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Functional analysis of poliovirus protein $2C^{ATPase}$ in viral RNA replication and encapsidation using alanine scanning mutagenesis

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Abstract of the Dissertation

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Polypeptide $2C^{ATPase}$ is one of the most thoroughly studied but least understood proteins in the life cycle of poliovirus. Within the protein, multiple functional domains, important for uncoating, host cell membrane alterations, RNA replication and encapsidation have previously been identified. In this study, charged to alanine scanning mutagenesis was used to generate conditional-lethal mutations in hitherto uncharacterized domains of the $2C^{ATPase}$ polypeptide, particularly those possibly involved in morphogenesis. Adjacent or clustered charged amino acids (2-4), scattered along the $2C^{ATPase}$ coding sequence, were replaced with alanines. RNA transcripts of mutant poliovirus cDNA clones were transfected into HeLa cells. Subsequently, ten lethal, one severely temperature-sensitive, two quasi-infectious, and three wild type-like mutants were identified. Using a Renilla luciferase reporter virus, all lethal and quasi-infectious mutants demonstrated RNA replication defects. Temperature-sensitive mutants were defective in

RNA replication only at the restricted temperatures. These mutants have led to the identification of several new sites within the $2C^{ATPase}$ polypeptide that are required for RNA replication. Interestingly, I characterized a quasi-infectious mutant (K_6A/K_7A) that produced a suppressor mutation (G_1R) and a novel $2B^{\wedge}2C^{ATPase}$ cleavage site (Q^R). Surprisingly, this cleavage site mutation did not interfere with normal processing of the polyprotein. Furthermore, analysis of the suppressor mutants of one quasi-infectious mutant and a detailed mutagenic analysis of its flanking Cysteine Rich regions have revealed a new domain near the C-terminus of $2C^{ATPase}$ that is involved in encapsidation possibly achieved through interacting with a spacer between A and B motifs of the NTP-binding domain of $2C^{ATPase}$. Most importantly, suppressor mutations were identified not only in PV nonstructural protein $2C^{ATPase}$ but also in PV capsid proteins VP3 and VP1 - the first demonstration of genetic suppression of a $2C^{ATPase}$ defect by capsid proteins in the background of PV genome. The data presented here reinforce our previous conclusion that an interaction between $2C^{ATPase}$ and the capsid proteins is required for viral encapsidation. In addition, I performed a yeast two hybrid screening of a HeLa cell cDNA library and identified several cellular factors that may interact with PV non-structural protein $2C^{ATPase}$. The roles of these candidates in PV replication and/or assembly of PV viral particles, if any, need further investigations.

Dedication

I dedicate this thesis to my dear family for their unconditional love and support.

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Chapter I. Introduction

I.1 History of poliomyelitis and global polio eradication

Poliovirus (PV) is a member of the large *Picornaviridae* family of small, non-enveloped, (+) stranded RNA viruses (**Figure 1.**). Picornaviruses are the most common viruses infecting humans. The virus family contains thirteen genera, of which the best-known: *Enterovirus* (including Rhinovirus). Enteroviruses gain entry into the host primarily via an oral-fecal contact. The annual rate of enteroviral infections has been estimated to be about six billion worldwide. Besides PV, rhinoviruses, enterovirus 71, many other members of this family that afflict humans include coxsackie B viruses, parechoviruses, and hepatitis A virus, which can cause diseases from mild (common cold) to severe, even life threatening, such as meningitis, encephalitis, myocarditis, paralysis, diarrhetic and respiratory diseases.

PV is the prototype of the *Enterovirus* genus of this family. PV, which consists of three serotypes (Burnet 1931; The-Committee-on-Typing-of-the-National-Foundation-for-Infantile-Paralysis 1951), establishes an infection by replicating in the gastrointestinal tract (GI) for up to 60 days post infection, usually producing no symptoms or very mild symptoms, which are usually unnoticed. The most severe form of disease commonly associated with PV, poliomyelitis, is the result of the invasion of the Central Nervous System (CNS) and subsequent destruction of motor neurons by the virus, resulting in flaccid paralysis typically in the limbs (Landsteiner and Popper 1909). Initial symptoms include fever, fatigue, headache, vomiting, stiffness in the neck, and pain in the limbs (http://www.polioeradication.org). Among those paralyzed, 5% to 10% die when their breathing muscles become immobilized (http://www.who.int/mediacentre/factsheets/fs114/en/). The invasion of the CNS is a rare secondary site infection and it occurs only at a rate of 1 per 100 infections for PV serotype 1 and 1 per 1000 infections for PV serotypes 2 and 3 to cause neurological disease. However, in the

latter half of the 19th century, due to the vast number of infections observed, poliomyelitis was a common occurrence. Later on two excellent PV vaccines were developed: the inactivated PV vaccine (IPV) by Jonas Salk (Salk 1954) and a live attenuated oral PV vaccine (OPV) by Albert Sabin (Sabin 1957). These two vaccines have been successfully used worldwide since 1960s.

In 1988, the World Health Assembly launched the Global Polio Eradication Initiative, which aimed to use large-scale vaccination with OPV to eradicate PV worldwide by the year 2000. The campaign has led to a drastic reduction of poliomyelitis and since then, 2.5 billion children have been immunized against polio (http://www.polioeradication.org). However, the problems intrinsic to OPV are challenging the goal and postponed the date of global eradication of PV to the future. Unexpected outbreaks still occur due to the emergence of neurovirulent, circulating vaccine-derived polioviruses (cVDPVs), which are believed to be generated by homologous recombination between PV and the closely related Coxsackieviruses (CAVs) (Arita, Zhu et al. 2005; Jiang, Faase et al. 2007). The other problem is due to a vaccine-induced PV carrier state, which can occur in selected individuals in the population or in vaccine recipients with primary immune deficiencies (Halsey, Pinto et al. 2004). These long-term persistent infections could lead to the evolution of highly neurovirulent PV strains, so-called immunodeficiency-related vaccine-derived polioviruses (iVDPVs) that may be disseminated to an immunologically naive population in the post eradication era, thereby inducing widespread outbreaks of poliomyelitis (Mueller, Wimmer et al. 2005).

I.2 Genome organization of poliovirus

The PV genome is a single (+) sense RNA molecule of about 7441 nucleotides (nt). As shown in **Figure 2.**, the viral genome can be divided into three regions: the 5' non-translated region (5'

NTR) of 742 nt, followed by a single open reading frame (Fuller, von Bonsdorff et al.) coding for a polyprotein of 2209 amino acids (aa), and finally the polyadenylated 3' non-translated region (3' NTR) of 70 nt (Kitamura, Semler et al. 1981).

The 5' NTR is composed of two structural elements: a cloverleaf (Rivera, Welsh et al. 1988; Andino, Rieckhof et al. 1990) and the downstream internal ribosomal entry site (IRES) (Jang, Krausslich et al. 1988; Pelletier and Sonenberg 1988). The cloverleaf has been implicated in the formation of the replication complex both during (+) strand and (-) strand RNA synthesis and in the switch from translation to replication (Andino, Rieckhof et al. 1990; Andino, Rieckhof et al. 1993; Parsley, Towner et al. 1997; Gamarnik and Andino 2000; Herold and Andino 2000; Perera, Daijogo et al. 2007) . Cap-independent translation of the PV genome is initiated by binding of the virally modified host translation machinery to the IRES (Pelletier and Sonenberg 1988). The PV genomic RNA does not carry a m7G cap structure, which is essential for translational initiation of eukaryotic mRNAs; but instead VPg (3B), a virus-encoded 22aa protein, is covalently linked to the 5' NTR by a bond between the O^4 of tyrosine 3 and the 5'-phospate of the terminal uridylic acid residue (Lee, Nomoto et al. 1977). This is important because PV inhibits host cell protein synthesis by $2A^{pro}$'s cleavage of eukaryotic translation initiation factor-4G (eIF4G), a component of the cap recognizing complex eIF4F that is utilized in cap-dependant translation (Kräusslich, Nicklin et al. 1987; Sonenberg 1987). Therefore, once poliovirus has infected a cell, only IRES-containing viral RNAs will be translated (Kräusslich, Nicklin et al. 1987; Sonenberg 1987). More recently a C-rich spacer region between the cloverleaf and the IRES was also shown to have an important function in PV proliferation (Toyoda, Franco et al. 2007).

The ORF of the PV genome encodes a 247 KDa polyprotein, subdivided into regions denoted as P1, P2 and P3 (**Figure 2.**), that is cleaved into precursor and mature proteins by two virus-encoded proteinases, $2A^{pro}$ and $3C^{pro}/3CD^{pro}$ (Leong, Selmer et al. 2002). The P1 region encodes the structural proteins, while P2 and P3 encode the nonstructural proteins. Following translation of the mRNA, the polyprotein is processed by virus-encoded proteinases: $2A^{pro}$, $3C^{pro}/3CD^{pro}$ in *cis* and in *trans*. The first cleavage of the polyprotein at a tyrosine^glycine (Y^G) dipeptide is catalyzed by $2A^{pro}$, which results in the separation of a 97 kDa P1 structural segment from the P2-P3 nonstructural domain (Toyoda, Nicklin et al. 1986). Subsequent cleavage of P1 at a glutamine^glycine (Q^G) dipeptide by $3CD^{pro}$ releases VP0, VP3 and VP1 (Ypma-Wong, Dewalt et al. 1988). Cleavage of the P2 and P3 regions is catalyzed at a Q^G dipeptide by $3C^{pro}$ and $3CD^{pro}$ (Hanecak, Semler et al. 1982). In addition, there is one maturation cleavage that yields VP4 and VP2 from VP0 by an unknown mechanism (Harber, Bradley et al. 1991). The poliovirus capsid is an icosahedron (Hogle, Chow et al. 1985) formed by 60 copies of each of the four structural proteins VP1, VP2, VP3, and VP4 (Rossmann and Johnson 1989).

The short 3' NTR contains a polyadenylated tail, which is genetically encoded (Dorsch-Hasler, Yogo et al. 1975), and two stem loops. Numerous genetic data showed that the entire 3' NTR is important for the initiation of efficient minus strand RNA synthesis (Pilipenko, Poperechny et al. 1996) but it can be deleted without loss of viral viability (Brown, Cornell et al. 2005).

I.3 The life cycle of poliovirus

PV is the prototype of the *Enterovirus* genus, which has been a great model for studies of picornavirus replication. The cellular life cycle of PV is schematically presented in **Figure 3**.

First PV binds to its cellular receptor CD155 and the virion is internalized into the cell and gets uncoated. The genome linked protein VPg is cleaved off and genomic RNA then serve as messenger RNA for translation which is initiated by the binding of cellular ribosomes to the IRES. The open reading frame of the RNA is translated 5' end-to-3' end into a single polyprotein, which is subsequently cleaved into precursors and mature viral proteins by viral proteases $2A^{pro}$ and $3C^{pro}$/ $3CD^{pro}$. Next intracellular membranes are remodeled and a membrane-associated replication complex is formed, where the viral RNA replication occurs. Newly synthesized plus stranded RNA has the choice of re-entering genome replication, serving as mRNA in translation or being encapsidated to form mature virions. In the last step, progeny viruses are released from the cell.

(1) Receptor binding and uncoating PV enters into the host cell through interaction of the viral capsids with the PV cellular receptor CD155 (also known as PVR), a Type I transmembrane glycoprotein that is a member of the immunoglobulin superfamily (Mendelsohn, Wimmer et al. 1989). CD155 inserts itself into a surface depression of the viral capsid known as the “canyon”, which surrounds each of the twelve five-fold axes of the icosahedral capsid (Bernhardt, Harber et al. 1994; Colston and Racaniello 1994; Harber, Bernhardt et al. 1995; Liao and Racaniello 1997).

(2) Translation and proteolytic processing of the polyprotein The viral particles are internalized and the viral RNA is released into the cytoplasm, the 5'-terminal VPg is cleaved off (Nomoto, Kitamura et al. 1977). Serving as mRNA, the viral RNA is translated into a single polyprotein by hijacked host cell ribosomes. The proteolytic cleavage products of the polyprotein serve as capsid precursors and replication proteins. After translation, $2A^{pro}$ carries out a *cis*-cleavage of the Y^G bond at its own N-terminus to release P1 from P2-P3. The second major

cleavage leads to the release of P3 from P2 through *cis*-cleavage by $3C^{pro}/3CD^{pro}$ (Lawson and Semler 1992). This is followed by highly ordered successive *trans*- cleavage events by $3C^{pro}/3CD^{pro}$, yielding the non-structural proteins, $2A^{pro}$, $2BC^{ATPase}$, 3AB, $3CD^{pro}$, 2B, $2C^{ATPase}$, 3A, 3B (VPg), $3C^{pro}$, $3D^{pol}$. The capsid proteins VP0, VP1 and VP3 are released from their P1 precursor by $3C^{pro}/3CD^{pro}$. This cascade of fast and slow proteolytic cleavages by viral proteinases $2A^{pro}$ and $3C^{pro}/3CD^{pro}$ allows PV a certain degree of processing control (**Figure 2.**). The last cleavage of VP0, which is required for the infectivity of the virus, occurs during the assembly of the virus particle, presumably by an autocatalytic mechanism involving the encapsidation of RNA genome, to yield VP4 and VP2 (Basavappa, Syed et al. 1994).

(3) RNA replication It has been proposed that there is an active switch from poliovirus translation to RNA replication mediated by changes in RNA-binding protein affinities. The cellular protein poly (rC)-binding protein (PCBP) which can bind to cloverleaf and IRES was identified as a primary candidate in this process (Perera, Daijogo et al. 2007). It is proposed that PCBP2 mediates the switch from viral translation to RNA replication through the loss of the KH3 domain, by cleavage with $3C^{pro}/3CD^{pro}$, and therefore loss of its ability to function in translation. The splicing factor polypyrimidine tract-binding protein (PTB) has also been suggested as a candidate (Back, Kim et al. 2002) to mediate the switch on the basis of the observation that PTB stimulates IRES-mediated translation and is also cleaved and inactivated during the late stages of a poliovirus infection. RNA replication occurs via a following simplified pathway: (+) strand virion RNA → (-) strand synthesis → RF → (+) strand synthesis → RI → (+) stand RNA, where RF means replicative form (double strand RNA), and RI means replicative intermediate { one (-) strand partially hybridized to numerous nascent (+) strands} (Wimmer, Hellen et al. 1993). RNA replication takes place on rosette-like membranous structures that are

induced by viral proteins. The newly synthesized (-) strand RNA serves as the template to generate progeny (+) strand RNAs. The newly synthesized (+) RNA can either re-enter genome replication, serve as template for protein synthesis or be packaged into new progeny virions.

(4) Encapsidation and release of mature virions Prior to exiting the cell, the capsid proteins encapsidate the newly synthesized single (+) stranded RNA genome and the VP0 precursor undergoes the maturation cleavage to form the infectious mature virions (Lee, Monroe et al. 1993). Virions are then released from the host cell. Again CD155 mediates the entry of PV into the new host cell to initiate another infectious cycle.

Among the fastest known viral growth cycles, a typical round of PV replication in a single cell lasts approximately 8 h at 37 ℃ in cell culture. It should be noted that the nucleus is not involved in the replication cycle of PV since the viral growth of PV occurs entirely in the cytoplasm of the host cell.

I.4 Viral proteins involved in poliovirus RNA replication.

The PV genome is translated into a 247 KDa polyprotein that is subdivided into regions denoted as P1, P2 and P3, as discussed above (**Figure 2.**). P1 is cleaved to generate four structural proteins, while P2 and P3 is cleaved into ten non-structural proteins including both precursor and mature proteins by two viral proteinases, $2A^{pro}$ and $3C^{pro}/3CD^{pro}$. Genetic and biochemical experiments have shown that all of the nonstructural proteins of the virus are involved at certain step of the genome replication (reviewed in (Paul 2002)). Moreover, the function of the precursors may be different from that of the cleavage products (for example $3CD^{pro} \rightarrow 3C^{pro}$ and $3D^{pol}$).

The proteins derived from the P3 domain are the ones that are most directly involved in the synthesis of viral RNA. 3AB is a small basic protein, with a membrane-binding domain in 3A, which is believed to anchor the RNA polymerase $3D^{pol}$ to membranes (Giachetti, Hwang et al. 1992; Towner, Ho et al. 1996). 3AB is thought to insert its hydrophobic domain into the membranes and recruit $3D^{pol}$ to the replication complex by means of its affinity to $3D^{pol}$ and $3CD^{pro}$ (Xiang, Cuconati et al. 1998; Fujita, Krishnakumar et al. 2007).

3AB has a variety of functions as shown *in vitro*: (1) it stimulates the activity of $3D^{pol}$ on a poly(A) template (Paul, Cao et al. 1994); (2) it is a nonspecific RNA binding protein, which in a complex with $3CD^{pro}$ binds specifically to the 5' cloverleaf and 3' NTR of PV RNA (Harris, Xiang et al. 1994; Paul, Cao et al. 1994); (3) it stimulates the auto-processing of $3CD^{pro}$ (Molla, Harris et al. 1994); (4) it has nucleic acid chaperone and helix destabilizing activity (DeStefano and Titilope 2006). $3CD^{pro}$ is a proteinase and both its proteinase and its RNA binding activities (Andino, Rieckhof et al. 1990) are located in the $3C^{pro}$ domain. This RNA binding activity is important at different stages of RNA replication.

The enzyme most directly involved in RNA synthesis, the RNA dependent RNA polymerase $3D^{pol}$, catalyzes primer dependent synthesis of both (-) and (+) strands. In addition to the elongation of the RNA/DNA primers on a suitable RNA template (Flanegan and Baltimore 1977), $3D^{pol}$ also uridylylates VPg either on a poly(A) or a *cre* (2C) template, yielding VPgpU(pU) *in vitro* (Paul, van Boom et al. 1998). PV VPg (3B) is a small peptide containing 22 amino acids of which tyrosine 3 serves as the attachment site to the 5'-end of the RNA (Rothberg, Harris et al. 1978). VPg (3B) serves as a protein primer for both (-) and (+) strand synthesis after being uridylylated on a conserved tyrosine residue to form VPg-pU-pU. For plus strand RNA synthesis, the template for the uridylylation of VPg is an internal RNA hairpin

structure termed cis-replicating element (cre) in the $2C^{ATPase}$ coding region (Goodfellow, Chaudhry et al. 2000) (**Figure 2.**). Whether the poly (A) tail or the cre (2C) serves as template for uridylylation for minus strand synthesis remains controversial (van Ooij, Vogt et al. 2006).

The proteins of the P2 domain are primarily involved in inducing the biochemical and structural changes that occur in the infected cells. $2A^{pro}$ is a proteinase whose function in RNA replication is not yet known (Molla, Paul et al. 1993; Li, Lu et al. 2001). Vesicular structures generated in picornavirus-infected cells appear to serve as compartments where synthesis of viral RNA takes place. Proteins $2BC^{ATPase}$ and its cleavage products 2B and $2C^{ATPase}$ are closely associated with replication complex-associated vesicles and it is believed that these rosette-like membranous structures are triggered by the viral precursor $2BC^{ATPase}$ and its mature polypeptide $2C^{ATPase}$ (Bienz, Egger et al. 1990; Cho, Teterina et al. 1994; Teterina, Gorbalenya et al. 1997). The membranes are derived from different cellular organelles involved in secretory pathways, primarily endoplasmic reticulum (ER) but also the Golgi complex, and possibly others as reviewed in (Tang, Yang et al. 2007). Host-encoded proteins may also play a role in the formation of these replication complex-associated vesicles.

I.5 Poliovirus encapsidation and viral proteins involved in this process

Encapsidation is the last and a crucial step of the virus's life cycle. Successful assembly not only provides newly synthesized viral genomes with a protective shell, but also assures proper uncoating which is required for attachment to and penetration into subsequent host cells to initiate the next round of viral infection. In addition, capsid proteins are one of the major determinants of tropism due to their ability to bind to specific receptors for cell entry (Racaniello 1990). As discussed above, PV encodes only one protein, the polyprotein, which is cleaved by

two virus-encoded proteinases, $2A^{pro}$ and $3C^{pro}/3CD^{pro}$, into functional precursor intermediates (e.g. $3CD^{pro}$) and into mature proteins (**Figure 2.**). First, the PV polyprotein is cleaved by $2A^{pro}$ and the P1 (structural region) is released from the polyprotein. Then with the help of cellular chaperone Hsp90 (Geller, Vignuzzi et al. 2007), P1 is further cleaved by $3CD^{pro}$ into capsid proteins VP0, VP3 and VP1 (**Figure 2.**) (Ypma-Wong, Dewalt et al. 1988), which will spontaneously form a 5S protomer (VP0, VP3, VP1). Five 5S protomer can pentamerize into the 14S pentamer $(VP0, VP3, VP1)_5$, twelve of which will oligomerize into a 75S empty capsid $[(VP0, VP3, VP1)_5]_{12}$, also called procapsid (Hellen and Wimmer 1995; Racaniello 2007). It is not known yet at what stage progeny RNA genome starts to be associated with capsid proteins and encapsidated. They may be inserted into the procapsids or, alternatively, protomer or pentamers may condense around the newly synthesized RNA genome at the replication complex. Either way, once provirions $\{[(VP0, VP3, VP1)_5]_{12}RNA\}$(Jacobson and Baltimore 1968; Nugent and Kirkegaard 1995; Pfister, Egger et al. 1995) are formed, the infectious virions will be generated after final maturation cleavage when VP0 is cleaved to VP4 and VP2, possibly by an RNA-dependent autocatalytic process (Hellen and Wimmer 1995; Racaniello 2007).

Encapsidation is highly specific to newly synthesized plus strand viral RNA genome (Molla, Paul et al. 1991; Nugent, Johnson et al. 1999). Thus elaborate mechanisms have evolved to discriminate against non-progeny nucleic acids including cellular RNAs, messenger viral RNA and negative strand viral RNA (Hellen and Wimmer 1995). It should be noted that different mechanisms have been adopted for different viruses. For some RNA viruses such as hepatitis B virus and alphaviruses (Bartenschlager, Junker-Niepmann et al. 1990; Frolova, Frolov et al. 1997), their specificity of encapsidation is dependent on an RNA encapsidation signal and RNA/protein interactions.

Although much is known about PV morphogenesis, as described above, the mechanism of viral assembly remains elusive (Semler and Wimmer 2002). Numerous studies searching for an RNA packaging signal for PV have so far been unsuccessful. As discussed by Liu et al. (Liu, Wang et al. 2010), the 5′ NTR of PV can be substituted with that of the distantly related coxsackie B3 virus (CVB3) (Johnson and Semler 1988) or CVB4 (Barclay, Li et al. 1998), and the resulting chimera proliferate with kinetics like wild type PV(Crowther and Melnick). The cloverleaf of PV can also be replaced by that of HRV2 (Xiang, Harris et al. 1995), the PV IRES has been exchanged with IRESes from other picornaviruses (Wimmer, Hellen et al. 1993; Alexander, Lu et al. 1994; Gromeier, Alexander et al. 1996) and even with that of HCV (Lu and Wimmer 1996) and generated chimeric viruses with normal encapsidation phenotypes. Additionally, the 3′ NTR of PV has been exchanged with that of HRV14, which yielded chimera proliferating with wild type kinetics too (Rohll, Moon et al. 1995). Combining all of these data, it is highly unlikely that the 5′- or 3′-NTRs of PV contain any packaging signal(s) that determines the specificity of encapsidation. The P1 capsid encoding region also cannot harbor an encapsidation signal since PV replicons, in which the entire P1 encoding sequence can be either deleted (Kajigaya, Arakawa et al. 1985) or replaced by foreign genes (Porter, Ansardi et al. 1995; Barclay, Li et al. 1998; Porter, Ansardi et al. 1998), not only can replicate but also can be efficiently encapsidated in *trans*. Recent experiments from our laboratory, employing a "scrambled" sequence (Mueller, Papamichail et al. 2006) of the P2 and P3 coding region (scramble of synonymous codons), have also eliminated these two regions from carrying an encapsidation signal (Song, Liu, Ward, Mueller, Futcher, Skiena, Paul and Wimmer, manuscripts in preparation). Finally, genetic modification of the PV VPg coding sequence (Reuer, Kuhn et al. 1990; Cao and Wimmer 1996) or engineering chimeric PVs carrying VPg sequences of other

picornaviruses (Reuer, Kuhn et al. 1990; Cheney, Naim et al. 2003; Paul, Peters et al. 2003) have also eliminated the VPg coding sequence from providing a specific encapsidation signal. VPg protein, however, may still play a role in encapsidation. Currently it seems unlikely that PV or other enteroviruses (including the rhinoviruses that have recently been reclassified as enteroviruses) harbor an RNA signal that would instruct the capsid components to bind to and enclose the viral genome in a species-specific manner. One exception is Aichi virus (*Kobuvirus* genus), a member of the extended family of *Picornaviridae,* which was reported to contain a 5′-terminal RNA stem loop with a role in particle assembly (Sasaki and Taniguchi 2003).

Available evidence suggests that genome replication is a precondition for PV encapsidation (Molla, Paul et al. 1991; Nugent, Johnson et al. 1999). Electron-microscopic studies also supported this hypothesis by showing that RNA replication complexes co-localize with capsid precursors on membranous vesicles during infection (Pfister, Pasamontes et al. 1992). Nugent et al. (Nugent, Johnson et al. 1999) hypothesized that encapsidation specificity may be determined by the spatial arrangement of replication complexes with the capsid precursors. This intriguing hypothesis lacked an essential component: what brings the capsid precursors into the vicinity of the replication complexes since PV replicons lacking the P1 domain altogether can be efficiently encapsidated in *trans*?

Among the nonstructural proteins of PV, $2C^{ATPase}$ and $3CD^{pro}$, have been reported to be involved in packaging. The involvement of $2C^{ATPase}$ in PV encapsidation has previously been shown by both drug inhibition studies and genetic experiments. Hydantoin, a drug which inhibits poliovirus morphogenesis yields resistant mutants that map to protein $2C^{ATPase}$. The resistant mutations (Vance, Moscufo et al. 1997) (Paul, Mugavero and Wimmer, unpublished data) are located in the N-terminus and central portions of the $2C^{ATPase}$ coding region. However,

there is no clear evidence showing whether these two regions are involved in encapsidation process separately or by interacting with other motifs within $2C^{ATPase}$ (**Figure 4.**). In addition, two types of genetic experiments also link $2C^{ATPase}$ to encapsidation. The first one is a temperature sensitive mutant with an insertion in $2C^{ATPase}$ (Li and Baltimore 1988). This mutant yielded suppressor mutations ($M_{293}V$, $K_{295}R$) in $2C^{ATPase}$ that proved to be cold sensitive in uncoating (Li and Baltimore 1990), an observation suggesting that $2C^{ATPase}$ has a role in determining unknown aspects of virion structure. In the second type of genetic experiment we used a chimera ($C_{20}PP$) containing the capsids of the closely related CAV20 and the nonstructural proteins of poliovirus. This chimera, which was shown to be defective only in encapsidation, yielded a suppressor mutation either in $2C^{ATPase}$ or in capsid protein VP3 of CAV20. These results suggested that the specificity of encapsidation is determined by an interaction between $2C^{ATPase}$ and the capsid VP3 (Liu, Wang et al. 2010).

In vitro translation/RNA replication studies have suggested that $3CD^{pro}$ functions at a late step in the encapsidation process just before or during the maturation cleavage of VP0 to VP2 and VP4 (Franco, Pathak et al. 2005).

I.6 Poliovirus non-structural protein $2C^{ATPase}$

One of the most conserved non-structural viral proteins common to all picornaviruses is $2C^{ATPase}$ (**Figure 4.**). A small RNA hairpin in the coding sequence of PV $2C^{ATPase}$, cis acting replication element, *Cre* (nt 4444-4504) (Goodfellow, Chaudhry et al. 2000), is required for RNA synthesis by serving as the template for the uridylylation of the terminal protein VPg (Paul, Rieder et al. 2000).

PV $2C^{ATPase}$ is a complex nonstructural protein that contains a nucleoside triphosphate-binding motif (Mirzayan and Wimmer 1992) and displays ATPase activity *in vitro* (Rodriguez and Carrasco 1993; Mirzayan and Wimmer 1994). This ATPase activity is inhibited by guanidine hydrochloride (GnHCl) (Pfister and Wimmer 1999), a well-known and potent inhibitor of PV RNA replication (Crowther and Melnick 1961). Specific mutations in $2C^{ATPase}$ have been shown to confer GnHCl resistance or dependence to PV (Pincus, Diamond et al. 1986). As shown in **Figure 4.**, three conserved motifs are located in the middle part of the protein including the A box: NTP binding; B box: magnesium binding; C-box: helicase motif with unknown function. Based on sequence analyses, protein $2C^{ATPase}$ was classified as a member of superfamily III helicases (Gorbalenya and Koonin 1993) and it forms ring-like hexamers typical of various helicases (Adams, Kandiah et al. 2009). These helicases contain a small putative helicase-domain, which has 3 conserved motifs, including the two classical ATP-binding motifs. The two sites common to other helicases is the A site (GxxxxGKS), which is involved in the binding of ATP and the B site (DD), which interacts with Mg^{++}. The third site, motif C, whose function is unknown, consists of an invariant N residue preceded by a stretch of moderately hydrophobic residues and is located downstream of motif B. However, numerous attempts to discover helicase activity associated with the $2C^{ATPase}$ protein have failed (Rodriguez and Carrasco 1993) (Kim and Wimmer, unpublished results). Downstream of motif C is residue N_{252}, which is involved in an interaction with capsid protein VP3 of coxsackie A virus 20 (CAV20), in the context of a chimeric virus (C_{20}PP) (Liu, Wang et al. 2010).

The *N*-terminal region of protein $2C^{ATPase}$, encompassing amino acids 21 to 54 is sufficient for binding the polypeptide to membranes (Echeverri, Banerjee et al. 1998). This region contains a conserved, predicted amphipathic helix between residues 10 and 32 (Paul,

Molla et al. 1994; Echeverri, Banerjee et al. 1998; Teterina, Gorbalenya et al. 2006) . Moreover, the *N*-terminal domain of $2C^{ATPase}$ possesses the ability to bind RNA (Rodriguez and Carrasco 1995). Specific binding of the protein to the 3'-terminal sequences of the minus strand RNA has also been demonstrated (Banerjee, Echeverri et al. 1997; Banerjee, Tsai et al. 2001). The protein also contains a second amphipathic helix at the C-terminus, which is also implicated in membrane binding (Teterina, Gorbalenya et al. 1997) and a cysteine-rich region, which is involved in RNA replication and binds Zinc (Pfister, Jones et al. 2000). The central and C-terminal domains of the polypeptide possess serpin (serine protease inhibitor) motifs and, indeed, the inhibition of $3C^{pro}$ protease activity *in vitro* and *in vivo* by $2C^{ATPase}$ was demonstrated (Banerjee, Weidman et al. 2004). The protein also has the ability to oligomerize (Adams, Kandiah et al. 2009) and to interact with viral proteins 2B, $2BC^{ATPase}$ (Cuconati, Xiang et al. 1998), 3A, 3AB (Yin, Liu et al. 2007), $3C^{pro}$ (Banerjee, Weidman et al. 2004) and VP3 (Liu, Wang et al. 2010).

In general, genetic studies have implicated $2C^{ATPase}$ in several different functions during the life cycle of poliovirus such as: virus uncoating (Li and Baltimore 1990); host cell membrane binding and rearrangement (Cho, Teterina et al. 1994; Aldabe and Carrasco 1995; Teterina, Gorbalenya et al. 1997; Suhy, Giddings et al. 2000); formation of the viral cytoplasmic replication vesicles, RNA binding and RNA synthesis (Kirkegaard and Baltimore 1986; Li and Baltimore 1988; Baltera and Tershak 1989; Teterina, Kean et al. 1992; Paul, Molla et al. 1994; Tolskaya, Romanova et al. 1994; Banerjee, Echeverri et al. 1997; Barton and Flanegan 1997; Banerjee, Tsai et al. 2001; Teterina, Levenson et al. 2006) and encapsidation (Vance, Moscufo et al. 1997; Verlinden, Cuconati et al. 2000; Liu, Wang et al. 2010). In infected cells, $2C^{ATPase}$

appears to be attached to viral RNA in the replication complexes on the surface of membranous vesicles (Bienz, Egger et al. 1987).

Through use of genetic and drug inhibition studies multiple functional domains have been identified in $2C^{ATPase}$, as discussed above, but the exact role(s) of the protein in the viral life cycle remains elusive. Previous mutational studies of the conserved functional domains in PV $2C^{ATPase}$ have primarily yielded lethal or poor growth phenotypes due to defects in RNA replication and/or encapsidation. So far only two sets of conditionally defective temperature-sensitive (*ts*) mutants have been identified, which are located near the C-terminus of the protein (Li and Baltimore 1988; Li and Baltimore 1990; Pfister, Jones et al. 2000). Additional *ts* PV $2C^{ATPase}$ mutants would be particularly helpful for identifying proteins/domains involved in the process of viral morphogenesis.

I.7 Alanine scanning mutagenesis of poliovirus non-structural protein $2C^{ATPase}$

Alanine scanning mutagenesis is a technique used to determine the contribution of specific amino acid residues to a protein's function by mutating the residues to alanine (small and mostly inert). The term scanning refers to the fact that mutagenesis is performed along the whole length of the protein. Since charged hydrophilic residues are usually located at the outer surface of the folded protein, they are likely to be involved in numerous protein-protein interactions. Substitution of a charged residue with a small and neutral alanine residue is likely to produce a viable mutation, since it allows the overall integrity of the protein to be preserved. However, small and uncharged side chain limits the possibility for protein interactions, thus likely altering the phenotype. Since protein folding is severely affected by heat, an essential protein/protein interaction that can no longer occur at the physiological temperature may be restored at low

temperature thereby allowing proper function of the mutant protein. Hence the mutant virus may express a temperature sensitive (ts) phenotype. A previous investigation by Diamond and Kirkegaard revealed that a "clustered charged-to-alanine mutagenesis" of poliovirus non-structural protein $3D^{pol}$ resulted in frequent temperature sensitive mutations (Diamond and Kirkegaard 1994).

As mentioned before, recently we have reported a direct interaction between $2C^{ATPase}$ and VP3, which is required for enterovirus morphogenesis. Amino acid N_{252} of PV $2C^{ATPase}$ is involved in an interaction with CAV20 VP3 protein (Liu, Wang et al. 2010). Surprisingly this N_{252} amino acid does not belong to any of the previously identified or putative domains of $2C^{ATPase}$. In order to further characterize the individual functions of other previously uncharacterized amino acids of $2C^{ATPase}$ and to enhance our understanding of the role $2C^{ATPase}$ in PV life cycle especially in the encapsidation process, it would be very useful to have additional ts mutant.

To achieve the above goal, by using alanine scanning mutagenesis, I performed a systematic genetic analysis of poliovirus non-structural protein $2C^{ATPase}$. Sixteen mutants of $2C^{ATPase}$ were generated that contain a minimum of 2 adjacent or closely positioned charged to alanine changes. In addition, another four conserved cysteine rich zinc binding sites (CRs) in PV $2C^{ATPase}$ were also subjected to alanine scanning mutagenesis. The *in vitro* translation and polyprotein processing of these $2C^{ATPase}$ mutants were analyzed in HeLa cell free extracts. The viability of the mutants was tested at different temperatures (33 ℃, 37 ℃ and 39.5 ℃) in HeLa cells and the growth phenotypes of the resulting viruses, if any, were analyzed by plaque assays (Chapter II). Furthermore, I also searched for and identified several revertants and particularly second site suppressor mutations in different proteins of the PV. The suppressor mutations were

found not only in the $2C^{ATPase}$ and the VP3 coding regions but also in the VP1 coding region. These variants are particularly useful in the identification of proteins that might interact with $2C^{ATPase}$ during PV RNA replication or encapsidation (Chapter III).

I.8 Yeast two-hybrid analyses of PV non-structural protein $2C^{ATPase}$ interaction with cellular protein(s)

It is generally believed that specific cellular factors, termed host factors, interact with the genomic RNA of picornaviruses and possibly with some viral non-structural proteins. Individual host factors may be involved in different processes of the viral life cycle, such as internal initiation of translation, proteolytic processing of the viral polyprotein, and shutdown of host cell protein synthesis, RNA replication, and encapsidation of the viral genome. A well-established example is the binding of polypyrimidine tract binding protein (PTB) to the IRES, which is implicated in internal initiation of cap-independent translation of several picornaviruses including poliovirus (Jang and Wimmer 1990; Hellen, Witherell et al. 1993; Hellen, Pestova et al. 1994). Another well-known example of a cellular protein involved in RNA synthesis is PCBP2 (poly (rC) binding protein 2) (Blyn, Chen et al. 1995), which forms complexes with the 5' NTR of the PV genome (Andino, Rieckhof et al. 1990; Andino, Rieckhof et al. 1993; Parsley, Towner et al. 1997) by binding to the 5'-terminal cloverleaf (Gamarnik and Andino 2000) and an adjacent C-rich spacer region (Parsley, Towner et al. 1997; Gamarnik and Andino 2000; Toyoda, Franco et al. 2007) in a complex with $3CD^{pro}$. This interaction is required for RNA replication (Andino, Rieckhof et al. 1990). PCBP2 can also bind to stem loop IV of the IRES and regulates the initiation of translation (Gamarnik and Andino 2000). Perera et al. proposed that PCBP2 mediates the switch from viral translation to RNA replication through the loss of the KH3 domain which is cleaved by $3C^{pro}/3CD^{pro}$, thus loss of its ability to function in translation (Perera,

Daijogo et al. 2007). Although the mechanism remains obscure, several other proteins have been proposed to be involved in PV proliferation such as La autoantigen (Meerovitch, Svitkin et al. 1993), unr (upstream of N-ras) (Hunt, Hsuan et al. 1999) and PABP (poly (A) binding protein), which interacts with the poly(A) tail (Herold and Andino 2001; Svitkin, Imataka et al. 2001) and might be involved in the IRES function of picornaviruses; Sam68 interacts with $3D^{pol}$ (McBride, Schlegel et al. 1996); hnRNPC binds to $3CD^{pro}$ and the minus strand cloverleaf (Brunner, Nguyen et al. 2005); nucleolin interacts with the 3' NTR (Waggoner and Sarnow 1998); reticulon 3 (RTN3) interacts with enterovirus 71 $2C^{ATPase}$ N-terminal domain (Tang, Yang et al. 2007).

The pioneering study by Fields and Song was the first demonstration of the applicability of yeast two-hybrid techniques to determine the possible protein/protein interactions by measuring transcription of a reporter gene (Fields and Song 1989). The Gal4 protein is a yeast transcription factor that normally controls genes responsible for galactose metabolism. Each Gal4-responsive gene contains a target site called an Upstream Activating Sequence, or UAS. When Gal4 binds the UAS, transcription is activated from a downstream promoter. By linking the GAL UAS with other metabolic genes (e.g., ADE2, HIS3, MEL1, and lacZ) and by eliminating the wild-type GAL4 gene, researchers have developed yeast strains that change their phenotypes when Gal4 is activated. A simple diagram of the Y2H principle can be seen in **Figure 5**.

To determine whether protein-protein interactions occur among the polypeptide cleavage products of the P2 and P3 regions of the PV polyprotein, previous lab members have carried out yeast two-hybrid analyses, including a protein linkage map of the P2 nonstructural proteins (Cuconati, Xiang et al. 1998), the P3 proteins (Xiang, Cuconati et al. 1998) and between P2 and P3 regions (Yin, Liu et al. 2007). In the P2 region two classes of interactions were observed,

which may be relevant to the functions of the P2 cleavage products during RNA replication. In the P3 region sixteen signals of homo- or hetero-dimer interactions have been observed. Most interesting of these interactions is the strong affinity of $3D^{pol}$ and VPg. Mutants of 3AB have been screened to map determinants of 3AB/$3D^{pol}$ interaction and 3AB/3AB interactions (Xiang, Cuconati et al. 1998). Between the P2 and P3 proteins (Yin, Liu et al. 2007), significant interaction between $2C^{ATPase}$ and 3AB; $2A^{pro}$ and 3A, $3C^{pro}$ or $3D^{pol}$; 2B and 3A or 3AB were observed. Results of these protein linkage maps of PV nonstructural protein are summarized in **Table 1**.

de Jong et al. investigated multimerization reactions of the 2B, $2C^{ATPase}$ and $2BC^{ATPase}$ proteins of the closely related coxsackie B3 virus (CBV3) using a mammalian two-hybrid (M2H) system (de Jong, Schrama et al. 2002). In their study homo-multimerization reactions were observed with the 2BC protein and weakly with the 2B protein but not with the $2C^{ATPase}$ protein. Hetero-multimerization reactions were observed between proteins $2BC^{ATPase}$ and 2B, and also between proteins $2BC^{ATPase}$ and $2C^{ATPase}$, but not between the 2B and $2C^{ATPase}$ proteins. To corroborate the interactions revealed by their genetic data, Teterina et al. also employed a M2H system to evaluate the binding between PV proteins containing 2B, $2C^{ATPase}$, and 3A sequences (Teterina, Levenson et al. 2006). Positive bindings were observed between the Bind-$2C^{ATPase}$ fusion protein with Act-$2BC^{ATPase}$ fusion protein and with Act-3A fusion protein; Bind-$2BC^{ATPase}$ fusion protein with Act-$2BC^{ATPase}$ fusion protein (homo-oligomerization of the $2BC^{ATPase}$ protein) and Act-3A fusion protein; Bind-3A fusion protein with Act-3A fusion protein; Bind-3AB fusion protein with Act-3A fusion protein (Bind: binding domain; Act: activation domain).

In yeast two-hybrid screening, separate bait and prey plasmids are simultaneously introduced into the mutant yeast strain usually by co-transformation. Recent advances in

biotechnology has introduced a new method that is yeast mating between a mating type a (MATa) and a mating type α (MATα) strains. This eliminates the need for library-scale yeast co-transformations (which can vary 10- to 100-fold in their efficiency) and makes the process much easier and more reproducible. Clontech Matchmaker Pretransformed Libraries are screened by yeast mating instead of co-transformation. Matchmaker™ Pretransformed Libraries are high-complexity cDNA libraries cloned into a GAL4 AD vector and transformed into yeast strain Y187 (MATα). To screen this pretransformed library, bait strain AH109 (MATa), which is transformed with a DNA-BD vector containing our bait construct, is combined and incubated for 20-24 hours before plating the mixture on synthetic drop-out (Fuller, von Bonsdorff et al.) medium. SD medium, which lacks one or more essential nutrients, selects for diploid clones expressing interacting proteins. If a positive interaction occurs, the diploid Y187-AH109 strain will express dual nutritional reporters (His and Ade) and both α- and β-galactosidase. Positive interactions can be quickly and easily identified using X-α-Gal in the selection plates (-His-Ade) causing positive yeast colonies to turn blue, or with a standard β-galactosidase colony filter-lift assay.

Recently an Y2H study conducted by Tang et.al (Tang, Yang et al. 2007) has demonstrated that reticulon 3 protein binds the 2C protein of Enterovirus 71 and is required for viral replication. In their study the N terminal region of 2C (5-43aa and 5-124aa, respectively) was fused with the GAL4-BD and used as the bait to screen a human fetal brain cDNA library.

Since PV invades and replicates well in human HeLa cells, genes that encode proteins important for replication should be represented in this HeLa cDNA library. Hypothetically, if any interaction between $2C^{ATPase}$ and a cellular partner is required, I should be able to detect it. To achieve this, I performed a yeast two hybrid screening of a HeLa cell cDNA library and

identified several cellular factors that may interact with PV non-structural protein $2C^{ATPase}$ (Chapter IV). The roles of these candidates in PV replication and/or assembly of PV viral particles, if any, need further investigations.

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FIGURES/TABLES

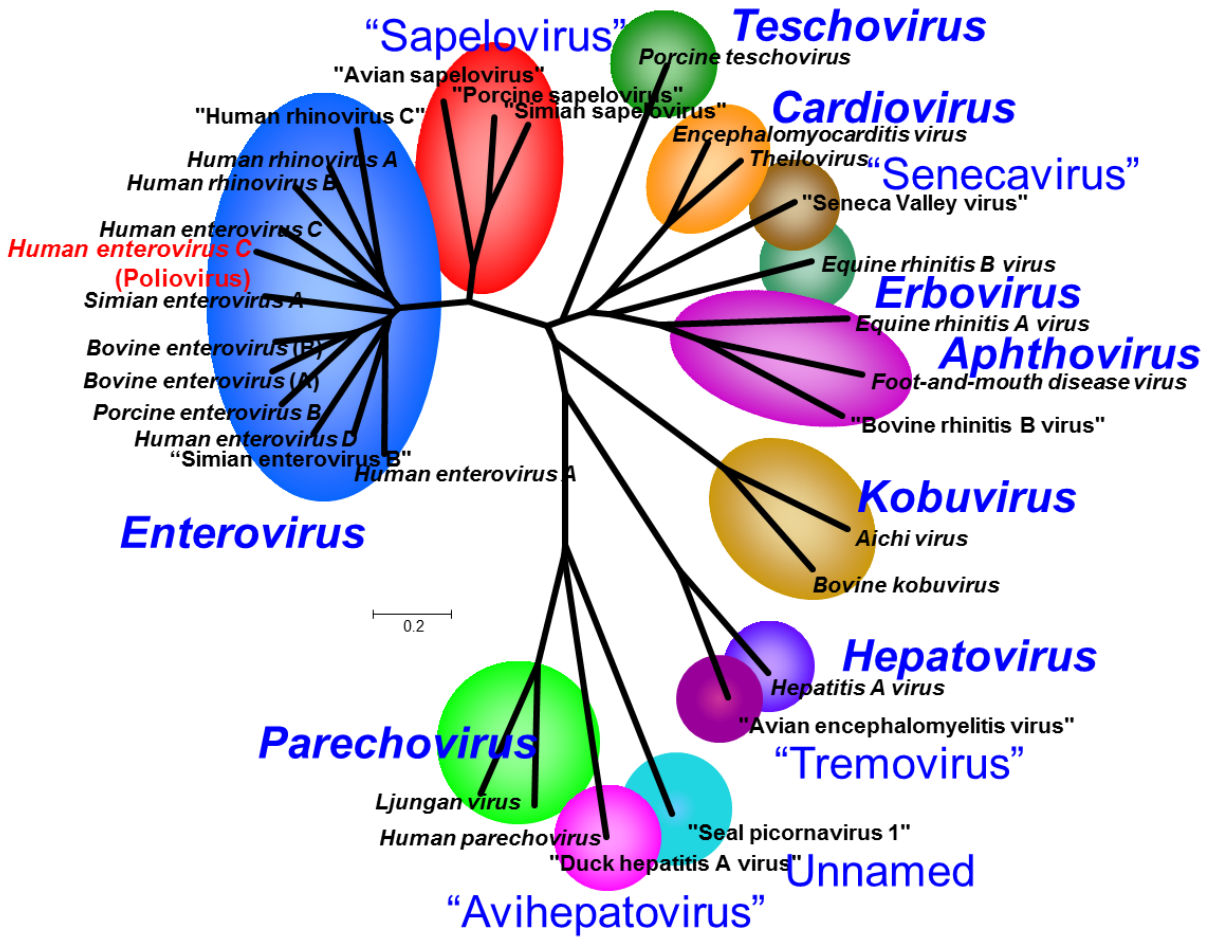

Figure1. Phylogenetic tree of *Picornaviridae*. *Picornaviridae* is a large family of small, non-enveloped (+) single stranded RNA viruses. The virus family contains thirteen genera including 8 current genera (shown in bold), 4 current proposals (shown in quotation marks) and one new unnamed genus proposals in preparation. Poliovirus (PV) belongs to the *Enterovirus* genus. Modified from www.picornastudygroup.com/posters/**europic_2008**.pdf

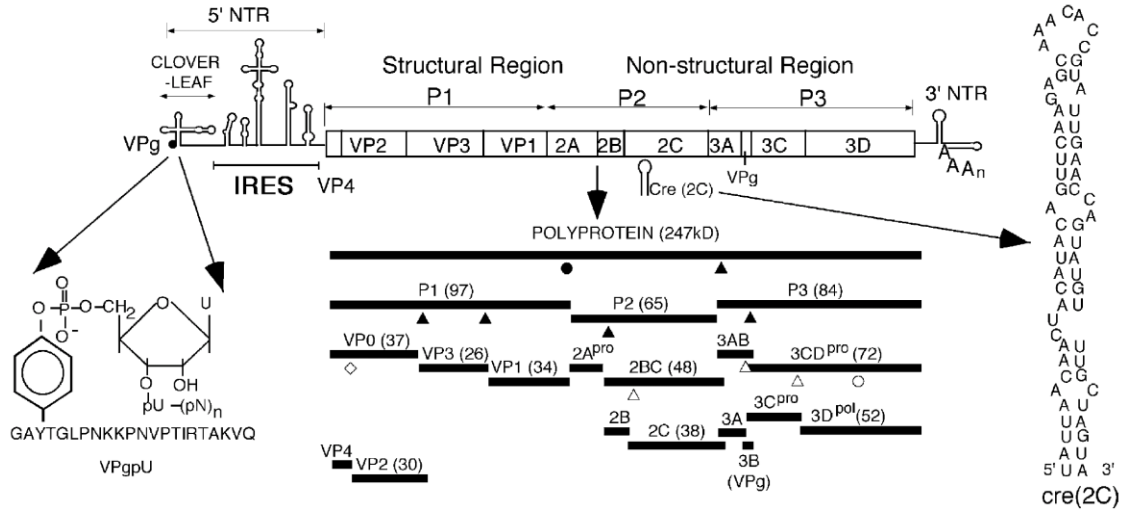

Figure 2. Genomic structure of PV RNA and proteolytic processing of the PV polyprotein. A terminal protein VPg (structure and sequence are indicated on the left) is linked covalently to the 5' NTR. The 5' NTR consists of several stem loops that form the 5' cloverleaf followed by the IRES element. The single open reading frame is translated into a polyprotein of 247 kDa that is processed by the virally encoded proteinases $2A^{pro}$ (circle) and $3C^{pro}/3CD^{pro}$ (triangles), where filled shapes indicate fast cleavages and open shapes indicate slow cleavages. The VP0 maturation cleavage, which occurs by an unknown mechanism, is marked by a diamond. The structure and sequence of the *cre* (2C) element, located in the coding sequence of protein $2C^{ATPase}$, is given on the right.

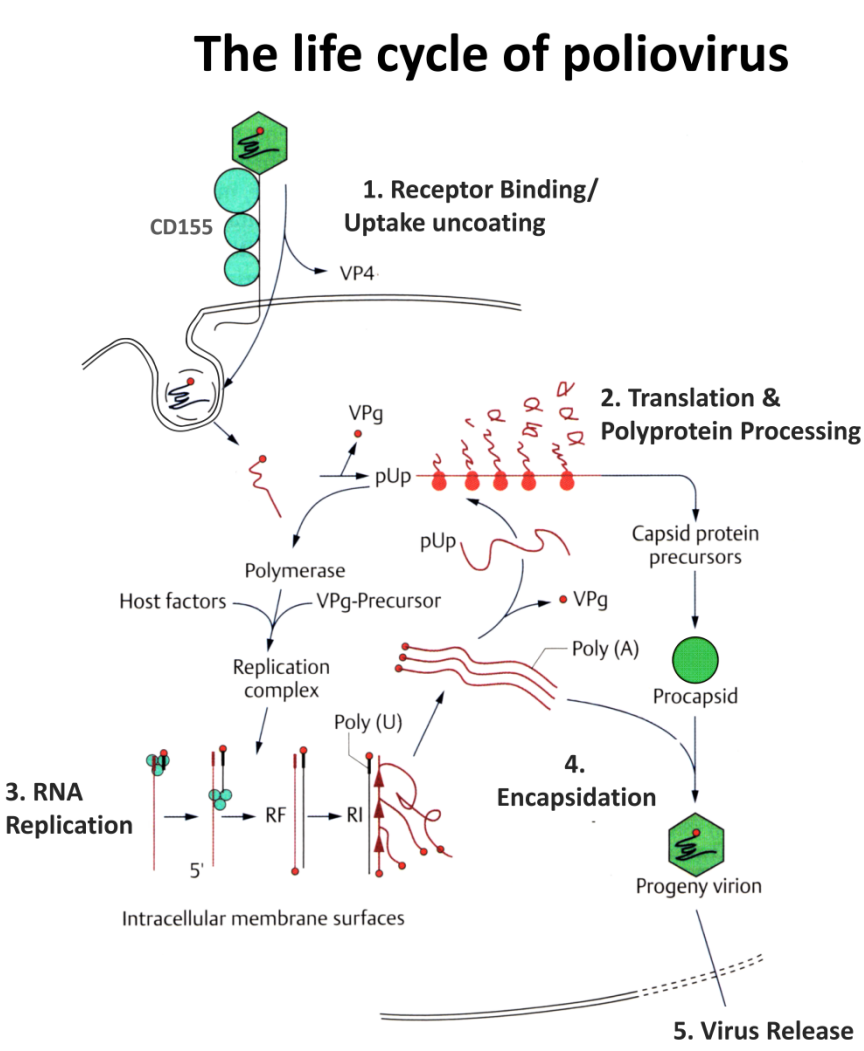

Figure 3. An overview of the PV life cycle. After binding to the receptor CD155 (only found on primate cells), the virion is internalized into the cell and uncoated. The genome linked protein VPg is cleaved off, after which translation is initiated by the binding of cellular ribosomes to the IRES. (2) The open reading frame (Fuller, von Bonsdorff et al.) of the RNA is translated end-to-end into a polyprotein, which is subsequently processed into precursors and mature proteins by viral proteases $2A^{pro}$ and $3C^{pro}$/ $3CD^{pro}$. (3) Next a membrane-associated replication complex is formed by viral and cellular proteins, where the plus stranded genome RNA is transcribed into a minus strand with the formation of a double stranded "replicative form" (RF). Minus strands then function as templates for the synthesis of plus strands. Newly synthesized plus stranded RNA has the choice of (a) re-entering genome replication, (b)serving as mRNA in translation or (c) associating with procapsids (4) to form mature virions that are released from the cell (5).

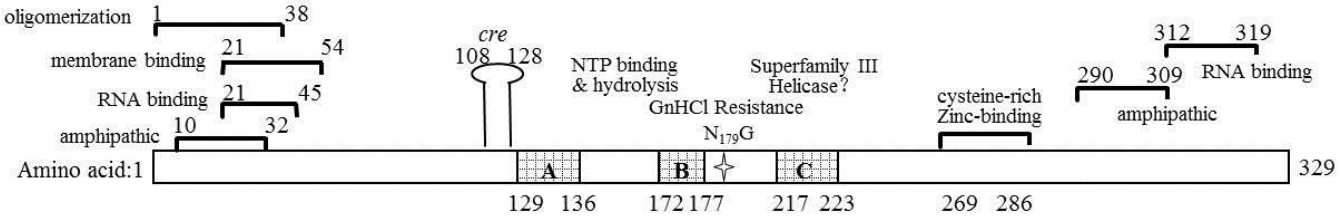

Figure 4. Putative domains of poliovirus nonstructural protein 2C^{ATPase}

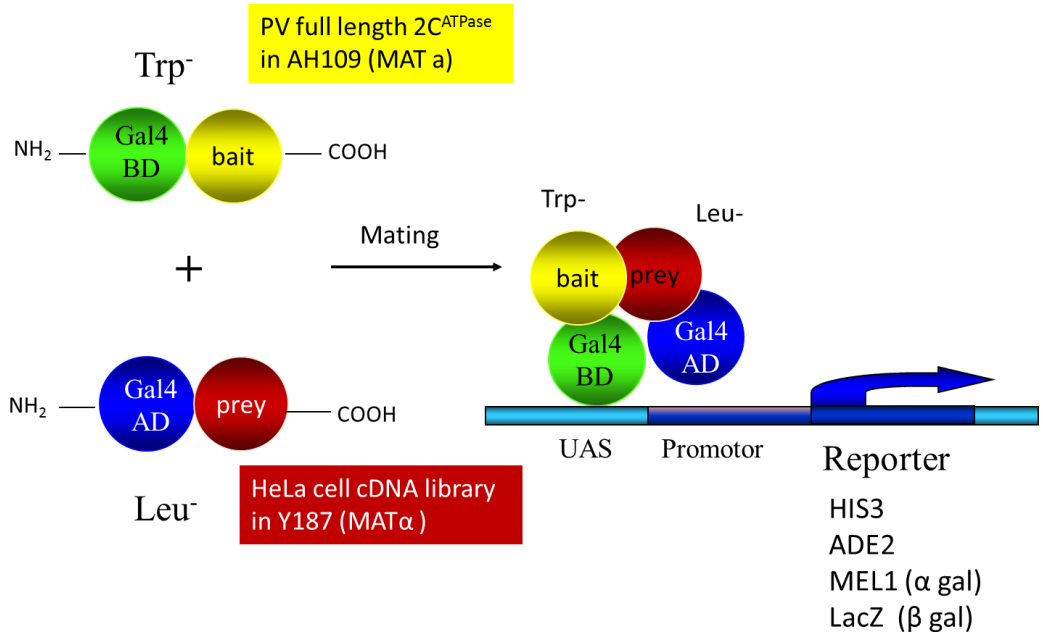

Figure 5. The principle of yeast two hybrid assay. In a two-hybrid assay, when the Gal4 DNA-binding domain (BD) and activation domain (AD), each expressed as fusions, are joined through a protein-protein interaction, Gal4 is activated, reporter genes are expressed, and yeast strains change phenotypes, as shown. Although the DNA-BD can bind the UAS, it cannot activate transcription by itself. Transcription is activated only when the other half of the protein, the Gal4 AD, joins the DNA-BD at the UAS. In the Matchmaker systems, the AD consists of amino acids 768–881 of the Gal4 protein; the DNA-BD, amino acids 1–147. In my experiment, the bait protein is BD-2C^{ATPase} and the prey is the AD fused HeLa cell cDNA library.

Table 1. A summary of protein linkage maps of the cleavage products of the P2 and P3 PV nonstructural proteins.

P2 (Cuconati et al.,1998)			P2&P3 (Yin et al.,2007)			P3 (Xiang et al.,1998)		
2A	↔	2A				3AB	↔	3AB
2B	↔	2B				3A	↔	3AB
2C	*↔	2C	2C	↔	3AB	3A	↔	3A
2BC	↔	2BC	2B	↔	3AB	3CD	↔	3CD
2C	→	2BC	2A	↔	3D	3D	↔	3CD
	→		2A	↔	3C	3D	↔	3D
2B	↔	2BC	2A	↔	3A	3CD	→	3AB
	→		2B	→	3A	3D	→	3AB
2C	→	2B	2C	→	3A	3B	→	3D
2B in 2BC	↻	2C in 2BC	2A	→	3CD	3B	→	3CD

Double arrows indicate positive reciprocal interactions (bait-prey/prey-bait), whereas single arrows denote unidirectional (bait-prey) interactions.

* Detected only *in vitro*

Chapter II. Alanine Scanning Mutagenesis of Poliovirus Nonstructural Protein $2C^{ATPase}$

INTRODUCTION

Poliovirus (PV) protein $2C^{ATPase}$ is a highly conserved nonstructural protein common to picornaviruses. Through the use of genetic and drug inhibition studies multiple functional domains have been identified in $2C^{ATPase}$ but the exact role(s) of the protein in the viral life cycle remains elusive. Previous mutational studies of the conserved functional domains in PV $2C^{ATPase}$ have primarily yielded lethal or poor growth phenotypes due to defects in RNA replication and/or encapsidation. So far only two conditionally defective temperature-sensitive (*ts*) mutants have been identified, which are located near the C-terminus of the protein (Li and Baltimore 1988; Li and Baltimore 1990; Pfister, Jones et al. 2000). Additional *ts* PV $2C^{ATPase}$ mutants would be particularly useful for identifying proteins/domains involved in the process of viral morphogenesis.

$2C^{ATPase}$ is a complex nonstructural protein, which contains a nucleoside triphosphate-binding motif (Mirzayan and Wimmer 1992) and displays ATPase activity *in vitro* (Rodriguez and Carrasco 1993; Mirzayan and Wimmer 1994). This ATPase activity is inhibited by guanidine hydrochloride (GnHCl) (Pfister and Wimmer 1999), a well-known and potent inhibitor of PV RNA replication (Crowther and Melnick 1961). Specific mutations in $2C^{ATPase}$ have been shown to confer GnHCl resistance and dependence (Pincus, Diamond et al. 1986). In infected cells this 329-amino acid protein complexes with other proteins to form viral RNA replication complexes on the surface of remodeled cytoplasmic vesicles (Bienz, Egger et al. 1987). Genetic studies have implicated $2C^{ATPase}$ in a number of different functions in viral growth, including virus uncoating (Li and Baltimore 1990), host cell membrane rearrangements (Cho, Teterina et al. 1994; Aldabe and Carrasco 1995; Teterina, Gorbalenya et al. 1997; Suhy, Giddings et al. 2000), RNA binding and replication (Li and Baltimore 1988; Paul, Molla et al. 1994; Banerjee,

Echeverri et al. 1997; Barton and Flanegan 1997; Banerjee, Tsai et al. 2001; Teterina, Levenson et al. 2006), and encapsidation (Vance, Moscufo et al. 1997; Verlinden, Cuconati et al. 2000; Liu, Wang et al. 2010).

The N-terminus of $2C^{ATPase}$, harboring an amphipathic helix (Paul, Molla et al. 1994), contains oligomerization (Adams, Kandiah et al. 2009) and RNA binding domains (Rodriguez and Carrasco 1995), and anchors the protein to membranes (Echeverri, Banerjee et al. 1998) (**Figure 1.B**). Near its C-terminus, $2C^{ATPase}$ contains a second amphipathic helix, also implicated in membrane binding (Teterina, Gorbalenya et al. 1997), and a cysteine rich region, which binds $Zinc^{++}$ (Pfister, Jones et al. 2000). The central and C-terminal domains of the polypeptide possess serpin (serine protease inhibitor) motifs and, indeed, $2C^{ATPase}$ can inhibit $3C^{pro}$ proteinase activity *in vitro* and *in vivo* (Banerjee, Weidman et al. 2004). The protein also has the ability to interact with viral proteins 2B, $2BC^{ATPase}$ (Cuconati, Xiang et al. 1998), 3A, 3AB (Yin, Liu et al. 2007), $3C^{pro}$ (Banerjee, Weidman et al. 2004) and VP3 (Liu, Wang et al. 2010).

Based on sequence analyses, protein $2C^{ATPase}$ was classified as a member of superfamily III helicases (Gorbalenya and Koonin 1993). Downstream of motif C is residue N_{252}, which is involved in an interaction with capsid protein VP3 of coxsackie A virus 20 (CAV20), in the context of a chimeric virus (C_{20}PP) (Liu, Wang et al. 2010). A small RNA hairpin in the coding sequence of $2C^{ATPase}$, cis acting replication element, *Cre* (nt 4444-4504) (Goodfellow, Chaudhry et al. 2000), is required for RNA synthesis as the template for the uridylylation of the terminal protein VPg (Paul, Rieder et al. 2000).

Many mutants of $2C^{ATPase}$ have been studied in the past but so far only two *ts* mutants have been identified. One of these displays cold-sensitivity, as described above, and is involved in uncoating (Li and Baltimore 1990). The other leads to a *ts* defect in RNA replication (Pfister,

Jones et al. 2000). The best-known and most commonly used method for the generation of *ts* mutants is clustered charged to alanine mutagenesis, which was used successfully to produce *ts* mutants in RNA polymerase $3D^{pol}$ of poliovirus (Diamond and Kirkegaard 1994). This method was originally designed to study the role of surface charges in the functions of proteins, as summarized by Diamond and Kirkegaard (Diamond and Kirkegaard 1994). It has been suggested that the production of *ts* phenotypes by this method is related to the fact that charged residues (particularly clusters) in proteins frequently reside on solvent exposed surfaces. Disruption of these residues is believed to interfere with electrostatic or hydrogen bonding interactions between molecules or with the solvent.

Since $2C^{ATPase}$ has been implicated in several processes of the PV life cycle, as mentioned above, alanine scanning of its charged residue clusters should be an effective approach to dissect its individual functions. I decided, therefore, to employ a technique similar to that used by Diamond and Kirkegaard (Diamond and Kirkegaard 1994) and conducted charged to alanine mutagenesis in PV $2C^{ATPase}$. In this chapter I have used the clustered charged to alanine mutagenesis method and generated sixteen mutants of $2C^{ATPase}$ that contain a minimum of two adjacent or closely positioned charged to alanine changes. The viability of the mutants was tested at different temperatures (33 ℃, 37 ℃ and 39.5 ℃) and the growth phenotypes of the resulting viruses, if any, were analyzed at the three temperatures. I have identified ten lethal, one *ts* and two quasi-infectious (*q.i.*) mutants. Only three mutants possessed wild type-like growth phenotypes.

RESULTS

Since *in vivo* PV RNA replication is tightly linked to translation, and encapsidation is tightly linked to RNA replication (Wimmer and Paul 2010), it is difficult to design experiments that clearly distinguish between defects in replication and encapsidation. We define encapsidation as the formation of mature virus particles capable of initiating an infectious cycle when added to fresh HeLa cells. Since poliovirus does not have an active mechanism of egress from HeLa cells, we always assay encapsidated virus by freeze-thawing of the host cells after infection, e.g. total virus will be harvested from inside and outside of the cells (note: Full CPE will eventually lead to a destruction of the host cells). It should be noted that all enteroviruses follow a strict rule: active translation is necessary in *cis* for RNA replication, active RNA replication is necessary in *cis* for encapsidation. Thus no RNA synthesis will result in no encapsidation of newly synthesized genomes. However, efficient translation does not guarantee efficient RNA replication, and efficient RNA replication does not guarantee efficient encapsidation.

In this study I subjected the PV $2C^{ATPase}$ protein to clustered charged to alanine mutagenesis, a method that has previously been shown to preferentially yield *ts* phenotypes in the poliovirus RNA polymerase $3D^{pol}$ (Diamond and Kirkegaard 1994). Such mutants might exhibit significant differences in the temperature-sensitivity of the protein's various functions, and facilitate studies of encapsidation separate from replication.

II.1 Construction of sixteen clustered charged to alanine mutants of PV $2C^{ATPase}$

Aim at discovering novel functional domains in the $2C^{ATPase}$ protein that are involved in encapsidation, I designed sixteen clustered charged to alanine scanning mutants spreading over the entire length of the polypeptide. I targeted clusters (2 to 4) of adjacent or nearby charged amino acids for substitution with alanine residues. The sixteen alanine scanning mutants of

$2C^{ATPase}$ and the corresponding amino acid and nucleotide changes are summarized in **Table 1**. The locations of the sixteen mutations on $2C^{ATPase}$ protein are indicated in **Figure 1.A**. Mutants were first constructed in a T vector and were subsequently subcloned into the pT7-PVM vector. Wt and mutant RNA transcripts derived from pT7-PVM were used for the following *in vitro* translations (section II.2) and transfections (section II.3) (Materials and Methods).

II.2 All sixteen clustered charged to alanine $2C^{ATPase}$ mutants exhibited normal translation and processing of the PV polyprotein

To examine the possibility that the mutants exhibit defects in translation or polyprotein processing, I translated the wt and all of the sixteen mutant RNA transcripts *in vitro* in HeLa cell free extracts (Molla, Paul et al. 1991). After 6 hrs, 8 hrs and 16 hrs incubation at 34 ℃, samples of the reactions were analyzed by SDS-polyacrylamide gel electrophoresis. All of the mutants exhibited normal translation and polyprotein processing profiles at 8 hrs incubation, as shown in **Figure 2.,** at 6 hrs and 16hrs incubation (data not shown). Some of the $2C^{ATPase}$-related polypeptides (P2, $2BC^{ATPase}$, and $2C^{ATPase}$) had aberrant migration patterns (**Table 2.**; **Figure 2.**), presumably due to changes in the charge of the protein resulting from substitutions of charged residues with alanine. I did not observe a correlation between the migration patterns and the growth phenotypes of the mutants. Thus I conclude that the sixteen mutations of $2C^{ATPase}$ do not have any effect on PV translation and polyprotein processing.

II.3 Growth phenotypes of the sixteen clustered charged to alanine $2C^{ATPase}$ mutants

To examine the growth properties of the mutants, wt and mutant RNA transcripts were transfected into HeLa R19 cells and incubated at 33 ℃, 37 ℃ and 39.5 ℃ for up to 72 hrs or until

full CPE (cytopathic effect) developed. Full CPE was defined as the point where 90-95% of the cells displayed CPE. Cell lysates of mutants producing no CPE on transfection were subjected up to 4 serial blind passages at the same temperatures. The time required for full CPE to develop was recorded (**Table 2.**) and the virus titers and plaque phenotypes of each viable mutant were determined at all 3 temperatures as necessary. Based on their growth phenotypes, the sixteen mutants were divided into four groups: group I, wild type-like (three mutants); group II, temperature sensitive, *ts* (one mutant); group III, lethal (nine mutants); and group IV, quasi-infectious, *q.i*. (two mutants) (**Figure 1.B**).

Group I mutants possessed wt-like growth phenotypes as evidenced by the appearance of full CPE after transfection (33 ℃, 37 ℃ and 39.5 ℃) (**Table 2.**), and wt-like virus titers and plaque sizes (**Figure 3.**). They were not further characterized. However, mutant #2 was slightly *ts* at 39.5 ℃ with 1-2 logs lower virus production compared to the wt or the other two mutants in this group.

Group II included the only mutant that was severely temperature sensitive. Transfection of HeLa cells with RNA transcripts of mutant #7 at 33 ℃ resulted in full CPE after 48 hrs (**Table 2.**), and it produced virus titers that were similar to that of the wt virus. The plaque size phenotype, however, was greatly reduced even at 33 ℃ (**Figure 4.**). Viral progeny of #7 isolated at 33 ℃ was *ts* at 37 ℃ and 39.5 ℃ (**Figure 4.**). No CPE was detected for mutant #7 after transfection at either 37 ℃ or 39.5 ℃. Full CPE may appear at 37 ℃ upon first passage, while full CPE was never observed at 39.5 ℃ even after four blind passages (**Table 2.**).

Group III mutants (ten constructs) exhibited lethal growth phenotypes at all three temperatures: 33 ℃, 37 ℃ and 39.5 ℃. No progeny viruses could be isolated, even after four blind serial passages (**Table 2.**).

Group IV mutants were quasi-infectious, which produced progeny viruses only after the appearance of suppressor mutations. Mutant #1 produced full CPE on HeLa R19 cells only after the 2nd passage (*P2*) at 33 ℃ (**Figure 5.**) or after the first passage (*P1*) at 37 ℃ and 39.5 ℃ (**Table 2.**). Two different kinds of suppressor mutations of #1 were identified, as will be discussed in Chapter III. Interestingly, some of the suppressor mutants of #11 were *ts* (**Figure 5.**). As expected for a *q.i.* phenotype, the suppressor mutants of #11 evolved only after the first passage and, although they had a *ts* phenotype (**Figure 5.**), exhibited full CPE at 33 ℃, 37 ℃ and sometimes even at 39.5 ℃ (**Table 2**). The genotypes and phenotypes of the suppressor variants of mutant #11 and their growth properties will also be described in a separate section (see Chapter III).

II.4 Two interesting observations using the HeLa S10 cell free *in vitro* translation system

During the process of making the sixteen mutants, there was one clone of mutant #11, mutant #11', with one base pair missing at *Hpa*I site (nt 5240, in 3A encoding region) which was used to make the clone. This messed up the ORF of P3 coding region and generated stop codon at nt 5289. The *in vitro* translation of this mutant #11' exhibited a defective translation and polyprotein processing pattern, with a normal P1 translated and cleaved from P2, while a longer P2+P3' (partial out-of-frame P3) (**Figure 6.A**). Very interestingly, this *in vitro* translation of RNA transcripts of this wrong #11' clone yielded useful information: the cleavage of between P1 and P2 is carried out as long as the 2A is translated, independent of the translation and proteolytic processing of the rest of the PV nonstructural polyprotein. This confirmed that cleavage by $2A^{pro}$ occurs co-translationally as summarized by Kräusslich et al. (Krausslich, Nicklin et al. 1988).

In addition, one cellular protein band is always observed in the mock (where no viral RNA is added) of the *in vitro* translation system (**Figure 6.B)**. So far I do not have an explanation for this weird band. Since the HeLa cell free extract was pretreated with S7 micrococcal nuclease during preparation of the extract, theoretically there should be no cellular mRNA left unless some cellular mRNAs are protected from S7 nuclease treatment by some unknown mechanism. More surprisingly, this band is also present in the wt PV *in vitro* translation products although sometimes weaker; suggesting that the corresponding encoding mRNA, if any, retained the ability of being translated during PV mediated inhibition of cap-dependent translation. In the future it will be interesting to determine the identity of this protein using mass spec and investigate whether this cellular protein contributes to the quality of HeLa cell free extracts concerning PV virus yields.

DISCUSSION

In order to identify novel functional domains of PV nonstructural protein $2C^{ATPase}$, I have carried out clustered charged to alanine scanning mutagenesis of residues primarily located in heretofore uncharacterized regions of the polypeptide. I was particularly interested in generating a conditional lethal mutant (e.g. a *ts* mutant) that would facilitate the dissection of individual steps in morphogenesis. Surprisingly, of the sixteen charged to alanine mutants only one (6%) exhibited a severely *ts* growth phenotype, a yield of conditional lethal mutants significantly lower than that observed in similar studies with PV RNA polymerase (35%) (Diamond and Kirkegaard 1994). This result is likely related to the multifunctional nature of the PV $2C^{ATPase}$ polypeptide and also because I used a stricter standard for definition of *ts* phenotype. If I use a less strict standard for *ts* phenotype, mutant #2 is also *ts*, which will give a yield of *ts* phenotype

12%. Most of the mutants were nonviable (63%) or *q.i.* (13%), indicating that charged residues in the protein are very important for its normal function(s).

In vitro translation in HeLa cell free extracts (Molla, Paul et al. 1991) indicated that none of the sixteen alanine mutations interfere with PV polyprotein synthesis or proteolytic processing (**Figure 2.**). Meanwhile, I have confirmed that *in vitro* translation system is a very useful tool to double check if the clone is correctly constructed. As mentioned in the results, I had one clone of mutant #11' with a missing base pair which I did not notice initially. The *in vitro* translation gave a very weird pattern (**Figure 6.A**), thus I double checked the clone and found that there is one base pair missing which presumably occurred during HpaI digestion and ligation. From here I learned a lesson for making constructs especially when blunt end enzymes were used such as *Sna*BI and *Hpa*I: 1. first digest the clone using the same restriction enzymes used to make the construct before 2. Send the clone to sequencing to confirm the mutation was successfully introduced. Meanwhile, very interestingly *in vitro* translation of this wrong clone also yielded useful information: it confirmed that the cleavage of the P1 and P2 is carried out as long as the 2A is translated, independent of the translation and proteolytic processing of the rest of PV polyprotein.

In addition, one cellular protein band is always present in the mock lane of the *in vitro* translation system where no viral RNA is added (**Figure 6.B)**. This band was observed before also (Paul and Wimmer, unpublished data) but so far we do not have an explanation for this weird band since the HeLa cell free extract was pretreated with S7 micrococcal nuclease during preparation of the extract, theoretically there should be no messenger RNA present to serve as template for translation. This band is also present in the wt PV *in vitro* translation as well; suggesting that the corresponding encoding mRNA, if any, retained the ability of being

translated during PV mediated inhibition of cap-dependent translation. Such example of cellular cap-independent translation does exist for example the eIF4GI IRES was activated by 2A protease cleavage of eIF4GI, *in vitro*, and its expression can continue under stress or infection conditions where eIF4GI is cleaved (Byrd, Zamora et al. 2005). In the future it will be interesting to determine the identity of this protein using mass-spec and investigate whether this cellular band has any contribution to the quality of the HeLa S10 extracts.

Most importantly, the current study has unveiled several new sites in PV $2C^{ATPase}$ essential for viral survival. Since RNA replication is a prerequisite for encapsidation, thus we cannot rule out the possibility that these sites may also be involved in encapsidation. Of the ten lethal mutations only one is located within a domain of known function (membrane binding) (**Figure 1.**) while the other lethal mutations are located upstream of motif A , between motifs B and C or downstream of motif C of the NTP binding domain (Mirzayan and Wimmer 1992). The remaining three lethal mutations are located downstream of the putative Zn^{++} binding domain near the C-terminus of the polypeptide. Surprisingly, two of the three mutations resulting in wt-like growth phenotypes are located near the N-terminus of the protein where several functional domains overlap (**Figure 1.**). In fact, mutant #2 exhibited mild *ts* phenotype at 39.5℃ with 1-2 logs lower virus production compared to the wt or the other two Group I mutants (**Figure 3.**), though this was not investigated further. The third wt-like mutant ($R_{256}A/D_{257}A$) was the most robust mutant with growth phenotype identical with wt PV (Fig.S1). #10 was mutated downstream of motif C (**Figure 1.**) near N_{252}, the site in $2C^{ATPase}$ known to interact with VP3 in the $C_{20}PP$ chimeric virus (Liu, Wang et al. 2010). Interestingly, the N_{252} residue itself can be mutated to G, S, or A without affecting PV morphogenesis (Ma, Asare et.al, unpublished data). These data suggest that a region downstream of motif C in PV $2C^{ATPase}$ near

R_{252}, R_{256}, R_{257} are tolerant to certain genetic manipulations. The region downstream of box C (S_{255}) has previously been shown to tolerate a linker insertion (Li and Baltimore 1988).

The suppressor mutants of quasi-infectious #1 and #11 evolved only after passaging (**Table 2.**). The genotypes and phenotypes of the suppressor variants and their growth properties will be described in a separate section (see Chapter III). To further characterize these defective mutants, I investigated their RNA replication and encapsidation phenotypes using a Renilla luciferase reporter virus (R-PPP) which will also be discussed in Chapter III.

MATERIALS AND METHODS

Cells. HeLa R19 cells were maintained in DMEM (Life Technology) supplemented with 10% bovine calf serum (BCS), 100 units of penicillin, and 100 mg/ml of streptomycin. Transfection or passages of HeLa cells were carried out with 2% BCS.

Plasmids. pT7PVM contains a full-length infectious cDNA of PV1(M). The pGEM®-T vector was obtained from Promega. pR-PPP is an infectious Renilla luciferase reporter virus construct in which Renilla luciferase (311 aa) is expressed as an N-terminal fusion to the PV polyprotein (Liu, Wang et al. 2010). pFPP is a reporter replicon in which the P1 capsid was replaced by a Firefly luciferase (550 aa) coding sequence.

T vector based site-directed mutagenesis. Site-directed mutagenesis was used to obtain the desired mutations. In each mutation, clustered positively charged Lysine (K) and Arginine(R), or negatively charged Aspartic acid (D) and Glutamic acid (E) residues (2-4) in the PV $2C^{ATPase}$ protein were replaced with neutral alanine by changing the corresponding codons. First, a wt $2C^{ATPase}$ nucleotide sequence flanking the *Sna*BI and *Hpa*I restriction sites in pT7PVM was amplified by PCR and ligated into the pGEM®-T vector, which was used as the template for site-directed mutagenesis in the $2C^{ATPase}$ coding sequence. Then sixteen pairs of oligonucleotide primers were designed containing the alanine scanning mutations and introduced into the pGEM®-T vector that contained the wt $2C^{ATPase}$ sequence, using the Stratagene QuikChange site-directed mutagenesis kit according to the instruction manual. The mutated sites and the corresponding codon changes are summarized in **Table 1**. After sequencing analysis, the designed $2C^{ATPase}$ mutations were then subcloned from the pGEM®-T vector into pT7PVM (Chapter II) or the Renilla Luciferase reporter virus (R-PPP) or F-Luc replicon (Chapter III) using restriction site *Sna*BI / *Xho*I (mutants #1-6) or *Xho*I / *Hpa*I (mutants #7-16).

Preparation of RNA transcripts Wt and mutant plasmids of pT7PVM were linearized at a unique *Eco*RI restriction site and used as templates for *in vitro* RNA synthesis using T7 RNA polymerase.

Protocol for *in vitro* translation using the HeLa cell free extracts HeLa cell S10 cytoplasmic extracts were prepared and *in vitro* RNA translations were performed with these cytoplasmic extracts at 34 ℃, as previously described (Molla, Paul et al. 1991) with slight modification.

Nuclease treatment:

1. 1.5 μl of 100 mM $CaCl_2$/200 μl of extract in 10% glycerol.

   Immediately add 1.5 μl of nuclease (1U/ μl)/200 μl of extract as titrated for each batch of nuclease.

2. Vortex gently, and incubate at RT for 15min.

3. Add 6 μl of 100 mM EGTA/200 μl of extract, vortex gently.

Master Mix:

50 μl cytoplasmic Hela extract

17 μl translation mix

9.17 μl 10×salt mix

8.3 μl ^{35}S-Methionine

1 μl RNase inhibitor (Promega, 40U/μl)

6.15 μl RNase free dH_2O

Total 91.62 μl, sufficient for 10 reactions×12.5 μl

Translation reaction: 12.5 μl

8.8 μl master mix

2.7 µl RNase free dH_2O or other additions

1.0 µl PV-RNA resuspended in RNase free dH_2O

34 ℃, O/N

Transfection of RNA transcripts into HeLa R19 cell RNA transcripts (3-10 µg) were transfected into 35-mm-diameter HeLa R19 cell monolayers by the DEAE-Dextran method as described previously (van der Werf, Bradley et al. 1986) with slight modification as shown below and incubated at 33 ℃, 37 ℃ and 39.5 ℃. Three days post transfection or at the time of full cytopathic effect (CPE), viruses, if any, were harvested. Full CPE was defined as the point where 90-95% of the cells displayed CPE. Lysates of cells transfected with mutants lacking CPE were inoculated into fresh 35-mm-diameter HeLa R19 cell monolayers for up to four subsequent serial passages. To assess the virus titers (PFU/ml) and plaque phenotypes of the viable alanine scanning mutants, any samples displaying full CPE at 33 ℃ were then subjected to plaque assays at 33 ℃, 37 ℃ and 39.5 ℃.

Summary of Alanine scanning experiments flow chart:

For mutant in pT7PVM vector:

1. Make sure the clone is correct:
    a. First do midi-prep, make sure to have the same batch of plasmid for all the following experiments.
    b. Digest the clone with the same Restriction Enzyme(s) that were used to make the clone, to make sure that the ligation site is correct thus the open reading frame is correct.
    c. Sequence the clone; make sure the mutation you introduced is indeed there and that the cloned fragment was the correct sequence.

2. Make fresh wt and mutant PVM RNA transcripts (do the 2hr linearization the first day, and make fresh RNA transcripts the 2nd day morning, and purify RNA transcripts and do the *in vitro* translation and transfection in the same afternoon using the fresh RNA transcripts).
    a. *In vitro* translation, to see the effect of mutation on the polyprotein translation and polyprotein processing.
    b. Use the same batch of RNA transcripts, do transfection into HeLaR19 at three temperatures: 33℃, 37℃ and 39.5℃. Record CPE phenotype at 24hrs, 48hrs and 72hrs post transfection.
    c. For those that did not show any CPE at any temperatures, blind passage the transfection samples four times (Passage1-Passage4).
    d. If ts or quasi-infectious (full CPE appears only after further passages), plaque purify at both low and high temperatures (33℃ and 39.5℃). Full length sequencing using different primers to cover the full length as will be described in detail in Chapter III.

Transfection of RNA transcripts to HeLa cells Using DEAE Dextran

Basically: 35mm plates contain approx. 1×10^6 cells

200-500 ng (or 2 μg-5 μg) of RNA per plate

1. Prepare Dextran and RNA Mix
    a. 200 ng RNA transcripts (or 10-20 μl transcription Rxn)
    b. Mix with 10×HeBSS and RNase free water to make 1× in 100 μl (or 150 μl) total, take 100μl for example:

RNA transcripts	10 μl/20 μl
10*HeBSS	10 μl

DEPC H2O 80 µl/70 µl

Total 100 µl

c. Add 100 µl (or 150 µl) of EDAE Dextran(1 mg/ml in 1×HeBSS)

2. Incubate on ice for 30 min before transfection
3. Wash cells 2 to 3 times with DMEM-serum or PBS (2 ml/well)
4. Add Dextran RNA mix from step1 to cells, make sure to spread the mixture over the surface, incubate on a rocking table slowly at RT for 30min
5. Aspirate off DEAE mix (toxic to cells)
6. Add 1.5 ml/well DMEM+2% calf serum (bovine calf)
7. Observe CPE (WT PV:24-48 hpi at 37℃/39.5℃; 48-72 hpi at 33℃)

10×HeBSS, pH 7.05 (50 ml recipe)

HEPES	2.5 g
NaCl	4 g
KCl	0.18 g
$Na_2HPO_4.2H_2O$	0.062 5g

Plaque assays. Plaque assays were performed on HeLa R19 monolayers using 0.6% tragacanth gum. After 72 hrs incubation at 33℃, 48 hrs at 37℃ or 39.5℃ the viral plaques were developed by 1% crystal violet staining.

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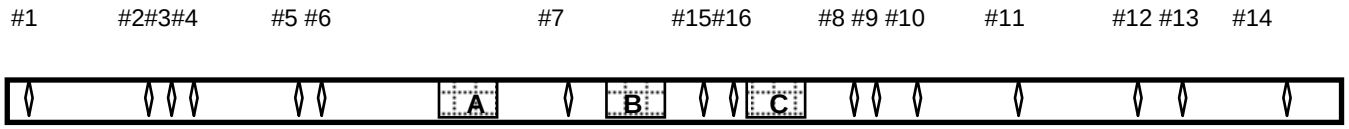
FIGURES/TABLES

A.



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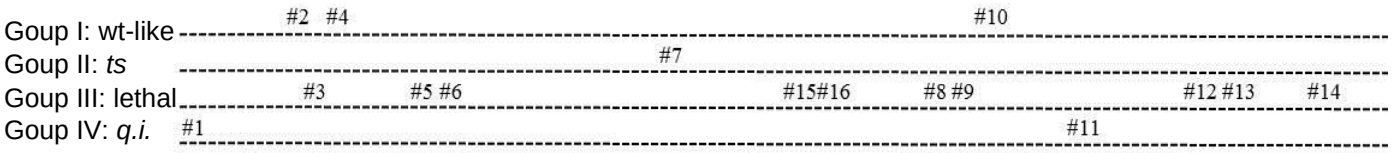

Figure 1. Locations and grouping of the sixteen alanine scanning mutants of PV nonstructural protein 2C^{ATPase}. (A) The locations of the sixteen charged to alanine mutations of 2C^{ATPase}. **(B)** Based on their growth phenotypes the sixteen mutants were divided into four groups: I, wild type-like (three); II, temperature sensitive, *ts* (one); III, lethal (nine); and IV, quasi-infectious, *q.i*. (two).

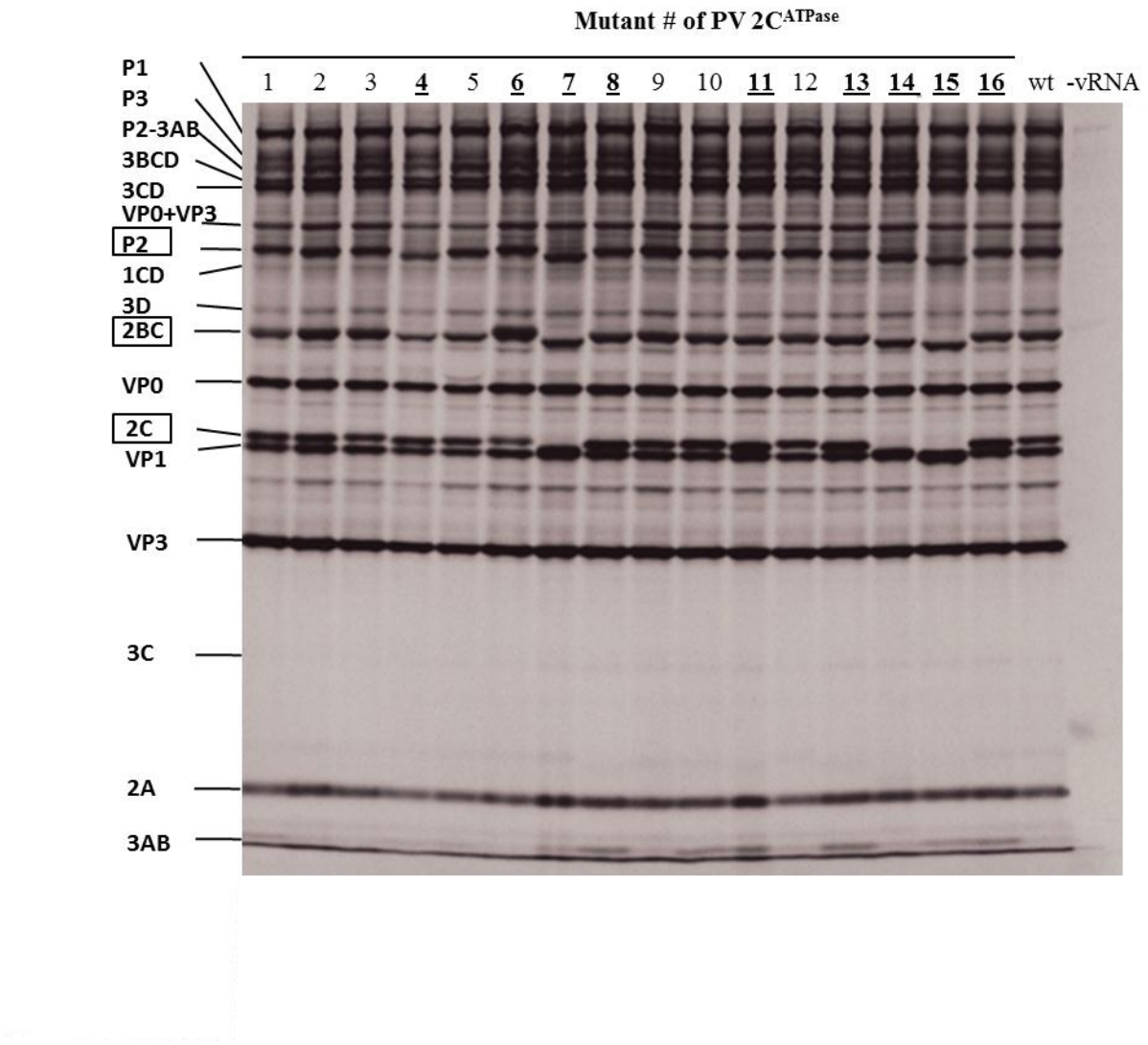

Figure 2. *In vitro* translation of wt and sixteen charged to alanine mutant RNA transcripts. RNA transcripts (200 ng) derived from wt and sixteen alanine scanning mutant constructs were translated *in vitro* in HeLa cell-free extracts at 34 ℃ for 8 hrs, as described in Materials and Methods. Positions of the precursor and mature proteins are indicated and the $2C^{ATPase}$-related proteins (P2, 2BC and $2C^{ATPase}$) are boxed. Those mutants that exhibit a shift in the migration of their $2C^{ATPase}$-related proteins are shown in bold and underlined.

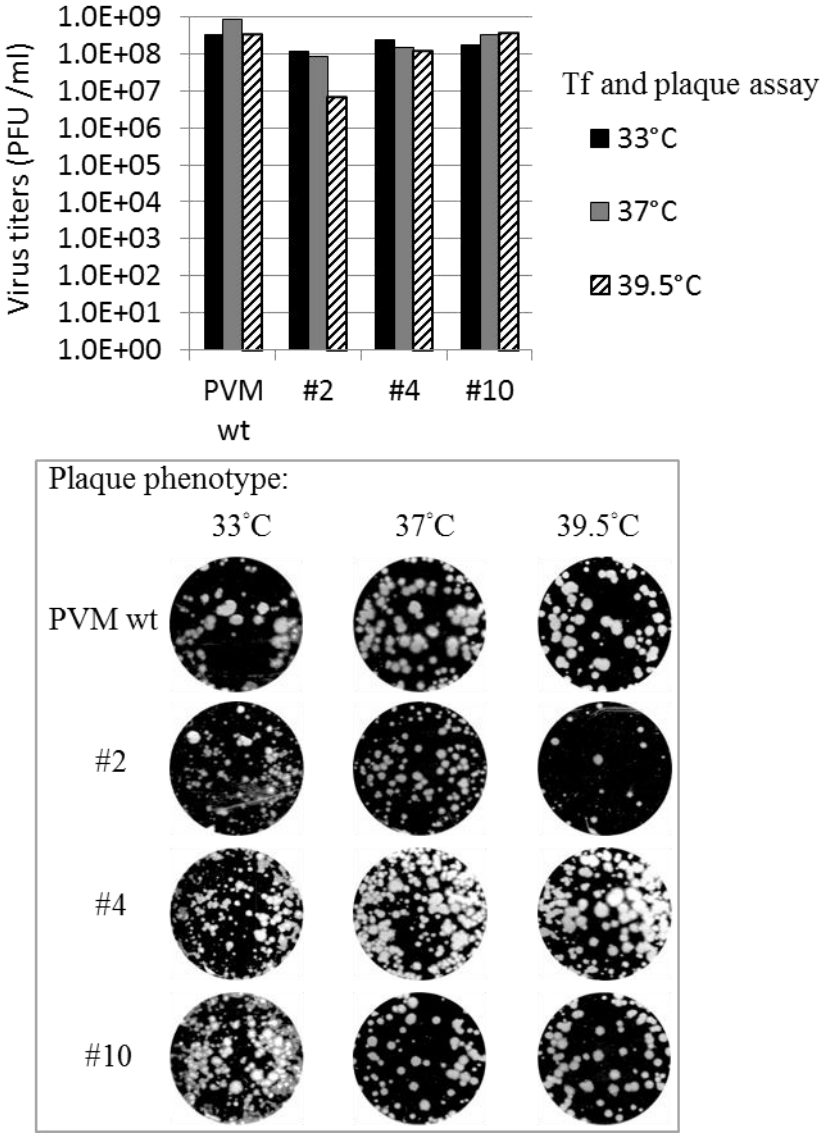

Figure 3. Three 2C^{ATPase} mutants exhibited wt-like growth phenotypes. RNA transcripts of PVM wt and mutants #2 ($K_{33}A/E_{34}A/K_{35}A$), #4 ($K_{49}A/R_{51}A$) and #10 ($R_{256}A/D_{257}A$) were transfected into HeLa cells at 33 ℃, 37 ℃ and 39.5 ℃. After transfection full CPE was observed at all three temperatures and the lysates were collected. Virus titers in lysates were determined by plaque assays at the same temperatures. Virus titers and plaque sizes are shown on the top and bottom panels, respectively.

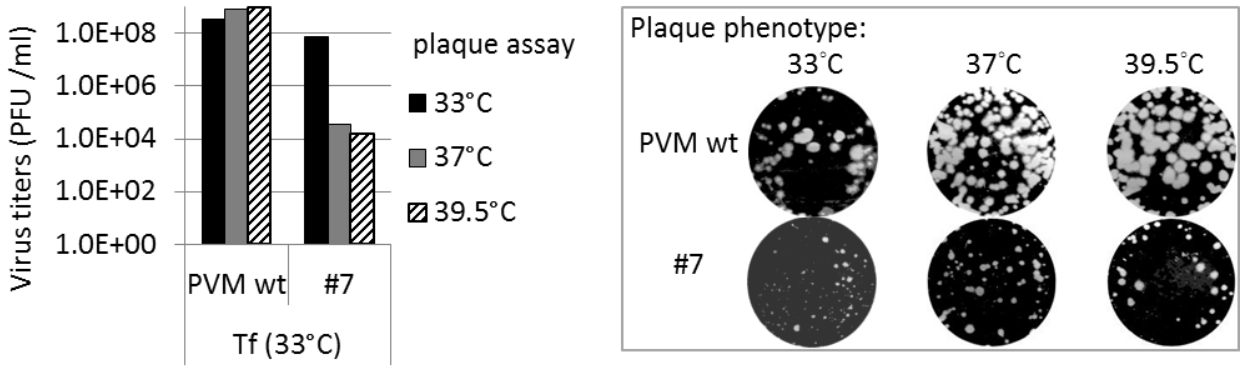

Figure 4. Growth phenotype of mutant #7 ($E_{148}A/R_{149}A/E_{150}A$)**: severely temperature sensitive.** Lysates of wt and mutant #7 ($E_{148}A/R_{149}A/E_{150}A$) from the 33 ℃ transfections (Tf) were titered at 33 ℃, 37 ℃ and 39.5 ℃ by plaque assay. Virus titers are shown on the left and plaque sizes on the right of the figure. For mutant #7, plaque pictures are taken from different dilutions factors: 10^{-5}, 10^{-2} and 10^{-2}, at 33 ℃, 37 ℃ and 39.5 ℃ respectively.

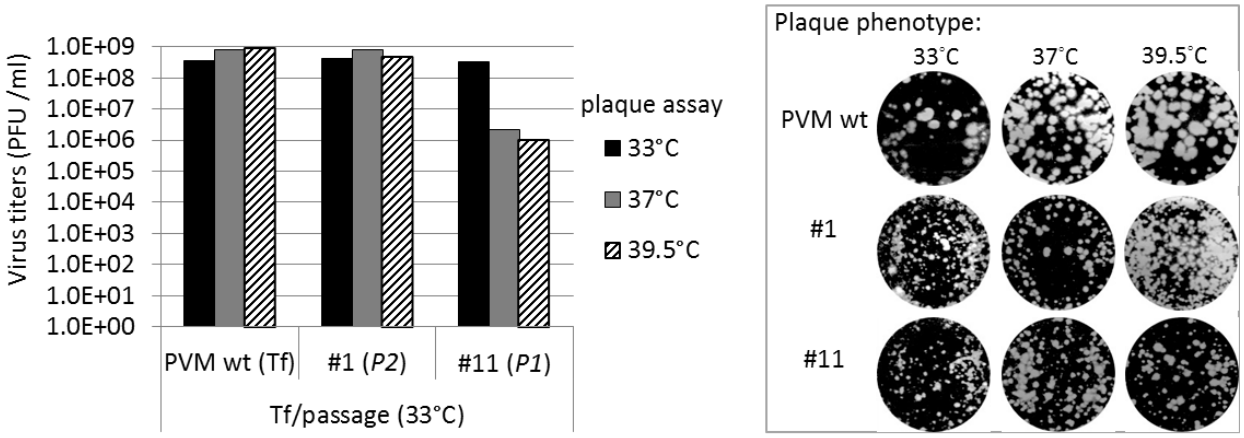

Figure 5. Growth phenotypes of two quasi-infectious mutants #1 (K_6A/K_7A) and #11 ($K_{279}A/R_{280}A$). At the time of full CPE (*P2* with mutant #11; *P1* with mutant #1) the transfection lysates at 33 ℃ were collected and the virus titers were determined by plaque assay at 33 ℃, 37 ℃ and 39.5 ℃. Virus titers are illustrated on the left panel and plaque sizes are shown on the right side of the figure. For mutants, plaque pictures are taken from different dilutions factors: mutant #1, 10^{-5}, 10^{-6} and 10^{-5}, at 33 ℃, 37 ℃ and 39.5 ℃ respectively; mutant #11, 10^{-5}, 10^{-3} and 10^{-3}, at 33 ℃, 37 ℃ and 39.5 ℃ respectively.

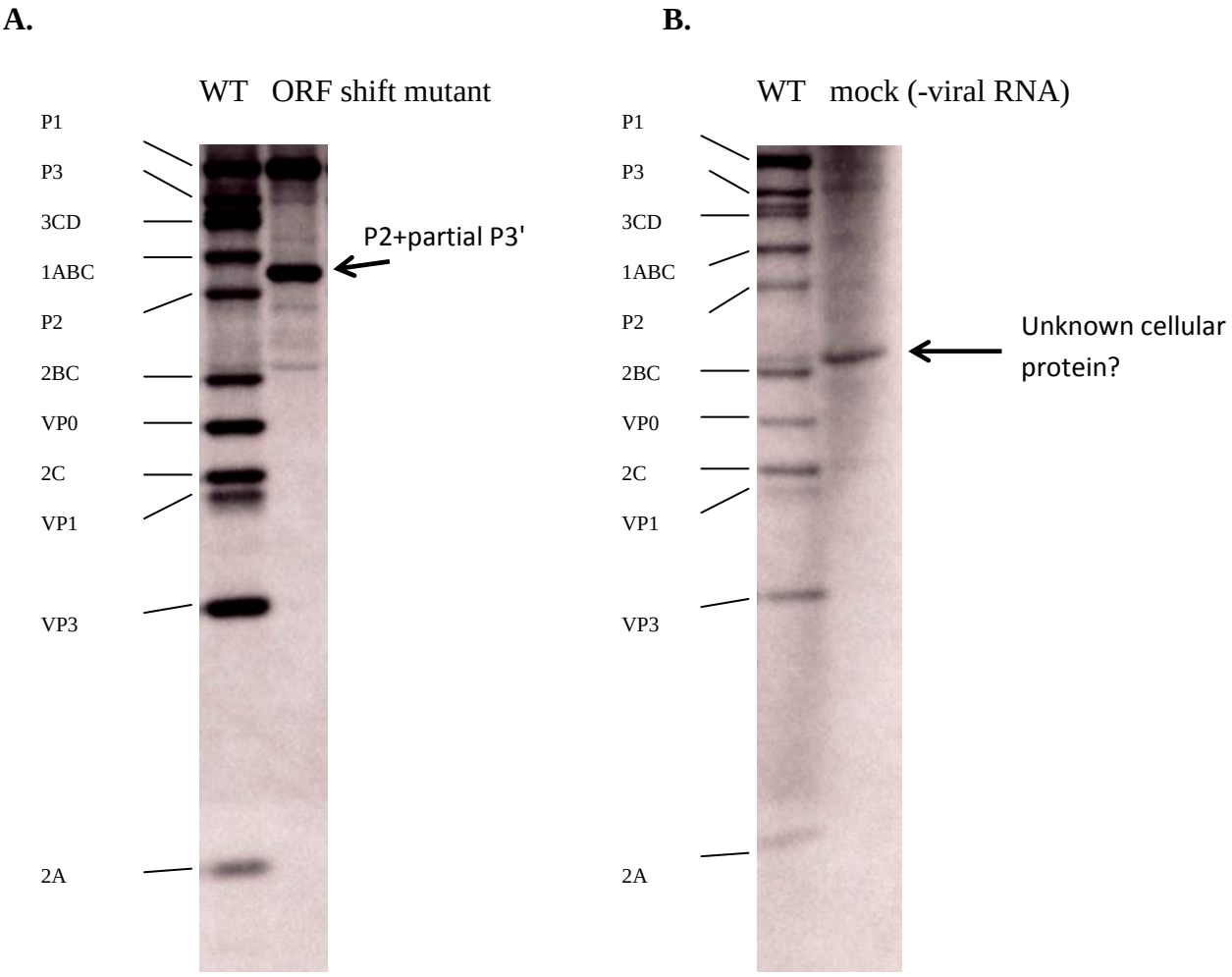

Figure 6. Two interesting observations using the HeLa S10 cell free *in vitro* translation system. *In vitro* translation of **(A)** wt and mutant #11' (a wrong clone of mutant #11, Hpa I site lost a base pair during digestion and ligation) or **(B)** wt and mock (without viral RNA templates) RNA transcripts**.** RNA transcripts (200 ng) derived from wt and mutant construct were translated *in vitro* in HeLa cell-free extracts at 34℃ for 16 hrs, as described in Materials and Methods. Positions of the precursor and mature proteins are indicated. The locations of the P2+partial P3' (where ' indicates wrong ORF which generated stop codons at nt 5289, nt 5307 and more following) and unknown cellular protein are indicated with arrow heads in Panel A and B, respectively.

Table 1. List of the sixteen clustered charged to alanine mutants of PV 2C^{ATPase} and the corresponding electronic charge, amino acids and nucleotides changes.

PV 2C^{ATPase} Mutants	Δ electronic charge	Wild type codons	Alanine codons
#1.(K_6A/K_7A)	-2	AAG AAG	gcG gcG
#2.($K_{33}A/E_{34}A/K_{35}A$)	-1	AAG GAG AAA	gcG GcG gcA
#3. ($R_{41}A/D_{42}A/K_{43}A$)	-1	AGA GAT AAG	gcA GcT gcG
#4. ($K_{49}A/R_{51}A$)	-2	AAA CTT AGA	gcA CTT gcA
#5. ($K_{88}A/R_{89}A$)	-2	AAG AGG	gcG gcG
#6. ($E_{97}A/K_{99}A/R_{100}A$)	-1	GAA GCC AAA AGA	GcA GCC gcA gcA
#7. ($E_{148}A/R_{149}A/E_{150}A$)	+1	GAA AGA GAA	GcA gcA GcA
#8. ($R_{240}A/R_{241}A$)	-2	AGG CGC	gcG gcg
#9. ($D_{245}A/D_{247}A$)	+2	GAC ATG GAC	GcC ATG GcC
#10. ($R_{256}A/D_{257}A$)	0	AGA GAT	gcA GcT
#11. ($K_{279}A/R_{280}A$)	-2	AAG AGA	gcG gcA
#12. ($D_{294}A/K_{295}A$)	0	GAC AAA	GcC gcA
#13. ($R_{298}A/R_{300}A$)	-2	AGA GTT AGA	gcA GTT gcA
#14. ($E_{313}A/R_{314}A/R_{316}A/R_{317}A$)	-2	GAG AGA AAC AGA AGA	GcG gcA AAC gcA gcA
#15. ($D_{183}A/D_{186}A/K_{188}A$)	+1	GAT GGT GCG GAC ATG AAG	GcT GGT GCG GcC ATG gcG
#16. ($E_{207}A/E_{208}A/K_{209}A$)	+1	GAG GAG AAA	GcG GcG gcA

Table 2. Summary of the growth phenotypes and *in vitro* translation phenotypes of the sixteen charged to alanine mutants of $2C^{ATPase}$.

Four groups	Alanine scanning mutants	Time of full CPE (Tf,*P1-P4*)			Translation and polyprotein processing	P2/2BC/2C migration on SDS-PAGE gel
		33 ℃	37 ℃	39.5 ℃		
Group I (3/16) Wt-like	#2. $K_{33}A/E_{34}A/K_{35}A$	Tf	Tf	Tf	+	+
	#4. $K_{49}A/R_{51}A$	Tf	Tf	Tf	+	Shift
	#10.$R_{256}A/D_{257}A$	Tf	Tf	Tf	+	+
Group II (1/16) *ts*	#7. $E_{148}A/R_{149}A/E_{150}A$	Tf	*-/P1*	-	+	Shift
Group III (10/16) lethal	#3. $R_{41}A/D_{42}A/K_{43}A$	-	-	-	+	+
	#5. $K_{88}A/R_{89}A$	-	-	-	+	+
	#6. $E_{97}A/K_{99}A/R_{100}A$	-	-	-	+	Shift
	#8. $R_{240}A/R_{241}A$	-	-	-	+	Shift
	#9. $D_{245}A/D_{247}A$	-	-	-	+	+
	#12.$D_{294}A/K_{295}A$	-	-	-	+	+
	#13.$R_{298}A/R_{300}A$	-	-	-	+	Shift
	#14.$E_{313}A/R_{314}A/R_{316}A/R_{317}A$	-	-	-	+	Shift
	#15. $D_{183}A/D_{186}A/K_{188}A$	-	-	-	+	Shift
	#16. $E_{207}A/E_{208}A/K_{209}A$	-	-	-	+	Shift
Group IV (2/16) *q.i.*	#1.K_6A/K_7A	*P2*	*P1*	*P1*	+	+
	#11.$K_{279}A/R_{280}A$	*P1*	*P1*	*P1/-*	+	Shift

ts: temperature sensitive
q.i.: quasi-infectious
Tf: transfection
P1, 2…: passages 1, 2…

Chapter III. Alanine Scanning Mutagenesis Revealed the Critical Roles of $2C^{ATPase}$ in Poliovirus RNA Replication and Encapsidation

INTRODUCTION

Although much is known about the complex life cycle of PV, including virus entry, translation, viral RNA replication, and release of the mature viruses from the host cell (Wimmer, Hellen et al. 1993), encapsidation of the viral genome remains the least understood step.

As discussed earlier in Chapter I, the ORF of the PV genome encodes a single polyprotein, which can be further divided into three regions: structural regions P1, nonstructural regions P2 and P3. P1 encodes a 97k Da capsid precursor, which can be processed into the capsid proteins (VP0-VP3-VP1) with the help of cellular chaperone Hsp90 (Geller, Vignuzzi et al. 2007).The capsid intermediates (5S, 14S, 75S, and 150S) in the assembly of poliovirus are summarized in **Table 1**. The nonstructural P2 domain encodes viral proteins $2A^{pro}$, $2BC^{ATPase}$ and $2C^{ATPase}$. $2BC^{ATPase}$ and $2C^{ATPase}$ are required for the formation of membranous vesicles where RNA replication takes place. The P3 domain encodes proteins directly (3AB, $3CD^{pro}$, $3D^{pol}$) or indirectly (3A, $3C^{pro}$) involved in genome replication.

Among the nonstructural proteins, available evidence suggests that $3CD^{pro}$ and $2C^{ATPase}$ are involved in virus morphogenesis. $3CD^{pro}$ appears to be involved at both the early and late stages of virion assembly. First, $3CD^{pro}$ but not $3C^{pro}$, is responsible for the cleavage of the P1 precursor to produce the protomer (Ypma-Wong, Dewalt et al. 1988). $3CD^{pro}$ has been observed to have a higher affinity to P1 than the mature $3C^{pro}$, suggesting that the $3D^{pol}$ domain of the precursor confers increased binding to the capsid protein (Parsley, Cornell et al. 1999). Second, studies with an *in vitro* translation/RNA replication system, which produces viable viruses, have suggested that $3CD^{pro}$ also functions at a late step in the assembly process just before or during the maturation cleavage of VP0 to VP2 and VP4 (Franco, Pathak et al. 2005). The multifunctional protein $2C^{ATPase}$ of PV has been implicated in virion formation through genetic

analysis of a cold-sensitive mutant (Li and Baltimore 1988), a chimeric virus C_{20}PP (Liu, Wang et al. 2010) or by determining escape mutants from drug (hydantoin) inhibition (Vance, Moscufo et al. 1997) (Paul et al. unpublished data). The locations of these previously identified mutations in PV $2C^{ATPase}$ involved in encapsidation or uncoating are summarized in **Figure 1**.

It is commonly accepted that several different factors are involved in the morphogenesis process, such as viral RNA, cellular factors, and viral (structural and nonstructural) proteins. Recently, the 90 KDa heat shock protein (Hsp90) was reported to be required for PV encapsidation (Geller, Vignuzzi et al. 2007). As mentioned in Chapter I, more recently we have reported a direct interaction between $2C^{ATPase}$ and VP3, which is required for enterovirus morphogenesis. Amino acid N_{252} of PV $2C^{ATPase}$ is involved in an interaction with CAV20 VP3 protein (Liu, Wang et al. 2010). Surprisingly, the N_{252} residue does not belong to any of the previously identified or putative domains of $2C^{ATPase}$. As described in Chapter I, in order to further characterize the individual functions of other previously uncharacterized amino acids of $2C^{ATPase}$ and to enhance our understanding of the role $2C^{ATPase}$ in the PV life cycle, especially in the encapsidation process, a systematic genetic mutagenesis of PV non-structural protein $2C^{ATPase}$ was performed. Genetic analysis of suppressor variants of some of the poliovirus non-structural protein $2C^{ATPase}$ mutants revealed the involvement of more than one capsid proteins: not only of VP3, but also of VP1 in an interaction with $2C^{ATPase}$ during the PV encapsidation process.

RESULTS

III.1 Analysis of RNA replication and encapsidation phenotypes of the defective $2C^{ATPase}$ mutants using a Renilla luciferase reporter virus

To further characterize our mutants described in Chapter II, I measured the RNA replication and encapsidation using a Renilla luciferase reporter virus (R-PPP) (**Figure 2.A**) (Liu, Wang et al. 2010) that contains the R-Luc coding sequence linked to the N-terminus of the PV polyprotein (PPP corresponds to the three domains in the PV polyprotein). During translation the reporter polypeptide is cleaved off from the polyprotein by $3CD^{pro}$ and gives off luciferase signal when substrate is added. To test for a defect in RNA replication, RNA transcripts of wt R-PPP (illustrated in **Figure 2.B**) or mutants R-PPP were transfected into HeLa cells, both in the absence and presence of GnHCl, a strong inhibitor of PV RNA synthesis (Pincus, Diamond et al. 1986). Luciferase activity was measured at 16 hrs post-transfection.

A schematic illustration of the reporter virus assay is shown in **Figure 3**.Samples incubated in the presence of GnHCl yielded a luciferase signal representative of input RNA translation. Luciferase signal obtained in the absence of GnHCl, on the other hand, is a measure of RNA replication. To test for encapsidation, cell lysates from transfections in the absence of GnHCl were passaged to fresh cells and the cells were incubated, again both in the absence and presence of GnHCl, followed by measurement of luciferase signal at 8 hrs post-infection. Encapsidation was inferred from the detection of luciferase activity in the lysates of the passaged cells.

The genomes of all thirteen defective mutants, including group II (one *ts* mutant), group III (ten non-viable mutants) and group IV (two *q.i.* mutants), were analyzed by the R-PPP strategy. Replication and subsequent encapsidation of *ts* mutant #7 ($E_{148}A/R_{149}A/E_{150}A$) were completely inhibited at the restrictive temperatures, 37 ℃ and 39.5 ℃, whereas at 33 ℃ replication and encapsidation were achieved albeit slightly less efficiently (**Figure 4.A**). All group III mutants were found to be severely defective in RNA replication and subsequent

encapsidation (**Figure 5.**) whereas quasi-infectious mutants #1 (K_6A/K_7A) and #11 ($K_{279}A/R_{280}A$) (group IV) exhibited less severe replication phenotype at 33 ℃ (**Figure 6.**). Since RNA replication is a precondition for encapsidation, ideally only when the replication is normal but the infectious virus yield is decreased, one can directly ascribe the defect to encapsidation. Thus to pinpoint the defects of mutants in group II (one *ts* mutant) and group IV (two *q.i.* mutants), we did further genetic analysis of the suppressor mutants as discussed below.

III.2 A partial reversion rescues the severely temperature sensitive phenotype of mutant #7 ($E_{148}A/R_{149}A/E_{150}A$)

Temperature-sensitive mutant #7 was collected from HeLa cells at 33 ℃ and the lysate was tested for the presence of revertants or suppressor mutants using plaque assays performed at either 33 ℃ or 39.5 ℃. Following reverse transcription and PCR (RT/PCR) of the total RNA, the full-length PV genome were sequenced. The full length sequencing strategy was summarized in **Table 2**. At 33 ℃, no revertants or suppressors of mutant #7 were found as expected. At 39.5 ℃, mutant #7, which contains three mutations ($E_{148}A/R_{149}A/E_{150}A$) produced a partial revertant at $A_{150}E$. This result suggests that the presence of an alanine residue at position 150 was the primary cause of the *ts* phenotype of mutant #7. This was confirmed by constructing an $E_{150}A$ $2C^{ATPase}$ mutant of wt PV that indeed exhibited a *ts* phenotype similar to that of mutant #7 (data not shown). Temperature shift and ^{35}S pulse label experiments of mutant #7 demonstrated that *in vivo* translation per se of mutant #7 is not affected at high temperature (**Figure 4.B**).

III.3 Suppressor mutations of quasi-infectious mutant #1 (K_6A/K_7A) generated a novel Q^R cleavage site at $2B^2C^{ATPase}$

Mutant #1 (K_6A/K_7A) yielded two suppressor mutations at G_1R and S_3R during the first passage on HeLa cells at 37 ℃, both of which had substituted a neutral residue (G_1 or S_3) upstream of the original mutated sites (K_6A/K_7A) for a positively charged arginine (R), whilst retaining the original (K_6A/K_7A) mutations (**Figure 7.A**). Interestingly, the G_1R suppressor mutation somehow generated an unusual Q^R 2B^2C^{ATPase} proteinase cleavage site for 3CD^{pro} (Q^G in wt, **Figure 7.A**). To confirm that the G_1R and S_3R mutations were responsible for rescuing the *q.i.* phenotype of the original mutant #1, I introduced these changes individually into #1 mutant background in pT7PVM and reconstructed two variants $R_1A_6A_7$ and $R_3A_6A_7$ **(Figure 7.A)**. In addition, to examine the effect of the G_1R mutation alone I constructed a $R_1K_6K_7$ mutant **(Figure 7.A)**.

First to investigate the processing efficiency of the novel Q^R cleavage site, I then *in vitro* translated the RNA transcripts of the reconstructed $R_1A_6A_7$ and $R_1K_6K_7$ variants and observed normal polyprotein translation, processing and especially normal production of 2C^{ATPase} (**Figure 7.B**). This suggests G_1R mutation confers normal translation and protein processing pattern to both $R_1A_6A_7$ (G_1R mutation in the context of #1 mutant) and $R_1K_6K_7$ mutant (G_1R mutation in the context of wt PV), indicating Q^R can be cleaved by 3CD^{pro}, a new observation for PV. Suppressor variant $R_3A_6A_7$ also showed normal *in vitro* translation and polyprotein processing (data not shown).

Then I tested the growth phenotype of these three reconstructed variants. The $R_1A_6A_7$ and $R_3A_6A_7$ reconstructed suppressor variants yielded progeny during the first passage on HeLa cells at 33 ℃. The virus titers were comparable to that of the wt at all 3 temperatures tested (33 ℃, 37 ℃ and 39.5 ℃) but the plaque size was small (**Figure 8.**). The $R_1K_6K_7$ mutant, however, yielded full CPE immediately after transfection and both virus titer and plaque size were normal

(**Figure 8.**). All three reconstructed mutants showed full CPE upon transfection at 37 ℃ and 39.5 ℃ (data not shown).

III.4 Suppressor mutations of quasi-infectious mutant #11 ($K_{279}A/R_{280}A$) revealed genetic suppression of PV $2C^{ATPase}$ encapsidation defects by capsid proteins VP1 or VP3

Mutant #11 ($K_{279}A/R_{280}A$) was shown to be quasi-infectious and, thus, by definition, progeny viruses isolated from HeLa cell lysates were always found to be genetic variants thereof. Direct reverion of the mutations in #11, however, were not observed presumably because the large number of nt mutations (four) substituted to the genome. To extend the scope of finding possible suppressor mutations, we analyzed the transfections of mutant #11 at two temperatures (33 ˚C and 39.5 ˚C) and selection of variants, also at the two different temperatures (Fig. 9A bottom panel). Mutant #11 RNA transcripts were transfected into HeLa cells and, after development of full CPE upon first passage (*P1*), I plaque purified the emerged viruses. Initial transfection of #11 RNA transcripts into HeLa cells and the subsequent passages were carried out either at 33 ℃ or 39.5 ℃, plaque assay and the enrichment of the emerged plaques were also carried out either at 33 ℃ or 39.5^{o}C. The full-length genomes of plaque purified variants were then sequenced. None other than those mutations shown in **Figure 9.A top panel** were identified, which had arisen during the selection procedures (**Table 3**). Note that all of the four suppressor variants of #11 still carried the original #11 mutation $K_{279}A/R_{280}A$, as indicated by the diamond in the schematic genomes (Fig. 9A).

The results of these experiments are summarized in **Figure 9**. The most intriguing variant identified is variant #11a, which was isolated by transfection, 1st passage and plaque purification at 33 ℃. Variant #11a contained a single suppressor mutation in capsid protein VP1 ($T_{36}I$)

(**Figure 9.A**). Although this variant was found to be *ts* at 37 ℃ and 39.5 ℃ it proliferated at 33 ℃ to titers equivalent to wt PV (**Figure 9.B**), demonstrating that encapsidation defect of mutant #11 was fully restored at this temperature by a "cross-talk" of unknown characters between VP1 and $2C^{ATPase}$.

Variant #11b, isolated after transfection and 1st passage at 33 ℃ and plaque purification at 39.5 ℃, carried a single suppressor mutation in $2C^{ATPase}$ ($E_{148}K$) (**Figure 9.A**). $E_{148}K$ fully rescued the growth phenotype of the parental mutant #11 at 37 ℃ and 39.5 ℃, although the rescue was slightly less robust at 33 ℃ (**Figure 9.B**). Just as in #11a, a single suppressor mutation in #11b rescued the q.i. phenotype of mutant #11, although the former was a capsid VP1 ($T_{36}I$) suppressor mutation while the latter was a $2C^{ATPase}$ ($E_{148}K$) suppressor mutation.

Two other interesting suppressor mutants, variants #11c and #11d, were isolated after transfection and 1st passage at 39.5 ℃, and plaque-purified at 33 ℃ (**Figure 9.A**). Both variants carried two suppressor mutations. They shared the same $C_{323}R$ suppressor mutation in $2C^{ATPase}$, but variant #11c had an additional mutation in VP3 ($K_{41}R$) whereas variant #11d had an additional mutation in VP1 ($N_{203}S$).

We were interested to test which of these suppressor mutations rescued RNA replication. Of particular interest were the suppressor mutations $E_{148}K$ of #11b and $C_{323}R$ of #11c/d, both mapping to $2C^{ATPase}$. These experiments were carried out with an F-Luc replicon (F-PP), in which the capsid-coding region of the PV polyprotein was replaced by the F-Luc coding sequence (**Figure 10.A**). This F-PP reporter replicon eliminates any effect of capsids and studies the mutations' effect on RNA replication only. F-PP (#11+$E_{148}K$) (subcloned from variant #11b) replicated well at 37 ℃ and 39.5 ℃ but less well at 33 ℃ (**Figure 10.B**) , an observation indicating rescue of RNA replication of #11 by the single suppressor mutation $2C^{ATPase}$ ($E_{148}K$).

Since suppressor variant #11b grew well at 37 ℃ and 39.5 ℃ with virus yields comparable to that of the wt (Fig.6B), we thus suspect that rescue of encapsidation defect of mutant #11 at these temperatures can also be achieved by $E_{148}K$.

The $C_{323}R$ mutation (from variants #11c and #11d), however, had no effect at 33 ℃ and only partially rescued #11's RNA replication defect at 37 ℃ and to a less extent at 39.5 ℃ as shown by FPP (#11+$C_{323}R$) in **Figure 10.B**. Indeed, mutation $C_{323}R$ was never isolated by itself but rather was always accompanied by a capsid mutation in either VP3 (#11c) or VP1 (#11d) in suppressor mutants with wt yields of mature virions (Fig. 6B). Just as $C_{323}R$ itself was unable to rescue encapsidation, the capsid mutation in #11c or #11d alone could neither rescue replication nor encapsidation of mutant #11 using R-PPP reporter virus (data not shown). Excitingly, the double suppressor mutants #11c and #11d exhibited growth properties similar to the wt virus when proliferated at all three temperatures (Fig. 6B). These data strongly suggests that either one of the two capsid mutations, VP1 ($N_{203}S$) or VP3 ($K_{41}R$), in cooperation with $C_{323}R$ in $2C^{ATPase}$, rescued encapsidation of #11. (**Figure 9.B**), suggests that the capsid mutations can rescue encapsidation. These genetic data strongly support the conclusion that $2C^{ATPase}$ acts in conjunction with capsid polypeptides during PV morphogenesis.

My interpretation of why different variants were isolated from mutant #11: (1) when transfection was performed at 33 ℃, mutant #11 can slowly replicate, but has encapsidation defect. It needs revertants in capsid to rescue encapsidation, as isolated variant #11a: #11+VP1 ($T_{36}I$). Or $E_{148}K$ in 2C can rescue both replication and encapsidation defect of mutant #11, as isolated variant #11b:#11+2C ($E_{148}K$). Upon passaged at 39.5 ℃, this #11b with $E_{148}K$ compete out because compared with variant #11+VP1 ($T_{36}I$), it replicate faster at 39.5 ℃, while at 33 ℃ there is no pressure since #11 replicate fine at low temperature. (2)When transfected at 39.5 ℃,

mutant #11 first has to gain the ability to replicate by suppressor mutation in 2C ($C_{323}R$). In addition, it has encapsidation defect, that's why $C_{323}R$ never appeared by itself thus both variants #11c and #11d need an additional suppressor mutations in capsid region, VP3 ($K_{41}R$) and VP1 ($N_{203}S$) respectively.

Either case, these genetic data strongly support the conclusion that $2C^{ATPase}$ is involved in encapsidation and it may act in conjunction with capsid polypeptides VP1 or VP3 to facilitate PV morphogenesis.

III.5 The more conserved the original charged amino acid at the sites of alanine scanning among enteroviruses, the more debilitated the PV $2C^{ATPase}$ alanine scanning mutants

Viabilities of the all our $2C^{ATPase}$ alanine scanning mutants, but one (#4), correlate with the extent of conservation of the corresponding original charged amino acid sequence among human enterovirus C cluster. The more conserved the original charged amino acid, the more debilitated the alanine scanning mutants (**Table 4.**). For example, the original charged amino acids of group III lethal mutants are all highly conserved. High conservation is defined when amino acids are 100% conserved in electronic charge, some of which may contain amino acid variations from K to R, R to K, D to E,G to S, or S to G as listed in **Table 4**. Low conservation is defined when amino acids vary in electronic charge.

Interestingly, the wild type sequence at aa 148, 279 and 280 of CAV1/CAV19/ CAV22, are K_{148} + S_{279}/T_{280}, where numbering of amino acid residues is according to the PV sequence. Very fittingly, one suppressor variant #11b (Fig.9A) generated a surprising $E_{148}K$ mutation in response to loss of two charged amino acids at aa 279 and 280 ($K_{279}A/R_{280}A$) in $2C^{ATPase}$. This strongly supports our proposal that there is an interaction between the C terminal (near mutant

#11, aa 272-286) and a spacer between A motif and B motif of the NTP-binding domain of $2C^{ATPase}$ (near mutant #7, aa 148) either within the $2C^{ATPase}$ protein or $2C^{ATPase}$ oligomers.

III.6 The cysteine rich region of PV $2C^{ATPase}$ is involved in PV encapsidation

In addition to the sixteen clusters of two or more charged residues, another four conserved cysteine rich zinc binding sites (CRs) of PV $2C^{ATPase}$, were also subjected to alanine mutagenesis (**Table 5**). The study of these CRs is based on two previous reports of CRs involved in virus encapsidation and my own data of alanine mutant #11. It was reported that HBV CRs is involved in pregenomic RNA encapsidation (Kim, Lee et al. 2009) and Cys-His boxes of HIV-1 nucleocapsid have been shown to play a critical role in RNA encapsidation as summarized by Schwartz et al. (Schwartz, Fiore et al. 1997). Pfister and Wimmer (Pfister, Jones et al. 2000) have shown that mutating the $C_{272}\&H_{273}$ of PV $2C^{ATPase}$ to serine yielded a mutant with a ts phenotype. The more relevant evidence is from my data of alanine mutant #11 ($K_{279}A/R_{280}A$), which has mutations located in the middle of the CRs region of PV $2C^{ATPase}$, and was shown to be involved in PV encapsidation. From all these data, it appears likely that the CRs of PV $2C^{ATPase}$ are also involved in encapsidation. Thus, to further characterize the function(s) of the four CR sites of PV, I made four new CR mutants by changing the cysteine/ histidine to alanines (**Table 5.**).

First I analyzed the *in vitro* translation and polyprotein processing properties of the four CRs mutants. As shown in **Figure 11.**, all four CRs mutants exhibited normal translation and polyprotein processing profiles. Then I did phenotypic analysis of the four CRs mutants using the same strategy as described with the above sixteen charged to alanine mutants. Mutants #CR1 and #CR3 are lethal at all three temperatures tested, no CPE was observed even upon four blind

passages; mutant #CR2 is *ts*, while mutant #CR4 is *q.i.*, as summarized in **Table 6.** and **Figure 12**.

Interestingly, viability of the four mutants correlates with the extent of conservation of the corresponding CR sequence among enteroviruses: the more conserved the four CRs among enteroviruses, the more debilitated the alanine scanning mutants. For example, mutant #CR1 ($C_{269}A$) with a cysteine at amino acid 269, which is 100% conserved cysteine among the members of the enterovirus genus, was mutated to alanine and the mutant is nonviable (**Table 6.**) and showed no replication when tested with the Renilla reporter virus (**Figure 13.A**). Mutant #CR2 ($C_{272}A/H_{273}A$) showed CPE at transfection with a ts phenotype (**Table 6**, **Figure 12.**) with replication and encapsidation defects at the nonpermissive temperatures (**Figure 13.B**). Very fittingly, this CR2 site is only partially (~50%) conserved within the enterovirus genus. Mutant #CR3 ($C_{281}A/C_{282}A$) is nonviable (**Table 6**) and exhibited no RNA replication (**Figure 13.A**), probably due to the necessity of the presence of both of the two cysteines at CR3, which are ~90% conserved in the enterovirus genus. Mutant #CR4 ($C_{286}A$) is quasi infectious and exhibited normal translation but had a replication defect (**Figure 13.A**). It showed CPE upon 1st passage, and it is not ts although the plaque size is small at 39.5 ℃ (**Figure 12.B**). This observation also correlates with the fact that the cysteine at CR4 is 100% conserved in the enterovirus genus. All the percentage numbers mentioned here are calculated according to the homologous alignment of the CR of enterovirus $2C^{ATPase}$ protein performed by Pfister et al. (Pfister, Jones et al. 2000).

More interestingly, mutants #CR2 and #CR4 yielded suppressor mutation at $K_{295}R$, $M_{293}V$ or $A_{138}V$ of $2C^{ATPase}$ (**Table 7.**). Coincidently, $K_{295}R$ and $M_{293}V$ both appeared in a cold sensitive, uncoating defective mutant virus, which can be rescued by an additional suppressor mutation ($N_{140}S$) (Li and Baltimore 1990). Since improper uncoating is a result of improper

encapsidation, the perfect match of the suppressor mutations of my CRs mutants and the uncoating defective mutants strongly supports that CRs of PV $2C^{ATPase}$ are involved in the encapsidation process and determines some unknown aspect(s) of virion structure.

III.7 Biochemical studies revealed a direct interaction between PV nonstructural protein $2C^{ATPase}$ and capsid protein VP3

Both the genetic data from the C_{20}PP chimeric virus and the capsid suppressor mutations of mutant #11 described above indicated that an interaction between the capsid proteins and $2C^{ATPase}$ might be a requirement for encapsidation. However, it was not clear from these genetic data whether this interaction occurs directly or is mediated by other factors. To distinguish between these two possibilities, I expressed in E. coli and purified PV proteins GST-$2C^{ATPase}$ (**Figure 14.A**) and His-VP3. The ATPase activity of purified GST-2C was confirmed and it was inhibited by 2mM GnHCl or heat inactivation by boiling (**Figure 14.B**). Using the two purified proteins I carried out a GST-pull down assay (**Figure 14.C**, lane 2). The same assay was performed with the purified GST protein, which is used as a control (**Figure 14.C**, lane 1). The results clearly showed that GST-$2C^{ATPase}$, but not GST, interacts directly with the His-tagged VP3 protein. Combined with the genetic analysis of the C_{20}PP chimera, the data suggest that $2C^{ATPase}$ is required for viral encapsidation through a direct interaction with capsid protein VP3 (Liu, Wang et al. 2010). This result also helps us to confirm that $2C^{ATPase}$ interacts with VP3 through protein–protein rather than RNA-protein interaction (Liu, Wang et al. 2010). Whether $2C^{ATPAse}$ also interacts with VP3 in the context of the capsid intermediates (5S, 14S, 75S and 150S) remains to be determined.

Due to the slight background derived from glutathione's affinity to PV viral particles (Jiang et al., manuscripts in preparation), future studies will employ MBP tagged-2C to investigate whether $2C^{ATPase}$ interacts with capsid protein VP1 instead of a GST tag, which requires the use of glutathione beads. MBP-$2C^{ATPase}$ fusion proteins and MBP proteins were purified (data not shown) and will be used in the future pull down of PV or mock infected HeLa cell lysates or a GST-VP1 fusion protein.

DISCUSSION

This alanine scanning mutagenesis study of PV nonstructural protein $2C^{ATPase}$ confirmed the critical roles of $2C^{ATPase}$ in poliovirus RNA replication and encapsidation. Encapsidation is the last and a crucial step of the virus's life cycle. Successful assembly not only provides newly synthesized viral genomes with a protective shell, but also assures proper uncoating which is required for attachment to and penetration into subsequent host cells to initiate the next round of viral infection. In addition, capsid proteins are one of the major determinants of tropism due to their ability to bind to specific receptors for cell entry (Racaniello 1990).

First, although *in vitro* translation in HeLa cell free extracts (Molla, Paul et al. 1991) indicated that none of the alanine mutations interfere with protein synthesis and proteolytic processing (Chapter II, **Figure 2.**), the suppressor mutations identified in this study provide novel insights into the proteolytic processing of PV. The suppressor mutation G_1R of mutant #1 (K_6A/K_7A), generated a unique $3C^{pro}/3CD^{pro}$ proteinase cleavage site at the 2B^$2C^{ATPase}$ cleavage site. Enteroviruses are purists in that all of their natural $3C^{pro}/3CD^{pro}$ cleavage sites are Q^G; thus the generation of the Q^R site was surprising. The only naturally occurring Q^R site amongst picornavirus polyproteins exists in the HAV polyprotein between proteins $3C^{pro}$ and $3D^{pol}$ (Tesar, Pak et al. 1994), as summarized in **Table 8**. Our *in vitro* translation assays

demonstrated that processing between 2B and $2C^{ATPase}$ in the Q^R suppressor polyprotein was just as efficient as at the original Q^G site in the wt polyprotein (**Figure 7.B**). It should be noted that the G to R mutation involves only a single nucleotide change instead of the two required for the A to R substitution (Chapter II, **Table 1**). This likely explains why the virus regenerated a positively charged residue at position 1 rather than reverting the nucleotides of the A codons at positions 6 or 7.

The other suppressor mutations revealed two novel functional domains in the $2C^{ATPase}$ polypeptide, which differ from those domains identified by the lethal mutations as discussed in Chapter II. These mutations, arising in the $2C^{ATPase}$ protein, always regenerated a charged amino acid that was originally eliminated by the charged to alanine substitution. These were either at the same ($A_{150}E$ of mutant #7) or at different ($E_{148}K$, $C_{323}R$ of mutant #11) locations to the original residue change. The first domain that I hypothesize to play a role in $2C^{ATPase}$ function(s) is located at the spacer between the A and B motifs of the NTP-binding domain, involving $A_{150}E$ and $E_{148}K$: $A_{150}E$ is a partial revertant of *ts* mutant #7 and $E_{148}K$ is a suppressor mutant of the $K_{279}A/K_{280}A$ mutant #11. The presence of a suppressor mutation ($E_{148}K$) at this location suggests a functional interaction between the C-terminus of the $2C^{ATPase}$ polypeptide and the spacer between motifs A and B of the NTP-binding domain (Mirzayan and Wimmer 1992) either within the $2C^{ATPase}$ protein or $2C^{ATPase}$ oligomers, which may contribute to the signature of different viruses. This is further supported by the $A_{138}V$ suppressor mutations of mutants #CR2 and #CR4. The second domain maps to the cysteine rich sites containing the original mutated site ($K_{279}A/R_{280}A$) of mutant #11. These mutations are localized close to the C-terminus of the $2C^{ATPase}$ polypeptide within the Zn^{++} binding domain that consists of five cysteines (C_{269}, C_{272}, C_{281}, C_{282}, and C_{286}) and one histidine (H_{273}) residue. Thus, to further characterize the functions

of the four CR sites of PV, I made four new CRs mutants by changing the cysteine/ histidine to alanines (**Table 5.**). Indeed the fours cysteine rich sites are also involved in the PV encapsidation process: mutant #CR2 and #CR4 yielded suppressor mutation at $K_{295}R$, $M_{293}V$ or $A_{138}V$ of $2C^{ATPase}$ (**Table 7**.). Coincidently, $K_{295}R$ and $M_{293}V$ both appeared in 2C-31R3 which is a cold sensitive, uncoating defective virus while $N_{140}S$ rescued the ts phenotype (Li and Baltimore 1990). This strongly supports the idea that CRs of PV $2C^{ATPase}$ are involved in the encapsidation process. It should be noted that residues $K_{279}A/R_{280}A$ of mutant #11 are located just upstream of M_{293} and K_{295}. Based on these results, I propose that the cysteine rich domain containing residues K_{279}/R_{280} is involved in encapsidation and there is a possible interaction between the spacer between box A and box B of the NTP-binding domain (such as A_{138}, E_{148}) and the C terminus of PV $2C^{ATPase}$, flanking the cysteine rich domain.

Very interestingly, viabilities of the all our $2C^{ATPase}$ alanine scanning mutants, but one (#4), correlate with the extent of conservation of the original charged amino acid sequence among human enterovirus C cluster: the more conserved the original charged amino acid among enteroviruses, the more debilitated the alanine scanning mutants (**Table 4.**). We thus predict that if we make the same alanine mutations at the corresponding highly conserved sites of related viruses, majority, if not all, of the mutations will be lethal. Interestingly, the suppressor variant #11b ($K_{279}A/R_{280}A+E_{148}K$) mimic the electronic charges of three CAVs (CAV1/CAV19/ CAV22) ($S_{279}/T_{280}+ K_{148}$) at aa 148, 279 and 280. This strongly supports our proposal that there is an interaction between the C terminal and the spacer between A motif and B motif of the NTP-binding domain of $2C^{ATPase}$ either within the $2C^{ATPase}$ protein or $2C^{ATPase}$ oligomers. One explanation is that these two regions might possibly form a high order structure which is required to interact with viral capsid proteins.

The most important conclusion derived from my work is that both VP3 and VP1 communicate with the C-terminal domain of $2C^{ATPase}$, and that this interaction is required for encapsidation. This conclusion is strongly supported by the emergence of suppressor variants of mutant #11: #11a, #11c and #11d (**Figure 9.**). This was achieved by a simple but powerful design, to extend the scope of finding suppressor mutations, of subjecting the mutants to different selection pressures by combination of low/high temperatures between transfections and plaque purification of variants (Fig. 6A bottom panel). My interpretation of why different variants were isolated from mutant #11: when transfection was performed at 33 ℃, mutant #11 could slowly replicate, but had encapsidation defect. (1) It needed revertants in capsid to rescue encapsidation, as isolated variant #11a: #11+VP1 ($T_{36}I$). (2) Or $E_{148}K$ in $2C^{ATPase}$ could rescue both replication and encapsidation defect of mutant #11, as isolated variant #11b:#11+2C ($E_{148}K$). Upon passaged at 39.5 ℃, this #11b with $E_{148}K$ competed out because it replicated faster compared with variant #11+VP1 ($T_{36}I$), while at 33 ℃ there was no selection pressure since #11 replicated fine at 33 ℃. (3)When initially transfected at 39.5 ℃, mutant #11 first had to gain the ability to replicate by suppressor mutation in 2C ($C_{323}R$) although weakly compared to wt (Fig. 6C). In addition, it had encapsidation defect, that's why $C_{323}R$ never appeared by itself thus both variants #11c and #11d needed an additional suppressor mutations in capsid region, VP3 ($K_{41}R$) and VP1 ($N_{203}S$) respectively. Either case, these genetic data strongly support the conclusion that $2C^{ATPase}$ is involved in encapsidation and it act in conjunction with capsid polypeptides VP1 or VP3 to facilitate PV morphogenesis.

However, I cannot at present determine whether VP1 binds directly to $2C^{ATPase}$, or whether it complexes with other capsid proteins to induce structural changes in VP3 through mutation while VP3 is the direct binding partner of $2C^{ATPase}$ in encapsidation (**Figure 14.C**) (Liu,

Wang et al. 2010). The capsid precursor P1 of PV is processed and assembled to form protomers (VP0, VP3, VP1). Available evidence suggests that the protomer does not dissociate into the individual cleavage products in the cytoplasm of the infected cell, rather, it will pentamerize to $(VP0, VP3, VP1)_5$ - the building block of the immature capsid, as discussed by Liu et al. (Liu, Wang et al. 2010). In the future, it will be interesting to determine 1) whether or not there is a direct physical interaction between VP1 and $2C^{ATPase}$; 2) At which stage of encapsidation (capsid intermediates 5S, 14S, 75S and 150S) is $2C^{ATPase}$ involved in the interaction with capsid proteins VP3 and VP1?

Interestingly, the VP1 and VP3 suppressor mutations involved the exchange of a polar residue to one with increased hydrophobicity (VP1: $T_{36}I$ or $N_{203}S$) or of one charged residue to another (VP3: $K_{41}R$). One of the VP1 residues (T_{36}) is located near the N-terminus of the VP1 protein, which was previously shown to be involved both in RNA release from the infecting virion and in the interaction/insertion of the progeny RNA into capsid proteins during morphogenesis (Kirkegaard 1990). On the basis of the study of two small N-terminal deletions of PV VP1, Kirkegaard and colleagues have concluded that RNA packaging and RNA release are genetically linked but can be mutated separately in different VP1 alleles. Whether an interaction between VP1 and $2C^{ATPase}$, if any, is required for these processes remains to be determined.

Although not further analyzed, I also isolated several additional suppressor variants of mutants #11, #CR2 and #CR4. First, suppressor mutations in VP3 ($I_{231}V$) +2C ($C_{323}R$), VP3 ($M_{52}I$), VP3 ($E_{45}Q$) + $3C^{pro}$ ($T_{20}A$), 3D ($M_{189}V$) and some silent mutations in 2C (F_{77}silent) or VP1 (S_{179}silent) of mutant #11 were identified. Second, mutant #CR2 yielded suppressor mutations in 2C ($N_{271}H/D$), ($M_{293}I$) or 3D ($I_{67}T$); silent mutations in VP1 (R_{243}silent) or 2A (N_{29}silent). Mutant #CR4 also yielded additional suppressor mutations in 2C ($R_{298}G$) and silent

mutations in VP2 (C_{257}silent), VP1 (L_{224}silent), 2A (L_{113}silent), 3C (G_{145}silent). In the future it will be interesting to further analyze these suppressor mutations especially those mutations in 3C or 3D which could contribute to PV encapsidation through its precursor $3CD^{pro}$. Whether or not any of the silent mutations will contribute to any steps of PV life cycle will also need to be investigated.

Our results clearly demonstrate, however, that the selection of revertants or suppressor mutants generated from alanine scanning mutagenesis is a useful method of identifying viral protein interacting components of the replication/encapsidation complex.

MATERIALS AND METHODS

Cells. HeLa R19 cells were maintained in DMEM (Life Technology) supplemented with 10% bovine calf serum (BCS), 100 units of penicillin, and 100 mg/ml of streptomycin. Transfection or passages of HeLa cells were carried out with 2% BCS.

Plasmids. pT7PVM contains a full-length infectious cDNA of PV1 (M). pR-PPP is an infectious Renilla luciferase reporter virus construct in which Renilla luciferase (311 aa) is expressed as an N-terminal fusion to the PV polyprotein (Liu, Wang et al. 2010). pFPP is a reporter replicon in which the P1 capsid was replaced by a Firefly luciferase (550 aa) coding sequence. The designed $2C^{ATPase}$ mutations were then subcloned from the pGEM®-T vector into pT7PVM or the Renilla Luciferase reporter virus (R-PPP) or F-Luc replicon using restriction site *Sna*BI / *Xho*I (mutants #1-6) or *Xho*I / *Hpa*I (mutants #7-16).

RNA transcription and transfection of pRPPP. Wt and mutant plasmids of pRPPP were linearized at a unique *Dra*I restriction site and used as templates for *in vitro* RNA synthesis using T7 RNA polymerase. RNA transcripts (3-10 μg) were transfected into 35-mm-diameter HeLa R19 cell monolayers by the DEAE-Dextran method as described previously (van der Werf, Bradley et al. 1986) and incubated at 33 ℃, 37 ℃ and 39.5 ℃ as necessary.

***In vitro* translation using the HeLa cell free extracts.** HeLa cell S10 cytoplasmic extracts were prepared and *in vitro* RNA translations were performed with these cytoplasmic extracts at 34 ℃, as previously described (Molla, Paul et al. 1991) with slight modification as shown in Chapter II Materials and Methods.

Plaque assays. Plaque assays were performed on HeLa R19 monolayers using 0.6% tragacanth gum. After 72 hrs incubation at 33 ℃, 48 hrs at 37 ℃ or 39.5 ℃ the viral plaques were developed by 1% crystal violet staining.

RT-PCR and sequencing analysis of viral RNAs isolated from purified plaques. The identity of the viruses was confirmed or determined by RT-PCR/sequencing analysis. Single plaques were isolated from the plaque assay plates before staining, and amplified by one passage at the same temperature in fresh 35-mm-diameter HeLa R19 monolayers. Total RNA was extracted from 200 μl lysates with 1 ml Trizol reagent (Invitrogen) and reverse transcribed into cDNA using SuperScript® III Reverse Transcriptase (Invitrogen). The PCR products generated by Expand Long Template PCR system (Roche) were purified and sequenced.

For ts or quasi-infectious (full CPE appears only after further passages) mutants, plaque purify at both low and high temperatures (33℃ and 39.5℃). Full length reverse transcribe - using primer P36, and then do Expand long templates PCR-dividing the full length PV into 3 PCR fragment, PCR fragment A using P1 and P14; PCR fragment B using P13 and P28; PCR fragment C using P27 and P36, then gel purify and sequencing using different primers to cover the full length as summarized in **Table 2**.

Luciferase assays. Dishes (35-mm diameter) of HeLa R19 monolayer cells were transfected with 5 μg of R-PPP RNA transcripts (linearized with *Pvu*I for pR-PPP) and were incubated at 33℃, 37℃ and 39.5℃ as needed in standard tissue culture medium (DMEM) with 2% BCS in the presence and absence of 2 mM GnHCl. Luciferase activity was determined in the lysates of cells harvested 16 hrs post-transfection. Cell lysates (20 μl) were mixed with 20 μl of Renilla luciferase (R-Luc) assay reagent (Promega luciferase assay system catalog no. E2810) and R-Luc activity was measured in an OPTOCOMP I luminometer (MGM Instruments, Inc.). Cell lysates (250 μl) from transfections in the absence of GnHCl were used to infect HeLa R19 cells in the presence and absence of 2 mM GnHCl, and luciferase activity was determined in the lysates of cells harvested 8 hrs post-infection. For the measurement of firefly luciferase (F-Luc)

(linearized with *Dra*I for pFPP), the F-Luc substrate (Promega luciferase assay system catalog no. E1501) was used. Cells were harvested at time points corresponding to the greatest F-Luc signal achievable at each temperature after transfection: 10 hrs at 33 ℃, 8 hrs at 37 ℃, and 6 hrs at 39.5 ℃ (Jahan, Wimmer et al. 2011).

Purification of GST-2C^{ATPase} and His-tagged VP3 proteins. GST-tagged 2C^{ATPase} and His-tagged VP3 recombinant proteins were expressed in *E. coli*. The GST-2C^{ATPase} proteins were expressed from pGEX-2C vector and purified by glutathione sepharose column (GE Healthcare) as described below. The His-VP3 proteins were purified by nickel column chromatography according to the manufacture's protocol (QIAGEN).

GST-2C purification protocol

1. Preparation:

   1). 2×YTA medium –Tryptone 16 g/l; Yeast extract 10 g/l; NaCl 5 g/l. To prepare as a solid medium, add 12–15 g of agar prior to autoclaving.

   Dissolve above ingredients in 900 ml of distilled H2O. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 liter. Sterilize by autoclaving for 20 minutes. Once the medium has cooled, aseptically add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration 100 μg/ml).

   2). pGEX2C transformed in BL21 competent cell

2. Culture growth

1st day night, inoculate one colony to 5ml×4tubes of 2×YTA+ Amp, 37 ℃ O/N

2nd day morning, transfer 8ml O/N culture to 800ml (1:100) 2×YTA+ Amp, 37 ℃, ~3h, till A600 of 0.6-0.7

RT, add 2 ml (200 mM) IPTG (final 0.5 mM), take out 1ml for SDS-PAGE.

RT, 4-6h

Take out 1ml, 3000 rpm×3min, run SDS-PAGE check induction.

The rest 798 ml, 3000 rpm×25min, measure pellet quantity (gram)

Store at -70℃

3. Sonication lysates

Thaw culture pellet at room temperature

Cells resuspended in lysis buffer(2-5ml per gram pellet):

7 μl	14.3M beta-ME	(final 10 mM)
4 μl	5mg/ml pepstatin A	(final 2 μg/ml)
10 μl	5mg/ml Leupeptin	(final 5 μg/ml)
1 ml	10%Trition X-100	(final 1%)
8.79 ml	PBS	

Total volume 10ml

Keep on ice, 30 min

Sonication: 10×10 times until no chunks, clear

Duty cycle 10; Output control 2-3; Timer: on and off

Take 100 μl whole cell lysate for final SDS-PAGE gel analysis

13,000 rpm×10min, keep S/N for the following purification, save pellet for final SDS-PAGE gel analysis.

4. Beads purification

1gram pellet=300 μl 75% beads slurry

For example 800 ml culture, 3.6 gram, 1066 μl 75% beads slurry.

(1) 1ml beads, wash 3 times with PBS

(2) S/N (for example 10ml), 4^C, incubate 2hrs (3000 rpm, 30 s, beads, wash with PBS 3times)

Keep s/n after binding for final SDS-PAGE gel analysis

Keep wash for final SDS-PAGE gel analysis

5. Elution

Elution buffer:

10mM reduced glutathione (glutathione elution buffer) 5ml

20mM beta-ME 7µl 14.3M

0.05% Triton X-100 25µl 10% stock

1ml beads, 1ml elution buffer, 30min cold room rock*3times,

Collect elution1, elution2, elution3 respectively in 1.5ml tubes.

Cold room rock O/N, elution 4. Make 25% glycerol stock.

6. Concentrate

Amicon ultra-4 5KDa ultracel-PL memb 8/PK #UFC 805008

3000 rpm, 4^C, 30min ×several times, may take hours, till desired concentration.

7. Final SDS-PAGE gel analysis

Do Bradford assay to get an rough concentration.

SDS-PAGE gel analysis:

WCL(whole cell lysate); S/N (supernatant before binding to beads); s/n' (supernatant after binding to beads); wash; E1(elution 1); E2(elution 2); E3(elution 3); E4(elution 4, O/N); BSA standard, , 1 µl, 2 µl, 5 µl, 10 µl, 20 µl, 50 µl ×1mg/ml which correspond to eluted protein concentration 0.1mg/ml, 0.2mg/ml,0.5mg/ml,1mg/ml,2mg/ml, 5mg/ml

GST pull-down assay. Briefly, 5 μg GST-2C^{ATPase} (or 2 μg GST as a control) were incubated with glutathione sepharose beads at 4 ℃ for 3 hr in buffer containing 50 mM Tris-HCl pH7.5, 140 mM NaCl, 0.1% TritonX-100 with protease inhibitor cocktail tablets (Roche). The protein bound GSH beads were washed with PBS 3 times and then 5 μg His-VP3 was added. After 1 hr incubation at 4 degree Celsium, the glutathione beads were washed 3 times and were boiled in 1x SDS sample buffer for 5 min. The samples were analyzed by SDS-polyacrylamide gel (12.5% acrylamide) electrophoresis and followed by western blot analysis using antibodies against PV VP3 (polyclonal, kindly contributed by Dr. Delpeyroux, Pasteur Institute, France).

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FIGURES/TABLES

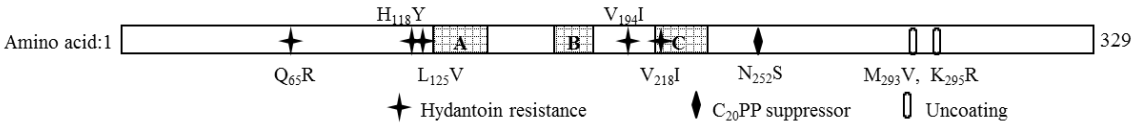
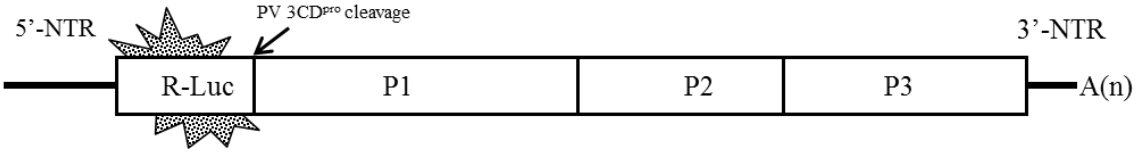


Figure 1. Locations of previously identified mutations in 2C^{ATPase} involved in encapsidation or uncoating. $H_{118}Y$ and $V_{194}I$ are newly discovered hydantoin resistant mutations (Paul, Mugavero and Wimmer, unpublished data).

A.



B.

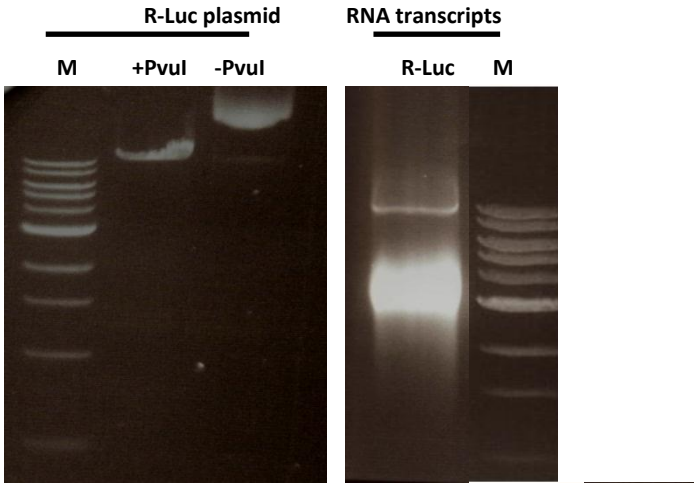


Figure 2. The Renilla luciferase reporter virus (A). The genome structure of the R-Luc reporter virus (R-PPP) is shown. R-PPP: Renilla Luciferase gene fused to three PV polyprotein domains. Linearization of wt R-PPP plasmid and *in vitro* transcription of R-Luc RNA is shown in the middle. **(B).** Preparation of R-PPP RNA transcripts.

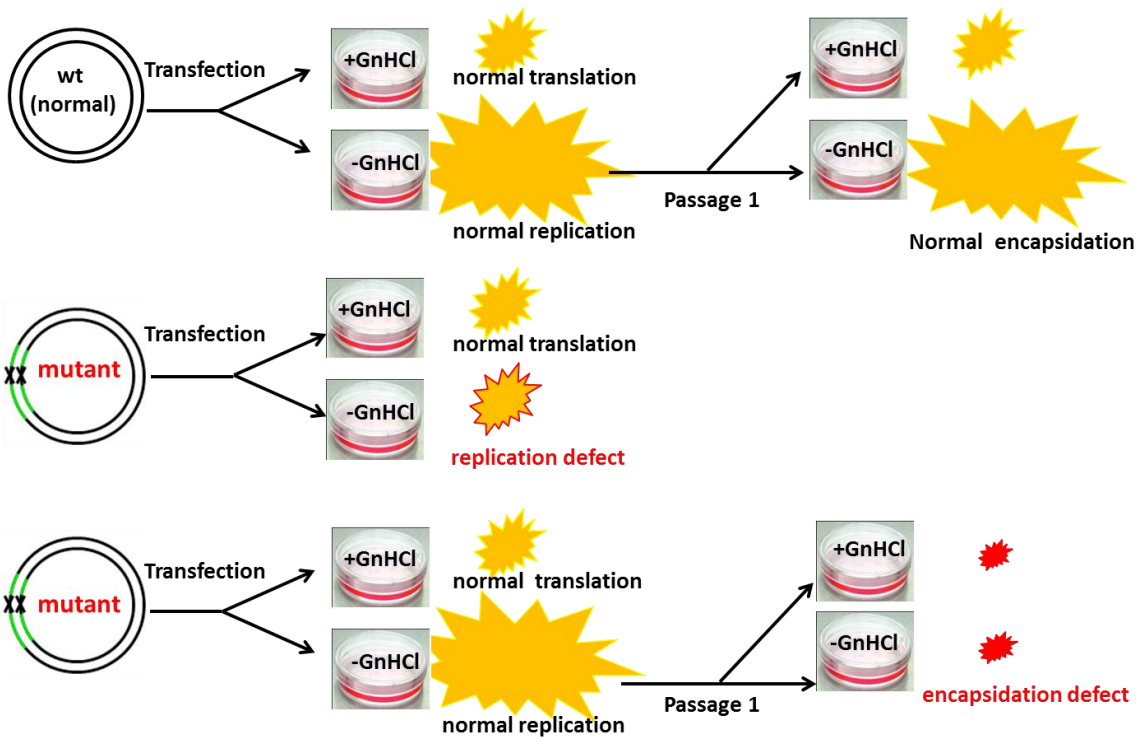
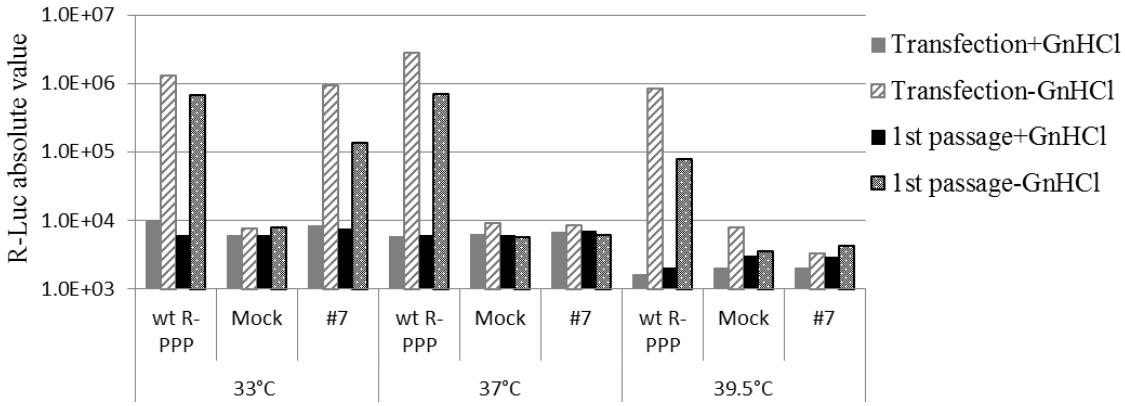


Figure 3. A schematic illustration of the Renilla luciferase reporter virus assay. HeLa R19 cells were transfected with 5 μg R-PPP RNA transcripts followed by one blind passage in HeLa R19 both in the presence (+GnHCl, signal from translation of input genome) and absence (-GnHCl, signal amplified by replication) of GnHCl.

A.



B.

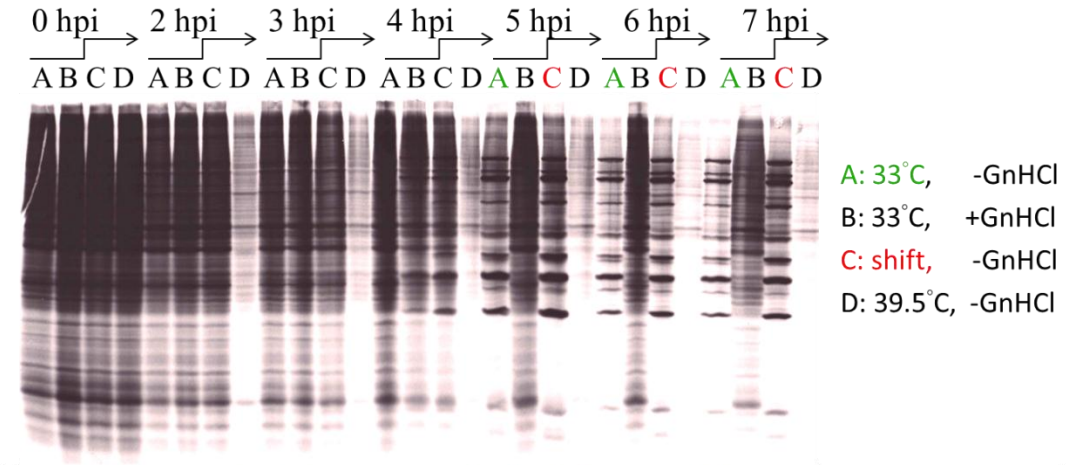


Figure 4. Analysis of RNA replication and *in vivo* translation phenotype of mutant #7. (A) RNA replication and encapsidation by the wt and mutant #7 was assayed at different temperatures using the luciferase reporter virus. Transfection of RNA transcripts and luciferase assays were performed as described in Materials and Methods. **(B)** *In vivo* temperature shift and ^{35}S pulse label experiments of mutant #7. Mutant #7 (P1), HeLa R19, 150 MOI (revertants 0.01MOI at 39.5 ℃). Shift at different time points as indicated and pulse for 30 min.

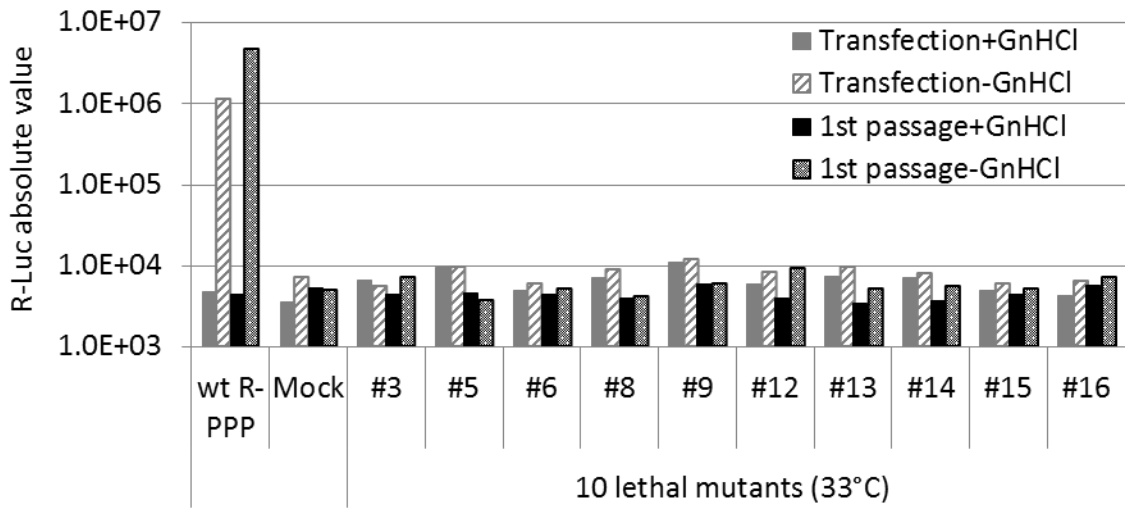


Figure 5. Ten lethal mutants possess RNA replication and encapsidation defects. HeLa R19 cells were transfected with 5 μg of wt or mutant Renilla luciferase reporter virus RNA transcripts. They were passaged once on HeLa cells at 33 ℃ both in the presence and absence of 2 mM GnHCl. Luciferase activities were determined as described in Materials and Methods.

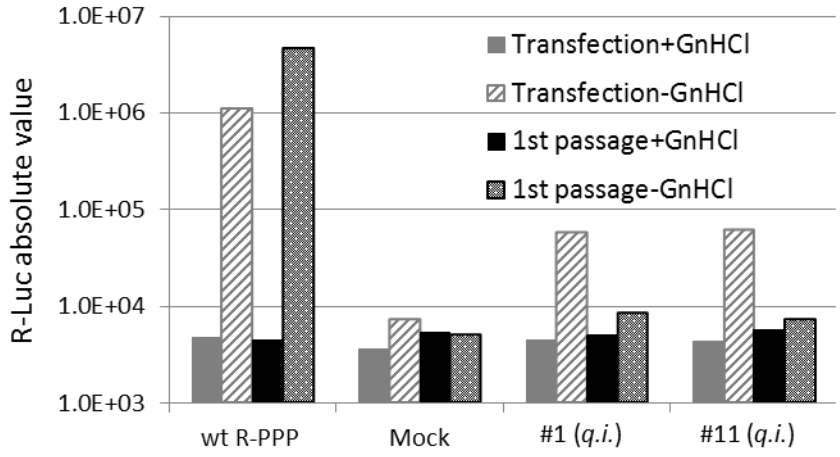
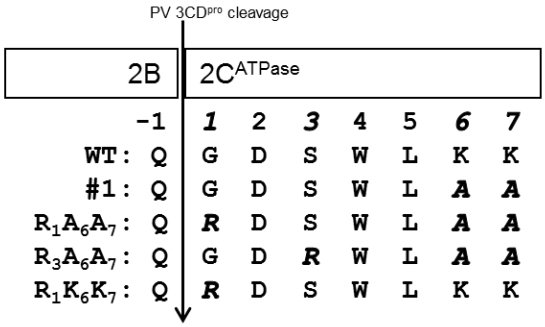


Figure 6. Quasi-infectious mutants #1 and #11 are defective in RNA replication. HeLa R19 cells were transfected with 5 μg of wt or mutant Renilla luciferase reporter virus RNA transcripts both in the presence and absence of 2 mM GnHCl and then they were passaged once at 33 ℃ on HeLa cells. Luciferase activities were determined as described in Materials and Methods.

A.



B.

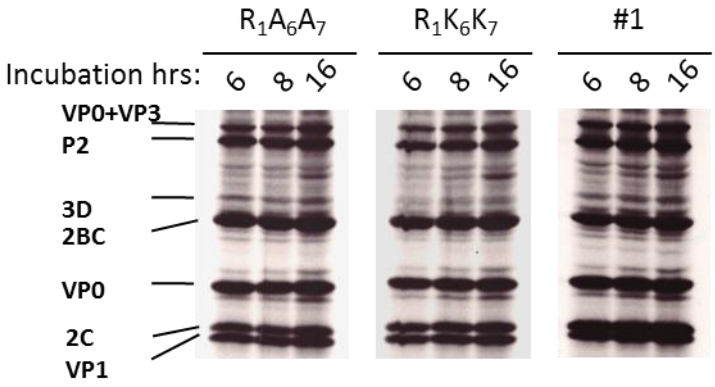


Figure 7. Quasi-infectious mutant #1 yielded suppressor mutants harboring a novel Q^R 3CD^{pro} protease cleavage site at 2B^2C^{ATPase}. (A) Location of suppressor mutations G_1R and S_3R of 2C^{ATPase} alanine scanning mutant #1. The amino acid sequence around the 2B^2C^{ATPase} cleavage site of the wt, mutant #1,$R_1A_6A_7$ (G_1R) and $R_3A_6A_7$ (S3R) suppressor mutants is shown. The G_1R mutation was introduced into the wt PV background ($R_1K_6K_7$) which is also included in the figure. A vertical arrow shows the 3CD^{pro} cleavage site at 2B^2C^{ATPase}. The last amino acid in 2B is indicated by -1, the amino acids of 2C^{ATPase} by 1 to 7. **(B)** *In vitro* translation assays of mutant #1 and two reconstructed suppressor mutants $R_1A_6A_7$ and $R_1K_6K_7$. Translations were carried out in HeLa cell-free extracts *in vitro* at 34 ℃ for 8 hrs (Materials and Methods).

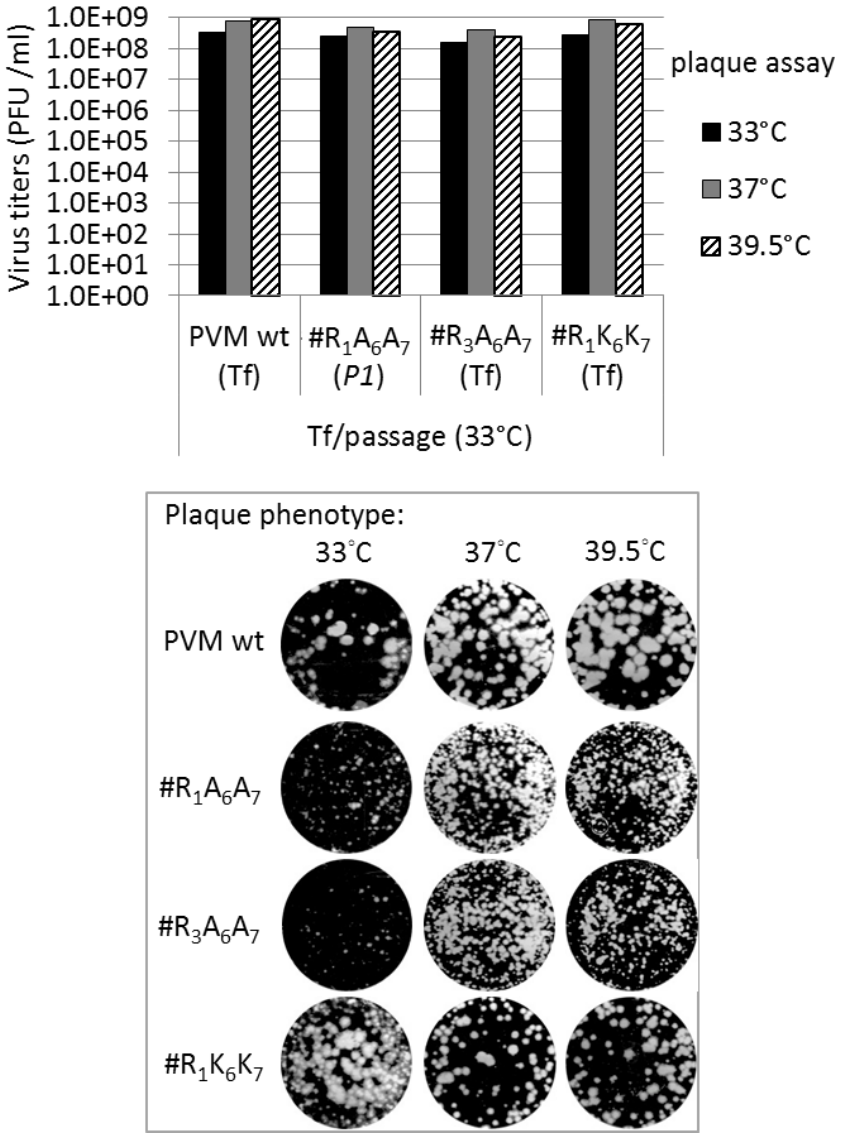
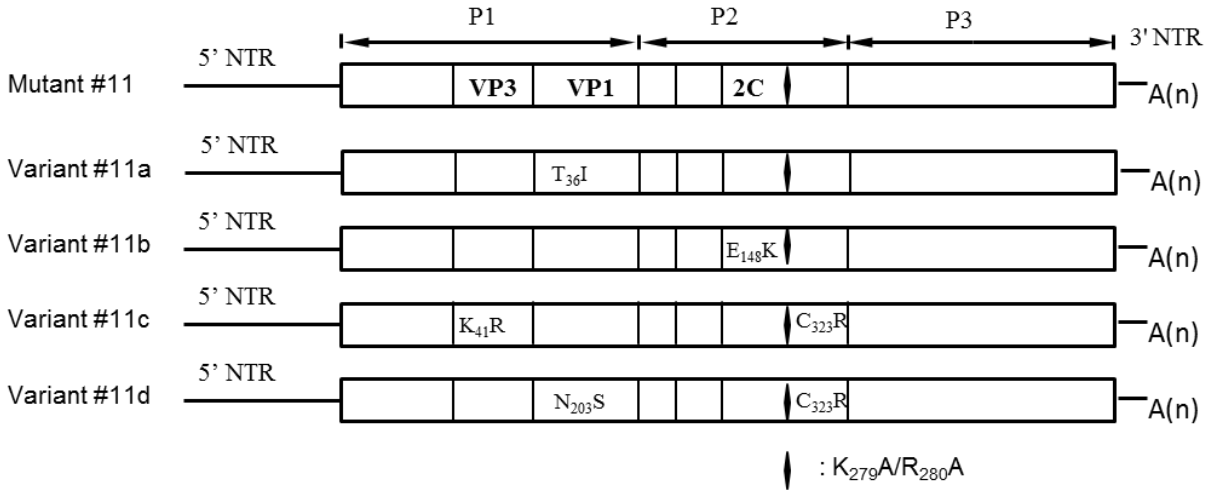


Figure 8. Growth phenotypes of wt and three derivatives of mutant #1. RNA transcripts were transfected into HeLa cells and the lysates were collected at time of full CPE. At 33 ℃ the $R_1A_6A_7$ mutant yielded full CPE only at P1. The lysates were titered by plaque assay at 33 ℃, 37 ℃ and 39.5 ℃. The virus titers are shown on the top and the plaque phenotypes at the bottom of the figure.

A



Variants	genotypes	Transfection ,*P1*	Plaque assay, enrichment	Replication /encapsidation phenotypes	*ts phenotype*
#11a	#11($K_{279}A/R_{280}A$)+VP1($T_{36}I$)	33˚C	33˚C	+	+
#11b	#11($K_{279}A/R_{280}A$)+2C($E_{148}K$)	33˚C	39.5˚C	+	-
#11c	#11($K_{279}A/R_{280}A$)+2C($C_{323}R$)+VP3($K_{41}R$)	39.5˚C	33˚C	+	-
#11d	#11($K_{279}A/R_{280}A$)+2C($C_{323}R$)+VP1($N_{203}S$)	39.5˚C	33˚C	+	-

B

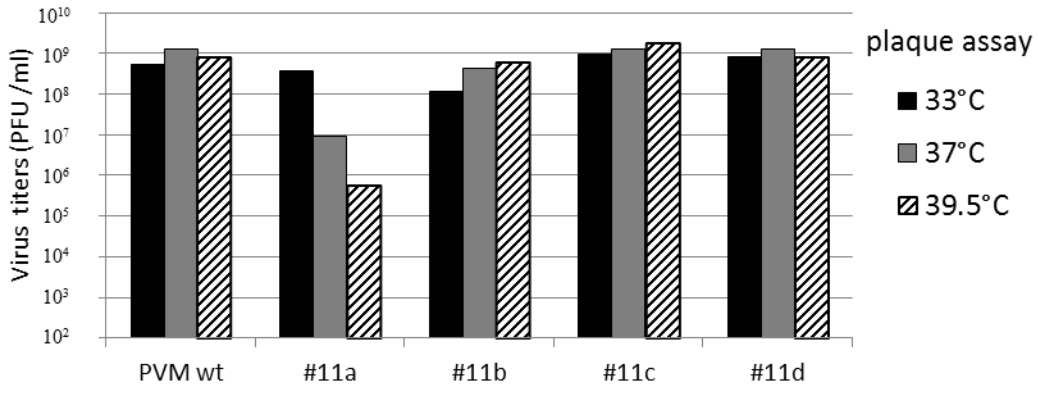
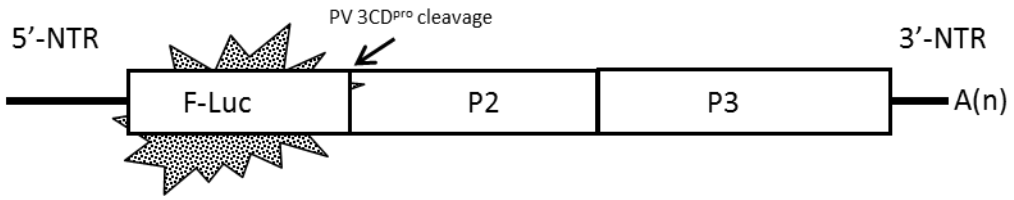


Figure 9. Genotypes and growth phenotypes of four suppressor variants of mutant #11 ($K_{279}A/R_{280}A$). (A) Four suppressor variants of mutant #11, which all keep the original #11 mutation at $K_{279}A/R_{280}A$, as indicated by the diamond in the schematic genomes. The location of suppressor mutations in $2C^{ATPase}$ (either $C_{323}R$ or $E_{148}K$) and/or capsid proteins VP3 ($K_{41}R$) and VP1 ($T_{36}I$ or $N_{203}S$) are indicated. The genomic structure of mutant #11 and its four suppressor variants are shown on the top. The genotypes and the selection temperatures and phenotypes of the four suppressor variants are summarized in the bottom. **(B)** Growth phenotypes of the four suppressor variants of mutant #11. Plaques were picked and amplified at the temperature indicated in Figure 6.A and the lysates were titered by plaque assay at 33 ℃, 37 ℃ and 39.5 ℃.

A



B

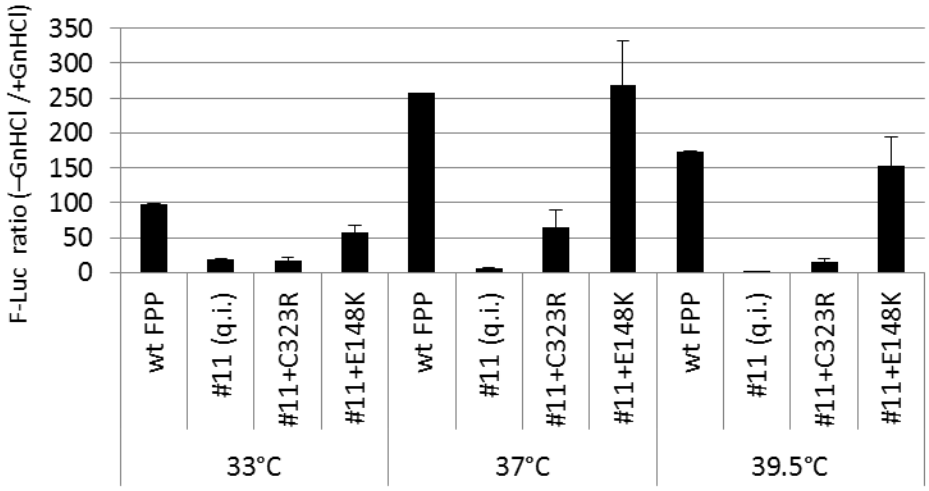


Figure 10. Analysis of $C_{323}R$ and $E_{148}K$ suppressor mutations on #11's replication. (A) The genome structure of the FPP replicon was shown. P1 replaced by F-Luc. **(B)** RNA replication of wt PV, mutant #11 and its variants with suppressor mutations in $2C^{ATPase}$, were assayed with a F-Luc replicon (FPP) at 33 ℃, 37 ℃, 39.5 ℃ in the absence and presence of GnHCl (Materials and Methods). The ratio of F-Luc observed in the absence and presence of GnHCl is plotted.

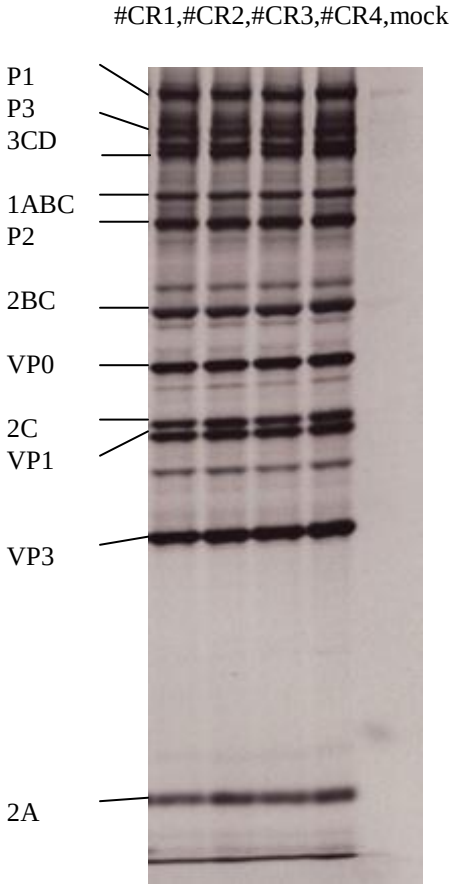
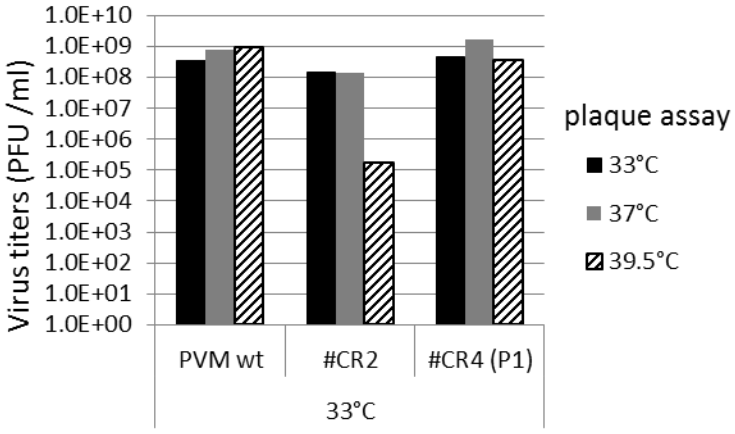


Figure. 11. *In vitro* translation of RNA transcripts of four cysteine rich sites mutants. RNA transcripts (200 ng) derived from four mutant constructs were translated *in vitro* in HeLa cell-free extracts at 34 ℃ for 8 hrs, as described in Materials and Methods. Positions of the precursor and mature proteins are indicated. None of the four mutants exhibit a shift in the migration of their $2C^{ATPase}$-related proteins (P2, 2BC and $2C^{ATPase}$).

A



B

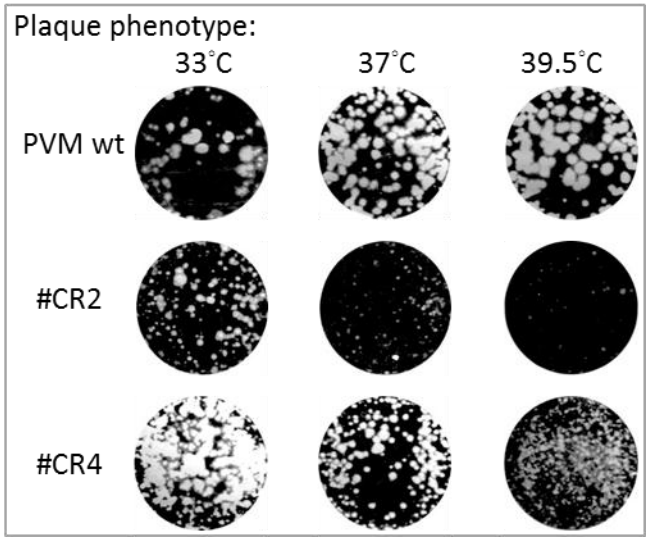
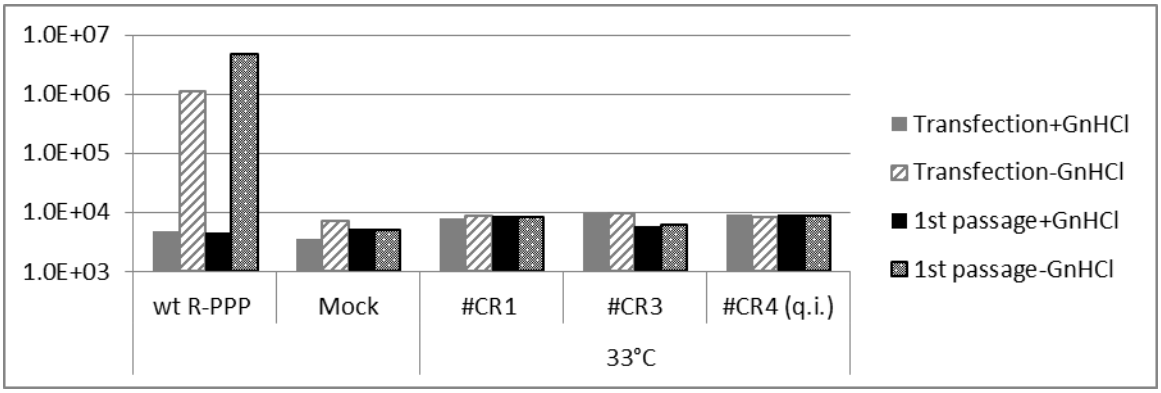


Figure 12. Growth phenotypes of two viable cysteine rich sites mutants #CR2 and #CR4. Growth phenotypes of mutants #CR2 and #CR4. Corresponding mutant RNA transcripts were transfected into HeLa cells and the lysates were collected at time of full CPE. At 33 ℃ mutant #CR2 yielded full CPE at transfection and #CR4 mutants yielded full CPE only at P1. The full CPE lysates at 33 ℃ were titered by plaque assay at 33 ℃, 37 ℃ and 39.5 ℃. The virus titers are shown in panel **(A)** and the plaque phenotypes in panel **(B)**.

A



B

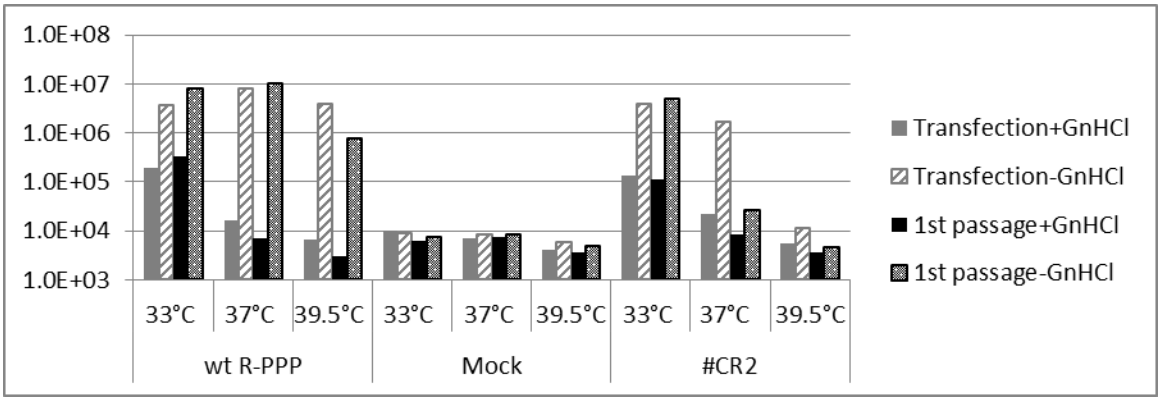


Figure 13. Renilla reporter virus assay of four cysteine rich sites mutants. (A). Mutants #CR1, #CR3 and #CR4 showed replication defects using Renilla reporter virus assay. **(B).** Mutant #CR2 has replication and encapsidation defects at higher temperature. HeLa R19 cells were transfected with 5 μg of wt or mutant Renilla luciferase reporter virus RNA transcripts both in the presence and absence of 2 mM GnHCl and then they were passaged once at 33 ℃, 37 ℃ and 39.5 ℃ as needed on HeLa cells. Luciferase activities were determined as described in Materials and Methods.

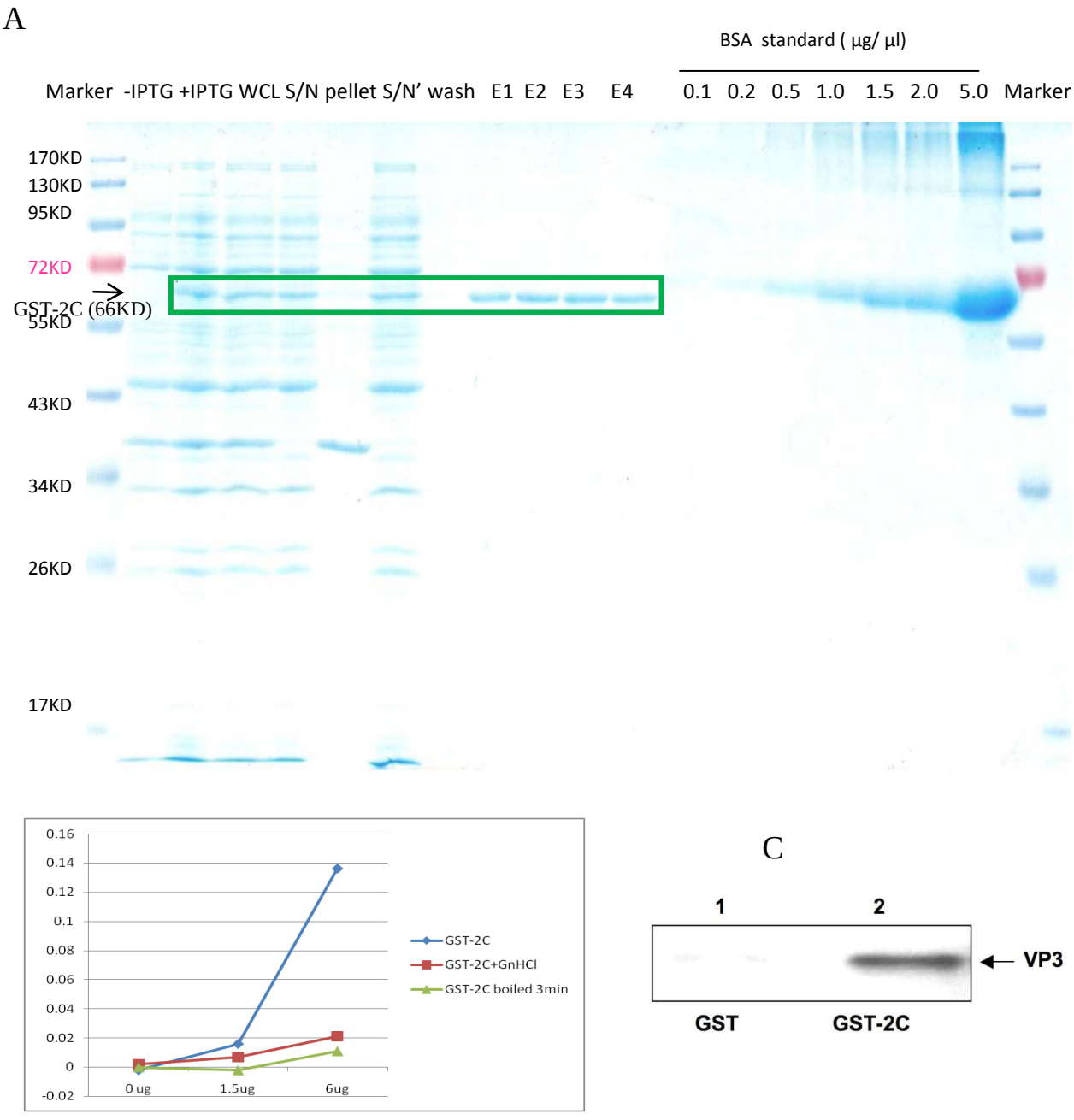


Figure 14. Direct interaction between PV GST2C^{ATPase} and VP3-His. (A) Coomassie-stained SDS-PAGE gel of purification of GST-2C. The location of GST-2C is highlighted in green box and indicated by arrow. (B) ATPase activity of GST-2C. (C) GST-2C^{ATPase} pull down VP3-His. GST was used as a negative control.

Table 1. Capsid intermediates in the assembly of poliovirus.

	precursor protein	Capsid intermediates	Sedimentation Coefficient
I	VP0,VP3, VP1	protomer	5S
II	$(VP0,VP3, VP1)_5$	pentamer	14S
III	$[(VP0,VP3, VP1)_5]_{12}$	empty capsid	75S
VI	$[(VP0,VP3, VP1)_5]_{12}$RNA	provirion	150S
V	$[(VP4,VP2, VP3, VP1)_5]_{12}$RNA	virion	150S

Table 2. Summary of the full length sequencing strategy and list of primers used.

Step 1. Full length reverse transcribe	Make cDNA using primer P36 (-PVM/ nt 7441-7424)										
Step 2. Expand long templates PCR	PCR fragment A				PCR fragment B				PCR fragment C		
	P1-P14 (2928bp) P14 (-PVM/ nt 3286-3267)				P13-P28 (2600bp)				P27-P36 (2181bp)		
Step 3. Primers used for sequencing (PVM nt)	P1	P5	P7	P11	P13	P15	P20	P28-	P27	P33-	P36-
	+ (358-379)	+ (1208-1226)	+ (1657-1678)	+ (2477-2496)	+ (2947-2968)	+ (3521-3543)	+ (4154-4172)	- (5567-5547)	+ (5260-5278)	- (6881-6861)	- (7441-7424)
Step 4. analyze sequencing data (PVM nt)	400-1300	1300-2200	2200-2600	2600-3200	3200-3900	3900-4400	4300-5000	5000-5400	5400-6300	6300-6800	6400-7400

Table 3. Suppressor mutations revealed by full length sequencing analysis in #11 (*q.i.*) mutants of PV 2C^{ATPase}

Alanine scanning mutant	Growth temperature		Suppressor mutations	
	Tf/*P1*	Plaque & amplify	in 2C^{ATPase}	in capsids
#11 (q.i.) ($K_{279}A/R_{280}A$)	33 ℃	33 ℃	-	VP1-T_{36}I (nt 2586)
		39.5 ℃	E_{148}K (nt 4565)	-
	39.5 0 ℃	33 ℃	C_{323}R (nt 5090)	+VP3-K_{41}R (nt 1887)
			C_{323}R (nt 5090)	+VP1-N_{203}S (nt 3087)

Table 4. Amino acid sequence conservation at the sites of alanine scanning of PV 2C^{ATPase} mutants among human C cluster enteroviruses.

Mutants/ suppressor mutations	Amino acids mutated	aa sequence conservation	Variations of aa among human C cluster enteroviruses
Group I (3/16) Wt-like	#2. $K_{33}/E_{34}/K_{35}$	Low[a]	$K_{33}R/E_{34}(N/T/C)/K_{35}R$
	#4. K_{49}/R_{51}	High[b]	only one $R_{51}K$
	#10.R_{256}/D_{257}	Low	$R_{256}(V/I/T)/D_{257}(K/R)$
Group II (1/16) *ts*	#7. $E_{148}/R_{149}/E_{150}$	Low	$E_{148}K/R_{149}(K/Q)/E_{150}A$
Group III (10/16) lethal	#3. $R_{41}/D_{42}/K_{43}$	High	$R_{41}K$
	#5. K_{88}/R_{89}	High	$K_{88}R/R_{89}K$
	#6. $E_{97}/K_{99}/R_{100}$	High	$K_{99}R$
	#8. R_{240}/R_{241}	High	None
	#9. D_{245}/D_{247}	High	$D_{247}E$
	#12.D_{294}/K_{295}	High	only one $K_{295}R$
	#13.R_{298}/R_{300}	High	None
	#14.$E_{313}/R_{314}/R_{316}/R_{317}$	High	$R_{314}K$
	#15. $D_{183}/D_{186}/K_{188}$	High	None
	#16. $E_{207}/E_{208}/K_{209}$	High	None
Group IV (2/16) *q.i.*	#1.K_6/K_7	High	None
	#11.K_{279}/R_{280}	Low	$K_{279}S/R_{280}(K/T)$
Suppressor mutations	G_1	High	Only one G_1S
	S_3	High	Only one S_3G
	E_{148}	low	$E_{148}K$ in CAV1/19/22
	C_{323}	High	None

[a] :low is defined as when amino acids vary in electronic charge.

[b] :high is defined as when amino acids are 100% conserved in electronic charge, some of which may have amino acid changes from K to R, R to K, D to E,G to S, or S to G as listed above. Numbering of amino acid residues is according to the PVM sequence.

Table 5. List of the four cysteine rich sites-to-alanine mutants of PV 2C^{ATPase} and the corresponding amino acid and nucleotide changes.

PV 2C^{ATPase} Mutants	Wild type codons	Alanine codons
#CR1. ($C_{269}A$)	TGT	gcT
#CR2. ($C_{272}A/H_{273}A$)	TGT CAC	gcT gcC
#CR3. ($C_{281}A/C_{282}A$)	TGC TGT	gcC gcT
#CR4. ($C_{286}A$)	TGT	gcT

Table 6. Summary of the growth phenotypes and *in vitro* translation phenotypes of the four cysteine rich sites-to-alanine mutants of PV 2C^{ATPase}.

Growth phenotypes	Alanine scanning mutants	Time of full CPE (Tf,*P1-P4*)			Translation and polyprotein processing	P2/2BC/2C migration on SDS-PAGE gel
		33 ℃	37 ℃	39.5 ℃		
ts	#CR2. $C_{272}A/H_{273}A$	Tf	Tf	*P1*	+	+
lethal	#CR1. $C_{269}A$	-	-	-	+	+
	#CR3. $C_{281}A/C_{282}A$	-	-	-	+	+
q.i.	#CR4. $C_{286}A$	*P1/-*	*P1/-*	*P1/-*	+	+

ts: temperature sensitive
q.i.: quasi-infectious
Tf: transfection
P1, 2…: passages 1, 2…

Table 7. Suppressor mutations revealed by full length sequencing analysis in #CR2 (*ts*) and #CR4 (*q.i.*) mutants of PV 2C^{ATPase}.

Alanine scanning mutants	Growth temperature		Reversion or suppressor mutations
	Tf/*P1*	Plaque & amplify	in 2C^{ATPase}
#CR2 (*ts*) ($C_{272}A/H_{273}A$)	33 ℃	33 ℃	-
		39.5 ℃	**$A_{138}V$** (nt4536,C/T)
			$M_{293}V$ (nt 5000,A/G) +/-$N_{271}H$ (nt 4934,A/C)
	39.5 ℃	39.5 ℃	**$K_{295}R$** (nt 5007,A/G)
#CR4 (*q.i.*) ($C_{286}A$)	33 ℃	33 ℃	$A_{286}D$ (nt 4980,C/A)
		39.5 ℃	$A_{286}D$ (nt 4980,C/A)
			$A_{286}D$ (nt 4980,C/A) +**$A_{138}V$** (nt 4536,C/T)
	39.5 ℃	39.5 ℃	$A_{286}D$ (nt 4980,C/A) +**$M_{293}V$** (nt 5000,A/G)
			$A_{286}D$ (nt 4980,C/A) +**$K_{295}R$** (nt 5007,A/G)

Table 8. The cleavage sites of 2A^{pro} and 3CD^{pro} among enteroviruses and HAV.

Proteinase and genus	**Cleavage site**	**Cleavage site**	**Reference**
	-1st AA	**+1st AA**	
2A(22 unique sites from entero- including rhinovirus)	Y>A>T>V>F>R	G	Protein Science (1996). 52203-2216.
3C(114 unique sites from entero- including rhinovirus)	Q>E	G>N>S>A	Protein Science (1996). 52203-2216.
3C(PV)	Q	G	natural PV,J Virol. 1999 December;
3CD(PV)(GG virus 3B^3C)	E	G	J Virol. 2009 Sep;83(18):9370-87.
3C(HAV) (3C^3D cleavage site)	**Q**	**R**	**J Virol. 1999 Dec;73(12):9867-78.**

Chapter IV. Yeast Two Hybrid Analyses of PV nonstructural protein $2C^{ATPase}$ Interaction with Cellular Protein(s)

INTRODUCTION

Because of the limited amount of genetic information available in the small genome of poliovirus (PV), it uses not only the mature viral proteins but also their precursors. In addition, PV utilizes not only viral proteins, *cis*-replicating RNA elements, and the cellular translation machinery but also cellular proteins for the proliferation of its genome.

It is commonly accepted that specific cellular factors termed host factors interact with the genomic RNA of picornaviruses and possibly with some viral structural/non-structural proteins. Individual host factors may be involved in different processes of the viral life cycle, such as internal initiation of translation, proteolytic processing of the viral polyprotein, and shutdown of host cell protein synthesis, RNA replication and encapsidation of the viral genome. A well-established example is the binding of polypyrimidine tract binding protein (PTB) to the IRES, which is implicated in internal initiation of cap-independent translation of several picornaviruses, including poliovirus (Jang and Wimmer 1990; Hellen, Witherell et al. 1993; Hellen, Pestova et al. 1994). Another well-known example of a cellular protein involved in RNA synthesis is PCBP2 (poly (rC) binding protein 2) (Blyn, Chen et al. 1995), which forms complexes with the 5' NTR of the PV genome (Andino, Rieckhof et al. 1990; Andino, Rieckhof et al. 1993; Parsley, Towner et al. 1997) by binding to the 5'-terminal cloverleaf (Gamarnik and Andino 2000) and an adjacent C-rich spacer region (Parsley, Towner et al. 1997; Gamarnik and Andino 2000; Toyoda, Franco et al. 2007) in a complex with $3CD^{pro}$. This interaction is required for RNA replication (Andino, Rieckhof et al. 1990). PCBP2 can also bind to stem loop IV of the IRES and regulates the initiation of translation (Gamarnik and Andino 2000). It is proposed that PCBP2 mediates the switch from viral translation to RNA replication through the loss of the KH3 domain, which is cleaved by $3C^{pro}/3CD^{pro}$, thus loss of its ability to function in translation (Perera, Daijogo et al.

2007). Although the mechanism remains obscure, several other proteins have been proposed to be involved in PV proliferation such as La autoantigen (Meerovitch, Svitkin et al. 1993), unr (upstream of N-ras) (Hunt, Hsuan et al. 1999) and PABP (poly (A) binding protein), which interacts with the poly(A) tail (Herold and Andino 2001; Svitkin, Imataka et al. 2001) and might be involved in the IRES function of picornaviruses; Sam68 interacts with $3D^{pol}$ (McBride, Schlegel et al. 1996); hnRNPC binds to $3CD^{pro}$ and the minus strand cloverleaf (Brunner, Nguyen et al. 2005); nucleolin interacts with the 3' NTR (Waggoner and Sarnow 1998).

As one of the most conserved non-structural viral proteins amongst picornaviruses, the 329 aa long $2C^{ATPase}$ polypeptide appears to be multifunctional and is involved in multiple protein/protein and protein/RNA interactions. All of the nonstructural proteins of PV, including their processing precursors, are required for the replication of the viral RNA. These proteins assemble into replication/encapsidation complexes in association with cellular factors and viral RNA. PV $2C^{ATPase}$ is an indispensable component of the replication/encapsidaiton complex and a number of functional domains have been identified. Since a single protein carries out so many different functions, it is likely that this is achieved by interaction with other viral and cellular proteins. Interestingly, Tang et al. (Tang, Yang et al. 2007) identified RTN3 as an interacting partner of enterovirus 71 (EV71) 2C using the N-terminal sequence of EV71 $2C^{ATPase}$ against a human fetal brain cDNA library in a yeast two hybrid (Y2H) analysis.

As described in Chapter I.8, previous lab members have carried out yeast two-hybrid analyses (results summarized in Chapter I, **Table 1.**) to determine whether protein-protein interactions occur among the polypeptide cleavage products of the P2 and P3 regions of the PV polyprotein, including a protein linkage map of the P2 nonstructural proteins (Cuconati, Xiang et al. 1998), the P3 proteins (Xiang, Cuconati et al. 1998) and between P2 and P3 regions (Yin, Liu

et al. 2007). Recent advances in biotechnology has introduced a new method that is yeast mating between a mating type a (MATa) and a mating type α (MATα) strains, which eliminates the need for library-scale yeast co-transformations. Clontech Matchmaker™ Pretransformed Libraries are high-complexity cDNA libraries cloned into a GAL4 AD vector and transformed into yeast strain Y187 (MATα). To screen this pretransformed library, bait strain AH109 (MATa), which is transformed with a 2C-BD vector containing our bait construct 2C, is combined and incubated for 20-24 hours before plating the mixture on synthetic drop-out (Fuller, von Bonsdorff et al.) (Fuller, von Bonsdorff et al.) medium. SD medium, which lacks one or more essential nutrients, selects for diploid clones expressing interacting proteins. If a positive interaction occurs, the diploid Y187-AH109 strain will express dual nutritional reporters (His and Ade) and both α- and β-galactosidase (Chapter I, **Figure 5.**). Positive interactions can be quickly and easily identified using X-α-Gal in the selection plates (-His-Ade) causing positive yeast colonies to turn blue, or with a standard β-galactosidase colony filter-lift assay.

Since human HeLa cells are the common cell line used for growing PV in the lab (Ackermann, Rabson et al. 1954; Syverton, Scherer et al. 1954), if any interaction between $2C^{ATPase}$ with its cellular partners is required, I should be able to detect it if genes that encode proteins important for replication are represented in this HeLa cDNA library. To achieve this, I used the Y2H assay using the full length $2C^{ATPase}$ sequence fused with the GAL4 binding domain (BD) vector as bait and screened the Clontech Matchmaker pretransformed HeLa cell cDNA library fused to the Gal4-activatin domain vector. Several cellular factors were identified that may interact with PV non-structural protein $2C^{ATPase}$. The roles of these candidates in PV replication and/or assembly of PV viral particles, if any, need further investigations.

RESULTS

IV.1 A PV 2C^{ATPase} bait plasmid was constructed and tested for its suitability in an Y2H analysis

The plasmid pGBKT7-PV2C was constructed as bait, in which the full length PV2C^{ATPase} coding sequence was fused to the DNA binding domain of GAL4 in vector pGBKT7.

Before doing the Y2H screening, some preliminary experiments were performed: Testing the bait plasmid for expression, toxicity effects and transcriptional activation. (a) First, I checked the expression of the bait plasmid. Yeast protein extracts were prepared using Urea/SDS Method according to the Yeast Protocols Handbook Protocol (Clontech). As shown in **Figure 1.A**, the fusion bait protein with the expected size of about 70KDa was detected in Western blot using monoclonal 2C^{ATPase} antibody. (b) Then I checked for toxicity of the bait protein on the host strain. Fortunately the fusion protein shows little, if any, toxicity effects on the host strain AH109 (MATa) by comparing the growth rate in liquid culture of cells transformed with the DNA-BD/bait plasmid and cells transformed with the empty DNA-BD vector (**Figure 1.B**). (c). In addition, I tested the BD-2C^{ATPase} bait protein for transcriptional activation and ruled out the possibility of auto-transcriptional activity by the Gal4 BD-2C^{ATPase} bait alone, or together with an empty Gal4 AD (data not shown).

IV.2 Y2H screening of a HeLa cell cDNA library by the PV 2C^{ATPase} bait

Since PV invades and replicates well in Human HeLa cells, genes that encode proteins important for replication should be represented in this HeLa cDNA library. Hypothetically, if any interaction between 2C^{ATPase} and a cellular partner is required, I should be able to detect it.

To achieve this, I performed Y2H assays using the full length $2C^{ATPase}$ sequence fused with the GAL4 binding domain (GAL4-BD), and screened the HeLa cell cDNA library fused with the GAL4 activating domain (GAL4-AD). The library titer is 10^7 clones with a mating efficiency of about 10%, so the final number of the clones tested is 10^6, which is representative of the mRNA population complexity (Ausubel 1994).

Putative positive clones were verified by the following strategy: retest phenotypes, isolate plasmid DNA from yeast, eliminate duplicates by PCR and restriction enzyme digestion, sequencing and perform yeast mating to confirm interaction. About 100 positive clones were identified, based on their ability to activate all of the reporters present in the yeast.

IV.3 Candidates that showed interaction with PV $2C^{ATPase}$ identified by Y2H assay

Among the positive clones, CREB3 and SPCS2 were detected in several clones. Another three candidates (RDH11, PMF1, and AARS) were detected only once but showed strong interactions with $2C^{ATPase}$. Two represent full-length proteins (RDH11, PMF1) while the third (AARS) was only a 161 aa long fragment of the complete polypeptide (968 aa). In addition, ARFIP2, GNL3 (guanine nucleotide binding protein-like 3 (nucleolar)), Damage-specific DNA binding protein 1, and PPARA (peroxisome proliferative activated receptor, alpha) were also identified as possible $2C^{ATPase}$ interacting partners by the Y2H assay. The open reading frames of these candidates were confirmed to be correct.

ADP-ribosylation factor interacting protein 2 (ARFIP2) / Partner of RAC1 (POR1)

ADP Ribosylation Factors (ARFs) are members of the ARF family of GTP-binding proteins of the Ras superfamily. ARF family proteins are ubiquitous in eukaryotic cells, and six highly conserved members of the family have been identified in mammalian cells. Although ARFs are

soluble, they generally associate with membranes because of N-terminus myristoylation. They function as regulators of vesicular trafficking between the endoplasmic reticulum and the cis-Golgi compartment and actin remodeling. For PV and CVB3, both members of the enterovirus genus of the picornavirus family, previous data have shown that GBF1, a guanine nucleotide exchange factor (GEF) of the small Ras-family GTPase Arf1, was required for enteroviral RNA replication (Belov, Altan-Bonnet et al. 2007; Belov, Feng et al. 2008; Lanke, van der Schaar et al. 2009). As summarized by Hsu et al. (Hsu, Ilnytska et al. 2010), "GBF1 catalyzes GDP/GTP exchange on Arf1, stabilizing membrane association, which in turn recruits various effectors to these membranes. In uninfected mammalian cells, GBF1 and Arf1 are both localized to the ER, ER-Golgi intermediate compartment (ERGIC), and the Golgi apparatus. Arf1's known major effectors at these sites include coat proteins such as COPI complex and clathrin, which regulate membrane budding, and phosphatidylinositol-4-kinase IIIβ (PI4- KIIIβ), which catalyzes the production of phosphatidylinositol-4- phosphate (PI4P) lipids at the membrane bilayer."

Arfaptin-2 is a protein that in humans is encoded by the ARFIP2 gene. ARFIP2 has many synonyms, partner of RAC1 (POR1) (Van Aelst, Joneson et al. 1996). ARFIP2 is a ubiquitously expressed protein implicated in mediating cross talk between RAC and ARF small GTPases. It has been shown that ARFIP2 binds specifically to GTP-bound ARF1 and ARF6, but binds to Rac-GTP and Rac-GDP with similar affinities. The X-ray structure of arfaptin reveals an elongated, crescent-shaped dimer of 3-helix coiled-coils.

Many RNA viruses, including PV, need to remodel the intracellular membranes for viral RNA replication. Brefeldin A (BFA), an inhibitor of intracellular vesicle-dependent secretory transport, is a potent inhibitor of poliovirus RNA replication both in infected cells and in the cell-free translation/replication and encapsidation system (Cuconati, Molla et al. 1998). This is

related to the ARF (ADP-ribosylation factor) family of GTP-binding proteins. Normally GBF1 activates the ADP-ribosylation factor (Arf) GTPases necessary for formation of COPI transport vesicles. BFA is an inhibitor of Arf activation by cellular protein GBF1. BFA targets the catalytic Sec7 domain of GBF1. A recent study has shown that PV replication requires the N-terminus but not the catalytic Sec7 domain of GBF1 (Belov, Kovtunovych et al. 2010). Lanke et al. reported a critical role of GBF1 in CVB3 RNA replication also (Lanke, van der Schaar et al. 2009). Recently Hsu et al. proposed a model for secretory pathway reorganization during enteroviral infection. Their data showed that GBF1/ARF1 in the context of enteroviral protein 3A may facilitate recruitment of PI4KIIIβ at the expense of coat protein COPI in infected cells, yielding PI4P lipid-enriched organelles which will in turn facilitate the recruitment of $3D^{pol}$ to the membrane thus essential for viral RNA replication (Hsu, Ilnytska et al. 2010).

Since PV $2C^{ATPase}$ and its precursor $2BC^{ATPase}$ are the major viral proteins to induce the intracellular membrane structures for RNA replication, in the future I am interested to find out whether $2C^{ATPase}$/$2BC^{ATPase}$'s interaction with ARFIP2, if any, will have any role in the reorganization of the secretory pathway to form the replication/encapsidation complex. It will also be interesting to find out whether there is a complex formed by both cellular and viral proteins like this: 3A—GBF1—ARF1—ARFIP2—2C—3A?

Luman/ cAMP responsive element binding protein 3 (CREB3)/ LZIP

Luman/CREB3, also known as LZIP, is an ER-bound cellular transcription factor that is a member of the leucine zipper family of DNA binding proteins. This protein has been implicated in the mammalian unfolded protein response (UPR). Under ER stress, CREB3 proteins are cleaved and translocated into the nucleus and activate downstream target genes as summarized in

(Audas, Li et al. 2008). It binds to the cAMP-responsive element (CRE), an octameric palindrome.

Luman/CREB3 was first identified through its association with herpes simplex virus-related host cell factor (HCF) (Lu, Yang et al. 1998). The protein interacts with host cell factor 1(HCF-1), which also associates with the herpes simplex virus (HSV) protein VP16 that induces transcription of HSV immediate-early genes (Lu, Yang et al. 1998). This protein and VP16 both bind to the same site on host cell factor 1. It is thought that the interaction between this protein and host cell factor 1 plays a role in the establishment of latency during HSV infection.

As reviewed by Asada et al (Asada, Kanemoto et al. 2011), the Luman transcript is present in a wide range of adult and fetal tissues, but its translated protein has only been found in trigeminal ganglional neurons and monocytes, and dendritic cells (DCs), which are the professional antigen-presenting cells (APCs). Processing of Luman at the transmembrane domain is highly stimulated by brefeldin A (BFA), the compound mentioned above that causes the reflux of the Golgi apparatus enzymes to the ER.

It was also reported that NS2 protein of HCV activates cellular cAMP-dependent pathways (Kim, Kwon et al. 2007).

Microsomal signal peptidase 25 kDa subunit (SPCS25), similar to Signal peptidase complex subunit 2 (SPCS2)

Microsomal signal peptidase 25 kDa subunit (SPC25), 226 aa long, is located in the ER. This family consists of several microsomal signal peptidase 25 kDa subunit proteins. Translocation of polypeptide chains across the endoplasmic reticulum (ER) membrane is triggered by signal sequences. Subsequently, signal recognition particle interacts with its membrane receptor and the ribosome-bound nascent chain is targeted to the ER where it is transferred into a protein-

conducting channel. At some point, a second signal sequence recognition event takes place in the membrane and translocation of the nascent chain through the membrane occurs. The signal sequence of most secretory and membrane proteins are cleaved off at this stage. Cleavage occurs by the signal peptidase complex (SPC) as soon as the luminal domain of the translocating polypeptide is large enough to expose its cleavage site to the enzyme. The signal peptidase complex is possibly also involved in proteolytic events in the ER membrane other than the processing of the signal sequence, for example the further digestion of the cleaved signal peptide or the degradation of membrane proteins. Mammalian signal peptidase is as a complex of five different polypeptide chains, including this family of the 25 kDa subunit (SPC25).

Retinol dehydrogenase (RDH11)/ RalR1 or RDH5

RDH11, also called, RalR1 or RDH5, belongs to a class of Short-chain dehydrogenases/reductases (SDR). SDR is a large family involved in the metabolism of steroids, prostaglandins, retinoids, aliphatic alcohols, and xenobiotics. RDH11 shares conserved motifs with the SDR family including (1) an N-terminal coenzyme-binding motif, NADP (H) and (2) a C-terminal catalytic domain.

RDH11 participates in retinol metabolism. Retinal, also known as retinaldehyde, can be reversibly reduced to produce retinol or it can be irreversibly oxidized to produce retinoic acid. RDH11 can recognize all-*trans*-retinal (vitamin A) and all-*trans*-retinol and is in charge of catalyzing this bidirectional reaction. For example, RDH11 catalyzes the oxidation of 11-cis-retinol to 11-*cis*-retinal, the chromophore of visual pigments. Retinoids (retinal and retinol) have many functions: (1) indispensable light-sensitive elements of vision; (2) maintains intact epithelial tissues as a physical barrier to infection; (3) maintains a number of immune cell types

(a) lymphocytes (B-cells, T-cells, and natural killer cells), (b) myelocytes (neutrophils, macrophages, and myeloid dendritic cells).

RDH11 is abundant in the retinal pigment epithelium of the eye. Western blot analysis showed that RalR1(RDH11) is expressed in a wide variety of normal human tissues such as liver, kidney, spleen, jejunum, lung, testis, prostate, brain and is present at detectable levels in various cancer cells such as LNCap prostate cancer cells, HepG2 hepatocytes, HEK-293embryonal kidney cells, and neuroblastoma cells lines, SK-N-SH and SH-SY5Y (Olga V. Belyaeva 2003). The mRNA of the RDH11 gene contains 2538 nucleotides. Our clone stands for the splicing isoform 2, which is 305aa. This enzyme is anchored in the ER membrane by the N-terminal signal-anchor domain, with the majority of the polypeptide chain located on the cytosolic side of the membrane. The cytosolic orientation suggested that, in intact cells, the enzyme would function predominantly as a reductase (RalR1) (Olga et al., 2003). Delayed dark adaptation in 11-*cis*-retinol dehydrogenase-deficient mice was observed, which indicate a role of RDH11 in visual processes *in vivo* (Kim, Maeda et al. 2005). Interestingly, RDH11 also interacts with the core protein of hepatitis C virus, a Flavivirus, closely related to picornaviruses (Li, Wang et al. 2003).

Polyamine-modulated factor 1 (PMF1)

PMF1 has been identified as a new interacting partner of the Nrf-2 transcription factor that, together with Nrf-2 regulates the polyamine analogue-induced transcription of SSAT (spermidine/spermine N1-acetyltransferase). The human PMF1 gene is located on chromosome 1; it yields an mRNA transcript of about 1.1 kilobases that codes for a 205 aa long polypeptide. In addition to the transcriptional regulation of SSAT, PMF1 might be involved in the regulation of other polyamine-dependent genes.

Alanyl-tRNA synthetase (AARS).

The human alanyl tRNA synthetase belongs to a family of tRNA synthetases (Class II), which recognize the RNA code and attach specific amino acids to the tRNA that contains the cognate trinucleotide anticodons. The full-length protein contains 968 aa but our clone codes only for a 161 residue long fragment near the C-terminus of the polypeptide (aa 718-938). The full-length protein contains 9 conserved domains of which only one (DHHA1, aa 886-955) is contained in a complete form in our protein fragment. The DHHA1 domain contains a conserved GG motif and probably has an RNA binding function.

IV.4 Identify the domain of $2C^{ATPase}$ that is required for interaction with some cellular candidates

The N-terminal part of the protein $2C^{ATPase}$ is particularly well conserved. It possesses the ability to bind RNA and cellular membranes and induces vesicle formation through its *N*-terminal amphipathic region in the absence of other viral proteins. Recently using the N-terminal of EV71 2C as bait in Y2H assays, Tang et al. (2007) has identified RTN3 as an important cellular partner of EV71 2C. In an effort to identify the interacting domain of $2C^{ATPase}$, we first made the following construct: $\Delta_{aa\ 10\text{-}32}\ 2C^{ATPase}$, a truncated form of the $2C^{ATPase}$ constructs, in which we deleted residues 10-32 that are located in the N-terminal part of the amphipathic helix. The preliminary Y2H experiments indicated that the truncated $2C^{ATPase}$ lost its ability to interact with two of the cellular polypeptides (RDH11, and PMF1) while it had no effect on the interaction with the AARS fragment (**Table 2.**). More specifically, Paul et al. (1994) have made several mutations in the PV protein $2C^{ATPase}$ amphipathic region including the $I_{25}K$ single amino acid change, and observed that they reduced or abolished RNA replication. Tang et al. (2007) created

the same mutations in the corresponding region of EV71 2C: EV71 2C1 $D_{10}V/E_{19}V$ was mutated at two conserved acidic amino acids that are located at the boundary of the hydrophobic and hydrophilic half. The second mutant (EV71 2C1 $I_{25}K$) contained an isoleucine-to-lysine conversion in the hydrophobic half, and in the third mutant (EV71 2C1 $K_{16}T/K_{24}T$), two lysines were converted to threonines in the hydrophilic half. To test if this isoleucine 25 is critical for the interaction of PV $2C^{ATPase}$ with the candidates, I also used this point mutation $I_{25}K$ (an isoleucine-to-lysine conversion at 25aa in the amphipathic helix of $2C^{ATPase}$). This same mutation was found to abolish the interaction of enterovirus 71 $2C^{ATPase}$ with reticulon 3 from neuronal cells (Tang, Yang et al. 2007). I observed that the $I_{25}K$ mutation of PV $2C^{ATPase}$ strongly reduced the interaction of the protein with one of its cellular partners RDH11 (**Table 1.**), although the mutation had no effect on the interaction with PMF1 and AARS. In addition, $2C^{ATPase}$ ($N_{252}G$) and $2C^{ATPase}$ ($N_{252}S$), in which the mutations are located in the middle part of the protein downstream of the C box, showed no effect at all on the interaction between the $2C^{ATPase}$ mutants and the three candidates. All the candidates are interesting; however, we have previously shown that an I25K mutation in the amphipathic helix of $2C^{ATPase}$ abolishes PV RNA replication (Paul, Molla et al. 1994), so I first concentrated working on RDH11.

To obtain further evidence for the interaction between PV2C and RDH11 detected by yeast two-hybrid analyses, I also performed an *in vitro* GST pull down assay with purified GST-$2C^{ATPase}$ and purified His-RDH11.

First, GST-$2C^{ATPase}$ fusion protein was purified. The pGEX plasmids are designed for inducible, high-level intracellular expression of proteins as fusions with *Schistosoma japonicum* GST. Previous lab members have constructed a plasmid pGEX-2C for the expression of GST fused $2C^{ATPase}$ (Pfister. 1999). I used this plasmid for the expression of GST-2C in E.coli. GST-

2C was purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B contained in the Bulk GST Purification module (GE Healthcare Life sciences). Conditions for the sonication and the elution steps were modified and optimized conditions (group (2)) were used for the final purification procedure (**Figure 2.A**, **Figure 2.B**).The purified GST-2C protein tested positive for ATPase activity (data not shown) following the protocol used by Pfister (1999).

Then pET21 (b+) RDH11was constructed and RDH11-His was purified as described in materials and methods. The GST-$2C^{ATPase}$ was used for the following pull down assays. The pull down assay confirmed the interaction of PV $2C^{ATPase}$ and RDH11 and as a control I used GST protein instead of GST-$2C^{ATPase}$ to demonstrate that the interaction between $2C^{ATPase}$ and the cellular protein is specific (**Figure 2.C**).

In addition, in order to evaluate the biological relevance of these interactions for PV infection I used shRNA knockdown of the 293T endogenous cellular proteins and studied their effect on PV growth. The results of the preliminary experiments showed that the knockdown effect by Origene shRNA for RDH11on the growth of PV is small but promising (data not shown). In the future, the conditions for the knockdown will be optimized such as (1) the amount of shRNA plasmid (0.1 μg, 0.2 μg, 0.5 μg, 1 μg, and 2 μg), (2) different combination out of the 4 constructs will be tested and (3) the best time point for PV infection after transfection will be determined.

IV.5 *In vitro* translation replication results showed that RDH11 is important for the growth of PV

The easiest and most direct way for us to assess the importance of the cellular proteins identified by either yeast two-hybrid analysis is to examine the effect of antibodies to these proteins in an *in vitro* translation/RNA replication system (Molla, Paul et al. 1991). Polyclonal antibodies to RDH11 are commercially available from Abcam. By adding various amounts of the antibodies to the *in vitro* system and check the effect of the antibodies on virus yield using plaque assays. As a negative control, I added heat-inactivated antibodies.

First, the stability of PV viral RNA in the presence of the RDH11 antibody was tested in the *in vitro* translation system. HeLa S10 extracts were prepared (**Figure 3.A**). PV was propagated in HeLa R19 cells and banded on 5–40% CsCl gradients and PV viral RNA was extracted by phenol chlorophyll extraction (**Figure 3.B**).Stability of PV viral RNA in the *in vitro* translation system showed no difference in the absence and presence of untreated or heat-inactivated RDH11 antibody as determined by running the viral RNA on gel (**Figure 3.C**) or quantified by real time PCR (**Figure 3.D**).

As shown in **Figure 4.**, *in vitro* results indicated that RDH11 is involved in either the replication or the encapsidation process. The effect is not at translation level as shown by *in vitro* translation assay. PV viral RNA (750ng) was *in vitro* translated and replicated for 15hr at 34 ℃, as described in materials and methods, in the presence of different amounts of RDH11 monoclonal antibody (50μg to 90μg/ml), untreated or heat inactivated (2 min at 95 ℃). Translation of PV RNA in Hela cell-free extracts was not affected by the presence of RDH11 antibody heat inactivated or untreated (**Figrue 4.A**). Virus yield in the samples were determined by plaque assay on HelaR19 cells. As shown by **Figure 4.B**, the yield of virus in the absence of any RDH11 antibody was 7.5×10^6 PFU/ml. With increasing amount of RDH11 antibody (50 μg/ml to90 μg/ml) added to the *in vitro* system, the virus yield dropped from 7.5×10^6 PFU/ml to

zero. Heat-inactivated antibody showed an unexpected decrease in virus yield, from 7.5×10^6 PFU/ml to 3.6×10^3 PFU/ml. This mild decrease in virus yield turned out to be due to the PBS buffer used for the storage of the antibody (data not shown).

DISCUSSION

The pioneering study by Fields and Song (Fields and Song 1989) was able to generate a novel genetic system to study protein-protein interactions by taking advantage of the properties of the GAL4 transcriptional activator of the yeast *Saccharomyces cerevisiae*. This study was indeed ground breaking as they suggested in the paper that this system may be applicable as a general method to identify proteins interactions by a simple selection. Two-hybrid screens can provide an important first hint for the identification of interaction partners and are now routinely performed in many labs. The BD and AD are each fused to the proteins to be investigated. If these proteins interact and bind, the AD domain is brought into proximity of the transcription start site of the reporter gene and thus activates transcription of genes in charge of the biosynthesis of certain nutrients (usually amino acids or nucleic acids). Whereas the bait protein is typically a known protein that the investigator is using to identify new binding partners, the prey protein can be either a known protein or a library of proteins. If the bait and prey proteins interact (i.e. bind), then the AD and BD domains of the transcription factor are indirectly connected and transcription of the reporter gene(s) occurs.

As part of a search for the *in vivo* function of $2C^{ATPase}$, I undertook the identification of cellular binding partners of this protein that might modulate its activity. To do this I used yeast two hybrid analysis, which we have used extensively in the past in our laboratory. For my current work I selected The Matchmaker Two-hybrid System 3 (Clontech), which is an advanced

GAL4-based two-hybrid system that reduces the incidence of false positives and allows the user to quickly identify and confirm protein interactions. A bait gene is expressed as a fusion to the GAL4 DNA-binding domain while a cDNA is expressed as a fusion to the GAL4 activation domain. I used the full-length $2C^{ATPase}$ bait construct transformed into yeast strain AH109 (MATa strain) as bait to screen the Pre-transformed Matchmaker HeLa cDNA library (Clontech), previously transformed into yeast strain Y187 (MATα).

I inserted the full length PV $2C^{ATPase}$ sequence into plasmid pGBKT7 and transformed the bait construct into yeast strain AH109 (MATa strain). The prey, HeLa cell cDNA library, was commercially available from Clontech. Matchmaker™ pretransformed libraries are high-complexity cDNA libraries cloned into a yeast GAL4 activation domain (AD) vector and pretransformed into *Saccharomyces cerevisiae* host strain Y187. This library is screened by yeast mating rather than by cotransformation, which significantly reduces the time and labor involved in performing two-hybrid screening and more importantly, the mating efficiency (10% in my experiment) is much higher than the co-transformation efficiency, which used to be the critical step for screening libraries. I used the Clontech's Matchmaker system to implement the systematic, genome-wide identification of host genes interacting with $2C^{ATPase}$, which might affect replication of PV. As a general positive two-hybrid control, Y187 (MATα) and AH109 (MATa), pretransformed with plasmids encoding known interacting proteins (SV40 T-antigen and murine p53, respectively), are provided by Clotech Company and included with each Y2H experiment.

Analysis and verification of putative positive clones were carried out by the following procedure: a) retest phenotypes; b) isolate plasmid DNA from Yeast; c) eliminate duplicates by

PCR and restriction enzyme digestion; d) sequencing; e) perform yeast mating to confirm interaction.

I have identified several positive clones that interact with PV $2C^{ATPase}$ in the Y2H assay. CREB3 and SPCS2 were detected in several clones. Another three candidates (RDH11, PMF1, and AARS) were detected only in one clone but showed strong interactions with $2C^{ATPase}$. In addition, ARFIP2, GNL3 (guanine nucleotide binding protein-like 3 (nucleolar)), Damage-specific DNA binding protein 1, and PPARA (peroxisome proliferative activated receptor, alpha) were also identified as possible $2C^{ATPase}$ interacting partners by the Y2H assay. GST pull down assay has shown there is a direct interaction between PV $2C^{ATPase}$ and RDH11, and *in vitro* translation/replication assay has indicated RDH11 is involved in some steps, other than translation, of PV life cycle. Based on previous publications, the most relevant candidates are ARFIP2 and CREB3. In the future it will be interesting to find out whether these candidates, for example ARFIP2 and CREB3, have any role(s) in PV life cycle.

It should be noted that although Tang et al. (2007) have identified reticulon 3 (RTN3) as an interacting partner of enterovirus 71 and of PV $2C^{ATPases}$ using the N-terminal sequence of $2C^{ATPases}$ against a human fetal brain cDNA library, this protein did not appear in our search using full length $2C^{ATPases}$ against HeLa cell cDNA library. One possible reason is that the virus selects different host factors from different tissues for the replication of its RNA. Thus the difference between our results and those of Tang et al. (2007) might be due to the differences between the HeLa cell and human fetal brain cDNA libraries used in the Y2H screening.

MATERIALS AND METHODS

Plasmid. The plasmid pGBKT7-PV2C was constructed as bait, in which the full length PV2C^{ATPase} coding sequence was fused to the DNA binding domain of GAL4 in the vector pGBKT7. Restriction sites adopted were *Eco*RI and *Sal*I.

Preparation of yeast protein extracts. Yeast protein extracts were prepared using the Urea/SDS Method according to the Yeast Protocols Handbook Protocol (Clontech).

Yeast two hybrid screening. Y2H assays were performed using the full length 2C^{ATPase} sequence fused with the GAL4 binding domain (GAL4-BD), and screened the HeLa cell cDNA library fused with the GAL4 activating domain (GAL4-AD) according to the Matchmaker pretransformed libraries user manual.

Purification of the GST-2C^{ATPase} fusion protein. I used plasmid pGEX-2C (Pfister and Wimmer 1999) for the expression of GST-2C in E.coli. GST-2C^{ATPase} was purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B contained in the Bulk GST Purification module (GE Healthcare Life sciences). Conditions for the sonication and the elution steps were modified and optimized conditions were used for the final purification procedure.

ATPase assay. The ATPase activity of purified 2C was tested following the protocol used by Pfister et al. (Pfister and Wimmer 1999)

Purification of a RDH11-His fusion protein. The coding sequence of RDH11 was amplified by PCR from the RDH11 plasmid which was isolated from a positive colony in the Y2H assay. The PCR product and pET21 (b+) vector were digested with *Eco*RI and ligated to form the pET21 (b+) RDH11. RDH11-His was purified using Ni-NTA (nickel-nitrilotriacetic acid) resin according to the manufacturer's instructions (the QIA*express* Ni-NTA Protein Purification

System). After the unbound proteins are washed away, the target protein is recovered by elution with imidazole.

GST-pull down assay. Purified GST-$2C^{ATPase}$ was bound to glutathione-Sepharose beads. Purified His-RDH11 was added to it and incubated for 2hrs; the bound proteins were eluted and electrophoresed on a SDS-polyacrylamide gel. The interaction was tested by Western blot using polyclonal antibody to RDH11 which was commercially available (Abcam). As a control I used GST protein instead of GST-$2C^{ATPase}$ to demonstrate that the interaction between $2C^{ATPase}$ and the cellular protein is specific.

***In vitro* translation/replication assay** PV viral RNA (750 ng) was translated and replicated in the presence of unlabeled methionine, 200 μM each CTP, GTP, UTP, and 1 mM ATP in a total volume of 25 μl. The reactions were carried out for 15 hr at 34 ℃. Virus yield in the samples were determined by plaque assay on HeLa R19 cells.

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FIGURES/TABLES

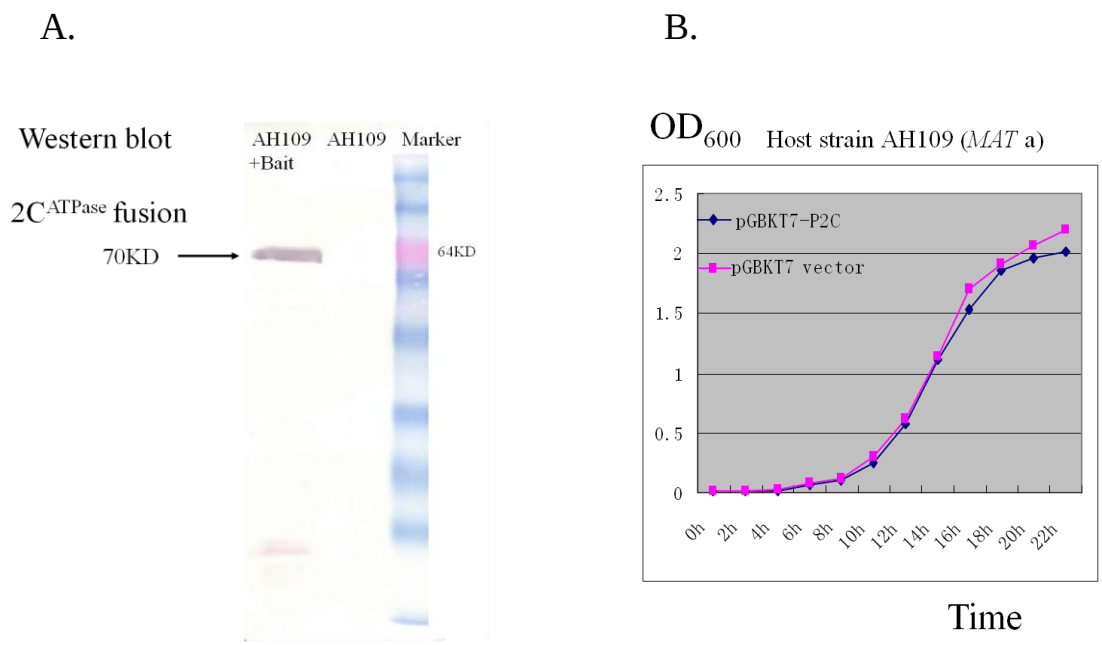


Figure 1. Bait protein (GAL4BD+ Full length Poliovirus 2C^{ATPase}) expression. (A) Bait plasmid was transformed into host strain AH109 and expressed. Yeast Cultures Protein Extracts were prepared using the Urea/SDS Method according to the Yeast Protocols Handbook Protocol (Clontech). The fusion bait protein was checked in a Western blot using monoclonal 2C^{ATPase} antibody. **(B).** The growth rate in liquid culture of cells transformed with the empty DNA-BD vector (pGBKT7) and cells transformed with the DNA-BD/bait plasmid (pGBKT7-P2C).

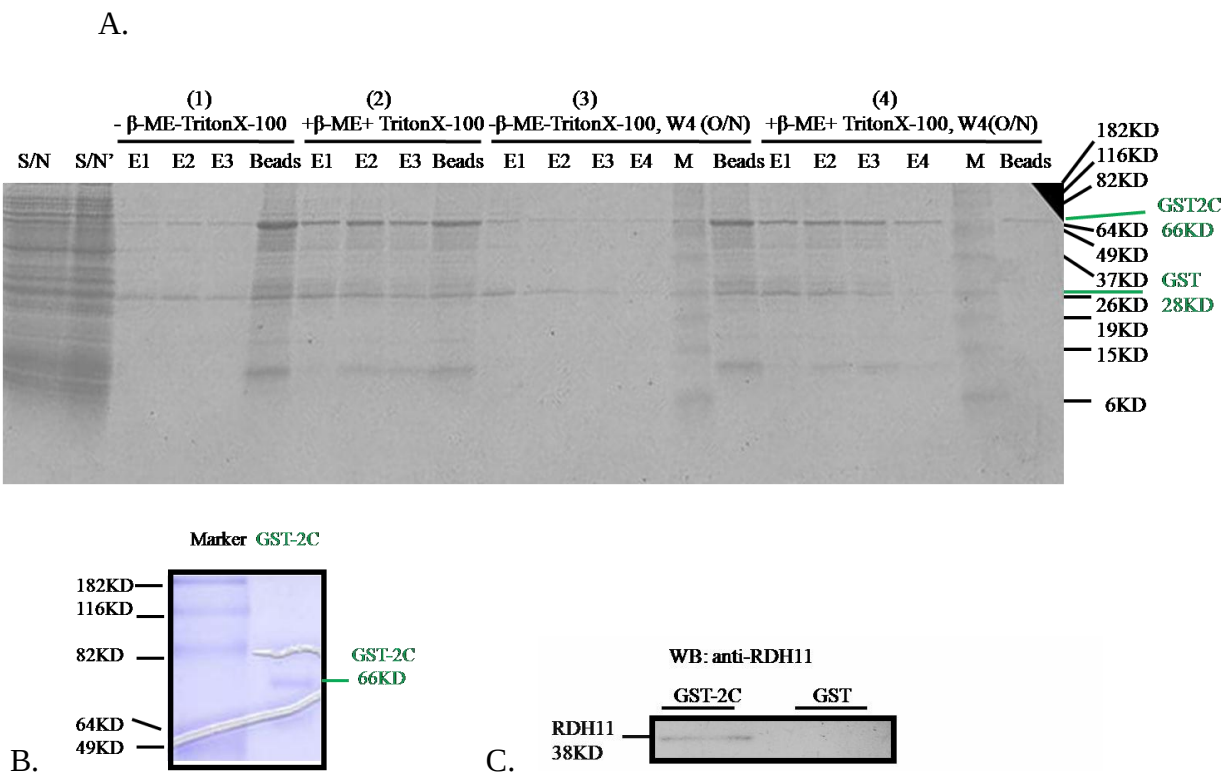
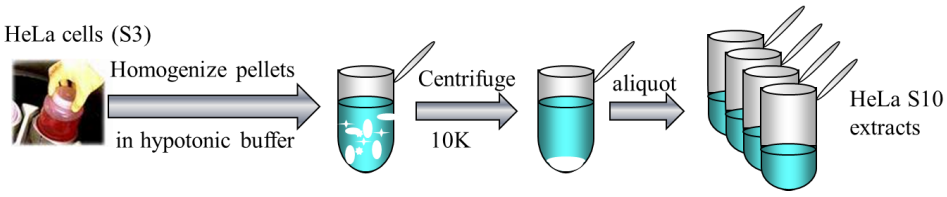


Figure 2. Purification of GST-2C^{ATPase} and pull down of RDH11-His. **(A)** Coomassie-stained SDS-PAGE gel. Elutions were carried out using 10 mM reduced glutathione buffers, in the absence ((1) and (3)) or presence ((2) and (4)) of 20 mM β-mercaptoethanol (β-ME) and 0.05% TritonX-100. S/N: supernatant, soluble fraction of E.coli lysates; S/N': soluble fraction after adsorption to glutathione-Sepharose beads, unbound proteins; E1, E2, E3: bound beads were incubated with elution buffer for 20 min at 4 ℃, elutions were repeated 3 times; E4: (3) and (4) has an additional elution (E4) after overnight incubation at 4 ℃; Beads: after elution, beads were boiled in Sample preparation buffer; M: marker, molecular masses in KDa are given to the right. **(B)** Coomassie-stained SDS-PAGE gel of purified GST-2C^{ATPase} using condition (2). C, GST-2C pull down RDH11-His, Western blotted with anti-RDH11 antibody.

A.



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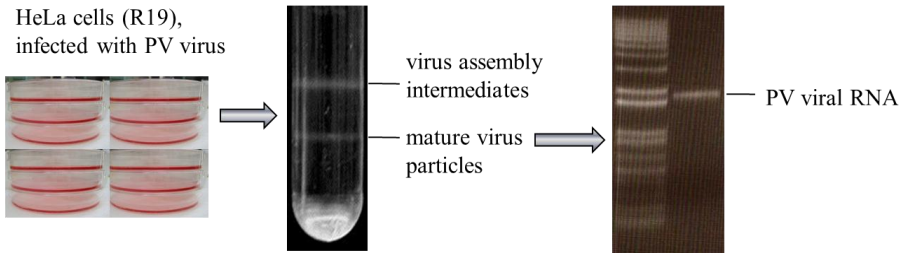

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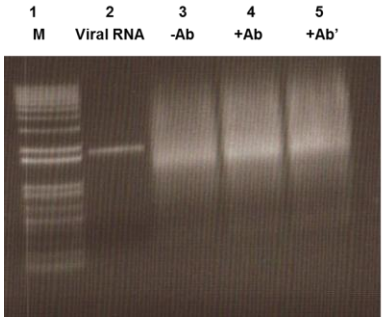

D.

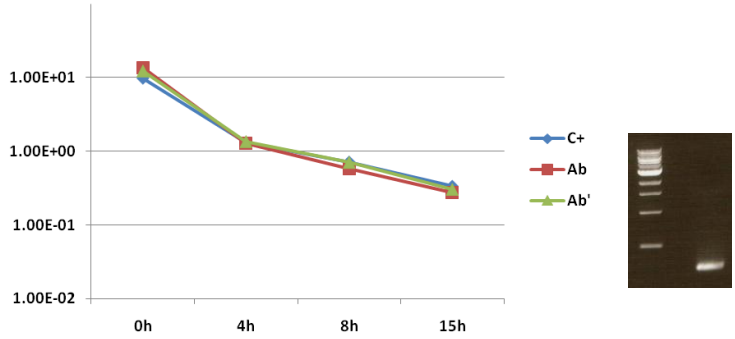

Figure 3. Stability of PV viral RNA in the *in vitro* translation system. (A) Preparation of HeLa S10 extracts and **(B)** PV viral RNA. **(C)** Stability of PV viral RNA in the absence and presence of untreated or heat-inactivated RDH11 antibody. PV viral RNA (750 ng) was incubated at 34 ℃ under translation conditions of 15 hr in a final volume of 25 µl. The samples were loaded on a 0.8% Tris-agarose gel (Tris-acetate). Lane 1: DNA molecular weight marker (Roche); Lane 2: 750 ng purified viral RNA loaded; Lane 3: 750 ng viral RNA in a translation mix in the absence of RDH11 antibody; Lane 4: 750 ng viral RNA in a translation mix in the presence of 60 µg/ml RDH11 antibody; Lane 5: 750 ng viral RNA in a translation mix in the presence of 60 µg/ml heat inactivated RDH11 antibody. **(D)** RNA amount determined by real time RT PCR. Agarose gel shows the amplified 2C fragment. 750 ng PV viral RNA in 25 µl Cell free translation- replication system, +2 mM GnHCl, no replication; 0 h, 4 h, 8 h, 15 h, take out 1 µl, then 1:30 dilution and use 1 µl out of 30 µl for real time PCR (final RNA dilution is 1:750).

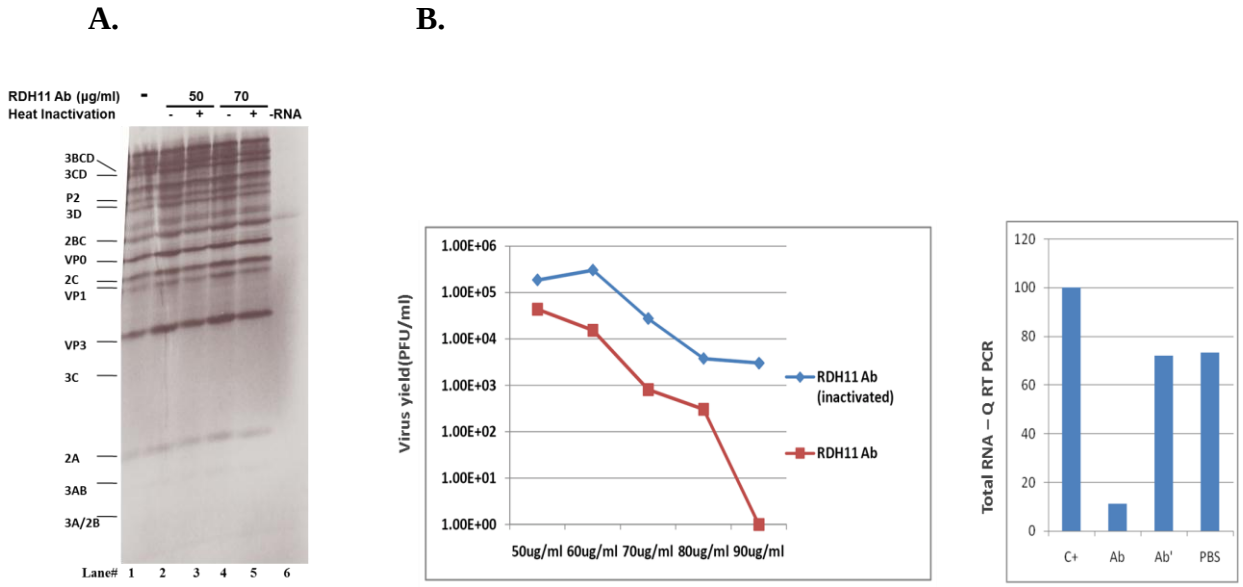

Figure 4. Inhibition of PV viral synthesis by adding RDH11 antibodies in an *in vitro* translation/RNA replication/encapsidation system. (A) *In vitro* translation of PV RNA in Hela cell-free extracts in the presence of RDH11 antibody. Samples were analyzed by SDS-PAGE and the ^{35}S-labeled bands were detected by autoradiography. **(B)** Inhibition of poliovirus synthesis in an *in vitro* translation/RNA replication system by adding RDH11 antibodies. Virus yield were determined by plaque assay on HelaR19 cells. Viral RNA yield is quantified by real time RT PCR.

Table 1. Interaction of cellular proteins with wt or mutant PV $2C^{ATPase}$ in the Y2H

	SPC 25	RDH11	PMF1	AARS
$2C^{ATPase}$	+++	+++	+++	+++
$\Delta_{aa\ 10\text{-}32}\ 2C^{ATPase}$	-	-	+	+++
$2C^{ATPase}\ (I_{25}K)$	+++	+	+++	+++
$2C^{ATPase}(N_{252}G)$	+++	+++	+++	+++
$2C^{ATPase}(N_{252}S)$	+++	+++	+++	+++

$\Delta_{aa\ 10\text{-}32}\ 2C^{ATPase}$: deletion of N terminal 10 aa-32 aa amphipathic domain of $2C^{ATPase}$

$I_{25}K$: an isoleucine-to-lysine conversion at 25 aa in the amphipathic helix of $2C^{ATPase}$

$N_{252}G$ and $N_{252}S$: in which the mutations are located in the middle part of $2C^{ATPase}$. N_{252} of PV $2C^{ATPase}$ was investigated because of its involvement with encapsidation (Liu, Wang et al. 2010).

Chapter V. Summary and Discussion

SUMMARY

$2C^{ATPase}$ of poliovirus (PV) has multiple functions during PV proliferation and encapsidation is the least understood step. In this study I systematically characterized the individual functions of twenty clusters of amino acids of $2C^{ATPase}$ to which no genetic phenotypes were previously assigned. The twenty mutants, consisting of sixteen clusters of charged residues and four conserved cysteine rich Zinc-binding sites (CRs) spanning the primary protein sequence of PV $2C^{ATPase}$, were subjected to alanine scanning mutagenesis analysis. The majority (17 / 20) of the mutants displayed severe phenotypes, such as lethal (12 /20), temperature sensitive (*ts*) (2/20) or quasi-infectious (*q.i.*) (3/ 20) which all highlighted the importance of the original charged amino acids or cysteine rich sites of PV $2C^{ATPase}$. Our data confirms essential roles of protein $2C^{ATPase}$ in PV RNA replication and encapsidation. These mutants have led to the identification of several new sites within the $2C^{ATPase}$ polypeptide that are required for RNA replication. One quasi-infectious mutant yielded escape mutant with a novel Q^R cleavage site at 2B^$2C^{ATPase}$. More interestingly, the involvement of more than one capsid protein: not only VP3, but also of VP1 in the coordination with $2C^{ATPase}$ during the encapsidation process was revealed by genetic evidence from several ts or quasi-infectious mutants, in which suppressor mutations were found in the coding regions of not only $2C^{ATPase}$ but also of capsid proteins VP1 and VP3. Indeed a direct interaction was detected between $2C^{ATPase}$ and VP3 by pull down assay. Using a Renilla luciferase reporter virus (R-PPP), I demonstrated that these quasi-infectious or ts mutants also showed defects in RNA replication, which suggest that these amino acids of $2C^{ATPase}$ are involved both in RNA replication and encapsidation. The 12 lethal mutants all have RNA replication defects demonstrated by the R-PPP reporter virus, since RNA replication is a prerequisite for encapsidation, thus I cannot rule out the possibility that these sites may also be

involved in encapsidation. Our data suggest that the cysteine rich motif and the flanking sequence are highly involved in the PV encapsidation process possibly achieved through interacting with a spacer between A and B motifs of $2C^{ATPase}$. In addition, I performed a yeast two hybrid screening of a HeLa cell cDNA library and identified several cellular factors that may interact with PV non-structural protein $2C^{ATPase}$. The roles of these candidates in PV replication and/or assembly of PV viral particles, if any, need further investigations.

DISCUSSION

$2C^{ATPase}$ is one of the most conserved non-structural viral proteins of the picornaviruses. $2C^{ATPase}$ of poliovirus (PV), a 329 amino acid long protein, has multiple functions during PV proliferation, such as modification of cellular organelles, RNA replication, and morphogenesis that are likely to require multiple interactions with viral and/or cellular proteins. Recently using a quasi-infectious chimera C_{20}PP consisting of the nonstructural proteins of PV and the capsid precursor of the closely related coxsackie A virus 20 (C-CAV20) we have reported a direct interaction between $2C^{ATPase}$ and VP3, which is required for enterovirus morphogenesis (Liu, Wang et al. 2010). Amino acid N_{252} of PV $2C^{ATPase}$ is involved in the interaction with the VP3 protein of CAV20 and this amino acid does not belong to any of the previously identified or putative domains of $2C^{ATPase}$. I thus suspect that other previously unidentified amino acids of $2C^{ATPase}$ may also carry out important functions especially in encapsidation.

As described in Chapter II and Chapter III, in order to further characterize the individual functions of $2C^{ATPase}$ and identify novel functional domains of $2C^{ATPase}$, especially in a search for more encapsidation components, I generated and analyzed 20 alanine mutants of $2C^{ATPase}$ in this study. Only three mutants generated viruses with normal growth phenotypes and most of the

alanine mutants (12 out of 20) were lethal with RNA replication defects, suggesting that some if not all of the aa of $2C^{ATPase}$ investigated at these 12 sites are important. *In vitro* translation in HeLa cell free extracts (Molla, Paul et al. 1991) indicated that none of the alanine mutations interfered with PV polyprotein synthesis and proteolytic processing. The genetic austerity of the PV genome and the close coupling of RNA replication and encapsidation make it difficult to dissect the defect on these two steps. Available evidence suggests that genome replication is a precondition of PV encapsidation, thus I cannot rule out the possibility that these 12 sites could also be involved in encapsidation.

This alanine scanning mutagenesis, described in Chapter II, highlighted the important roles of the charged amino acids of $2C^{ATPase}$ that I investigated. In Chapter II, I have used the clustered charged to alanine mutagenesis method and generated sixteen mutants of $2C^{ATPase}$ that contain a minimum of two adjacent or closely positioned charged to alanine changes. The viability of the mutants was tested at different temperatures (33 ℃, 37 ℃ and 39.5 ℃) and the growth phenotypes of the resulting viruses, if any, were analyzed at the three temperatures. Based on their growth phenotypes the sixteen mutants were divided into four groups: I, wild type-like (three); II, temperature sensitive, *ts* (one); III, lethal (nine); and IV, quasi-infectious, *q.i.* (two) (Chapter II, **Figure 1.B**).

The current study has unveiled several new sites in PV $2C^{ATPase}$ essential for viral survival. Of the ten lethal mutations only one is located within a domain of known function (membrane binding) (Chapter II, **Figure 1.**) while the other lethal mutations are located upstream of motif A , between motifs B and C or downstream of motif C of the NTP binding domain (Mirzayan and Wimmer 1992). The remaining three lethal mutations are located downstream of the putative Zn^{++} binding domain near the C-terminus of the polypeptide. Surprisingly, two of

the three mutations resulting in wt-like growth phenotypes are located near the N-terminus of the protein where several functional domains overlap (Chapter II, **Figure 1.**). In fact, mutant #2 exhibited mild *ts* phenotype at 39.5 ℃ with 1-2 logs lower virus production compared to the wt or the other two Group I mutants (Chapter II, **Figure 3.**), though this was not investigated further. The third wt-like mutant ($R_{256}A/D_{257}A$) was the most robust mutant with growth phenotype identical with wt PV (Fig.S1). #10 was mutated downstream of motif C (Chapter II, **Figure 1.**) near N_{252}, the site in $2C^{ATPase}$ known to interact with VP3 in the $C_{20}PP$ chimeric virus (Liu, Wang et al. 2010). Interestingly, the N_{252} residue itself can be mutated to G, S, or A without affecting PV morphogenesis (Ma, Asare et.al, unpublished data). These data suggest that a region downstream of motif C in PV $2C^{ATPase}$ near R_{252}, R_{256}, R_{257} are tolerant to certain genetic manipulations. The region downstream of box C (S_{255}) has also previously been shown to tolerate a linker insertion (Li and Baltimore 1988).

As discussed in Chapter II, I have confirmed that the *in vitro* translation system is a very useful tool to double check if the clone is correctly constructed. From here I also learned a lesson for making constructs: 1. first digest the clone using the same restriction enzymes used to make the construct and make sure the insert can be cut out 2. Send the clone to sequencing to confirm the mutation was successfully introduced. Meanwhile, very interestingly, *in vitro* translation of this wrong clone also yielded useful information: it confirmed that the cleavage of the P1 and P2 is carried out as long as the 2A is translated, independent of the translation and proteolytic processing of the rest of PV nonstructural polyprotein.

Another interesting observation is that one cellular protein band, with a size slightly bigger than PV $2BC^{ATPase}$, is always present in the mock lane of the *in vitro* translation system where no viral RNA is added (Chapter II, **Figure 6.B**). In addition, sometimes there are also

several other cellular protein bands in the mock lane. This major band was observed before also (Paul and Wimmer, unpublished data) but so far we do not have an explanation for this weird band since the HeLa cell free extract was pretreated with S7 micrococcal nuclease during preparation of the extract, theoretically there should be no messenger RNA present to serve as template for translation. This band is also present in the wt PV *in vitro* translation as well; suggesting that the corresponding encoding mRNA, if any, retained the ability of being translated during PV mediated inhibition of cap-dependent translation. Such cellular cap-independent translations do exist. For example the eIF4GI IRES was activated by $2A^{pro}$ protease cleavage of eIF4GI, *in vitro*, and its expression can continue under stress or infection conditions where eIF4GI is cleaved (Byrd, Zamora et al. 2005). In the future it will be interesting to determine the identity of this protein using mass-spec and investigate whether this cellular band has any contribution to the quality of the HeLa S10 extracts used for virus synthesis.

Studies in Chapter III confirmed the critical roles of $2C^{ATPase}$ in poliovirus RNA replication and encapsidation. To further characterize the mutants described in Chapter II, I measured RNA replication and encapsidation using a Renilla luciferase reporter virus (R-PPP) that contains the R-Luc coding sequence linked to the N-terminus of the PV polyprotein (PPP corresponds to the three domains in the PV polyprotein). The genomes of all thirteen defective mutants, including group II (one *ts* mutant), group III (ten non-viable mutants) and group IV (two *q.i.* mutants), were analyzed by the R-PPP strategy. Replication and encapsidation of *ts* mutant #7 were completely inhibited at the restrictive temperatures, 37 ℃ and 39.5 ℃, whereas at 33 ℃ encapsidation was achieved albeit less efficiently (Chapter III, **Figure 4.A**). All group III mutants were found to be severely defective both in RNA replication and encapsidation (Chapter III, **Figure 5.**) whereas *q.i.* mutants #1 and #11 (group IV) exhibited less severe replication

phenotype at 33 ℃ (Chapter III, **Figure 6.**). The genotypes and phenotypes of the suppressor variants of mutant #7, #1 and #11 and their growth properties were also described in detail.

Although all twenty $2C^{ATPase}$ mutants exhibited normal translation and processing of the PV polyprotein, the suppressor mutations identified in this study provide novel insights into the proteolytic processing of PV. The suppressor mutation G_1R of mutant #1 (K_6A/K_7A), generated a unique $3C^{pro}/3CD^{pro}$ proteinase cleavage site at the 2B^$2C^{ATPase}$ cleavage site. Enteroviruses are purists in that all of their natural $3C^{pro}/3CD^{pro}$ cleavage sites are Q^G; thus the generation of the Q^R site was surprising. The only naturally occurring Q^R site amongst picornavirus polyproteins exists in the HAV polyprotein between proteins $3C^{pro}$ and $3D^{pol}$ (Tesar, Pak et al. 1994), as summarized in (Chapter III, **Table 8)**. Our *in vitro* translation assays demonstrated that processing between 2B and $2C^{ATPase}$ in the Q^R suppressor polyprotein was just as efficient at the Q^R cleavage site as at the original Q^G site in the wt polyprotein (Chapter III, **Figure 7.B**). Our explanation for the appearance of this novel Q^R cleavage site is that an N terminal positive charge is important. Since K_6/K_7 were mutated to alanine, mutant #1 lost two positive charges at amino acid positions 6 and 7 of $2C^{ATPase}$, thus mutant #1 reverted to gain the positive charge back, even at the expense of losing the original cleavage site. It should be noted that the G to R mutation involves only a single nucleotide change instead of the two required for the A to R substitution (Chapter III, **Table 5.**). This likely explains why the virus regenerated a positively charged residue at position 1 rather than reverting the nucleotides of the A codons at positions 6 or 7.

The other suppressor mutations as described in Chapter III, revealed two novel functional domains in the $2C^{ATPase}$ polypeptide, which differ from those domains identified by the lethal mutations as discussed in Chapter II. These mutations, arising in the $2C^{ATPase}$ protein, always

regenerated a charged amino acid that was originally eliminated by the charged to alanine substitution. These were either at the same ($A_{150}E$ of mutant #7) or at different ($E_{148}K$, $C_{323}R$ of mutant #11) locations to the original residue change. The first domain that I hypothesize to play a role in $2C^{ATPase}$ function(s) is located in the spacer between the A and B motifs of the NTP-binding domain, involving $A_{150}E$ and $E_{148}K$: $A_{150}E$ is a partial revertant of *ts* mutant #7 and $E_{148}K$ is a suppressor mutant of the $K_{279}A/K_{280}A$ mutant #11. The presence of a suppressor mutation ($E_{148}K$) at this location suggests a functional interaction between the C-terminus of the $2C^{ATPase}$ polypeptide and the spacer between motifs A and B of the NTP-binding domain (Mirzayan and Wimmer 1992) either within the $2C^{ATPase}$ protein or $2C^{ATPase}$ oligomers, which may contribute to the signature of different viruses. This is further supported by the $A_{138}V$ suppressor mutations of mutants #CR2 and #CR4. The second domain maps to the cysteine rich sites containing the original mutated site ($K_{279}A/R_{280}A$) of mutant #11. These mutations are localized close to the C-terminus of the $2C^{ATPase}$ polypeptide within the Zn^{++} binding domain that consists of five cysteines (C_{269}, C_{272}, C_{281}, C_{282}, and C_{286}) and one histidine (H_{273}) residue.

Very interestingly, viabilities of all our $2C^{ATPase}$ alanine scanning mutants, but one (#4), correlate with the extent of conservation of the original charged amino acid sequence among human enterovirus C cluster (Chapter III, **Table 4.**). We thus predict that if we make the same alanine mutations at the corresponding highly conserved sites of related viruses, majority, if not all, of the mutations will be lethal. Interestingly, the suppressor variant #11b ($K_{279}A/R_{280}A+E_{148}K$) mimic the electronic charges of three CAVs (CAV1/CAV19/ CAV22) ($S_{279}/T_{280}+ K_{148}$) at aa 148, 279 and 280. This strongly supports our proposal that there is an interaction between the C terminal and the spacer between A motif and B motif of middle region of $2C^{ATPase}$ either within the $2C^{ATPase}$ protein or $2C^{ATPase}$ oligomers.

Thus, to further characterize the functions of the four CR sites of PV, I made four new CRs mutants by changing the cysteine/ histidine to alanines (Chapter III, **Table 5**.). Interestingly, viability of the four mutants correlates with the extent of conservation of the corresponding CRs sequence among enteroviruses: the more conserved the four CRs among enteroviruses, the more debilitated the alanine scanning mutants. Indeed the four cysteine rich sites are also involved in the PV encapsidation process: mutants #CR2 and #CR4 yielded suppressor mutation at $K_{295}R$, $M_{293}V$ or $A_{138}V$ of $2C^{ATPase}$ (Chapter III, **Table 7.**). Coincidently, $K_{295}R$ and $M_{293}V$ both appeared in 2C-31R3 which is a cold sensitive, uncoating defective virus while $N_{140}S$ rescued the ts phenotype (Li and Baltimore 1990). This strongly supports the idea that CRs of PV $2C^{ATPase}$ are involved in the encapsidation process. It should be noted that residues $K_{279}A/R_{280}A$ of mutant #11 are located just upstream of M_{293} and K_{295}. Based on these results, I propose that the cysteine rich domain containing residues K_{279}/R_{280} is involved in encapsidation and there is a possible interaction between the spacer between box A and box B of the NTP-binding domain (such as A_{138}, E_{148}) and the C terminus of PV $2C^{ATPase}$, flanking the cysteine rich domain.

The most important conclusion derived from our work is that both VP3 and VP1 communicate with the C-terminal domain of $2C^{ATPase}$, and that this interaction is required for encapsidation. This conclusion is strongly supported by the emergence of suppressor variants of mutant #11: #11a, #11c and #11d (Chapter III, **Figure 9.**). This was achieved by a simple but powerful design, to extend the scope of finding possible suppressor mutations, of subjecting the mutants to different selection pressures by combination of low/high temperatures between transfections and plaque purification of variants (Fig. 6A bottom panel). Our interpretation of why different variants were isolated from mutant #11: when transfection was performed at 33 ℃, mutant #11 can slowly replicate, but has encapsidation defect. (1) It needs revertants in capsid to

rescue encapsidation, as isolated variant #11a: #11+VP1 ($T_{36}I$). (2) Or $E_{148}K$ in $2C^{ATPase}$ can rescue both replication and encapsidation defect of mutant #11, as isolated variant #11b:#11+2C ($E_{148}K$). Upon passaged at 39.5 ℃, this #11b with $E_{148}K$ compete out because compared with variant #11+VP1 ($T_{36}I$), it replicate faster at 39.5 ℃, while at 33 ℃ there is no selection pressure since #11 replicate fine at 33 ℃. (3)When initially transfected at 39.5 ℃, mutant #11 first has to gain the ability to replicate by suppressor mutation in 2C ($C_{323}R$) although weakly compare to wt (**Figure. 10**). In addition, it has encapsidation defect, that's why $C_{323}R$ never appeared by itself thus both variants #11c and #11d need an additional suppressor mutations in capsid region, VP3 ($K_{41}R$) and VP1 ($N_{203}S$) respectively. Either case, these genetic data strongly support the conclusion that $2C^{ATPase}$ is involved in encapsidation and it act in conjunction with capsid polypeptides VP1 or VP3 to facilitate PV morphogenesis.

However, based on current data I cannot determine whether VP1 binds directly to $2C^{ATPase}$, or whether it complexes with other capsid proteins to induce structural changes in VP3 through mutation while VP3 is the direct binding partner of $2C^{ATPase}$ in encapsidation (Chapter III, **Figure 14.**) (Liu, Wang et al. 2010). The capsid precursor P1 of PV is processed and assembled to form protomers (VP0, VP3, VP1). Available evidence suggests that the protomer does not dissociate into the individual cleavage products in the cytoplasm of the infected cell, rather, it will pentamerize to $(VP0, VP3, VP1)_5$ - the building block of the immature capsid, as discussed by Liu et al. (Liu, Wang et al. 2010). In the future, it will be interesting to determine 1) whether or not there is a direct physical interaction between VP1 and $2C^{ATPase}$; 2) At which stage of encapsidation (capsid intermediates 5S, 14S, 75S and 150S) is $2C^{ATPase}$ involved in the interaction with capsid proteins VP3 and VP1?

Interestingly, the VP1 and VP3 suppressor mutations involved the exchange of a polar residue to one with increased hydrophobicity (VP1: $T_{36}I$ or $N_{203}S$) or of one charged residue to another (VP3: $K_{41}R$). One of the VP1 residues (T_{36}) is located near the N-terminus of the VP1 protein, which was previously shown to be involved both in RNA release from the infecting virion and in the interaction/insertion of the progeny RNA into capsid proteins during morphogenesis (Kirkegaard 1990). On the basis of the study of two small N-terminal deletions of PV VP1, Kirkegaard and colleagues have concluded that RNA packaging and RNA release are genetically linked but can be mutated separately in different VP1 alleles. Whether an interaction between VP1 and $2C^{ATPase}$ is required for these processes remains to be determined.

Although not further analyzed, I also isolated several additional suppressor variants of mutants #11($K_{279}A/R_{280}A$), #CR2 ($C_{272}A/H_{273}A$) and #CR4 ($C_{286}A$). First, suppressor mutations in VP3 ($I_{231}V$) +2C ($C_{323}R$), VP3 ($M_{52}I$), VP3 ($E_{45}Q$) + $3C^{pro}$ ($T_{20}A$), 3D ($M_{189}V$) and some silent mutations in $2C^{ATPase}$ (F_{77}silent) or VP1 (S_{179}silent) of mutant #11 were identified. Second, mutant #CR2 yielded suppressor mutations in $2C^{ATPase}$ ($N_{271}H/D$), ($M_{293}I$) or 3D ($I_{67}T$); silent mutations in VP1 (R_{243}silent) or 2A (N_{29}silent). Mutant #CR4 also yielded additional suppressor mutations in $2C^{ATPase}$ ($R_{298}G$) and silent mutations in VP2 (C_{257}silent), VP1 (L_{224}silent), 2A (L_{113}silent), 3C (G_{145}silent). In the future it will be very interesting to further analyze these suppressor mutations especially those mutations in 3C or 3D which could contribute to PV encapsidation through its precursor $3CD^{pro}$. Whether or not any of the silent mutations will contribute to any steps of PV life cycle will also need to be tested out.

The location of three mutants ($K_{279}A/R_{280}A$, $C_{272}A/H_{273}A$ and $C_{286}A$) and second site mutations at 2C: $K_{295}R$, $A_{138}V$, $M_{293}V$, $A_{286}D$, $R_{298}G$, $E_{148}K$, $C_{323}R$, combined with our previously identified encapsidation mutation of $2C^{ATPase}$ ($N_{252}G$) (Liu, Wang et al. 2010), suggested that

many sites in the middle and C terminal of the $2C^{ATPase}$ protein are involved in encapsidation. Especially the cysteine rich motif and the flanking sequence near the C terminal, is highly involved in PV encapsidation process. In contrast, hydantoin (encapsidation drug) resistant mutations are located to either the middle part or the N terminal part of $2C^{ATPase}$ (Chapter III, **Figure 1.**).

The cysteine rich motif has been shown to bind Zinc, thus I suspect that Zinc maybe somehow needed for PV encapsidation. Since the efficiency of virus yield using the cell-free *in vitro* translation-replication system is always an uncertainty and the missing factors is always a mystery, it is worthwhile to test Zinc in the cell-free *in vitro* translation-replication system to see whether the virus yield will be increased by adding the right concentration of Zinc to the HeLa S10 extract. In fact a colleague did some preliminary experiments and data suggested that adding 500-800 μM $Zinc^{++}$ indeed improved the virus yield up to 2-log. In the future to study Zinc's effect to improve virus yield in the *in vitro* translation/replication system, it is best to test it out in different batches of HeLa extracts, especially those with poor virus yields when previously used without Zinc. Also it is worthwhile to test the helicase activity of $2C^{ATPase}$ by adding different amount of Zinc.

As described in Chapter II and Chapter III, I have identified mutant #7 ($E_{148}A/R_{149}A/E_{150}A$) with a severely ts phenotype and replication defects. This mutant #7 ($E_{148}A/R_{149}A/E_{150}A$) will be a good candidate for further studies of PV RNA replication. A partial reversion rescues the severely temperature sensitive phenotype of mutant #7 ($E_{148}A/R_{149}A/E_{150}A$), thus in the future I will modify this mutant by mutating more nucleotides to lower the chance of direct reversion.

Among the PV nonstructural proteins, $3CD^{pro}$ and $2C^{ATPase}$ have been previously reported

to play a role in viral encapsidation. $3CD^{pro}$ appears to be involved in both the early and late stages of virion assembly: $3CD^{pro}$ cleaves P1 precursor yielding the protomer (Ypma-Wong, Dewalt et al. 1988) and $3CD^{pro}$ has a high affinity to P1 (Parsley, Cornell et al. 1999) while studies with an *in vitro* translation/RNA replication system have suggested that $3CD^{pro}$ also functions at a late step in the assembly process just before or during the maturation cleavage of VP0 to VP2 and VP4 (Franco, Pathak et al. 2005). In this study through genetic analysis of the alanine scanning mutants, I found suppressor mutations not only in $2C^{ATPase}$ and VP3, but also in VP1. Here I propose that $2C^{ATPase}$ and other viral/cellular proteins may induce the membranous complex not only for replication but also for encapsidation; and during the late stages of PV life cycle, the replication complex cooperate with the capsid protein for encapsidation. Thus I prefer the term "replication/encapsidation complex". This could provide many advantages for the virus. For example the replication/encapsidation complex might provide the newly synthesized viral RNA a safe microenvironment: as soon as one round of replication is complete, the nearby $2C^{ATPase}$ and possibly also $3CD^{pro}$ will help the capsid proteins and the newly made viral RNA to form a complex. Near the end stage of the packaging, capsid proteins, VPg, and RNA remain stayed and forms the progeny virions while $2C^{ATPase}$ and $3CD^{pro}$ was removed from the encapsidation complex. Although $2C^{ATPase}$ has the helicase motif but its helicase activity has never been proved. Thus I am not sure during this process whether the helicase activity, if any, will contribute to unwinding the secondary structure of PV genome and stuff the genome into the empty capsids. I also suspect that besides serving as the protein primer, VPg may also play a role in the encapsidation, for example VPg may finally dock the viral genome RNA inside the capsids. Since $2C^{ATPase}$ interact with 3AB (Yin et al., 2007) which is a precursor of 3B (VPg), it would be interesting to know the exact mechanism of VPg during the encapsidation process: whether

during the encapsidation process $2C^{ATPase}$ mediates the interaction of capsid proteins with VPg or VPg directly interacts with one of the capsid proteins to dock the viral genome inside the capsid shell. Also a related question, *in vitro* translation/replication system the viral RNA is always better than the RNA transcripts concerning virus yield. A major difference between viral RNA and RNA transcripts is the presence of VPg in viral RNA. If this difference contributes to different virus yields in the *in vitro* translation/replication system, what's the underlining mechanism?

The studies of this thesis have opened many new paths for future studies. Our results clearly demonstrate, however, that the selection of revertants or suppressor mutants generated from clustered charged amino acid to alanine mutagenesis is a useful method of identifying viral protein interacting components of the replication/encapsidation complex.

Because of the limited amount of genetic information available in the small genome of poliovirus (PV), it uses not only the mature viral proteins but also their precursors. In addition, PV utilizes not only viral proteins, *cis*-replicating RNA elements, and the cellular translation machinery but also cellular proteins for the proliferation of its genome. As one of the most conserved non-structural viral proteins amongst picornaviruses, the 329 aa long $2C^{ATPase}$ polypeptide appears to be multifunctional and is involved in multiple protein/protein and protein/RNA interactions. Since a single protein carries out so many different functions, it is likely that this is achieved by interaction with other viral and cellular proteins.

Thus, as part of a search for the *in vivo* function of $2C^{ATPase}$ I performed an Y2H screening of a HeLa cDNA library with the aim of finding the cellular factors interacting with PV non-structural protein $2C^{ATPase}$ that are required for PV replication and/or assembly of PV viral particles, which were described in Chapter IV. Two-hybrid screens can provide an

important first hint for the identification of interaction partners and are now routinely performed in many labs and it was used extensively in the past in our laboratory. For my current work I selected The Matchmaker Two-hybrid System 3 (Clontech), which is an advanced GAL4-based two-hybrid system that reduces the incidence of false positives and allows the user to quickly identify and confirm protein interactions. I used the full-length $2C^{ATPase}$ bait construct transformed into yeast strain AH109 (MATa strain) as bait to screen the Pre-transformed Matchmaker HeLa cDNA library (Clontech), previously transformed into yeast strain Y187 (MATα). This library is screened by yeast mating rather than by cotransformation, which significantly reduces the time and labor involved in performing two-hybrid screening and more importantly, the mating efficiency (10% in my experiment) is much higher than the co-transformation efficiency, which used to be the critical step for screening libraries. I used the Clontech's Matchmaker system to implement the systematic, genome-wide identification of host genes interacting with $2C^{ATPase}$.

I have identified several positive clones that interacted with PV $2C^{ATPase}$ in the Y2H assay. CREB3 and SPCS2 were detected in several clones. Another three candidates (RDH11, PMF1, and AARS) were detected only once but showed strong interactions with $2C^{ATPase}$. In addition, ARFIP2, GNL3 (guanine nucleotide binding protein-like 3 (nucleolar)), Damage-specific DNA binding protein 1, and PPARA (peroxisome proliferative activated receptor, alpha) were also identified as possible $2C^{ATPase}$ interacting partner by the Y2H assay. Based on previous publications, the most relevant candidates are ARFIP2 and CREB3. In the future it will be interesting to find out whether these candidates, for example ARFIP2 and CREB3, have any role(s) in the PV life cycle. GST pull down has shown there is a direct interaction between PV

$2C^{ATPase}$ and RDH11 and *in vitro* translation/replication assay has indicated RDH11 is involved in some steps, other than translation, of PV life cycle.

It should be noted that although Tang et al. (2007) have identified reticulon 3 (RTN3) as an interacting partner of enterovirus 71 and of PV $2C^{ATPases}$ using the N-terminal sequence of $2C^{ATPases}$ against a human fetal brain cDNA library, this protein did not appear in our search using full length $2C^{ATPases}$ against HeLa cell cDNA library. One possible reason is that the virus selects different host factors from different tissues for the replication of its RNA. Thus the difference between our results and those of Tang et al. (2007) might be due to the difference between HeLa cell and human fetal brain cDNA library used in the Y2H screening.

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