear localization of active RNR prevents eukaryotic RNA virus polymerases from having to select against utilization of 2’-dNTPs, then prokaryotic RNA virus polymerases may require a more stringent selection mechanism.

Asn-297 is the primary determinant of ribose specificity in PV 3Dpol (14). This residue is located in the ribose-binding pocket and hydrogen bonds to the ribose 2’-OH (Fig. 3-1A). The capacity for this derivative to distinguish between ATP and 2’-dATP incorporation is reduced by 10 fold relative to wild-type 3Dpol (14). Asn is present at the structurally homologous position in all plant and animal RNA virus polymerases (Fig. 3-1B), suggesting that this residue functions in ribose selection for these enzymes as well. Curiously, RNA phage polymerases have a Glu at this position (Fig. 3-1B), consistent with a difference in ribose/2’-deoxyribose selection for these enzymes.

We show that Glu-297 3Dpol does not have a more stringent selection against 2’-dNMP incorporation than wild-type (WT) 3Dpol, although the affinity for 2’-dNTPs is reduced relative to WT 3Dpol. However, nucleotide incorporation by Glu-297 3Dpol was slower and less faithful than WT 3Dpol. Glu-297 3Dpol represents the first RdRp
derivative with a mutator phenotype. Glu-297 PV was inviable due to an RNA synthesis defect in cells that was on par with that of a catalytically inactive 3Dpol derivative. This phenotype could be explained by a severe reduction in the yield of product produced by protein-primed initiation of RNA synthesis. We conclude that a requirement for decreased replication speed and/or incorporation fidelity imposes a constraint on the evolution of the polymerase active site that restricts the variety of initiation mechanisms that can be employed. Thus phage polymerases employ strictly a de novo mechanism for initiation, whereas animal RNA viruses can utilize de novo and protein-primed mechanisms for initiation (Fig. 3-1B). The capacity of a single 3Dpol derivative to exhibit significant differences between RNA- and protein-primed RNA synthesis suggests that the structural organization of the corresponding elongation complexes is different. We propose that inhibitors specific for the PV 3Dpol initiation complex may be more efficient than those targeting the elongation complex.
A

Asp-233
ATP
Thr-293 Gly-289
Asn-297
Asp-238
Ser-288
Cys-290

B

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host</th>
<th>5'-end of RNA</th>
<th>Motif A</th>
<th>Motif B</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV</td>
<td>A</td>
<td>VPg</td>
<td>DYTGY-D</td>
<td>SGCSGTSIN</td>
</tr>
<tr>
<td>HRV14</td>
<td>A</td>
<td>VPg</td>
<td>DYSNF-D</td>
<td>SGCSGTSFIN</td>
</tr>
<tr>
<td>CVB3</td>
<td>A</td>
<td>VPg</td>
<td>DYSGY-D</td>
<td>SGCSGTSIFN</td>
</tr>
<tr>
<td>FMDV</td>
<td>A</td>
<td>VPg</td>
<td>DYSAF-D</td>
<td>SGCSATIIIN</td>
</tr>
<tr>
<td>HAV</td>
<td>A</td>
<td>VPg</td>
<td>DFSAF-D</td>
<td>SGSPCATALLN</td>
</tr>
<tr>
<td>RHDV</td>
<td>A</td>
<td>VPg</td>
<td>DYSKW-D</td>
<td>SGMPFTSVIN</td>
</tr>
<tr>
<td>MHV</td>
<td>A</td>
<td>Cap</td>
<td>DYPKC-D</td>
<td>SGDATTAFAN</td>
</tr>
<tr>
<td>BEV</td>
<td>A</td>
<td>Cap</td>
<td>DYTKC-D</td>
<td>SGDATTAHSN</td>
</tr>
<tr>
<td>FCV</td>
<td>A</td>
<td>VPg</td>
<td>DYSKW-D</td>
<td>SGMPLTSVIN</td>
</tr>
<tr>
<td>CPMV</td>
<td>P</td>
<td>VPg</td>
<td>DYSKF-D</td>
<td>SGFPMTVIVN</td>
</tr>
<tr>
<td>BWVV</td>
<td>P</td>
<td>VPg</td>
<td>DCSSF-D</td>
<td>SGSYNTSSSN</td>
</tr>
<tr>
<td>SMBV</td>
<td>P</td>
<td>VPg</td>
<td>DSIFG-D</td>
<td>SGSCSTSTSN</td>
</tr>
<tr>
<td>BVDV</td>
<td>A</td>
<td>ppp?</td>
<td>DTKAW-D</td>
<td>SGQPDTSAGN</td>
</tr>
<tr>
<td>YFV</td>
<td>A</td>
<td>Cap</td>
<td>DTACW-D</td>
<td>SGQVVTYALN</td>
</tr>
<tr>
<td>CarMV</td>
<td>P</td>
<td>Cap</td>
<td>DMSRF-D</td>
<td>SCDMTNLGN</td>
</tr>
<tr>
<td>MS2</td>
<td>B</td>
<td>ppp</td>
<td>DLSSASD</td>
<td>MCNGFTFELE</td>
</tr>
<tr>
<td>GA</td>
<td>B</td>
<td>ppp?</td>
<td>DLSSASD</td>
<td>MCNGFTFELE</td>
</tr>
<tr>
<td>QBETA</td>
<td>B</td>
<td>ppp</td>
<td>DLSSASD</td>
<td>MCNGFTFELE</td>
</tr>
<tr>
<td>SP</td>
<td>B</td>
<td>ppp?</td>
<td>DLSSASD</td>
<td>MCNGFTFELE</td>
</tr>
<tr>
<td>HCV</td>
<td>A</td>
<td>ppp</td>
<td>DTRCY-D</td>
<td>SGVLDTSICG</td>
</tr>
<tr>
<td>HEV</td>
<td>A</td>
<td>Cap</td>
<td>DFSEF-D</td>
<td>SGEPGLTLWN</td>
</tr>
<tr>
<td>RuV</td>
<td>A</td>
<td>Cap</td>
<td>DFTEF-D</td>
<td>SGEPATLLHN</td>
</tr>
<tr>
<td>BSMV</td>
<td>P</td>
<td>Cap</td>
<td>DFSKF-D</td>
<td>SGNCSTYGSN</td>
</tr>
<tr>
<td>PVX</td>
<td>P</td>
<td>Cap</td>
<td>DYTAF-D</td>
<td>TGEAPTPFAN</td>
</tr>
</tbody>
</table>
Fig. 3-1: Differences in ribose/2'-deoxyribose specificity for RNA phage polymerases relative to animal and plant RNA virus polymerases predicted from structure/sequence comparison. **(A) Structural model for the ribose-binding pocket of the RdRp from PV, 3Dpol (14).** The 3Dpol ribonucleotide-binding pocket is shown with bound ATP. This structural model is based on homology modeling because a crystal structure for this complex is still not available for any RdRp (14). This pocket is composed of residues from conserved structural motifs A (Asp-233 to Asp-238) and B (Ser-288 to Asn-297). The extensive hydrogen bonding network shown links the position of the triphosphate (catalytic efficiency) to the nature of the bound nucleotide - that is, whether or not a correct base and sugar configuration are present. Asn-297 permits the enzyme to distinguish between ribose and 2'-deoxy ribose by hydrogen bonding to the 2'-OH group of ribose. A 3Dpol derivative containing Ala at this position instead of Asn fails to distinguish rNTPs from 2'-dNTP as well as wild-type 3Dpol (14). The image was created by using the program WebLab Viewer (Molecular Simulations Ins., San Diego, CA). **(B) Conserved Asn in animal and plant RNA virus polymerases is a Glu in RNA phage polymerases.** RNA virus polymerases can be organized into three supergroups: I, II and III. Representative viruses, their host (A: animal; P: plant; B: bacteria) and mechanism of initiation of RNA synthesis based on the structure of the 5'-end of the genome (VPg: protein primed; Cap and ppp: de novo) are indicated. In those instances in which the 5'-end has not been analyzed directly, the structure of the 5'-end appears with a question mark and represents the most logical structure based on available biochemical data or relationship to other viruses. Residues 233 and 238 (3Dpol numbering) of conserved structural motif A are conserved in all animal and plant RNA virus polymerases. Residues 288, 290, 293 and 297 (3Dpol numbering) of conserved structural motif B are conserved in all animal and plant RNA virus polymerases. Variability exists for the RNA phage polymerases at positions 288 and 297. Abbreviations used are: PV - poliovirus, HRV14 – human rhinovirus 14, CVB3 – coxsackie virus B3, FMDV – foot-and-mouth disease virus, HAV - hepatitis A virus, RHDV – rabbit hemorrhagic disease virus, MHV – mouse hepatitis virus, BEV - Berne virus, FCV – feline calici virus, CPMV - cowpea mosaic virus, BWYV - beet western yellow virus, SBMV - southern bean mosaic virus, BVDV – bovine viral diarrhea virus, YFV - yellow fever virus, CarMV - carnation mottle virus, bacteriophages MS2, GA, SP, QBETA, HCV - hepatitis C virus, HEV - hepatitis E virus, RuV - rubella virus, BSMV - barley stripe mosaic virus, PVX - potato virus X. (adapted from (51, 52)).
Results

Rationale

Previous studies identified Asn-297 as a key player in ribonucleotide selection by the RdRp from PV, 3Dpol. Asn-297 hydrogen bonds to the 2’-hydroxyl of the incoming ribonucleotide (rNTP) (Fig. 3-1A). Changing Asn-297 to Ala prevented 3Dpol from distinguishing an rNTP with correct basepairing capacity from a 2’-dNTP with correct basepairing capacity, a lethal change for PV (Table 3-1) (14). Asn-297 is conserved in all plant and animal RNA virus polymerases; however, this residue is a Glu in RNA phage polymerases (Fig. 3-1B). This study was initiated to address the possibility that the observed difference in the ribose-binding pocket between eukaryotic and prokaryotic RNA viruses may reflect the need for different levels of rNTP/dNTP selection in the different cellular environments.

Glu-297 3Dpol Incorporates 2’-deoxyribonucleotides as Efficiently as WT 3Dpol

3Dpol-coding sequence was changed to encode a glutamic acid at position 297 (Glu-297 3Dpol). This derivative was purified to >90% purity exactly as described for WT 3Dpol, suggesting no significant change in the structure of the enzyme (23). In addition, this derivative retained at least half of the poly(rU) polymerase activity of WT 3Dpol (Table 3-2).
Table 3-1: Glu-297 3Dpol Incorporates 2’-deoxyribonucleotides as Efficiently as WT 3Dpol

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Nucleoside</th>
<th>$K_{d,app}$ $\mu$M</th>
<th>$k_{pol}$ s$^{-1}$</th>
<th>$k_{pol}/K_{d,app}$ s$^{-1}/\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>GCAUGGGCCC</td>
<td>ATP</td>
<td>130 ± 20</td>
<td>90 ± 10</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>sym/sub-U</td>
<td></td>
<td>530 ± 120</td>
<td>8 ± 1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>CCCGGGUAACG</td>
<td></td>
<td>180 ± 30</td>
<td>5 ± 1</td>
<td>0.03</td>
</tr>
<tr>
<td>Glu-297</td>
<td>dATP</td>
<td></td>
<td>280 ± 60</td>
<td>0.8 ± 0.1</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>sym/sub-A</td>
<td></td>
<td>1700 ± 400</td>
<td>0.4 ± 0.02</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>GCUAGGGCCC</td>
<td></td>
<td>260 ± 30</td>
<td>0.2 ± 0.05</td>
<td>0.0008</td>
</tr>
<tr>
<td>Ala-297$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT$^a$</td>
<td>GTP</td>
<td></td>
<td>210 ± 70</td>
<td>0.01 ± 0.001</td>
<td>0.00005</td>
</tr>
<tr>
<td></td>
<td>sym/sub-A</td>
<td></td>
<td>290 ± 90</td>
<td>0.005 ± 0.001</td>
<td>0.00002</td>
</tr>
<tr>
<td>Glu-297</td>
<td>UTP</td>
<td></td>
<td>100 ± 10</td>
<td>270 ± 20</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>sym/sub-A</td>
<td></td>
<td>380 ± 80</td>
<td>70 ± 6</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>GCUAGGGCCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT$^a$</td>
<td>dUTP</td>
<td></td>
<td>190 ± 40</td>
<td>6 ± 1</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>sym/sub-A</td>
<td></td>
<td>1100 ± 200</td>
<td>2 ± 0.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Glu-297</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Values are taken from (26).
$^b$ Values are taken from (14).
Table 3-2: Activity of Glu-297 3Dpol was not Enhanced by Introduction of Residues Conserved in Phage RdRps

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/µg</td>
</tr>
<tr>
<td>WT</td>
<td>800</td>
</tr>
<tr>
<td>WT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>400</td>
</tr>
<tr>
<td>Glu-297</td>
<td>300</td>
</tr>
<tr>
<td>Met-288, Glu-297&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Asn-290, Glu-297&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Met-288, Asn-290, Glu-297&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> This enzyme was only purified to the ammonium sulfate precipitation step.

Poly r(U) polymerase activity was measured in purified or partially purified preparations of the indicated 3Dpol derivatives as described under Experimental Procedures. The specific activity is reported to one significant figure; the standard error of the measurement was less than 20%.
In order to evaluate the kinetics and mechanism of 3Dpol, we use a 10-nt symmetrical RNA substrate referred to as sym/sub (S/S). S/S-U, for example, has uracil as the first templating base (Fig. 3-2A) (12). 3Dpol binds tightly to the duplex portion of this substrate and can utilize either 3’-end as a primer for nucleotide addition. The rate constant for dissociation of Glu-297 3Dpol was 0.0001 s\(^{-1}\), corresponding to a half-life for the complex of ~2 h as observed for WT 3Dpol (12).

We evaluated the dependence of the rate constant for AMP incorporation on ATP concentration. For this experiment, 2 \(\mu\)M 3Dpol was first incubated with 2 \(\mu\)M 5’-\(^{32}\)P-labeled S/S-U (1 \(\mu\)M duplex) in reaction buffer in order to establish the enzyme-RNA complex. The assembled complex was then rapidly mixed with ATP of the appropriate concentration in reaction buffer, as described under Experimental Procedures. Mixing resulted in a 2-fold dilution. The reaction was stopped at various times by addition of EDTA to 0.3 M final concentration. The product, 11-mer RNA, was separated from the substrate, 10-mer RNA, by electrophoresis through a denaturing, 23% polyacrylamide gel. Substrate and product were visualized by using a phosphoroimager and quantitated by using the ImageQuant software (Molecular Dynamics) (Fig. 3-2B).

The concentration of 11-nt product formed was plotted as a function of time and fit to a single exponential equation (eq. 2-1), yielding the observed rate constant for AMP incorporation templated by U (Fig. 3-2C). The observed rate constants were then plotted as a function of ATP concentration, and the data were fit to a hyperbola (eq. 2-2), yielding an apparent dissociation constant for ATP, \(K_{d,app}\), of 530 ± 120 \(\mu\)M, and a maximal rate constant for AMP incorporation, \(k_{pol}\), of 8 ± 1 s\(^{-1}\) (Fig. 3-2D, Table 3-1).
A

\[ \begin{align*}
5' & \text{GCAUGG GCCC}^{3'} \\
3' & \text{CCCGGGUACG} \text{ 5'}
\end{align*} \]

B

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>0</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-mer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-mer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

[11-mer RNA (\text{\textmu}{\text{M}})] vs Time (sec)

D

[\text{ATP}] (\text{\textmu}{\text{M}}) vs \text{k}_{\text{obs}} (\text{s}^{-1})
Fig. 3-2: Kinetic analysis of Glu-297 3Dpol. (A) RNA primer-template substrate employed. A 10-nt self-complementary RNA referred to as sym/sub (S/S). Shown is S/S-U. The “U” indicates that uracil is the templating base. (B) Product analysis. In this experiment, 2 µM Glu-297 3Dpol was incubated with 2 µM sym/sub (1 µM duplex) and rapidly mixed with 200 µM ATP. Reactions were quenched at various times up to 1 sec by addition of EDTA. Reaction products were resolved by electrophoresis on a denaturing 23% polyacrylamide gel and visualized by using a PhosphorImager (Molecular Dynamics). (C) Concentration dependence of the kinetics of nucleotide incorporation. Experiments were performed as described above in the presence of: 50 (●), 100 (○), 250 (■), 500 (□), 1000 (▲) or 2000 µM (△) ATP. The solid line represents the fit of the data to a single exponential (eq. 2-1), yielding $k_{obs}$ values of: 0.6 ± 0.03, 1.3 ± 0.1, 2.3 ± 0.15, 3.53 ± 0.3, 5.6 ± 0.5, 5.7 ± 0.4 s$^{-1}$, for 50-2000 µM ATP, respectively. (D) Kinetic constants for AMP incorporation by Glu-297 3Dpol. Values for $k_{obs}$ were plotted as a function of ATP concentration. The solid line represents the fit of data to a hyperbola (eq. 2-2), yielding values for $K_{d,app}$ for ATP of 530 ± 120 µM and $k_{pol}$ of 8 ± 1 s$^{-1}$. 
The ability to fit the data to a hyperbola is in good agreement with the minimal kinetic mechanism proposed for WT 3Dpol (Fig. 3-3) (26). The \( K_{d,\text{app}} \) value for ATP was increased by 4-fold and the maximal rate constant for AMP incorporation by Glu-297 3Dpol was reduced by 11-fold relative to WT 3Dpol (Table 3-1).

Next, we evaluated the kinetics of dAMP incorporation for Glu-297 3Dpol. The \( K_{d,\text{app}} \) and \( k_{\text{pol}} \) values for dAMP incorporation into S/S-U were 1700 ± 400 \( \mu \text{M} \) and 0.4 ± 0.02 s\(^{-1}\), respectively (Table 3-1). Relative to WT 3Dpol, these values reflect a 6-fold increase in the \( K_{d,\text{app}} \) value for dATP and a 2-fold decrease in the \( k_{\text{pol}} \) value for dAMP incorporation. These data are consistent with Glu-297 3Dpol selecting against dAMP incorporation better than WT 3Dpol (Table 3-1). In order to show that this observation was not unique to dAMP incorporation, we evaluated dUMP incorporation into S/S-A. For Glu-297 3Dpol, the \( K_{d,\text{app}} \) value for dUTP increased by 6-fold and the \( k_{\text{pol}} \) value decreased by 3-fold relative to WT 3Dpol (Table 3-1), consistent again with this derivative selecting against dNMP incorporation better than WT 3Dpol. If we define the overall efficiency of nucleotide incorporation as \( k_{\text{pol}}/K_{d,\text{app}} \), then the efficiency of dNMP incorporation relative to the efficiency of AMP incorporation for Glu-297 3Dpol was essentially the same as WT 3Dpol (Table 3-1).

Glu-297 3Dpol exhibited a 14- to 35-fold reduction in the efficiency of rNMP incorporation relative to WT 3Dpol (Table 3-1). Unexpectedly, the fidelity of base selection (GMP incorporation into S/S-U) by Glu-297 3Dpol was decreased by 10-fold relative to WT 3Dpol. This is the first RdRp derivative with a mutator phenotype.
Fig. 3-3: Minimal kinetic mechanism for 3Dpol-catalyzed nucleotide incorporation. ERₙ (3Dpol–sym/sub substrate complex); NTP (nucleotide); ERₙNTP (catalytically competent ternary complex); ERₙ₊₁ (3Dpol-sym/sub product complex).
**Glu-297 PV is Quasi-Infectious**

The rate constant for rNMP incorporation by Glu-297 3Dpol was decreased by 4- to 11-fold relative to WT 3Dpol. Therefore, this mutant virus should reveal the impact, if any, of replication speed and fidelity on virus multiplication and fitness. Glu-297-encoding changes were engineered into the infectious cDNA for PV. RNA was produced by *in vitro* transcription and the infectivity of the RNA evaluated by infectious center assay at both 37 °C and 32 °C (14). Glu-297 PV RNA did not produce virus at either temperature (Fig. 3-4A). Under the same conditions, WT PV RNA was able to establish a productive infection (Fig. 3-4A).

If the change of Asn-297 to Glu is indeed lethal, then viable virus will never be isolated. However, if this change permits some low level of replication (quasi-infectious), then viable virus may be recovered after serial passage. In this experiment, HeLa cells were electroporated with Glu-297 PV RNA and plated onto a fresh monolayer of HeLa cells. Plates were incubated at 37 °C. Every two days, a fraction of the media was used to infect a fresh monolayer. This process was repeated reiteratively until the monolayer was killed in two days. This took three serial passages. The 3Dpol gene from this virus was amplified by using RT-PCR and sequenced. A single nucleotide substitution was observed that converted the GAA codon encoding Glu-297 to a GGA codon encoding Gly, a virus isolated by us previously (Fig. 3-4B) (27). Together, these data permit us to conclude that Glu-297 PV is quasi-infectious.
**Fig. 3-4: Glu-297 PV is quasi-infectious.** (A) PV genomic RNA encoding Glu-297 3Dpol is not infectious. An infectious center assay (see Experimental Procedures) was performed to evaluate the infectivity of WT or Glu-297 PV RNA after electroporation into HeLa cells. Virus was not recovered from cells containing Glu-297 PV at 37 ºC or 32 ºC. **(B) Isolation of Gly-297 PV as a pseudo-revertant of Glu-297.** After transfection of Glu-297 PV RNA into HeLa cells, a fraction of the media was transferred to a fresh monolayer every two days for six days at which point cytopathic effect was observed. Therefore, Glu-297 PV is quasi-infectious. Shown is the electropherogram and sequence for cDNA prepared from viral RNA by using RT-PCR. The underlined codon is for position 297 and encodes a Gly. The original sequence was GAA.
**Glu-297 PV is Severely Impaired for RNA Synthesis in Cells**

The polymerase domain (3D) has functions in the context of precursors that are unrelated to RNA elongation. For example, the 3CD precursor is a protease that processes the polyprotein into “mature” forms required for virus multiplication (7,24,28,29). 3CD is an RNA-binding protein that binds to the cis-acting element required for initiation of genome replication, possibly recruiting 3Dpol to this location (30-32). Finally, 3CD appears to function after RNA synthesis as well in a step related to virion maturation that does not require 3CD protease activity (33). In order to determine to what extent RNA synthesis was attenuated for Glu-297 PV, mutations encoding this change were engineered into the PV subgenomic replicon. This replicon consists of a PV genome in which the capsid-coding sequence has been replaced by a luciferase reporter gene (Fig. 3-5A), permitting RNA synthesis to be monitored indirectly by measuring luciferase activity (14).

WT and Glu-297 PV replicons were evaluated at 37 °C and 32 °C (Fig. 3-5B and data not shown). Exponential replication of the WT PV replicon was observed for 5 h after transfection, leading to greater than $10^5$-fold increase in luciferase activity. In contrast, only a 2-log increase in luciferase activity was observed for the Glu-297 PV replicon. This level of luciferase activity was also observed for the GAA PV replicon that encodes a polymerase completely lacking polymerase activity due to a change in the active site GDD motif to GAA (34,35). Luciferase activity observed for the GAA PV replicon derives from translation of the transfected RNA. Such a substantial replication defect was not expected for Glu-297 PV given the significant polymerase activity...
Fig. 3-5: Replication of Glu-297 PV in cells is as debilitated as a PV encoding a catalytically inactive polymerase. (A) PV subgenomic replicon (14). This replicon has the capsid-coding sequence replaced by a luciferase reporter gene. After transfection of the RNA into HeLa cells, translation of the open reading frame produces a luciferase-P2-P3 polyprotein from which luciferase is released by the 2A protease activity encoded by the P2 region of the genome. In the absence of replication, translation occurs, yielding a 1-2 log increase in luciferase activity. Mutations which affect translation or RNA stability would be scored in this manner. Replication is monitored indirectly by luciferase activity that accumulates to levels higher than observed for translation. (B) Glu-297 PV replicon translates but does not replicate. In this experiment the Glu-297 (●) PV replicon was compared to WT (■) and GAA (○) PV replicons at 37 °C. The GAA replicon encodes an inactive polymerase and serves as a control for translation and RNA stability in the absence of RNA synthesis. This experiment was performed three times; a representative experiment is shown.
associated with this Glu-297 3Dpol (Tables 3-1 and 3-2).

**The Product Yield and Processivity of VPg-primed RNA Synthesis by Glu-297 3Dpol is Severely Impaired**

Another aspect of PV genome replication in which 3Dpol plays a crucial role is VPg uridylylation. VPg is a 22 amino acid-long peptide that is found covalently attached to the 5’-end of PV RNA. VPg or some precursor thereof is used to prime RNA synthesis (35-38). Uridylylation of VPg leads to production of VPg-pUpU, which serves as a primer for viral RNA synthesis (31,36,39). This reaction is catalyzed by 3Dpol, templated by an adenylate residue in the 2C-cre (cis-replicating element) by using a slide-back mechanism and is stimulated by protein 3CD (31,40,41). This reaction can be mimicked in vitro as shown in Fig. 3-7.

For WT 3Dpol, both VPg-pU and VPg-pUpU products are observed at UTP concentrations below 5 µM. Above 5 µM UTP, however, VPg-pUpU is the primary product (Fig. 3-7A). For Glu-297 3Dpol, VPg-pU is the primary product at low UTP concentrations and both VPg-pU and VPg-pUpU are still equimolar at concentrations of UTP as high as 300 µM (Fig. 3-7B). Quantitative evaluation of the UTP concentration dependence of UMP incorporation into VPg showed that the maximal value of UMP incorporated by Glu-297 3Dpol was reduced by approximately half relative to WT 3Dpol (Fig. 3-7C and D). Moreover, the concentration of UTP required for half maximal UMP incorporation ($K_{0.5}$) by Glu-297 3Dpol was increased by 14-fold relative to WT 3Dpol (Fig. 3-7C and D). These data suggest that the capacity for the Glu-297 3Dpol uridylylation complex to bind UTP is compromised significantly relative to WT 3Dpol.
Fig. 3-6: VPg uridylylation reaction. Incorporation of two UMP residues into VPg by 3Dpol is templated by a single AMP residue in the 2C-cre RNA using a slide-back mechanism (41). The reaction is capable of processive synthesis of VPg-pUpU but under sub-optimal conditions VPg-pU also accumulates (32, 42). The reaction is stimulated by 3CD, presumably as a recruitment/retention factor for 3Dpol (32, 36, 42).
while the incorporation rate of Glu-297 3Dpol uridylylation complex was much less affected. These observations are different than those made for the Glu-297 3Dpol RNA elongation complex where incorporation rate constant \( (k_{pol}) \) was affected more than UTP binding \( (K_{d,app}) \) (Table 3-1).

The 5’-ends of PV plus- and minus-strands contain VPg-pUpU, likely the result VPg-pUpU produced at the 2C-cre being used as the primer for extension from the 3’-end of PV plus- and minus-strand RNAs (36,37,39,42). While WT 3Dpol produced as much VPg-pUpU product at saturating concentrations of UTP (Fig. 3-7E), Glu-297 3Dpol produced half as much VPg-pUpU product, even at apparently saturating concentrations of UTP (Fig. 3-7F). Moreover, the \( K_{0.5} \) value for UTP for production of VPg-pUpU by Glu-297 3Dpol was still 7-fold higher than that observed for WT 3Dpol. We conclude that both the overall product yield and the processivity of the Glu-297 3Dpol uridylylation complex is reduced relative to WT 3Dpol and that, when coupled with the reduced elongation rate of Glu-297 3Dpol, provides a biochemical explanation for the severe RNA synthesis defect observed biologically.
The finding that processive VPg-primed RNA synthesis was reduced for Glu-297 3Dpol suggested the possibility that processive RNA-primed RNA synthesis might also be reduced, contributing to the lack of RNA synthesis observed in cells. Processive RNA synthesis was evaluated by using S/S-U, the same RNA substrate utilized for single-nucleotide-addition experiments, in the presence of all four ribonucleotides (125 µM each). Upon rapid mixing of Glu-297 3Dpol-S/S-U complex with all four nucleotides, formation of 11-, 12-, 13- and 14-mer RNA products were observed (Fig. 3-8). The net rate constants for sequential incorporation of all four nucleotides were comparable and no
strong stops (Fig. 3-8A) or long lags (inset to Fig. 3-8B) were observed. We conclude that processivity of RNA-primed RNA synthesis by Glu-297 3Dpol is normal. This result was expected because the rate constant for dissociation of 3Dpol from S/S-U is less than 0.0001 s\(^{-1}\) (12); therefore, a 35-fold reduction in incorporation efficiency would not be observed.

**Efficient and Processive Uridylylation of VPg by Gly-297 3Dpol**

Serial passage of Glu-297 PV uncovered a pseudo-revertant virus, Gly-297 PV, that we isolated previously (27). This virus exhibits delayed growth kinetics but reaches titers within one log of wild type (27). Interestingly, growth of this virus could be stimulated at least 5-fold by growth in the presence of Mn\(^{2+}\) (27). RNA-primed RNA synthesis by Gly-297 3Dpol was similar to WT 3Dpol; VPg-primed RNA synthesis was not evaluated (27). If the reduced yield of uridylylated VPg products and/or processivity of VPg uridylylation by Glu-297 3Dpol was indeed the reason that Glu-297 PV did not replicate, then Gly-297 3Dpol should exhibit enhanced production of uridylylated VPg products, processivity or both. Indeed, VPg uridylylation by Gly-297 3Dpol was essentially indistinguishable from WT 3Dpol (Fig. 3-9).
Fig. 3-8: Processive RNA-primed RNA synthesis by Glu-297 3Dpol is normal. Nucleotide-addition experiments were performed as described in the legend to Fig. 3-2 in the presence of all four nucleotides. The phosphorimage of the gel (A) and quantification thereof (B) are shown. The kinetics of formation and disappearance of 11-mer (○), 12-mer (□), 13-mer (●), and 14-mer (■) were simulated (solid lines) to net rate constants of 1 s⁻¹, 4 s⁻¹, 7 s⁻¹, and 2 s⁻¹ for formation of each of the individual products. The inset is an enlargement of the data between 0 and 2.5 s.
Attempts to Rescue Glu-297 3Dpol RNA-primed Elongation Rate by Rational Design

The initial observation that the rate constant for AMP incorporation into S/S-U by Glu-297 3Dpol was 10-fold slower than WT 3Dpol was first interpreted to mean that the context into which Glu-297 was placed was suboptimal. Structure/sequence comparisons identified two other changes in the ribose-binding pocket between RdRps from RNA viruses of eukaryotes and prokaryotes. These changes were at positions 288 and 290 using the PV 3Dpol numbering (Fig. 3-1). We changed Ser-288 to methionine and Cys-290 to asparagine individually and in combination in the context of Glu-297 3Dpol. None of these derivatives rescued the activity of Glu-297 3Dpol as all changes yielded derivatives that retained less than 3% of the poly(rU) polymerase activity observed for WT 3Dpol (Table 3-2).
Fig. 3-9: VPg-primed RNA synthesis by Gly-297 3Dpol is normal. The yield of uridylylated VPg products and processivity of VPg uridylylation by Gly-297 3Dpol was compared to that of the WT and Glu-297 enzymes as described in the legend to Fig. 3-7. Gly-297 3Dpol uridylylation activity was qualitatively and quantitatively indistinguishable from WT 3Dpol. The standard error on the values for processivity was no greater than 10%.
Discussion

Previous studies of the RdRp from PV, 3Dpol, established a role for Asn-297 in ribonucleotide selection (14). By changing Asn-297 to Ala, a 3Dpol derivative was created that was incapable of distinguishing an rNTP with correct basepairing capacity from a 2’-dNTP with the same capacity (14). A structural model for the 3Dpol elongation complex has been developed based on homology modeling because a crystal structure for this complex is still not available (14). Many of the features of this model are consistent with biochemical data, in particular a hydrogen bonding interaction between Asn-297 and the 2’-hydroxyl of the rNTP (Fig. 3-1A) (14). Interestingly, PV containing substitutions at position 297 of 3Dpol, including Ala, Gln and Asp, were all inviable, despite the fact that all of these 3Dpol derivatives exhibited demonstrable levels of RNA-primed elongation activity (14). Consistent with the essential nature of this residue was the finding that all animal and plant RNA virus polymerases have an Asn at this position (Fig. 3-1B). Therefore, it was quite surprising to find that RNA phage polymerases have a Glu at this position (Fig. 3-1B).

We hypothesized that the cytosolic availability of 2’-dNTPs in prokaryotes may account for this difference. Perhaps a Glu at this position would confer upon the polymerase the capacity to be more stringent in its selection against 2’-dNTP utilization and perhaps even permit the virus to grow. A Glu-297 3Dpol was indeed active, and a decrease in 2’-dNTP affinity was noted. However a decrease in efficiency of 2’-dNMP incorporation relative to WT 3Dpol was not observed (Table 3-1). Glu-297 3Dpol
exhibited two differences relative to WT 3Dpol in the RNA-primed synthesis of RNA. The observed rate constant for correct rNMP incorporation was decreased by 4 to 11 fold to the 10-100 s⁻¹ regime (Table 3-1). The efficiency of GMP misincorporation was increased by 14 fold, corresponding to a mutation frequency of 1 X 10⁻³.

Recent studies of the kinetics of nucleotide incorporation by the phage Qβ RdRp have shown that the average rate constant for nucleotide incorporation by this enzyme is ~10 s⁻¹ (43), with projected maximal rates being on the order of 30 s⁻¹ (Kazuhumi Hosada, personal communication). In addition, Drake has shown that the mutation rate of Qβ in cells is 1.5 x 10⁻³, 10- to 100-fold higher than measured for PV in the same study (44). Our data would suggest that the presence of Glu instead of Asn in the ribose-binding pocket contributes to both the reduction in replication speed and fidelity.

Do RNA bacteriophage require a slower replication rate, greater population diversity, both or neither? Given the substantially faster doubling time of prokaryotic cells relative to mammalian cells, it appears unlikely that a slower replication rate for RNA bacteriophage would increase the yield of progeny virus per cell at a given time. Unlike mammalian host cells, prokaryotic host cells exhibit substantial phenotypic diversity, for example the presence of phage-resistant cells (45). It is possible that the increased mutation frequency of the RNA phage permits phage evolution to keep pace with host cell evolution, thus precluding virus extinction caused by the selection of phage-resistant cells. The capacity for prokaryotes like E. coli to grow to much higher cell densities than mammalian cells permits RNA phage to grow to higher titers than animal RNA viruses. Because viral population dynamics theory predicts a log-linear relationship between virus titer and mutation frequency, the high titers of RNA phage
should absorb the increased mutational load because of a higher error threshold, thus preventing error catastrophe (46).

To our knowledge, Glu-297 3Dpol represents the first RdRp derivative with a mutator phenotype. As discussed below, studying the biological consequences of decreased fidelity is not possible in the PV system. However, construction of this derivative in the context of other animal RNA viruses may be possible. Similarly, construction of a Qβ mutant encoding an RdRp with an Asn in the ribose-binding pocket instead of Glu should alter phage evolutionary capacity relative to the host, providing important insight into the role of parasite/pathogen evolvability in host-parasite co-evolution.

Our previous biochemical and biological studies of position 297 of PV 3Dpol led us to conclude that subtle (2.5- to 5-fold) decreases in the elongation rate measured in vitro cause substantial RNA replication defects in vivo due to tight coupling between translation and replication (14). Since these initial studies were published, there have been two advances in the field. First, an in vitro reaction that mimics VPg-primed initiation of PV genome replication has been developed (Fig. 3-6) (31,47). Second, it has become clear that not all 3Dpol alleles that confer a reduced elongation rate in vitro confer a more substantial defect to RNA replication in vivo (48).

Evaluation of Glu-297 3Dpol in the VPg uridylylation reaction revealed that this derivative had a substantially reduced affinity for UTP (14-fold) relative to WT 3Dpol (compare Fig. 3-7C to Fig. 3-7D), without a significant difference on the observed rate of uridylylation, perhaps a reflection of the fact that nucleotidyl transfer is not rate limiting in this reaction. In addition, there was a decrease in uridylylation processivity in that
only approximately half of the Glu-297 3Dpol complexes yielded VPg-pUpU even under apparently saturating concentrations of UTP (Fig. 3-7F). In the Glu-297 3Dpol elongation complex, affinity for UTP was only affected minimally (Table 3-1), elongation rate was decreased substantially (10-fold) (Table 3-1) and processivity was not affected at all (Fig. 3-8).

The opposite effects of the Glu-297 substitution on the two different reactions: VPg uridylylation and RNA elongation, suggest differences in the structural role and biochemical function of Asn-297 in these reactions. The structure of the FMDV 3Dpol-VPg-pU complex has been solved (49). Based upon this structure, Asn-297 of PV 3Dpol would interact with the 3’-OH in the uridylylation complex (Fig. 3-10) but the 2’-OH in the elongation complex (Fig. 3-1A). The finding that relatively modest reduction in yield of uridylylated VPg (10-15 fold) and processivity (2-fold) have such dramatic effect on genome replication and virus production suggests that the drugs targeting initiation may be more efficacious than those targeting RNA elongation.

The inability of Glu-297 3Dpol to support both protein-primed RNA synthesis and RNA-primed RNA synthesis may suggest that the pressure to be more selective against dNTP utilization also limits the nature of the initiation mechanisms employed by RNA phages. For example, both plant and animal RNA viruses employ both protein-primed (VPg in Fig. 3-1B) and de novo (Cap and ppp in Fig. 3-1B) mechanisms of initiation; however, RNA phages only employ a de novo mechanism (Fig. 3-1B).

Glu-297 PV was quasi-infectious as a pseudo-revertant PV (Gly-297 PV) was isolated (Fig. 3-4). This virus was also isolated in the past and shown to be a Mn$^{2+}$-stimulated PV mutant (27). Gly-297 3Dpol restored the defects to uridylylation observed
Fig. 3-10: Asn-297 interacts with the 3’-OH of the VPg-pU product. The model for 3Dpol-VPg-pU complex shown is that published for the FMDV enzyme (PDB entry 2F8E). PV 3Dpol numbering has been employed to preclude confusion. In this complex, Asn-297 hydrogen bonds to the 3’-OH instead of the 2’-OH observed for the RNA-primed elongation complex shown in Fig. 3-1 (The structural model shown in Fig. 3-1 is based on homology modeling because a crystal structure for this complex is still not available for any RdRp (14). Biochemical/biological validation of this complex has not been performed.
for Glu-297 3Dpol, suggesting that the uridylylation defect is the cause of the severe RNA synthesis defect in cells observed here and with other 297 alleles (14). Why do Gly-297 3Dpol and PV function (27) and Ala-297 3Dpol and PV not function (14)? We propose that a water molecule can be accommodated in the pocket formed by Gly-297 but not in the pocket formed by Ala-297, thus permitting hydrogen bonding as observed for Asn-297. If our interpretation is correct, then the temperature sensitivity of RNA synthesis observed for Asp-297 PV (14) and the Mn\(^{2+}\) dependence of RNA synthesis observed for Gly-239, Ala-297 PV (27) are a result of an initiation (2C-cre-dependent VPg uridylylation) defect. To our knowledge, these PV mutants represent the first polymerase mutants with a specific defect to VPg uridylylation for any picornavirus or any RNA virus that employs a protein-primed mechanism of initiation. These mutants will be very useful for studying initiation and clarifying some of the controversies surrounding the role of the 2C-cre-templated uridylylation in picornavirus minus- and plus-strand synthesis (37,50).
Acknowledgements

I would like to thank Dr. Harsh B. Pathak for purification of the 3CD and 2C-cre RNA.


References

88
42. Lyons, T., Murray, K. E., Roberts, A. W., and Barton, D. J. (2001) J Virol 75(22), 10696-10708
Chapter 4

Proofreading by Glu-297 3Dpol: use of the First and Second Conformational-Change Steps in Nucleotide Selection

Summary

Investigation of the structure-function relationships in the RNA-dependent RNA polymerases (RdRp) is one of the major research focuses of our laboratory. PV 3Dpol represents a model system for all RdRps, and the complete kinetic mechanism was solved for this enzyme (1,2). The mechanism of 3Dpol-catalyzed nucleotide incorporation includes five kinetically defined steps: nucleotide binding; first conformational-change step; phosphoryl transfer; conformational-change step after the chemistry step; and finally, pyrophosphate release (1,2). In this chapter we continued evaluation of the Glu-297 3Dpol derivative, in which the absolutely conserved Asn-297 of the ribose-binding pocket was substituted by glutamic acid, the amino acid residue found to be conserved in the phage RdRps at the homologous position. In the previous chapter we showed that Glu-297 3Dpol, selected against 2’-deoxyribonucleotide incorporation 15-fold better than the WT enzyme. In this chapter we focused on determining which steps during the nucleotide incorporation process were affected by Glu-297 substitution. We found that the conformational-change step preceding the chemistry step became more rate limiting for the Glu-297 3Dpol derivative. Analysis of the successive incorporation of two nucleotides suggested the conformational-change step after chemistry became less favorable for Glu-297 3Dpol as well, especially after deoxyribonucleotide incorporation.
Remarkably, Glu-297 3Dpol was able to excise dAMP after its incorporation much more efficiently, suggesting that a proofreading strategy may be employed by phage RdRps in the case of deoxyribonucleotide incorporation.

**Introduction**

RNA-dependent RNA polymerase from RNA viruses is a key enzyme involved in the genome replication. In spite of substantial sequence diversity between the enzymes from various RNA viruses the overall shape of RdRps resemble all other single subunit replicases. The overall shape of the enzyme is often compared to a cupped right hand with the fingers, palm and thumb subdomains (Fig. 1-4). Seven sequence-structure motifs are conserved among RdRps: A, B, C, D, E and F. Four of this motifs, A to D, form the core of the palm subdomain, motif E is located in the hinge between the palm and thumb subdomains, and motif F resides within the fingers subdomain. Motifs A and C posses amino acid residues essential for catalysis, the function of motif D was mostly described as structural, although some evidence for its role in catalysis has been recently proposed (3). Motif F is almost certainly involved in nucleotide and RNA binding; role of motif E, although it bears strong structural resemblance to the primer/template grip found in RTs, is still unclear; structural motif B was implicated in nucleotide selection (4-7). In poliovirus polymerase two key residues of both motifs A and B, Asp-238 and Asn-297, have been characterized (8). Alanine substitution of Asp-238 in motif A significantly reduced the enzyme’s ability to carry on RNA synthesis; Asn-297 was found to modulate selection of ribonucleotides over deoxyribonucleotides (8). In the previous
chapter we described a Glu-297 3Dpol derivative. Substitution of the WT residue Asn-297 for Glu, allowed this enzyme to discriminate deoxyribonucleotides more efficiently than the WT polymerase. Improved fidelity was mostly attributed to the substantial increase in the apparent binding constant, $K_d,\text{app}$, for dNTPs. In this chapter we addressed the question of nucleotide selection on a different level of detail – what steps in the kinetic mechanism for nucleotide incorporation were affected by this mutation.

The complete mechanism for single nucleotide incorporation catalyzed by 3Dpol was previously solved by our group (1,2). This mechanism includes at least five kinetically distinct steps: nucleotide binding to the polymerase-RNA binary complex; conformational-change step; phosphoryl transfer or chemistry step; conformational-change step after chemistry; and, finally, pyrophosphate release (Fig. 1-5). The conformational-change step prior to the chemistry and the chemistry step are the rate limiting steps for the WT 3Dpol in the presence of Mg$^{2+}$ as a metal cofactor (1). Use of Mn$^{2+}$ as a metal cofactor relaxes the first conformational-change step and leaves only chemistry as the rate limiting step. Both the conformational-change step and the chemistry step are thought to be used as fidelity check-points for the WT polymerase in the presence of Mg$^{2+}$, whereas only the conformational change contributes to the 3Dpol fidelity in the presence of Mn$^{2+}$ (1,2). 3Dpol is not a unique polymerase in this respect; both T7 DNA pol and KF were found to use the first conformational-change and chemistry for fidelity (9-13). Nevertheless, some controversy on whether the conformational change is involved in nucleotide discrimination still exists. Thus, a computational approach showed that for T7 DNA polymerase only the chemistry step was capable to account for the enzyme’s fidelity, and the first conformational change was
not essential (14). Because the conformational change prior to chemistry occurred at a much faster rate constant than the phosphoryl transfer for DNA polymerase β, Tsai’s group concluded that only the chemistry step defined the fidelity of the enzyme as well (15). Only the chemistry step was reported to be rate limiting and to modulate fidelity for HIV-RT-catalyzed nucleotide incorporation based on the lack of the observed elemental effect (16). Despite the debates on the use of the conformational-change step preceding chemistry as a key determinant of polymerase fidelity, we are confident that for PV 3Dpol this step plays a crucial role in nucleotide selection (1,2). Kinetic evaluation of the Glu-297 3Dpol derivative, which discriminates deoxyribonucleotides 15-fold better than the WT 3Dpol, showed that the conformational-change step was indeed affected, and the overall equilibrium for this step was strongly shifted to the left.

The existence of the second conformational-change step following the phosphoryl transfer was observed for KF and T7 DNA polymerase, and it has been suggested that this step may be the translocation of the polymerase along the growing RNA chain (11,12,17). This step was kinetically resolved for the PV 3Dpol as well, and it was proposed to be the translocation of the enzyme into the next register for the subsequent nucleotide incorporation (1). Nevertheless, the physical nature of this step is yet unresolved. It is feasible to imagine that the efficiency of polymerase translocation across a distorted base-pair would be compromised after incorrect nucleotide incorporation. If it were indeed the case, then reduced efficiency of translocation would shift the equilibrium to the left and might provide a kinetic opportunity for proofreading to occur. Hence, incorporation of the incorrect nucleotide may facilitate the movement of the enzyme back into pre-translocation mode. Existence of a slow step following
phosphoryl transfer was documented for a number of DNA polymerases – HIV-RT (as DdDp), KF, T7 DNA pol (11,12,16,17). Guajardo et al., suggested a model for coupling conformational transitions and translocation and proposed the potential use of translocation for proofreading (18). In spite the fact that the conformational-change step prior to chemistry determines the overall reaction rate and modulates enzyme fidelity for DNA pol I and KF, the conformational change after phosphodiester bond formation slowed dissociation of the incorrect DNA product and in conjunction with 3’-5’ exonuclease activity increased fidelity; in addition, the extension of a mismatch was significantly slowed down for this enzyme (10). This chapter describes attempts to elucidate the role of the second conformational-change step as a modulator of 3Dpol fidelity.

Results

Apparent Dissociation Constants for Glu-297 3Dpol are Elevated in the Case of Deoxyribonucleotide Incorporation

Our previous biochemical evaluation of Glu-297 3Dpol derivative revealed that this polymerase indeed exhibited a substantial decrease in the specificity for 2’-deoxyribonucleotides when compared to its WT counterpart. In the case of Glu-297 3Dpol the apparent dissociation constants ($K_{d,app}$) for both dAMP and dUMP incorporation were significantly increased (Table 4-1). The mechanism of a single nucleotide incorporation by PV 3Dpol is comprised of at least five kinetically distinct steps (Fig. 1-5) (1). First, nucleotide (NTP) binds to the enzyme-RNA (ER$_n$) complex to
form ternary complex (ERₙₐNTP); the initial binding is thought to be driven by the triphosphate moiety of the nucleotide. After the nucleotide is bound, the first conformational-change step occurs that leads to the formation of the activated ternary complex (*ERₙₐNTP). During this step the nucleotide assumes an orientation proper for catalysis. For PV 3Dpol and other polymerases this step serves as a first fidelity checkpoint, it is possible that during this step the enzyme also “tests” the base of the incoming nucleotide against the template. After the nucleotide acquires the most competent orientation for catalysis, the chemistry step or phosphoryl transfer occurs forming a ternary product complex (*ERₙ₊₁PPi). The next step in the mechanism is the second conformational-change. Though there is no structural evidence for what occurs during this step, it is generally accepted that it involves translocation of the polymerase along the growing RNA strand (1,11,19). The last step in the mechanism is the release of the pyrophosphate moiety (PPi), after this step the enzyme-RNA complex is ready to undergo the next round of catalysis.

Given the relationship between the $K_{d,app}$ and the true dissociation constant, $K_d = K_{d,app}^*(1+K_2)$, and the 5-step complete kinetic mechanism for a single nucleotide incorporation, it is reasonable to propose that the conformational-change step prior to the chemistry step is affected by Glu-297 mutation.
Table 4-1: Kinetic and Thermodynamic Constants for 3Dpol-Catalyzed Nucleotide Incorporation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Kinetic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleic Acid</td>
<td>Metal Cofactor</td>
</tr>
<tr>
<td></td>
<td>sym/sub-U</td>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td></td>
<td>GCAUGGGCCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glu-297</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT$^a$</td>
<td>dATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mn$^{2+}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glu-297</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT$^b$</td>
<td>dATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sym/sub-UA</td>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td></td>
<td>GCAUGGGCCCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glu-297</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT$^a$</td>
<td>dATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sym/sub-UdA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCAUGGGCCCdA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glu-297</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Values are taken from (1).

$^b$ Values are taken from (2).
**Phosphorothioate Effect Suggests that Steps other than Chemistry are more Rate Limiting for Glu-297 3Dpol**

In order to evaluate what steps may have been affected by the presence of glutamic acid at position 297 during AMP incorporation we measured the phosphorothioate effect by employing a thiosubstituted analog of ATP, ATPαS ($S_P$ isomer). For the WT 3Dpol the maximal observed elemental effect in the presence of Mn$^{2+}$, when chemistry is solely rate limiting, was equal to 9 (Table 4-2). Since use of Mg$^{2+}$ as a metal cofactor makes the first conformational-change step partially rate limiting along with the chemistry step, the elemental effect is expected to be lower than the elemental effect observed in the presence of Mn$^{2+}$. This was indeed the case, with an observed value of 5 in the presence of Mg$^{2+}$ (Table 4-2). Decrease in the value of the observed elemental effect when magnesium is employed points out to the conformational-change step prior to the chemistry step becoming more rate limiting (1). In the case of the Glu-297 3Dpol derivative, the observed elemental effects for AMP incorporation in the presence of both Mg$^{2+}$ and Mn$^{2+}$ were lower, 3 and 7, respectively (Table 4-2). The decrease in the observed elemental effect in the presence of both metal cofactors suggests that other steps, besides chemistry, are becoming more rate limiting for the Glu-297 derivative and is consistent with the first conformational-change step evolving to be more pronounced for Glu-297 3Dpol as a fidelity checkpoint.
EDTA-HCl Quench Analysis of the Glu-297 3Dpol-Catalyzed Nucleotide Incorporation Supports the Hypothesis that the Conformational-change step Prior to the Chemistry Step Becomes more Rate Limiting

In order to directly evaluate the equilibrium constant for the first conformational-change step, $K_2$, we performed EDTA-HCl quench analysis for both AMP and dAMP incorporation catalyzed by both WT and Glu-297 3Dpol (Fig. 4-1). When Mn$^{2+}$ is used as a metal cofactor, EDTA can quench only the activated enzyme-product complex (*ER$_{n+1}$PP$_i$) but not the activated enzyme-nucleotide complex (*ER$_n$NTP), whereas HCl quenches both complexes (Fig. 4-1A) (2). In the case when the rate constant of the phosphoryl transfer step ($k_{chem}$) is relatively slow, and the equilibrium constant, $K_2$, favors the forward reaction a definite amount of the activated ternary complex (*ER$_n$NTP) can be observed (Fig. 4-1A). If the equilibrium constant $K_2$ favors the reverse reaction and/or chemistry is fast, no activated species (*ER$_n$NTP) would be detected. For WT 3Dpol in the presence of Mn$^{2+}$ only the chemistry step stays rate limiting, while the conformational-change step is strongly shifted in the forward direction with $K_2$ equal to 3 (Fig. 4-1B) (2). The combination of a relatively large $K_2$ and a small $k_{chem}$ (30 s$^{-1}$) for the WT 3Dpol led to a visible lag for the HCl-quenched trace (○) when compared to the EDTA-quenched trace (●) (Fig. 4-1B) (2). The solid lines in the graph represented the simulation of the experimental data to the simplest mechanism, shown in Fig. 4-1A. These data showed that for the WT 3Dpol a large amount of the activated complex accumulated, and the equilibrium for the first conformational-change step strongly favored the forward reaction. These data are consistent with the conformational-change
step prior to the chemistry step not being rate limiting when Mn$^{2+}$ is utilized as the metal cofactor (2).
Table 4-2: Observed Phosphorothioate Effect for 3Dpol-catalyzed Nucleotide Incorporation in the Presence of Mg$^{2+}$ and Mn$^{2+}$

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactor</th>
<th>Nucleotide</th>
<th>ATP $k_{obs}$, s$^{-1}$</th>
<th>ATPαS $k_{obs}$, s$^{-1}$</th>
<th>Elemental effect$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^b$</td>
<td>Mg$^{2+}$</td>
<td>ATP</td>
<td>100 ± 10</td>
<td>22 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>Mn$^{2+}$</td>
<td>ATP</td>
<td>26 ± 5</td>
<td>3 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Glu-297</td>
<td>Mg$^{2+}$</td>
<td>ATP</td>
<td>5.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Mn$^{2+}$</td>
<td>ATP</td>
<td>2.8 ± 0.5</td>
<td>0.4 ± 0.5</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>dATP</td>
<td>dATP$^c$</td>
<td>0.80 ± 0.1</td>
<td>0.13 ± 0.01</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td>Mn$^{2+}$</td>
<td>dATP$^c$</td>
<td>9 ± 1</td>
<td>0.8 ± 0.04</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Glu-297</td>
<td>Mg$^{2+}$</td>
<td>dATP$^c$</td>
<td>0.7 ± 0.1</td>
<td>0.2 ± 0.02</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td>Mn$^{2+}$</td>
<td>dATP$^c$</td>
<td>0.4 ± 0.02</td>
<td>0.04 ± 0.004</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

$^a$ Observed elemental effect is calculated as $(k_{obs})_{NTP}/(k_{obs})_{NTPαS}$, at 1mM nucleotide.

$^b$ Value taken from (1,2).

$^c$ $k_{obs}$ was determined at 5 mM dATP and dATPαS.
Fig. 4-1: The amount of intermediate that accumulated prior to chemistry step for Glu-297 3Dpol was reduced relative to WT 3Dpol. (A) Minimal kinetic mechanism for EDTA-HCl quench analysis. ERₙNTP (ternary complex); *ERₙNTP (activated elongation complex); ERₙ₊₁PPᵢ (product complex). (B, D) Kinetics of AMP (B) and 2’-dAMP (D) incorporation by WT 3Dpol in the presence of Mn²⁺ quenched by either EDTA (●) or HCl (○). The solid line represents the kinetic simulation of the data fit to the mechanism shown in panel (A) with $K₂$ of 3 for AMP and 0.4 for 2’-dAMP incorporation, and $k_{+3}$ of 30 s⁻¹ in both cases. Data for AMP and 2’-dAMP incorporation is taken from (2). (C, E) Kinetics of AMP (C) and 2’-dAMP (E) incorporation by Glu-297 3Dpol in the presence of Mn²⁺ quenched by either EDTA (●) or HCl (○). The solid line represents the kinetic simulation of the best data fit to the mechanism shown in panel A with $K₂$ of 0.15 for AMP and 0.01 for 2’-dAMP incorporation, and $k_{+3}$ of 30 s⁻¹ in both cases. For this experiment 2µM 3Dpol was incubated with 2 µM sym/sub (1 µM duplex) and rapidly mixed with 1000 µM ATP (or 2’-dATP). After mixing the concentration of all reagents was reduced 50%. At indicated times the reaction was either quenched by addition of EDTA to a 300 mM final concentration or by addition of 2N HCl to a final concentration of 1.2 N. Immediately after addition of the HCl solution the reaction was neutralized by addition of 3M KOH in 1M Tris pH 8.0.
The EDTA-HCl quench analysis was performed only in the presence of Mn\(^{2+}\), because in Mg\(^{2+}\) the rate constant for the chemistry step is too fast to allow the detectable amount of the activated complex to accumulate.

In the case of Glu-297 3Dpol the difference between EDTA-quenched reaction (●) and HCl-quenched reaction (○) became much less pronounced (Fig. 4-1C). Collapse of the EDTA-quenched and HCl-quenched traces argued for the substantial decrease in the amount of the activated complex being formed, pointing to changes in \(K_2\). As the Glu-297 3Dpol derivative showed a significant decrease in the observed reaction rate constants for both ribo- and deoxyribonucleotides incorporation in the presence of either Mg\(^{2+}\) or Mn\(^{2+}\) (Table 4-1), we can eliminate the possibility of the phosphoryl transfer step being more efficient for the Glu-297 3Dpol derivative. Therefore, during the data simulation to the mechanism shown in Fig. 4-1A, the rate constant for the chemistry step, \(k_{\text{chem}}\), was kept at the WT 3Dpol level, at 30 s\(^{-1}\), and only \(K_2\) value was modulated (2). Simulation of the data resulted in a significant decrease in the equilibrium constant; the value of \(K_2\) was 0.15, which is 20-fold lower than WT \(K_2\). These data provides convincing evidence that for the Glu-297 3Dpol the conformational-change step prior to the chemistry step becomes much more rate limiting than in the case of the WT enzyme even for AMP incorporation.

When dATP was employed as a substrate for the EDTA-HCl analysis by the WT 3Dpol, the equilibrium constant, \(K_2\), was diminished 7.5-fold and equals to 0.4 (Fig. 4-1D) (2). For dAMP incorporation \(k_{\text{chem}}\) was kept at the same value of 30 s\(^{-1}\), since we showed previously, that in manganese the chemistry step was not involved in the modulation of fidelity of the enzyme as no significant amount of the activated
intermediate (EDTA-resistant) is accumulated; $K_2$ was set at 0.4, as was determined earlier by both calculations and simulation (2).

When dAMP incorporation was evaluated for the Glu-297 3Dpol derivative, the data points from EDTA-quench (●) and HCl-quench (○) essentially overlapped (Fig. 4-1E). The equilibrium constant for the conformational-change step preceding the chemistry, $K_2$, was set at 0.01 in order to achieve a good fit of the experimental data to the simulation traces; $k_{chem}$ was again left unchanged. Thus, the equilibrium constant for the conformation-change step prior to chemistry, $K_2$, for the dAMP incorporation by Glu-297 3Dpol is 40-fold lower than $K_2$ for the WT enzyme for deoxyribonucleotide incorporation. Comparing changes in the equilibrium constant for the first conformation-change step for AMP versus dAMP incorporation, we see that for the WT 3Dpol $K_2$ changed 7.5-fold, going from 3 to 0.4, respectively; whereas for the Glu-297 this change was more dramatic - $K_2$ for dAMP incorporation was decreased 15-fold relative to the equilibrium constant for the AMP incorporation by the same derivative. As a result, for the dAMP incorporation catalyzed by the Glu-297 3Dpol derivative, the conformational-change step prior to the chemistry step emerges into a serious roadblock on the pathway of the deoxyribonucleotide incorporation.

The Rate Constant of the Dissociation of a Nucleotide Analog, AMPCPP, from a Ternary Complex Suggests that Decrease in $K_2$ was due to Change in $k_{+2}$, not in $k_{-2}$

AMPCPP ($\alpha,\beta$-methyleneadenosine 5’-triphosphate) is a non-hydrolysable ATP analog that can form a ternary complex with 3Dpol-sym/sub (20). The overall stability of this
complex depends on both $K_1$ and $K_2$ (Fig. 1-5). Because the initial binding of a nucleotide to the polymerase-sym/sub complex is most likely driven by its triphosphate moiety, we can attribute any changes in the dissociation rate of the AMPCPP from the ternary complex to $K_2$ (20). For this experiment, preassembled 3Dpol/sym-sub complex was first incubated with AMPCPP in order to establish the ternary complex (ER$_n$AMPCPP) (Fig. 4-2A). This ternary complex was then rapidly mixed with ATP solution and the rate constant of AMP incorporation was monitored (Fig. 4-2B, C). The rate constant of AMP incorporation was attenuated to a similar extent for both WT and Glu-297 3Dpols by the rate of AMPCPP dissociation from the ternary complex (Fig. 4-2B, C). The data was simulated to the mechanism shown in Fig. 4-2A, with $k_{+1}$ values set at 1 and 0.7 for WT and Glu-297, respectively. In our previous experiment, EDTA-HCl quench analysis, we examined changes in the equilibrium constant for the 1st conformational-change step, $K_2$, and found that it was significantly decreased for the Glu-297 derivative. Because AMPCPP dissociation rate constants from the ternary complex were essentially the same for WT and Glu-297 3Dpols, and at the same time $K_2$ was much smaller for the Glu-297 3Dpol, we can argue that only the forward rate constant over the conformational-change step was affected by the Glu-297 substitution. (In other words, $K_2 = k_{+2}/k_{-2}$, $K_2$ was decreased for Glu-297, but $k_{-2}$ was the same, therefore, $k_{+2}$ also had to be decreased).
A

\[ \text{ER}_n \text{ AMPCPP} \xrightarrow{k_{+1}} \text{ER}_n + \text{AMPCPP} + \text{ATP} \]

\[ K_d \uparrow \]

\[ \text{ER}_n \text{ ATP} \]

\[ k_{pol} \downarrow \]

\[ \text{ER}_{n+1} + \text{PP}_i \]

B

![Graph showing [11-mer RNA] vs Time for WT](image)

C

![Graph showing [11-mer RNA] vs Time for Glu-297](image)
Fig. 4-2: Stability of the Glu-297 3Dpol ternary complex with AMPCPP was similar to WT. (A) Minimal kinetic mechanism for dissociation of the 3Dpol-sym/sub-AMPCPP complex monitored by AMP incorporation. ERnAMPCPP (3Dpol – sym/sub-AMPCPP complex); ERn (3Dpol – sym/sub complex); ERnATP (ternary complex); ERn+1 (3Dpol – sym/sub product complex); PPi (pyrophosphate). (B, C) Kinetics of AMP incorporation in the presence (○) or absence of AMPCPP (●) for WT (B) and Glu-297 3Dpol (C). The solid lines represent the kinetic simulation of the data fit to the mechanism shown in panel A, with the rate constant of dissociation for AMPCPP \( k_{+1} \) of 1 s\(^{-1}\) and 0.7 s\(^{-1}\) for WT and Glu-297 3Dpol, respectively. 2µM 3Dpol was first assembled with 2µM sym/sub (1µM duplex) at room temperature for 90 sec in the reaction buffer (50 mM HEPES pH 7.5, 60 µM ZnCl\(_2\), 5 mM MnCl\(_2\), 10 mM 2-mercaptoethanol). Then either buffer or 200 mM AMPCPP solution was added to the assembled complex, after 1 min incubation the reaction mix was loaded into the rapid mixing/quenching apparatus. Reactions were initiated by mixing with an equal volume of 2mM ATP solution and allowed to react at 30 ºC for the indicated time. The solid lines represent kinetic simulation of the data to a mechanism shown in panel A, using \( K_d \) and \( k_{pol} \) values listed in Table 4-1, and the apparent dissociation rate constant of AMPCPP, \( k_{+1} \), of 1 and 0.7 for WT and Glu-297 3Dpol.
Successive Incorporation of Two Nucleotides

In order to evaluate the steps that follow chemistry we examined the kinetics of 2 successive nucleotides incorporation (Fig. 4-3). Two pairs of nucleotides, ATP and UTP, and their deoxyribonucleotide analogs, dATP and dUTP were employed. Because Glu-297 3Dpol has substantially higher $K_{d,app}$ values, especially for deoxyribonucleotides, the concentrations of nucleotides used in these reactions were set at 5 X $K_{d,app}$ value (Table 4-1), in order to reach saturating conditions for each nucleotide-enzyme-substrate combination. The results of this experiment are shown in Fig. 4-3B-E. When two ribonucleotides were used as substrates for successive nucleotide incorporation, transient accumulation of 11-mer RNA was observed, in both the WT and Glu-297 3Dpol-catalyzed reaction (Fig. 4-3B, C). There was no visible block for extension of 11-mer to 12-mer. In the case of Glu-297 3Dpol the amount of 11-mer accumulated was more pronounced (Fig. 4-3C), yet this phenomenon could probably be explained by significantly lower polymerization rate constants displayed by this 3Dpol derivative (Table 4-1). In order to obtain estimation for the translocation step rate constant the experimental data was simulated to the simplified mechanism shown in Fig. 4-3A. In this mechanism $K_d$ and $k_{pol}$ values were experimentally determined, and only translocation rate constants (or equilibrium constant over translocation step) were defined by the simulation. When the complete kinetic mechanism was solved for the WT 3Dpol, the rate constants for the second conformational-change step were identified to be $k_{+transl} = 500 \text{ s}^{-1}$ and $k_{-transl} = 0.0025 \text{ s}^{-1}$ (in the complete mechanism $k_{+transl}$ and $k_{-transl}$ correspond to $k_{+,4}$ and $k_{-,4}$, respectively) (1). It is worth mentioning, that the value for $k_{transl}$ was a lower
limit, set up based on the pyrophosphate exchange and pyrophosphorolysis rate constants (1). The simulation of the experimental data to the complete kinetic mechanism was insensitive to changes in $k_{\text{transl}}$ (1). During the simulation of the successive AMP and UMP incorporation catalyzed by WT 3Dpol to a 2-nucleotide mechanism (Fig. 4-3A) $k_{\text{transl}}$ can range from 0 to 500 s$^{-1}$, with $k_{+\text{transl}}$ fixed at 500 s$^{-1}$. Therefore, we can say, that the overall equilibrium constant for this step, $K_{\text{transl}}$, had a lower limit of 1 ($k_{+\text{transl}}/k_{-\text{transl}}$), its highest limit was published to be $2 \times 10^5$ (1). For Glu-297 3Dpol neither $k_{+\text{transl}}$ nor $k_{-\text{transl}}$ values were fixed; however, the lower limit for $k_{+\text{transl}}$ was 50 s$^{-1}$. In spite of some flexibility in the forward and reverse rate constants for the translocation step during simulation, their ratio, the equilibrium constant $K_{\text{transl}}$, was fixed at 0.07.

When successive incorporation of two deoxyribonucleotides was evaluated, a substantial fraction of 11-mer RNA (the product of the first nucleotide incorporation) accumulated for both WT and Glu-297 3Dpol (Fig. 4-3D, E). The incorporation rate constants of dAMP into S/S-U and dUMP into S/S-UdA were comparable for both enzymes (Table 4-1), but in the case of the Glu-297 3Dpol-catalyzed reaction the kinetics of disappearance of 11-mer was significantly slower (Fig. 4-3E). Besides the reduced rate of 11-mer conversion into a 12-mer RNA observed for Glu-297 3Dpol-catalyzed reaction, a large fraction of 11-mer failed to be extended at all, suggesting a serious block for 11-mer elongation (Fig. 4-3E).

When the dAMP-dUMP data for the WT 3Dpol was simulated both rate constants for the translocation step, $k_{+\text{transl}}$ and $k_{-\text{transl}}$, were again flexible, however the best fit was achieved when $K_{\text{transl}}$ was $0.13 - 0.05$ (Fig. 4-3D). For Glu-297 3Dpol the best fit of the data was achieved at $K_{\text{transl}}$ of $0.05 - 0.001$ (Fig. 4-3E).
Although a very simplified mechanism was used to analyze two successive nucleotides incorporation, apparent block at the translocation step after first nucleotide incorporation appeared in the case of Glu-297 3Dpol, especially when two deoxyribonucleotides are utilized. Addition of a stalling step either before or after translocation and two steps accounting for binding of incorrect nucleotide (as both nucleotide were present at very high concentration) significantly improved fit of the data to simulation (Appendix A, Fig. A-1 - Fig. A-4).
A

\[
ER_n + X \xrightleftharpoons{K_d, X} ER_n^X \xrightarrow{k_{\text{pol, } X}} ER_{n+1} \xrightleftharpoons{k_{\text{transl}}} FR_{n+1} \xrightarrow{K_d, Y} FR_{n+1}^Y \xrightarrow{k_{\text{pol, } Y}} FR_{n+2}
\]

B

[11, 12-mer RNA] (µM)

<table>
<thead>
<tr>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>ATP + UTP</td>
</tr>
</tbody>
</table>

C

[11, 12-mer RNA] (µM)

<table>
<thead>
<tr>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>ATP + UTP</td>
</tr>
</tbody>
</table>

D

[11, 12-mer RNA] (µM)

<table>
<thead>
<tr>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>WT</td>
</tr>
</tbody>
</table>

E

[11, 12-mer RNA] (µM)

<table>
<thead>
<tr>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>Glu-297</td>
</tr>
<tr>
<td>dATP + dUTP</td>
</tr>
</tbody>
</table>
Fig. 4-3: Successive incorporation of two nucleotides suggested block in translocation for Glu-297 3Dpol after first dNMP incorporation. (A) Kinetic mechanism used in simulation of 2 nucleotides incorporation experiment. In this mechanism $K_d$ and $k_{pol}$ values were experimentally determined values listed in Table 4-1, and $k_{+trans}$ and $k_{trans}$ are the only values obtained through simulation. (B, C) Subsequent incorporation of AMP and UMP into S/S-U by WT and Glu-297 3Dpol. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (B) or Glu-297 (C) 3Dpol. The solid lines represent simulation to the mechanism shown in panel A. For WT 3Dpol $k_{+trans}$ was fixed at 500 s$^{-1}$ and $k_{trans}$ was set at lower limit of 0.0025 s$^{-1}$, therefore $K_{trans}$ for WT 3Dpol had a lower limit of 1. In the case of Glu-297 values for $k_{+trans}$ and $k_{trans}$ were flexible and only the equilibrium constant, $K_{trans}$, was fixed at 0.07. (D, E) Consecutive incorporation of dAMP and dUMP into S/S-U by WT and Glu-297 3Dpol. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (D) or Glu-297 (E) 3Dpol. The solid lines represent simulation to the mechanism shown in panel A. For both WT and Glu-297 3Dpol in the case of successive incorporation of the two deoxyribonucleotides $k_{+trans}$ and $k_{trans}$ were flexible with the best fit of data to the simulation was achieved at $K_{trans}$ of 0.13 – 0.05 for WT 3Dpol and $K_{trans}$ of 0.05 – 0.001 for Glu-297 3Dpol, respectively.
Evaluation of the Reverse Reaction Showed that Glu-297 3Dpol can Proofread after Deoxyribonucleotide Incorporation

In order to evaluate the reverse reaction for both WT and Glu-297 enzymes we performed the pyrophosphate exchange experiment with both ribo- and deoxyribo-nucleotides. The experimental design for this experiment is shown in Fig. 4-4. After 3Dpol-sym/sub complex was assembled, supplemental MgCl₂ was added to the reaction in order to keep the concentration of free Mg²⁺ at 5 mM after addition of the nucleotide and pyrophosphate. The reaction was initiated by addition of a nucleotide (ATP or dATP) simultaneously with the [³²P]-labeled pyrophosphate. Thus, the 3Dpol-sym/sub-product complex was formed in the presence of [³²P]pyrophosphate. At indicated time points the reaction was quenched by addition of EDTA to a 125 mM final concentration. At low pyrophosphate concentrations nucleotide incorporation is essentially irreversible, in spite of every single step in the complete kinetic mechanism being reversible (Fig. 4-4B). However, when exogenous pyrophosphate is added to the reaction, the equilibrium over every step is shifted towards the initial substrates. When radiolabeled pyrophosphate is used, it gets ‘incorporated’ through the reverse reaction back into the nucleotide triphosphate (Fig. 4-4B). The rate constant of the pyrophosphate exchange (reverse reaction rate constant) was monitored by the rate of the appearance of the [γ-³²P]ATP (Fig. 4-4C, D) or [γ-³²P]dATP (Fig. 4-4E, F). The rate constant of the pyrophosphate exchange was very similar for both WT and Glu-297 3Dpol when AMP incorporation was evaluated (Fig. 4-4C, D). For both enzymes linear accumulation of [γ-³²P]ATP was observed at comparable rate constants of 4.8 × 10⁻⁴ µM s⁻¹ and 1.0 × 10⁻⁵
µM s⁻¹ for WT and Glu-297 3Dpol, respectively (Fig. 4-4G). Consequently, rate constants for pyrophosphate exchange are 6 x 10⁻⁴ s⁻¹ for WT and 1.3 x 10⁻³ s⁻¹ for the Glu-297 derivative, as only 0.8 µM of the enzyme-sym/sub-product ternary complex should have been formed under the experimental conditions employed (1). However, significant difference in the kinetics and the amount of [γ-³²P]dATP generated was observed for WT and Glu-297 3Dpol when dATP was utilized as a substrate for establishing 3Dpol-sym/sub-product complexes (Fig. 4-4E, F). In the case of WT 3Dpol no detectable amount of [γ-³²P]dATP was produced (Fig. 4-4E). On the other hand, for Glu-297 the amount of [γ-³²P]dATP generated was 5-fold higher when compared to the amount of [γ-³²P]ATP (compare Fig. 4-4D and 4-4F). For the Glu-297 derivative the rate of the [γ-³²P]dATP accumulation was determined by fitting the data to a line and is 6 x 10⁻³ µM s⁻¹. Thus the rate constant of the pyrophosphate exchange for this derivative was 7.5 x 10⁻³ s⁻¹, a 6-fold increase when compared to the rate constant of the reverse reaction in the event of AMP incorporation. Thus, substitution of asparagine at position 297 for glutamic acid allowed this 3Dpol derivative to employ the reverse reaction in order to excise misincorporated deoxyribonucleotides.
Fig. 4-4: Pyrophosphate exchange. (A) Experimental design. 2µM 3Dpol was incubated with 20 µM sym/sub (10 µM duplex) for 90 sec to assemble enzyme-RNA complex (0.8 µM), supplemental MgCl₂ was added to the reaction in order to keep concentration of free Mg²⁺ at 5mM after nucleotide and pyrophosphate addition. After addition of MgCl₂ the reaction was initiated by addition of either 1mM ATP (C, D) or 1 mM dATP (E, F) and 2mM [³²P]PPi. Reactant concentrations were reduced by 50% after mixing. Reactions were quenched by addition of EDTA to a final concentration of 125 mM. (B) Complete kinetic mechanism for nucleotide incorporation. ERₙ (3Dpol – sym/sub complex); NTP (nucleotide); ERₙNTP (ternary complex); *ERₙNTP (activated elongation complex); *ERₙ₊₁PPi (activated product complex); ERₙ₊₁PPi (product complex); ERₙ₊₁ (3Dpol – sym/sub product complex); P Pi (pyrophosphate). The kinetic mechanism is divided into two parts, forward (top line) and reverse (bottom line) reactions. [³²P]PPi is shown in red, [³²P]PPi can bind to the ERₙ₊₁ complex and through the reverse reaction get reincorporated into the nucleotide triphosphate moiety. (C, D) [γ⁻³²P]ATP, pyrophosphate exchange product, accumulated with similar kinetics for WT (C) and Glu-297 (D) 3Dpol catalyzed reactions. Products from the reactions described above were resolved by thin-layer chromatography on polyethyleneimine cellulose F plates. The TLC plates were developed in 1M LiCl buffer (first 6 cm), and then switched to a buffer containing 1.5M LiCl, 4 M urea, 0.2 M sodium acetate. TLC plates were then dried and product formation was visualized by phosphorimaging. (E, F) [γ⁻³²P]dAMP was accumulated only in the reaction catalyzed by Glu-297 (F) 3Dpol, no product of pyrophosphate exchange was observed for WT 3Dpol (E). (G, H) Kinetics of [γ⁻³²P]ATP (G) and [γ⁻³²P]dATP (H) accumulation. Kinetics of pyrophosphate exchange were monitored by the increase in [γ⁻³²P]ATP (G) or [γ⁻³²P]dATP (H) production at 1mM PPi. The solid lines represent the fit of the data to a straight line. In the case of AMP incorporation, the rates of pyrophosphate exchange were 4.8 x 10⁻⁴ µM s⁻¹ and 1.0 x 10⁻⁵ µM s⁻¹ for WT and Glu-297 3Dpol, respectively (G); when dATP was employed in the reaction [γ⁻³²P]dATP formation was observed only for Glu-297 3Dpol, at 6 x 10⁻³ µM s⁻¹ (H). Although the solid lines in the graphs represent the best data fit to a straight line, they essentially overlap with the lines generated during the kinetic simulation of the data to a complete mechanism shown in panel B. The reverse rate constants (k₋₄) over the second conformational-change step derived from the simulation were equal to 0.007, 0.0065 and 0.03 s⁻¹ for WT and Glu-297 in the case of AMP incorporation, and Glu-297 in the case of dAMP incorporation, respectively.
Discussion

In the previous chapter the basic biochemical and biological characteristics of Glu-297 3Dpol were evaluated. In this 3Dpol derivative, Asn-297, an absolutely conserved amino acid residue involved in nucleotide selection was mutated to a glutamic acid. Asn-297 is absolutely conserved among animal RNA virus RdRps, however, in phage RNA polymerases there is a glutamic acid residue present in the homologous position (Fig. 3-1). We hypothesized that higher dNTP pools present in the prokaryotic organisms may be responsible for this difference (Table 3-1). Presence of Glu in phage RdRps might have altered their specificity for dNTPs, restricting the polymerase from utilizing deoxyribonucleotides. Indeed, Glu-297 3Dpol derivative exhibited higher discrimination against deoxyribonucleotides, which was mainly manifested in a large increase in the apparent dissociation constants for dNTPs (Table 4-1). Despite the improved selection against dNTPs exhibited by Glu-297 3Dpol, substitution of Asn-297 for Glu, however, resulted in a reduced incorporation rate constants for rNMPs.

Previous studies on RdRp mechanism accomplished in our lab identified the conformational-change step preceding phosphoryl transfer as a major fidelity checkpoint for PV 3Dpol. In the past we had used the tosylsubstituted ATP analog, ATPαS, in order to obtain evidence of the conformational-change step being rate limiting. We compared the observed rate constants of AMP, dAMP, AMPαS and dAMPαS incorporation by Glu-297 3Dpol to those of the WT 3Dpol; the observed phosphorothioeffects for Glu-297 3Dpol derivative were slightly decreased, both in the presence of Mg²⁺ and Mn²⁺ (Table 4-2). This observation was consistent with our hypothesis that a step other than
chemistry, most likely the first conformational change, became more rate limiting for the Glu-297 3Dpol.

Comparison of EDTA-quenched versus HCl-quenched reactions for both AMP and dAMP incorporation yielded a more direct evaluation of the first conformational-change step. This step follows initial nucleotide binding and leads to the formation of the activated ternary complex (*ERₙNTP in Fig. 4-1A), which is ready to undergo phosphoryl transfer. The amount of the activated species that accumulates is governed by the rate of their formation ($K_2$) and disappearance (chemistry step, $k_{chem}$). For WT 3Dpol there was a substantial amount of the activated complex formed during AMP incorporation, which is consistent with the equilibrium over the conformational-change step favoring formation of the activated enzyme-nucleotide complex. For Glu-297 3Dpol, as expected, significant reduction in $K_2$ was observed. For the Glu-297 3Dpol derivative the decrease in the equilibrium constant for the conformational-change step, $K_2$, was 20- and 40-fold for AMP and 2’-dAMP incorporation, respectively, when compared to the WT values (Fig. 4-1).

AMPCPP inhibition studies showed that the reverse rate over the conformational-change step was very similar for both enzymes (Fig. 4-2B, C). AMPCPP is capable of forming relatively stable ternary complex with 3Dpol-sym/sub (20). If a pre-established 3Dpol-sym/sub-AMPCPP ternary complex is mixed with ATP, a slow AMP incorporation is observed. The rate constant of AMP incorporation is an estimation of AMPCPP dissociation rate constant. Because AMP incorporation rate constants were comparable for WT and Glu-297 3Dpol, we concluded that only forward rate constant of the conformational-change step was affected by introducing Glu at position 297.
Although the first conformational change has been implicated in polymerase fidelity for various polymerases (T7, KF, HIV-RT), there still exists some debate on whether this step is universally used to tune fidelity among various classes of polymerases (9-11,14-16). For example, only the phosphoryl transfer step was proposed to modulate fidelity for DNA polymerase β, and a computational study by Florian et al., suggested that in the T7 DNA polymerase system just the binding and chemistry steps were essential for enzyme fidelity (14,15). In this study, we showed that for PV 3Dpol the conformational-change step preceding chemistry is an important determinant of RdRp fidelity. In addition, we demonstrated that for 3Dpol derivatives with higher fidelity, such as Glu-297, the conformational change becomes more rate limiting.

The conformational-change step after chemistry is also not unique to the RdRp. This step has been identified for other classes of polymerases as well, such as DNA-dependent DNA polymerase (KF), DNA-dependent RNA polymerase (T7 RNAP), and reverse-transcriptase (MLV-RT) (9,16,17,21-24). Whether this step can be crucial for fidelity is a controversial question. In polymerases with 3′-5′ exonuclease activity this step may allow a time frame for mismatched nucleotide excision, provided it is slower after incorrect base incorporation (10). For polymerases lacking the exonuclease domain this conformational change could be linked to translocation; the possibility for proofreading at this step has been suggested, given that there is an equilibrium between the pre-translocation and the post-translocation state (18). Also, a single-molecule study performed with *E.coli* RNAP showed that the enzyme could cleave the nascent base in the backtracked mode by exonucleolytic activity (25).
In a complete kinetic mechanism for PV 3Dpol-catalyzed nucleotide incorporation the second conformational-change step occurs after phosphoryl transfer and before pyrophosphate release (Fig. 1-5) (1). For the WT enzyme this step was not identified to be involved in fidelity, though it substantially attenuated the rate constants of incorporation consecutive nucleotides. In the case of Glu-297 3Dpol the equilibrium for translocation was decreased to a large extent. Interestingly, when successive incorporation of two dNMPs was evaluated, the equilibrium constants for those steps were notably affected for both WT and Glu-297 3Dpols, suggesting that after misincorporation of the deoxyribonucleotides the polymerase may experience some kind of block, which may allow, perhaps, for nucleotide excision.

An intriguing result was observed during the pyrophosphate exchange experiment. In this experiment addition of exogenous pyrophosphate drives the reaction in the reverse direction (Fig. 4-4A, B). When ATP was used as a substrate in the ‘incorporation part’, for both WT and Glu-297 3Dpols pyrophosphate exchange occurred at comparable rate constants, giving rise to slow accumulation of $[\gamma^{-32}\text{P}]\text{ATP}$ (Fig. 4-4C, D). Pyrophosphate exchange was only 2-fold more efficient for Glu-297 3Dpol, the observed rate constants for pyrophosphate exchange were $1.3 \times 10^{-3} \text{ s}^{-1}$ and $6 \times 10^{-4} \text{ s}^{-1}$ for WT and Glu-297 3Dpol, respectively. However, in the case of dAMP incorporation WT and mutant polymerases behaved very differently. For the WT enzyme no $[\gamma^{-32}\text{P}]\text{dATP}$ accumulation was observed (Fig. 4-4E). Glu-297 3Dpol, on the other hand, was capable of pyrophosphate exchange after dAMP incorporation; $[\gamma^{-32}\text{P}]\text{dATP}$ accumulated for Glu-297 derivative at a much faster rate constant than after AMP incorporation, at $7.5 \times 10^{-3} \text{ s}^{-1}$ (Fig. 4-4F, H). Interestingly, the efficiency of pyrophosphate exchange was increased
for Glu-297 3Dpol only after deoxyribonucleotide incorporation. Perhaps, phage RdRpS use this residue to facilitate deoxyribonucleotide excision? Based on the complete kinetic mechanism for a single nucleotide addition, pyrophosphate binding to the product complex (ER_{n+1}) should be a relatively fast step (1). Therefore we can suggest that either chemistry and/or the second conformational change were affected by introducing Glu at position 297. The possibility of the increase in the reverse rate constant over the conformational-change step preceding chemistry can be ruled out based on similar rate constants for AMPCPP dissociation from the activated ternary complex (*ER_{n}NTP) (Fig. 4-2B, C). The complete kinetic mechanism was employed to simulate pyrophosphate exchange, yet the mechanism was separated into two parts: the forward and the reverse direction (Fig. 4-4B). In order to simplify the simulation we assumed that neither \([\gamma-^{32}P]ATP\) nor \([\gamma-^{32}P]dATP\) get reincorporated. This probably was the case, because pyrophosphate exchange rate constants were much slower than either AMP or dAMP incorporation rate constants for both of the enzymes, and the concentration of generated labeled nucleotides should have been negligible, compared to the unlabeled nucleotides added in the beginning of the experiment. We were able to simulate experimental data to the complete mechanism by only changing the reverse rate constant over the second conformational-change step. For both enzymes simulation resulted in straight lines that essentially overlapped with the ones represented in Fig. 4-4G and H. In the case of AMP incorporation the values for \(k_{-4}\) derived through simulation were similar for WT and Glu-297 3DpolS, and were 0.007 and 0.0065 s\(^{-1}\), respectively (Fig. 4-4G). When dAMP pyrophosphate data for Glu-297 3Dpol was simulated, the reverse rate constant over the second conformational change was significantly increased, and was
0.03 s$^{-1}$ (Table 4-3). Interestingly, the simulations were insensitive to increase in the $k_{-3}$ values, therefore we can propose that introduction of Glu at position 297 in PV 3Dpol affected only the second conformational-change step in the reverse direction. Increase in the $k_{-4}$ value exhibited by Glu-297 3Dpol derivative may suggest that in phage polymerases the second conformational-change step is employed in deoxyribonucleotide discrimination.
Table 4-3: Kinetic parameters for the kinetic mechanism used in simulations of pyrophosphate exchange data$^a$

<table>
<thead>
<tr>
<th>Enzyme/ Nucleotide</th>
<th>$k_{+1}$</th>
<th>$k_{-1}$</th>
<th>$k_{+2}$</th>
<th>$k_{+3}$</th>
<th>$k_{+4}$</th>
<th>$k_{-4}$</th>
<th>$k_{+5}$</th>
<th>$k_{-5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ATP</td>
<td>10</td>
<td>2140</td>
<td>300</td>
<td>500</td>
<td>520</td>
<td>375</td>
<td>500</td>
<td>0.0065</td>
</tr>
<tr>
<td>Glu-297-ATP</td>
<td>10</td>
<td>5450</td>
<td>300</td>
<td>10000</td>
<td>1000</td>
<td>375</td>
<td>500</td>
<td>0.007</td>
</tr>
<tr>
<td>Glu-297-dATP</td>
<td>10</td>
<td>17034</td>
<td>300</td>
<td>150000</td>
<td>460</td>
<td>375</td>
<td>500</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$^a$ The values were either taken from (2) or were determined using the following equations:

$$K_d = k_{-1}/k_{+1} \quad \text{(4-1)}$$

$$K_d = K_{d, \text{app}} \times (1 + K_2) \quad \text{(4-2)}$$

$$k_{+3} \text{ or } k_{\text{chem}} = k_{\text{pol}} \times \frac{\Lambda_2 \times (\sigma - 1)}{E_{\text{obs}} - 1} \quad \text{(4-3)}$$

$$K_2 = 1/(\Lambda_2 - 1) \quad \text{(4-4)}$$

$$K_2 = k_{+2}/k_{-2} \quad \text{(4-5)}$$

$$K_{2,Mg^{2+}} = K_{2,Mn^{2+}} / 5 \quad \text{(4-6)}$$

where $K_d$ is true dissociation constant, $K_{d, \text{app}}$ is an apparent dissociation constant, $E_{\text{obs}}$ is an observed elemental effect and $\sigma$ is the maximal elemental effect (7.9) (2). Equation 4-6 was used to calculate $K_{2,Mg^{2+}}, K_{2,Mn^{2+}}$ was an experimentally determined value (2).
References

*Nature* 426(6967), 684-687
Chapter 5

Biological Evidence for Post-replication Function of Picornavirus Protein 3CD

Summary

PV RNA-dependent RNA polymerase is known for establishing very stable elongation complexes with its RNA substrate. We have hypothesized that the strong interaction between the fingers and thumb subdomains, observed in crystal structure, was responsible for the unprecedented stability of the RdRp-RNA binary complex (Fig. 5-1). Two phenylalanine residues, at positions 30 and 34, were identified that lock the hydrophobic fingertips-thumb interface. In attempt to gently disrupt this interface we changed Phe-30 to tyrosine. Neither 3Dpol-RNA primer-template complex stability, nor its assembly rates were affected in the Tyr-30 3Dpol derivative (Fig. 5-2). Surprisingly, when the Tyr-30 substitution was engineered into the viral cDNA, Tyr-30 PV exhibited a small plaque phenotype. RNA replication was eliminated as a cause of the small plaque phenotype, as the Tyr-30 subgenomic replicon showed the same kinetics of RNA accumulation as the WT replicon. By performing an in vitro translation experiment using viral Tyr-30 RNA, we were able to conclude that the Tyr-30 mutation within the 3D domain affected 3CD protease function, and resulted in delayed kinetics of structural protein processing, which in turn may have caused the small plaque phenotype. The observation that Tyr-30 3CD derivative was unable to stimulate virus maturation in the cell-free translation/replication reactions was unexpected; however this finding strongly
suggests an important role for 3CD in the virus life cycle after translation and/or replication steps.

**Introduction**

Binding of 3Dpol to its RNA primer/template substrate is a relatively slow step. However, the resulting binary complex is extremely stable, with a half-life on the order of two hours at room temperature (1). Binding of DNA polymerases and reverse transcriptases to their primer/templates is much more complicated, where the binding event seems to be branched, and the polymerase can relatively easily dissociate from the nucleic acid (2-5). For both DNA polymerases and RTs large subdomain movements were observed upon binding of the nucleic acid (6-10). In contrast, all RdRps for which crystal structures have been solved appear to have a fully encircled active site even in the apo-form, and the overall fold of the enzyme is not affected by primer/template binding (11-20). The unique interface between the fingertips and thumb subdomains is a conserved feature among RdRps (Fig. 5-1) (11). We hypothesized that this interface exists in a dynamic motion, and opening and closing motions of the fingertips subdomain occur upon primer/template binding. If, indeed, the tight interaction of the fingertips and thumb is essential for establishing a stable elongation complex, disruption of this interface should result in destabilization of the 3Dpol-primer/template complex. For PV 3Dpol the fingertips-thumb interface is comprised of the hydrophobic pocket of the thumb subdomain lined up by the following amino acid residues: Ile-393, Ile-401,
Fig. 5-1: PV 3Dpol crystal structure revealed an interface between fingertips and thumb subdomains. (A) Two tyrosine residues, Tyr-30 and 34, from the fingers subdomain were inserted into the hydrophobic pocket of the thumb subdomain. Polymerase backbone is shown as a gray ribbon; Tyr-30 and Tyr-34, inserted into the hydrophobic pocket of the thumb subdomain, are shown in black. The image was created using the program WebLab Viewer (Molecular Simulations Ins., San Diego, CA), (PDB access code 1RA6). (B) Enlargement of the fingers-thumb interface shown in (A). Tyr-30 and Tyr-34 are shown in black. Amino acid residues which create the hydrophobic pocket of the thumb subdomain are depicted in gray. These amino acids are highly conserved among picornavirus RdRps, most likely due to their essential function. (C) Partial sequence alignment of the fingers and thumb subdomains. Both Tyr-30 and Tyr-34, as well as hydrophobic residues, which form the pocket of the thumb, are highly conserved among picornaviruses. The sequence alignment was generated using the Clustaw 1.8 algorithm. The output was generated using the program SHADEBOX. Tyr-30 and Tyr-34 are shown in bold. PV - poliovirus, CVB3 – coxsackie virus B3, HRV14 – human rhinovirus 14, HRV16 – human rhinovirus 16, FMDV – foot-and-mouth disease virus.
Val-414, Phe-432, Val-439, Ile-436, and Ile-441; and two phenylalanines from the fingertips, Phe-30 and Phe-34, which are inserted into the cavity of the thumb pocket (Fig. 5-1). In order to slightly destabilize the interface we introduced Tyr at position 30. Tyrosine differs from phenylalanine only by the presence of the hydroxyl group, and we assumed that introduction of a single hydroxyl group into the hydrophobic cavity of the thumb should be sufficient to promote a slight opening of the fingertips-thumb interface. In this chapter we describe biochemical and biological analysis of the Tyr-30 3Dpol derivative. Unexpectedly, the biochemical properties of Tyr-30 3Dpol were identical to the WT enzyme. On the other hand, virus bearing the Tyr-30 substitution displayed a small plaque phenotype. Analysis of the Phe-30 to Tyr mutation in the 3D domain in the context of virus cell-free translation/replication reactions resulted in discovery that the fingers-thumb interface somehow alters 3CD protease function. The exact function of the 3D domain in the context of the 3CD protein has not been yet elucidated. Because the protease and RNA-binding activities of the 3C domain are essential for 3CD-catalyzed capsid protein cleavage, we suggest that the 3D domain mainly functions in determining 3CD specificity to the P1 region, within which the structural proteins reside (Fig. 5-4). Potential roles of 3CD in the posttranslation and postreplication events during virus infection are discussed as well.
Results

*Kinetic Properties of Tyr-30 3Dpol were Unaffected by Mutation*

Underlying our rationale to change WT hydrophobic phenylalanine to tyrosine was a desire to gently disrupt the fingertips-thumb interaction. Our reasoning was that while tyrosine is very similar in size to phenylalanine, its hydroxyl group should be excluded from the hydrophobic pocket of the thumb (Fig. 5-1A). The 3Dpol-coding sequence was therefore changed to encode a tyrosine residue at position 30 (Tyr-30), and the Tyr-30 3Dpol derivative was expressed and purified to more than 90% purity. In order to evaluate if Tyr-30 is an active enzyme we first performed a steady state experiment - active site titration (Fig. 5-2A). In this experiment sym/sub RNA was present at 5-fold higher concentration than enzyme and, therefore enzyme concentration was a limiting factor for the reaction. The fraction of active enzyme can be estimated by monitoring the amount of 11-mer RNA produced. As seen from Fig. 5-2A, Tyr-30 3Dpol was essentially as active as WT enzyme. The y-intercept on the graph indicates the amount of active enzyme present in the reaction mixture, and was 1.6 µM for the WT 3Dpol and 1.8 µM for Tyr-30 3Dpol. The nominal enzyme concentration, based on the absorbance reading at 280 nm, should have been 2 µM for both enzymes. Thus, we can conclude that the concentration of active enzyme constituted 80 and 90% for WT and Tyr-30 3Dpol, respectively. The steady state rate of AMP incorporation ($k_{cat}$) was determined by fitting the data to a straight line, and was $0.0002 \pm 0.00004$ s$^{-1}$, equal for
Fig. 5-2: Kinetic properties of Tyr-30 3Dpol were unaffected by the mutation. (A) Active site titration. For this experiment, 20 µM S/S-U RNA (10 µM duplex) was incubated in a reaction buffer (500 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 µM ZnCl₂) containing 500 µM ATP. The reaction was initiated by addition of the enzyme to a 2 µM final concentration. At indicated time points the reaction was quenched by addition of EDTA to a final concentration of 50 mM. The data fit best to a straight line with y-intercepts representing concentrations of the active enzyme, and were 1.6 µM for WT 3Dpol (■) and 1.8 µM for Tyr-30 3Dpol (□), which corresponded to 80 and 90% of the total enzyme being active, respectively. The steady state rate of AMP incorporation was 0.0002 ± 0.00004 s⁻¹ for both WT and Tyr-30 3Dpols. (B) Kinetics of 3Dpol-sym/sub complex assembly. The reaction contained 2 µM S/S-U (1 µM duplex), 500 µM ATP and 1 µM 3Dpol. The reaction was initiated by addition of 3Dpol. The solid lines represent the best fit of the data to a single exponential with assembly rates of 0.038 ± 0.01 s⁻¹ and 0.035 ± 0.01 s⁻¹ for WT (■) and Tyr-30 (□) 3Dpols, respectively. (C) Kinetics of 3Dpol dissociation from 3Dpol-sym/sub complex. 3Dpol-sym/sub complex stability was evaluated. First, 2 µM 3Dpol was incubated in the reaction buffer (500 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 µM ZnCl₂) with 2 µM 5'-32P-labeled S/S-U (1 µM duplex) for 90 sec to assemble enzyme-RNA complex, at which point unlabeled sym/sub (trap) was added to a final concentration of 100 µM. At various times after addition of trap RNA, the amount of complex remaining was determined by taking a 5 µL reaction aliquot and rapidly mixing it with an equal amount of a 1 mM ATP solution. After mixing with ATP the reactions were allowed to proceed for an additional 30 sec and then were quenched by addition of EDTA to a 50 mM final concentration. Formation of 11-mer product RNA was monitored. The solid lines represent the best fit of the data to a single exponential with dissociation rates of 0.00035 ± 0.00002 s⁻¹ and 0.00037 ± 0.00002 s⁻¹ for WT (■) and Tyr-30 (□) 3Dpols, respectively.
both enzymes (Fig. 5-2A). Thus, introducing Tyr-30 mutation did not affect steady state activity of the polymerase.

We next evaluated the rate of enzyme-sym/sub complex assembly. The Tyr-30 mutation was created with the goal of disrupting the fingers-thumb interface; disruption of this interface may in turn affect enzyme-RNA complex stability. It is known that the WT 3Dpol enzyme-sym/sub complex assembly step is relatively slow and, therefore, for most pre-steady state experiments we preassemble the enzyme on its RNA substrate (1). In order to form the enzyme-RNA complex 3Dpol might need to go through an “open” conformation state, in which reorientation of the fingers subdomain occurs, similar to RT or DNA polymerases (8,21-23). If opening of the fingers-thumb interface is required for efficient 3Dpol assembly on its RNA substrate, then Tyr-30 polymerase, which presumably has a perturbed interface, may have an enhanced rate of assembly. In order to determine the 3Dpol-sym/sub complex assembly rate, 2 µM S/S-U (1 µM duplex) was incubated in reaction buffer containing 500 µM ATP. The reaction was initiated by addition of 3Dpol to a final concentration of 1 µM. The reaction was quenched at indicated times by addition of EDTA to a final concentration of 50 mM, and formation of 11-mer RNA product was monitored as a function of time (Fig. 5-2B). In this experiment, Tyr-30 3Dpol displayed a rate of complex assembly essentially equal to WT enzyme (Fig. 5-2B). The rates were 0.038 ± 0.01 s\(^{-1}\) and 0.035 ± 0.01 s\(^{-1}\) for WT (■) and Tyr-30 (□) 3Dpol, respectively. Thus, changing Phe-30 to tyrosine did not affect the enzyme-RNA complex assembly rate.

Our next goal was to evaluate 3Dpol-sym/sub complex stability. Tyr-30, due to its hydroxyl group, should be expelled from the hydrophobic pocket of the thumb (Fig. 5-
Displacement of the fingertips from the thumb subdomain could lead to decreased stability of 3Dpol on its RNA primer/template complex. In order to determine the rate constant for dissociation of this complex directly, we performed the following experiment. First, 3Dpol (2 µM) and 2 µM 5'-32P-labeled S/S-U (1 µM duplex) were mixed in reaction buffer and allowed 90 sec to assemble polymerase-RNA complex. At that time 100-fold molar excess of unlabeled sym/sub was added to trap any free enzyme. At the indicated times after trap addition a 5 µL reaction aliquot was taken and rapidly mixed with an equal volume of 1 mM ATP solution. The reaction was allowed to proceed for an additional 30 sec and was then quenched. The amount of the complex remaining was determined by following production of 11-mer RNA (Fig. 5-2C). Although we expected Tyr-30 3Dpol-sym/sub complex to be less stable, both complexes exhibited similar stability with the 5 half-lives of about 3 hrs and basically the same rates of dissociation - 0.00035 ± 0.00002 s⁻¹ and 0.00037 ± 0.00002 s⁻¹ for WT and Tyr-30, respectively (Fig. 5-2C). Thus, contrary to our expectations, Tyr-30 3Dpol demonstrated basic biochemical properties essentially unchanged from the WT 3Dpol.

**Biological Evaluation of the Tyr-30 Allele**

*Tyr-30 PV Conferred a Small Plaque Phenotype in Cell Culture*

Although basic biochemical evaluation of Tyr-30 3Dpol did not reveal any intriguing phenotype, we decided to assess whether changing Phe-30 to Tyr within 3Dpol will have any effect on poliovirus fitness and replication. Tyr-30 encoding changes were
engineered into infectious PV cDNA, as described under Experimental Procedures. Tyr-30 PV RNA was produced by *in vitro* transcription and its infectivity was evaluated at both 31 and 37 °C (Fig. 5-3A). Tyr-30 viral RNA was able to establish productive infection at both temperatures evaluated (Fig. 5-3A). Although the number of plaques observed for Tyr-30 PV in infectious center assay was essentially equal to the number of plaques produced by WT PV, the size of the plaques differed substantially; at both temperatures Tyr-30 PV exhibited a small-plaque phenotype (Fig. 5-3A).

**RNA Synthesis by Tyr-30 PV was Identical to WT PV**

Use of a single gene product, as a mature protein or in the context of its precursors, at multiple steps during virus replication is a common strategy employed by RNA viruses. The major function of PV 3Dpol is viral RNA synthesis, but it also plays an essential role in the protein (VPg) primer uridylylation reaction, and functions in the context of 3CD protease (25-29). Although basic biochemical properties of Tyr-30 3Dpol did not differ from WT 3Dpol, its function in replication as 3Dpol alone or in the context of precursors may have been affected. Therefore the Tyr-30 coding mutation was introduced into a PV subgenomic replicon, and its replication was analyzed (Fig. 5-3B, C). WT and Tyr-30 replicons were evaluated at 31 and 37 °C (Fig. 5-3C). Exponential replication with similar kinetics was observed for both replicons at the temperatures tested. As a negative control a GAA replicon was used. This replicon codes for the polymerase with absolutely essential Asp-328 and Asp-329 residues replaced by alanines, and any luciferase signal detected for the GAA replicon comes from translation of input
Fig. 5-3: Biological evaluation of the Tyr-30 allele. (A) Small plaque phenotype was observed for Tyr-30 poliovirus. Infectious center assays were performed at both 31 and 37 °C, as described under Experimental Procedures. After electroporation of viral RNA into HeLa cells 1/100 and 1/1000-fold dilutions were plated onto fresh HeLa cell monolayers. The number of plaques observed for WT and Tyr-30 viruses were essentially the same at both temperatures evaluated. However, Tyr-30 virus produced plaques of a much smaller size at both 31 and 37 °C. (B) PV subgenomic replicon (24). This replicon has the capsid-coding sequence replaced by a luciferase reporter gene. After transfection of RNA into HeLa cells, translation of the open reading frame produces a luciferase-P2-P3 polyprotein from which luciferase is released by the 2A protease activity encoded by the P2 region of the genome. In the absence of replication, translation occurs, yielding a 1-2 log increase in luciferase activity. Mutations which affect translation or RNA stability would be scored in this manner. Replication is monitored indirectly by luciferase activity that accumulates to levels higher than observed for translation. (C) Tyr-30 PV replicon replicated as effectively as WT. In this experiment the Tyr-30 (●) PV replicon was compared to WT (■) and GAA (○) PV replicons at both 31 and 37 °C. The GAA replicon encodes an inactive polymerase and serves as a control for translation and RNA stability in the absence of RNA synthesis. This experiment was performed three times; a representative experiment is shown.
RNA. The Tyr-30 replicon showed no defect in RNA synthesis. Therefore, RNA replication can be ruled out as a cause of small-plaque phenotype observed for Tyr-30 PV.

**The Tyr-30 Mutation Caused Delay in Structural Proteins Processing During Viral RNA Translation in vitro**

As mentioned above, 3Dpol plays crucial roles in the virus life cycle not only in the form of 3D, but also as a part of its precursor proteins, for example 3CD, which may not be directly involved in RNA elongation. While in the context of 3CD, the 3D domain cannot perform the polymerization reaction and instead modulates 3C activity. 3CD is a multifunctional protein. Some of its known functions include viral RNA binding and viral polypeptide cleavage (Fig. 5-4) (27,28,30-32). 3CD was also implicated in processes after RNA synthesis, such as virion maturation and RNA packaging (33,34). During viral polypeptide processing 3CD cleaves only structural proteins (Fig. 5-4). The combination of the small-plaque phenotype observed for Tyr-30 PV, and the fact that 3CD is involved in structural protein maturation led us to the hypothesis that the Tyr-30 mutation in 3Dpol affects 3CD activity, either as a protease or in the virion maturation process. In order to test this hypothesis we performed *in vitro* translation experiments with WT and Tyr-30 viral RNAs (Fig. 5-5A). Host protein synthesis is severely down regulated in PV infected cells because viral protein 2A cleaves eukaryotic elongation factor eIFG4 (35,36). This protein is essential for host protein synthesis, but is not required for IRES-mediated viral RNA translation. Therefore, during virus infection
Fig. 5-4: **Processing cascade of the poliovirus polyprotein.** PV genomic RNA is comprised of one open reading frame flanked by 3' and 5'-non-translated regions (NTRs). 2A protease releases the P2-P3 precursor from the polypeptide upon translation (shown by curved arrow). Further processing of the remaining polypeptides is carried out by 3C protease in the form of either 3C (△) or 3CD (▽). Alternative processing of 3CD by 2A protease (shown by a gray arrow ▼) generates 3C' and 3D' proteins. Their function in virus replication is unknown. The stable precursors and mature proteins that accumulate during PV infection are shown along with their corresponding molecular weights (kDa).
mostly viral proteins are accumulated in the cells. *In vitro* translation of viral RNA in HeLa cell-free extracts mimics the processes that occur in infected cells. However, if translation is carried out in the presence of guanidine, viral RNA replication is inhibited and only translation takes place. WT and Tyr-30 viral genomes were translated in the presence of guanidine and $[^{35}\text{S}]$methionine at 37 °C for 3 to 4 hours. Labeled proteins were separated through a 12.5% SDS-PAGE and visualized with a Phosphorimager (Molecular Dynamics) (Fig. 5-5A). Precursor proteins P1 and P3 accumulated to comparable levels in WT and Tyr-30 translation reactions (Fig. 5-5A). However, a significant delay in structural protein processing (VP3-VP1, VP0, VP1, VP3) was observed for Tyr-30 PV. Although the amount of 3CD protein by itself was slightly lower for Tyr-30 PV, the decrease in structural proteins accumulation and slower kinetics of their cleavage were much more pronounced for the Tyr-30 mutant virus in order to be explained only by lower level of 3CD.

To rule out a potential decrease in Tyr-30 3CD stability we performed *in vitro* translation with WT and Tyr-30 PV subgenomic replicons (Fig. 5-5B). As expected, no significant differences were observed in protein translation levels for WT and Tyr-30 replicons; Tyr-30 3CD accumulated to a comparable level with similar kinetics. Thus, Tyr-30 PV exhibited a delay in structural protein processing, perhaps due to altered 3CD activity. This delay in capsid protein accumulation/processing was most likely the cause of the small-plaque phenotype, observed for Tyr-30 PV in cell culture.
Fig. 5-5: Delay in structural protein processing was observed during *in vitro* translation of the Tyr-30 viral RNA.  

(A) *In vitro* translation of WT and Tyr-30 pMovRA in the HeLa S10 extracts. In vitro translation was performed with WT and Tyr-30 viral RNA as described under Experimental Procedures. A 12.5% SDS-PAGE gel of a typical *in vitro* translation reaction is shown. Positions of markers and 3CD protein was determined by staining with coumassie blue protein stain. The positions of structural proteins and their precursors were deduced based on their molecular weight. P1, P3 and 3CD polypeptides were accumulated at a similar level in both WT and Tyr-30 pMovRA translation reactions. However, a significant delay in capsid protein processing was observed for Tyr-30 viral RNA; compare the amount of VP3-VP1, VP0, VP1 and VP3 accumulated for WT and Tyr-30 PV.  

(B) *In vitro* translation of WT and Tyr-30 PV replicons showed no difference in non-structural protein processing. When pRLucRA subgenomic RNA was used in in vitro translation experiments no significant differences in the polyprotein processing was observed. Luciferase, 3CD and P3 proteins accumulated at comparable rates. A representative 12.5% SDS-PAGE is shown. Positions of the markers and viral proteins 3CD, 3D and 3ABC were determined by staining with coumassie blue protein stain.
Tyr-30 3CD was Incompetent in in vitro Virus Maturation Stimulation Assay

Besides its function as a protease and an RNA binding protein, 3CD has been implicated in virus assembly and maturation (33,34,37). Although the process of virus assembly, maturation and RNA packaging remains unclear, 3CD was shown to enhance virus production by up to 100-fold in in vitro translation experiments by increasing RNA synthesis and stimulating virus maturation (33,34). The small-plaque phenotype observed for Tyr-30 PV may have been caused not only by slower capsid protein cleavage but also by 3CD malfunctioning in virus maturation. The 3CD-coding sequence in the 3CD expression vector was changed to encode a tyrosine residue at position 30 (Tyr-30 3CD). Tyr-30 3CD was then expressed and purified as described under Experimental Procedures. In order to achieve stimulation of virus maturation, purified WT and Tyr-30 3CD proteins were added to a translation reaction programmed by WT PV RNA (Fig. 5-6). Surprisingly, Tyr-30 3CD was not able to increase virus production during an in vitro translation experiment, whereas addition of WT 3CD resulted in 100-fold increase in the titer of virus produced (Fig. 5-6). Thus, the single-point mutation Phe-30 to Tyr in 3Dpol did not affect any biochemical characteristics of the polymerase, but significantly altered functions of a 3Dpol precursor, the multifunctional protein 3CD.
Fig. 5-6: Tyr-30 3CD was incompetent in in vitro virus maturation stimulation assay. Purified WT 3CD protein can stimulate virus production in in vitro translation assays (34). When purified Tyr-30 3CD protein was added to the in vitro translation reaction no stimulation was observed. The amount of the virus produced from the Tyr-30 3CD-stimulated reaction was essentially identical to the non-stimulated reaction, whereas addition of the purified WT 3CD increased virus production up to 100-fold. This experiment was performed by Dr. David Franco.
Discussion

Picornavirus polymerases are capable of establishing extremely stable complexes with primer-template substrates; the half-life of these complexes is on the order of two hours (1). This level of stability is much greater than observed for reverse transcriptases and DNA polymerases. One clear difference between picornavirus polymerases and other classes of nucleic acid polymerases is that the picornavirus polymerases have an interface between the fingers and thumb subdomains that should restrict the conformational flexibility of these domains (Fig. 5-1A). We hypothesized that dissociation of polymerases from primer-template requires movement of the fingers subdomain relative to the thumb subdomain. In order to test this hypothesis, we engineered a mutation into this region of poliovirus polymerase that should have destabilized the interaction between the fingers and thumb subdomains. The first derivative we have evaluated had the highly conserved phenylalanine at position 30 replaced by tyrosine. Surprisingly, this change had no effect on the biochemical properties of the polymerase (Fig. 5-2). Tyr-30 3Dpol-RNA binary complex assembly rate and complex stability were essentially indistinguishable from the WT complex. The steady state rate of AMP incorporation was unperturbed for this derivative as well. These results were unexpected. Recently solved crystal structure of FMDV 3Dpol with bound primer/template RNA showed that the fingertips-thumb interface remained essentially unchanged from the interface observed in the unliganded complex; conservation of this interface in the apo enzyme and in its binary complex confirms the importance of the fingertips-thumb interaction (12). Presence of the tyrosine residue at the tips of the
fingers therefore should have made the interface more open due to the electrostatic repulsion by the hydrophobic pocket of the thumb. Explanation of the lack of a biochemical phenotype for Tyr-30 3Dpol may be as follows. First, the fingers-thumb interface perhaps undergoes dynamic movement, and is not static. Mutation of Phe-30 to tyrosine might have facilitated “opening” of the fingers-thumb interface. At the same time a second nearby phenylalanine residue, Phe-34, was unchanged, and its presence may have been sufficient to stabilize “closing” of the fingers-thumb interface. Interestingly, aliphatic amino acids, Ala-29 and Val-33, precede both Phe-30 and Phe-34, with their side chains facing the hydrophobic fingers-thumb interface, which is consistent with strong hydrophobic interaction between the fingertips and thumb subdomains. Surprisingly, the loop region at the fingertips that is in close proximity to the thumb subdomain does not contain only aliphatic amino acid residues. Between two highly conserved phenylalanines that lock the fingertips-thumb interface, two charged or bulky amino acid residues are found in most picornavirus polymerases (Fig. 5-1C). In poliovirus RdRp these are His-31 and Tyr-32, both of which were highly ordered in the crystal structure with their side chains rotated out of the thumb pocket (Fig. 5-1A). If Tyr-30 could not be accommodated inside the hydrophobic pocket of the thumb subdomain, it is feasible to imagine that the Tyr-30 side chain could be flipped out of the hydrophobic cavity of the thumb. If this were indeed the case, then remaining aliphatic residues (Ala-29, Val-33 and Phe-34) would most likely be sufficient to retain the fingers-thumb interface, and the activity of the 3Dpol would not be perturbed. Perhaps, mutating both phenylalanines (Phe-30 and Phe-34) simultaneously would produce a stronger effect on the stability of the fingers-thumb interface. Clustered-aliphatic to
charged scanning mutagenesis may be considered as another experimental approach to effectively disrupt the fingers-thumb interface. If, for example, we engineer either aspartic or glutamic acid residues instead of Phe-30 and Phe-34 and aspartic or glutamic acid residues instead of Val-414, Ile-401, Phe-432, electrostatic repulsion between these side chains would probably make the fingertips-thumb interaction unfavorable, and would prevent formation of a tight interface contact between the fingertips and the thumb. However, it is difficult to predict whether strong disruption of the interface will yield viable polymerase derivatives. Integrity of the fingers-thumb interface may be essential for the proper alignment of the active site residues. In addition, a large structural reorganization of the thumb domain may occur after introduction of a few charged amino acids that may lead to loss of 3Dpol activity.

Thus, contrary to our expectations substitution of Phe-30 with tyrosine did not have any impact on the biochemical properties of the polymerase, and the only difference observed for Tyr-30 PV in cell culture was the small plaque phenotype, which still remained unsolved. The infectivity of Tyr-30 viral RNA was very similar to WT RNA at both 31 and 37 °C, although the size of the plaques formed was significantly smaller for Tyr-30 PV at both temperatures (Fig. 5-3A). Evaluation of the subgenomic replicon bearing the Tyr-30 mutation showed that the mutant polymerase could support WT levels of RNA replication (Fig. 5-3C). Thus RNA synthesis was ruled out as a potential cause of the small plaque phenotype.

Activity of the 3D domain is not limited to the RNA polymerization reaction performed by 3Dpol. The 3D domain functions in the context of partially processed viral precursor proteins, such as 3CD, as well. 3CD is a protease, and does not possess
polymerase activity. Interestingly, in the 3CD structure, both 3C and 3D domains are joined by a polypeptide linker and retain very similar structural organization to those of fully processed 3C protease and 3Dpol (38). The fingers-thumb interface, observed in a 3Dpol crystal structure, remained intact in the context of 3CD molecule (38). As a protease, 3CD mainly cleaves structural proteins from their precursor, P1, whereas 3Cpro cleaves the rest of the polypeptide, P2-P3 (Fig. 5-4) (27,28). Thus presence of the 3D domain in 3CD in some way increases 3CD specificity for capsid proteins. Semler’s group found that 3CD protease activity was strongly facilitated by an unidentified cellular protein cofactor present in HeLa S10 cytoplasmic extracts (39). At the same time 3C protease activity was not dependent on cellular cofactors. Therefore, we propose that the 3D domain of 3CD may be involved in recruiting the cellular cofactor to 3CD. Flanked by two phenylalanines (Phe-30 and Phe-34) that lock the fingertips within the thumb subdomain of the 3Dpol, two amino acid residues, His-31 and Tyr-32, caught our attention previously. The side chains of these two amino acids were located on the surface of the fingertips and appeared to be highly ordered in both 3D and 3CD crystal structures (Fig. 5-1A). The high degree of conservation between the various picornavirus RdRps suggested that these amino acids might possess an important function either in the context of 3D or 3CD proteins (Fig. 5-1C). Hydroxyl group of Tyr-32 was solvent accessible, and 3Dpol sequence analysis suggested that it could be a phosphorylation site as well. If the 3D domain within 3CD were responsible for binding to the cellular cofactor, essential for 3CD proteolytic function, surface residues His-31 and Tyr-32 would be reasonable candidates for establishing such an interaction. If this were the case, the mutation of Phe-30 to tyrosine could have perturbed their location and accessibility
and thus, influenced 3CD interaction with the cellular cofactor. Analysis of the cell-free translation reactions programmed with WT and Tyr-30 viral RNAs showed substantial delay in the capsid protein processing for the mutant virus; a result consistent with distorted 3CD proteolytic activity (Fig. 5-5A). Interestingly, P1 polyprotein, within which structural proteins reside, accumulated to comparable levels for both viruses evaluated, arguing that translation of Tyr-30 RNA was most likely not changed. However subsequent cleavage of P1 to yield partially or fully processed capsid proteins was delayed for Tyr-30 PV. A translation reaction performed with WT and Tyr-30 subgenomic replicons showed no difference in the amount and kinetics of viral proteins accumulation (Fig. 5-5B). WT and Tyr-30 3CD proteins exhibited very similar kinetics of accumulation and stability, suggesting that the delay in structural protein processing observed for cell-free translation reactions programmed with viral RNAs was due to altered 3CD protease function and not to its degradation. In order to test the hypothesis that His-31 and Tyr-32 are involved in establishing contacts between 3CD and the cellular cofactor, I propose that both of these amino acid residues be mutated to alanines. This change should completely abolish the interaction of the 3D domain with the cofactor, but at the same time the polymerase function should remain intact.

Another intriguing observation was made with Tyr-30 3CD in virus maturation assay. Previously, Franco et. al., showed that addition of purified 3CD protein or its messenger RNA to cell-free in vitro translation/replication reactions programmed by viral RNA significantly increased virus production (33,34). At the same time, 3CD did not have any affect on RNA translation or polyprotein processing. The mechanism by which 3CD stimulated virus maturation was not solved. However, for the stimulatory function
exerted by 3CD, integrity of the 3Cpro RNA binding site was found to be essential, whereas 3Cpro protease activity was not required (34). In the virus production enhancement experiments with WT and Tyr-30 3CD, protease function of 3C was disrupted by changing catalytic Cys-147 to glycine. 3Cpro alone was found to be inactive in virus stimulation assay, but together with 3CD it strongly inhibited 3CD enhancing activity, arguing that both 3C and 3CD may be competing for the same RNA sequence or structure. 3CD is known to interact with viral RNA at least at three different sites within the poliovirus genome. 3CD forms a ribonucleoprotein complex at the cloverleaf structure, which comprises part of the 5’ NTR, and it binds to a 2C-cre (cis-replicating element); efficient binding to these two sites is important for RNA replication (29,30,40-43). 3CD was also shown to interact with the 3’NTR poly(A) tail, but it is still unknown whether this interaction has any biological significance (32). Another indirect interaction of 3CD with 3’NTR can be mediated by poly(A)-binding protein PABP1, that binds both the poly(A) tail and 3CD (44). Changing Phe-30 for tyrosine did not affect RNA replication or viral RNA infectivity; however 3CD was inactive in the virus maturation assay. These results suggested that 3CD function after translation and replication steps is affected by the Tyr-30 mutation. Newman et. al., discovered that some non-structural proteins 2C, 3CD, 3D and 3C could be found in highly purified preparation of FMDV and PV (37,45). Interestingly, these proteins remained bound to viral RNA after RNA was extracted from viruses (37). These data, taken together with known RNA-binding properties and enhancement of virus production in cell-free extracts exhibited by 3CD, suggest that the 3CD protein may function during RNA recruitment into virions or in virion maturation.
The mechanism of poliovirus RNA packaging is still undetermined and neither sequence signals within viral RNA nor viral or host proteins involved in this process have been identified. Nevertheless, only positive-sense strand viral RNA is packaged inside the virions; negative-sense strand, messenger, ribosomal and transfer RNAs are somehow excluded from the virions (46,47). Only newly synthesized RNAs are recruited into virions suggesting that there is functional coupling between RNA replication and packaging (48). Because reported 3CD interactions mostly constitute contacts with the \textit{cis}-replicating RNA structures of the positive-sense strand RNA and 3CD is found to be associated with purified viral particles and RNA isolated from those, we hypothesized that 3CD may be a mediator of RNA packaging. The mechanism by which Tyr-30 substitution disrupted this function of 3CD needs to be elucidated.

Given the conserved nature of the fingers-thumb interface and its potential importance for RNA packaging and virus maturation, disruption of the interface is likely to have a significant impact on virus production and infectivity. Thus, the fingers-thumb interface represents a novel and intriguing target for antiviral drug development.

In addition, in order to distinguish between the possible role the fingers-thumb interface and the top surface of the fingertips (mainly residues His-31 and Tyr-32) may play in virus replication/maturation it would be interesting and important to evaluate 3DPol/3CD derivatives with more disruptive changes in the region of interest.
Acknowledgements

I would like to thank Dr. Ian Goodfellow for generously sharing HeLa S10 extracts and teaching me the technique of in vitro translation/replication assays.

References

Chapter 6

Residue Arg-273 as a Modulator of the Polymerase Fidelity

Summary

Structure-function relationships in the fidelity of RdRp have been an issue of interest for our research group for a number of years. In this chapter, a 3Dpol derivative with a true mutator phenotype is described. Surprisingly, a His-273 to Arg mutation which conferred a lower fidelity phenotype was located about 20 Å from the active site of the polymerase. X-ray analysis performed for this 3Dpol derivative did not reveal any significant perturbations in the 3Dpol crystal structure, suggesting that altered fidelity of this enzyme can most likely be attributed to the dynamics of the enzyme during the polymerization reaction. A substantial decrease in the fidelity of this polymerase was manifested in both relaxed nucleotide binding and increased polymerization rates for correct and incorrect nucleotides. Detailed kinetic analyses of the purified Arg-273 3Dpol revealed that the conformational-change step prior to chemistry, a key fidelity checkpoint in 3Dpol-catalyzed nucleotide incorporation, was affected. In order to examine how decreased fidelity of the polymerase could affect virus replication, fitness and pathogenicity, we introduced changes encoding Arg-273 mutation into the PV genome. Surprisingly, Arg-273 PV was able to function indistinguishably from the WT PV in tissue culture when no selective pressure was applied. However, when challenged with the mutagenic drug ribavirin, Arg-273 virus proved to be much more sensitive than
the WT virus. In PV-susceptible mice, Arg-273 PV was shown to be much less neuropathogenic. Use of Transmission Electron Microscopy analysis enabled us to get a first glimpse of the potential connection between 3Dpol fidelity and RNA packaging.

Introduction

The discovery story of the low fidelity mutant described in this chapter is an unusual one. There was no structural rationale for mutating His-273, located about 20 Å from the active site, to Arg. This mutation was an artifact of the PCR step during cloning of a Glu-297 mutation into the 3Dpol gene and was unnoticed by the person who performed the cloning. When I started working on the Glu-297 project, I did not suspect that the 3Dpol-coding sequence of the pET26Ub-3D-BPKN-I92T-Glu-297 contained another mutation that changed His-273 for Arg. This secondary mutation was discovered only during subcloning of Glu-297 mutation into viral subgenomic replicon and cDNA vectors. Thus, the first set of biochemical data obtained for what I thought to be a Glu-297 3Dpol derivative was performed with the double mutant, Arg-273/Glu-297. Interestingly, biochemical and thermodynamic characteristics of the Arg-273/Glu-297 3Dpol derivative were very similar to the Glu-297 3Dpol derivative (Appendix B, Table B-1). First, when the secondary mutation, His-273 to Arg, was discovered, we hoped it would not have any effect on polymerase activity, as the mutation was in the hinge of the fingers, very distant from the active site, which is found within the palm subdomain (Fig. 6-1). However, an amino acid residue does not have to be in proximity to the active site in order to modulate the fidelity of the enzyme. A 3Dpol derivative
bearing a Gly-64 to Ser mutation was identified by serial passaging of the poliovirus in the presence of the antiviral drug ribavirin (1-3). The Gly-64-Ser mutation conferred resistance to ribavirin due to increased fidelity of Ser-64 3Dpol in both biochemical and cell culture assays (3,4). This mutation was located far from the active site of the polymerase as well, and somewhat close to residue 273 (Fig. 6-1). Solution of the complete crystal structure of the poliovirus polymerase by Peersen’s group shed some light on the role of residue 64 in the fidelity of the polymerase (5). The backbone amide and carbonyl groups of the Gly-64 residue make two hydrogen bonds with Gly-1 and link together the two ends of the index finger sequence. This hydrogen-bond network in turn anchors residues 238-241, thus stabilizing the nucleotide-binding pocket of the polymerase (5). Recently, Hogle’s group solved the crystal structure of the Ser-64 3Dpol mutant (6). From this structure it was clearly seen that the hydrogen-bond network that involves the N-terminus of the polymerase was modified compared to the WT polymerase (6). The Ser-64 side chain formed an additional hydrogen bond with the side chain of the carboxylate group of Glu-2 and promoted formation of an extra hydrogen bond between the Glu-2 and Gln-4 residues. Because no large structural rearrangements were observed in the Ser-64 3Dpol structure, Hogle’s group suggested that fidelity of the Ser-64 polymerase might have been affected due to the N-terminus being locked in a more stable formation. His-273 is located in the vicinity of residue 64, and thus, in theory, changing His-273 to Arg may affect the hydrogen-bond network that stabilizes the fingers subdomain and connects to the active site of the polymerase. Intriguingly, in the crystal structure solved for the Arg-273 3Dpol derivative no obvious perturbation was observed in the hydrogen-bond network connecting the fingers subdomain with the active
Fig. 6-1: Location of Arg-273 and Gly-64 in the PV 3Dpol crystal structure. The conserved structural motifs are colored as follows: motif A, red; motif B, green; motif C, yellow; motif D, blue; motif E, magenta; and motif F, purple. Van der Waal's projections of Arg-273 and Gly-64 are shown in magenta and light blue, respectively. The image was generated using the program WebLab Viewer (Molecular Simulations Inc., San Diego, CA).
site and the ribose-binding pocket (Fig. 6-5). However, from the first biochemical experiments it was obvious that Arg-273 3Dpol possessed lower fidelity than the WT 3Dpol (Fig. 6-2). At the same time this 3Dpol derivative was highly active (Table 6-1) and the Arg-273 PV was infectious and able to replicate with kinetics similar to the WT virus.

Recently, a lethal mutagenesis approach became an accepted strategy in the battle against various viruses (2,3,7-9). Both lethal mutagenesis and error threshold strategy terms stemmed out from Eigen’s theory of molecular evolution, where the term “error catastrophe” was originally introduced. However, despite the popularity of lethal mutagenesis, the theoretical basis for it was mostly undeveloped (10). Regardless of the lack of theory behind lethal mutagenesis, a large amount of empirical data supported the principle of lethal mutagenesis, and artificial nucleosides were shown to severely reduce virus titer or even drove certain viruses to extinction (8,11-17). Because the use of antiviral compounds that can increase mutation rates in virus populations became a promising strategy for curing viral diseases a great deal of theoretical research that ties virus fitness, quasispecies diversity and error threshold appeared during last few years (7,18-22). A recent paper by Wilke and colleagues states that the lethal mutagenesis threshold is not merely an error rate beyond which virus survival is impossible, but instead is defined by both mutation rate and the size of the viral population within a given host organism (20). The relation between mutation rate and fecundity (or inoculum size) is log-linear, with mutation rate displaying a higher effect for achieving extinction (20). Addition of various antiviral compounds affects the mutation rate exhibited by a given virus. However, it is difficult to quantitate the increase in the mutation rate, and more
importantly, to distinguish the increased mutation rate from other changes that the artificial nucleoside compound can implement – such as altering the replication environment by changing the nucleotide pools present in the cytoplasm, by potentially altering viral genomic RNA stability, etc. In the case of Arg-273 and Ser-64 PV we have two unique viruses which intrinsically display lower and higher mutation rates, respectively (Fig. 6-10). Thus, WT, high fidelity Ser-64 and low fidelity Arg-273 PV represent an exceptional experimental system for elucidating the connection between mutation rates and virus population fitness.
Results

Biochemical Evaluation of Arg-273 3Dpol, a Low Fidelity Polymerase

Arg-273 3Dpol is a Functional Polymerase with Decreased Fidelity

A mutation encoding Arg-273 was introduced into the 3Dpol coding region of the pET26Ub-3D-BPKN-I92T expression vector as described under Experimental Procedures. Arg-273 3Dpol was expressed and purified essentially as WT enzyme to more than 90% purity. In order to evaluate kinetic characteristics of 3Dpol we use a 10-nt symmetric RNA primer/template substrate, termed sym/sub, or S/S-U (Fig. 6-2A). “U” in S/S-U stands for uracil, the first templating base. WT 3Dpol binds very tightly to the double-stranded portion of sym/sub and either 3’-end can be utilized as a primer in a nucleotide incorporation reaction. Products of the nucleotide incorporation can be separated from the input RNA by electrophoresis through denaturing polyacrylamide gels, visualized by phosphorimaging and quantified (Fig. 6-2B, C, D). First, we evaluated the ability of Arg-273 3Dpol to incorporate the correct nucleotide templated by uracil, AMP (Fig. 6-2B). Arg-273 3Dpol incorporated AMP into S/S-U as efficiently as WT enzyme. Next, we decided to evaluate the ability of Arg-273 3Dpol to misincorporate. For this experiment we used GTP in combination with S/S-U. As seen from Fig. 6-2C, Arg-273 polymerase misincorporated GMP much more efficiently than WT with more product observed at early time points (Fig. 6-2C). When ribavirin
A

5'–GCAUGGGCCC–3'
3'–CCC GGGUACG–5'

B

+ATP

Time (sec) 0 600
WT Arg-273

C

+GTP

Time (sec) 0 600
WT Arg-273

D

+RTP

Time (sec) 0 900
WT Arg-273
Fig. 6-2: Arg-273 3Dpol has reduced fidelity relative to WT 3Dpol in vitro. (A) RNA primer-template substrate employed in this study. A 10-nt self-complementary RNA substrate referred to as sym/sub-U. The “U” indicates that uracil is the templating base for the first round of nucleotide addition. (B) Arg-273 3Dpol is an efficient polymerase. In this experiment, 2 µM enzyme solution was preincubated with 2 µM S/S-U (1 µM duplex) in reaction buffer (as described under Experimental Procedures) in order to establish an elongation complex. The reaction was initiated by addition of ATP to a final concentration of 500 µM and then was quenched at various time points by addition of 2N HCl to a final concentration of 1.2 N. Immediately after addition of HCl, the solution was neutralized by addition of 3 M KOH in 1 M Tris pH 8.0. Reaction products were resolved by electrophoresis on a denaturing 23% polyacrylamide gel and visualized by using a PhosphorImager (Molecular Dynamics). (B) GMP was misincorporated into S/S-U much more efficiently by the Arg-273 derivative than the WT 3Dpol. The experiment was performed essentially as described in panel (A) with minor modifications. The reaction was quenched at various time points by mixing 5 µL of the reaction solution with an equal amount of 100 mM EDTA instead of quenching with HCl. (C) A nucleotide analog, ribavirin triphosphate, is a better substrate for Arg-273 3Dpol. The unnatural nucleotide, ribavirin, is utilized by Arg-273 3Dpol much more efficiently: formation of 11-, 12- and 13-mer RNA is readily observed even at early time points.
triphosphate, an unnatural nucleotide analog, was used in the reaction 11-, 12- and 13-mer RNA products were readily observed in Arg-273 3Dpol catalyzed reaction, whereas for the WT 3Dpol only a slight amount of 11- and 12-mer RNA was observed on the same timescale. Together, these experiments showed that the 3Dpol bearing the Arg-273 mutation is a functional polymerase with altered fidelity.

**Decreased Arg-273 3Dpol Fidelity Originates from Tighter Binding and Increased Rate of Nucleotide Incorporation**

In order to determine the cause of altered fidelity for Arg-273 polymerase we evaluated the dependence of the rate constants for correct and incorrect nucleotide incorporation on nucleotide concentration. For this experiment, 2 μM 3Dpol was first incubated with 2 μM S/S-U (1 μM duplex) in the reaction buffer (50 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 60 μM ZnCl₂) in order to establish the enzyme-RNA complex. The complex was then rapidly mixed with the appropriate nucleotide (ATP, GTP or RTP) at various concentrations in reaction buffer and reactions were quenched by addition of 2 N HCl to a final concentration of 1.2 N. Immediately after addition of HCl, the solution was neutralized by addition of 3 M KOH in 1 M Tris pH 8.0. Reaction products were resolved on denaturing PAGE and visualized by phosphorimaging. Data analysis was then performed as described under Experimental Procedures. The results of this analysis are shown in Table 6-1. Interestingly, both the apparent dissociation constant \(K_{d,\text{app}}\) and the maximal rate of nucleotide incorporation \(k_{pol}\) were affected for Arg-273 3Dpol with all three nucleotides evaluated. The apparent
Table 6-1: Decreased Fidelity of Arg-273 3Dpol Originates from Tighter Binding and Increased Rate of Nucleotide Incorporation.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Nucleic Acid</th>
<th>Nucleotide</th>
<th>$K_{d, app}$</th>
<th>$k_{pol}$</th>
<th>$k_{pol}/K_{d, app}$</th>
<th>$(k_{pol}/K_{d, app})_{WT}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>sym/sub-U</td>
<td>GCAUGGGGCC</td>
<td>ATP</td>
<td>130 ± 20</td>
<td>90 ± 10</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCGGU</td>
<td>ACG</td>
<td>30 ± 10</td>
<td>160 ± 10</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Arg-273</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT$^a$</td>
<td>GTP</td>
<td></td>
<td>ATP</td>
<td>210 ± 70</td>
<td>0.01 ± 0.001</td>
<td>5 x 10$^{-5}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GTP</td>
<td>120 ± 40</td>
<td>0.04 ± 0.003</td>
<td>3 x 10$^{-4}$</td>
<td>6</td>
</tr>
<tr>
<td>Arg-273</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT$^b$</td>
<td>RTP</td>
<td></td>
<td>RTP</td>
<td>500 ± 20</td>
<td>0.01 ± 0.001</td>
<td>2 x 10$^{-5}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ± 30</td>
<td>0.05 ± 0.004</td>
<td>5 x 10$^{-4}$</td>
<td>25</td>
</tr>
</tbody>
</table>

$^a$ Values are taken from (23).

$^b$ Values are taken from (24).
dissociation constants were decreased for both correct and incorrect nucleotides and were on average 2- to 5-fold lower than the corresponding constants for the WT enzyme (Table 6-1). At the same time, $k_{pol}$ was increased to the same extent, 2- to 5-fold, when compared to the WT numbers (Table 6-1). Importantly, the simultaneous decrease in the apparent dissociation constant and increase in the observed polymerization rate enhanced the overall efficiency ($k_{pol}/K_{d,app}$) of the Arg-273 3Dpol 6- to 25-fold (Table 6-1). To our knowledge, Arg-273 3Dpol is the first highly active RdRp that exhibits a mutator phenotype.

**Arg-273 3Dpol Exhibits an Increase in the Equilibrium Constant for the Conformational-Change Step Preceding Phosphoryl Transfer**

The kinetics of single nucleotide incorporation follows the five-step kinetic mechanism depicted in Fig. 1-5. First, incoming nucleotide binds to the 3Dpol-primer/template complex. This complex then undergoes a conformational-change step to yield a catalytically competent complex. Phosphoryl transfer occurs, followed by a second conformational-change step and pyrophosphate release. The observed decrease in the apparent dissociation constant and increase in the polymerization rate for Arg-273 3Dpol could be a result of perturbation of either step 2 or 3. For WT 3Dpol both steps 2 and 3 are partially rate limiting in the presence of Mg$^{2+}$. In the presence of Mn$^{2+}$, however, only the chemistry step is rate limiting (23,25). Because the conformational-change step is partially rate limiting in the presence of Mg$^{2+}$, the observed phosphorothioate effect for the WT 3Dpol is smaller when Mg$^{2+}$ is used as a metal cofactor than when Mn$^{2+}$ is used (Table 6-2) (23,25). To obtain evidence that the first
conformational-change step is at least partially rate limiting for Arg-273 3Dpol we evaluated the efficiency of AMPαS incorporation into S/S-U relative to AMP incorporation into the same RNA substrate in the presence of Mg\textsuperscript{2+} or Mn\textsuperscript{2+}. The results of this experiment are summarized in the Table 6-2. In general, the observed phosphorophioate effects for Arg-273 3Dpol were very similar to those of the WT enzyme and are consistent with the conformational-change step prior to chemistry being rate-limiting. Interestingly, the observed reaction rates for AMP and AMPαS incorporation for both WT and Arg-273 were essentially identical in the presence of Mn\textsuperscript{2+}, whereas in the presence of Mg\textsuperscript{2+} Arg273 3Dpol performs the reaction almost 2-fold faster. We know that for the WT enzyme the conformational-change step is no longer rate limiting in the presence of Mn\textsuperscript{2+}, and the observed reaction rate more closely resembles the chemistry step. Based on the similarity of the observed incorporation rates for these two enzymes in the presence of Mn\textsuperscript{2+}, we argue that the Arg-273 mutation does not affect the chemistry step rate, but most likely affects the conformational-change step prior to chemistry.

To obtain additional support that the conformational-change step preceding the chemistry step is altered for the Arg-273 3Dpol we performed pulse-chase pulse-quench analysis. Recently we have been using combination of HCl- and EDTA-based quench to imitate the pulse-chase pulse-quench experiment (25). However, reactions catalyzed by Arg-273 3Dpol even in the presence of Mg\textsuperscript{2+} were not quenched efficiently by EDTA (Fig. 6-3); for WT 3Dpol in the presence of Mg\textsuperscript{2+} no difference between EDTA and HCl quench was observed (25). Therefore, all the reactions for Arg-273 3Dpol reported in this chapter were quenched with HCl. Pulse-chase pulse-quench analysis allows one to
Table 6-2: Observed Phosphorothioate Effects for 3D\textsuperscript{pol}-catalyzed Nucleotide Incorporation Suggest that the Conformational-Change Step Remains Partially Rate Limiting for Arg-273 3Dpol in the Presence of Mg\textsuperscript{2+}.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactor</th>
<th>Nucleotide</th>
<th>Elemental effect\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATP\textsubscript{b} (k_{o, s}^{-1})</td>
<td>ATP\textalpha S\textsubscript{b} (k_{o, s}^{-1})</td>
</tr>
<tr>
<td>WT\textsuperscript{c}</td>
<td>Mg\textsuperscript{2+}</td>
<td>100 ± 10</td>
<td>22 ± 1</td>
</tr>
<tr>
<td></td>
<td>Mn\textsuperscript{2+}</td>
<td>26 ± 5</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Arg-273</td>
<td>Mg\textsuperscript{2+}</td>
<td>170 ± 20</td>
<td>40 ± 8</td>
</tr>
<tr>
<td></td>
<td>Mn\textsuperscript{2+}</td>
<td>23 ± 3</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Observed elemental effect is calculated as \((k_{o, s})_{\text{NTP}}/(k_{o, s})_{\text{NTP\alpha S}}\).

\textsuperscript{b} Observed reaction rates were determined at 1mM ATP and ATP\textalpha S.

\textsuperscript{c} Value taken from (23,25).
evaluate directly the equilibrium constant for the first conformation-change step, $K_2$ (Fig. 6-4A). In the pulse-quench part of the experiment, preassembled 3Dpol-sym/sub complex is rapidly mixed with [$\alpha$-$^{32}$P]ATP and quenched at various time points by the addition of 2N HCl (Fig. 6-4B). For the pulse-chase part of the experiment the reaction is chased with excess unlabeled nucleotide (Fig. 6-4C). In this case, if the rate of the phosphoryl transfer step ($k_{chem}$) is relatively slow, and equilibrium constant $K_2$ favors the forward reaction, a definite amount of the activated ternary complex (*ER$_n$NTP) can be observed (Fig. 6-4A). If the equilibrium constant $K_2$ favors the reverse reaction and/or the chemistry step is fast, no activated species (*ER$_n$NTP) would be detected. The pulse-chase pulse-quench analysis was performed only in the presence of Mn$^{2+}$, because in Mg$^{2+}$ the rate for the chemistry step is too fast to allow detectable amounts of activated complex to accumulate.

For WT 3Dpol in the presence of Mn$^{2+}$ only the chemistry step stays rate limiting; the conformational-change step is strongly shifted in the forward direction with $K_2$ equal to 3 (Fig. 6-4D) (25). The combination of a relatively large $K_2$ and small $k_{chem}$ (30 s$^{-1}$) for WT 3Dpol leads to a visible lag for the pulse-quench trace (○) when compared to the pulse-chase trace (●) (25). These data provided convincing evidence that for the WT 3Dpol a large amount of the activated complex is accumulated, and the equilibrium for the first conformational-change step strongly favored the forward reaction. These data are consistent with the conformational-change step prior to the chemistry step not being rate-limiting when Mn$^{2+}$ is utilized as the metal cofactor (25). In the case of Arg-273 3Dpol the difference between pulse-chase trace (●) and pulse-quench trace (○) became much more pronounced (Fig. 6-4E). The best fit of the data to the minimal mechanism
Fig. 6-3: The observed kinetics of Arg-273 3Dpol-catalyzed AMP incorporation in the presence of Mg$^{2+}$ is quench agent-dependent. For this experiment 1 µM 3Dpol was incubated with 1 µM sym/sub (0.5 µM duplex), the reaction was initiated by the addition of AMP to a 500 µM final concentration. At various time points the reaction was quenched by addition of either EDTA (●) or HCl (○). EDTA fails to quench the reaction efficiently, especially at the early time points. This results in an artificially elevated observed rate constant for the nucleotide incorporation. The solid lines represent the fit of the data to a single exponential with $k_{obs}$ of 340 ± 60 s$^{-1}$ and 140 ± 10 s$^{-1}$, when quenched with EDTA and HCl, respectively.
A

\[ \text{ER}_n \text{NTP} \xrightleftharpoons[k_{-2}]{k_{+2}} ^* \text{ER}_n \text{NTP} \xrightarrow{k_{\text{chem}}} \text{ER}_{n+1} \text{PP}_i \]

B

Pulse-Quench

\[ 3D_{\text{pol}}^\text{sym/sub} \]

\[ [\alpha^{32}\text{-P}]\text{ATP} \]

Quench

\[ \Delta t \]

(O)

C

Pulse-Chase

\[ 3D_{\text{pol}}^\text{sym/sub} \]

\[ [\alpha^{32}\text{-P}]\text{ATP} \]

ATP

\[ \Delta t \]

30 sec

(●)

D

WT

\[ \text{[\alpha^{32}\text{-P}]AMP incorporated (\muM)} \]

\[ \text{Time (sec)} \]

E

Arg-273

\[ \text{[\alpha^{32}\text{-P}]AMP incorporated (\muM)} \]

\[ \text{Time (sec)} \]
Fig. 6-4: The conformational-change step preceding phosphoryl transfer is relaxed for Arg-273 3Dpol. (A) Minimal kinetic mechanism employed for simulation of the data obtained in pulse-chase pulse-quench experiment. ER$_n$NTP (ternary complex); *ER$_n$NTP (activated elongation complex); ER$_{n+1}$PPi (product complex). (B, C) Experimental design for the pulse-chase pulse-quench analysis. 4 µM 3Dpol was incubated with 20 µM sym/sub (10 µM duplex) and rapidly mixed with 100 µM [γ-$^{32}$P]ATP (3.8 Ci/mmol) (final concentration) as described under Experimental Procedures. At the indicated time, reactions were either quenched by addition of HCl or chased by addition of ATP to a final concentration of 20 mM. After addition of the chase ATP solution the reaction was allowed to proceed for an additional 30 s, at which time the reaction was quenched with HCl. (D) Kinetics of AMP incorporation by the WT 3Dpol. The solid line represents the kinetic simulation of the data fit to the mechanism shown in panel (A) with $K_2$ of 3 and $k_{chem}$ of 30 s$^{-1}$ for AMP incorporation. Data for WT 3Dpol-catalyzed AMP incorporation are taken from (25). The simulated curve of the pulse-quench data (○) predicts the rate of formation of all ER$_{n+1}$-containing species; the simulated curve of the pulse-chase (●) data predicts the rate of formation of *ER$_n$NTP and all ER$_{n+1}$-containing species. (E) Pulse-chase pulse-quench analysis of AMP incorporation catalyzed by Arg-273 3Dpol. The solid line represents the kinetic simulation of the best data fit to the mechanism shown in panel (A) with $K_2$ of 6 and $k_{chem}$ of 15 s$^{-1}$ for AMP incorporation catalyzed by Arg-273 3Dpol. The end-point in this experiment corresponds to the amount of the enzyme-sym/sub complex formed; higher salt concentration in the Arg-273 3Dpol preparation was the cause of the lower end-point observed for this enzyme.
shown in Fig. 6-4A is achieved only when the equilibrium constant over the conformational-change step, $K_2$, is set at 6 and $k_{-3}$ is set at 15 s$^{-1}$ (Fig. 6-4E). The observed increase in the equilibrium constant $K_2$ is consistent with our proposal that the conformational-change step preceding chemistry is more relaxed in the Arg-273 3Dpol. Relaxation in the equilibrium over the first conformational-change step, which usually serves as the first fidelity check-point during nucleotide incorporation, results in the decreased fidelity of the Arg-273 polymerase.

**Relaxed Conformational-change Step Results in Altered 3Dpol-RNA Complex Assembly Rate and Complex Stability**

To this day, the conformational-change step has been detected for the PV RdRp only kinetically. The physical nature of this step remains unclear. In order to form a complex competent for phosphoryl transfer, subdomain movements of the polymerase, along with reorientation of the bound nucleotide and RNA primer/template in the 3Dpol active site must occur during this step. Kinetic and thermodynamic constants, the observed phosphorothioate effect and pulse-chase pulse-quench analysis obtained for Arg-273 3Dpol led us to the conclusion that the conformational-change step favors the forward direction more strongly for this 3Dpol derivative. To evaluate if a relaxed conformational-change step can affect 3Dpol-RNA complex performance we examined the 3Dpol-sym/sub complex assembly rate, complex stability and inactivation of the Arg-273 3Dpol. Experiments were performed as described under Experimental Procedures; the results are summarized in Table 6-3. Intriguingly, both the observed complex assembly and its stability were affected. There was a 2-fold increase in the observed
assembly rate; however, the dissociation rate of Arg-273 3Dpol from sym/sub RNA was increased 3-fold (Table 6-3). Arg-273 3Dpol also exhibited lower thermal stability when assayed at 30 ºC in the presence of 500 μM ATP. The observed inactivation rate was of 0.02 s⁻¹, 2-fold faster than the rate of the WT 3Dpol inactivation. No structural information is available on how the enzyme binds to its RNA substrate, whether it has to go through an “open” conformation or not, but our kinetic study of the Arg-273 3Dpol gives us a glimpse of the connection between a lower apparent dissociation constant and relaxed conformational-change step subsequent to nucleotide binding with the enzyme-RNA complex stability.

X-ray Crystallographic Analysis of Arg-273 3Dpol Confirms only Minimal Changes to the Polymerase Structure

The Arg-273 3Dpol crystal structure was solved by Laura Marcotte in Dr. J.M. Hogle’s laboratory.

The complete crystal structure of the WT PV 3Dpol has been solved recently (5). Peersens’ group showed that disruption of the interface between the front of the thumb of one polymerase molecule and the back of the palm of the other (interface I) significantly increases 3Dpol solubility without affecting polymerase function (5). We took advantage of their findings and introduced mutations coding for Arg-273 into the 3Dpol expression vector with Lys-446 and Arg-455 changed to aspartic acid residues. The two later mutations efficiently disrupt the interface between the fingers and thumb subdomains of
Table 6-3: Rate of the Complex Assembly ($k_{ass}$) and Dissociation ($k_{off}$) and Enzyme Inactivation for WT and Arg-273 3Dpol in the Presence of Mg$^{2+}$.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{ass}$ s$^{-1}$</th>
<th>$k_{off}$ s$^{-1}$</th>
<th>$k_{inact}$ s$^{-1}$&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.04 ± 0.005</td>
<td>0.0002 ± 0.00006</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Arg-273</td>
<td>0.08 ± 0.01</td>
<td>0.0006 ± 0.0001</td>
<td>0.02 ± 0.003</td>
</tr>
</tbody>
</table>

<sup>a</sup> At 30 °C, at 500 µM ATP
3Dpol. The Arg-273 3Dpol crystal structure was solved. Surprisingly, it appeared essentially unchanged from the WT 3Dpol structure (Fig. 6-5). Even at the location of the Arg-273 mutation amino acid residues seemed to be unperturbed (Fig. 6-5B). Upon more rigorous structure examination a few places were identified in the Arg-273 crystal structure which exhibited slight changes. Both of these locations are identified by asterisks in Fig. 6-5A, and shown in detail in Fig. 6-5C. Only positions of a few amino acid side chains (Lys-133, Asn-132, Gln-134, and Tyr-267) where slightly modified when compared to the WT 3Dpol structure; meanwhile the backbone of the protein stayed essentially unchanged. Thus, only modest perturbations to the crystal structure of Arg-273 3Dpol were observed. Taken together with the altered rates for 3Dpol-RNA complex assembly and dissociation, we propose that the Arg-273 mutation mostly affects dynamics of 3Dpol, which cannot be assessed by the static crystal structure. The nature of these dynamic movements remains unsolved.
Fig. 6-5: X-ray crystallographic analysis of Arg-273 3Dpol confirms subtle (minimal) effects on the polymerase structure. (A) Superimposition of WT 3Dpol and Arg-273 3Dpol crystal structures. WT 3Dpol is in black (PDB code 1RA6) and Arg-273 3Dpol is in gray. Location of the Arg-273 mutation and areas that are perturbed the most are indicated by asterisks. Alignment of the two structures and the images were generated by using WebLab Viewer software (Molecular Simulations, Inc., San Diego, CA). (B) Close-up of the Arg-273 mutation area shown in panel (A). The Arg-273 mutation does not introduce any noticeable changes in the position of the protein backbone or adjacent amino acid residue side chains. (C) Close-up of the perturbed areas indicated in panel (A). The Arg-273 mutation causes only very minor changes in the amino acid residue positions located in the hinge of the fingers subdomain. The Arg-273 3Dpol crystal structure was solved by Laura Marcotte.
Biological Evaluation of the Arg-273 3Dpol Allele

Poliovirus with Arg-273 3Dpol Resembles WT PV in Tissue Culture

Arg-273 3Dpol exhibited an exciting phenotype in the biochemical assays. It is the first RdRp with a mutator phenotype which has activity in polymerization assays comparable to, if not better than, WT 3Dpol (Table 6-1). PV bearing the Arg-273 mutation in its 3Dpol gene would reveal to us the impact of a less faithful polymerase on virus fitness. Arg-273 PV could also be an ideal tool to test the error catastrophe theory (20).

RNA viruses exist as quasispecies, that is, viral genomes in a given virus population are extremely heterogeneous, with each particular member of the population differing from another by a discrete number of mutations. However, there is a limit to virus genome diversity. When the limit is exceeded the virus loses its fitness and is thought to be driven into error catastrophe (20). Various viral mutagens, such as ribavirin, could force a virus into error catastrophe by increasing the number of mutations in the viral genome (8). The Arg-273 virus would be a unique system for investigating error catastrophe. In contrast to use of synthetic mutagens (e.g. ribavirin), which can alter the replication environment for the virus thus complicating outcome interpretations, mutations in the Arg-273 virus will be brought about by the virus itself (2,26-28).

Arg-273 encoding changes were engineered into an infectious cDNA for PV. RNA was produced by in vitro translation and infectivity of viral RNA was evaluated by an infectious center assay at 39.5, 37 and 34 °C (Fig. 6-6A). Surprisingly, Arg-273 PV was able to establish productive infections comparable to that of WT PV (Fig. 6-6A). No
temperature sensitivity was detected. Arg-273 PV was able to replicate as efficiently as WT PV at both permissive temperatures, 34 and 37 °C, as well as at the elevated temperature, 39.5 °C.

The kinetics of virus growth was evaluated by a one-step growth curve assay, and again no difference between WT and Arg-273 PV was observed (Fig. 6-6B). Thus, we showed that poliovirus with a less faithful polymerase was viable, and did not display any complications in establishing an infection, at least when assayed in cell culture. The question of whether Arg-273 PV is attenuated in mice (our animal model for PV infection) will be addressed later in this chapter.

In order to determine whether RNA synthesis would be affected for Arg-273 PV, changes encoding Arg-273 were introduced into the PV subgenomic replicon (Fig. 6-6C). Viral RNA synthesis was evaluated using a PV subgenomic replicon assay at 37 and 32 °C. Exponential replication for both WT and Arg-273 replicons was observed, yielding more than a $10^5$ increase in luciferase activity at both temperatures tested (Fig. 6-6D). Thus, in spite of substantial biochemical differences discovered for Arg-273 3Dpol, Arg-273 PV exhibited infectivity and ability to establish a productive infection similar to WT PV. In addition, equivalent virus growth kinetics were observed in one-step growth curve experiments for the Arg-273 and WT PV, and no defect in viral RNA synthesis was detected in Arg-273 PV by subgenomic replicon assays.
Fig. 6-6: Arg-273 PV kinetics of growth in cell culture is similar to WT PV. (A) Arg-273 is not temperature sensitive. HeLa cells were infected with 50 PFU of either WT or Arg-273 virus and incubated at 39.5, 37 and 34 °C essentially as described under Experimental Procedures (infectious center assay). Both viruses were able to establish productive infections over the range of tested temperatures. (B) One-step growth curve experiment shows similar kinetics of virus production for Arg-273 and WT PV. For this experiment HeLa cells were infected at MOI (multiplicity of infection) of 10 with either WT or Arg-273 PV. Cells were incubated at 37 °C. At various time points, cells were harvested and produced virus was titered by infectious center assay. Both viruses revealed identical kinetics of virus production in tissue culture. (C) PV subgenomic replicon. This replicon has capsid-coding sequence replaced by a luciferase reporter gene. (D) Arg-273 replicon shows no defect in RNA synthesis at both 37 and 32 °C. Arg-273 (▲) replicon was compared to WT (■) and GAA (○) PV replicons at 37 and 32 °C. Translation of the replicon RNA produces a luciferase-polyprotein fusion, from which luciferase is released by 2A protease activity encoded in the P2 region of the PV genome. Replication is monitored indirectly by luciferase activity. The GAA replicon encodes inactive 3Dpol and functions as a control for luciferase activity derived only from the translation of the input RNA. This experiment was performed twice, with a representative experiment shown.
**Arg-273 PV Exhibits a Mutator Phenotype in Cell Culture**

Infectious center assays, one-step growth curve experiments and subgenomic replicon assays showed that Arg-273 mutation in the 3Dpol part of the PV genome did not cause any noticeable decrease in virus fitness. Nevertheless, Arg-273 3Dpol was much less faithful than the WT 3Dpol in elongation assays (Table 6-1), and the nucleotide analog ribavirin was a better substrate for Arg-273 3Dpol than for WT 3Dpol (Fig. 6-2D and Table 6-1). Therefore, we should see a more pronounced effect of ribavirin on virus replication when tested in cell culture. Indeed, as expected, the Arg-273 virus was more sensitive to ribavirin than WT PV (Fig. 6-7A). At the highest ribavirin concentration tested more than 100-fold difference in the virus production was observed.

Virus replication in the presence of guanidine chloride is another cell culture assay that is commonly used to test the viral mutation frequency. Guanidine chloride inhibits poliovirus replication; however, the resistance to guanidine can be conferred by a single mutation in the 2C-coding region (29). Because Arg-273 viral polymerase is more error prone than its WT counterpart, mutations conferring guanidine resistance should arise more frequently in the case of Arg-273 PV. In fact, about a 3-fold increase in the number of guanidine resistant viruses emerging during Arg-273 virus replication in the presence of 3 mM guanidine was observed (Fig. 6-7B). Elevated sensitivity of the Arg-273 PV to ribavirin and an apparent increase in guanidine resistance frequency exhibited by this virus provide sufficient evidence that Arg-273 PV displays a mutator
A

![Graph showing virus titer (PFU/ml) vs Ribavirin (µM) with data points for WT and Arg-273.](image)

B

![Bar chart showing Guanidine resistance frequency for WT and Arg-273.](image)

C

<table>
<thead>
<tr>
<th>Virus</th>
<th>Total number of mutations</th>
<th>Mutations per genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>22/86,700</td>
<td>1.9</td>
</tr>
<tr>
<td>Arg-273</td>
<td>65/161,330</td>
<td>3.0</td>
</tr>
<tr>
<td>Ser-64</td>
<td>4/86,700</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Fig. 6-7: Arg-273 has a mutator phenotype in cell culture. (A) Arg-273 PV is more sensitive to the RNA mutagen ribavirin. For this experiment HeLa cells were infected with either Arg-273 or WT virus at an MOI of 0.01 and treated with increasing concentrations of ribavirin. Upon cytopathic effect (CPE), progeny virus was harvested and titered on fresh cell monolayers. The mean and standard deviation of 3 independent experiments is shown. (B) Arg-273 shows increased guanidine resistance. Poliovirus resistance to guanidine can be conferred by a point mutation in the 2C-coding region (29). By measuring the number of resistant viruses in a population, an estimation of error frequency can be obtained. The error frequencies of WT and Arg-273 viruses were measured by a guanidine resistance assay (8). The apparent error frequencies were calculated as the titer (PFU/mL) on guanidine chloride treated plates divided by the titer on control plates. Arg-273 PV showed a 3-fold increase in the mutational error frequencies. (C) Sequencing analysis confirms an increased mutation rate for Arg-273 PV in tissue culture. In order to determine the mutation frequency in each poliovirus population, 24 independent poliovirus cDNA clones were obtained. Poliovirus cDNAs were generated by RT–PCR from viral RNA isolated from single plaques in a plaque assay. A significant difference in the number of mutations was observed between WT, Arg-273 and Ser-64 viruses (P<0.002, Mann-Whitney U-test). Values reported for WT and Ser-64 viruses are taken from (3). Experiments reported in panels (A) and (C) were performed by Dr. Marco Vignuzzi.
phenotype in tissue culture. Direct sequencing of viral isolates was also performed to confirm the increased mutation rate displayed by Arg-273 PV. WT and Ser-64 virus, which codes for a less error prone polymerase, were used as controls (3,4). It was shown previously, that Ser-64 virus population had 6-fold fewer mutations than WT population (Fig. 6-7C) (3). If Arg-273 3Dpol is a mutator polymerase, than the number of mutations observed among Arg-273 PV isolates should be greater than that for WT PV. Indeed, Arg-273 exhibited a 1.6-fold increase in mutational frequency, in good agreement with our biochemical studies.

Arg-273 PV was not Extinguished during Serial Passage

Both biological and biochemical studies of Arg-273 3Dpol confirmed its mutator phenotype. Thus, Arg-273 PV should intrinsically accumulate more mutations in its genome during virus replication. If the error catastrophe theory is correct, even small increase in mutation frequency could drive a virus to extinction (20). In order to test whether Arg-273 virus can “eliminate” itself, due to increased mutation rate of the Arg-273 3Dpol, serial passage of this virus was performed. For this experiment a fresh HeLa cell monolayer was infected with 2000 PFU of Arg-273 PV. WT virus was used as a positive control. CPE was observed for both viruses within 2 days of incubation at 37 °C. This result was not unexpected, as Arg-273 PV had the same kinetics of virus growth when tested in one-step growth experiments. For WT PV, when infection is allowed to go to CPE, the titer of the produced virus usually stays in the 2 x 10⁸ PFU/mL range. Therefore, the assumption was made for both WT and Arg-273 PV that viruses produced
and collected in the first passage had a titer of $2 \times 10^8$ PFU/mL. Accepting this number, 2000 PFU of passage 1 (P1) were used to inoculate a fresh HeLa cell monolayer for passage 2. If the fitness of Arg-273 virus would be compromised, then a lower titer of virus would be obtained during the first serial passage. Therefore, our assumption that the titer remains at $2 \times 10^8$ PFU/mL would not hold. This, in turn, would lead to a delay in the observed CPE. During the second passage, the same kinetics of CPE were observed for WT and Arg-273 PV. Serial passaging was repeated 2 more times. As no differences in CPE kinetics were detected, viral RNA from P1 and P4 was isolated and RT-PCR amplified in order to verify that the mutations coding for Arg-273 were still present in the viral genome. Electropherograms of the corresponding cDNAs are shown in Fig. 6-8. Codon AGG, which encodes Arg-273 was present in both passage 1 and passage 4 viruses. Thus, no virus extinction was observed for Arg-273 virus under the experimental conditions tested.

Arg-273 PV Exhibited Reduced Fitness Relative to WT PV in the Coinfection Experiment

In spite of Arg-273 3Dpol being much more error prone than WT 3Dpol and Arg-273 PV exhibiting mutator phenotype in cell culture experiments, no fitness defect was observed for Arg-273 PV in tissue culture. Previously, we may have failed to notice a defect in Arg-273 virus fitness because the virus had been evaluated in isolation. Therefore we decided to perform serial passage experiments, coinfecting HeLa cells with both WT and Arg-273 PV simultaneously. For this coinfection experiment, a mixture of WT and Arg-273 viruses in a ratio 1:10 (WT PV: Arg-273 PV) was generated. This virus
Arg-273 PV was not extinguished during serial passage. For this experiment, 0.5 x 10⁶ HeLa cells were plated in a well on a 6-well plate and infected the next day with 2000 PFU of Arg-273 PV and incubated at 37 °C until CPE. 2000 PFU of the virus obtained from passage 1 were then used to inoculate a fresh cell monolayer (titer of the virus from passage 1 was estimated at 2 x 10⁸ PFU/mL). A total of 4 passages were performed. Viral RNA from passages 1 and 4 was isolated and sequenced, as described under Experimental Procedures. Electropherograms of the viral RNA obtained from the Arg-273 PV serial passage experiment (passage 1 (P1) and passage 4 (P4)) are shown. The AGG codon, which codes for Arg-273 is underlined.
mixture was used to infect HeLa cells at an MOI of 10 (passage 1). Infection was allowed to proceed to CPE, which took 2 days at 37 °C. 120 µL of the virus from passage 1 were used to carry out the infection for passage 2. After CPE, virus was collected, and again 120 µL were used for the next passage. A total of 4 passages were performed. At that point viral RNA was isolated, RT-PCR amplified and sequenced. Electropherograms of the viral RNA obtained from serial passages 1 to 4 are shown (Fig. 6-9A). As expected, at passage 1 mostly Arg-273 RNA was detected, as the Arg-273 RNA constituted 90% of the viral RNA mix. At passage 2 (P2), however, peaks for the CAC codon (which codes for the WT residue His-273) appeared above the baseline. At passage 3 (P3) the amount of WT and Arg-273 viral RNA was approximately equal, and at passage 4 (P4) the fraction of WT RNA exceeded that of Arg-273 RNA. The ability of WT PV to easily outcompete Arg-273 virus shows that the mutant virus has substantially reduced fitness. When the coinfection experiment was performed at lower MOIs (1 and 0.1) WT PV was also able to outcompete Arg-273 PV, though with slightly slower kinetics (Fig. 6-9B, C). The fact that at lower MOI less PFU per cell is used for infection (MOI of 1 and 0.1 implies 1 PFU and 0.1 PFU per cell, respectively) could explain the slower kinetics of Arg-273 disappearance. At low MOI, technically no or very little direct competition occurs between WT and Arg-273 viruses, as even at an MOI of 1, a single viral particle (or PFU) is localized to a single cell. As we did not observe a fitness defect for Arg-273 PV when it was evaluated by itself, in isolation, its ability to withstand “competition” with WT PV at low MOI is not surprising.
Fig. 6-9: Arg-273 PV exhibits reduced fitness relative to WT PV in a coinfection experiment. (A) WT virus quickly out-competed Arg-273 PV during coinfection at MOI of 10. HeLa cells were infected with a 1:10 mixture of WT PV and Arg-273 PV at an MOI of 10 as described under Experimental Procedures. Electropherograms of the viral RNA obtained from the serial passage of the viral mixture are shown. At passage 1 (P1), mostly Arg-273 viral RNA is detected (underlined codon AGG codes for Arg-273). However, during the next three passages (P2-P4) WT RNA accumulates to a higher degree, slowly displacing Arg-273 viral RNA (codon CAC codes for the WT amino acid residue His-273). (B, C) Coinfections at MOI of 1 and 0.1 resulted in slower Arg-273 PV disappearance. In this experiment the coinfections were performed at MOI of 1 and 0.1, panels (B) and (C), respectively. The coinfections were not allowed to proceed to CPE, but instead were terminated at 8 hrs postinfection. This was done in order to limit the infections to only one round of replication. At an MOI of 1 each cell should be infected with only one virus; at an MOI of 0.1 – only 10% of cells should be infected. Therefore, WT and Arg-273 virus infections/replications should be separated and localized to a single cell. The rate of disappearance of Arg-273 PV, monitored by viral RNA isolation and sequencing, was slower at lower MOIs, pointing to the fact that the Arg-273 PV fitness defect can be observed only when in competition with a more fit virus (WT) and is less pronounced at lower multiplicities of infection (MOI of 1 and 0.1).
Arg-273 PV is greatly Attenuated in cPVR Mice

Arg-273 PV showed a defect in fitness only when in competition with a more fit virus (WT). In cell culture experiments, such as a one-step growth curve experiment, no difference between WT and mutant virus was observed. However, Arg-273 3Dpol was less faithful than WT 3Dpol in elongation assays. As previously mentioned, RNA viruses exist as quasispecies, and some diversity between individual viral genomes occurs. Because Arg-273 3Dpol is a more error prone polymerase, a population of virus generated during Arg-273 PV infection should be more diverse than a WT PV population. Previously, it was shown that for Ser-64 virus that encodes the more faithful Ser-64 3Dpol, a restricted quasispecies population was observed (3). Decreasing the quasispecies diversity for Ser-64 PV resulted in a substantial reduction of viral pathogenicity (Fig. 6-10B) (3). An Arg-273 virus population should be more diverse then either WT or Ser-64 virus populations. On one hand, greater sequence heterogeneity, characteristic of RNA viruses, is considered an advantage, allowing RNA viruses to adapt better to environmental pressure selection. On the other hand, increased levels of mutations can drive the virus to its error threshold. In other words, RNA viruses are adapted to the mutation frequency which allows them to rapidly respond to the changing environment but, at the same time, the number of mutations does not exceed the threshold value. Direct genome sequencing of Arg-273 PV showed that this virus had an elevated number of mutations compared to WT PV (Fig. 6-7C). Coinfection experiments with WT PV demonstrated that its fitness was compromised. The next question was whether Arg-273 PV would be able to establish a productive infection in an animal
model, and whether pathogenicity of the virus would be comparable to WT. Surprisingly, when assayed in an animal model, Arg-273 PV exhibited a highly attenuated phenotype (Fig. 6-10A, B). All of the cPVR mice, which were inoculated intramuscularly with the highest titer (4 x 10⁸ PFU) of Arg-273 virus, survived. In contrast, no mice survived infection when WT virus was used for inoculation, even at a 10-fold lower dilution (Fig. 6-10A, B). Arg-273 virus also showed restricted tissue tropism after intravenous inoculation into cPVR mice (Fig. 6-10C). Both WT and Arg-273 PV were readily isolated from spleen and muscle tissues; however, the titer of the produced virus was substantially lower for the Arg-273 PV. Spinal cord and brain tissue constitute primary sites of WT PV replication with very high viral load of the WT PV detected in these tissues after intravenous inoculation (Fig. 6-10C, D). Arg-273 PV was not able to spread and establish a productive infection in these tissues; no virus was detected in either spinal cord or brain (Fig. 6-10C). Thus, Arg-273 exhibits a greatly attenuated phenotype in the animal model.

**Transmission Electron Microscopy (TEM) Revealed that Arg-273 PV produced more Empty Viral Particles than WT PV**

Recently our lab began using electron microscopy for virus particle analysis. During virus infection both infective and non-infective viral particles are produced. Loss of infectivity by a viral particle can be potentially attributed to deleterious mutations in the viral RNA (which make it non-infectious), or simply to lack of RNA inside the particle. It is currently unknown how PV RNA is packaged inside virions, which viral or host proteins maybe involved in the packaging process, and which RNA determinants are
**A**

![Graph showing % of survival over days post inoculation.](image)

**B**

<table>
<thead>
<tr>
<th>Virus</th>
<th>LD₅₀ (PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.2 x 10⁶</td>
</tr>
<tr>
<td>Arg-273</td>
<td>&gt; 4 x 10⁸</td>
</tr>
<tr>
<td>Ser-64</td>
<td>3.9 x 10⁸</td>
</tr>
</tbody>
</table>

**C**

![Graph showing PFU/g in spleen, muscle, spinal cord, and brain.](image)

**D**

![Graph showing PFU/g in spleen, muscle, spinal cord, and brain.](image)
Fig. 6-10: Arg-273 PV was attenuated in cPVR mice. (A) Arg-273 PV is less neuropathogenic than WT PV. cPVR mice expressing the human poliovirus receptor were inoculated intramuscularly with serial dilutions of either WT or Arg-273 virus. Survival curves were generated by monitoring the percentage of surviving mice each day following infection. Values reported for Ser-64 viruses are taken from (3). The dose that is lethal to 50% of mice (LD$_{50}$) was determined by the Reed and Muench method, and is listed in panel (B). (B) H273R was attenuated in mice. LD$_{50}$ values for each virus stock are reported. Values reported for WT and Ser-64 viruses are taken from (3). (C, D) Arg-273 virus exhibited restricted tissue tropism. Virus titers in PFU per gram from tissue of mice infected intravenously with the WT, Arg-273 or Ser-64 virus. Mean values and standard deviations of three experiments are shown. Data reported for WT and Ser-64 viruses in panel (D) are taken from (3). Experiments reported in this figure were performed by Dr. Marco Vignuzzi.
essential for this process. In the case of Arg-273 PV we have a virus that displayed a higher intrinsic mutation rate, resulting in slightly increased genomic RNA sequence diversity. However, when no selection pressure was applied, this virus functioned similar to WT PV. We decided to evaluate whether an increased mutation rate would affect the empty vs. filled virus particle distribution. Unfortunately, we could not distinguish the possibilities that the increased proportion of empty particles was due to an increased number of mutations present in the viral RNA or due to viral proteins (potentially essential for RNA packaging) being affected by the increased mutation rate. When Arg-273 virus was visualized by TEM about 55 ± 5% of viral particles appeared to be empty (Fig. 6-11B). WT virus, on the other hand, had only 35 ± 5% of empty particles (Fig. 6-11A). Whether this difference in the ratio of empty/filled particles is important for virus survival/fitness needs to be determined. We also evaluated Ser-64 PV by using TEM. This PV, unlike the Arg-273 PV, encodes less error prone polymerase. Therefore, if the ratio of empty to filled particles is different for this mutant virus, we can begin establishing a correlation between the polymerase fidelity and RNA packaging. Surprisingly, Ser-64 virus had a slightly lower proportion of empty particles in purified virus preparations, around 33 ± 5% (Fig. 6-11C). These results require further investigation. However, use of TEM opens new doors to understanding processes such as RNA packaging. The increased proportion of empty viral particles observed for Arg-273 PV may suggest the possibility of another fidelity checkpoint which lies beyond the RNA synthesis step, fidelity control at the stage of RNA packaging.
Fig. 6-11: Arg-273 virus presents bottlenecks in RNA packaging. Transmission electron microscopy (TEM) analysis of purified viruses was performed as described under the Experimental Procedures. Representative TEM images at a magnification of 30X are shown. The white bar in each image represents 500 nm. White arrows point to virus particles containing RNA whereas black arrows point to empty viral particles. (A) TEM image of WT PV. Particles with no RNA constitute 35 ± 5% of total viral particles. (B) TEM image of Arg-273 PV. For Arg-273 PV empty particles constitute 55 ± 5% of total viral particles. (C) TEM image of Ser-64 PV. For Ser-64 PV empty particles constitute 33 ± 5% of total viral particles.
Discussion

RNA viruses are often characterized as viruses with high mutation rates. Usually RNA viruses exist as a quasispecies population. The lower fidelity of the RNA virus polymerase is thought to be the cause of the diversity seen among viral genomes in a given virus population. The ability of RNA viruses to mutate rapidly and easily is considered beneficial for the virus. Thus RNA viruses are able to escape immune responses and adapt quickly to new environmental conditions. The increased rate of mutation in RNA viruses is the cause of drug resistant strain emergence and ability of RNA viruses to cross species barriers, as in flu pandemics, where swine or bird flu can become infectious in humans. High mutation rates also make RNA viruses a difficult target for vaccine development. On the other hand, decreased fidelity of an RdRp can be turned against the virus. Thus, nucleoside analogs are efficiently incorporated by viral RdRps, and RNA virus infections can be treated with this class of the drugs (2,8). The mechanism of action of nucleoside analogs, such as ribavirin, has been termed lethal mutagenesis (8,20,28). It is generally accepted that due to the ability of these artificial compounds to be incorporated into an RNA viral genome, the virus can be driven to error catastrophe, that is, the number of mutations being introduced into the viral genome becomes higher than the virus can tolerate without losing its fitness (7-9,11,13,21).

First PV virus with an RdRp with altered fidelity was discovered by growing the virus in the presence of ribavirin (1,2). The polymerase from this virus contained a mutation; instead of Gly at position 64 there was Ser. This mutation conferred resistance
to ribavirin. Ser-64 3Dpol was shown to be more faithful than the WT polymerase (4). Both Ser-64 3Dpol and the virus have been characterized (2,3).

In this chapter, we describe biochemical and biological properties of Arg-273 3Dpol, a low fidelity polymerase. As mentioned previously, creation of Arg-273 mutation was a mere PCR error. The mutation was relatively far from the active site (~20 Å) (Fig. 6-1), and it was reasonable to propose that it would have minimal effect on polymerase activity. However, as mentioned above, the Ser-64 amino acid was also found at a site remote from the active site of the polymerase (Fig. 6-1) and this mutation strongly affected enzyme fidelity (4).

Basic biochemical evaluation of Arg-273 3Dpol showed that changing wild type residue His-273 to Arg resulted in a relaxed nucleotide selection. Arg-273 3Dpol was as efficient as the WT polymerase when correct nucleotide (AMP) incorporation was assayed using a sym/sub RNA primer/template complex (Fig. 6-2B). Nevertheless, Arg-273 3Dpol was able to incorporate incorrect nucleotides, GMP or RMP, much more effectively than the WT enzyme (Fig. 6-2C, D). As seen from Table 6-1, diminished fidelity of the Arg-273 mutant was reflected in both apparent dissociation constants, $K_{d,app}$, and maximal polymerization rate, $k_{pol}$, for correct and incorrect nucleotides. Interestingly, Arg-273 3Dpol exhibited an almost 2-fold faster polymerization rate in the case of correct ribonucleotide incorporation. However, Arg-273 virus had growth kinetics identical to WT PV (Fig. 6-6B). Perhaps a 2-fold increase in polymerization rate is not sufficient to produce an observable difference in the virus growth. Alternatively, Arg-273 3Dpol, or 3Dpol-containing precursors, may be compromised in their functions at stages of virus replication other than RNA synthesis, such as establishment of the
initiation complex. It is also possible that Arg-273 3Dpol is less processive than WT 3Dpol. Thus Arg-273 3Dpol had a 2-fold faster rate of complex assembly, but a 3-fold faster rate of the complex dissociation (Table 6-3). Along with the increased rate of 3Dpol-sym/sub complex assembly and decreased complex stability, Arg-273 3Dpol displayed an additional biochemical property unique to this enzyme. All Mg$^{2+}$-containing reactions catalyzed by any 3Dpol derivative evaluated to date can be efficiently quenched by addition of EDTA. Only when Mn$^{2+}$ is used as a metal cofactor is HCl quench required (23,25). Arg-273 3Dpol was an interesting exception in this respect; EDTA was incapable of efficiently stopping the reactions (Fig. 6-3). Therefore HCl quench was performed in all biochemical assays for the Arg-273 3Dpol derivative. The reason for the inability of EDTA to stop reactions catalyzed by Arg-273 3Dpol remains unclear.

If we consider the 5-step complete mechanism for single nucleotide incorporation (Fig. 1-5), it is clear that the maximal rate of nucleotide incorporation in the presence of Mg$^{2+}$ as a metal cofactor is governed by two steps, conformational-change step preceding phosphoryl transfer (step 2), and phosphoryl transfer (step 3) (23). For WT 3Dpol both of these steps are rate limiting, when Mg$^{2+}$ is employed as a metal cofactor; when Mn$^{2+}$ is employed, only chemistry step remains rate limiting (23,25). If we would compare the observed reaction rates for AMP incorporation both in the presence of Mg$^{2+}$ and Mn$^{2+}$ (Table 6-2) we would see that in the presence of Mg$^{2+}$ Arg-273 polymerase catalyzed reaction almost 2 times faster than the WT enzyme. However, when Mn$^{2+}$ was employed as a divalent cofactor, the rates for both enzymes became essentially the same (Table 6-2). The fact that phosphoryl transfer is the sole rate-limiting step in the presence of Mn$^{2+}$,
and the rates for the WT and Arg-273 were equal suggested that chemistry step should not be affected for the Arg-273 3Dpol derivative, and only conformational-change step preceding it should have been perturbed. Detailed evaluation of this step showed that the value of $K_2$ for Arg-273 3Dpol was, indeed, increased 2-fold relative to WT 3Dpol. Interestingly, Ser-64 3Dpol, the polymerase with higher fidelity, had the value of $K_2$ equal to 2; that is 3-fold reduction relative to WT 3Dpol. Thus, we concluded that conformational-change step preceding chemistry is the key determinant of 3Dpol fidelity, as only change in the equilibrium over this step was sufficient enough to alter fidelity of the polymerase either way.

Although Arg-273 mutation is located far away from the active site, somehow it affected the equilibrium over the conformational-change step. Molecular basis of the conformational change still remains unsolved. With all the crystal structures of DNA polymerases solved for apo enzymes and ternary complexes it is tempting to believe that the conformational changes observed crystallographically upon nucleotide binding could reflect the changes happening during catalysis (30-33). However, there is some evidence, that this might not be the case. Thus, the mutational analysis on DNA polymerase β suggested that rotation of the fingers domain observed crystallographically was not the conformational-change step detected kinetically (34). Instead, Vande Berg et al., proposed that the conformational-change step in DNA β polymerase was triggered by the open-to-close conformation switch and the subdomain closer was found to be fast, and not rate-limiting (35). In addition, Wilson’s group used fluorescence anisotropy decay to study microenvironment and dynamics of α-helix N in the polymerase β. Upon binding of the nucleotide N-helix interacts with the nascent base pair. The researchers found that
the segmental motions of the N-helix occur on a time scale of ~1 nsec and far more rapid than the step that limits chemistry (36). Additional evidence that open-to-close transition did not constitute the conformational change step was acquired during stopped-flow analysis for KF DNA polymerase by Purohit et al. (37). The authors proposed that the binding of the nucleotide (which in itself is a very fast step that cannot be resolved using stopped-flow apparatus) promotes open-to-close transition, which occurs at a very fast rate. Thus, an early rapid step was observed with a dideoxyterminated primer, suggesting that closed ternary complex forms immediately upon dNTP binding (37).

In the case of PV RdRp conformational-change step preceding chemistry has been identified only kinetically. As ternary complex structure is not yet available for this class of the enzymes, no structural information exists on the character of the subdomains or single amino acid residues movement upon nucleotide binding. For Arg-273 3Dpol the first conformational-change step was found to be more relaxed when compared to the WT 3Dpol. With the hope to gain some explanation on the change in the conformational change step observed kinetically for this 3Dpol derivative we performed an X-ray analysis. However, the crystal structure of an unliganded enzyme did not reveal any significant changes in the overall organization of the polymerase and was essentially identical to the WT 3Dpol structure, with only minor changes in the position of a few amino acid residues detected (Fig. 6-5). Although it was somewhat disappointing, but not a completely unexpected result, as crystal structure represents only a static picture of the enzyme three-dimensional organization. Conformational-change step by definition is a dynamic step that may involve but not limited to movements of the subdomains, rotations of single amino acid residues in the nucleotide-binding site, realignment of the
bound nucleotide that would result in the reorganization of the ternary complex into the conformation appropriate for the catalysis. Overall molecular flexibility of the 3Dpol may play crucial role in the polymerase fidelity, rather than structural rearrangements of its subdomains. Perhaps, use of other experimental techniques, such as circular dichroism anisotropy, or stopped-flow analysis could help us to elucidate the molecular details of the conformational change.

After confirming mutator phenotype for Arg-273 3Dpol biochemically we decided to investigate what impact low fidelity polymerase will have on the virus replication. Interestingly, Arg-273 PV did not exhibit any fitness defects, when it was evaluated alone (Fig. 6-6). Thus, Arg-273 PV was able to establish productive infection at 34, 37 and 39.5 °C as efficiently as WT PV (Fig. 6-6A). The kinetics of virus production under one-step growth curve conditions was undistinguishable from the WT counterpart (Fig. 6-6B). When RNA replication was evaluated using WT and Arg-273 PV genomic replicons, no difference in RNA accumulation was detected (Fig. 6-6D). Although Arg-273 PV functioned very similar to WT virus during basic biological evaluation, Arg-273 still exhibited mutator phenotype in cell culture. As expected, Arg-273 PV was much more sensitive to ribavirin, a nucleoside analog used to treat RNA viral infections (Fig. 6-7A) (9,15,17,24). At highest concentration of ribavirin tested (600 μM), the titer for Arg-273 PV was reduced more than 102-fold, when compared to the titer of WT virus (Fig. 6-7A). Consistent with the mutator phenotype, Arg-273 PV had ~ 3-fold increase in the guanidine resistance frequency (Fig. 6-7B). Direct sequencing of viral genomes showed that, indeed, Arg-273 PV accumulated, on average, 3 mutations per genome, while for the WT and Ser-64 viruses these numbers were 1.9
and 0.3 mutations per genome, respectively. Thus, in spite of similarity between WT and Arg-273 virus behavior in tissue culture, Arg-273 virus did accumulate more mutations in its genome. Although no obvious differences were observed during Arg-273 PV replication, the increased mutation rate of this mutant virus may slowly reduce virus fitness, if the mutations would accumulate over time. We made an attempt to test this hypothesis by serially passaging the Arg-273 virus. Under the experimental conditions tested, Arg-273 PV did not extinguish itself. The presence of the mutation coding for arginine at position 273 was verified by RT-PCR sequencing (Fig. 6-8). While we are still unsure why we do not see Arg-273 virus extinction, or at least gradual decrease in the titer, there are some feasible explanations to the results observed. First, the viral infection was allowed to achieve CPE, and this gave enough time for the virus to spread out to multiple cells, and the coinfection of a single cell by multiple viral particles could have occurred. If a deleterious mutation is introduced into a viral genome during coinfection, this genome can be rescued by other viral genomes replicating within the same cell. In order to decrease genetic complementation we are currently performing serial passage experiment at low multiplicity of infection (MOI of 0.1 and 0.01). In addition, the infection cycle was limited to 8 hrs to eliminate virus spread and coinfection. Although these experiments are still underway, some promising results were obtained. When serial passage was performed at MOI of 0.01 Arg-273 PV showed the decrease in titer after six consecutive passages. The experiments to confirm this observation are being conducted.

In spite of terms “error catastrophe” and “lethal mutagenesis” being used among virologists for decades (2,8,11,13-16), the theoretical base for the field was lacking.
Recently, J. J. Bull, R. Sanjuàn and C.O. Wilke published a “Theory of lethal mutagenesis for viruses” (20). In order to define the extinction threshold, the authors embrace its evolutionary component, based on the mutation rate, and its ecological component, virus population within an infected host organism. In this work, lethal mutagenesis threshold is defined by both mutation rate and fecundity (inoculum size). The relationship between mutation rate and fecundity are log-linear, and changes in the mutational rate should have much larger effect on extinction than changes in fecundity. According to this theory Arg-273 PV, with an increased mutation rate should achieve extinction at a higher than the WT PV inoculum size. Evaluation of higher fidelity mutant virus, Ser-64, paired with WT and Arg-273 PV, gives us a unique opportunity to test the new theory of lethal mutagenesis for viruses.

While no fitness defect was observed for Arg-273 PV when it was examined alone, it did exhibit decreased fitness in competition with the WT PV. In coinfection experiment, when the Arg-273 PV was mixed with WT PV in a ratio 10:1, WT PV was slowly outcompeting Arg-273 virus over a few serial passages (Fig. 6-9). Interestingly, Arg-273 PV was disappearing much faster at MOI of 10 than at lower MOIs of 1 or 0.1 (compare Fig. 6-9A to B, C). During coinfection experiment at MOIs of 1 and 0.1 we limited the time of infection to 8 hrs in order to prevent CPE and virus spread to the neighboring cells. The reason for this change was coming from slower than expected kinetics of Arg-273 PV disappearance. In similar experiment, performed with Ser-64 PV at MOI of 10, Ser-64 virus was outcompeted by WT virus by passage 2 (4). In the case of Arg-273 PV, even by passage 4 the mutant virus was not completely displaced by WT virus (Fig. 6-9A). Originally, we thought that decrease in MOI and shortened time for
the infection will help us to eliminate any genomic complementation of Arg-273 virus by
the WT virus. If indeed, Arg-273 viral genomes were rescued by the presence of the WT
genomes during coinfection at MOI of 10, an infection at low MOI for a limited time
should prevent virus complementation and we would see more rapid Arg-273 PV
disappearance. However the result was totally opposite to what we expected; Arg-273
virus exhibited slower kinetics of disappearance at lower MOIs (Fig. 6-9B, C).
Therefore, we can conclude, that WT virus did not help Arg-273 PV to survive for longer
time at MOI of 10 by genetic complementation. And we observed slower kinetics of
Arg-273 PV disappearance at low MOIs, because at multiplicity of infection of 1 and 0.1,
there should have been 1 or 0.1 viruses replicating per cell, respectively, and Arg-273
virus did not face competition from WT virus. Overall, these results showed us, that the
defect in Arg-273 PV fitness could be detected only in the competition with the WT.

The next question addressed was the behavior of Arg-273 virus in an animal
model. Virus interaction with the host organism defines most selective pressures that
determine virus survival and spread. The high fidelity virus, Ser-64, showed attenuated
phenotype in PV susceptible mice (3). However, decreased diversity in the viral genome
population was the cause of diminished pathogenicity observed for this virus (3). In the
case of Arg-273 PV a more broad genomic diversity of virus population should exist due
to the lower fidelity polymerase. It is though that for an asexually reproducing organism
an optimal genomic mutation rate should exist (38,39). The optimal mutation rate
depends on the correlation of mutation rate and fitness (38). The example of Ser-64 PV
showed that the virus with increased fidelity paid a fitness cost (3). If the WT virus
evolutionary evolved to exist at an optimal mutation rate, than even slight increase in the
mutation rate detected for Arg-273 PV should result in its fitness decrease (Fig. 6-7C). Intriguingly, Arg-273 PV was severely attenuated in mice (Fig. 6-10). Arg-273 PV was not lethal to mice, even at highest PFU dilution employed. Although mice developed some signs of paralysis, all animals were able to recover from the disease. To the contrast, Ser-64 PV proved to be more pathogenic than Arg-273, with an LD$_{50}$ of 3.9 x 10$^8$ PFU, and WT virus was most deleterious, with LD$_{50}$ of 1.2 x 10$^6$ PFU (Fig. 6-10B). Arg-273 PV, like Ser-64 PV, exhibited restricted tissue tropism after intravenous inoculation into cPVR mice (Fig. 6-10C, D). Both mutant viruses were readily detected in spleen and muscle tissues, but failed to establish infection and replicate effectively in the primary sites of WT PV replication, in spinal cord and brain (Fig. 6-10C, D). Arg-273 PV appeared to be even more attenuated than Ser-64, the titers of virus recovered from spleen and muscle tissue were at least 10-fold lower than the titer for Ser-64. It was shown, that in the case of Ser-64 virus the attenuated phenotype was due to the restricted nature of quasispecies, characteristic of this virus (3). Artificial expansion of the genomic diversity for Ser-64 PV completely restored its pathogenicity and tissue tropism to the WT level (3). Arg-273 PV intrinsically should have a more heterogeneous genomic population due to the error prone polymerase. But higher mutation rate exhibited by Arg-273 PV was not beneficial for virus replication in the animal host. The attenuated phenotypes displayed by the low and high fidelity viruses evaluated suggest that the WT virus has evolutionarily evolved to have an optimal mutation rate and, as a consequence of correlation between mutation and adaptation rates, to demonstrate better fitness.
Another interesting difference was observed for Arg-273 PV during transmission electron microscopy (Fig. 6-11). During infection WT virus generates both infectious and non-infectious particles. Lack of infectivity can be caused by either mutated or damaged RNA, or simply by absence of RNA in virions. Empty viral particles can be distinguished from filled particles by transmission electron microscopy (Fig. 6-11) (40). The ratio of filled to open particles has not been yet investigated. When we used sucrose cushion-purified WT virus preparation, on average, 35 ± 5% of viral particles appeared to be empty (Fig. 6-11A). Interestingly, for Arg-273 virus the proportion of empty particles increased to 55 ± 5% (Fig. 6-11B), while higher fidelity Ser-64 virus showed distribution similar to WT virus, around 33 ± 5% empty particles (Fig. 6-11C). The TEM data suggests that Arg-273 PV may have a defect at the stage of viral RNA packaging. The process of RNA packaging and virion maturation is still highly unexplored. It appears that translation, replication and RNA packaging are all coupled events (41,42). Based on the experimental data obtained for Arg-273 PV, the virus did not have any defect in either replication or virus growth (Fig. 6-6). Therefore an intriguing hypothesis to explain a larger fraction of empty particles observed for Arg-273 would be an existence of some kind of proofreading mechanism at the stage of RNA packaging. Of cause, further investigation is required in order to explain different empty to filled virions ratio, observed for Arg-273 poliovirus.
References

Chapter 7

General Discussion and Future Directions

General conclusions

Despite the tremendous progress in vaccine and antiviral drugs development that has been made during the last few decades, the diseases caused by RNA viruses, such as hepatitis C, West Nile virus and influenza still impose serious economic and health threat to our society (1-9). In the United States alone, more than 3.9 million of its citizens are estimated to be infected with HCV (2). Current HCV therapy, which includes combination of ribavirin and interferon alfa, is at best effective only in ~ 50% of all patients; the majority of patients develop chronic infection that can lead to cirrhosis and hepatocellular carcinoma; 40% of chronic liver disease cases are HCV related (2,3). Another severe problem arises from coinfection with various viruses. Over the last ten years treatment of HIV infection was taken to a new level after introduction of a number of very effective antiretrovirals; current strategies, that usually include combination of at least three antiviral drugs, have significantly reduced morbidity and mortality (10,11). But more than 300,000 HIV patients are coinfected with HCV, and liver disease has become a leading cause of death among HIV-infected patients (12). In addition, emergence of new viruses, like SARS, and re-emergence of yellow fever, Hanta, Dengue and avian influenza viruses has a growing impact worldwide. Therefore there is still desperate need for the creation of more robust antiviral therapeutics and effective and
safe vaccines. Understanding the mechanisms of virus replication, virus-host interaction, and functioning of viral enzymes can strongly benefit new antiviral drug discovery.

A common strategy in battling viral diseases is to inhibit virus genome replication. In the case of RNA viruses, viral RNA polymerases represent an excellent target, as these enzymes are solely responsible for virus genomic RNA replication. In addition, mammalian cells lack RdRp activity, and drugs aimed at inhibition of the viral enzymes may have lower cellular toxicity, leading to fewer side effects, commonly associated with DNA polymerase inhibitors.

Polymerase inhibitors generally are divided into two large groups: nucleotide mimics, compounds that resemble natural nucleotides that can serve as substrates for viral polymerases; and non-nucleoside inhibitors, small molecules that can bind to the polymerase and alter/inhibit its function. Understanding the major principles of nucleotide selection will definitely facilitate rational drug design and also shed light on potential mechanisms of drug resistance that could arise when nucleotide analogues are employed as antiviral agents.

Research performed in our lab has illuminated some aspects of the PV RdRp fidelity and nucleotide selection (13-16). The work presented in this thesis is a logical continuation of our studies that target structure-function relationships in PV 3Dpol and a mechanistic basis for fidelity (chapters 3 and 4). Chapters 5 and 6 represent two exciting stories that branched out of the 3Dpol structure-function relationships studies and expanded to the level of virus replication, virus-host interaction, and to the field of lethal mutagenesis.
In chapter 3, the studies that continue the evaluation of the ribose-binding pocket of PV 3Dpol are presented. We created the Glu-297 3Dpol derivative in anticipation to make an enzyme that would be able to discriminate deoxyribonucleotides more efficiently than WT 3Dpol. However, Glu-297 3Dpol had exhibited only a modest change in nucleotide selection; elevated discrimination of deoxyribonucleotides was mainly manifested by an increase in the apparent dissociation constants for dNTPs. However, despite only a 10-fold decrease in the polymerization rate in the elongation assays for rNTPs, the Glu-297 mutation was detrimental for the virus. Analysis of the VPg uridylylation reaction catalyzed by Glu-297 3Dpol revealed that this 3Dpol derivative is much less efficient and processive during uridylylation. The defect in uridylylation exhibited by this 3Dpol derivative was suggested to be the cause of the observed severe virus replication defect.

In chapter 4 the detailed kinetic evaluation of Glu-297 3Dpol is summarized. We showed that the conformational-change step preceding phosphoryl transfer is the key determinant of an increased selection against deoxyribonucleotides exhibited by this 3Dpol derivative. Analysis of the reverse reaction catalyzed by Glu-297 3Dpol resulted in an exciting finding that substitution of Asn-297 in the nucleotide-binding pocket for Glu (an amino acid residue found in phage polymerases) resulted in a polymerase capable of dNTP excision. This discovery could, perhaps, explain the presence of Glu in phage polymerases. Phage replication occurs inside the prokaryotic cells, where phage RdRp faces mixed pools of dNTPs and rNTPs; on average dNTP concentrations in bacterial cells are a few orders of magnitude higher than in mammalian cells, and perhaps, phage
RdRps utilize glutamic acid in their ribose-binding pocket for dNTP removal (Appendix C, Table C-1).

The objective of the studies presented in chapter 5 was to evaluate the importance of the fingers-thumb interface integrity. However, the Phe-30 to Tyr amino acid substitution did not affect 3Dpol activity. Contrary to our expectations neither polymerase-RNA complex assembly nor its stability was affected. On the other hand, biological evaluation of the Tyr-30 PV revealed an exciting discovery that the fingertips-thumb interface modulates 3CD function during virus replication. A small plaque phenotype and significant decrease in the kinetics of capsid proteins were observed for Tyr-30 PV. The mechanism of PV maturation and RNA packaging still remains unknown. In this chapter we present both biochemical and biological evidence that 3CD is an important player in this process. In addition, the fingers-thumb interface is highly conserved among picornaviruses and represents a unique and novel target for antiviral drug development.

Finally, in chapter 6 the first polymerase and PV with mutator phenotype are described. As mentioned above, the discovery of the Arg-273 mutation within 3Dpol was a mere serendipity. Biochemical evaluation of Arg-273 3Dpol revealed that the conformational-change step prior to the chemistry step is the key determinant of the relaxed fidelity exhibited by this 3Dpol derivative. X-ray analysis of Arg-273 3Dpol, however, did not unveil any structural features in the enzyme that may have caused mutator phenotype. This finding suggests that dynamic movements of the enzyme during nucleotide incorporation are essential in modulating its fidelity.
PV bearing the Arg-273 mutation exhibited mutator phenotype as well. Thus, the Arg-273 PV was much more susceptible to treatment with ribavirin; this virus also demonstrated a 3-fold increase in guanidine resistance. Because Arg-273 3Dpol has higher misincorporation rates, Arg-273 PV can be utilized as a model for screening antiviral compounds in tissue culture experiments. Although, Arg-273 PV showed kinetics of replication identical to those of WT PV when assayed in tissue culture alone, Arg-273 PV displayed a strongly attenuated phenotype in the competition assays and in an animal model. The latter finding, taken together with the similar phenotype displayed by the Ser-64 PV, implies that WT PV exhibits optimal mutational frequency, and either an increase (Arg-273 PV) or decrease (Ser-64 PV) in the mutational frequency considerably compromises virus fitness. In light of the recently proposed theory of lethal mutagenesis for viruses Arg-273 PV (in combination with WT and Ser-64 PV) represents a unique empirical system to provide experimental evidence either in support or opposition for the theory (17). Understanding relationships between virus mutation frequency and fidelity in the light of virus-host interactions is essential for successive development of new antiviral strategies and treatments.

**Future directions**

*Additional studies of the conformational-change step preceding phosphoryl transfer*

We recently developed a stopped-flow fluorescence assay for 3Dpol using an RNA template containing 2-aminopurine for monitoring the rate of single nucleotide
incorporation (18). For WT 3Dpol at 30 °C only a single decay of the fluorescence signal occurs, the rate of the decay corresponds to the rate of nucleotide incorporation, reported by a chemical-quench-flow assay (18). For other polymerases usually more than one fluorescent transition occurs; the rate of one transition that differs substantially from the polymerization rate determined by chemical-quench-flow technique is frequently assigned to the conformational-change step rate (19-23). In the case of WT 3Dpol the fast rate of the chemistry step masks a slower conformational change, resulting in only one detectable fluorescence transient. However, it was observed that for the 3Dpol derivatives with lower $k_{pol}$ values (His-359, Arg-359) there is a rapid increase in the fluorescent transient, followed by decay (Castro, C., personal communication). The rate of the fluorescent decay corresponded to the polymerization rate; whereas the rate of the first fluorescence transient may reflect the rate of the conformational change step. I propose to further investigate the phenomenon of the first fluorescence transient, using Glu-297 and Ser-64 3Dpol derivatives. For both of these enzymes it was shown biochemically that the equilibrium constant over the conformational-change step preceding chemistry was decreased when compared to WT 3Dpol; and that both enzymes exhibited lower than WT 3Dpol polymerization rates (chapter 4 and (15)). In addition, decreasing the temperature at which reactions are performed could significantly slow down the chemistry step, and allow the detection of the conformational-change step even for the WT 3Dpol. Recently, use of various viscogenes and thiosubstituted nucleotide analogs (NTPαS) in the study of the conformational-change and chemistry steps in DNA polymerase β have been reported (19). Increase in the reaction buffer viscosity should decrease the rate of the conformational-change step, without affecting chemistry, whereas
α-thiosubstituted nucleotide analogues could influence the rates of both steps, since conformational change and chemistry are both involved in modulating 3Dpol fidelity (13,14). Another experimental approach for investigating the conformational-change step may involve the utilization of a 3’-deoxyterminated primer-template substrate; this substrate may allow us to eliminate potential complications arising from the chemistry or translocation steps. Unfortunately, the Arg-273 3Dpol, a low fidelity derivative, was not amenable to stopped-flow analysis; the rate of nucleotide incorporation was too fast to monitor by this technique (data not shown). However, performing incorporation assays using the stopped-flow technique at decreased temperatures and employment of thiosubstituted nucleotide analogs may help to overcome this difficulty.

**Further analysis of the structural basis for 3Dpol fidelity**

Crystal structures for all three polymerases with different fidelity – WT, Arg-273 and Ser-64 3Dpol, are now available (24,25). Because the crystal structure of Arg-273 3Dpol did not reveal any significant changes that could have accounted for the observed mutator phenotype, exhibited by this 3Dpol derivative, we proposed that the dynamics of the protein during catalytic cycle were affected by His-273 to Arg mutation. One of the ways to assess protein dynamics is the use of computational simulations (26). The simulations can be performed for all three polymerase derivatives in order to predict the range of subdomain movements (WT, Arg-273, and Ser-64). If indeed the polymerases with altered fidelity (Arg-273 and Ser-64) exhibit noticeable differences in the subdomain movement dynamics, then a 3Dpol library of variants could be created and
evaluated *in silico*. The mutations that would produce similar to Arg-273 and Ser-64 phenotypes would be then selected for further biochemical evaluation. If this approach proves itself useful it could be extrapolated to analysis of the polymerase interactions with potential inhibitors and/or surface-binding molecules that could alter polymerase fidelity. Thus prospective drug candidates could be first screened by using computer simulations.

**Theory of lethal mutagenesis**

Recently a theoretical framework for the lethal mutagenesis for viruses has been developed (17). This theory ties up two components of lethal mutagenesis: evolutionary, that involves deleterious mutation rate; and demographic, an inoculum size, specific to the infection or fecundity. The theory makes a few intriguing predictions for virus behavior within an infected host organism. Lethal mutagenesis is considered as a progressive decline and not a sudden drop in the viral fitness; the fitness of viral population can stay above the extinction threshold for a few generations. According to this theory, the extinction threshold for lethal mutagenesis represents a dynamic relationship between the deleterious mutation rate \(U_d\) and the fecundity of the infection (Fig. 7-1). Thus, the size of the viral population within the infected host is an essential determinant of the extinction threshold. After this threshold is reached the virus multiplication still continues, but at the rate that is not sufficient to maintain virus population size. According to this theory, even WT PV could be driven to the extinction at a very low inoculum size. Because the Arg-273, Ser-64 and WT PV exhibit different
mutation frequencies, their extinction should occur at various inoculum sizes; that is, for Arg-273 PV the inoculum size should be higher than for the WT and Ser-64 PV, because Arg-273 virus encodes a more error prone polymerase, and the intrinsic mutation rate of the Arg-273 virus is higher than the mutational rates of the WT and Ser-64 PV. In order to test this hypothesis we are currently performing serial passage experiments at the MOIs of 0.1, 0.01 and 0.001. The preliminary results were promising, as Arg-273 PV showed decrease in the titer of the generated virus after a few passages (Appendix D, Fig. D-1). Performing serial passage assays at low MOI for Arg-273, WT, Ser-64 at an elevated temperature (39 °C) will add an extra selection pressure on the replicating viruses and may help us achieve extinction even more quickly. Availability of the three replication competent viruses with a normal (WT), low (Arg-273) and high fidelity (Ser-64) polymerases represent a unique biological system for addressing the issues of virus evolution, virus-host interaction, and lethal mutagenesis as an antiviral strategy.
Fig. 7-1: Determination of the lethal mutagenesis and virus extinction threshold. Lethal mutagenesis threshold according to mutation rate $U_d$ and maximum fecundity, $R_{\text{max}}$, from inequality 7-1:

$$e^{-U_d R_{\text{max}} < 1}$$  \hfill (7-1)

The relationship is log-linear, so that changes in mutation rate have a much larger effect on extinction than changes in fecundity. In turn, modest increases in mutation rate, especially for RNA viruses, may be especially amenable to achieving extinction (taken from (17)).
Biochemical and biological evaluation of the Ser-64/Arg-273 3Dpol

Gly-64 to Ser mutation resulted in a much more faithful polymerase than the WT 3Dpol. The mutation that changed His-273 to Arg produced a mutator polymerase. Both of these mutations were shown to change fidelity of the 3Dpol by altering the conformational-change step preceding chemistry (chapter 6 and (15)). It would be appealing to combine these two mutations together. It is difficult to predict whether combination of these two mutations will be additive and yield a polymerase with close to the WT 3Dpol phenotype or whether one of the mutations’ phenotype will prevail. In 3Dpol Arg-273/Glu-297 derivative Arg-273 mutator phenotype was masked by the second mutation; this double mutant exhibited a phenotype very similar to the single mutant, Glu-297 3Dpol (Appendix B, Table B-1). Evaluation of the double mutant Ser-64/Arg-273 3Dpol, both biochemically and in the context of the poliovirus, will promote our better understanding of the mechanisms that tune polymerase fidelity.

References

Appendix A

Introduction of Additional Binding Steps and Stalling into Kinetic Mechanism for Successive 2 Nucleotides Incorporation Significantly Improves Fit of the Data

In Fig. A-1 one of the new mechanisms is shown. Because both nucleotides were present at very high concentrations (5 x $K_{d,app}$ value), we first added two extra steps that accounted for misincorporation events. Addition of these two steps significantly improved fit of the data to simulation traces (Fig A-1). Addition of the translocation step either before or after translocation alone also improved the fit of the data (Fig. A-2 and Fig. A-3). However, the best fit of the data to simulations was achieved when misincorporation steps were combined with the stalling step either before or after translocation (Fig. A-4).
Fig. A-1: Introduction of additional binding steps into kinetic mechanism for successive 2 nucleotides incorporation significantly improves fit of the data. (A) Modified kinetic mechanism for 2-nucleotide incorporation analysis. Because the nucleotides were present at very high concentrations (5 x $K_{d,app}$) in the reactions examined, additional steps were introduced into the mechanism in order to account for misincorporation events. Introduction of two binding steps placed some restrictions on the flexibility of the translocation step rates. (B, C) Subsequent incorporation of AMP and UMP into S/S-U by WT and Glu-297 3Dpols. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (B) or Glu-297 (C) 3Dpols. The solid lines represent simulation to the mechanism shown in panel A. For WT 3Dpol $k_{+transl}$ was fixed at 250-500 s$^{-1}$ and $k_{-transl}$ was insensitive to the simulation and in the range from 0 to 1000, with lower limit estimated at 0.0025 s$^{-1}$ (1). Thus, the upper limit for $K_{transl}$ was 0.5. In the case of Glu-297 values for $k_{+transl}$ and $k_{-transl}$ were more flexible, the lower limit for $K_{transl}$ was 5-10, and $K_{transl}$ fixed at 5. (D, E) Consecutive incorporation of dAMP and dUMP into S/S-U by WT and Glu-297 3Dpols. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (D) or Glu-297 (E) 3Dpols. The solid lines represent simulation to the mechanism shown in panel A. For both WT in the case of successive incorporation of the two deoxyribonucleotides $k_{+transl}$ was fixed at 30 s$^{-1}$, $k_{-transl}$ was varied from 0 to 100 s$^{-1}$, therefore the upper limit for $K_{transl}$ was 0.3. For Glu-297 3Dpol $k_{+transl}$ was fixed at 0.07 s$^{-1}$, and $K_{transl}$ were flexible with the best fit of data to the simulation achieved at $K_{transl}$ of 0.07.
Fig. A-2: Introduction of additional stalling step prior to translocation into kinetic mechanism for successive 2 nucleotides incorporation improves fit of the data. (A) Modified kinetic mechanism for 2-nucleotide incorporation analysis with stalling happening before translocation step. Addition of the stalling step (\(k_{\text{stall}}\)) before translocation allows achieving good agreement between the simulation traces and the experimental data. (B, C) Subsequent incorporation of AMP and UMP into S/S-U by WT and Glu-297 3Dpols. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (B) or Glu-297 (C) 3Dpols. The solid lines represent simulation to the mechanism shown in panel A. For WT 3Dpol \(k_{\text{transl}}\) was fixed at 250-500 s\(^{-1}\) and \(k_{\text{transl}}\) was insensitive to the simulation and in the range from 0 to 1000, with lower limit estimated at 0.0025 s\(^{-1}\) (1). Thus, the upper limit for \(K_{\text{transl}}\) was 0.5. The rate constant for the stalling step, \(k_{\text{stall}}\), was set at 10 s\(^{-1}\). In the case of Glu-297 values for \(k_{\text{transl}}\) and \(k_{\text{transl}}\) were more flexible, the lower limit for \(k_{\text{transl}}\) was 5-10, and \(K_{\text{transl}}\) fixed at 5. The rate constant for the stalling step, \(k_{\text{stall}}\), was set at 50 s\(^{-1}\). (D, E) Consecutive incorporation of dAMP and dUMP into S/S-U by WT and Glu-297 3Dpols. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (D) or Glu-297 (E) 3Dpols. The solid lines represent simulation to the mechanism shown in panel A. For WT 3Dpol in the case of successive incorporation of the two deoxyribonucleotides \(k_{\text{transl}}\) was fixed at 30 s\(^{-1}\), \(k_{\text{transl}}\) was varied from 0 to 100 s\(^{-1}\), therefore the upper limit for \(K_{\text{transl}}\) was 0.3; \(k_{\text{stall}}\), was 2 s\(^{-1}\). For Glu-297 3Dpol \(k_{\text{transl}}\) was fixed at 0.07 s\(^{-1}\), and \(k_{\text{transl}}\) were flexible with the best fit of data to the simulation achieved at \(K_{\text{transl}}\) of 0.07; \(k_{\text{stall}}\), was 0.025 s\(^{-1}\).
**A**

\[
\begin{align*}
&K_{d, X} \quad k_{\text{pol, } X} \quad K_{d, Y} \quad k_{\text{pol, } Y} \\
&ER_n + X \rightleftharpoons ER_nX \rightarrow ER_{n+1} \rightleftharpoons FR_n + Y \rightarrow FR_{n+1} \rightarrow FR_{n+2}
\end{align*}
\]

**B**

- [11, 12-mer RNA] (nM) vs. Time (sec) for WT, ATP + UTP

**C**

- [11, 12-mer RNA] (nM) vs. Time (sec) for Glu-297, ATP + UTP

**D**

- [11, 12-mer RNA] (nM) vs. Time (sec) for WT, dATP + dUTP

**E**

- [11, 12-mer RNA] (nM) vs. Time (sec) for Glu-297, dATP + dUTP
Fig. A-3: Introduction of additional stalling step after translocation into kinetic mechanism for successive 2 nucleotides incorporation significantly improves fit of the data. (A) Modified kinetic mechanism for 2-nucleotide incorporation analysis with stalling step following the translocation. Addition of the stalling step ($k_{stall}$) after translocation allows achieving reasonably good agreement between the simulation traces and the experimental data. (B, C) Subsequent incorporation of AMP and UMP into S/S-U by WT and Glu-297 3Dpol. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (B) or Glu-297 (C) 3Dpol. The solid lines represent simulation to the mechanism shown in panel A. For WT 3Dpol $k_{transl}$ was fixed at 250-500 s$^{-1}$ and $k_{transl}$ was insensitive to the simulation and in the range from 0 to 1000, with lower limit estimated at 0.0025 s$^{-1}$ (1). Thus, the upper limit for $K_{transl}$ was 0.5. The rate for the stalling step, $k_{stall}$, was set at 90 s$^{-1}$, with stalling happening after translocation. In the case of Glu-297 values for $k_{transl}$ and $k_{transl}$ were more flexible, the lower limit for $k_{transl}$ was 5-10, and $K_{transl}$ fixed at 5. The rate constant for the stalling step, $k_{stall}$, was set at 0.2 s$^{-1}$, with stalling happening after translocation step. (D, E) Consecutive incorporation of dAMP and dUMP into S/S-U by WT and Glu-297 3Dpol. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (D) or Glu-297 (E) 3Dpol. The solid lines represent simulation to the mechanism shown in panel A. For both WT in the case of successive incorporation of the two deoxyribonucleotides $k_{transl}$ was fixed at 30 s$^{-1}$, $k_{transl}$ was varied from 0 to 100 s$^{-1}$, therefore the upper limit for $K_{transl}$ was 0.3; $k_{stall}$, was 10 s$^{-1}$. For Glu-297 3Dpol $k_{transl}$ was fixed at 0.07 s$^{-1}$, and $k_{transl}$ were flexible with the best fit of data to the simulation achieved at $K_{transl}$ of 0.07; $k_{stall}$, was set at 0.15 s$^{-1}$. 
Fig. A-4: Introduction of additional binding steps and stalling into kinetic mechanism for successive 2 nucleotides incorporation significantly improves fit of the data. (A) Modified kinetic mechanism for 2-nucleotide incorporation analysis. Because the nucleotides were present at very high concentrations (5 x $K_{d,app}$) in the reactions examined, additional steps were introduced into the mechanism in order to account for misincorporation events. Addition of the stalling step ($k_{\text{stall}}$) either before or after translocation allows achieving very good agreement between the simulation traces and the experimental data. Introduction of two binding steps placed some restrictions on the flexibility of the translocation step rates. (B, C) Subsequent incorporation of AMP and UMP into S/S-U by WT and Glu-297 3Dpol. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (B) or Glu-297 (C) 3Dpol. The solid lines represent simulation to the mechanism shown in panel A. For WT 3Dpol $k_{\text{trans}}$ was fixed at 250-500 s$^{-1}$ and $k_{\text{transl}}$ was insensitive to the simulation and in the range from 0 to 1000, with lower limit estimated at 0.0025 s$^{-1}$ (1). Thus, the upper limit for $K_{\text{transl}}$ was 0.5. The rate for the stalling step, $k_{+\text{stall}}$, was set at 10 s$^{-1}$ and 90 s$^{-1}$, with stalling happening before and after translocation, respectively. In the case of Glu-297 values for $k_{\text{transl}}$ and $k_{\text{transl}}$ were more flexible, the lower limit for $k_{+\text{transl}}$ was 5-10, and $K_{\text{transl}}$ fixed at 5. The rate constant for the stalling step, $k_{+\text{stall}}$, was set at 50 s$^{-1}$ and 0.2 s$^{-1}$, with stalling happening before and after translocation step, respectively. (D, E) Consecutive incorporation of dAMP and dUMP into S/S-U by WT and Glu-297 3Dpol. Kinetics of 11-mer (●) and 12-mer (■) RNA accumulation and disappearance in the reaction catalyzed by WT (D) or Glu-297 (E) 3Dpol. The solid lines represent simulation to the mechanism shown in panel A. For both WT in the case of successive incorporation of the two deoxyribonucleotides $k_{+\text{transl}}$ was fixed at 30 s$^{-1}$, $k_{\text{transl}}$ was varied from 0 to 100 s$^{-1}$, therefore the upper limit for $K_{\text{transl}}$ was 0.3; $k_{+\text{stall}}$, were 2 s$^{-1}$ and 10 s$^{-1}$, with stalling before and after translocation step, respectively. For Glu-297 3Dpol $K_{\text{transl}}$ was fixed at 0.07 s$^{-1}$, and $k_{\text{transl}}$ were flexible with the best fit of data to the simulation achieved at $K_{\text{transl}}$ of 0.07; $k_{+\text{stall}}$, were 0.025 s$^{-1}$ and 0.15 s$^{-1}$, with stalling before and after translocation step, respectively.

Appendix B

Kinetic and Thermodynamic Constants for Arg-273/Glu-297 3Dpol-Catalyzed Nucleotide Incorporation

Kinetic and thermodynamic parameters for single nucleotide incorporation for double polymerase mutant, Arg-273/Glu-297 are summarized in Table B-1.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Nucleic Acid</th>
<th>Metal Cofactor</th>
<th>Nucleotide</th>
<th>K_{d,app}</th>
<th>k_{pol}</th>
</tr>
</thead>
<tbody>
<tr>
<td>sym/sub-U</td>
<td>GCAUGGGCCC</td>
<td>Mg^{2+}</td>
<td>ATP</td>
<td>200 ± 20</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>CCCGGGUACG</td>
<td>dATP</td>
<td>820 ± 60</td>
<td>0.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTP</td>
<td>210 ± 60</td>
<td>0.002 ± 0.0002</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATPαS</td>
<td>60 ± 10</td>
<td>3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>sym/sub-A</td>
<td>GCUAGGGCCC</td>
<td>Mn^{2+}</td>
<td>ATP</td>
<td>4 ± 0.3</td>
<td>1.4 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>CCCGGGΔUCG</td>
<td>dATP</td>
<td>8 ± 1</td>
<td>0.4 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATPαS</td>
<td>2 ± 0.5</td>
<td>0.2 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>UTP</td>
<td>130 ± 10</td>
<td>40 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>dUTP</td>
<td>340 ± 70</td>
<td>4 ± 1</td>
<td></td>
</tr>
</tbody>
</table>
### Appendix C

**Intracellular Concentrations of Nucleotides Differ between Mammals and Bacteria**

Table C-1: Intracellular Concentrations of Nucleotides Differ between Mammals and Bacteria

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Humans(^a) (\mu\text{M})</th>
<th>Mammals(^a) (\mu\text{M})</th>
<th>Bacteria(^b) (\mu\text{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>2102</td>
<td>3152</td>
<td>3000</td>
</tr>
<tr>
<td>dATP</td>
<td>2.4</td>
<td>24</td>
<td>175</td>
</tr>
<tr>
<td>GTP</td>
<td>305</td>
<td>468</td>
<td>923</td>
</tr>
<tr>
<td>dGTP</td>
<td>2.7</td>
<td>5.2</td>
<td>122</td>
</tr>
<tr>
<td>CTP</td>
<td>91</td>
<td>278</td>
<td>515</td>
</tr>
<tr>
<td>dCTP</td>
<td>4.5</td>
<td>29</td>
<td>65</td>
</tr>
<tr>
<td>UTP</td>
<td>253</td>
<td>567</td>
<td>894</td>
</tr>
<tr>
<td>dUTP</td>
<td>0.7</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>dTTP</td>
<td>17</td>
<td>37</td>
<td>77</td>
</tr>
</tbody>
</table>


Appendix D

WT and Arg-273 PV: Serial Passage at Low MOIs.

Serial passaging of the WT and Arg-273 PV was performed by Stephen Hemperly. The Arg-273 virus, when passaged at MOI of 0.01, showed significant decrease in the amount of virus generated at passages 4, 5 and 6 (Fig. D-1).

Fig. D-1: Serial passage of the WT and Arg-273 PV at MOI of 0.1 and 0.01. To determine dose-response ratios at MOI of 0.1 and 0.01 fresh HeLa cell monolayers were infected with the appropriate amount of virus, the infection was allowed to proceed for 8 h, and then viruses were harvested and titered (passage 2). The amount of the virus produced during passage 2 for each virus-MOI pair was assumed to be constant (i.e. dose-response ratio), and this titer was then used to calculate the amount of the virus required to achieve MOI of 0.1 or 0.01 for subsequent passage infections. After all six passages were completed; viruses collected after each passage were titered. For WT PV at both MOIs the titer of virus generated was unchanged (■ and ●); for Arg-273 PV at MOI of 0.1 only a slight change in titer was observed (○), whereas at MOI of 0.01 a substantial decrease in the amount of the virus produced during P4, P5 and P6 was observed (■).
Appendix E

Pulse-Chase Pulse-Quench Analysis of Glu-297 3Dpol-Catalyzed Nucleotide Incorporation

Pulse-chase pulse-quench analysis of Glu-297 3Dpol-catalyzed nucleotide incorporation supports the hypothesis that the conformational-change step prior to chemistry becomes more rate limiting compared to WT

In order to directly evaluate the equilibrium constant for the first conformation-change step, $K_2$, we performed pulse-chase pulse-quench analysis for both AMP and dAMP incorporation (Fig. E-1). In the pulse-quench part of the experiment, preassembled 3Dpol-sym/sub complex is rapidly mixed with the $\alpha$-32P-ATP (or $\alpha$-32P-dATP) and quenched at the various time points by the addition of 2N HCl as described under Experimental Procedures (Fig. E-1B). For the pulse-chase part of the experiment the reaction is chased with excess of unlabeled nucleotide (Fig. E-1C). In this case, if the rate of the phosphoryl transfer step ($k_{chem}$) is relatively slow, and the equilibrium constant, $K_2$, favors the forward reaction a definite amount of the activated ternary complex (*ER_nNTP) can be observed (Fig. E-1A). If the equilibrium constant, $K_2$, favors the reverse reaction and/or chemistry is fast, no activated species (*ER_nNTP) would be detected. For the WT 3Dpol in the presence of Mn$^{2+}$ only chemistry is rate limiting, the conformational-change step is strongly shifted in the forward direction with $K_2$ equal to 3 (Fig. E-1D) (1). The combination of relatively large $K_2$ and small $k_{chem}$ (30 s$^{-1}$, (1)) for WT 3Dpol leads to a visible lag for the pulse-quench trace (○) when compared to the
pulse-chase trace (●). These data provides convincing evidence that for the WT 3Dpol a large amount of the activated complex is accumulated, and the equilibrium for the first conformational-change step strongly favors the forward reaction. These data is consistent with the conformational-change step prior to the chemistry step not being rate-limiting when Mn$^{2+}$ is utilized as the metal cofactor (1).

In the case of Glu-297 3Dpol the difference between the pulse-chase trace (●) and pulse-quench trace (○) becomes much less pronounced (Fig. E-1E). Collapse of the pulse-quench and pulse-chase traces argues for the substantial decrease in the amount of the activated complex being formed, pointing to changes in $K_2$. Taking into account significant decreases in the observed reaction rates for Glu-297 3Dpol for both ribo- and deoxyribonucleotide incorporation in the presence of either Mg$^{2+}$ or Mn$^{2+}$ we can eliminate the possibility of the phosphoryl transfer step becoming more efficient for this derivative. Therefore, during the data simulation to the mechanism shown in Fig. E-1A, the rate for the chemistry step, $k_{chem}$, was kept at the WT 3Dpol level, 30 s$^{-1}$, and only the $K_2$ value was modulated (1). Simulation of the data resulted in a significant decrease in the equilibrium constant, $K_2$, giving a value of 0.15, which is 20-fold lower than WT $K_2$.

When dATP is employed as a substrate for the pulse-chase pulse-quench analysis for the WT 3Dpol-catalyzed reaction, a difference in the end-points is observed for the pulse-chase (●) and pulse-quench (○) data (Fig. E-1F). The only way to explain the fact that less product is formed when the reaction is quenched (○) is to introduce the back-reaction rate into the simulation mechanism. In our minimal mechanism (Fig. E-1A), only if the product complex (ER$_{n+1}$PP$_i$) can undergo phosphoryl transfer in the reverse step a smaller amount of the product can be accumulated in the pulse-quench (○) part of
the experiment. When this mechanism is used (Fig. E-1A), the data can no longer be simulated upon addition of the rate in the reverse direction of the chemistry step ($k_{chem}$). Therefore, complete kinetic mechanism (Fig. 1-5) was used in order to simulate the pulse-chase pulse-quench data for dAMP incorporation by WT 3Dpol (Fig. E-1F). For this simulation the kinetic constants determined for WT 3Dpol in our earlier studies were utilized (1,2). $K_2$ was kept at 0.4, as was previously determined by both calculations and simulation approach (1,2). However, in order to achieve a good fit for the dAMP incorporation data $k_{3}$ and $k_{4}$ had to be changed from 22 s$^{-1}$ and <0.0001 s$^{-1}$ to 45 s$^{-1}$ and 10000 s$^{-1}$, respectively (1) ($k_{4}$ had to be fixed at 10000 s$^{-1}$ in order to achieve line separation). For $k_{3}$ the change was modest – only 2-fold, whereas $k_{4}$ was considerably increased; $k_{4}$ was previously insensitive to any manipulations during simulations and was set at a limit of <0.0001s$^{-1}$, the later lower rate limit was derived from the pyrophosphate exchange experiment (3). Thus, for the first time, this data analysis pointed us to the possibility of the chemistry and/or the conformational-change step after chemistry in the reverse direction serving as an additional fidelity check-point.

When dAMP incorporation was evaluated for the Glu-297 3Dpol derivative, the data points from pulse-chase (●) and pulse-quench (○) parts essentially overlapped (Fig. E-1G). The solid lines in the graph represent the simulation of the experimental data to the simplest mechanism, shown in Fig. E-1A. The equilibrium constant for the conformational-change step prior to chemistry, $K_2$, was set at 0.01 in order to achieve a reasonable fit of the data to the simulation traces. Thus, for the dAMP incorporation by the Glu-297 3Dpol derivative, the conformational-change step prior to the chemistry step
emerges into a serious roadblock on the pathway of the deoxyribonucleotide incorporation.

The pulse-chase pulse-quench analysis was performed only in the presence of Mn$^{2+}$, because in Mg$^{2+}$ the rate for the chemistry step is too fast to allow a detectable amount of the activated complex to accumulate.
A

\[
\begin{align*}
ER_n NTP & \rightleftharpoons ^*ER_n NTP \\
& \xrightarrow{k_{+2}} ER_{n+1} PP_i \\
& \xrightarrow{k_{-2}}
\end{align*}
\]

B

\[
\text{Pulse-Quench} \quad \text{Quench} \quad \Delta t \quad (O)
\]

C

\[
\text{Pulse-Chase} \quad \text{ATP} \quad \text{Quench} \quad 30 \text{sec} \quad (●)
\]

D

\[
\begin{align*}
[\alpha^{32}\text{P}]\text{(d)ATP} \quad \text{incorporated (μM)} \\
\text{Time (sec)}
\end{align*}
\]

E

\[
\begin{align*}
[\alpha^{32}\text{P}]\text{AMP incorporated (μM)} \\
\text{Time (sec)}
\end{align*}
\]

F

\[
\begin{align*}
[\alpha^{32}\text{P}]\text{AMP incorporated (μM)} \\
\text{Time (sec)}
\end{align*}
\]

G

\[
\begin{align*}
[\alpha^{32}\text{P}]\text{dAMP incorporated (μM)} \\
\text{Time (sec)}
\end{align*}
\]
Fig. E-1: The amount of the intermediate that accumulates prior to chemistry for the Glu-297 derivative is reduced relative to WT 3Dpol. (A) Minimal kinetic mechanism for pulse-chase pulse-quench analysis. ERₐNTP (ternary complex); *ERₐNTP (activated elongation complex); ERₐ₊₁PPi (product complex). (B, C) Experimental design. 4 µM 3Dpol was incubated with 20 µM sym/sub (10 µM duplex) and rapidly mixed with 100 µM [α-³²P]ATP or [α-³²P]dATP. At the indicated times the reaction was either chased by addition of ATP to a final concentration of 20 mM or quenched by addition of HCl to a final concentration of 1 M. After addition of the chase solution the reaction was allowed to proceed for an additional 30 s, at which time the reaction was quenched with HCl. Immediately after addition of HCl, the solution was neutralized by addition of 1 M KOH in 300 mM Tris. (D-G) Kinetics of AMP and 2′-dAMP incorporation by the WT (D, F) and Glu-297 3Dpol (E,G) in the presence of Mn²⁺. Kinetics of pulse-chase (●) and pulse-quench (○) experiments as described in (B, C). The solid lines represent kinetic simulation of the data to the mechanism shown in panel (A), with the following kinetic parameters: $K_2$ of 3 and $k_{+3}$ of 30 s⁻¹ for AMP (D) incorporation. Data for AMP incorporation is taken from (1). Pulse-chase pulse-quench analysis of 2′-dAMP incorporation by the WT 3Dpol is shown in panel F. These data were simulated to a complete kinetic mechanism for single nucleotide incorporation (Fig. 1-5). See text for details. (E, G) Kinetics of AMP (E) and 2′-dAMP (G) incorporation by Glu-297 3Dpol in the presence of Mn²⁺; pulse-chase (●) and pulse-quench (○). The solid line represents the kinetic simulation of the best data fit to the mechanism shown in panel (A) with $K_2$ of 0.15 for AMP (E) and 0.01 for 2′-dAMP (G) incorporation, and $k_{+3}$ of 30 s⁻¹ in both cases. Experiments were performed in triplicates, the representative data is shown.

Appendix F

Supplemental Materials for Chapter 4

A

5'- GGGCCC - 3'
3'- AAAACCCGGGAAUUUAGGGACCCA - 5'
4-nt
14-nt

B

Time (sec) 0 10 Glu-297 300 10 WT 300

dNTPs Mn^{2+}

C

Time (sec) 0 10 Glu-297 300 10 WT 300

rNTPs Mn^{2+}

6-mer
Fig. F-1: Substantial block is observed for Glu-297 3Dpol-catalyzed dNMP incorporation even in the presence of Mn$^{2+}$. (A) 6/24-mer RNA substrate employed in this study. (B) In spite of the relaxed specificity conferred by using Mn$^{2+}$ for WT 3Dpol, Glu-297 3Dpol was significantly delayed in producing full-length (20-nt) product. (C) A similar block was not observed in Mn$^{2+}$ for rNMP incorporation catalyzed by Glu-297 3Dpol. RNA duplex (1 µM) was incubated at 30°C with all four deoxyribonucleotides or ribonucleotides (100 µM each), (B) and (C), respectively. Reactions were initiated by addition of 3Dpol (1 µM). Reactions were quenched by addition of EDTA with the gel-loading buffer. Products were resolved by electrophoresis on a denaturing 22% polyacrylamide - 39% formamide gel and visualized by using a Phosphorimager (Molecular Dynamics). Experiment was performed on 083106, gel was run on 090506 (N297E book V, pp. 111-117).
A

5' - GGGCCC - 3'  
3' - AAAACCCCGGGAAUUUAGGGACCCA - 5'

4-nt  14-nt

B

Time (sec) 0 10 300 10 300

Glu-297  WT

dNTPs  Mg^{2+}

6-mer

C

Time (sec) 0 10 300 10 300

Glu-297  WT

rNTPs  Mg^{2+}

6-mer
Fig. F-2: Deoxy- and ribonucleotide incorporation by Glu-297 and WT 3Dpol in the presence of Mg\(^{2+}\). (A) 6/24-mer RNA substrate employed in this study. (B) A substantial block for deoxyrobonucleotide incorporation was observed for Glu-297 3Dpol-catalyzed reaction in the presence of Mg\(^{2+}\). Although strong stops were observed for WT 3Dpol-catalyzed reaction as well, the WT polymerase was able to overcome the stops, producing small amount of full-length products. (B) When ribonucleotide incorporation was evaluated in the presence of Mg\(^{2+}\) both Glu-297 and WT 3Dpol were capable of full-size product synthesis. Experimental design: RNA duplex (1 µM) was incubated at 30 °C with all four deoxyribonucleotides or ribonucleotides (100 µM each), (B) and (C), respectively. Reactions were initiated by addition of 3Dpol (1 µM). Reactions were quenched by addition of EDTA with the gel-loading buffer. Products were resolved by electrophoresis on a denaturing 22% polyacrylamide - 39% formamide gel and visualized by using a PhosphorImager (Molecular Dynamics). Experiment was performed on 090706 (N297E book V, pp. 118-119).
**Fig. F-3: dATP excision by Glu-297 3Dpol upon addition of exogenous pyrophosphate.** (A) **Experimental design.** First, 3Dpol-RNA complex was assembled by preincubating 1 μM 3Dpol with 1 μM S/S-U RNA in the reaction buffer for 90 s at 30 °C. Then dATP was added to the complex (100 μM), followed in 3 minutes by either pyrophosphate (1 mM), ATP (10 μM) or both pyrophosphate and ATP (1 mM and 10 μM, respectively). Reactions were quenched at the indicated times by addition of EDTA to a final concentration of 50 mM, and products of the reactions were separated by electrophoresis on a 23% denaturing polyacrylamide gel and visualized using a PhosphorImager (Molecular Dynamics). (B, C) **Reactions described in (A) are shown in panels (B) and (C) for Glu-297 3Dpol and WT 3Dpol, respectively.** (D) **Quantitation of the images shown in panels (B) and (C).** The amount of 11-mers formed due to both AMP and dAMP incorporation is reported, in the presence of 10 μM ATP alone and 10 μM ATP with 1 mM pyrophosphate. Amount of the 11-mer RNA formed due to dAMP incorporation is indicated by black and gray circles for Glu-297 and WT 3Dpol-catalyzed reactions, respectively; the amount of the 11-mer RNA produced by AMP incorporation is shown by black and gray squares, for Glu-297 and WT 3Dpol-catalyzed reactions. When only exogenous pyrophosphate or ATP were added to the reactions, the amount of 11-mer produced due to either AMP or dAMP incorporation events, were essentially identical for Glu-297 and WT 3Dpol. However, when ATP was added together with the pyrophosphate, the amount of 11-mer RNA formed due to initial dAMP incorporation decreased (from 0.1 μM to 0.7 μM) in the case of Glu-297 3Dpol-catalyzed reaction (●); at the same time, the 11-mer RNA, formed due to AMP incorporation, accumulated to a higher degree and at faster rate (■), suggesting that in the presence of exogenous pyrophosphate AMP could be incorporated instead of previously incorporated dAMP, which could then be excised in the presence of pyrophosphate. Experiment was performed on 092006 (N297E book V, pp. 127-133). Similar experiment was performed on 100306, but final ATP concentration was decreased to 1 μM; the data were noisy and inconclusive (N297E book V, pp. 137-144).
Fig. F-4: dNTP incorporation in the presence of Mg$^{2+}$, catalyzed by Glu-297 and WT 3Dpol, (A) and (B), respectively. 3Dpol (1 µM) was incubated with S/S-U RNA (1 µM) for 180 s, then all four dNTPs were added to the reaction to a final concentration of 100 µM. At indicated times reactions were quenched by addition of EDTA (50 mM final concentration), and reaction products were separated through a 23% denaturing polyacrylamide gel and visualized by PhosphorImager. Data analysis did not reveal the presence of any severe block for dNTP incorporation for Glu-297 3Dpol. Experiment was performed on 100306 (N297E book V, pp. 137, 145-145f).
A

3Dpol (2µM) → S/S-U (2µM) → a. + ATP (10 µM) + 3'-dATP (100 µM) → b. + GTP (10 µM) + UTP (10 µM) + PPI (1 mM) → c. + GTP (10 µM), + UTP (10 µM), + PPI (1 mM) → Quench → 180 sec → 5 min → Δt

B

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>300</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>a, b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Glu-297

- +GMP
- +dUMP
- +AMP
- 10-mer

C

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>300</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>a, b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

WT

- +GMP
- +dUMP
- +AMP
- 10-mer
Fig. F-5: GMP incorporation after 3’dUMP by Glu-297 and WT 3Dpol, (B) and (C), respectively. (A) Experimental design. For this experiment 2 µM 3Dpol was preincubated with 2 µM S/S-U RNA for 2 min at 30 ºC in order to establish the enzyme-RNA complex. The reaction was then initiated by adding ATP and 3’-dUTP to final concentrations of 10 and 100 µM, respectively. The reaction was allowed to proceed for 5 min, at which point either GTP and UTP were added (to a 10 µM final concentration), or GTP, UTP and pyrophosphate were added (to final concentrations of 10 µM for both nucleotides and 1000 µM for pyrophosphate). The reactions were quenched by addition of EDTA. Products were resolved by electrophoresis on a denaturing 23% polyacrylamide gel and visualized by using a PhosphorImager. Experiment was performed on 100906 (N297E book V, pp. 149-157). Incorporation of GMP after dUMP was evaluated for both Glu-297 and WT 3Dpol as well. No stalling was observed for GMP incorporation for either WT or Glu-297 3Dpol-catalyzed reactions (100306, N297E book V, p. 136).
Fig. F-6: dNTP incorporation in the presence of Mg$^{2+}$ catalyzed by Glu-297 and WT 3Dpols. For this experiment 4 µM 3Dpol was preincubated with 4 µM S/S-U RNA for 2 min at room temperature in order to establish the enzyme-RNA complex. The reaction was then initiated by adding all four dNTPs to a final concentration of 100 µM. Reactions were quenched by addition of 2N HCl, and immediately neutralized with 3M KOH in 1M Tris solution. Products were resolved by electrophoresis on a denaturing 23% polyacrylamide gel and visualized by using a PhosphorImager. Experiment was performed on 101306 (N297E book V, pp. 159-167d).
VITA

Victoria S. Korneeva

Education
Ph.D. candidate in the Biochemistry, Microbiology, and Molecular Biology graduate program, The Pennsylvania State University, University Park, PA, August 2000-present
Diploma, Chemistry and English, Herzen State Pedagogical University of Russia, St.-Petersburg, Russia, June 1998

Research Experience
Graduate Research Assistant, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, 2000-present
Graduate Research Assistant, Department of Chemistry, University of Northern Iowa, 1999-2000
Undergraduate Research Assistant, Department of Chemistry, University of Northern Iowa, 1997

Honors and Awards
The Roberts/Braddock Graduate Student Award, 2000
Pela Fay Braucher Fellowship, 2001
American Society for Virology Graduate Travel Award, 2003
American Society for Biochemistry and Molecular Biology Graduate Travel Award, 2004

Publications