ABSTRACT

Title of Dissertation: MUTATIONAL ANALYSIS OF POLIOVIRUS PROTEIN 3AB TO IDENTIFY REGIONS CRITICAL TO NUCLEIC ACID CHAPERONE ACTIVITY

Divya Gangaramani, Doctor of Philosophy, 2013

Dissertation Directed By: Dr. Jeffrey J. DeStefano, Professor
Department of Cell Biology and Molecular Genetics

Poliovirus 3AB protein was the first picornavirus protein demonstrated to have nucleic acid chaperone activity. Current results demonstrate that chaperone activity requires the C-terminal 22 amino acid (3B region (also referred to as VPg), amino acid 88-109) of the protein as mutations in this region abrogated nucleic acid binding and chaperone activity. Protein 3B alone had no chaperone activity as determined by established assays testing chaperone activity including: the ability to stimulate nucleic acid hybridization in a primer-template annealing assay, or helix-destabilization in a nucleic acid unwinding assay, or aggregation of nucleic acids. In contrast, the putative 3AB C-terminal cytoplasmic domain (N terminal amino acid 81-109, 3B + the last 7 C-terminal amino acids of 3A, termed 3B+7 in this report) possessed strong activity in these assay, albeit at much higher concentrations than 3AB. Results from several mutations in 3B+7 are described and a model proposing that 3B+7 possesses the “intrinsic” chaperone activity of 3AB while the 3A N-terminal region (amino acid 1-58) and/or membrane anchor domain (amino acid 59-80) serve to increase the effective concentration of the 3B+7 region leading to the potent chaperone activity of 3AB. Two mutations with

reduced chaperone activity *in vitro*, K81A and F83A in 3AB were tested in tissue culture. Viruses with these mutations produced near wildtype and minute plaques, respectively. F83A gave rise to revertants with either wildtype 3AB sequences or additional nearby compensatory mutations. Translation and polyprotein processing were not affected by these mutations but RNA synthesis compared to wildtype, was slightly lower for K81A and significantly lower for F83A. This data suggests that mutations that decrease chaperone activity of 3AB may lead to decreased RNA synthesis, although the exact steps that are affected need to be determined.

MUTATIONAL ANALYSIS OF POLIOVIRUS PROTEIN 3AB TO IDENTIFY REGIONS CRITICAL TO NUCLEIC ACID CHAPERONE ACTIVTY

By

Divya Gangaramani

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2013

Advisory Committee:
Professor Jeffrey J. DeStefano, Chair
Professor James N. Culver
Assistant Professor Brenda Fredericksen
Assistant Professor Nicole LaRonde-LeBlanc
Professor Siba K. Samal, Dean's Representative

Dedication

This thesis is dedicated to my family for all their love and support; to Asit, for his unconditional love and to my friends who stood by me through thick and thin.

Acknowledgements

My journey through graduate school has been the most humbling phase of my life till now. It is through this journey that I have met some of the most wonderful people and I would like to take this opportunity to thank each one of them. Firstly, I would like to thank my advisor, Dr. Jeffrey DeStefano for being an excellent mentor through my graduate work. He has helped me hone my scientific skills and never gave up on me in my innumerable attempts with protein purification and FPLC. Jeff is a genuine and fair individual and has been a great example of maintaining work-life balance.

I would like to thank my committee members Drs. Jim Culver, Brenda Fredericksen, Nicole LaRonde-LeBlanc and Siba Samal for their valuable suggestions throughout my research and for being available whenever I was ready for a meeting. Their constant encouragement and acknowledgement of my efforts kept me going.

I would like to especially thank each and every member of the Destefano lab, starting with Dr. Deena Jacob and Dr. Yi-Tak(Megan) Lai, the two wonderful people who trained me during my rotation and played a big role in my decision of joining the lab. Both of them were very patient in training me and helped me adapt to the first year of graduate school. Megan, with her detailed information about every course either to take or to teach, along with details about the professors, guided me to make the right choices. Deena for introducing me to fun games on the computer while we would wait for research to happen and for having meaningful conversations with me about life in general. To Dr. Gauri Nair, for being a very dear friend and an excellent mentor in lab.

She taught me cloning and everything I know about microbiology. Thank you for encouraging me to come out of my shell and grow professionally by believing in me. To JeffO, for just being beside me in everything we did during grad school, I had the best time in lab when you were around, our friendship kept me going. To Katherine, for expanding my knowledge about American culture, making the best figures for me, being the best roomie at a conference and for helping me through, in my trying times. To Vasu, for being a little brother in lab and providing me with awesome food during lunch that I could trade mine for. To Katherine, Vasu, Jeff and Kanika for wine Fridays. To Irani for her warmth and eagerness to help me and to Elizabeth, for the preliminary work with the 3B+7 protein.

My family has been a great support all my life. They have always encouraged my decisions in life and made me an independent person. To my dad, who has been my hero in more than one way and to my mom for her tender love and care. Thank you for making me the person I am today.

I would like to thank my friends Neha, Divya, Kanika, Sangeetha, Mayank and Ashwin for standing by me. To Emma, Priyanka, Joceline and Dipti for being the best 'all girls' group that kept each one of us sane.

And last but not the least, my biggest thank you to Asit, my best friend, companion and my would be life partner, for loving me the way I am and for keeping me humble and balanced by being his simple, genuine self.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

CNS: Central nervous system
DMEM: Dulbecco’s modified Eagle's medium
DTT: Dithiothreitol
EDTA: Ethylenediaminetetraacetic acid
FBS: Fetal bovine serum
FRET: Fluorescence resonance energy transfer
GuHCl: Guanidine hydrochloride
HIV: Human immunodeficiency virus
hnRNP A1: Heterogeneous nuclear ribonuclear protein
IPV: Inactivated polio vaccine
IRES: Internal ribosome entry site
kDa: Kilodalton
Kb: Kilobase
Min: Minutes
NC: Nucleocapsid
NTR: Non-translated region
NMR: Nuclear magnetic resonance
OPV: Oral polio vaccine
PV: Poliovirus
PCR: Polymerase chain reaction
RF: Replicative form
RI: Replicative intermediate
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
VAPP: Vaccine-associated poliomyelitis
VDPV: Vaccine-derived poliovirus

Chapter 1: Introduction

1.1 A Brief History of Poliomyelitis

Poliomyelitis is a highly infectious viral disease that causes diarrhea, vomiting, fatigue, muscle stiffness and in rare cases partial or complete paralysis (80). The disease can strike at any age but is more common among children under the age of five. The earliest clinical description of poliomyelitis was provided in 1789 by British physician Michael Underwood. Initial outbreaks in Europe and the United States were recorded in the early 19th century. Evidence from Egyptian mummies suggests that polio existed as far back as 3700 B.C.

Karl Landsteiner and Erwin Popper identified the causative agent of poliomyelitis, a poliovirus, in 1908, long after the disease had established itself as globally endemic. The disease reached a peak in the United States in 1952, with more than 21,000 cases of polio paralysis (21, 47). There was an urgent need for a vaccine to stop the spread of this dreaded disease. Different laboratory findings across the globe contributed immensely to development of an effective polio vaccine. In 1952, Jonas Salk successfully developed a formalin-inactivated viral vaccine that was able to trigger the body's immune system without potentially causing an infection. Following this, massive field trials were organized across the country to test its efficacy. The March of Dimes, a nonprofit organization established by Franklin D. Roosevelt to fight polio, successfully mobilized nearly two million children to participate in these field trials (58). The success of the trial led to the release of the vaccine for the public by 1955. As a result of

vaccination there was a dramatic decrease in the incidence of polio by 85-90% in the United States between 1955-1957 (21, 52).

Despite this great achievement, efforts to develop more effective vaccines continued. In 1957, Dr. Albert Sabin began testing a live, attenuated, oral form of the vaccine. In addition to being more cost effective due to oral administration, this version of the vaccine proved to confer long lasting immunity and was thus released to the public in 1962 (39). With the development of two successful vaccines, a global effort to eradicate polio was initiated by the World Health Organization, UNICEF, Rotary Club International, and the Centers for Disease Control and Prevention in 1988 (48). It was one of the largest and most successful public health initiatives in the history of mankind. At the onset of the global polio eradication initiative, polio existed in 125 countries worldwide with 350,000 cases of infection in 1988. Twenty-four years later, the numbers have dropped by greater than 90% with just four countries (India, Pakistan, Afghanistan, and Nigeria) being endemic (48). Recently, in February 2012, India was declared polio-free by the World Health Organization (Morbidity and Mortality Weekly Report May 2012, CDC).

1.2 Classification of Poliovirus

Poliovirus is an enterovirus that belongs to the family *Picornaviridae.* There are nine genera within the family (Table 1-1), which consists of many viruses that adversely affect humans, agriculture and livestock. Poliovirus, Hepatitis A virus and Rhinovirus are important human pathogens while the Foot and Mouth Disease virus was the first discovered animal virus and remains one of the most important animal pathogens (97). Poliovirus is the most extensively researched member of the family (80) and hence serves

as prototype model system for plus-strand RNA viruses. The study of poliovirus has contributed extensively not only to the field of virology but biology in general. Some notable examples are the discovery of mammalian uncapped messenger RNA (72), the internal ribosome entry sire (IRES) (51), a 5′-terminal genome linked protein, and a 3′-terminal poly A tail (26).

There are three known antigenically distinct serotypes of poliovirus, Mahoney (type 1), MEF-1 (type 2) and Sabin (type 3). The entire nucleotide sequence for all three serotypes has been determined and the total genome consists of 7440, 7440, and 7435 nucleotides, respectively (107). All known poliovirus isolates can be grouped into these three categories based on their ability to react to a reference panel of antisera. Although the antigenic structure of poliovirus is identical for all serotypes, each of the virus serotypes fails to elicit neutralizing antibodies against viruses of the other two serotypes. The virus displays four distinct neutralization antigenic sites on the capsid (1, 2, 3A and 3B) that bind neutralizing antibodies (46, 68, 73). All three serotypes are capable of causing paralytic disease (80). In an unimmunized population, the majority of poliomyelitis cases were caused by type 1 poliovirus, followed by type 3, and then type 2 (27, 54).

TABLE 1-1

Genus	Representative species	Diseases	Natural hosts
Enterovirus	Poliovirus	Poliomyelitis	Human
Rhinovirus	Rhinovirus	Common cold	Human
Cardiovirus	Theiler's murine encephalomyelitis virus	Encephalomyelitis	Mouse
Aphthovirus	Foot-and-mouth-disease virus	Foot-and-mouth disease	Cloven-hooved Ungulates
Erbovirus	Equine rhinitis B virus	Acute respiratory disease	Horses
Kobuvirus	Aichi virus	Gastroenteritis	Human
Teschovirus	Porcine teschovirus	Encephalomyelitis	Pigs
Hepatovirus	Hepatitis A virus	Hepatitis	Human
Parechovirus	Human parechovirus	Gastroenteritis, respiratory disease	Human

Table 1-1: The family *Picornaviridae*: genera, some representative species and the diseases that they cause.

Table adapted from J. Lindsay Whitton, Christopher T. Cornell and Ralph Feuer. (108)

1.3 Epidemiology of Poliomyelitis

Humans are the only natural reservoirs of poliovirus. The virus can be found in fecal-contaminated food, water, and sewage (31), although the virus does not survive long in the environment outside the human body. Poliomyelitis occurs year round in the tropics, while it is mostly limited to summer and fall in the temperate zones. Winter outbreaks are rare (63). Transmission of the virus mainly occurs through the fecal-oral route. Oral-oral transmission is very rare. Once the virus enters the human body, it binds to CD155 poliovirus receptors and replicates in the pharyngeal and intestinal tract on follicle-associated epithelium and microfold or M cells of the Peyer's patch (49). Poliovirus may be found in specimens from the oropharynx for 1-2 weeks and from the intestine for 3-6 weeks after infection. Shedding of the virus in the stool is intermittent and the degree of shedding is affected by the immune status and immunocompetence of the individual (27, 80). In about 1-2% of infections, the virus may enter the central nervous system and replicate in motor neurons causing muscle paralysis (detailed below) (80). A person infected with the virus is most infectious between 7-10 days before and 3-4 weeks after onset of paralysis (2). The epidemiological pattern of the disease is associated with the socioeconomic development and health care services of a country. Crowded conditions and poor hygiene promote recurrent outbreaks of polio, thereby hampering global efforts to eradicate the disease.

1.4 Pathogenesis of Poliovirus

Upon ingestion, poliovirus infects the oropharynx and the gut, where it may cause mild symptoms but is often asymptomatic (14, 91). It is excreted in the oropharyngeal secretions and the stool for several weeks following infection. The virus has been isolated

from the lymphatic tissues of the gastrointestinal tract, including the tonsils, Peyer's patch of the ileum and mesenteric lymph nodes, as well as the feces, prior to the onset of illness (90), suggesting that susceptible cells in these tissues may be primary sites of replication. Following initial replication of the virus in the lymphoid tissue of the gut, most infected individuals develop a transient viremia with no neurological complications. Such infections are mainly asymptomatic. As the infection progresses further, the virus may spread to other sites of the reticuloendothelial system. Only about 4-8% of infected individuals that develop primary viremia progress to a later stage of secondary viremia associated with minor non-specific illness. This phase is also known as abortive poliomyelitis. The clinical manifestations of this nonspecific illness include a) upper respiratory tract infection characterized by sore throat and fever, b) a gastrointestinal illness characterized by nausea, vomiting, abdominal discomfort, and constipation or diarrhea; and/or c) headache and general malaise. Furthermore, in a small number of individuals who develop secondary viremia, the virus may invade the central nervous system, causing either non-paralytic aseptic meningitis or paralytic poliomyelitis. Non-paralytic aseptic poliomyelitis is characterized by rigidity of the neck, lower back, and lower limbs in addition to increased levels of leukocytes (from 0-5 cells/mm^3 to 10-200 cells/mm^3) and slightly elevated levels (from 15-45 mg/dL to 40-50 mg/dL) of protein in the cerebrospinal fluid. Paralytic poliomyelitis occurs in 0.1-1% of all poliovirus infections, depending on the specific serotype. Paralytic poliomyelitis can be classified into i) spinal poliomyelitis, characterized by acute flaccid paralysis, destruction of motor neurons, and denervation of associated skeletal muscles; ii) bulbar poliomyelitis

characterized by paralysis of respiratory muscles; iii) bulbospinal poliomyelitis which affects both the brain stem and the spinal cord (13, 21, 63).

There are three possible routes suggested for the spread of the virus to the central nervous system: (1) the circulating virus enters a peripheral nerve and is carried to the central nervous system by retrograde axonal transport (83) and/or (2) is able to permeate the blood brain barrier independent of the presence of the poliovirus receptor (113). Finally (3) poliovirus has been shown to replicate in primary human monocytes, suggesting this may be a third route for the spread of the virus from the primary site of infection to the central nervous system (30, 32). Poliovirus upon infection of a vaccinated human encounters secretory IgA present at the apical surface of mucosal epithelial cells and IgG and IgM in the blood stream. These antibodies complex with the invading virus and prevent the spread of virus via the bloodstream to target organs. Virus-antibody complexes are eliminated by phagocytosis, digestion, and excretion (114). The role of cell-mediated immunity in protection against viral infection and dissemination is less clear (18).

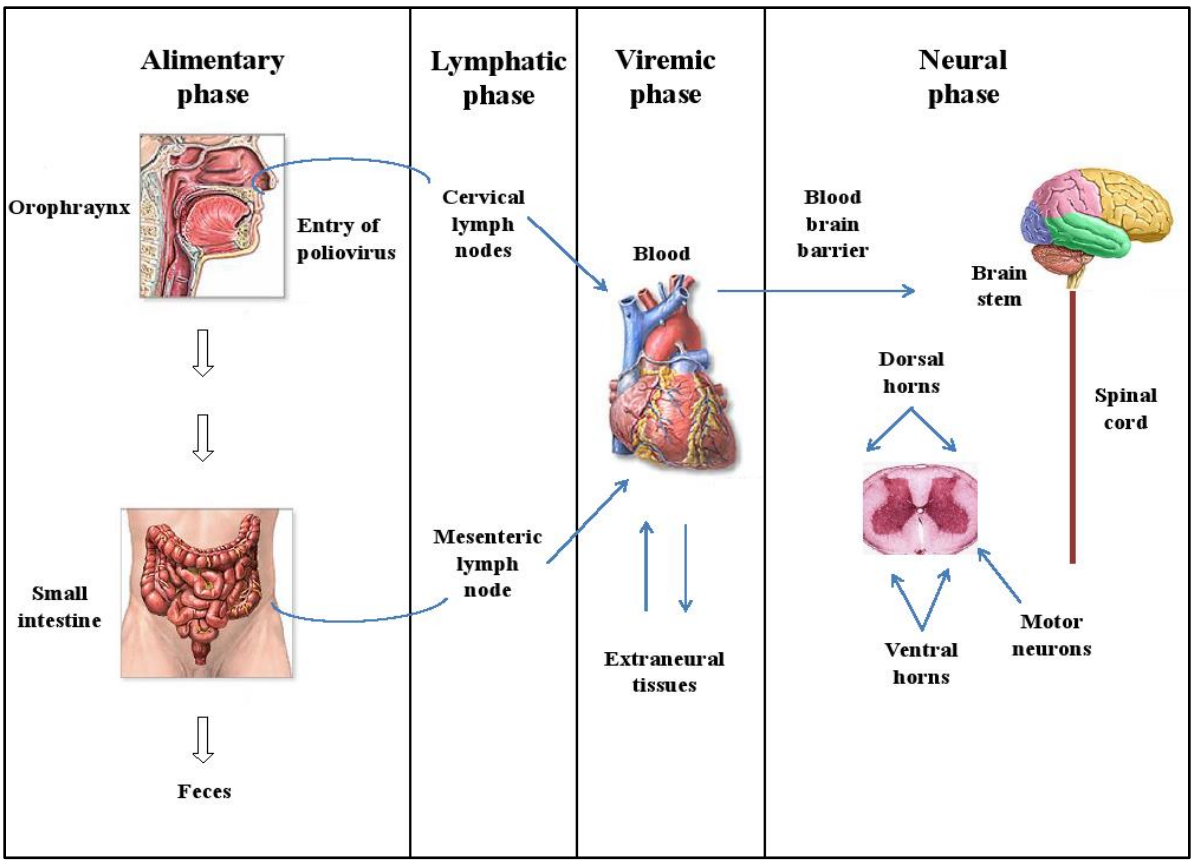


Figure 1-1: Poliomyelitis pathogenesis. Poliovirus infection occurs through the fecal-oral route. The virus replicates efficiently in intestinal tract and is shed in the stool for two to four weeks. The lymphatic system, especially the Peyer's patch of the ileum and the tonsils are invaded, and the virus enters the blood leading to transient viremia. In some cases, the virus enters the central nervous system and replicates in the motor neurons within the spinal cord and brain stem and causes muscle paralysis. Figure adapted from Blondel, B., Colbere-Garapin, F., Couderc, T., Woritius, A., Guivel-Benhassine, F. (13).

1.5 Vaccines against Poliovirus

Two different types of polio vaccines have been developed: Jonas Salk's formalin inactivated polio vaccine (IPV) and Albert Sabin's live, attenuated oral polio vaccine (OPV). Widespread immunization with IPV and OPV led to the eradication of polio from developed countries. IPV was the first polio vaccine to be licensed and is prepared by formalin-inactivation of three wildtype, virulent reference strains: Mahoney (type 1), MEF-1 (type 2), and Saukett (type 3). IPV was licensed for use in the United States, Canada, and Western Europe in 1955 and was the only polio vaccine available until licensure of OPV in 1961–1962. IPV is administered as an intramuscular injection and confers IgG immunity in the blood stream against all three strains of the virus. These antibodies prevent the progression of the infection to viremia and spread of virus to the central nervous system (54). Since IPV contains inactivated strains of poliovirus, it cannot cause poliomyelitis and is safe for use in immunocompromised people. One of the disadvantages of IPV is that it can only be administered by injection. This increases the effective cost of vaccination, which is a major concern in developing countries. The IPV also induces a lower level of gastrointestinal immunity (63). This protects the individual from paralytic poliomyelitis but cannot curtail the spread of virus to other people.

OPV consists of a mixture of live, attenuated poliovirus strains of each of the three serotypes that are able to mimic the immunological response to a wild type poliovirus infection. They are less neurovirulent and therefore are unable to spread to the central nervous system. Like IPV, OPV also produces antibodies against all three strains of the virus, which protects the individual in the event of an infection. It also produces a local immune response at the site of the virus replication, i.e. the mucous membrane of

the intestine. This additional immune response protects the individual from any incoming poliovirus infection. OPV confers passive immunity to people who have not been vaccinated. For several weeks post vaccination, the vaccine virus replicates in the gut and is excreted in the feces and can spread to people in close contact. OPV is more cost effective compared to IPV, as it is administered in the form of drops by mouth and can be given by volunteers without the additional requirement of healthcare workers, needles, or syringes. OPV remains the vaccine of choice in areas where wild poliovirus is endemic, but has been replaced with IPV in many countries where polio has been eradicated (70)

1.6 The Poliovirus Endgame

Since its launch in 1988, the Global Polio Eradication Initiative (GPEI) has come very close to its long term goal of a polio-free world. Polio existed in 125 countries across the globe in 1988 and by 2006 just four countries remained endemic. Of the four countries, India was recently declared as a polio-free nation (7). The remaining three countries i.e., Pakistan, Afghanistan and Nigeria are taking vigorous steps to stop the transmission of wild type poliovirus, mainly strains 1 and 3. In 1999, the GPEI achieved a major milestone of successfully eradicating type 2 poliovirus, leaving only the lab stocks for vaccine research. Unfortunately, ten years later in 2009 a new form of type 2 poliovirus emerged in Nigeria (85).

One of the main problems encountered on the road to polio eradication is the imperative use of oral polio vaccine in endemic regions. The risk associated with OPV is that, in rare cases (1 in 2.6 million doses), the genetically modified vaccine virus reverts via mutations or recombination to a more neurovirulent form while replicating in the gut, causing vaccine-associated poliomyelitis (VAPP) in some individuals. Since OPV viruses

are capable of person-to-person transmission, these unattentuated *vaccine-derived polioviruses* (VDPVs) can spread by person-to-person contact as well. For any given area to be remain polio free, it is necessary to eliminate indigenous wild type poliovirus transmission, control the importation of wild type poliovirus cases, and adopt a change in vaccine policy (i.e., from live, attenuated oral polio vaccine [OPV] to inactivated polio vaccine [IPV]) to eliminate the possibility of VAPP cases.

The final push towards eradication is at its peak but factors such as war, poverty, political instability, and isolated communities decrease the efficiency of widespread immunization programs required to block the spread of the virus. The recent successful eradication effort in India gives hope to the overall goal of a polio-free world. In early 2012, India was removed from the list of polio-endemic countries after it went without reporting a single case of polio in a span of one year. Since India was considered a hotbed for polio, this milestone is a big motivator for the remaining countries to make the final push towards eradication (53, 54).

1.7 Poliovirus Structural Characteristics

Poliovirus is a non-enveloped virus made up of capsid proteins enclosing a single-stranded RNA genome. The capsid is an icosahedron (45) that consists of 60 copies of each of the proteins VP1, VP2, VP3, and VP4, which are produced by proteolysis of a viral polyprotein (Figure 1-2). The capsid precursor P1 is cleaved into VP0, VP3, and VP1, which remain associated as protomerss but rapidly aggregate to form pentamers [(VP0, VP3, VP1)$_5$]. Twelve pentamers, in turn, assemble to form the "procapsid" [(VP0, VP3, VP1)$_5$]$_{12}$. It is unclear if the viral RNA is inserted into an empty capsid or if the pentamers condense around the RNA to enclose it. The final maturation step occurs with

the cleavage of VP0 to VP4 and VP2 (43). The three large capsid proteins (VP1, VP2, and VP3; ~ 30 kDa) are folded into eight-stranded, antiparallel beta barrels, a pattern that is highly conserved among eukaryotic RNA viruses (87). The smaller, myristoled capsid protein VP4 (7 kDa), lines the inside of the capsid (19). The amino acid loops that join the beta strands in the beta barrel structures constitute the neutralizing antigenic sites of the virion. There are four major neutralizing sites on the poliovirus. There are only three unique sets of these four antigenic sites hence there are only three serotypes of poliovirus; type 1, type 2 and type 3. Antibodies elicited against any one type are unable to neutralize the infectivity of the other two types (46, 65).

1.8 Poliovirus Genetic Organization and Protein Functions

The poliovirus type 1 (Mahoney) genome consists of a single copy of plus-sense single-stranded RNA which is approximately 7.5 kb long. The genome has a relatively long, highly structured 5' non-translated region (NTR) of 742 nucleotides with a covalently linked 22 amino acid peptide primer referred to as VPg or 3B at the 5' terminus. The 5' non- translated region contains sequences that control genome replication and translation. It contains a terminal clover-leaf structure that is indispensable to replication regulating initiation of translation. It also contains the internal ribosome entry site (IRES) that directs cap-independent translation of viral mRNA by internal ribosome binding. The 3' NTR at the other end is relatively short (70 nucleotides) and is followed by a polyA tail of 60 residues (21). The 3' NTR also contains secondary structures, including a pseudoknot that has been implicated in controlling viral RNA synthesis (50). Deletion of the 3' NTR has minimal effects on the propagation of the virus in HeLa cells but it significantly reduces the ability of the virus to propagate in

cells of neuronal origin *in vitro* and *in vivo* (16). Between these two regions lies a single open reading frame that codes for a 250 kDa polyprotein of 2209 amino acids. The polyprotein is processed into various precursor proteins and stable processed proteins by virally encoded proteases (21).

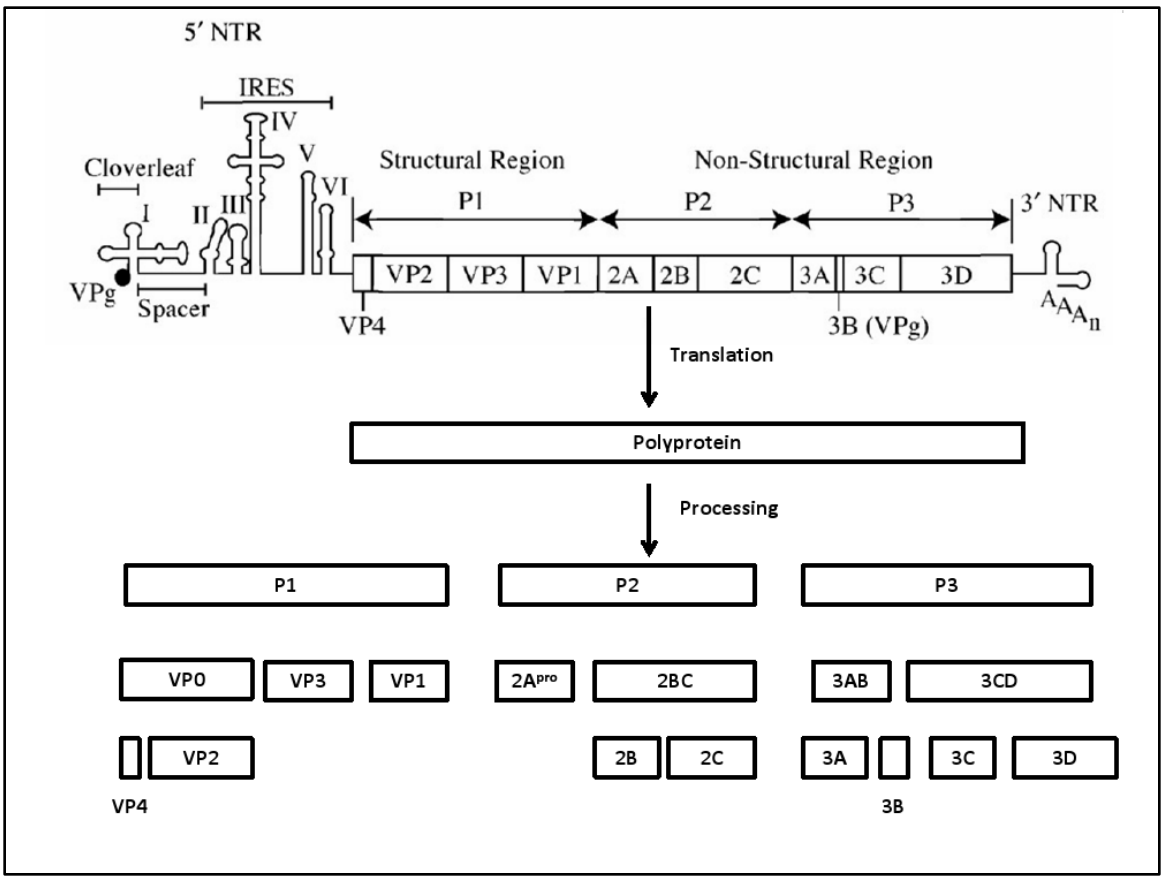


Figure 1-2: Genomic structure of poliovirus type 1 (Mahoney strain) and proteolytic processing of its polyprotein. The poliovirus genome consists of a single stranded, positive sense RNA molecule encoding a single polyprotein. The 5′ NTR harbors two functional domains, the cloverleaf structure and the IRES, and is covalently linked to a short highly basic protein, 3B (VPg). The 3′ NTR has a pseudoknot structure and a poly A tail. The polyprotein derived from the single mRNA transcript is cleaved by virus encoded proteases into ten fully processed proteins in addition to various intermediate precursor proteins. Figure is adapted from De Jesus, N.H (21).

TABLE 1-2

Protein	Properties and functions
3A	Required for RNA replication Membrane associated Inhibits host protein secretion Substrate for glycoslysation Interacts with 3AB Dimerizes
3B	Required for RNA replication Covalently linked to plus- and minus- strand RNA Substrate for uridylation Weakly stimulates 3D activity in vitro Weakly stimulates $3CD^{pro}$ autoprocessing Interacts with $3D^{pol}$ or $3CD^{pro}$
$3C^{pro}$	Processes viral proteins Cleaves host proteins Weakly binds to 5′ cloverleaf with 3AB
3Dpol	Required for RNA replication Uridylylates Vpg Elongates RNA chains Unwinds double stranded RNA during polymerization Binds to RNA Has terminal adenylyltransferase activity Can switch templates Interacts with cellular protein Sam68 Interacts with $3CD^{pro}$, 3B, 3AB Dimerizes
3AB	Required for RNA replication Associated with membranes Binds to RNA (nonspecific) Binds to 5′ cloverleaf structure with $3CD^{pro}$ Binds to 3′ NTR with $3CD^{pro}$ or 3Dpol Substrate for gyclosylation Stimulates $3D^{pol}$ activity invitro Stimulates $3CD^{pro}$ autoprocessing invitro Interacts with $3D^{pol}$, $3CD^{pro}$ Oligomerizes
3CD	Required for RNA replication Processes viral proteins Processes cellular protein Binds to 5′ cloverleaf with PCBP2 Interacts strongly with 3B, 3AB Interacts weakly with 3Dpol, $3CD^{pro}$ Binds to 3′ NTR with 3AB Binds to PV1 cre (2C)

Table 1-2: Properties and functions of proteins encoded by the P3 region of the poliovirus genome

1.9 Life Cycle of Poliovirus

The life cycle of the poliovirus is divided into four main steps (Figure 1-3)

1. Virus attachment and entry: Replication of poliovirus is initiated by attachment of the virus to its cognate receptor, CD155, found only on the surface of primate cells. The human poliovirus receptor belongs to the immunoglobulin superfamily and may function as a cell-adhesion molecule (64). Once the virus particle is internalized by either receptor-mediated endocytosis or through pore formation in the cell membrane, the genome is injected into the cytoplasm through vesicles located very close to the plasma membrane (34, 61). Within the host cell, the 5' NTR linked viral protein VPg may be cleaved by an unknown host cell phosphodiesterase termed VPg unlinkase (38, 89), although the enzyme causing the cleavage still remains controversial. The poliovirus genomic RNA now serves as an mRNA ready to be translated.
2. Protein synthesis: The host translational machinery binds to a highly structured RNA *cis*-acting element termed the internal ribosome entry site (IRES). The absence of a 7-methyl guanosine cap structure at the 5' end of the genome results in cap-independent translation of the polyprotein via the IRES. This polyprotein is co- and post-translationally cleaved into three main precursor proteins, P1, P2 and P3, by viral encoded proteases $2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$ (Table1-2). The P1 region encodes capsid proteins, VP1-VP4, while the P2 region encodes non-structural proteins ($2A^{pro}$, 2B, 2BC and $2C^{ATPase}$) that induce biochemical and structural changes in the host cell. The proteins of the P3 region are mainly involved in

RNA synthesis. They include highly stable precursor proteins 3AB and $3CD^{pro}$ that function in replication and may also be further processed into mature proteins 3A, 3B (VPg), $3C^{pro}$ and $3D^{pol}$ (21). These viral proteins cause disruption of a number of cellular functions, including inhibition of host-cell transcription and translation and the rearrangement of the host-cell endoplasmic reticulum to form membranous vesicles throughout the cytoplasm (17, 112).

3. RNA replication: With the synthesis of viral proteins, the virus now begins its replication process (Figure 1-3). Replication is believed to take place on the surface of host-cell endoplasmic reticulum derived rosette-like membranous structures, the formation of which is induced by viral encoded proteins 2BC and 2C (12). A replication complex comprised of the genomic RNA, viral proteins, and cellular proteins (Figure 1-4) is formed on the membranous structures that help increase the local concentration of these components and provide a scaffold for the assembly of the complex. 3AB is thought to associate with these membranous vesicles through its hydrophobic domain and also recruit $3CD^{pro}$ and $3D^{pol}$ owing to its affinity for these proteins. Genome replication is initiated by $3D^{pol}$ catalyzed uridylation of VPg. Uridylated VPg (Vpg-pU-pU) serves as a primer for negative strand synthesis, which results in a double stranded RNA known as the replicative form, RF. The RF is continuously formed during infection at a relatively low rate and is generally believed to be an intermediate of replication rather than an artifact of isolation procedures (59). Positive strands presumably originate from single stranded negative strands rather than RFs. Each

negative strand produces many new positive strands in a complex called a replicative intermediate (RI).

Negative strands are always associated with either the RF or RI and are never found free in the cell. The newly synthesized positive strand RNA can be used as templates for further translation of proteins or can associate with capsid precursors to undergo encapsidation, forming progeny virions that are then released upon lysis of infected cells (21).

4. Packaging and release: Encapsidation of Vpg - linked positive strand RNA molecules appears to be linked to RNA synthesis at the interface of membranous structures in the cytoplasm of infected cells. The capsid proteins VP0, VP1 and VP3, formed as a cleavage product of the P1 region of the polyprotein, assemble as promotmers to form pentamers. Twelve pentamers constitute a procapsid. The VPg - linked genomic RNA maybe encapsidated by condensation of pentamers about the viral RNA or by incorporation of viral RNA into the capsid. The final step involves the autocatalytic cleavage of VP0 into VP2 and VP4 that stabilizes the capsid and converts the provirion into a mature virus particle. Subsequent lysis of the infected cells leads to the release of mature virions of which about 1% can further initiate an infection in permissive host cells (21).

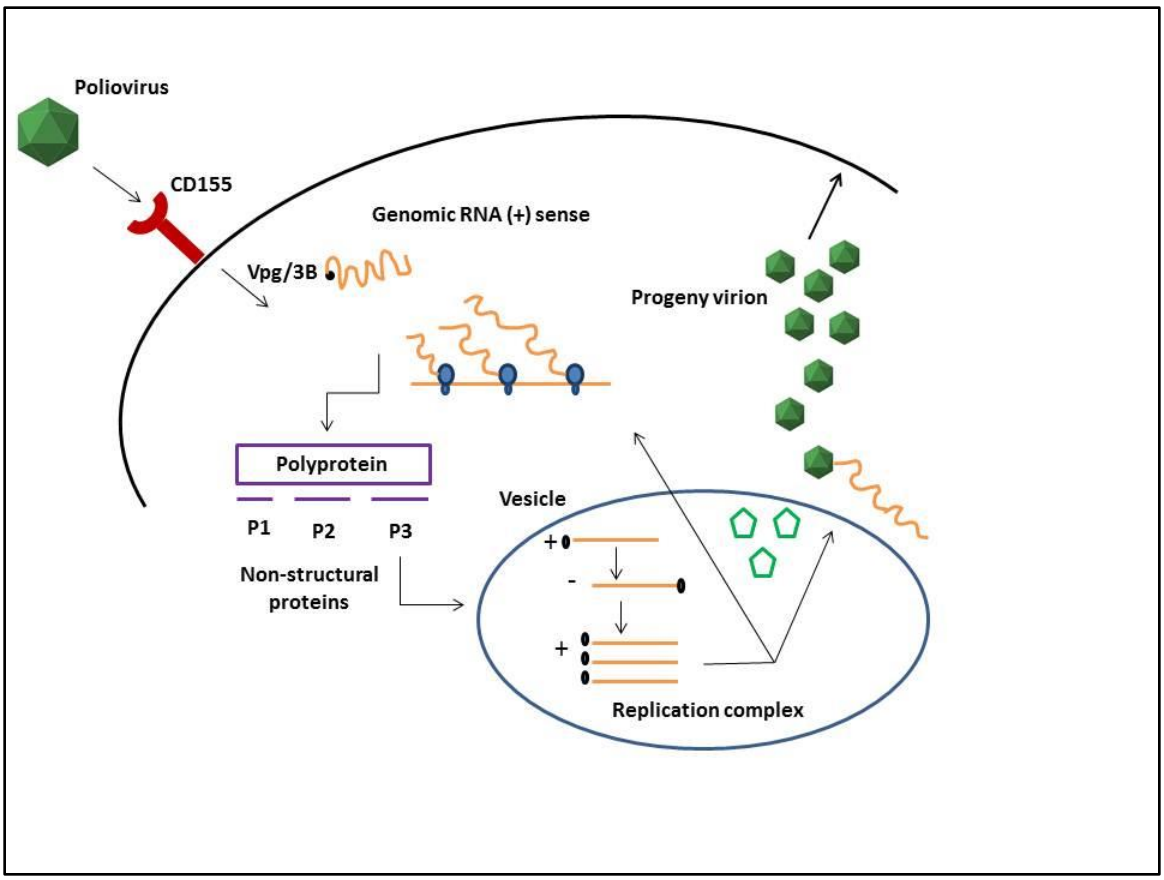


Figure 1-3: Schematic representation of the cellular life cycle of poliovirus. The cellular life cycle of poliovirus is initiated by binding to its cognate receptor CD155. Uncoating of viral RNA is followed by cap-independent translation giving rise to various proteins upon polyprotein processing. These proteins are utilized for positive-sense RNA synthesis which is formed from a negative strand intermediate. The newly synthesized positive strands can serve as templates for translation or associate with capsid proteins to undergo encapsidation and induce maturation cleavage of VP0, ultimately generating progeny virions. Lysis of the infected cell results in release of infectious progeny virions. Figure adapted from De Jesus, N.H. (21).

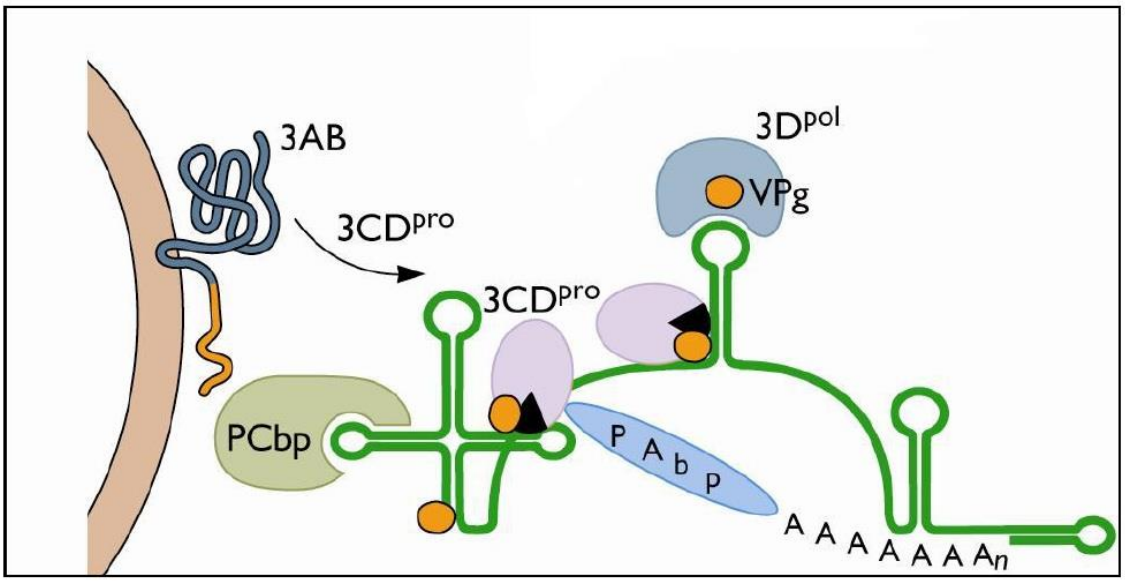


Figure 1-4: Schematic representation of poliovirus replication complex on membrane vesicles. Poliovirus replication occurs on the surface of intracellular membrane vesicles which, are induced by virus encoded proteins, mainly $2C^{pro}$ and 2BC. 3AB is thought to anchor the replication complex on the surface of membrane vesicles via its hydrophobic domain. 3AB interacts with other P3 proteins to form protein-protein bridges that allow non-RNA binding proteins required for replication, to interact with RNA. For example 3CD binds to 5' NTR in the presence of 3AB along with a cellular factor, Poly (C)-binding protein, (PCBP). Poly (A)-binding protein, (PABP), also a cellular factor, binds to the 3′ NTR which in turn interacts with the 5' NTR. This leads to genome circularization that brings the viral polymerase $3D^{pol}$ in close proximity of the 3′ poly A tail and allow for initiation of negative strand synthesis. Figure taken from Paul, A.V. Molecular Biology of Picronaviruses (75) .

1.10 Poliovirus Protein 3AB and its Role in Poliovirus Replication

Poliovirus 3AB is a 12 kDa protein with a hydrophobic 3A region (N-terminal 87 amino acids) and a highly basic 3B (VPg) region (C-terminal 22 amino acids). It is formed as a cleavage product of an 83 kDa precursor protein encoded by the P3 region of the viral genome, which in turn is derived from a 247 kDa viral polyprotein translated by a single open reading frame mRNA transcript (Figure1-2). 3AB is a multifunctional protein and one of the key players of the viral replication process. Infection of cells with poliovirus leads to accumulation of new membrane vesicles derived either from intracellular organelle rearrangement or *de novo* membrane synthesis. These rosette-like membranous structures support the viral replication complex on their surface, similar to what occurs in other plus sense RNA viruses. 3AB is thought to anchor the replication complex on these membrane vesicles through the hydrophobic domain in the 3A region of 3AB (35).

3AB interacts with other P3 proteins to form protein-protein bridges that allow non-RNA binding proteins required for replication, to interact with RNA. For example 3CD binds to the 3' and 5' non-translated region in the presence of 3AB (40, 111). Interaction between 3AB and 3CD stimulate the autoprocessing of 3CD to produce $3C^{pro}$ and $3D^{pol}$ and allows the membrane-associated 3AB to be cleaved to produce 3A and 3B (66). It has been shown that the processing of 3CD requires membrane bound 3AB (56). 3AB by itself is a non-specific RNA binding protein but in the presence of 3CD binds specifically to the 5' end of the genome (40, 111). 3AB also binds to $3D^{pol}$ and helps localize the replication complex to membranous vesicles. 3AB is known to stimulate the

synthetic activity of $3D^{pol}$ *in vitro,* especially when low concentrations of the polymerase are used (57, 76, 79, 84, 86).

3AB is cleaved to 3A and 3B by $3CD^{pro}$. 3A is a critical component of the replication complex and is a putative target of enviroxime, an antiviral drug that block viral replication (42). 3A inhibits host cell endoplasmic reticulum – Golgi apparatus transport, a function that may play a role in viral evasion from the host immune system (24, 25, 71). Although 3A differs from its precursor 3AB by 22 amino acids, the two proteins have distinct functions in viral replication. 3A does not stimulate the activity of $3D^{pol}$ or $3CD^{pro}$ *in vitro*; however mutations in the 3A region of 3AB produce replication-deficient viruses. The solution structure of a truncated form of 3A reveals that it is a homodimer, with the internal region of each mononmer adopting a helical hairpin fused to unstructured N- and C-termini (98).

3B (VPg) is covalently attached to all newly synthesized plus and minus strand RNAs via a phosphodiester bond between the conserved tyrosine residue at position 3 (Y3) of VPg and the terminal UMP in the RNA genome (3, 88). The uridylated form of this protein serves as a primer for RNA synthesis (74). Two uridine residues are added to the tyrosine residue, Y3, of VPg, in a reaction catalyzed by $3D^{pol}$ using a template referred to as CRE (*cis*-acting replication element) located in the 2C region of the genome (77, 97). Uridylated VPg is then transferred to the 3' end of the genome or minus sense copy of the genome by an unknown mechanism, and is extended by $3D^{pol}$ to initiate RNA replication.

1.11 Nucleic acid chaperone proteins

Nucleic acid chaperones are proteins that assist in folding nucleic acids to their biologically active and thermodynamically most stable conformations by resolving misfolded structures and kinetically trapped species (44, 81, 119). They do so by lowering the energy barrier for breakage and re-formation of base pairs. The interaction of a nucleic acid molecule with a chaperone protein results in transient unpairing of bases within the chain, making them available for repairing in alternate combinations (82).

Nucleic acid chaperone possess weak helix destabilizing (unwinding) activity that disrupts secondary structures and promote the annealing of complementary nucleic acids strands. Some chaperones can drive the formation of nucleic acid aggregates *in vitro*, thus further enhancing hybrid formation (20, 44). These activities of chaperone proteins are thought to encourage efficient viral genome replication within the host. Exactly how this occurs *in vivo* has not been established. The multifunctional and ubiquitous nature of chaperone proteins has made it difficult to assign discrete activities using standard genetic mutation based approaches. For plus sense viruses chaperone activity could be involved at several stages of the viral life cycle including: helping $3D^{pol}$ traverse the highly structured genome, promoting recombination, and facilitating structural switches that may be important for the genome to function in translation, replication and packaging (Figure 1-5).

The helix destabilizing activity of a chaperone protein can be explained by the entropy transfer model. According to this model, a chaperone protein that is predicted to have a region of disorder, unwinds secondary structures and refolds them into their thermodynamically stable conformation in a series of disorder-order promoting steps (

Figure 1-7) (119). Highly flexible regions of the protein undergo disorder to order transitions upon binding to the RNA, melting secondary structures within the RNA through entropy exchange process. The destabilized RNA could now adopt its most stable conformation. The interaction between the chaperone protein and the RNA is thought to be transient and non-specific. These proteins have to presumably get on and off to allow RNAs to fold properly and retain structure.

Chaperone proteins are known to promote hybridization of complementary nucleic acids. They destabilize weak secondary structures without the use of ATP hydrolysis and promote aggregation of complementary strands either by directly promoting binding or by reducing charge repulsion by coating nucleic acid strands (60). It has been well established that 3AB promotes hybridization of complementary nucleic acids in the absence of nucleotides (as an energy source). Hybrid formation is concentration-dependent and requires enough 3AB to at least partially coat nucleic acid strands (23). 3AB's chaperone function is similar to other chaperone proteins, such as human immunodeficiency virus nucleocapsid protein (HIV NC) and heterogenous nuclear ribonucleoprotein A1 (hnRNP A1). Both HIV NC and hnRNP A1 are known to bind and coat nucleic acids. HIV NC displays both specific as well as non-specific nucleic acid binding. The helix destabilizing activity of NC appears to be due to the physical binding of NC's zinc fingers to the unpaired bases of nucleic acids strands whereas the formation of nucleic acid aggregates is facilitated by coating nucleic acid strands and reducing inter-strand repulsion. The formation of nucleic acid aggregates increases the probability of finding complementary strands and facilitates the nucleation step of annealing. As a result, these properties are expected to greatly accelerate the rate

of hybridization between complementary sequences (60). Nucleic acid chaperone proteins tend to be positively charged. HIV NC has 16 positively charged amino acid residues out of a total of 55 while 3AB has not zinc fingers and only 14 positively charged amino acid residues out of its 109 total amino acids. In 3AB, these charges are grouped into discrete clusters at the amino and the carboxyl terminal regions. Mutations of positively charged bases at the carboxyl terminal region (3B region) greatly affect binding to RNA and stimulation of $3D^{pol}$. Therefore, this region is very important to RNA binding as well 3AB/3B-$3D^{pol}$ interactions (115). While chaperone proteins do not have a conserved motif that defines their activity, most of them are known to have intrinsically disordered domains, i.e. a cluster of amino acids that do not have a well-defined conformation under physiological conditions *in vitro* (102). These flexible regions probably allow the proteins to bind to a wide range of nucleic acid structures and sequences as well as promote rapid association and dissociation with target molecules. Based on GlobProt, an online program for predicting disordered protein regions, 3AB has an intrinsically disordered domain in the highly basic and hydrophilic 3B region, (see Figure 1-6).

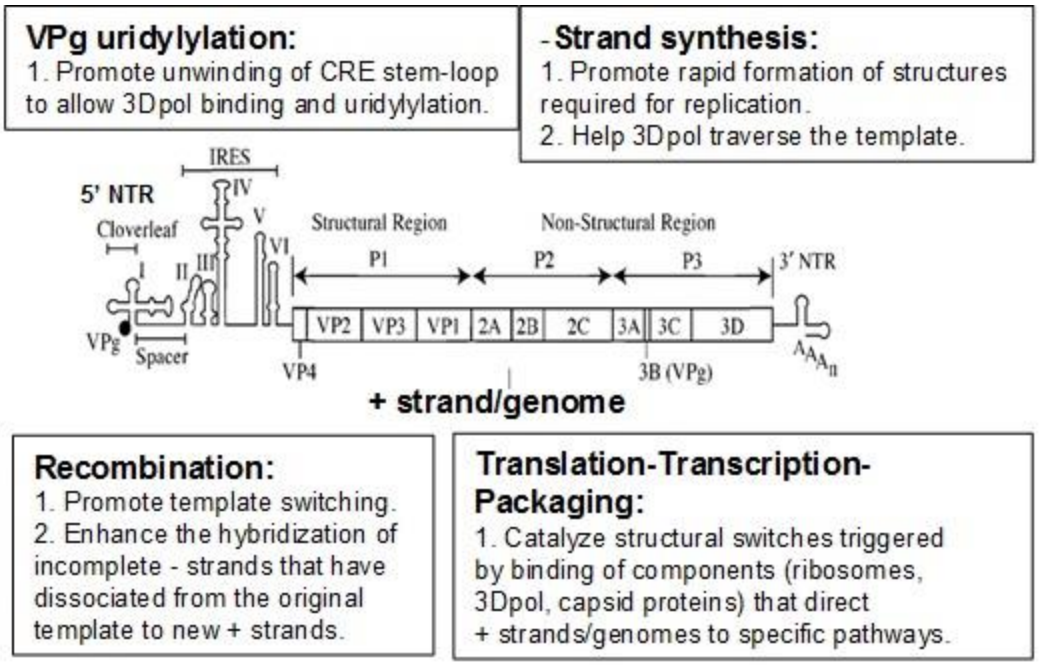


Figure 1-5: Possible roles for chaperone activity in poliovirus/picornavirus replication. A schematic representation of the poliovirus genome along with important secondary structures in the non-translated regions is shown. Possible roles of 3AB or other chaperones are noted in the boxed area. Figure adapted from De Jesus, N.H (21).

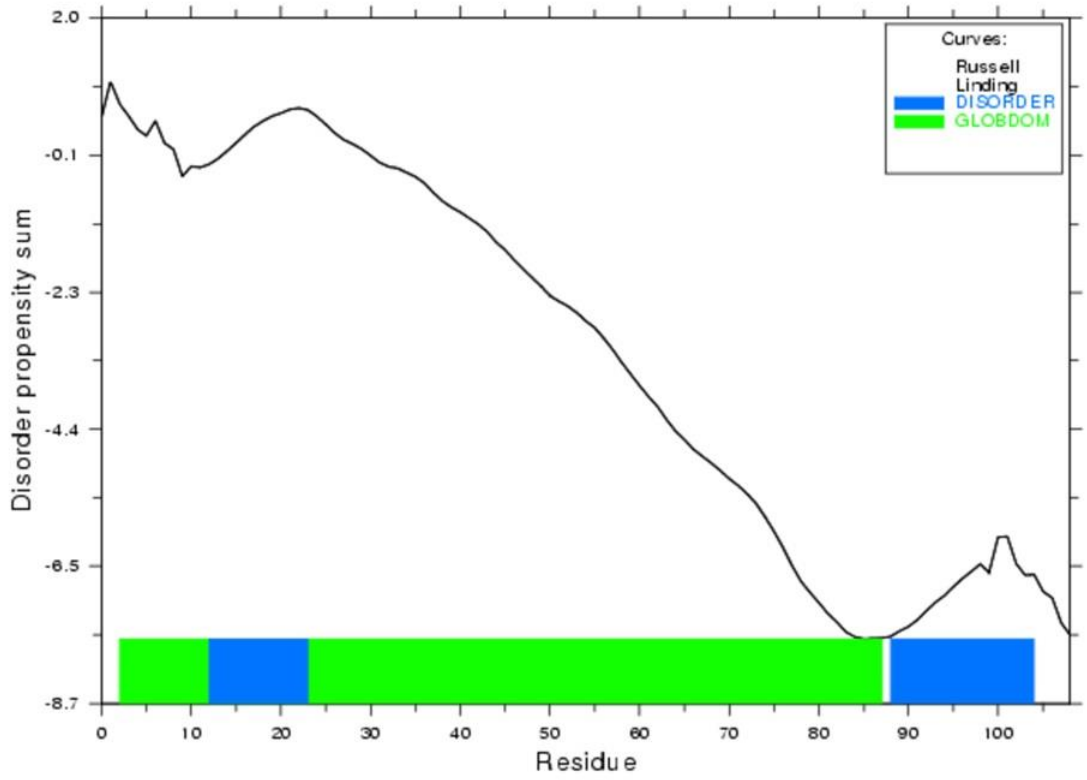


Figure 1-6: Predicted disordered domain of PV 3AB protein using GlobProt: Virus encoded chaperone proteins do not share a common domain or motif but display a high degree of disorder, suggesting that disorder might be a mechanistic requirement for chaperone activity. According to this prediction, a region of disorder lies in the N-terminus of 3A region and the entire 3B region (indicated in blue). Our results indicate that 3B region does not possess chaperone activity by itself but is required for chaperone activity of 3AB (Chapter 2). It is possible that the disordered region of 3B contributes to the chaperone function of the 3AB. Figure generated using GlobProt intrinsic disorder prediction online software by Linding, R., Russell, R.B., Neduva, V., Gibson, T.J. (62).

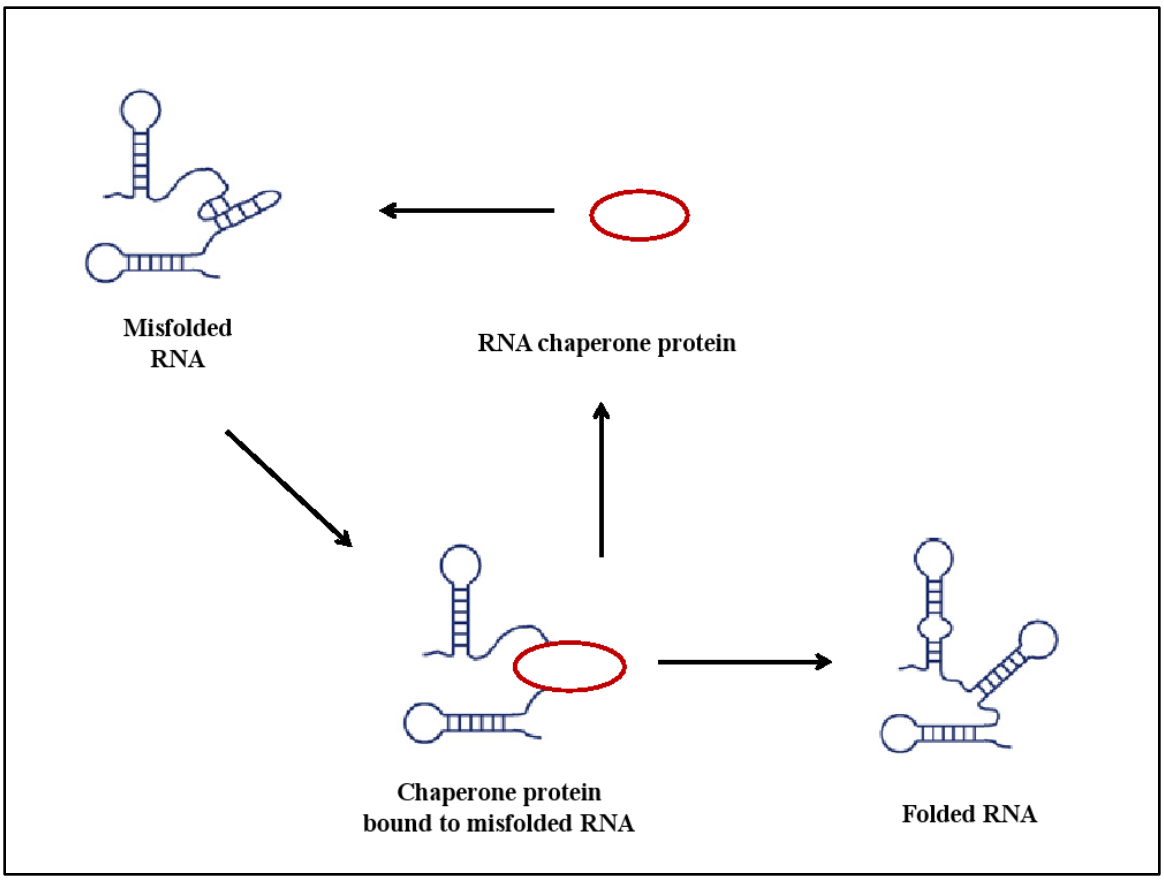


Figure 1-7: Model for RNA chaperone mechanism based on entropy transfer. A fully disordered RNA chaperone (red) binds to a partially misfolded substarte RNA in a non-specific manner. Disordered domains of the protein become ordered while domains of RNA are unfolded (entropy transfer from protein to RNA). The RNA is correctly folded while the protein becomes disordered again (entropy transfer from RNA to protein). The correctly folded RNA is released after multiple cycles of order-disorder, and the chaperone protein is free to act again. This process occurs in the absence of ATP hydrolysis. Figure adapted from Zuniga, S., Sola, I., Cruz, J., Enjuanes, L. (119)

1.12 Chaperone proteins in viruses

The first reported viral chaperone was the HIV NC protein in 1988. Since then the list of chaperones among viruses has been growing rapidly leading to an increased interest in this field. The most well studied viral chaperone protein is HIV NC. As a chaperone protein NC facilitates dimerization of genomic RNA, initiation of reverse transcription, strand transfer (template switching) events during reverse transcription, and also promotes recombination. In addition, NC is required for packaging, tRNA association with the genome for initiation of replication, and possibly integration of the proviral DNA (60). This illustrates the difficulty in assigning specific functions to this chaperone as single mutations may affect several protein functions. Some host proteins like hnRNP A1, also possess chaperone function that is thought to be essential for viral RNA synthesis. Interestingly, while chaperone proteins occur in many species, they do not share any common motifs or conserved sequence. Also, chaperone proteins from the same virus genus have little sequence similarity (119). Table 1-3 lists the chaperone proteins found in viruses to date.

The goal of the study is to identify what regions of 3AB are intrinsic to chaperone activity and to test the effect of mutations in the putative chaperone domain on replication of the virus in tissue culture.

TABLE 1-3

[a]Protein	[b]Virus	Function
Virus Encoded		
NC	Retrovirus	Reverse Transcription, Template switch
SdAg	HDV	Replication
Core	Flaviviridae	Replication
N	Coronavirus	RNA synthesis
N	Hantavirus	Replication
3AB	Poliovirus	Replication, Recombination
Vif	HIV-1	Temporal regulation of reverse transcription
Tat	HIV-1	Viral DNA transcription
Host Factors		
PTB	Picornavirus, HCV, Coronavirus, Calicivirus	Translation, RNA synthesis
hnRNP A1	Coronavirus	RNA synthesis
La	HCV, Poliovirus	Translation, Replication
Unr	HRV-2	Translation
PARBP33	ASBVd	Replication

a NC, nucleocapsid protein; SdAg, small delta antigen; N, nucleocapsid protein; Vif, viral infectivity factor; Tat, transcription activation factor; PTB, polypyrimidine tract binding protein; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; Unr, upstream of N-ras.

b HDV, hepatitis delta virus; HIV-1, human immunodeficiency virus-1; HCV, hepatitis C virus; HRV-2, human rhinovirus-2; ASBVd, avocado sunblotch viroid.

Table 1-3: A list of RNA chaperone proteins involved in virus life cycle

Chapter 2: The C terminal cytoplasmic domain of 3AB is intrinsic to the chaperone activity of 3AB

2.1 Introduction

Poliovirus replication involves complex secondary structures at the viral genome terminal regions, and both host and viral proteins. Replication occurs on the surface of intracellular membrane vesicles derived from the ER (11). 3AB is an integral part of the viral replication machinery as it anchors replication complexes to internal cellular membranes, interacts with several proteins required for genome replication and stimulates the viral polymerase ($3D^{pol}$) *in vitro* (reviewed in section 1.10). Details of the mechanism(s) by which 3AB accomplishes these functions remain vague. 3AB is a small hydrophobic protein with a membrane-binding region near the C-terminus of its 3A region. It has been shown to possess biochemical characteristics of an integral membrane protein (103). 3AB is proteolytically cleaved by 3C/3CD viral proteases to yield 3A and 3B (VPg) (56). The 3A protein is 87 amino acids in length, consisting of a soluble N terminal domain that forms a symmetrical dimer in solution (58 residues), a 22 amino acid hydrophobic domain (59-81) followed by seven amino acids at the C-terminus. The hydrophobic domain is subdivided into two domains (I, aa 64-72 and II, aa 73-80), the latter being the main membrane-anchoring determinant. The first 15 amino acids of the hydrophobic domain are predicted to form an amphiphatic helix. Even though the membrane association of protein 3A and 3AB has been known for years, the precise arrangement of these proteins in the hydrophobic lipid environment remains unclear (103). Recent work by Fujita et al. suggests a model in which 3AB interacts with

membranes forming a non-trans membrane configuration while the soluble regions of 3A and 3B (VPg) remain on the same cytosolic side (35).

In 2006, our lab showed that bacterially expressed purified 3AB protein possesses properties consistent with chaperone proteins - i.e. helix destabilizing activity and promotion of hybridization of complementary nucleic acids in the absence of nucleotides (as an energy source) (23) classifying 3AB as a nucleic acid chaperone. The effect was nonspecific as enhancement of annealing occurred for both RNA and DNA and even highly structured nucleic acids could be annealed. Hybrid formation was concentration-dependent and required enough 3AB to coat the nucleic acid strands. Conditions for optimal activity were established and included low divalent cation (1 mM Mg^{2+}) and salt (20 mM KCl) concentrations although 3AB also showed modestly reduced chaperone activity with higher more physiological salt concentrations.

In the current report we show that the 3B region of 3AB is necessary but not sufficient for chaperone activity as specific mutations in this domain abolish chaperone activity while 3B alone has no chaperone activity even at very high concentrations. Interestingly, the putative C-terminal cytoplasmic domain of 3AB, consisting of 3B and the last 7 amino acid at the C-terminal end of 3A (termed 3B+7 in this report), does have chaperone activity at high concentrations. These results form the basis of a working model of 3AB chaperone function presented in this report.

GPLQYKDLKIDIKTSPPPECINDLLQANDSQEVRDYCEKKGWIVNITSQVQTERNI
1 56

NR***AMTILQAVTTPAAVAGVVYVMY***KLFAGHQGAYTGLPNKKPNVPTIRTAKVQ
59 81 88 109

Figure 2-1: Amino acid sequence of 3AB. Amino acids 1-58 make up the 3A region of 3AB. The bolded letters indicate the hydrophobic region (amino acids 59-80). VPg or 3B is indicated by letters in red (amino acids 88-109). The table below indicates the different domains or regions of 3AB.

Region	Amino acids
3AB	1 – 109
3A	1 – 87
3B (VPg)	88 – 109
Hydrophobic membrane anchor sequence	59 – 80
C-terminal cytoplasmic domain of 3AB	81 – 109
Connection domain	81 – 87

Table 2-1: Regions of 3AB

2.2 Materials and Methods

2.2.1 Materials

T3 RNA polymerase, calf intestinal phosphatase (CIP), DNase I (RNase free), and ribonucleotides, were obtained from Roche Applied Science. RNasin was obtained from Promega, restriction enzymes and T4 polynucleotide kinase from New England Biolabs and Proteinase K from Stratagene. All synthetic oligonucleotides were custom – ordered from Integrated DNA technologies Inc. (IDT). Chemically synthesized proteins including 3B, 3B+7, and all 3B+7 proteins with mutations were from GenScript Inc. and were of 90% purity or greater. Point mutations in 3AB were made using the Quickchange Lightening Site-Directed Mutagenesis kit from Agilent technologies. Sephadex G-25 spin columns were from Harvard Apparatus. RNeasy mini kits were from Qiagen Inc. Radiolabeled compounds were from Perkin Elmer. All other chemicals were obtained from Sigma Chemical Co., Fisher Scientific, or VWR scientific.

2.2.2 Methods

Preparation of recombinant proteins poliovirus 3AB and HIV NC by expression in *Escherichia coli*. Protein 3AB of poliovirus type 1 (Mahoney strain) and its mutant forms 3AB-Y90A, 3AB-R104E and 3AB-Y90A/R104E were expressed in *E. coli* using plasmid pGEX-3AB, kindly provided by Dr. Stephen Plotch (formerly of Wyeth-Ayerst Research). Mutagenic primers for 3AB mutations are listed in Table 2-2. Expression and purification was performed as previously described (79). Purified 3AB was stored at -80^{o}C in buffer containing 50 mM Tris-HCl (pH=8), 1 mM DTT, 0.05% Triton X-100, and 10% glycerol (3AB buffer). HIV NC protein was produced using a vector kindly

provided by Dr. Charles McHenry (Univ. of Colorado). The protein was expressed in *E. coli* and purified as previously described (116).

Preparation of mutant forms of poliovirus 3AB with point mutations in the connection domain. Protein 3AB has a tendency to form insoluble aggregates/inclusion bodies during protein purification. We experienced difficulty using the pGEX expression system used for wildtype 3AB and specific mutants noted above. Although expression was successful, nearly all of the truncation and connection domain mutants formed insoluble aggregates. This was not resolved using various detergents and expression temperatures. SUMO-based expression systems have been reported to greatly aid in the expression of difficult proteins. We were successful in optimizing expression and purification of SUMO tagged wildtype 3AB and mutant forms of the putative C-terminal cytoplasmic domain of 3AB. Mutagenic primers for 3AB mutations are listed in Table 2-2. *E.coli* BL21[DE3] containing pSUMO-3AB was grown at 37°C in one liter 2xYT broth containing 40µg/ml Kanamycin to an optical density at 600 nm of 0.6 at which time IPTG (isopropylthiobetagalactoside) was added to a final concentration of 0.25mM. After 4h at 30°C cells were harvested by centrifugation and the pellet was either stored at -80°C or suspended immediately in 20 ml of 50 mM Tris-HCl pH 7.0, 200 mM NaCl, 1 mM DTT, 0.1% Triton X-100 and 10% glycerol (buffer I) per liter of original culture. The suspension was sonicated at 0°C using a Branson sonifier 450 with a tapered microtip (setting 6, 50% duty cycle) pulsing 6 x 30 seconds with 30 second intervals. The lysate was centrifuged at 25,000 x *g* for 20 min. The pellet was re-extracted in 10 ml of buffer I containing 1M NaCl. The lysate was centrifuged as before and the obtained supernatant was diluted fivefold with buffer I. The two supernatants were pooled together (70 ml) and incubated

with 3.0 ml of PerfectPro Ni-NTA agarose resin (5 PRIME) equilibrated in buffer I. After incubating for 2.5h at room temperature, the agaorse gel was washed with 50 column volumes (75 ml) of buffer I + 10 mM imidazole. The SUMO-tagged protein was eluted in 4 ml buffer I + 300 mM imidazole and dialyzed for several hours against one liter of 50 mM Tris-HCl pH 7.0, 30 mM NaCl, 1.0 mM DTT, 0.1% Triton X-100 and 10% glycerol (buffer A) at 4°C in presence of 50 units of SUMO protease 1. Any precipitate was removed by centrifugation and clear supernatant was loaded onto a 5 ml mini profinity IMAC Ni cartridge pre-equilibrated with buffer A using a Bio-Rad Biologic DuoFlow FPLC. After being washed with 10 ml of buffer A, the column was developed with a 25 ml linear gradient of 30 mM to1 M NaCl in buffer A. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and assayed for unwinding activity by FRET (see below). The protein minus the SUMO tag elutes at low salt concentrations while the SUMO tag, which contains a histidine nickel binding sequence, remains bound to the column. Fractions containing untagged 3AB were aliquoted and stored at -80°C.

5' end-labeling of oligonucleotides. Reactions for primer labeling were done in a 50 µl volume containing 70 mM Tris-HCl, pH=7.6, 10 mM $MgCl_2$, 5 mM DTT, 10 µl of γ-^{32}P ATP (3000 Ci/mmol, 10 µCi/µl) and 2 µl (20 units) of T4 polynucleotide kinase. The reaction mixture was incubated for 30 minutes at 37^{o}C and then the T4 polynucleotide kinase was heat inactivated for 10 minutes at 70^{o}C according to manufacturer's recommendation. The material was then run through a Sephadex G-25 spin column to eliminate free nucleotides and exchange buffer.

Preparation of RNA templates. Run-off transcripts were synthesized with T3 RNA polymerase using the manufacturer's protocol. Plasmid pBSM13+ was cleaved with Bgl I or Nde I was used to prepare run-off transcripts ~230 or 765 nucleotides in length, respectively. After transcription for 2 hours, 15 units of DNase I (RNase-free) was added and incubation was continued for 20 min. Reactions were then processed with a RNeasy mini kit according to the manufactures' protocol. The length and purity of the RNA was evaluated by gel electrophoresis to assure that it was full-length (data not shown). The RNA was then quantified by spectrophotometric analysis. The equation used to calculate the molecular weight was: ([A x 382.2] + [G x 344.2] + [C x 304.2] + [U x 305]). The molecular weight was used to calculate the molar concentration of RNA using the standard conversion of 1 $OD_{260} \approx 40$ µg/ml for single stranded RNA.

Fluorescence resonance energy transfer (FRET) unwinding assay. Two complementary 42 nucleotide DNAs, one with a 5' fluorescein-6-carboxamidohexyl (FAM) (FAM-CAT TAT CGG ATA GTG GAA CCT AGC TTC GAC TAT CGG ATA ATC-3') group and the second with a 3' 4-[[(4-dimethylamino)phenyl]-azo] benzenesulfonicamino (DABCYL) group (5'- GAT TAT CCG ATA GTC GAA GCT AGG TTC CAC TAT CCG ATA ATG-3'- DABCYL) were used in the assays. Using mfold(118) and conditions of 20 mM KCl, 1 mM $MgCl_2$, and 30^{o}C, each strand was predicted to form a stem-loop structure with a ΔG value of -7.2 kcal/mol (Fig.). Annealing assays were completed at 30^{o}C using a Cary Eclipse fluorescent spectrophotometer (Varian). FAM and DABCYL DNAs (10 and 20 nM, respectively.) were separately incubated for 5 min at 30^{o}C in the presence or absence of 3AB or other proteins at the concentrations indicated in 35 µl of buffer containing 50 mM HEPES

(pH=7), 20 mM KCl, 5 mM DTT, 1 mM $MgCl_2$, 6 mM Tris-HCl (pH=8), 0.014% Triton X-100, and 2.9% glycerol. The reactions were started by mixing the FAM and DABCYL samples in a quartz cuvette (final concentrations 5 and 10 nM, for FAM and DABCYL DNAs, respectively). The excitation wavelength was 494 nm with a bandwidth of 5 nm. The emission bandwidth was 10 nm and the spectrum was observed at 520 nm. The emission spectrum was taken every 15 seconds for up to 16 minutes. An intensity ratio (I_r) was determined by dividing the peak intensity at a given time (I_t) by the peak intensity at time zero (I_0) ($I_r = I_t/I_0$). This value was plotted versus time for the different concentrations of protein used. For some proteins rate constant values (*k*) describing the rate of annealing for the complementary strands were calculated. These values were calculated from the $t_{1/2}$ values by dividing 0.693 by $t_{1/2}$ as described.(117)

Primer-template annealing assay. The 230 nucleotide RNA transcript (10 nM final concentration) (described above), and a 25 nucleotide complementary 5′ ^{32}P-labeled RNA oligonucleotide (10 nM final concentration, 5'-CCU CUU CGC UAU UAC GCC AG -3') that hybridized to based 155-179 from the 5' end of the transcript were incubated in 50 mM HEPES (pH=7), 5 mM DTT, 1.4 mM $MgCl_2$ and 28.5 mM KCl. This material was pre-incubated for 3 mins at 30^{o}C in 7 µls total volume. Three µls of 3AB or other derivatives (concentration as indicated) or 3AB buffer (see above) was added after the pre-incubation to start the reaction which was continued for 15 minutes. One µl of proteinase K (final concentration 1 µg/µl) in 10 mM Tris-HCl pH=8, 50 mM EDTA pH=8 was then added to each sample and incubation was continued for 10 minutes at 37^{o}C. After this, 5 µls of stop mix (20% glycerol, 20 mM EDTA pH=8, 0.2% SDS, 0.4 µg/µl tRNA, 0.1% bromophenol blue) was added. There were also two controls. One

control contained the pre-incubated reaction and 3AB buffer and was heated at 65^{o}C for 5 mins and then slow cooled. This control was used to show where the hybrid migrated on the gel. A second control contained BSA (final concentration 0.1 µg/µl) in 3AB buffer instead of 3AB. All controls were processed as above. Products were analyzed on a 6% native polyacrylamide gel as describe below. Dried gels were exposed to an imaging screen and visualized and quantified using a Fujifilm FLA-7000.

Gel-shift assay to test nucleic acid binding. A 40 nucleotide 5′ ^{32}P-labeled RNA (2 nM final concentration, 5′-GAG UGC ACC AUA UGC CAU UCA GGC UAC GCA ACU GUU GGG A -3') was mixed with different amounts of 3AB or other proteins (see Fig. 5) in 10 µl of a buffer containing 10 mM HEPES pH=7, 5 mM KCl and 5 mM DTT. Two µls of 6X loading buffer (40% sucrose, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) was added and the samples were loaded onto a 6% native gel prepared in 0.5X Tris-borate-EDTA buffer (92). Samples were electrophoresed at 15 mA for approximately 1.5 hours and gels were processed as described above.

Polyacrylamide gel electrophoresis. Six % (w/v) native polyacrylamide (29:1 acrylamide:bisacrylamide) gels were prepared and electrophoresis was performed as described (92). Sixteen % (w/v) SDS-polyacrylamide gels were prepared for protein detection and electrophoresis was performed as described.

TABLE 2-2

Primer name	Sequence 5′-3′
R104E sense	ACAAAAAACCCAACGTGCCCACCATTGAAACAGCAAAGGTACAATAAC
R104E antisense	GTTATTGTACCTTTGCTGTTTCAATGGTGGGCACGTTGGGTTTTTTGT
Y90A sense	GTTTGCTGGACACCAGGGAGCAGCGACTGGTTTACCAAACAAAAA
Y90A antisense	TTTTTGTTTGGTAAACCAGTCGCTGCTCCCTGGTGTCCAGCAAAC
K81A sense	TGGAGTTGTCTATGTCATGTATGCGCTGTTTGCTGGACACCAGGGA
K81A antisense	TCCCTGGTGTCCAGCAAACAGCGCATACATGACATAGACAACTCC
F83A sense	GAGTTGTCTATGTCATGTATAAACTGGCGGCTGGACACCAGGGAGCA
F83A antisense	TGCTCCCTGGTGTCCAGCCGCCAGTTTATACATGACATAGACAACTC
H86A sense	CATGTATAAACTGTTTGCTGGAGCGCAGGGAGCATACACTGGTTTAC
H86A antisense	GTAAACCAGTGTATGCTCCCTGCGCTCCAGCAAACAGTTTATACATG

Table 2-2: List of primers used for mutagenesis

2.3 Results

Assays used to examine 3AB chaperone activity. The chaperone activity of 3AB was evaluated using three different assays that measure annealing of complementary nucleic acids and aggregation. The primer-template annealing assay indirectly tests helix destabilization and aggregation activities by testing the ability of a chaperone to stimulate annealing of a primer to a larger template using gel-shift (23). Chaperone proteins presumably accelerate annealing in the assay through aggregation and partial unwinding of the large template. The aggregation assay tests the chaperone's ability to induce aggregate formation, a common, though not exclusive property of chaperones. Incubation of chaperones with nucleic acid can result in the formation of large aggregates consisting of the chaperone and nucleic acid (which is generally labeled with radioactivity in the assay). Aggregated material can be isolated by slow speed centrifugation in a microfuge (4). The fluorescence resonance energy transfer (FRET) unwinding assay measures unwinding and annealing stimulation. The assay illustrated in Figure 2.2 was developed in the DeStefano lab for examining chaperones (23, 41, 69). It is rapid and produces immediate results that typically correlate well with the primer-template annealing assay. Complementary nucleic acid strands that have little structure or form stem-loops are used in the assay. One complement has a fluorescing group (FAM) at the 5′ end and the second has a quenching group (DABCYL) at the 3′ end. The thermodynamic stability of the strands can be varied by changing the G-C content of the stem (69). When the individual complements form stem-loops, they hybridize poorly at low temperature (30-37°C is typically used). In the presence of a chaperone, partial unwinding of the stem structures enhances annealing by exposing complementary regions.

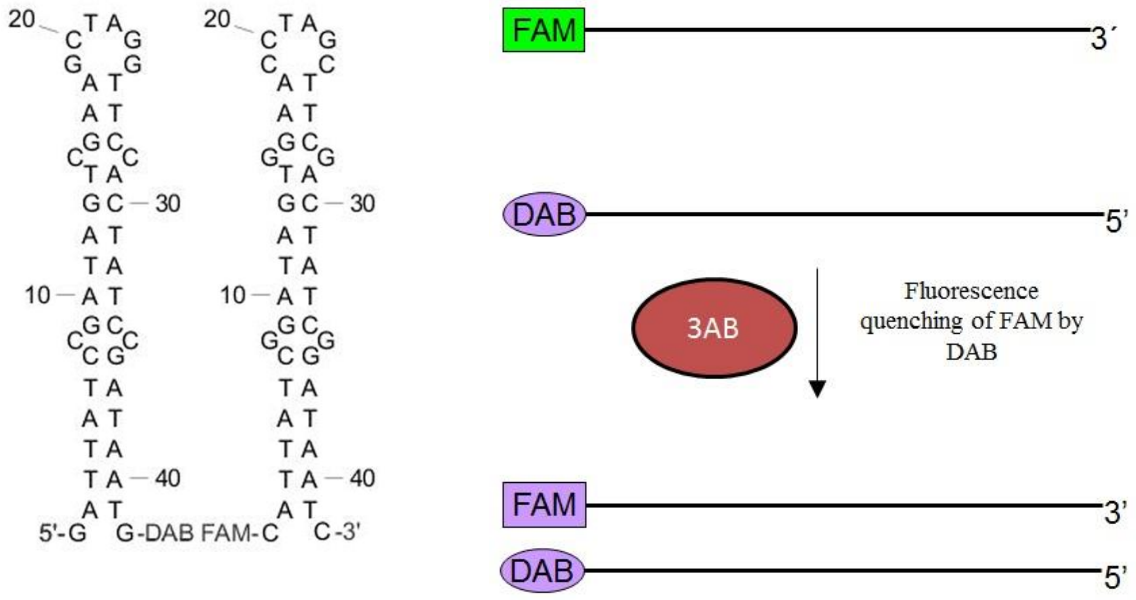


Figure 2-2: Schematic representation of FRET unwinding assay and predicted structure of complementary substrates: Folded structure of complementary 42-nucleotide DNA structures had ΔG values of approximately -7.2 kcal/mol as predicted from DNA fold (118). One complement had a FAM group (fluorophor) at the 5′ end, while the other had a DBCYL group (quencher) at the 3′ end. A chaperone protein like 3AB unwinds complementary nucleic acid strands and promotes hybridization. This brings the DAB and FAM in close proximity and results in quenching of the fluorescence signal.

Annealing is measured by the quenching of the fluorescence signal that occurs when the FAM and DABCYL groups become juxtaposed upon hybrid formation. In assays using complements with highly stable stem regions (as in the current report), the major parameter tested is the ability of the chaperone to unwind the stem. Gel-shift assays will be used to evaluate the affinity of the mutated and wild type proteins for nucleic acid.

Mutations in the 3B region of 3AB inhibit chaperone activity while 3B by itself shows no chaperone activity. Amino acids in the 3B region of 3AB are known to be pivotal for 3AB binding to nucleic acids and complex formation with $3D^{pol}$. Previous results showed that the R104E (arginine to glutamate) mutation severely disrupts binding to nucleic acid (110). The effect of this mutation on chaperone activity was tested using the FRET unwinding assay and, as expected, no significant chaperone activity was detected (Figure 2-3). A second mutation, Y90A (tyrosine to alanine), was also tested. Tyrosine 90 is the amino acid that is uridylylated by $3D^{pol}$ during replication (see chapter 1). In addition, aromatic amino acids are pivotal to the chaperone activity of HIV nucleocapsid protein (NC) and are presumed important in the disruption of secondary strucutures. The Y90A mutation also decreased chaperone activity although a low level of activity was detected. A double mutant with R104E and Y90A (Y90A/R104E) was also tested and found to be similar to R104E.

It was clear from the above results that the 3B region of 3AB was pivotal to chaperone acitivity. To test its role further the 22 amino acid 3B protein was chemically synthesized and tested for chaperone activity. No activity was observed, even at very high concentrations that were ~50 times greater than the concentration where 3AB

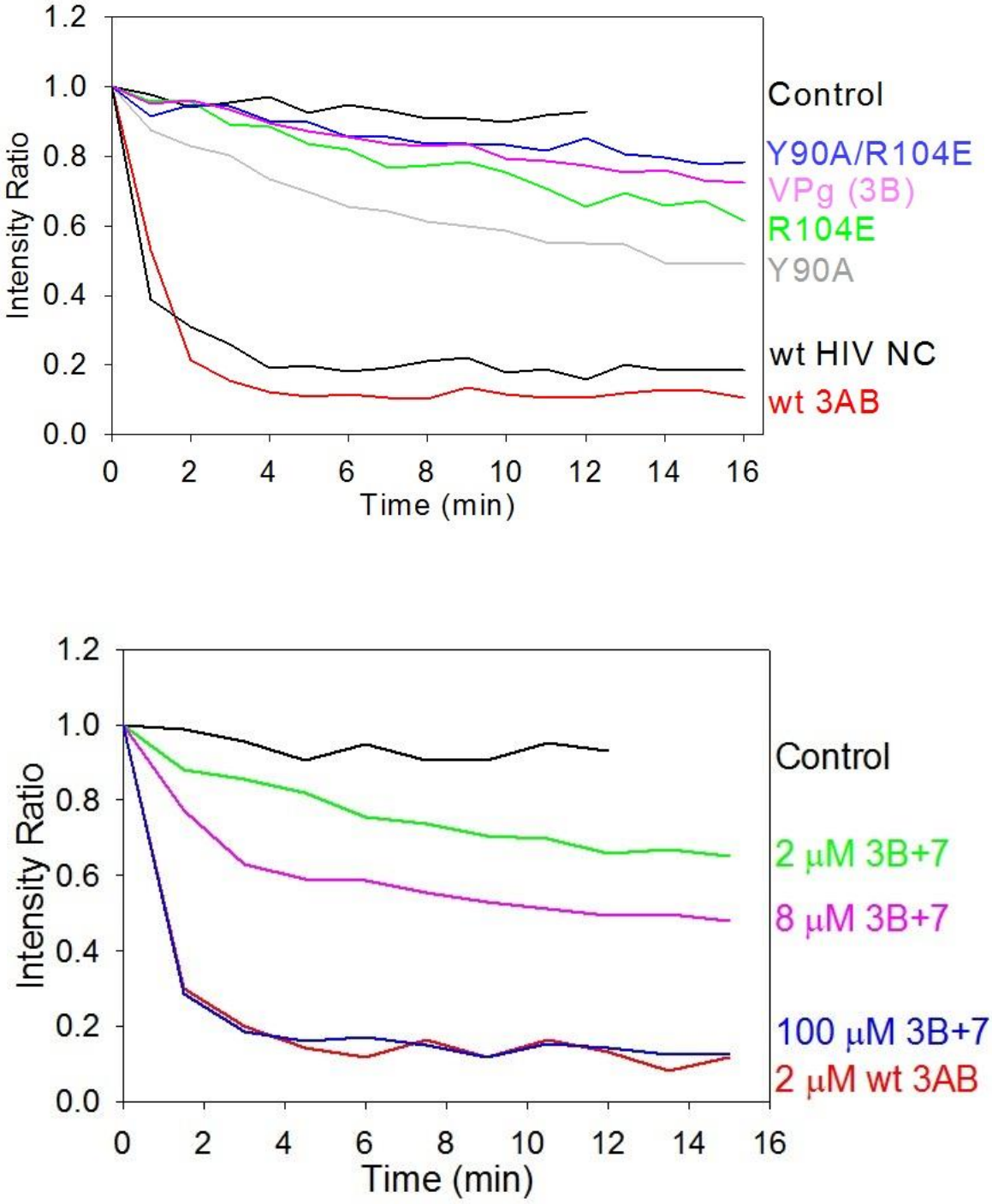


Figure 2-3: FRET unwinding assay results: Top panel shows the FRET unwwinding time course assay with wildtype 3AB, 3AB-R104E, 3AB-Y90A, 3AB-R104E/Y90A, 3B (VPg) and HIV NC all at 2μM. Bottom panel shows FRET unwinding assay for wildtype 3AB at 2 μM and and 2, 8 and 100 μM 3B+7.

showed maximal activity (Table 2-3). Based on these results, we concluded that the 3B region of 3AB was necessary but not sufficienct for chaperone activity.

The putative C-terminal cytoplasmic domain of 3AB possess chaperone activity at high concentrations. Since 3AB is a membrane protein, it is likely that the intrinsic chaperone activity resides in a portion of the protein that is cyctoplasmic allowing direct intereaction with nucleic acids. Models for 3AB interactions with membranes suggest that both the first 58 amino acid in the N-terminus and the last 29 amino acids in the C-terminus are cytoplasmic (Figure 4-1). Since interactions with nucleic acids map mostly to the N-terminus, this was clearly the region most likely to contain intinsic chaperone activity, alhtough none was detected in 3B alone (see above). The last 7 amino acid of 3A are proposed to lie just outside the membrane and are connected to the membrane insertion domain. These 7 amino acids and the 22 of 3B compose the C-terminal cytoplasmic domain. Chemically synthesized 3B+7 was tested in the FRET unwinding assay at various concentrations. Although very low chaperone activity was detected at concentrations where 3AB showed high activity (2 μM), activity increased at higher concentrations (8 μM) and high activity was observed with 100 μM 3B+7 (Figure 2-3).

Several 3B+7 proteins containing mutations were chemically synthesized and tested in the FRET unwinding assay (Table 2-3). Proteins containing the same mutation as those tested in 3AB were tested first (3B+7 R104E and Y90A, as well as R104A) to see if they had a similar affect on the activity of 3B+7. Similar to what was observed with 3AB, R104E dramatically reduced the chaperone activity of 3B+7 while Y90A was inhibitory but to a lesser extent. An R104A mutation was also strongly inhibitory.

Additional mutations residing in the connection domain including F83A (phenylalanine to alanine) and K81A (lysine to alanine) showed results similar to Y90A while H86A (histidine to alanine) showed greater chaperone activity than those mutants but slighlty less than wild type. From the limited number of mutations that were tested, 3B+7 appeared to mimic wild type 3AB's behavior, albeit at the much higher concentration that was required to observe activity.

Full length 3AB proteins containing the same mutations in the connection domain (F83A, K81A and H86A) as those tested in 3B+7 were tested to see if they had a similar affect on the chaperone activity of 3AB (Table 2-3). Similar to what was observed in 3B+7, H86A showed greater chaperone activity than the other two mutants and was comparable to wildtype at a lower concentration. K81A showed results similar to Y90A, even in the context of the full length 3AB protein. F83A in 3AB on the other hand showed a slightly increased chaperone activity but still less than wildtype.

Wild type 3AB is much more potent than 3B+7 in the primer-template annealing assay. Proteins were next compared in the primer-template annealing assay using a fixed time point protein titration. Wild type 3AB stimulated annealing strongly as the concentration of the protein was increased (Figure 2-4). Peak activity occurred at ~1 µM under the conditions used, similar to what was previously observed (23). In contrast, the Y90A and Y90A/R104E mutants showed no annealing activity in this assay (Table 2-3). Protein 3B+7 showed some annealing activity in the assay though reduced compared to wild type protein, even when very high concentrations were used (Figure 2-5). The H86A mutation in 3B+7 resulted in annealing that was detectable but lower than non-mutated 3B+7, consistent with the modestly lower level of activity in the FRET

unwinding assay for these proteins (Table 2-3). Other tested mutations in 3B+7 including Y90A and R104E and Y90A/R104E, showed no activity in the assay as did 3B (Table 2-3).

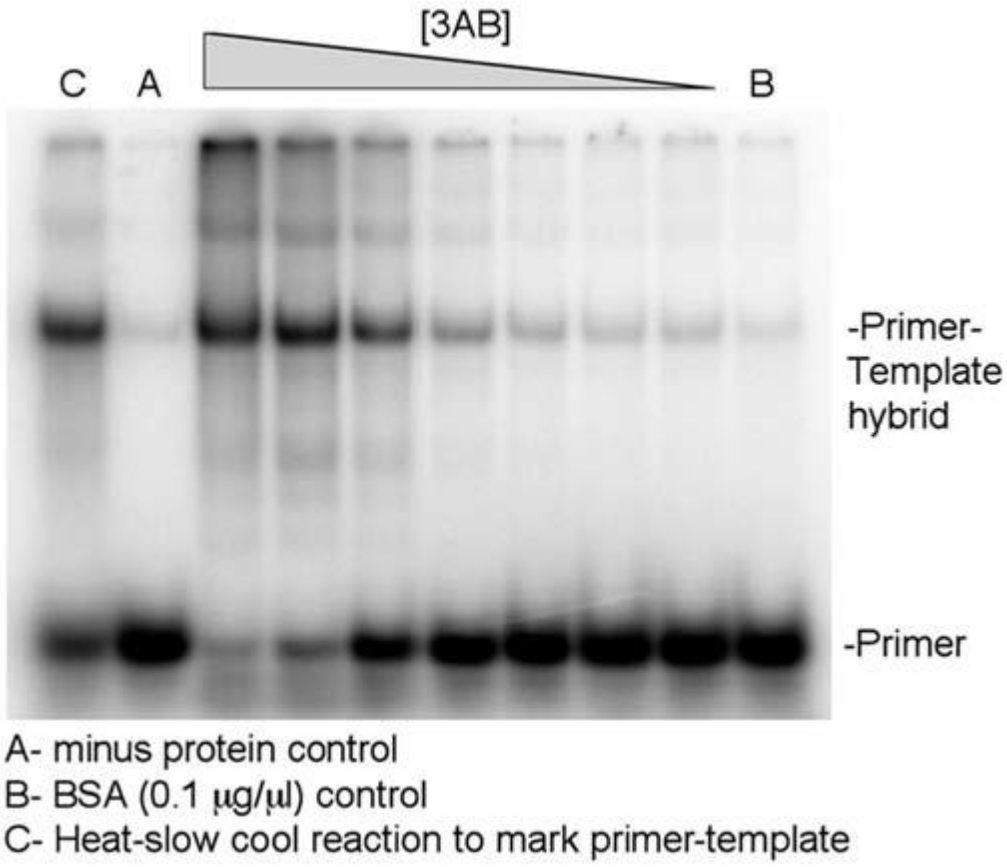


Figure 2-4: Primer template annealing assay with wildtype 3AB. A 25 nucleotide 5' end-labeled RNA primer that was complementary to a 230 nucleotide RNA template was used in the assay. The primer and template were incubated with decreasing amounts of wild type 3AB (l-r: 2, 1, 0.5, 0.25, 0.013, 0.0063 or 0.0031 μM). Samples were processed and run on a 6% native polyacrylamide gel as described under section 2.2. The positions of the primer and annealed primer-template hybrid are indicated. Three controls (A–C) are as described below the panel.

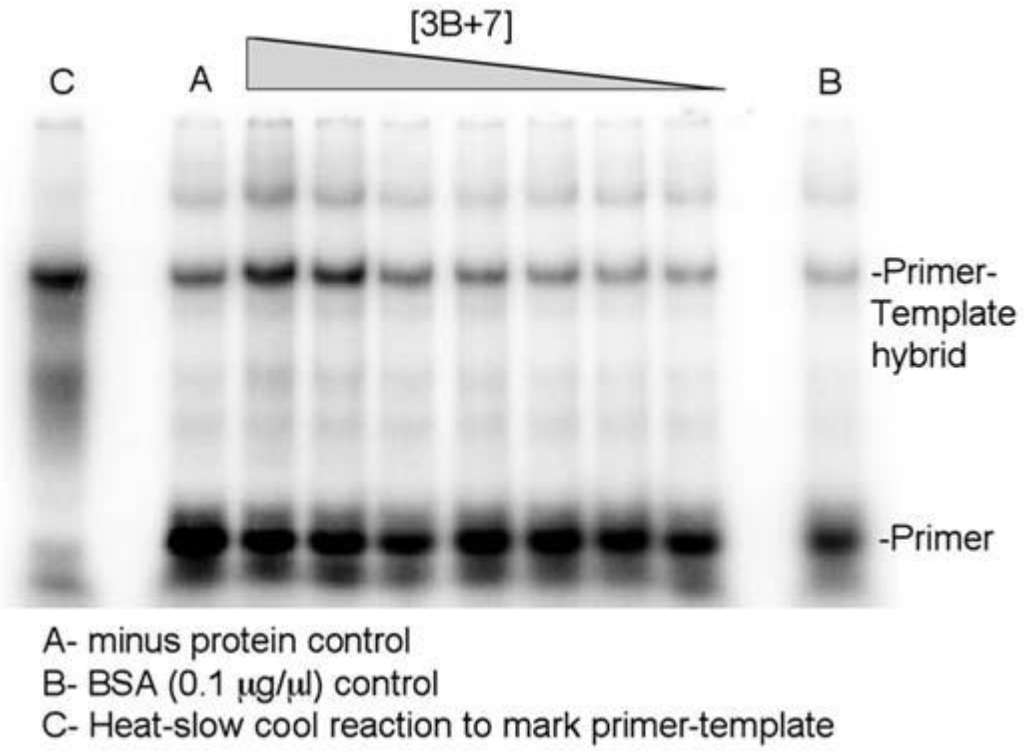


Figure 2-5: Primer-template annealing assay with wild type 3B+7. The amount of 3B+7 protein used was: l-r, 200, 100, 50, 25, 12.5, 6.3 or 3.1 μM. See figure 2-4 and section 2.2 for details.

Wild type 3AB and 3B+7 induce aggregate formation while mutated versions of these proteins with low unwinding and primer-template annealing activity do not. Large aggregates that can form in the presence of chaperone proteins and nucleic acids can help to induce the formation of hybrids (4, 100). An aggregation assay was performed with wild type 3AB, Y90A, Y90A/R104E, 3B+7 (and various mutations), and 3B. Wild type 3AB induced aggregate formation in a concentration-dependent manner (data not shown), similar to what was observed in the primer-template annealing assay. Full-length proteins were tested at 2 μM and 3B or 3B+7 at 100 μM. Of those proteins tested wild type 3AB showed the highest level of aggregation and was essentially equal to HIV NC, a protein known to aggregate efficiently (Table 2-3). In addtion, 3B+7 showed aggregation activity slighlty lower than 3AB while 3B+7-H86A showed some activity. All other tested proteins did not aggregate the RNA template.

Affinity of 3AB and mutated proteins for nucleic acid. Gel-shift assays can be used to estimate the binding affinity of 3AB for nucleic acids. Although this method does not yield an accurate equilibrium binding constant, it is useful for comparing various 3AB mutations (110). A simpler nitrocellulose filter binding assay proved unreliable in our hands as the amount of nucleic acid retained on the filters in the presence of 3AB was low and variable. Wild type 3AB was able to gel shift nucleic acid in this assay consistent with previous reports (Figure 2-6). In contrast, mutations Y90A, R104E, and Y90A/R104E did not shift any nucleic acid indicating that they have lower affinity for nucleic acid (Figure 2-6 and Table 2-3). The result is completely expected for R104E and Y90A/R104E as the R104E mutation was previously shown to abrogate nucleic acid binding (110). Since the Y90A mutation showed low but measurable activity in the

unwinding assay it would be expected that nucleic acid binding would occur; however, it was not detected by gel shift, perhaps because binding was too weak (see Discussion). Protein 3B+7 was unable to shift nucleic acids in the gel shift assay or reliably retain nucleic acid on nitrocellulose filters (data not shown).

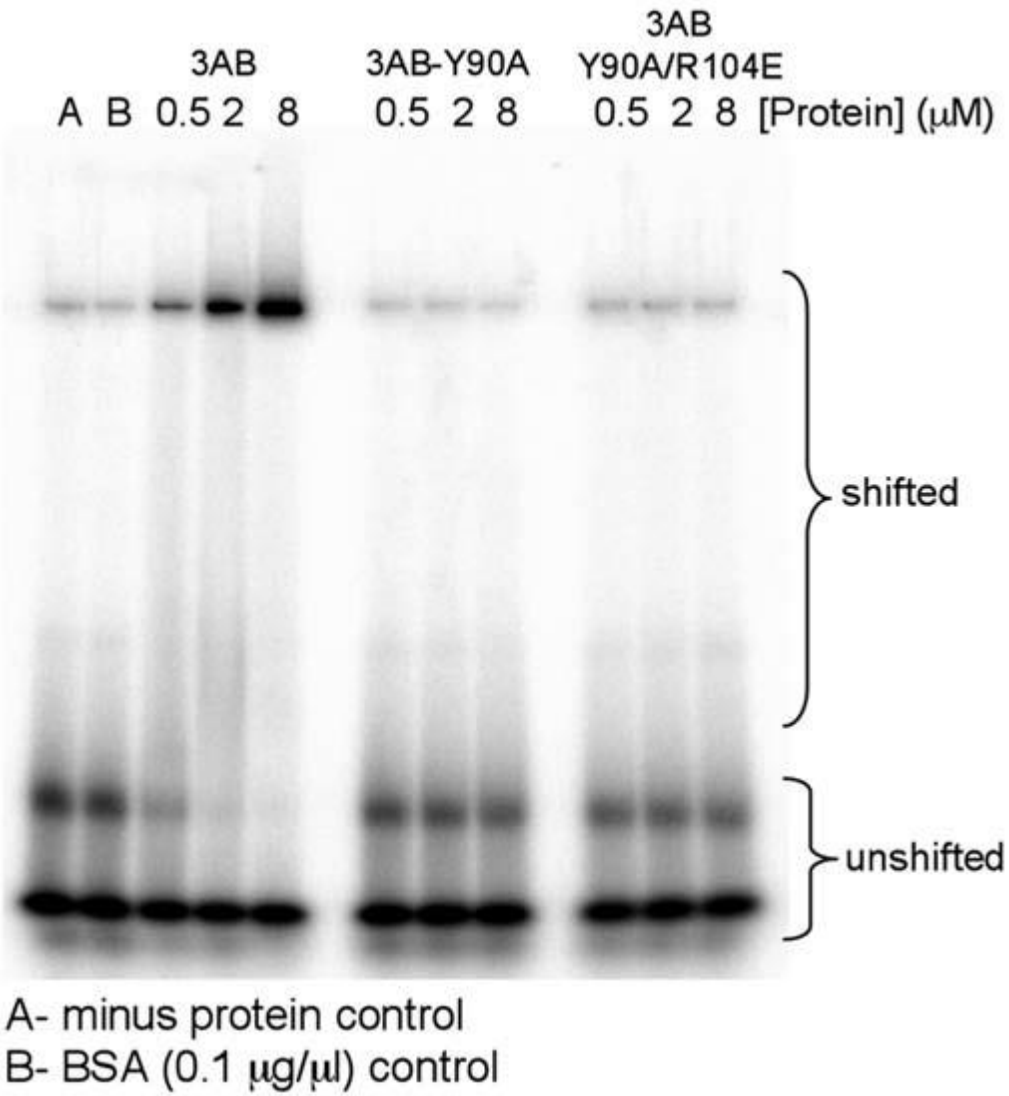


Figure 2-6: Gel-shift assay to test protein-nucleic acid binding. Proteins (type and amount as indicated) were incubated with a 40 nucleotide 5' end-labeled RNA and run on a 6% native polyacrylamide as described in section 2.2. The positions of the unshifted 40-mer and shifted material are indicated. Two controls are as described below the part.

TABLE 2-3

[a]Protein and Concentration	[b]FRET	[b,c]Aggregation	[b,d]P-T Ann.	[e]FRET rate constant
3AB (2μM)	+++	+++	+++	0.79 ± 0.21
3AB-Y90A (2μM)	+	_	_	ND
3AB-R104E (2μM)	_	ND	_	ND
3AB-Y90A/R104E (2μM)	_	_	_	ND
3AB-K81A (2μM)	+	+	+	ND
3AB-F83A (2μM)	++	++	++	ND
3AB-H86A (2μM)	+++	+++	+++	ND
3B (100μM)	_	_	_	0.030 ± 0.001
[f]3B+7 (100μM)	+++	++	++	0.59 ± 0.07
3B+7-K81A (100μM)	+	ND	ND	0.13 ± 0.02
3B+7-F83A (100μM)	+	ND	ND	0.095 ± 0.049
3B+7-H86A (100μM)	++	+	+	0.21 ± 0.06
3B+7-Y90A (100μM)	+	_	_	0.15 ± 0.02
3B+7-R014A (100μM)	_	ND	ND	ND
3B+7-R014E (100μM)	_	_	_	0.031 ± 0.016

Table 2-3: [a]3B and 3B+7 proteins were made by chemical synthesis. All others were expressed and purified from *E. coli.* [b]+++, ~75–100%; ++, ~25–74%; +, ~5–24%; -, <5%; ND, Not Determined. All relative to 3AB wild type. See Figure 2-3 for FRET unwinding assay. [c]Aggregation, determined by formation of a nucleic acid pellet using slow speed centrifugation (Section 2-2). [d]Primer-template annealing (see Figures. 2-4 and 2-5). [e]Rate constant (*k*) for FRET assays was determined as described in Methods. Results are an average of two or more experiments +/- standard deviation. [f]In FRET assays, 2 μM 3B+7 showed little stimulation while 8 μM showed some stimulation (+).

2.4 Discussion

This report presents the most recent advances in the understanding of how poliovirus 3AB functions as a nucleic acid chaperone *in vitro*. The importance of the 3B region of the protein in chaperone activity has been clearly demonstrated as mutations in this region severely weaken or abolish chaperone activity (Table 2-3). Thus far all of the 3B region mutations tested in the context of the full-length protein (Y90A, R104E, and Y90A/R104E) showed highly reduced nucleic acid binding, which explains the low chaperone acitivity. The 3B region of 3AB is known to be pivotal for nucleic acid binding so the results are not surprising (110). For single point mutations to have such a dramatic effect on nucleic acid binding, it is likely that they disrupt the structure of the 3B region. Both Y90A and R104E make additional contacts with other amino acid in the 3B (VPg) solution structure and the mutations tested would likely disrupt some or all of the interactions as seen in Figure 2-7 (94, 95). In fact, R104 interacts with nearly every amino acid residue in the C-terminal end of 3B in the proposed solution structure. Both R104 and Y90 lie on the same face of 3B along with several other amino acid residues that are conserved among picornaviruses (G88, G92, K96, K97, P101 and Q109). Future work would include evaluating the effect of mutations in these conserved residues on chaperone activity.

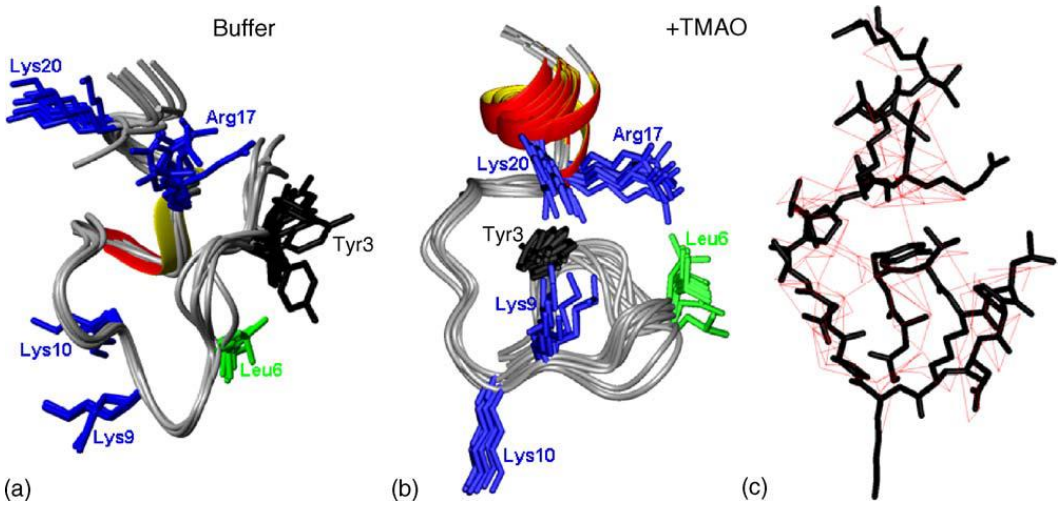


Figure 2-7: Structural location of amino acid tyrosine and arginine in the context of Vpg or 3B. Comparison of the structures calculated for PV-VPg in buffer (a) and in the presence of 1 M TMAO. (b) The top 10 structures in buffer alone show much more conformational flexibility, both in the backbone and in the position of the conserved side chains. (c) Inter-residue constraints (automatically assigned) used in determining the VPg structure in TMAO. Reprinted from Peptides, 27, Catherine H. Schein, Numan Oezguen,David E. Volk, Ravindranath Garimella, Aniko Paul, Werner Braun, NMR structure of the viral peptide linked to the genome (VPg) of poliovirus, 1676-1684, Copyright (2006), with permission from Elsevier. This figure shows the structural location of mutations Y90A and R014E as Tyr 3 and Arg 17 respectively in the context of VPg or 3B protein.

The most interesting result from this work was the finding that the putative C-terminal cytoplasmic domain of 3AB (termed 3B+7 in this work), but not 3B by itself, possessed chaperone activity. In the FRET unwinding assay, which measures mostly the ability of a chaperone to unwind nucleic acids, 3B+7 showed activity that was approximately 50-fold lower than 3AB on a per mole basis. This was based on saturation of the rate of annealing in the FRET unwinding assay requiring ~2 μM 3AB and ~100 μM 3B+7. Still, each protein was able to stimulate unwinding to about the same maximal rate. Mutations in 3B+7 behaved similar to the same mutations in the wild type protein for the few that were examined including R104E, Y90A, and Y90A/R104E, with the R104E and the double mutant showing essentially no activity in the FRET unwinding assay and Y90A a low level. This suggests that 3B+7 may be a reliable though incomplete (see below) model for examining the chaperone activity of 3AB. A R104A mutation in 3B+7 also showed very little activity indicating that the added minus charge of R104E was not responsible for the loss of activity.

Since 3B+7 showed chaperone activity while 3B did not, it was clear that the 7 additional amino acids from the C-terminal end of 3A were pivotal. Three mutations were made in this region and tested in the context of 3B+7 and 3AB. Two of them, K81A and F83A, showed reduced activity similar to the Y90A mutation in 3B+7. In contrast, H86A showed more activity but still less than wild type 3B+7. These results were similar when tested in the context of full length 3AB protein as well, although there was a slight increase in activity of H86A and F83A when tested in full length 3AB. The results reiterates the importance of postively charged and aromatic amino acid in chaperone acitivity.

It is also interesting that the 29 amino acids of 3B+7 comprise the entire putative C-terminal cytoplasmic domain of 3AB. This means that intrisic chaperone activity in vitro requires only that part of the C-terminal region of the protein that would be available for nucleic acid binding in vivo. The membrane anchor domain (amino acid 59-80) would presumably be embeded in membranes in vivo and this would prevent direct access to nucleic acid. The N-terminal 58 amino acids of 3A are also predicted to be cytoplasmic and could therefore be involved with direct contacts to nucleic acids. However, judging from the limited affect of mutation in this region on nucleic acid binding (110), a direct role in chaperone activity seems less likely, although an indirect role that enhances activity is possible for this region and the membrane anchor domain (see below)

At this point, it is difficult to speculate what roles the 3B and +7 region play in chaperone acitivity or how these region interact. One possible explanation is that 3B+7 simply binds nucleic acid better than 3B. While this remains possible, we were unable to detect binding in gel-shift or filter binding assays with either protein and these proteins did not aggregate nucleic acids. Still, it is clear that 3B+7 does interact with nucleic acids as it would not show activity in the FRET unwinding and primer-template annealing assays if it did not. Possible explanations for the lack of binding in gel-shift assays are that that 3B+7 is too small to produce a reliable shift, the binding is too weak to survive this assay, or the protein simply does not work in this type of assay, as is the case for many other nucleic acid binding proteins. As for the filter binding assay, even full-length 3AB did not work well in this assay, so it was not surprising that 3B+7 did not. We are currently working on assays that use fluorescently labled nucleic acid to test binding.

These assays should help in analyzing difference in binding between various mutants and wild type protein.

Another possible explanation of why both 3B and the additional 7 amino acids are required for activity is that they form a folded complex together that has intrisic chaperone activity. Although possible, this explanation is weakened by the fact that 3B by itself forms a defined structure in solution that has been solved using NMR (93, 95). In addition, chemically synthesized 3B is active in uridylylation assays in which two UMP residues are added to tyrosine 90 by $3D^{pol}$ in the presence of poly(rA) or the CRE genome region, the natural template for uridylylation (see Introduction). These findings suggest 3B forms an active complex, at least for uridylylation, in the absence of the 7 amino acid from 3A, and that chemically synthesized 3B folds correctly.

It was interesting that the various assays used to test chaperone activity gave different results with particular 3AB mutations. Wild type 3AB showed high activity in the FRET unwinding assay while 3AB-Y90A showed low but measurable activity. The same senario was observed with 3B+7 and 3B+7-Y90A, albeit at higher protein concentrations. In contrast, only wild type 3AB gel-shifted nucleic acid and only 3AB, 3B+7, and 3B+7-H86A aggregated RNA template. In the primer-template annealing assay wild type 3AB was most active and 3B+7 also showed modest activity and 3B+7-H86A low activity. None of the other mutated proteins, even those that showed some activity in the FRET unwinding assay, were able to anneal primer-template. This may be due to the primer-template annealing assay requiring both unwinding and aggregation activity while the FRET unwinding assay requires mostly the former. Both wild type 3AB and 3B+7 unwind and aggregate well based on the FRET unwinding and

aggregation assays, respectivley. This leads to greater annealing in the primer-template assay which is dependent on both activities. The only other mutated protein to show some activity in the primer-template annealing assay was 3B+7-H86A. This protein had low but measurable aggregation activity and reasonably high activity in the FRET-unwinding assay. This may have resulted in the low level of primer-template annealing that was observed. These results also point out the importance of measuring chaperone acitivity in more than one type of assay and using assay that focus on particular aspects of that activity (ie. unwinding, aggregation, or both).

Chapter 3: Effect of mutations in the putative chaperone domain of 3AB on replication of virus in tissue culture

3.1 Introduction

The molecular mechanism of viral RNA replication in poliovirus infected cells has been a subject of much investigation. Protein 3AB is a multifunctional protein that is indispensable for virus replication (Section 1-10). The first genetic evidence for the role of 3AB in virus replication was reported by Berstein and Baltimore (10). A cold sensitive mutant of poliovirus having a single amino acid insertion in the 3A region was isolated. This mutant formed small plaques and was defective in RNA replication at the restrictive temperature. Since then, several mutants defective in RNA synthesis have been isolated (36, 37, 101, 103, 104). Many of these were mapped to the hydrophobic domain of 3AB, which anchors the replication complex on membrane vesicles. In addition to affecting RNA synthesis, some mutations in the hydrophobic domain lead to aberrant polyprotein processing when viral RNAs obtained from transcription of plasmid DNA containing mutations are used to program HeLa cell extracts for *in vitro* translation. This suggests that 3AB may be involved in polyprotein processing as well as RNA synthesis. Mutations in the 3B (VPg) region of the protein lead to both lethal and non-lethal mutants with severe defects in RNA synthesis, as this region is important for uridylation and $3D^{pol}$ binding (55).

Work in our lab has recently established 3AB as a nucleic acid chaperone protein, although the importance of its chaperone activity in the virus life cycle has not been established. As explained earlier, 3ABs chaperone activity could play a role in helping

$3D^{pol}$ traverse the highly structured genome, promoting recombination and facilitating structural switches that maybe important for the genome to function in translation, replication and packaging. Exactly how chaperones function to enhance virus replication *in vivo* remains unclear. An ideal assay for RNA chaperone activity would be the *in vivo* demonstration of chaperone activity with its natural RNA partner, however, the technology for such an assay is lacking. The development and analysis of RNA chaperone activity *in vivo/in situ* is complicated by the ubiquitous nature of viral chaperones, all of which appear to have multiple functions at various steps of the virus life cycle. These reasons are in part responsible for the lack of appropriate *in vivo* systems for the analysis of RNA chaperone activity, and for a tendency to use complex *in vitro* systems with defined components.

In this report, we describe the effect of point mutations within the putative chaperone domain of 3AB (3B+7 domain described in Chapter 2) on replication of the virus in tissue culture. Specifically, we focused on the 7 amino acid connection domain at the C-terminus of 3A (+7 region of 3B+7) since mutations in this region have not been extensively characterized. As there are no defined *in vivo* assays to directly test chaperone function on viral RNA, these mutations are tested for their effect on plaque formation (viral infectivity), *in vitro* translation/polyprotein processing and RNA replication using HeLa cell extracts. These same mutations were also tested in *in vitro* chaperone assays as shown in Chapter 2. We observed that viruses containing 3AB-K81A and 3AB-F83A produced small and minute plaques respectively compared to wild-type 3AB. *In vitro* translation and polyprotein processing were not affected by these mutations; however, RNA synthesis was decreased severely by F83A and modestly by

K81A compared to wild-type. Future work would include evaluating particular steps in virus replication that are affected, specifically positive/negative strand synthesis and VPg uridylation.

3.2 Materials and Methods

3.2.1 Materials

All synthetic oligonucleotides (Table 2-2) were custom – ordered from Integrated DNA technologies Inc. (IDT). Point mutations in 3AB were made using the Quickchange Lightening Site-Directed Mutagenesis kit from Agilent technologies. T4 DNA ligase was from Invitrogen. Restriction enzymes were from New England Biolabs. Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were from Lonza.

3.2.2 Methods

Random mutagenesis of the poliovirus P2-P3 region: Point mutations within the connection domain (3B+7) of 3AB were introduced in plasmid pXpA-P2P3 using Quickchange Lightening Site-Directed Mutagenesis kit according to the manufacturer's protocol. Plasmid pXpA-P2P3 contains the cDNA of poliovirus RNA lacking structural protein coding sequences, under control of a T7 RNA polymerase promoter. All plasmids were analyzed by DNA sequencing using specific primers that flank the mutation. Polio-specific RNA produced from this plasmid is capable of autonomous replication but does not produce infectious virus. The mutation containing fragment was then recloned into pXpA-SH. pXpA-SH contains full-length cDNA of poliovirus type 1 under control of the T7 promoter, with the hammerhead ribozyme coding sequence at the 5′end of the poliovirus sequence and additional unique restriction sites within the viral cDNA (9).

RNA transcription: Full length plasmids pXpA-SH containing wild-type 3AB or mutations within the connection domain (3B+7) were linearized with restriction enzyme

Eco RI. Linearized plasmids were purified using phenol chloroform extractions and precipitated with ethanol. Transcription reactions were performed using a MEGAscript *in vitro* transcription kit (Ambion, Inc). RNAs were purified using phenol-chloroform extractions and precipitated with ethanol. Following precipitation, RNAs were further purified using Clontech Chroma Spin-100 columns. The RNA was run on an agaorse gel to visualize its quality and its concentration was estimated by A_{260}.

RNA transfection and characterization of virus infectivity: RNA transfections were performed using the TransIT-mRNA transfection kit (Mirus) according to the manufacturer's protocol. One microgram of RNA was used to transfect HeLa cells ($1.5x10^6$ cells) at 80% confluency in 35-mm 6-well plates. A ten – fold serial dilution of RNA was carried out to compare the infectivity of the virus produced from wildtype or mutant transcript. Following incubation at room temperature for 1 h, the monolayers were overlaid with 0.5% agarose in high glucose DMEM supplemented with 0.5mM sodium pyruvate, 0.5X penicillin/streptomycin, 1mM L-glutamine and 10 % (0.2%) FBS. Visible plaques were formed following 2-3 days post transfection.

Extraction of viral RNA and synthesis of cDNA: Viral RNA was extracted from plaques using the QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's specifications. Full length cDNAs were prepared using Random Nona-mer (9 mer) primer supplied with the reverse transcriptase MonsterScript 1^{st} Strand cDNA Synthesis Kit (EpiCenter). The fragments containing the 3AB sequence were amplified by PCR using specific primers (Table 2-2). These fragments were then sequenced to identify reversions or compensatory mutations within the 3AB region.

Coupled *in vitro* translation-replication assay: *In vitro* translation assays were performed using reactions mixtures containing the following components: 51% (vol/vol) HeLa cell S_{10} cytoplasmic extract, 20% (vol/vol) HeLa ribosomal salt wash, 10% (vol/vol) ATP –generating mixture containing creatine phosphate, creatine kinase and all nucleotides except CTP, and 2mM guanidine hydrochloride. CTP is excluded out of this mixture as it is substituted by [α-^{32}P] radiolabelled CTP to detect RNA synthesis *in vitro.* Guanidine hydrochloride is a reversible inhibitor of poliovirus replication and is used to completely inhibit RNA synthesis in an *in vitro* translation-replication reaction (8, 67). Reaction mixtures (50ul) were programmed with 2.5µg full length viral RNA. HeLa ribosomal salt wash was prepared as described by Brown and Ehrenfeld (15). For analysis of translation products, 9µl aliquots (out of the 50µl reaction mixture) were supplemented with 1µl of [S^{35}] methionine and incubated at 34°C for 3.5 h. At the end of 3.5 h, the 10µl translation reactions were heated to 95°C for 5 mins in presence of 2X sample buffer. Five microliters of sample was loaded for separation of proteins by electrophoresis in sodium dodecyl sulfate (SDS)-12% polyacrylamide gel. The gel was fixed with 10% glacial acetic acid-40% methanol in water, dried and subjected to autoradiography on HyBlot *CL* film. The remaining 40µl of the reaction mixture was used for analysis of RNA synthesis. PV RNA synthesis was assayed using preinitiation RNA complexes formed in translation-replication reactions at 34°C for 3.5 h. These preinitiation RNA complexes were obtained by centrifugation of the above reaction mixture for 20 min at 15,000 x g at 4°C. Supernatants were removed and the replication complexes were resuspended in replication mix containing the following components: 50% (vol/vol) S10 buffer, 4% (vol/vol) of 2.5mg/ml puromycin, 10% (vol/vol) ATP –

generating mixture containing creatine phosphate, creatine kinase and all nucleotides except CTP and 400μCi of [α-^{32}P] CTP. After 1h incubation at 37°C, total RNA was isolated using an RNeasy Mini Kit (Qiagen) and precipitated with ethanol. RNAs were denatured by incubation in glyoxal-dimethly sulfoxide buffer at 65°C for 45 mins and separated by electrophoresis on a native 1% agarose NorthernMax Glyoxal gel containing ethidium bromide. Equal loading of samples on the gel was confirmed by UV examination of 18S and 28S rRNA in each lane. The gel was dried and ^{32}P-labelled RNA was detected by autoradiography.

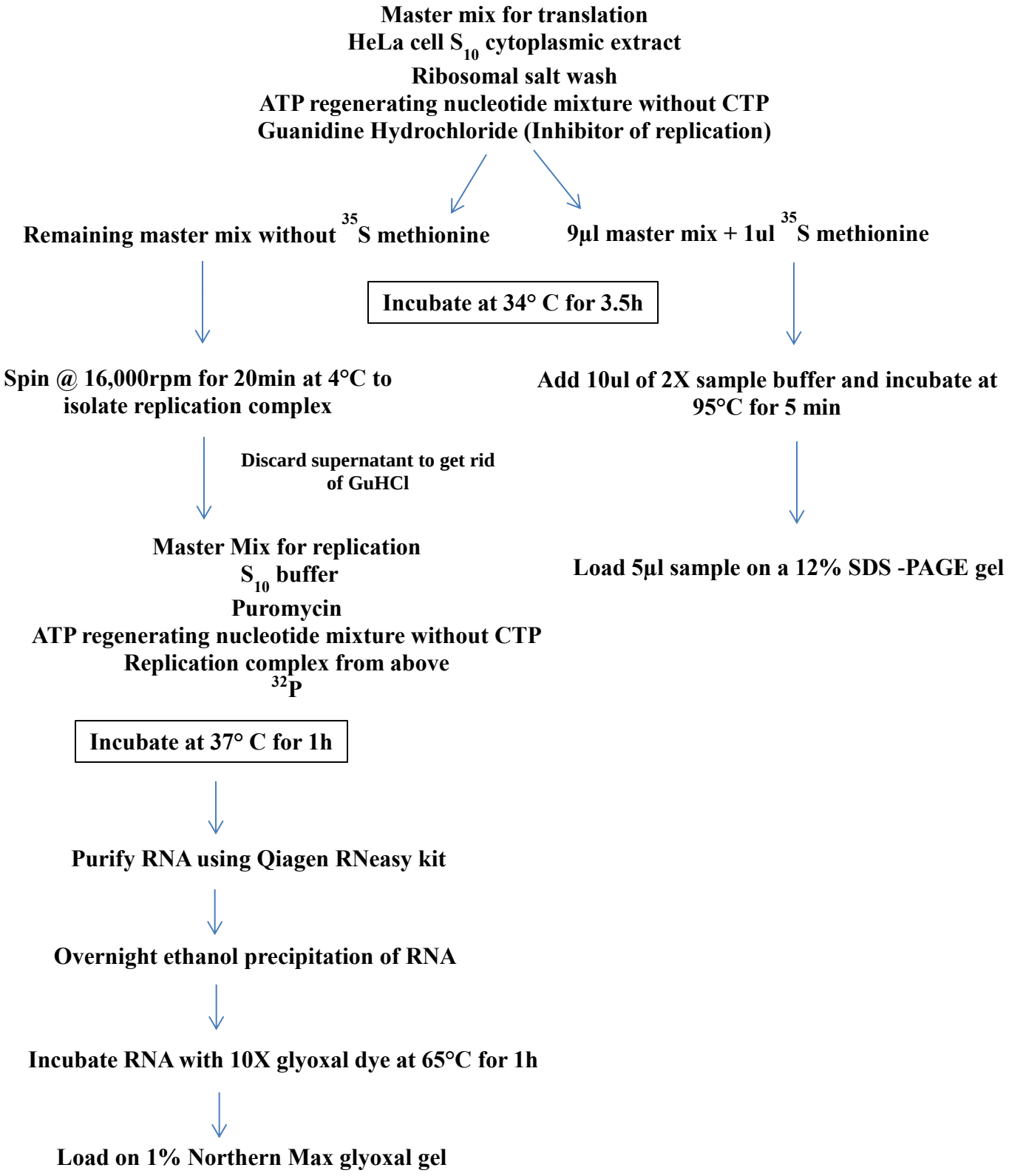


Figure 3-1: Schematic representation of coupled *in vitro* translation-replication assay

PCR primers	Sequence 5′-3′
B3	TAGAGACTTGTATGCCTACG
E4	GATGGAGTCTTGATCGTGAA
Sequencing primers	
Pr4	ACCAGCAAACTTTAAGAGATG
E4	GATGGAGTCTTGATCGTGAA

Table 3-1: Primers used to amplify the 3AB region of the PV cDNA and sequence the same.

3.3 Results

Infectivity of poliovirus RNAs with mutations in the putative chaperone domain of 3AB. Amino acids lysine and phenylalanine at position 81 and 83 respectively are highly conserved among picornavirus 3AB proteins and may be important for virus replication (99). Mutations K81A and F83A were introduced within 3AB into plasmid pXpA-SH. pXpA-SH contains full-length cDNA of poliovirus type 1 under control of the T7 promoter, with the hammerhead ribozyme coding sequence at the 5′end of the poliovirus sequence and additional unique restriction sites within the viral cDNA (9). HeLa cell monolayers were transfected with serial dilutions of full length RNA transcripts obtained from these constructs as described in the methods section. Viral infectivity, defined as number of plaque forming units per microgram of RNA was determined for each of these mutants. Our results indicate that K81A mutation had an infectivity of $2.2X10^5$ which is almost similar to wildtype at $3.7X10^5$, and produced slightly smaller plaques (Figure 3-2) in comparison to the wildytpe. F83A mutation had very low infectivity and produced minute plaques (Figure 3-2). These results are slightly different from what we observed *in vitro*. The effect of K81A mutation on chaperone activity *in vitro* was more severe than the effect of F83A mutation in the context of full length 3AB protein. However in tissue culture, K81A showed only a slight decrease in infectivity and plaque size compared to wildtype 3AB.

On the other hand F83A showed slightly better chaperone activity than K81A *in vitro* but much lower infectivity of 15 and a more severe effect on plaque phenotype (Figure 3-2). It is possible that the F83A mutation could have severe effects on other steps involved in virus replication, such as VPg uridylation and/or initiation of negative

and positive strand synthesis, which may or may not require the chaperone activity of 3AB. The effect of K81A mutation in tissue culture suggests that a decrease in chaperone activity *in vitro* does not severely affect infectivity and plaque phenotype of the virus.

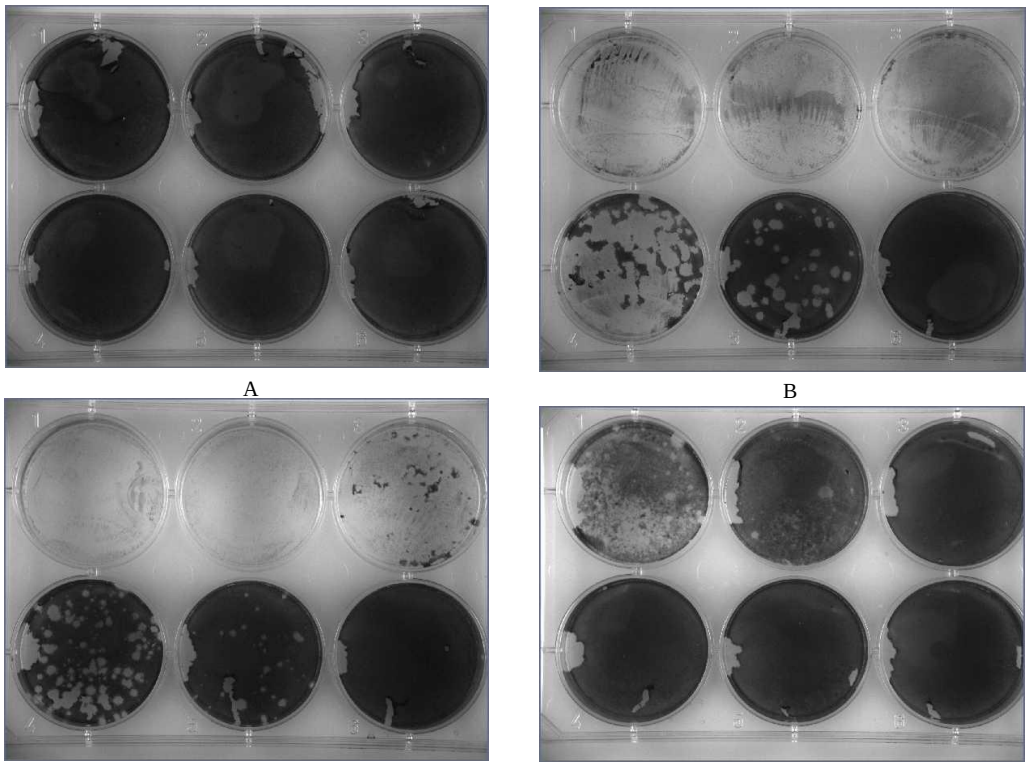
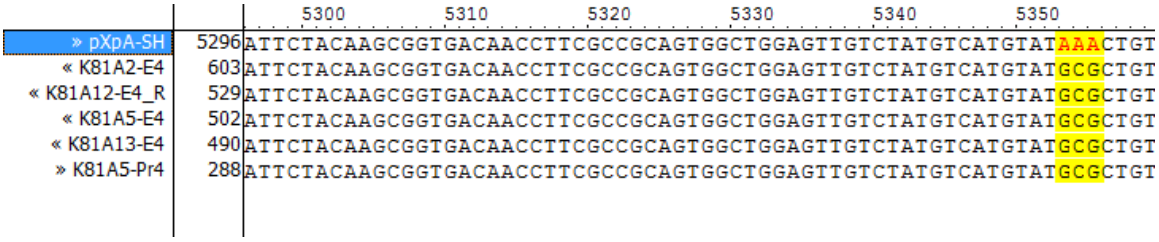


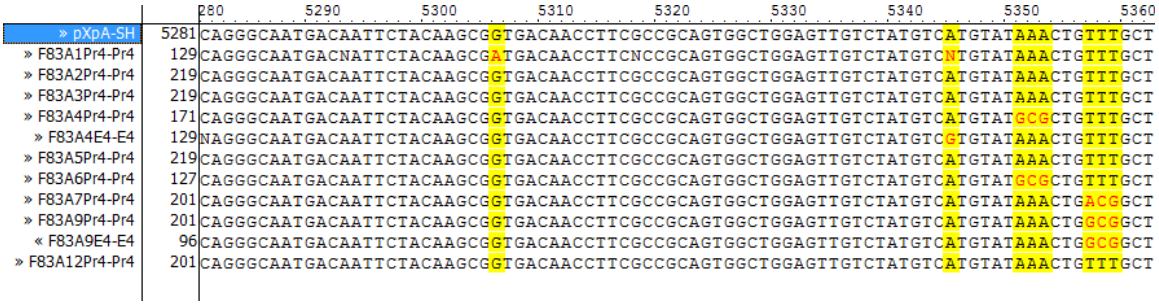
Figure 3-2: Plaque morphology of wildtype PV-3AB, PV-3AB-K81A and PV-3AB-F83A mutants. HeLa cell monolayers were transfected with serial dilutions of RNA transcript and overlaid with high glucose DMEM containing 0.5% agaorse 1 h after transfection. Plates were incubated at 37°C and stained with crystal violet 48 h after transfection. A) Mock transfection carried out in the absence of viral RNA. This was done to test the transfection reagents for contamination. B) Transfection with wildtype PV-3AB. C) Transfection with PV-3AB-K81A. D) Transfection with PV-3AB-F83A. The amount of RNA in µg in each well is as follows, starting left to right top panel is undiluted, $1X10^{-1}$, $1X10^{-2}$ and left to right bottom panel is $1X10^{-3}$, $1X10^{-4}$ and no RNA control. Infectivity for wildtype is $3.7X10^{5}$, K81A is $2.2X10^{5}$ and F83A is 15 plaque forming units per microgram of RNA used. Each of the transfection plate has a well that contains no RNA to control for no contamination between wells within the same plate.

Comparison of cDNA sequences derived from plaque purified viral RNA containing mutations within the putative chaperone domain of 3AB. 3AB sequence of poliovirus cDNA obtained from plaques generated by transfection with K81A RNA transcripts was determined. 3AB sequence of poliovirus cDNA obtained from revertants as a result of transfection with F83A RNA transcript was also determined. All primers for PCR and sequences are listed in Table 3-1. Comparison of five cDNA sequences from plaques derived from K81A mutation containing transcript showed that the virus maintained the original K81A mutation (AAA to GCG) indicating that this change does not apply any selective pressure on the virus to survive and hence gave rise to no compensatory mutations within the 3AB region of the genome.

Transfection with F83A RNA did not form clear plaques. Upon incubating the plates for a longer duration (4 days), some revertants developed. Comparison of nine cDNA sequences from these revertants showed that the virus reverts back to the wildtype in most cases, indicating that this mutation was not well tolerated by the virus. In one case, the virus retains the original F83A mutation (TTT to GCG) (Figure 3-3) and in another case, there is a mixed population of the virus containing the original mutation or converting to ACG (Figure 3-3, 3-4) which codes for threonine. These results do not eliminate the existence of other mutations in the rest of the viral genome that may contribute to the observed plaque phenotype, infectivity and difference in RNA synthesis compared to wildtype (explained below, Figure 3-6).



A



B

Figure 3-3: Comparison of cDNA sequences derived from plaque purified viral RNA containing mutations within the putative chaperone domain of 3AB.

Top panel A: The yellow highlighted region between nucleotides 5351-5353 indicates the K81A mutation compared to wildtype (topmost sequence). All five plaques sequenced retain the K81A mutation.

Bottom panel B: The yellow highlighted region between nucleotides 5358-5360 indicates the F83A mutation compared to wildtype (topmost sequence). Seven of the nine plaques sequenced reverted to wildtype. The other two substituted an alanine and a threonine at position 83. Other regions of the genome have not been sequenced.

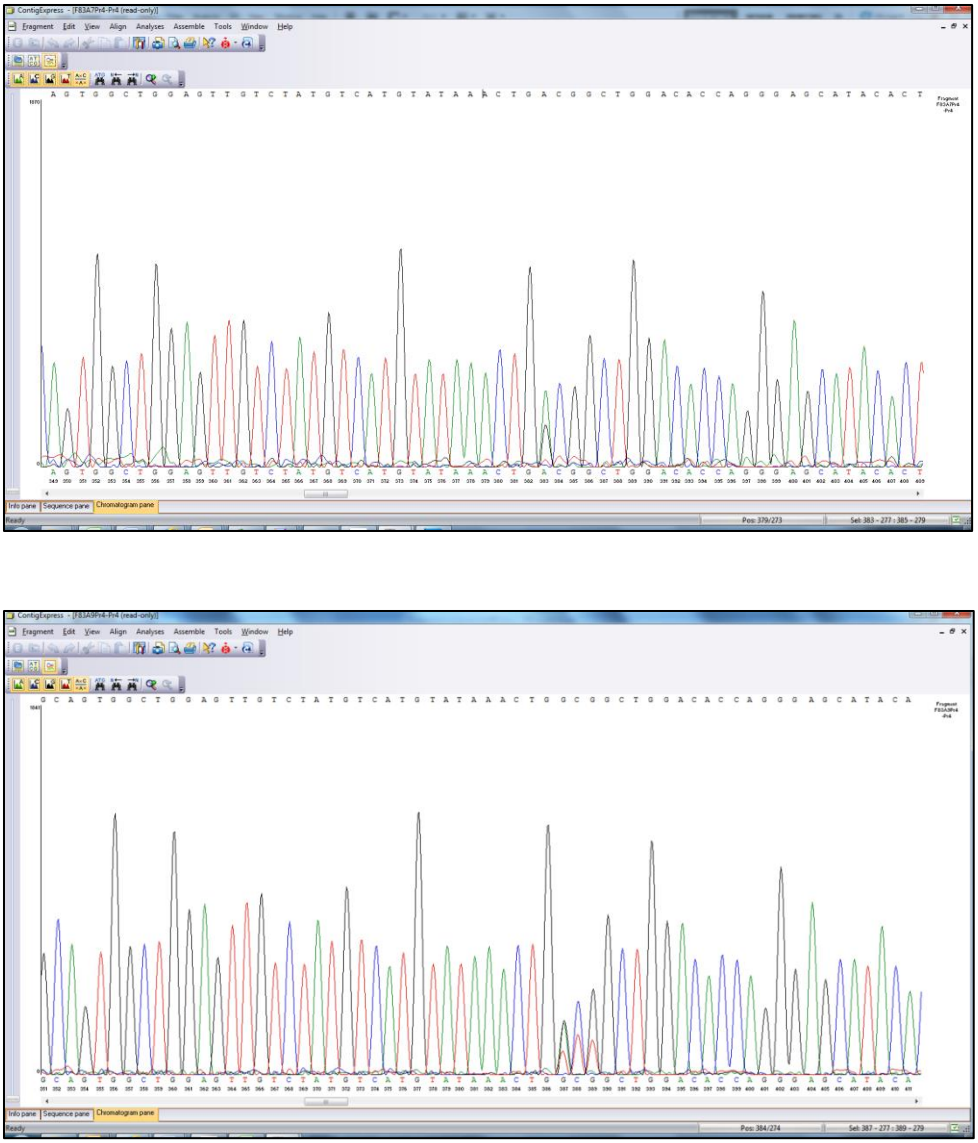


Figure 3-4: DNA sequence analysis of specific revertants from F83A mutations.

Top panel: Position 383-385 clearly indicates ACG, which codes for threonine

Bottom panel: Position 387-389 indicates a mixed population of viruses mostly containing ACG or GCG which codes for threonine and alanine respectively.

Effect of mutations in the putative chaperone domain of 3AB on various steps in viral replication. To test the effect of amino acid substitution at positions 81 and 83 on various steps in virus replication, we performed an *in vitro* translation-replication assay in HeLa cell extracts. The HeLa cell extract contains the necessary components required for PV RNA replication and is assumed to mimic the steps that occur in infected cells. In this assay, RNA transcripts from wild type pXpA-SH-3AB, pXpA-SH-K81A and pXpA-SH-F83A were used to program HeLa S10 extracts supplemented with ribosomal salt wash in the presence of [^{35}S] methionine, which allows the analysis of protein translation and polyprotein processing independent from other viral processes (Figure 3-1). This part of the assay was performed in the presence of guanidine hydrochloride in the reaction mixture, a known inhibitor of poliovirus RNA replication. Analysis of labeled viral protein by SDS-PAGE showed that there was no difference in the pattern or levels of synthesized proteins between wildtype and mutant 3AB constructs (Figure 3-5), indicating that these mutations do not have any effect on protein synthesis and processing of precursor proteins.

To determine whether these mutations alter the RNA production, we examined the ability of the HeLa cell extracts containing the proteins translated from wildtype or mutants RNA transcripts to synthesize RNA *in vitro*. Replication complexes were isolated by centrifugation after the translation reaction. This step is required to get rid of guanidine hydrochloride, an inhibitor of poliovirus replication (Figure 3-1). Analysis of ^{32}P labeled RNA by agaorse gel electrophoresis showed a difference in the levels of RNA synthesized by wildtype and mutant transcripts. K81A showed slightly lower levels of RNA synthesized while F83A showed a modest decrease in the level of RNA synthesized

compared to wildtype. These results indicate that the K81A and F83A mutations affect RNA synthesis. However, this system does not allow us to distinguish which steps in RNA replication are affected. Further work needs to be done to determine the effect of these mutations on different stages of viral RNA replication such as VPg uridylyation and or positive/negative strand synthesis.

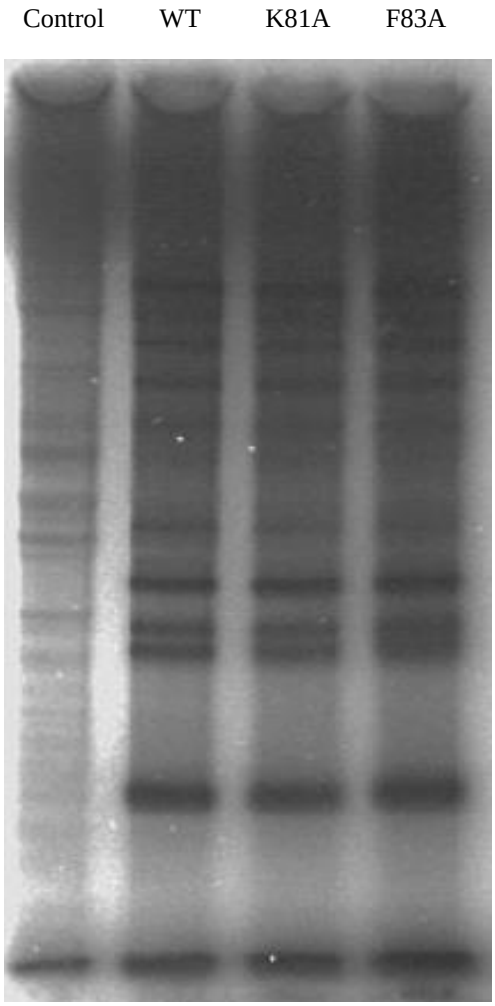


Figure 3-5: *In vitro* translation-replication assay; protein synthesis and polyprotein processing. Wildtype and mutant RNA transcripts were used to program translation in HeLa S10 extracts supplemented with ribosomal salt wash in the presence of ^{35}S methionine. Lane 1-4 (l-r): No RNA, F83A, K81A and wildtype 3AB. Reaction products were subjected to electrophoresis on a 12% polyacrylamide-SDS gel. Results indicate no difference in protein synthesis and polyprotein processing between wildtype and mutants.

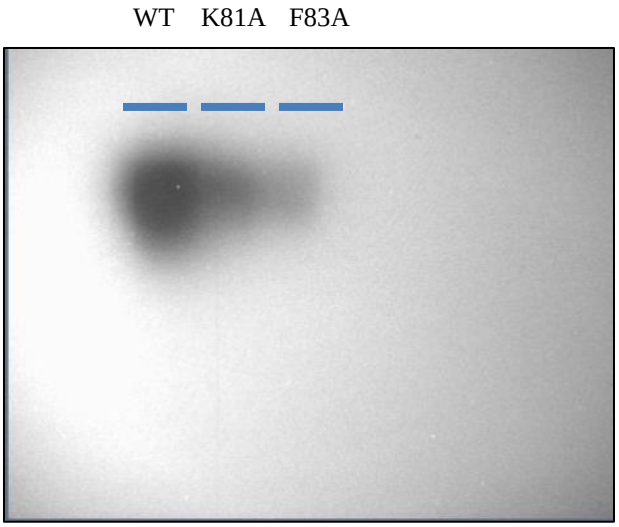


Figure 3-6: *In vitro* translation-replication assay; RNA synthesis. Replication complexes were isolated by centrifugation following translation in the *in vitro* translation- replication reaction. The ^{32}P labeled RNA was analyzed by agaorse gel electrophoresis. Lanes 1-3 (l-r): Wildtype 3AB, K81A and F83A. The results indicate a difference in RNA levels between the mutants and the wildtype. K81A is slightly lower than wildtype while F83A is lower.

3.4 Discussion

This report presents the effect of mutations within the putative chaperone domain (3B+7) of 3AB on replication of the virus in tissue culture. Here we have analyzed mutations that are associated with reduction in chaperone activity of 3AB *in vitro* (discussed in chapter 2). The two mutations tested, K81A and F83A, reside in the connection domain that consists of 7 amino acids at the C-terminus of the 3A (+7 region of 3B+7). Mutations in this region have not been extensively characterized.

From our tissue culture assays, we found that K81A did not dramatically affect the infectivity and plaque phenotype of the virus in comparison to the wildtype. Sequence analysis of virus produced as a result of this mutation showed that the virus retained the original mutation (AAA to GCG) at position 81, indicating that this mutation is well tolerated. The *in vitro* translation-replication assay shows wildtype levels of protein synthesis and polyprotein processing ability. This is possible as chaperone activity is based on nucleic acid binding of the protein and polyprotein synthesis and processing does not require the nucleic acid interaction of 3AB. The reduced RNA levels indicate that K81A does have an effect on RNA replication but the exact step at which replication is affected has to be further analyzed (VPg uridylation and/or initiation of negative and positive strand synthesis).

F83A on the other hand drastically affected viral infectivity and did not form clear plaques. Sequence analysis of revertants that appeared showed that the virus switches back to the wildtype in most cases. In vitro protein synthesis and polyprotein processing was unaffected by this mutations while RNA synthesis was severely reduced. 3AB is a

multifunctional protein and interacts with many proteins that are involved in replication (Section 1-10). The F83A mutation maybe critical for 3AB binding to other proteins that are involved in virus replication.

It is quite possible that in both cases, the stage at which viral replication is affected may or may not require the chaperone activity of 3AB. K81A is a change from positive amino acid to a neutral amino acid. This change would possibly reduce RNA binding purely based on charge interaction and that may be the reason why a decrease in chaperone activity of 3AB was observed *in vitro*. Phenylalanine is a bulky amino acid and maybe involved in base stacking of nucleic acid molecules. This change could have an effect on RNA binding ability of 3AB and hence could be the reason for a decrease in chaperone activity of 3AB *in vitro*. These effects may have caused a decrease in RNA synthesis levels by affecting one or more steps in virus replication like VPg uridylation and/or initiation of negative and positive strand synthesis.

3AB may be involved in recombination which occurs in high frequency in polio and other plus sense RNA viruses (1, 6, 28, 29). Recombination presumably occurs by a copy-choice mechanism that involves transfer (often referred to as “strand transfer” or “template-switching”) of an RNA being synthesized on one viral genome (generally during synthesis of the minus strand in polio) to another where synthesis continues. Chaperone proteins like HIV-NC can stimulate copy-choice recombination in reconstituted *in vitro* systems through accelerating the binding of complementary nucleic acids. Since the mutations tested above show reduced chaperone activity *in vitro,* they could decrease recombination in the virus. It will be interesting to identify mutations that decrease recombination without affecting replication of the virus. Such a virus can be

used to develop an effective vaccine that eliminates the problem of recombination in current vaccine strains (see general discussion). Based on our data from this report, K81A is a likely candidate to be tested for recombination as it produces viable virus, normal level of proteins and only slightly reduced RNA levels *in vitro.* F83A may not be a suitable candidate as this mutation is not tolerated well by the virus and reverts back to wiltype. Viruses produced from the K81A mutation should be further tested for growth kinetics. If this virus does not recombine effectively but has high growth kinetics, it could be incorporated into current vaccine strains and tested for recombination and growth of the resultant virus.

Chapter 4: General Discussion

Proteins with RNA chaperone activity are ubiquitous in nature and play an important role in various cellular processes. They are important partners of RNA as they help maintain its correct structure by rearrangement of misfolded structures in an ATP-independent manner at almost every stage of RNA metabolism including transcription, transport, storage and translation (20, 44, 81). The range of RNA chaperone proteins that exists varies from virus-encoded RNA chaperones, *E. coli* transcriptional regulator (StpA), many ribosomal proteins, cold-shock proteins, and *E. Coli* translation initiation factor 1 (IF1) among others (96). The field of virus-encoded chaperone proteins is growing rapidly and is of special interest to us (119). Our lab was the first to demonstrate chaperone activity of a picronavirus protein, poliovirus 3AB (23). It is important to note that chaperone activity is measured in the test tube so it is not always clear what role, if any, the observed activity is playing in the cell. For ribosomal proteins the observed activity is consistent with the putative function of the proteins in ribosome assembly, however, for viral chaperones like 3AB and HIV NC, the role is less defined.

The aim of this dissertation was to identify the regions intrinsic to the nucleic acid chaperone activity of 3AB. Previous work from our lab has shown that bacterially expressed purified protein 3AB possessed helix destabilizing activity and was able to promote hybridization of complimentary nucleic acid strands in the absence of nucleotides (as an energy source) (23). In Chapter 2, we were able to demonstrate that the 3B region of 3AB is pivotal to chaperone activity but 3B by itself does not possess activity. Since this region is indispensable for nucleic acid binding, this finding is not

surprising. Single point mutations, R104E and Y90A, within the 3B region of the 3AB protein showed severely reduced chaperone activity. These residues make additional contact with other amino acids in the 3B solution structure and it is possible that the mutations disrupt some or all of these interactions (94, 95). In fact these residues lie on the same face of 3B as other amino acids that are conserved among picronaviruses. This face is critical to nucleic acid binding as well as 3AB-$3D^{pol}$ protein-protein interactons (99). The mutations may directly disrupt nucleic acid binding, especially R104E which changes a basic amino acid to an acidic residue. They may also disrupt the structure in this region leading to abrogation of chaperone activity. Analysis of these mutation using circular dichroism (CD) or NMR may help reveal structural changes.

The most interesting result from this work is the finding that the putative C–terminal cytoplasmic domain of 3AB, 3B+7 possess chaperone activity at high concentrations. Since 3B+7 showed chaperone activity while 3B alone did not, it is clear that the additional seven amino acids from the C–terminal end of 3A are pivotal for the chaperone activity of 3AB. Three single point mutations were made in this region and were tested in the context of 3B+7 and full length 3AB protein. Two of them, K81A and F83A, showed reduced chaperone activity similar to the Y90A mutation in 3B+7. In contrast H86A showed more activity but still less than wildtype 3B+7. These results reiterate the importance of positively charged and aromatic amino acids in chaperone activity. In the context of the full length 3AB protein, these mutations had similar effects on chaperone activity. The dramatic change from essentially no activity for 3B to significant activity for 3B+7 could result from the addition of aromatic and basic amino acids in the +7 region as these are known to be important to chaperone activity in other

proteins. It is also possible that the +7 region is needed to stabilize the structure of 3B or this entire region of the protein. Although an NMR structure for 3B has been solved, stabilizing reagents were required to obtain a structure and the protein was unstructured without these agents. It would be interesting to see if 3B+7 has a stable structure, even in the absence of stabilizing agents.

The current results and previous results from others have allowed us to formulate a working model to explain the *in vitro* chaperone activity of 3AB (Figure 4-1). We hypothesize that the intrinsic chaperone activity of 3AB resides in the 3B+7 region based on the chaperone activity of this protein at high concentrations (Table 2-3). The membrane anchor domain of the 3A region (amino acid 59-80) serves to "concentrate" the 3B+7 region by stacking on itself. This leads to an increase in the effective concentration of the 3B+7 region such that a lower concentration of the full-length protein, in comparison to 3B+7 alone, is required to obtain high chaperone activity. In Figure 4-1, the membrane anchor domain is shown as the major stacking agent, although the N-terminal cytoplasmic domain (amino acids 1-58, labeled 3A in figure) could also be involved. This region has been shown by NMR to dimerize when expressed in the absence of the rest of the protein so interactions leading to multimer formation are plausible.(98) In addition to being consistent with the current results, the proposed model is also consistent with 3AB's ability to form homomultimeric complexes.(78, 109) Note that no membranes are required for the proposed stacking as the assays were conducted in the absence of phospholipids. Still, cellular membranes may help catalyze stacking and this will be examined in the future using membranes in in vitro reactions. We are currently testing this model by making point mutations in the membrane anchor and N-

terminal region of 3AB. Truncations and deletions will also be tested permitting that these proteins are able to be produced.

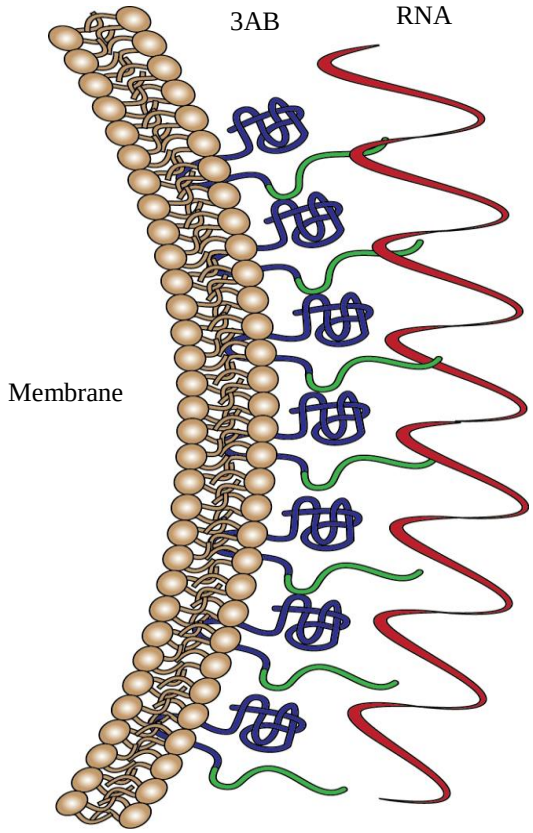


Figure 4-1: Model for in vitro 3AB chaperone activity. The intrinsic chaperone activity of 3AB resides in the 3B+7 region based on the chaperone activity of this protein at high concentrations (Table 2-3). The membrane anchor domain of the 3A region (amino acid 59–80) serves to "concentrate" the 3B+7 region by stacking on itself. This leads to an increase in the effective concentration of the 3B+7 region such that a lower concentration of the full-length protein, in comparison to 3B+7 alone, is required to obtain high chaperone activity.

We have successfully identified several mutations in 3AB (R104E, Y90A, R104E/Y90A, K81A, F83A) that negatively affect chaperone activity. Previous reports by other groups have shown that R104E and Y90A mutations are lethal in tissue culture infections. We tested a few of the remaining mutations (K81A and F83A) for their effect on replication of the virus in tissue culture. Based on our observation (Chapter 3), a decrease in chaperone activity does reduce RNA synthesis *in vitro.* However, since 3AB serves many functions in viral replication, it is not clear if RNA synthesis reduction resulted only from a decrease in chaperone activity. No clear role for F83 or K81 has been deciphered in the literature. It will be necessary to examine specific steps in replication to try to correlate chaperone activity with a specific viral function. For a protein with such ubiquitous function, this may be difficult. This work is ongoing and we plan to determine the individual steps in virus replication that are affected (VPg uridylation, negative and positive strand RNA synthesis) as well as assay the level of recombination in the virus. It has been proposed that chaperones promote recombination in viruses and there is some evidence that mutations in HIV NC protein can reduce recombination. Alteration in recombination could be a good readout for chaperone function as it may be possible to produce mutants that minimally effect replication while dramatically reducing recombination. This could be important as one problem with the current virus strains used in the oral polio vaccine is the possibility of reversion via mutation or recombination to a more neurovirulent form while they replicate in the gut. There is a need for developing a modified live attenuated vaccine but with lower recombination or mutation frequencies. Specific mutations in poliovirus 3Dpol have been shown to reduce mutation frequency and decrease recombination and these have recently

been added to the vaccine strain (5, 22, 33, 105, 106). The chaperone activity of 3AB can be used towards developing new effective vaccines that could lead to eradication poliovirus. Since recombination is not strictly "required" for replication, mutations that inhibit recombination may not necessarily inhibit replication. Therefore, we would be presented with a unique opportunity to identify mutations that could *decrease recombination without affecting replication*. Even if the mutations had a modest deleterious influence on replication kinetics, they could still find a potentially powerful use in the construction of attenuated poliovirus vaccine strains.

1. **Agut, H., K. M. Kean, C. Bellocq, O. Fichot, and M. Girard.** 1987. Intratypic recombination of polioviruses: evidence for multiple crossing-over sites on the viral genome. J Virol **61:**1722-5.

2. **Alexander, J. P., Jr., H. E. Gary, Jr., and M. A. Pallansch.** 1997. Duration of poliovirus excretion and its implications for acute flaccid paralysis surveillance: a review of the literature. J Infect Dis **175 Suppl 1:**S176-82.

3. **Ambros, V., and D. Baltimore.** 1978. Protein is linked to the 5' end of poliovirus RNA by a phosphodiester linkage to tyrosine. J Biol Chem **253:**5263-6.

4. **Anthony, R. M., and J. J. Destefano.** 2007. In vitro synthesis of long DNA products in reactions with HIV-RT and nucleocapsid protein. J Mol Biol **365:**310-24.

5. **Arias, A., J. J. Arnold, M. Sierra, E. D. Smidansky, E. Domingo, and C. E. Cameron.** 2008. Determinants of RNA-dependent RNA polymerase (in)fidelity revealed by kinetic analysis of the polymerase encoded by a foot-and-mouth disease virus mutant with reduced sensitivity to ribavirin. J Virol **82:**12346-55.

6. **Arnold, J. J., and C. E. Cameron.** 1999. Poliovirus RNA-dependent RNA polymerase (3Dpol) is sufficient for template switching in vitro. J Biol Chem **274:**2706-16.

7. **Bari, S., and T. Jasarevic** 2012, posting date. India records one year without polio cases. World Health Organization. [Online.]

8. **Barton, D. J., and J. B. Flanegan.** 1993. Coupled translation and replication of poliovirus RNA in vitro: synthesis of functional 3D polymerase and infectious virus. J Virol **67:**822-31.

9. **Belov, G. A., M. H. Fogg, and E. Ehrenfeld.** 2005. Poliovirus proteins induce membrane association of GTPase ADP-ribosylation factor. J Virol **79:**7207-16.

10. **Berstein, H. D., and D. Baltimore.** 1988. Poliovirus mutant that contains a cold-sensitive defect in viral RNA synthesis. J Virol **62:**2922-8.

11. **Bienz, K., D. Egger, and L. Pasamontes.** 1987. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. Virology **160:**220-6.

12. **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990. Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region. J Virol **64:**1156-63.

13. **Blondel, B., F. Colbere-Garapin, T. Couderc, A. Wirotius, and F. Guivel-Benhassine.** 2005. Poliovirus, pathogenesis of poliomyelitis, and apoptosis. Curr Top Microbiol Immunol **289:**25-56.

14. **Bodian, D.** 1956. Poliovirus in chimpanzee tissues after virus feeding. Am J Hyg **64:**181-97.

15. **Brown, B. A., and E. Ehrenfeld.** 1979. Translation of poliovirus RNA in vitro: changes in cleavage pattern and initiation sites by ribosomal salt wash. Virology **97:**396-405.

16. **Brown, D. M., S. E. Kauder, C. T. Cornell, G. M. Jang, V. R. Racaniello, and B. L. Semler.** 2004. Cell-dependent role for the poliovirus 3' noncoding region in positive-strand RNA synthesis. J Virol **78:**1344-51.

17. **Bushell, M., and P. Sarnow.** 2002. Hijacking the translation apparatus by RNA viruses. J Cell Biol **158:**395-9.

18. **Cello, J., O. Strannegard, and B. Svennerholm.** 1996. A study of the cellular immune response to enteroviruses in humans: identification of cross-reactive T cell epitopes on the structural proteins of enteroviruses. J Gen Virol **77 (Pt 9):**2097-108.

19. **Chow, M., J. F. Newman, D. Filman, J. M. Hogle, D. J. Rowlands, and F. Brown.** 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature **327:**482-6.

20. **Cristofari, G., and J. L. Darlix.** 2002. The ubiquitous nature of RNA chaperone proteins. Prog Nucleic Acid Res Mol Biol **72:**223-68.

21. **De Jesus, N. H.** 2007. Epidemics to eradication: the modern history of poliomyelitis. Virol J **4:**70.

22. **DeStefano, J. J.** 2010. Effect of reaction conditions and 3AB on the mutation rate of poliovirus RNA-dependent RNA polymerase in a alpha-complementation assay. Virus Res **147:**53-9.

23. **Destefano, J. J., and O. Titilope.** 2006. Poliovirus Protein 3AB Displays Nucleic Acid Chaperone and Helix-Destabilizing Activities. J Virol **80:**1662-71.

24. **Dodd, D. A., T. H. Giddings, Jr., and K. Kirkegaard.** 2001. Poliovirus 3A protein limits interleukin-6 (IL-6), IL-8, and beta interferon secretion during viral infection. J Virol **75:**8158-65.

25. **Doedens, J. R., and K. Kirkegaard.** 1995. Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A. EMBO J **14:**894-907.

26. **Dorsch-Hasler, K., Y. Yogo, and E. Wimmer.** 1975. Replication of picornaviruses. I. Evidence from in vitro RNA synthesis that poly(A) of the poliovirus genome is genetically coded. J Virol **16:**1512-7.

27. **Dowdle, W. R., and M. E. Birmingham.** 1997. The biologic principles of poliovirus eradication. J Infect Dis **175 Suppl 1:**S286-92.

28. **Duggal, R., A. Cuconati, M. Gromeier, and E. Wimmer.** 1997. Genetic recombination of poliovirus in a cell-free system. Proc Natl Acad Sci U S A **94:**13786-91.

29. **Duggal, R., and E. Wimmer.** 1999. Genetic recombination of poliovirus in vitro and in vivo: temperature-dependent alteration of crossover sites. Virology **258:**30-41.

30. **Eberle, K. E., V. T. Nguyen, and M. S. Freistadt.** 1995. Low levels of poliovirus replication in primary human monocytes: possible interactions with lymphocytes. Arch Virol **140:**2135-50.

31. **Farrah, S., C. Wallis, P. T. Shaffer, and J. L. Melnick.** 1976. Reconcentration of poliovirus from sewage. Appl Environ Microbiol **32:**653-8.

32. **Freistadt, M. S., H. B. Fleit, and E. Wimmer.** 1993. Poliovirus receptor on human blood cells: a possible extraneural site of poliovirus replication. Virology **195:**798-803.

33. **Freistadt, M. S., J. A. Vaccaro, and K. E. Eberle.** 2007. Biochemical characterization of the fidelity of poliovirus RNA-dependent RNA polymerase. Virol J **4:**44.

34. **Fricks, C. E., and J. M. Hogle.** 1990. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J Virol **64:**1934-45.

35. **Fujita, K., S. S. Krishnakumar, D. Franco, A. V. Paul, E. London, and E. Wimmer.** 2007. Membrane topography of the hydrophobic anchor sequence of poliovirus 3A and 3AB proteins and the functional effect of 3A/3AB membrane association upon RNA replication. Biochemistry **46:**5185-99.

36. **Giachetti, C., S. S. Hwang, and B. L. Semler.** 1992. cis-acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication. J Virol **66:**6045-57.

37. **Giachetti, C., and B. L. Semler.** 1991. Role of a viral membrane polypeptide in strand-specific initiation of poliovirus RNA synthesis. J Virol **65:**2647-54.

38. **Gulevich, A. Y., R. A. Yusupova, and Y. F. Drygin.** 2002. VPg unlinkase, the phosphodiesterase that hydrolyzes the bond between VPg and picornavirus RNA: a minimal nucleic moiety of the substrate. Biochemistry (Mosc) **67:**615-21.

39. **Hampton, L.** 2009. Albert Sabin and the Coalition to Eliminate Polio from the Americas. Am J Public Health **99:**34-44.

40. **Harris, K. S., W. Xiang, L. Alexander, W. S. Lane, A. V. Paul, and E. Wimmer.** 1994. Interaction of poliovirus polypeptide 3CDpro with the 5' and 3' termini of the poliovirus genome. Identification of viral and cellular cofactors needed for efficient binding. J Biol Chem **269:**27004-14.

41. **Heath, M. J., S. S. Derebail, R. J. Gorelick, and J. J. DeStefano.** 2003. Differing roles of the N-terminal and C-terminal zinc fingers in HIV-1 nucleocapsid protein enhanced nucleic acid annealing. J. Biol. Chem. **278:**30755-30763.

42. **Heinz, B. A., and L. M. Vance.** 1995. The antiviral compound enviroxime targets the 3A coding region of rhinovirus and poliovirus. J Virol **69:**4189-97.

43. **Hellen, C. U., and E. Wimmer.** 1992. Maturation of poliovirus capsid proteins. Virology **187:**391-7.

44. **Herschlag, D.** 1995. RNA chaperones and the RNA folding problem. J Biol Chem **270:**20871-4.

45. **Hogle, J. M., M. Chow, and D. J. Filman.** 1985. Three-dimensional structure of poliovirus at 2.9 A resolution. Science **229:**1358-65.

46. **Hogle, J. M., and D. J. Filman.** 1989. The antigenic structure of poliovirus. Philos Trans R Soc Lond B Biol Sci **323:**467-78.

47. **Horstmann, D. M.** 1985. The poliomyelitis story: a scientific hegira. Yale J Biol Med **58:**79-90.

48. **Hull, H. F., M. E. Birmingham, B. Melgaard, and J. W. Lee.** 1997. Progress toward global polio eradication. J Infect Dis **175 Suppl 1:**S4-9.

49. **Iwasaki, A., R. Welker, S. Mueller, M. Linehan, A. Nomoto, and E. Wimmer.** 2002. Immunofluorescence analysis of poliovirus receptor expression in Peyer's patches of humans, primates, and CD155 transgenic mice: implications for poliovirus infection. J Infect Dis **186:**585-92.

50. **Jacobson, S. J., D. A. Konings, and P. Sarnow.** 1993. Biochemical and genetic evidence for a pseudoknot structure at the 3' terminus of the poliovirus RNA genome and its role in viral RNA amplification. J Virol **67:**2961-71.

51. **Jang, S. K., H. G. Krausslich, M. J. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J Virol **62:**2636-43.

52. **Juskewitch, J. E., C. J. Tapia, and A. J. Windebank.** 2010. Lessons from the Salk polio vaccine: methods for and risks of rapid translation. Clin Transl Sci **3:**182-5.

53. **Kew, O.** 2012. Reaching the last one per cent: progress and challenges in global polio eradication. Curr Opin Virol **2:**188-98.

54. **Kew, O. M., R. W. Sutter, E. M. de Gourville, W. R. Dowdle, and M. A. Pallansch.** 2005. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. Annu Rev Microbiol **59:**587-635.

55. **Kuhn, R. J., H. Tada, M. F. Ypma-Wong, B. L. Semler, and E. Wimmer.** 1988. Mutational analysis of the genome-linked protein VPg of poliovirus. J Virol **62:**4207-15.

56. **Lama, J., A. V. Paul, K. S. Harris, and E. Wimmer.** 1994. Properties of purified recombinant poliovirus protein 3aB as substrate for viral proteinases and as co-factor for RNA polymerase 3Dpol. J Biol Chem **269:**66-70.

57. **Lama, J., M. Sanz, A , and P. Rodriguez, L.** 1995. Role of 3AB protein in poliovirus genome replication. Journal of Biological Chemisitry **270:**14430-14438.

58. **Lambert, S. M., and H. Markel.** 2000. Making history: Thomas Francis, Jr, MD, and the 1954 Salk Poliomyelitis Vaccine Field Trial. Arch Pediatr Adolesc Med **154:**512-7.

59. **Larsen, G. R., A. J. Dorner, T. J. Harris, and E. Wimmer.** 1980. The structure of poliovirus replicative form. Nucleic Acids Res **8:**1217-29.

60. **Levin, J. G., J. Guo, R. Ioulia, and K. Musier-Forsyth.** 2005. Nucleic acid chaperone activity of HIV-1 nucleocapsid protein: critical role in reverse transcription and molecular mechanism. Prog. Nucleic Acids Re. **In Press**.

61. **Li, N., Z. Q. Cui, J. K. Wen, Z. P. Zhang, H. P. Wei, Y. F. Zhou, and X. E. Zhang.** 2008. Live cell imaging of protein interactions in poliovirus RNA replication complex using fluorescence resonance energy transfer (FRET). Biochem Biophys Res Commun **368:**489-94.

62. **Linding, R., R. B. Russell, V. Neduva, and T. J. Gibson.** 2003. GlobPlot: Exploring protein sequences for globularity and disorder. Nucleic Acids Res **31:**3701-8.

63. **Melnick, J. L.** 1996. Current status of poliovirus infections. Clin Microbiol Rev **9:**293-300.

64. **Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56:**855-65.

65. **Minor, P. D.** 1990. Antigenic structure of picornaviruses. Curr Top Microbiol Immunol **161:**121-54.

66. **Molla, A., K. Harris, S , A. Paul, V , S. Shin, H , J. Mugavero, and E. Wimmer.** 1994. Stimulation of poliovirus proteinase 3Cpro -related proteolysis by the genome-linked protein VPg and its precursor 3AB. Journal of Biological Chemistry **269:**27015-27020.

67. **Molla, A., A. V. Paul, and E. Wimmer.** 1991. Cell-free, de novo synthesis of poliovirus. Science **254:**1647-51.

68. **Murdin, A. D., and E. Wimmer.** 1989. Construction of a poliovirus type 1/type 2 antigenic hybrid by manipulation of neutralization antigenic site II. J Virol **63:**5251-7.

69. **Narayanan, N., R. J. Gorelick, and J. J. DeStefano.** 2006. Structure/function mapping of amino acids in the N-terminal zinc finger of the human immunodeficiency virus type 1 nucleocapsid protein: residues responsible for nucleic acid helix destabilizing activity. Biochemistry **45:**12617-28.

70. **Nathanson, N.** 2011. Eradication of poliovirus: fighting fire with fire. J Infect Dis **203:**889-90.

71. **Neznanov, N., A. Kondratova, K. M. Chumakov, B. Angres, B. Zhumabayeva, V. I. Agol, and A. V. Gudkov.** 2001. Poliovirus protein 3A inhibits tumor necrosis factor (TNF)-induced apoptosis by eliminating the TNF receptor from the cell surface. J Virol **75:**10409-20.

72. **Nomoto, A., Y. F. Lee, and E. Wimmer.** 1976. The 5' end of poliovirus mRNA is not capped with m7G(5')ppp(5')Np. Proc Natl Acad Sci U S A **73:**375-80.

73. **Page, G. S., A. G. Mosser, J. M. Hogle, D. J. Filman, R. R. Rueckert, and M. Chow.** 1988. Three-dimensional structure of poliovirus serotype 1 neutralizing determinants. J Virol **62:**1781-94.

74. **Paul, A., V, J. Boom, H, D. Filippov, and E. Wimmer.** 1998. Protein-primed RNA synthesis by purified RNA polymerase. Nature **393:**280-284.

75. **Paul, A. V.** 2002. Molecular Biology of Picornaviruses. ASM Press, Washington, DC.

76. **Paul, A. V., X. Cao, K. S. Harris, J. Lama, and E. Wimmer.** 1994. Studies with poliovirus polymerase 3Dpol. Stimulation of poly(U) synthesis in vitro by purified poliovirus protein 3AB. J Biol Chem **269:**29173-81.

77. **Paul, A. V., E. Rieder, D. W. Kim, J. H. van Boom, and E. Wimmer.** 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the In vitro uridylylation of VPg [In Process Citation]. J Virol **74:**10359-70.

78. **Plotch, S., J , and O. Palant.** 1995. Poliovirus protein 3AB form a complex with and stimulates the activity of the viral RNA polymerase, $3D^{pol}$. Journal of Virology **69:**7169-7179.

79. **Plotch, S. J., and O. Palant.** 1995. Poliovirus protein 3AB forms a complex with and stimulates the activity of the viral RNA polymerase, 3Dpol. J Virol **69:**7169-79.

80. **Racaniello, V. R.** 2006. One hundred years of poliovirus pathogenesis. Virology **344:**9-16.

81. **Rajkowitsch, L., D. Chen, S. Stampfl, K. Semrad, C. Waldsich, O. Mayer, M. F. Jantsch, R. Konrat, U. Blasi, and R. Schroeder.** 2007. RNA chaperones, RNA annealers and RNA helicases. RNA Biol **4:**118-30.

82. **Rein, A., L. E. Henderson, and J. G. Levin.** 1998. Nucleic-acid-chaperone activity of retroviral nucleocapsid proteins: significance for viral replication. Trends Biochem Sci **23:**297-301.

83. **Ren, R., and V. R. Racaniello.** 1992. Poliovirus spreads from muscle to the central nervous system by neural pathways. J Infect Dis **166:**747-52.

84. **Richards, O., C, and E. Ehrenfeld.** 1998. Effects of poliovirus 3AB protein on 3D polymerase-catalyzed reaction. Journal of Biological Chemistry **273:**12832-12840.

85. **Roberts, L.** 2009. Public health. Type 2 poliovirus back from the dead in Nigeria. Science **325:**660-1.

86. **Rodriguez-Wells, V., S. J. Plotch, and J. J. DeStefano.** 2001. Primer-dependent synthesis by poliovirus RNA-dependent RNA polymerase (3D(pol)). Nucleic Acids Res **29:**2715-24.

87. **Rossmann, M. G., and J. E. Johnson.** 1989. Icosahedral RNA virus structure. Annu Rev Biochem **58:**533-73.

88. **Rothberg, P. G., T. J. Harris, A. Nomoto, and E. Wimmer.** 1978. O4-(5'-uridylyl)tyrosine is the bond between the genome-linked protein and the RNA of poliovirus. Proc Natl Acad Sci U S A **75:**4868-72.

89. **Rozovics, J. M., R. Virgen-Slane, and B. L. Semler.** 2011. Engineered picornavirus VPg-RNA substrates: analysis of a tyrosyl-RNA phosphodiesterase activity. PLoS One **6:**e16559.

90. **Sabin, A. B.** 1956. The significance of viruses recovered from the intestinal tracts of healthy infants and children. Ann N Y Acad Sci **66:**226-30.

91. **Sabin, A. B., and R. Ward.** 1941. The Natural History of Human Poliomyelitis : I. Distribution of Virus in Nervous and Non-Nervous Tissues. J Exp Med **73:**771-93.

92. **Sambrook, J., and D. W. Russell.** 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

93. **Schein, C. H., N. Oezguen, G. J. van der Heden van Noort, D. V. Filippov, A. Paul, E. Kumar, and W. Braun.** 2008. NMR solution structure of poliovirus uridylyated peptide linked to the genome (VPgpU). Peptides.

94. **Schein, C. H., N. Oezguen, G. J. van der Heden van Noort, D. V. Filippov, A. Paul, E. Kumar, and W. Braun.** 2010. NMR solution structure of poliovirus uridylyated peptide linked to the genome (VPgpU). Peptides **31:**1441-8.

95. **Schein, C. H., N. Oezguen, D. E. Volk, R. Garimella, A. Paul, and W. Braun.** 2006. NMR structure of the viral peptide linked to the genome (VPg) of poliovirus. Peptides **27:**1676-84.

96. **Semrad, K.** 2011. Proteins with RNA chaperone activity: a world of diverse proteins with a common task-impediment of RNA misfolding. Biochem Res Int **2011:**532908.

97. **Steil, B. P., and D. J. Barton.** 2009. Cis-active RNA elements (CREs) and picornavirus RNA replication. Virus Res **139:**240-52.

98. **Strauss, D. M., L. W. Glustrom, and D. S. Wuttke.** 2003. Towards an understanding of the poliovirus replication complex: the solution structure of the soluble domain of the poliovirus 3A protein. J Mol Biol **330:**225-34.

99. **Strauss, D. M., and D. S. Wuttke.** 2007. Characterization of protein-protein interactions critical for poliovirus replication: analysis of 3AB and VPg binding to the RNA-dependent RNA polymerase. J Virol **81:**6369-78.

100. **Tanchou, V., T. Delaunay, M. Bodeus, B. Roques, J. L. Darlix, and R. Benarous.** 1995. Conformational changes between human immunodeficiency virus type 1 nucleocapsid protein NCp7 and its precursor NCp15 as detected by anti- NCp7 monoclonal antibodies. J Gen Virol **76:**2457-66.

101. **Teterina, N. L., M. S. Rinaudo, and E. Ehrenfeld.** 2003. Strand-specific RNA synthesis defects in a poliovirus with a mutation in protein 3A. J Virol **77:**12679-91.

102. **Tompa, P., and P. Csermely.** 2004. The role of structural disorder in the function of RNA and protein chaperones. FASEB J **18:**1169-75.

103. **Towner, J. S., T. V. Ho, and B. L. Semler.** 1996. Determinants of membrane association for poliovirus protein 3AB. J Biol Chem **271:**26810-8.

104. **Towner, J. S., M. M. Mazanet, and B. L. Semler.** 1998. Rescue of defective poliovirus RNA replication by 3AB-containing precursor polyproteins. J Virol **72:**7191-200.

105. **Ward, C. D., M. A. Stokes, and J. B. Flanegan.** 1988. Direct measurement of the poliovirus RNA polymerase error frequency in vitro. J Virol **62:**558-62.

106. **Wells, V. R., S. J. Plotch, and J. J. DeStefano.** 2001. Determination of the mutation rate of poliovirus RNA-dependent RNA polymerase. Virus Res **74:**119-32.

107. **White, D. O., and F. J. Fenner.** 1994. Medical Virology, 4th ed. Academic Press.

108. **Whitton, J. L., C. T. Cornell, and R. Feuer.** 2005. Host and virus determinants of picornavirus pathogenesis and tropism. Nat Rev Microbiol **3:**765-76.

109. **Xiang, W., A. Cuconati, D. Hope, K. Kirkegaard, and E. Wimmer.** 1998. Complete protein linkage map of poliovirus P3 proteins: interaction of polymerase 3Dpol with VPg and with genetic variants of 3AB. J Virol **72:**6732-41.

110. **Xiang, W., A. Cuconati, A. V. Paul, X. Cao, and E. Wimmer.** 1995. Molecular dissection of the multifunctional poliovirus RNA-binding protein 3AB. Rna **1:**892-904.

111. **Xiang, W., K. S. Harris, L. Alexander, and E. Wimmer.** 1995. Interaction between the 5'-terminal cloverleaf and 3AB/3CDpro of poliovirus is essential for RNA replication. J Virol **69:**3658-67.

112. **Yalamanchili, P., U. Datta, and A. Dasgupta.** 1997. Inhibition of host cell transcription by poliovirus: cleavage of transcription factor CREB by poliovirus-encoded protease 3Cpro. J Virol **71:**1220-6.

113. **Yang, W. X., T. Terasaki, K. Shiroki, S. Ohka, J. Aoki, S. Tanabe, T. Nomura, E. Terada, Y. Sugiyama, and A. Nomoto.** 1997. Efficient delivery of circulating poliovirus to the central nervous system independently of poliovirus receptor. Virology **229:**421-8.

114. **Yin-Murphy, M., and J. W. Almond.** 1996. Medical Microbiology, 4th ed. University of Texas Medical Branch at Galveston.

115. **Yin, J., Y. Liu, E. Wimmer, and A. V. Paul.** 2007. Complete protein linkage map between the P2 and P3 non-structural proteins of poliovirus. J Gen Virol **88:**2259-67.

116. **You, J. C., and C. S. McHenry.** 1993. HIV nucleocapsid protein. Expression in Escherichia coli, purification, and characterization. J Biol Chem **268:**16519-27.

117. **You, J. C., and C. S. McHenry.** 1994. Human immunodeficiency virus nucleocapsid protein accelerates strand transfer of the terminally redundant sequences involved in reverse transcription. J Biol Chem **269:**31491-5.

118. **Zuker, M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res **31:**3406-15.

119. **Zuniga, S., I. Sola, J. L. Cruz, and L. Enjuanes.** 2009. Role of RNA chaperones in virus replication. Virus Res **139:**253-66.