Roles of Rubella virus nonstructural proteins in RNA replication

By

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Genetics Program

We accept this thesis as conforming to the required standard

The University of British Columbia

September, 2000

Xiaojie Wang
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The University of British Columbia
Vancouver, Canada

Date Oct 12, 2000
Abstract

Rubella virus (RV) is an enveloped, positive-strand RNA virus in the family Togaviridae. The 9759-nucleotide genomic RNA contains two long open reading frames (ORFs). One-third of the 3'-proximal ORF encodes the three structural proteins required for virus assembly. The other two-thirds encode the nonstructural proteins (NSPs) that function in RNA replication. The primary NSP polyprotein p200 is cleaved by a viral encoded protease into p150 and p90. The aim of my thesis project is to identify the functional roles of NSPs in RV replication with respect to individual NSPs required for plus-strand RNA synthesis and the effect of highly conserved GDD motif within p90 region on viral replication.

Both protease-inactive (pBRM33/C1152S) and cleavage-defective (pBRM33/G1301S) mutants can initiate minus-strand RNA but not plus-strand RNA synthesis (Liang and Gillam, 2000). In this study, individual RV NSPs were expressed by either a recombinant vaccinia virus or by co-electroporation of helper genomes. Complementation experiments were carried out by providing individual RV NSPs with either pBRM33/C1152S or pBRM33/G1301S mutant as a template. Rescue of pBRM33/C1152S by either p200 or p150 indicates that functional protease can be provided in trans. Rescue of pBRM33/G1301S by either p200 or p150/p90 suggests that the p150/p90 replication complex functions in plus-strand RNA synthesis.

Site-directed mutagenesis was employed to investigate the effect of putative RV RNA-dependent RNA polymerase GDD motif on viral replication. Substitution of glycine by
alanine (G1966A) resulted in impaired virus infectivity, reduced by $1.7 \times 10^4$-fold. Alteration of either aspartate residue completely abolished virus replication. A revertant was isolated from the passaged G1966A virus and its sequence confirmed. These results are consistent with the prediction that p90 is the RdRp of RV.
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney cell line</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cpm</td>
<td>count per minute</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
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</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>μg</td>
<td>microliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholno]-propanesulfonic acid</td>
</tr>
<tr>
<td>NSP-pro</td>
<td>nonstructural protease</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside triphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------------------------------------------</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<td>PAGE</td>
<td>polyarylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N, N’-bis[2-ethane-sulfonic acid]</td>
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<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>RV</td>
<td>rubella virus</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
</tr>
<tr>
<td>SIN</td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>Tris</td>
<td>trishydroxymethylamino methane</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
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Introduction

I-1. General information

I-1.1. Background

Rubella virus (RV) is the pathogen of the human disease, known as rubella or German measles. Transmission of the virus is via the respiratory route and initiation of virus multiplication occurs in the respiratory tract. Viremia occurs one week later, followed by the skin rash. Rubella occurs worldwide and mostly affects children in whom causes a mild illness. A more serious health impact of RV is that it is a teratogenic agent. The virus can cross the placenta and replicate in the fetus during the first trimester of pregnancy. This can cause a variety of birth defects, collectively called Congenital Rubella Syndrome (CRS), most commonly involving heart defects, cataracts, deafness, or mental retardation. The immune response generated by natural infection in most cases provides life-long immunity (Assad et al, 1985)

Vaccination with live, attenuated virus has been successful in reducing the incidence of CRS (Perfins et al, 1985). However, rubella-associated arthritis and the consequence of viral persistence in vaccinees resulting from RV vaccination remain major medical concerns (Chantler et al, 1982). Significant progress has been made in the molecular biology of RV structural proteins (for a review see Gillam, 1994). However, the function of nonstructural proteins in RV replication remains poorly characterized. Recently, the
construction of a full-length infectious clone has allowed us to study the functional roles of NSPs (Yao and Gillam, 1999).

I-1.2. Classification

RV is the sole member of the genus Rubivirus within the family Togaviridae (Francki et al., 1991). This family includes the well-studied prototype alphaviruses, Sindbis virus (SIN) and Semliki Forest virus (SFV). RV is the unique non-arthropod-borne human pathogen in the Togaviruses. Humans are the only natural host for RV, making experimental studies difficult since there is no animal model available that can mimic human disease.

Togaviruses are distinguished from the other positive-strand RNA virus families in that their genomic RNAs have an O cap at their 5’ proximal regions (without ribose methylation) and a poly (A) tail at the 3’ end. They contain two long open reading frames (ORFs): the 5’ proximal ORF encodes NSPs whereas the 3’ proximal ORF codes for SPs (Domínguez et al., 1990).

Based on virion structure, genome organization and replication strategy, four major groups of animal positive-strand RNA viruses have been classified, picornaviruses, togaviruses, flaviviruses, and coronaviruses. Generally speaking, picornaviruses have only one long ORF within the genome. The generated polyprotein is cleaved by a viral protease 3C into P1, P2 and P3. Capsid protein P1 is located at the N-terminus of the polyprotein (Wimmer et al., 1993). Internal ribosomal entry sites (IRES) instead of cap
structures in polioviruses may facilitate efficient cap-independent translation. The genome organization of flavivirus is much more similar to that of togavirus in that there are two ORFs in genomic RNA and a 5' terminal type O cap. The differences between flavivirus and togavirus are that there is no 3'-terminal poly A tail in flavivirus, and the locations of structural proteins (SPs) versus nonstructural proteins (NSPs) are reversed. One of the most important human pathogens hepatitis C virus (HCV) is within flavivirus family (Nicolas and Rice, 1999). The replicases of coronaviruses are encoded by two long ORFs. The ORF 1a and ORF 1b polyproteins are both processed by a number of ORF1a-encoded proteinases. A key feature of coronavirus replication is the 3' coterminall discontinuous subgenomic RNA transcription (for a review see Brown and Brierley, 1995).

I-2. Biology of Rubella virus

I-2.1. Virion structure

RV virion contains a nucleocapsid consisting of a 40S genomic RNA and multiple copies of a capsid protein (C). The nucleocapsid is surrounded by a host-derived envelope in which two membrane glycoproteins, E1 and E2, are embedded (Frey, 1994).

The virus particles are approximately 60 nm in diameter and are roughly spherical (for a review see Murphy et al, 1980). The two membrane glycoproteins, E1 and E2, form the virion fringe of 5-8 nm diameter. T=3 icosahedral symmetry has been shown by rotational analysis of thin-sections of RV virions (Matsumoto and Higashi, 1974), although alphavirus capsids have a T=4 symmetry. (Paredes et al., 1993).
1-2.2. *Genome organization and expression strategy of RV and SIN*

RV is an enveloped, positive-strand RNA virus. The RV genomic RNA is 9762-nt in length exclusive of 3'-terminal poly (A) tail (Dominguez *et al.*, 1990). The genome contains (from 5' to 3'), a 40-nt 5’untranslated (UTR); a 6345-nt ORF that encodes NSPs; a 123-nt UTR between the two genomic ORFs; an ORF of 3189-nt that encodes SPs, and a 62-nt 3'UTR (Pugachev *et al.*, 1997). The two thirds of 5' proximal ORF, from nt 41 to nt 6388, encodes NSPs that function mainly in viral RNA replication and the other one third of the 3' proximal ORF, from nt 6512 to nt 9701, encodes the virion SPs (C, E2, and E1) that function in virus assembly (Clark *et al.*, 1987; Gillam, 1994). In RV-infected cells, the NSP-ORF is translated from the genomic RNA whereas the SP-RF is translated from the subgenomic RNA that is co-linear with the 3’ terminal region. The primary translation product p200 of the NSP-ORF is cleaved by a viral protease into two mature products: p150 and p90 (Chen *et al.*, 1996; Marr *et al.*, 1994; Yao *et al.*, 1998). The translation product p110 of the SP-ORF is cleaved by cellular signal peptidases into C-E2-E1 (Clarke *et al.*, 1987) (Fig. 1A). In these aspects of genome organization and replication strategy, RV is similar to the alphaviruses that have been extensively characterized (Fig. 1A and 1B).
The 5'-proximal ORF encodes nonstructural proteins that function primarily in viral RNA replication. The 3'-proximal ORF encodes the virion structural proteins.
The termination at the opal codon (♀) produces p123. VcRNA is the minus-strand complement of the genomic RNA.
In SIN, a member of the *Alphavirus* genus in the *Togaviridae* family, the nsPs are translated as two large polyproteins (p123 and p1234) from the 5'-promixal ORF (Fig. 1B). These polyprotein precursors are processed by a papain-like protease residing in the C-terminal of nsP2 to generate several intermediate polyproteins and four individual nsPs (Strauss and Strauss, 1986).

Comparison of the 5'-proximal ORFs of RVs and alphaviruses reveals only one short region (122 amino acids) of significant homology in the X domain with unknown function (Gorbalenya et al., 1991) (Fig. 2). The relative positions of the short homology region (X-domain) and helicase within the 5'-proximal ORFs of RV and alphaviruses suggest that a rearrangement in this region of the genome has occurred (Dominguez et al., 1990).
Fig. 2. Genome organization of RV compared with that of Sindbis Virus

A number of domains with sequence homology to Sindbis virus have been identified. These include the methyltransferase, helicase, papain-like proteinase, X-domain of unknown function, and RNA-dependent RNA polymerase (RdRp). The order of the functional motifs in these two viruses is different. The inverted positions for helicase and the X-domain are indicated. (Modified from Frey, 1994)
I-2.3. *RV genome sequence information*

The G/C content of RV genomic RNA is the highest of any RNA virus, approximately 70%. This feature makes it difficult to determine RNA sequence and to construct RV infectious clones. Studies of RV have been delayed relative to those of alphaviruses for this major reason.

The first 65-nt of genomic and 78-nt in the middle of the genome around the subgenomic RNA start site show the remarkably low G/C contents of 49% and 47% respectively. (Domínguez *et al.*, 1990). This may facilitate recognition and initiation of the genomic and subgenomic RNAs.

The high G/C content in RV determines its unique codon usage. Firstly, there is a preference for G and C residues (80.9%) in the third position of codons compared to that of 54.8% in SIN ORFs (Strauss and Strauss, 1986) and 60.8% in human genes (Maruyama *et al.*, 1986). Secondly, in the RV ORFs isofunctional amino acids with G/C rich codons were favored. Arginine (CGC, AGA, and AGG) (87%) is more abundant than lysine (AAA and AAG) (13%) among the basic residues compared to similar amount of both in human genes and SIN ORFs (45% of arginine and 55% of lysine) (Domínguez *et al.*, 1990). The reason for this codon usage strategy remains to be characterized.

There are two well studied wild types of RV: the Therien strain (Frey *et al.*, 1986) and M33 strain (Yao *et al.*, 1998). Currently, there are two vaccine strains: HPV-77 (derived
from the M33 strain) (Zheng et al, 1989) and the RA 27/3 (whose parental strain is unclear). Infectious cDNA clones of Therien and M33 strains have been obtained recently (Pugachev et al, 1997b; Yao and Gillam, 1999). The successful construction of infectious clones allows us to pursue functional studies on RV.

I-2.4. life cycle

RV replicates noncytopathically in a number of primary cell line cultures and continuous cell lines of vertebrates with no apparent cytopathic effect (CPE) (Frey, 1994). However, in two cell lines, BHK-21 and Vero cells, CPE is induced and virus titers can be at a high level: $10^7$ pfu/ml in BHK-21 cells and $10^8$ pfu/ml in Vero cells (for a review see Frey, 1994).

Normally, viruses shut off and/or take over cellular transcription/translation machinery for the purpose of viral replication. However, RV does not shut down the cellular macromolecular machinery and replicates at a long latency period. Interestingly, both cell lines lack a functional interferon system. This may be a reason why these two cell lines can support RV replication.

Time course studies show that RV infection can reach a peak level by 24 h after a 12 h latent period (Hemphill et al., 1988). In contrast, alphavirus has a 2 h latent period and reaches peak level by 4 to 8 h p.i. (Strauss and Strauss, 1994). Both genomic and subgenomic RNA of RV are first detectable at 12 h p.i. and the rate of synthesis of both species is maximal by 26 h p.i. (Hemphill et al., 1988).
Like SFV, RV infects cells via receptor-mediated endocytosis and an acid-triggered fusion step (Baron and Forsell, 1991; Vaananen and Kaariainen, 1980). Following fusion of the endocytosed vesicle, the low pH environment of the endosome triggers conformational change of viral glycoproteins, resulting in release of viral nucleocapsid into the cytoplasm. In the cytoplasm, RV can complete its life cycle and produce more infectious progeny. The cellular receptor for RV has not yet been identified so far.

I-3. Nonstructural proteins

I-3.1. Nonstructural proteins of SIN

Studies of the well-characterized members of the Alphavirus genus of the Togavirus family, such as SIN and SFV, show that the NSP ORFs function mainly in RNA replication (Lemm and Rice, 1993a; 1993b; Lemm, 1994; 1998) and viral cytopathogenesis (Kuhn et al., 1992). The molecular biology of RV structural proteins is well-studied (for a review see Gillam, 1994) whereas that of NSPs is poorly understood. A review of SIN NSPs will be presented before RV NSPs.

nsP1 of SIN possesses methyltransferase and guanylyltransferase activities (Ahola and Kaariainen, 1995; Mi and Strollar, 1991) and is thought to function in capping of the genomic 40S and subgenomic 26S mRNA as well as in the initiation of minus-strand RNA synthesis. This latter function may involve the binding of a N-terminal tyrosine residue (Wang et al., 1991; Hahn et al., 1896; Sawicki et al., 1981).
nsP2 of SIN has helicase and protease domains at its N- and C-terminal regions, respectively. The N-terminal region contains the conserved sequence motifs found in NTP-binding helicases, and its ATPase activity is stimulated by RNA (Rikkonen et al., 1994). The C-terminal region contains the thiol protease activity and processes nonstructural polyprotein precursors (Gorbalenya and Koonin, 1993; Ding and Schlesinger, 1989; Hardy and Strauss, 1989). It also encodes a nuclear localization signal and may function to shut down host transcription in infected cells.

nsP3 of SIN is phosphorylated by a casein kinase II-like activity (Li et al., 1990) but its function is unclear. Flanking the papain protease domain, the N-terminus of alphavirus nsP3 contains a sequence motif of unknown function which is also found in RV and hepatitis E virus genome (Gorbalenya et al., 1991). The role of this “X” domain, which is conserved in most positive-strand RNA viruses is not well defined.

nsP4 of SIN contains the conserved GDD motif of RNA-dependent RNA polymerase (RdRp) (Gorbalenya and Koonin, 1993) and functions in elongation of both positive and negative-strand RNAs.

I-3.2. Functions of RV NSPs

The functions of RV NSPs are poorly understood. Most of our knowledge of their roles in viral RNA replication was either derived from sequence analysis or deduced from studies
on alphaviruses (Strauss and Strauss, 1994). Computer-assisted alignment predicted four conserved enzyme motifs in the RV NSP sequence (Fig. 2).

The p150 contains the predicted methyltransferase and protease sequences at its N- and C- regions, respectively (Marr et al., 1994). The p90 contains the proposed helicase and RdRp sequences at its N- and C-termini, respectively. Experimental data for the protease (Chen et al., 1996) and helicase (Gros and Wengler, 1996) activities associated with the predicted motifs have been presented. No evidence for RV putative RdRp and methyltransferase has so far been reported.

I-3.3. Protease

Polyprotein processing is a strategy employed by a number of positive-strand RNA viruses for genome expression in order to economize on coding capacity. Four groups of proteases are distinguished by their active sites as aspartate, cysteine, metallo-, or serine-proteases (Gorbalenya et al., 1991). Polyprotein cleavage is mediated by both cellular- and viral proteases. Studies from both herpes simplex virus and cucumber mosaic virus (CMV) revealed that there is a large superfamily of virus-encoded proteases related to chymotrysin-like cellular serine proteases (Gorbalenya et al., 1989). Recently, the existence of cysteine proteases related to papain-like cellular enzymes has been found for several positive-strand RNA viruses (Baker et al., 1989; Snijder et al., 1992; Strauss et al., 1992). Experimental data came from studies in several positive-strand RNA viruses including alphavirus nsP2 protease (Hardy and Strauss, 1989), equine arteritis virus (EAV; family Coronaviridae) nsP1 protease (Snijder et al., 1992), the potyvirus HC-pro
(Oh and Carrington, 1989), the p29 and p48 proteases of hypovirulence-associated virus (HAV) of chestnut blight fungus (Shapira and Nuss, 1991), and foot-and-mouth disease virus (FMDV; genus Aphthovirus, family Picornaviridae) (Piccone et al., 1995).

In general, papain-like proteases have a catalytic dyad consisting of a cysteine and a histidine residue. The catalytic domain of RV protease (RV-pro) is between residues 1005 and 1507 as determined by deletion mapping studies (Marr et al., 1994). On the basis of computer alignment analysis, Cys-1151 and His-1272 in RV-NSP-ORF were proposed to form the catalytic dyad (Gorbalenya et al., 1991). Mutations of Cys to Gly at residue 1151, or His to Leu at residue 1272 completely abolished proteolytic activity (Chen et al., 1996; Marr et al., 1994). Thus, Cys-1151 and His-1272 represent the catalytic dyad of RV NS protease (NS-pro).

In RV polyprotein p200 the single cleavage occurs after Gly-1301 in the sequence G1300-G1301-G1302 (Chen et al., 1996). Changing any one of the three glycine residues to valine at residue 1300, 1301 or 1302 results in the complete abolition of cleavage. In contrast, changing glycine to alanine at residues 1300, 1301 or 1302 impaired but did not eliminate cleavage, though substitution at residue 1301 resulted in only minimally detectable cleavage (Chen et al., 1996).

There are two viral papain-like protease families separated on the basis of their ability to function in cis or in trans (Gorbalenya et al., 1991). Main or M-proteases exhibit both trans and cis activity, cleave at several sites, whereas leader or L-proteases cleave the
viral polyprotein only in cis at a single site. In addition to the different trans- and cis-activities of these two groups, their locations are also dissimilar. Normally, L protease domains are located outside the domains and directly involved in genome expression. In contrast, M protease domains are located in the central region of the polyprotein. Most of the virus papain-like proteases belong to L-protease. Gorbalenya et al. (1991) predicted that RV NS-pro would be an M-protease by comparative sequence analysis. Yao and Gillam (1998) provided the first experimental data to show that RV protease was a Main protease that can function both in cis and in trans by using pSFV-1 eukaryotic expression system. Further characterization of RV NS-pro trans- versus cis-domain was accomplished recently using an in vitro transcription/translation system (Liang et al., 2000). The domain responsible for trans-activity was found within residues 920 to 1296, whereas the domain for cis-cleavage activity was mapped to residues 920 to 1020. It was also found that the X-domain had no effect on cis-cleavage activity, although it was important for trans-activity (Liang and Gillam, 2000). Thus far, the alphavirus nsP2 protease (Hardy and Strauss, 1989), the murine coronavirus mouse hepatitis virus (MHV) PLP-1 (Baker et al., 1993), and RV NS-pro (Chen et al., 1996; Yao et al., 1998) are the only defined M-proteases within the papain-like cysteine protease family.

SIN NS-pro is also an M-protease that is mapped to residues from 460 to 807 in the nsP2 region (Hardy and Strauss, 1989). C481 and H558 form the catalytic dyad and can cleave both in trans and in cis, as shown by mutational analysis (Strauss et al., 1992).
The relationship between SIN NS-pro cleavage and viral RNA replication has been established (Lemm et al., 1994). After infection, SIN genomic RNA is translated to produce p123 (200 kDa) and a small amount of p1234 (240 kDa) by readthrough of an opal termination codon at nsP3/nsP4 (Fig. 4). Cis-cleavage at the 3/4 site leads to the production of p123 and nsP4 complex. Cleavage at the 3/4 site is essential for the formation of functional polymerase activity by releasing nsP4. Minus-strand replication complex containing p123 and nsP4 binds to the 3' end and elongate minus strands. A complex containing nsP1, nsP2, nsP3 and nsP4 functions to initiate plus strand RNA synthesis. It is believed that cleavage between nsP1 and nsP2 occurs in response to the accumulation of p123. This trans-cleavage at the 1/2 site forms nsP1+p23+nsP4 complex that is active both in minus and plus strand synthesis (Lemm et al., 1997; 1994). Further trans-cleavage at 2/3 site results in inactivating the minus strand replicase activity and forms a new transcriptase activity to initiate both plus-strand genomic and subgenomic RNA synthesis. (Shirako and Strauss, 1994; Lemm et al., 1994). The formation of a stable replication complex allows the continuous production of plus-strand RNA throughout the viral life cycle.

Studies of the relationship between NSP processing and RV RNA synthesis are underway. Protease-inactive and cleavage-defective mutants that are composed of the uncleaved p200 function only in minus-strand RNA synthesis (Liang and Gillam, 2000). The functional role of p150 and p90 in RNA replication remains to be characterized.
I-3.4. RNA-dependent RNA Polymerase

RNA-dependent RNA polymerases (RdRps) are required for the replication of all positive-strand RNA viruses. They function as the catalytic subunit and form viral replication complex with host factor(s). Polymerase proteins in most positive-strand RNA viruses have been identified mainly on the basis of sequence conservation and mutational analysis. Catalytic activity has been demonstrated biochemically in several viral proteins, including: Qβ replicase subunit II (Landers et al., 1974), poliovirus 3D\textsuperscript{pol} protein (Neufeld et al., 1991), Hepatitis C virus NS5B protein (Behrens et al., 1996), and tobacco vein mottling virus (TVMV) nuclear inclusion protein NI6 (Hong and Hunt, 1996).

Kamer and Argos (1984) identified several common sequence motifs among animal and plant positive-strand RNA viruses. Currently, there are eight conserved RdRp motifs (Koonin, 1991). Four of these eight conserved motifs A, B, C and D are present in all polymerases and reside in the catalytic portion of the “palm” domain (Fig. 3).
Fig. 3. Sequence alignments of motifs A-E of RNA-dependent RNA polymerase

The polymerase subdomains including the palm motifs (A-E) are indicated below the polymerase alignments. The highly conserved motif GDD (shown in bold letters) is located at the core region of the “palm” domain.

The highly conserved GDD motif found in RdRps of all plant, animal and bacterial viruses is located at motif C which forms a “β-strand, turn, β-strand” structure (Kamer and Argos, 1984). This structure is very similar in all classes of polymerases and positions the two DD residues close to the conserved D of motif A (Hansen et al., 1997). The first aspartate of the GDD motif is thought to be involved in coordination of a second divalent cation, usually Mg++. Mutational analysis shows a strict requirement for this aspartate. Any changes to this position, both in vivo viral replication and/or in vitro RNA
synthesis (Jablonski and Morrow, 1993; Lohmann et al., 1997; Longstaff et al., 1993) are never tolerated. The second aspartate of the GDD motif is not absolutely conserved in all classes of polymerases, suggesting some flexibility at this position. However, mutation analysis indicates a fairly strict requirement for this second aspartate. The analogous changes in poliovirus 3D\textsuperscript{pol}, D329N and D329E (Jablonski and Morrow, 1995), or D334E in EMC 3D\textsuperscript{pol} (Sankar and Porter, 1992) were not accommodated. Subtle changes to D319N or D319E in hepatitis C virus (HCV) NS5B were tolerated only at low level (\(\approx 8\%\)) \textit{in vitro} (Lohmann et al., 1997).

The requirement for the glycine residue of the GDD motif is somewhat flexible \textit{in vitro}. Substitution in the glycine residue results in different levels of polymerase activity. Substitution of alanine for glycine in poliovirus, HCV, and tobacco vein mottling virus (TVMV) were tolerated polymerase activities from 5\% to 12\% of wildtype (Hong and Hunt, 1996; Jablonski et al, 1991; Lohmann et al., 1997). Substitution of cysteine for glycine in HCV NS5B or serine for glycine in poliovirus 3Dpol gave 8\% and 20\% of wildtype levels of RNA synthesis \textit{in vitro}, respectively (Jablonski et al., 1991; Lohmann et al., 1997).

The GDD motif is absolutely conserved in all positive-strand RNA viruses, although in other types of viruses, the G residue may be replaced (Table 1). The two aspartate residues are conserved in all viral families. Therefore, it is of interest to investigate the effects of the changes in the GDD motif on RV replication.
Table 1. Sequence alignments of RdRp motif C GDD region in viruses

<table>
<thead>
<tr>
<th>Polymerase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genome&lt;sup&gt;b&lt;/sup&gt;</th>
<th>lineage</th>
<th>virus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>motif C</th>
</tr>
</thead>
<tbody>
<tr>
<td>RdRp</td>
<td>RNA(+)</td>
<td>Picorna</td>
<td>PV</td>
<td>M I A Y G D D V I A S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YFV</td>
<td>M A V S G D D C V V R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HCV</td>
<td>M L V C G D D L V V I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BVDV</td>
<td>I H V C G D D G F L I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TMV</td>
<td>G A F C G D D S L L Y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SIN</td>
<td>A A F I G D D N I I H</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RV</td>
<td>G I F Q G D D M V I F</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA(-)</td>
<td></td>
<td>FLUA</td>
<td>G L Q S S D D F A L I</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA(ds)</td>
<td>IBDV</td>
<td>I E R S I D D I R G K</td>
<td></td>
</tr>
<tr>
<td>RdDp</td>
<td>RNA(+)</td>
<td>HIV</td>
<td>I Y Q Y M D D I L I A</td>
<td></td>
</tr>
<tr>
<td>RdDp</td>
<td>DNA</td>
<td>HBV</td>
<td>A F S U M D D V V L G</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>RdRp: RNA-dependent RNA polymerase; RdDp: RNA-dependent DNA polymerase (reverse transcriptase);
<sup>b</sup>RNA(+), positive-strand RNA; RNA(ds), double-strand RNA; RNA(-), negative-strand RNA.
<sup>c</sup>Abbreviations: PV, poliovirus; YFV, yellow fever virus; HCV, hepatitis C virus; BVDV, bovine diarrhea virus; TMV, tobacco mosaic virus; FLUA, influenza A virus; IBDV, infectious bursal disease virus; HIV, human immunodeficiency virus; HBV, hepatitis B virus;
Interestingly, oligomerization of the poliovirus polymerase is found to be necessary for polymerase function. Polymerase-polymerase interactions have been observed in vitro (Pata et al., 1995) and in the two-hybrid system (Hope et al., 1997).

Upon infection, the first event for RNA viruses is to translate viral RdRp by using genomic RNA as a mRNA. Simultaneously, the minus-strand initiates transcription through RdRp recognition of a conserved promoter region at the 3' end. In turn, the newly synthesized minus-strand serves as the template for generating multiple copies of genomic, and in some viral species, subgenomic plus-strand RNAs. Both minus-strand and plus-strand synthesis rely on the replicase, which is composed of virally encoded RdRp, along with viral/cellular proteins. It is obvious that RdRp is the key component for viral replication. Therefore, identification of RdRp itself will reveal many of the mysteries of viral RNA replication and viral pathogenesis.

I-3.5. Helicase

RNA helicase functions in unwinding duplex RNA structures formed during RNA replication to allow strands to act as templates for further replication. It may also play a role in removing the secondary structure from RNA templates. Presumably, the energy utilized in the unwinding reaction to disrupt the hydrogen bonds between the two strands derives from hydrolysis of high energy phosphates. There is no evidence how the two reactions are coupled.
Three superfamilies (SF) of helicases have been classified by comparative sequence analysis (Gorbaleya et al., 1989, 1990). SF1 represents the alphavirus-like (nsP2-like) proteins, SF2 are classified as the potyvirus-flavivirus-pestivirus group (NS3-like proteins) (Suzich et al., 1993) and SF3 includes picornovirus-like (2C-like) proteins (Mirzayan and Wimmer, 1992). In SF1, RNA helicase unwinding activity has not been detected thus far, although NTP-binding activity has been reported. One of the possibilities is that these proteins exhibit helicase unwinding activity only when they form replication complexes with other viral and/or cellular proteins.

All established viral helicases exhibit NTPase activity, although different groups of helicase show different level of NTPase activity. This activity is dependent on the presence of an NTP and a divalent cation, usually Mg$^{++}$. The level of nucleic acid stimulation of the ATPase activity varies among the three groups of SFs. The highest stimulatory effect (3 to 15-fold) has been found for the enzymes belonging to SF2 (Lain et al., 1990; Tamura et al., 1993; Warrener et al., 1993). In contrast, only a twofold stimulation was described for the ATPase of SFV (Rikkonen et al., 1994), RV (Gros et al., 1996), turnip yellow mosaic virus (members of SF1), and poliovirus (members of SF3). However, helicase activity has not been demonstrated for SF1 so far.

RNA virus helicases are RNA-dependent NTPases. Their functional domains are identified by one major motif: G/A×××GKS/T (Gorbalenya et al., 1989). Comparative sequence analysis suggests that the RV NS region (residues 1300 to 1600) represents a viral helicase. Gros (Gros et al., 1996) showed that the p70 glutathione S-transferase
fusion protein expressed by *Escherichia coli*. was stimulated by binding to a single-strand RNA. The highest stimulation of 1.7-fold was observed in the GTPase reaction. The role of RV conserved GXGKT sequence in NTP binding is predicted by sequence comparisons and is confirmed by mutational analysis. Experimental data (Gros et al., 1996) showed that no detectable NTPase activity was observed in this mutant p70 protein when the K residue was mutated to Q. Thus, the K residue is associated with NTPase activity of RV helicase. Unfortunately, no unwinding activity can be detected in p70 protein although NTPase can be easily characterized. This finding is consistent with that of the other SF1 family members such as alphaviruses. The reason behind this phenomenon remains to be investigated.

I-4. Viral structural proteins

RV virions contain three structural proteins: capsid protein (C) together with 40S RNA forms a nucleocapsid inside the virion; the two *trans*-membrane proteins, E2 and E1, are embedded in the viral envelop (Oker-Blom et al., 1983). The structural proteins are synthesized as a polyprotein precursor which is proteolytically processed into C, E2, and E1 (Oker-Blom, 1984). This polyprotein is translocated into the ER by two independent signal peptides at the N-termini of C and E2 (Hobman and Gillam, 1988) and is cleaved by cellular signal peptidases into three products C, E2 and E1 (Hobman *et al*., 1990). E2 and E1 form a heterodimer and is transported to the Golgi as a complex. At the same time, the capsid protein associates with membranes in the cytoplasm and interacts with 40S genomic RNA to form nucleocapsids (Hobman *et al*., 1994; Qiu *et al*., 1994). In this
respect, the mechanism of capsid cleavage in RV is completely different from that of alphavirus. In alphavirus, the capsid protein is an autoprotease and releases itself from the polyprotein precursor within the cytoplasm (Melancon and Graroff, 1987).

I-4.1. C protein

The molecular weight of C is between 33 and 38 kDa. Amino acid sequence analysis showed that the N-terminal half of the C protein is highly hydrophilic and is rich in positively charged arginine residues. This region may be involved in binding to the negatively charged genomic RNA to form nucleocapsids (Clark et al., 1987). The C-terminal 23 aa are highly hydrophobic and function as the signal sequence for E2 translocation into the ER (Hobman and Gillam, 1989). Marr reported that C was phosphorylated although the extent of phosphorylation was not determined (Marr et al., 1991).

I-4.2. E2 and E1 glycoproteins

Most viral glycoproteins contain multiple glycosylation sites. Normally, animal viruses utilize host cell glycosylation machinery to synthesize and process viral glycoproteins. Generally, oligosaccharides on glycoproteins play a functional role in initiation and maintenance of correct folding into a biologically active conformation, protecting polypeptides against proteolysis, and influencing the antigenicity and immunogenicity of glycoproteins (Elbein et al., 1987; Qiu et al., 1992b). Both E2 and E1 contain three N-linked glycosylation sites (Asn-X-Ser/Thr). Site-directed mutagenesis analysis shows that
all three are utilized (Hobman et al., 1991; Qiu et al., 1992a). Deletion of any one of the oligosaccharide side chains from E1 results in less immunogenicity, although a single mutation in the E1 glycosylation sites does not reduce immunogenicity of RV (Qiu et al., 1992b).

Both E2 (42-47 kDa) and E1 (57 kDa) are type I transmembrane proteins, containing a single transmembrane domain at their C-terminal regions. E1-E2 form heterodimers in the ER and are transported as a complex to the Golgi apparatus, where they mediate intracellular budding (Hobman et al., 1993). Transport of E1 is completely dependent on the coexpression of E2, whereas E1 is not essential for E2 transport to the cell surface (Hobman et al., 1993).

I-4.3. **Immunological determination on the virion proteins**

E1 is the dominant surface molecule of the RV virion. The evidence is that the majority of anti-E2 monoclonal antibodies (McAbs) do not react with intact virions whereas anti-E1 McAbs do (Waxham and Wolinsky, 1985). E1 glycoprotein has been suggested to be the immunodominant antigen since it can induce viral neutralization antibodies and hemagglutination (HA) activities (Terry et al., 1988). Thus, E1 possesses the major protective epitope against RV infection. In addition, human T and B cell epitopes have been localized to capsid and E2 (Ou et al., 1992a,b). Therefore, a candidate subunit vaccine should contain all three RV structural proteins.

I-5. **Regulation of viral RNA replication**
I-5.1. Regulatory signals for viral replication

Mutational analysis of cis-acting elements of the RV and alphavirus genomes revealed that three highly conserved regions in the genomic RNAs were the regulatory signals for viral RNA replication (Strauss and Strauss, 1986).

The first of these regions serves as a promoter for initiation of genomic plus-strand RNA by the viral RdRp and forms a stem-and-loop structure at the 5' end of the genomic RNA [5'(+) SL] (Strauss and Strauss, 1994). Site-directed mutagenesis of these structures in SIN infectious cDNA clone revealed that most of the deletions were tolerated. Only deletions at the immediate end of the genomic RNA (nt 5 or nt 2 to 4) were lethal. Deletions downstream from this site of 1 to 15-nt resulted in viable virus but had variable effects on virus replication (Niesters and Strauss, 1990). Similar results were obtained for RV. Pugachev and Frey (1998) showed that only a few nucleotides at the extreme 5' end of the RV genome are essential for RV viability. Moreover, the 5'(+) SL is important for efficient translation of the NSP ORF (Pugachev and Frey, 1998). Additionally, two host factors were found to interact with 5'(+) SL. One of these was the La autoantigen (Pogue et al., 1996). Interestingly, this La autoantigen was also found to be present at increased levels in rubella patients.

The second conserved region is the 3' terminus preceding the poly A tail. Most of the 3' UTR was indispensable for RV replication, in contrast to that of alphaviruses, in which large deletions can be tolerated (Chen and Frey, 1999; Levis et al., 1986). Three host
factors were found to bind to the 3'(+) SL RNA of RV. One of these was calreticulin (CAL), a calcium binding protein found in most eukaryotic cells (Singh et al., 1994).

The third conserved region is upstream nucleotides from the subgenomic RNA start site. Interestingly, SIN SG RNA can be detected when this region was replaced by several alphaviruses, whereas no SG RNA can be detected when this region was substituted by rubella virus (Hertz and Huang, 1992). These differences between RV and other togaviruses remain to be characterized.

Replication is a fundamental activity of all viruses. Initiation of replication requires the specific recognition of cis-acting elements by the viral RdRp and/or host factor(s). The cis-acting elements in Togaviridae are highly conserved. They play a critical role in the regulation of virus replication and selection.

I-5.2. Model for the temporal regulation of SIN RNA synthesis

In alphavirus replication, minus-strand synthesis occurs only at early stages of infection and ceases at 4 h p.i., whereas plus-strand synthesis remains constant throughout the viral life cycle (Sawicki and Sawicki, 1980). The minus-strand replicases are unstable and require continuous protein synthesis (Sawicki and Sawicki, 1980). The short-lived unstable replication complex (RC_{initial}) forms first from nascent polyproteins and is converted into long-lived stable RC_{stable} by processing of nonstructural polyproteins including p23 cleavage.
Several models to explain the temporal cessation of alphavirus minus-strand RNA synthesis have been proposed. One of these models proposes that the formation of new \( \text{RC}_{\text{init}} \) complexes limits minus-strand RNA synthesis early in infection. The accumulation of protease during infection leads to rapid cleavage of newly synthesized p1234. Further cleavages result in conformational change that may determine template specificity. Therefore, early in infection, minus-strand \( \text{RC}_{\text{init}} \) can form. No further \( \text{RC}_{\text{init}} \) complexes can be formed because of alteration of initial template later on. Stable replication complex generates plus-strand RNA synthesis throughout the viral life cycle.

The relationship between the NSP processing and minus- and plus-strand RNA synthesis was established by mapping temperature-sensitive mutants (Barton et al., 1988; 1991; Hardy et al., 1989; 1990; Shirako and Strauss, 1994) and by genetic complementation in a recombinant vaccinia virus system (Lemm and Rice, 1993; Lemm et al., 1994). The same results are obtained from both approaches. Polyproteins play a role in minus-strand synthesis, whereas cleaved products function in plus-strand synthesis (Fig. 4).
Fig. 4. Model for the temporal regulation of SIN minus- and plus-strand RNA synthesis.

Following translation of p123 and p1234, *cis* cleavage at the 3/4 site produces p123 and nsP4 to form initiation complex for minus-strand RNA synthesis. Accumulation of nsP2-containing proteinases, capable of *trans*-cleavage at the 1/2 and 2/3 sites, converts the p123 into nsP1, nsP2, and nsP4. These cleavages may result in conformational changes which shift the template preference of the complex to minus strands, leading to efficient plus-strand RNA. During this process, the conversion from polyproteins into individual proteins determines the initiation of plus-strand RNA and shut off the minus-strand RNA synthesis. (Modified from Lemm, 1994)
I-6. Project Rationale and Thesis Objective

The fundamental activity of all viruses is to replicate themselves. More RNA templates must be made in order to produce more progeny viral particles. Different viruses employ different strategies for the purpose of replication. SIN, the prototype of Togaviridae, controls the RNA template preference by changing viral encoded protease activity. The uncleaved polyproteins initiate minus-strand RNA synthesis, whereas the processed products produce plus-strand RNAs. RV NSP precursor p200 has been shown to function in minus-strand RNA synthesis, whereas the function of the two cleavage products p150 and p90 remains to be investigated.

Four functional motifs have been proposed within RV NSP ORF on the basis of computer-assisted alignment analysis. No studies have been reported to demonstrate the activities of the methyltransferase and RdRp. Since GDD is conserved in all positive-strand RNA viruses, it is possible to demonstrate RdRp activity by detecting the effect of RV GDD itself on viral replication.

The objective of this thesis is to define the functional roles of RV nonstructural proteins in RNA synthesis. Two specific aims have been achieved. One is to characterize the functional role of the cleavage products p150 and p90 in RNA synthesis by complementation assay. The other is to analyze the effect of RV GDD motif on viral replication by mutational analysis. The significance of this finding will be discussed.
II. Materials and Methods

II-1. Material

Restriction endonucleases and DNA modifying enzymes were purchased from commercial suppliers and used according to manufactures' specifications. The companies are including Bethesda Research Laboratories (BRL), Promega, New England Biolabs, Boehringer Mannheim, Sigma, Pharmacia and United States Biochemical Corporation. L-[\textsuperscript{35}S]methionine (10 mCi/ml) and α-[\textsuperscript{35}S]-CTP (12.5 mCi/ml) are from New England Nuclear (NEN). RV NSPs peptide-specific antibodies NS1 and NS5 were generated in our laboratory using rabbits. NS1 encompasses RV nonstructural proteins from 1 to 36, whereas NS5 from residues 1598 to 1637. Human anti-rubella serum was a gift from Dr. Aubrey Tingle (Department of Pediatrics, University of British Columbia). Human 143, BHK-21, Vero cells and M33 strain were from the American Type Culture Collection (ATCC). Vaccinia wild type virus and recombinant vTF7-3 virus that expresses T7 RNA polymerase and plasmid pTM3 were gifts from Dr. Moss B. (NIH). Cell culture reagents were from GIBCO BRL.

II-2. Methods

II-2.1. Bacterial strains and Growth of Bacteria

\textit{E. coli} strain WM1100 was used for the propagation of recombinant clones. WM1100 cells containing recombinant plasmids were grown in 2×YT medium (16 g/l tryptone; 10 g/l yeast extract; 5 g/l NaCl) with addition of 100 μg/ml ampicillin for selection of antibiotic resistance. \textit{E. coli} strain DH5α F' from BRL was used for the propagation of
M13 vector (for sequence analysis). DH5α F' cells were grown in LB (10 g/l tryptone; 5 g/l yeast extract; 5 g/l NaCl).

II-2.2. Transformation of E. coli

Competent cells were prepared according to the method described in Promega biotec Technical Bulletin. Briefly, E. coli cells were inoculated in 100 ml 2xYT medium and grew for about 3 h at 37°C with moderate shaking until OD₆₀₀ to 0.50. Cells were recovered by centrifuge at 5000 rpm for 5 min at 4°C. The cell pellets were washed once with Sol A (10 mM MOPS [pH 7.0], 10 mM rubidium chloride). Then the pellets were suspended in 10 ml of ice-cold Sol B (10 mM MOPS [pH 6.5], 10 mM rubidium chloride, 50 mM Calcium Chloride) and incubated on ice for 30 min. Finally, the pellets were resuspended in 1 ml ice-cold Sol B with additional DMSO (35 μl/ml). For transformation, recombinant DNA was mixed with 100 μl competent cells and incubated on ice for 30-45 min. Heat-shocked was performed for 90 seconds at 42°C. After that, reaction mixture was transferred immediately to ice. Competent cells were diluted with 0.8 ml 2xYT and incubated at 37°C for recovery of antibiotic resistant. These cells were then dispersed on 2xYT agar plate containing 100 μg/ml ampicillin.

For transformation of bacteriophage M13 DNA, the heat-shocked DH5α F' cells were diluted into the mixture of 3 ml of prewarmed LB top agar (0.7% agar) (at 47°C water bath), containing 200 μl DH5α F' cells, 4 μl of 200 mM IPTG, and 20 μl of 50 mM X-Gal. And pour onto a LB agar plate immediately.
II-2.3. *Preparation of recombinant DNA*

Small-scale isolation of DNA (5-10 ml) was performed as described in QIAprep Spin Plasmid Kit. After suspension in 250 µl Buffer P1, the cells were lysed with Buffer P2. Chromosomal DNA and proteins were precipitated by 350 µl of Buffer P3. The solution was bound to QIAprep columns by centrifuge. The columns were washed with 500 µl of Buffer PB followed by additional wash with Buffer PE. The purified DNA was eluted by 100 µl H₂O and collected by centrifuge.

Mini-prep procedure was performed as described in Maniatis *et al* (1982) for screening of recombinant plasmids. Approximately 1.2 ml of overnight culture was spun down in a micro-tube for 1 min, and the pellet was resuspended in 100 µl of Sol I (0.2 N NaOH; 1% glucose; 25 mM Tris/Cl [pH 8.0]; 10 mM EDTA). Then 200 µl of Sol II was added with gentle mixing and the mixture was incubated on ice for 5 min. Chromosomal DNA and proteins were precipitated by Sol III (0.15 ml of 3 M KAC; 5 M HAC). The supernatant was collected by centrifuge and extracted once with phenol/chloroform mixture (1:1). The DNA was obtained by precipitation with 2 volumes of ethanol. The pellets were dissolved in 50 µl of H₂O. For each restriction enzyme digestion, 10 µl of the preparation was used.

II-2.4. *Isolation of single strand bacteriophage M13DNA containing RV sequence*

To 1.5 ml of LB broth, 50 µl of plating bacteria and a single plaque was added and the infected LB culture was grown for 4-6 h at 37°C. After centrifugation to remove cell pellets, the phage particles in the culture supernatant was precipitated by addition of 200
μl of PEG solution (20% PEG 8000, 2.5 M NaCl) and stood on ice for 30 min. The precipitated particles were removed by centrifugation at 14,000g for 10 min at RT. The pellet was resuspended in 100 μl of H₂O and extracted once with 100 μl of phenol and 100 μl of chloroform, respectively. Single-stranded DNA (ssDNA) was precipitated with 240 μl of 95% ethanol containing 80 μl of 3 M sodium acetate [pH 5.2]. The ssDNA was recovered by centrifugation and the pellet washed with 75% ethanol. The pellet was dissolved in 50 μl of H₂O for sequencing.

II-2.5. Purification of DNA fragment

The PCR products were purified with QIAquick PCR Purification Kit. Briefly, 5 volumes of Buffer PB were added to 1 volume of the PCR reaction mixture. To bind DNA, the samples were applied to the QIAquick column and centrifuged 1 min. The column was washed once with 0.75 ml Buffer PE. The purified PCR products were eluted by 50 μl of H₂O.

DNA fragment was purified with GENECLEAN II Kit (Bio 101, Inc.). Briefly, the excise band from agarose gel were mixed with 3 volumes of NaI and incubated at 55°C to melt the agarose gel. After 5 min incubation, GLASSMILK suspension was added. Pelleted GLASSMILK/DNA complex was centrifuged for 10 seconds. The pellet was washed three times with NEW WASH, resuspended in 10 μl of water and incubated for 5 min at 55°C. Purified DNA fragment was obtained by centrifugation.

II-2.6. PCR-mediated mutagenesis
A series of mutants were introduced into p90 GDD motifs by making single mutations at residues 1966, 1967, and 1968, respectively. The synthetic deoxyribonucleotides used in the mutagenesis were synthesized at UBC sequencing service center and listed in Table 2. HindIII linearized pBRM33 plasmid was used as the template for PCR. To construct three mutants, fusion PCR was employed. For mutant G1966A1, a pair of primers JSY18 plus XJW1 was used to amplify approximately 0.6 kb fragment from nt 5919-5946. The downstream mutant G1966A2 containing 0.4 kb fragment from nt 6491-5951 was obtained by a second PCR with a pair of primers XJW2 and DC2. The 1.1 kb fragment containing the desired G→A mutation was generated by the fusion PCR with G1966A1 and G1966A2 as templates and JSY18 and DC2 as a pair of primers. To construct D1967A mutation, two pairs of primers JYS18 plus XJW3 and DC2 plus XJW4 were used. The other two pairs of primers JSY18 plus XJW5 and DC2 plus XJW6 were used for construction of the D1968A mutation. The PCR products were purified with QIAquick PCR Purification Kit. Then these purified products were cut with FseI/BglIII and recovered the desired fragment by GENECLEAN Kit. The respective fragment was then used to replace the BglII/FseI fragment of the infectious clone pBRM33 to form the mutant clones: pBRM33G1966A, pBRM33D1967A, and pBRM33D1968A.

PCR cycling parameters: pfu polymerase was added after 100°C 2 min denaturing. Then ran the 25 cycles of 98°C 30 sec, 55°C 45 sec, and 72°C 1 min and 30 sec. Finally, the reaction was extended at 72°C for 6 min.
### Table 2. PCR primers used in the generation of mutants

<table>
<thead>
<tr>
<th>Primer</th>
<th>polarity(^a)</th>
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<th>Amino acid change</th>
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- \(^a\) polarity of primers on RV M33 genome, +, sense; -, anti-sense
- \(^b\) positions of primers on RV M33 genome
- \(^c\) sequences of primers. The mutated nucleotides are underlined.

![Diagram of PCR-mediated mutagenesis on RV genome](image)

Fig. 5. Positions of PCR-mediated mutagenesis on RV genome
Fusion PCR was employed to create RdRp GDD mutations. For the pBRM33G1966A mutant, a pair of primers, JSY18 plus XJW1 and DC2 plus XJW2 were used. For the pBRM33D1967A, a pair of primer, JSY18 plus XJW3 and DC2 plus XJW4 were used. For the pBRM33D1968A construct, JSY18 plus XJW5 and DC2 plus XJW6 were employed. All primers are shown as arrows to indicate their polarities.

II-2.7. Plasmid constructs

An RV infectious cDNA clone, pBRM33 (Yao and Gillam, 1999) was used in the plasmid construction. All DNA manipulation were conducted by using standard methods (Sambroock et al., 1989)

pTM3/T7 (Moss et al., 1990) is a pUC-derived plasmid that contains the T7 RNA polymerase promoter, the 5’ untranslated region (UTR) of encephalomyocarditis virus (EMCV) which facilitates cap-independent ribosome binding, a polylinker region, and a T7 transcription terminator. The AUG within the NcoI site in pTM3 is the AUG at which cap-independent translation is initiated. In RV M33 genome, the initiation codon AUG starts at nt 41 within the NcoI site. RV cDNAs were positioned to the unique NcoI site in pTM3 (Fig. 6A). The cloning sites used in the construction of recombinant plasmids are shown in Fig. 6B.
The multiple cloning site is located between the EMC and T7 regions. pT7: the T7 RNA polymerase promoter, EMC: the 5' untranslated region (UTR) of EMCV, T7: the T7 transcription terminator, Eco gpt: *E. coli* guanine phosphoribosyltransferase (gpt) gene for the isolation of recombinants.

![Diagram](image)

Fig. 6A. The structure of pTM3

The inserted RV NSP-ORF is shown as a dash line. The EMCV UTR region is shown as a bold line. pT7 is resided upstream of EMCV UTR region to direct the transcription of foreign gene expression. The translation initiation site is located at NcoI site. The location of NS1 (residues 1-36) and NS5 (residues 1598-1637) are indicated (■). The cleavage site between p150 and p90 is shown with an arrowhead.

![Diagram](image)

Fig. 6B. The schematic diagram of RV NSP-ORF used in the construction of recombinant vaccinia viruses.
To construct pTM3/200, the NcoI-PstI fragment (nt 41-2632) of pBRM33 was subcloned into the NcoI and PstI sites of pTM3 resulting pTM3RV2632. A PstI-KpnI fragment (nt 2632-6470) (KpnI site was blunt with T4 DNA polymerase) was then subcloned into the PstI-Stul sites of pTM3RV2632. The resulting construct was named pTM3/200.

pTM3/150 was constructed by insertion of PstI-EcoRI fragment (nt 2632-4213) of pBR-150 (Liang et al., 2000) into pTM3RV2632 which had been digested with PstI and Stul sites. pBR-150 contains a stop codon at the cleavage site (nt 3945) in the NSP-ORF (Liang et al., 2000).

To construct pTM3/90, the NcoI-PstI fragment of pCMV5p90 was subcloned into pTM3 that had been digested with NcoI and PstI restriction enzymes. pCMVp90 contains the RV 5'-untranslated region (nt 1-40) and cDNA encoding p90 (nt 3945-4023). Since there are two NcoI sites (at nt 39 and 4023) within the cDNA, partial digestion with NcoI was carried out to isolate the fragment that retained the second NcoI site.

pTM3/200/C1152S and pTM3/200/G1301S were constructed by insertion of PstI-Stul fragment (nt 2632-6470) of pBRM33C1152S (full-length protease-inactive mutant) or pBRM33G1301S (full-length cleavage-defective mutant) into the PstI and Stul sites of pTM3RV2632.
A. Full-length genome

\[ \text{NSP-ORF} \quad \rightarrow \quad \text{SP6} \quad \rightarrow \quad \text{SP-ORF} \]

\[ \text{X} \quad \text{P} \quad \text{H} \quad \text{R} \quad \rightarrow \quad \text{C} \quad \text{E2} \quad \text{E1} \quad \rightarrow \quad \text{SG RNA} \]

\[ \text{P150} \quad \rightarrow \quad \text{P90} \]

\[ \text{SP6} \quad \rightarrow \quad \text{TCW} \rightarrow \text{TSW} \quad \rightarrow \quad \text{poly A} \quad \text{pBRM33} \]

\[ \text{GGG} \rightarrow \text{GSG} \quad \rightarrow \quad \text{poly A} \quad \text{pBRM33G1301S} \]

\[ \text{GDD} \rightarrow \text{ADD} \quad \rightarrow \quad \text{poly A} \quad \text{pBRM33G1966A} \]

\[ \text{GDD} \rightarrow \text{GAD} \quad \rightarrow \quad \text{poly A} \quad \text{pBRM33D1967A} \]

\[ \text{GDD} \rightarrow \text{GDA} \quad \rightarrow \quad \text{poly A} \quad \text{pBRM33D1968A} \]
B. *vaccinia virus* recombinants

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Fig. 7. Schematic representation of RV and various modified genomes used in this study

A. Full-length genome. NSP-ORF, open reading frame of nonstructural proteins; SP-ORF, open reading frame of structural proteins; SG RNA, subgenomic RNA; pBRM33, infectious RV cDNA clone; pBRM33C1152S, full-length protease-inactive mutant; pBRM33G1301S, full-length cleavage-defective mutant. B. Vaccinia virus recombinants expressing p200, p150 and p90 RV nonstructural proteins.
II-2.8. Generation of vaccinia virus recombinant

Recombinant vaccinia viruses were generated by marker rescue on human TK\(^{-}\) 143 cells, and identified by the gpt selection method (Falkner and Moss, 1988). Viruses were plaque purified three times under selective conditions (25 \(\mu\)g/ml of mycophenolic acid (MPA), 250 \(\mu\)g/ml of xanthine, and 15 \(\mu\)g/ml of hyperxanthine). Recombinants were identified by protein analysis of infected cell lysates. The recombinant viruses expressing p200, p150 and p90 were named vTM3/200, vTM3/150 and vTM3/90, respectively.

II-2.9. Protein analysis of recombinant vaccinia virus-infected cells

Monolayers of BHK cells in 35-mm tissue culture dishes were co-infected with vTF7-3 and vaccinia recombinant viruses at a multiplicity of infection (MOI) of 2 pfu per cell. For immunoprecipitation (IP), at 12 h post-infection (p.i.), the media was removed and cells were washed and starved in DMEM lacking methionine and cysteine and containing 5% dialyzed FCS for 30 min. \(^{35}\)S]methionine (20 \(\mu\)Ci/ml) was added and incubated at 37°C for 45 min. After labeling, the cells were lysed in lysis buffer (25 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) and nuclei were removed by centrifugation. RV NSPs were immunoprecipitated with NS1 and NS5 antibodies. NS1 is the rabbit serum against the N-terminal region of NSP-ORF (residues 1-36). NS5 is the rabbit serum against the C-terminal region of NSP-ORF (residues 1598-1637). After incubation at 4°C for 1 h, 40 \(\mu\)l of 50% protein A-Sepharose beads (Pharmacia Biotech) was added and incubated for a further 1 h at room temperature with constant shaking. The
beads were washed three times with 1 ml TNE buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), resuspended in SDS-gel loading buffer (50 mM Tris-HCl [pH 7.2], 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue) and boiled for 5 min. After centrifugation, the immunoprecipitates were collected and then analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8%). Radiolabeled proteins were visualized by fluorescence autoradiography.

In pulse-chase experiments, at the end of labeling period, the medium containing the radiolabel was removed, and D-MEM containing the normal concentrations of methionine and cysteine was added. The cells monolayers were scraped and suspended in PBS at various chase times and lysed in 200 µl of lysis buffer.

II-2.10. *In vitro RNA transcription*

Recombinant plasmids were linearized at the *Hind*III site and RNA transcripts were synthesized with SP6 RNA polymerase (Promega) in the presence of m$^7$Gpp(5')G cap analog (Promega) in the reaction mixture (40 mM Tris-HCl [pH 7.9], 6 mM MgCl$_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 0.05% Tween-20, 0.5 mM NTP, 1 mM cap, and 1U RnAsin Ribonuclease Inhibitor) at 37°C for 1 h.

II-2.11. *RNA transfection*

BHK cells were transfected by electroporation as described previously (Yao and Gillam, 1999). Briefly, BHK cells were trypized and washed with cold phosphate-buffered saline
(PBS) (without Ca\(^{++}\) and Mg\(^{++}\)) twice and resuspended at a concentration of 10\(^7\) cells/ml. RNA transcripts (10-20 \(\mu\)g) were mixed with 0.45 ml of cells, and the mixture was transferred to a 2-mm diameter cuvette. Electroporation was done with two consecutive 1.5-kv, 25-\(\mu\)F pulses. After electroporation, the cells were distributed into four culture dishes (35-mm).

II-2.12. Plaque assay

For one-step growth rate analysis, the culture medium was harvested and replaced with fresh medium every 24 h until indicated times. Vero cells were infected with the released viruses at a serial dilution for 2 h at 37°C. The monolayers were overlaid with 0.5% agarose in MEM-5% FCS and stained with 5% neutral red in MEM medium after incubation of six days.

II-2.13. Extraction of total RNA

Total cellular RNA was isolated from cell cultures with TRIzol Reagent (GIBCO BRL) followed the protocol recommended by the manufacturer. For each 35-mm cell culture dish, 0.8 ml of TRIzol was added. The lysates were passed through several times through a pipette. The sample was extracted with additional 0.16 ml of chloroform and then incubated on ice immediately for 2 min. The aqueous phase was transferred to a 1.5 ml microtube with 0.4 ml of isopropanol. The RNA was precipitated by centrifugation after a 10 min incubation. The pellet RNA was washed with 70% ethanol and dried by air. RNA sample was dissolved in 100% formamide (deionized) and stored at -70°C.
II-2.14. *RNase Protection Assay (RPA)*

For RPA experiments, a minus-polarity RNA probe (pb18) (Liang and Gillam, 2000) was used. pb18 was synthesized with SP6 RNA polymerase from *EcoRI*-digested plasmid pSPT18-pb which can protect 301-nt positive-strand genomic RNA and 188-nt subgenomic RNA. The $^{35}$S-labeled pb18 probe was synthesized with SP6 polymerase in a 20 µl *in vitro* transcription reaction mixture and incubated for 1-2 h at 37°C. The reaction mixture contains transcription buffer (2 mM spermidine; 10 mM DTT; 6 mM MgCl$_2$; 40 mM Tris-HCl; 0.05% Tween-20), 0.5 mM each of ATP, GTP, and UTP, 10 µM of CTP, 2.5 mCi/ml of [$\alpha$-$^{35}$S] CTP. DNasel (7500u/ml) was added and incubated for 15 min at 37°C. The probe was precipitated with ethanol after phenol/chloroform extraction and resuspended in hybridization buffer.

For positive-strand RNA assay, approximately 2 µg of total cytoplasmic RNA was hybridized with $5\times10^5$ cpm pb18 probe (Liang and Gillam, 2000) in 30 µl of hybridization buffer (40 mM PIPES [pH 6.4], 1 mM EDTA, 400 mM NaCl, 80% deionized formamide) overnight at 55°C. RNase digestion was for 45 min at 30°C in an RNase mixture (RNase A at 10 µg/ml, RNase T1 at 70 u/ml, 300 mM sodium acetate, 10 mM Tris-HCl [pH 7.5]). The samples were treated with SDS-protease K for 15 min at 37°C, extracted with phenol-chloroform and ethanol precipitated with 5 µg of *E. coli* tRNA at -70°C. Samples were denatured in boiling water and analyzed on a 5% polyarylamide-7M urea sequencing gel. The gel was fixed in 10% acetate acid infiltrated with Enhancer (DuPort), dried and exposed to X-ray film overnight at -70°C.
II-2.15. Image analysis

Image analysis was employed to quantitate the relative amount of protected RNAs. Program for image analysis was downloaded from the Scion Image program for windows at http://www.scioncorp.com.

I-2.16. Isolation of RV particles

Monolayers of Vero cells grown in 150-mm-diameter dishes were infected with RV M33 and mutant virus pBRM33G1966A, respectively. At day 3 post-infection, the culture medium was harvested and virions were precipitated by using PEG solution (40% PEG, 2.5 M NaCl). The pelleted virions are resuspended in 1 ml of TNE buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA). Virion RNA was extracted with TRIzol reagent as described previously and used for first-strand cDNA synthesis.

II-2.17. Synthesis of first-strand cDNA strand

First-strand cDNA synthesis was carried out at 42°C for 1 h in a 20 μl reaction mixture containing viral RNA, 100 pmol of oligo (dT), 200 u of Supercrypt RT RNase H reverse transcriptase (RT) and 40 u of RNase inhibitor in a RT buffer. The synthesized first-strand cDNA was used as the template for the PCR reaction.

II-2.18. PCR and sequencing analysis

A pair of primers, XJW7 and JSY18, was used in the reaction for the synthesis of cDNA covering the NSP-ORF nt 5310-6022. The purified PCR fragment was blunt-ended with Klenow, cut with Bgl II and then inserted into M13 vector at BamH I and EcoR I (blunt-
ended with Klenow) sites. After transformation into DH5α F', recombinant plasmids were identified by blue/white plaque selection and restriction enzyme mapping. Three individual clones were then sent to UBC sequencing service center for sequencing analysis.
III. Results and Discussion

III-1. Identification of components for plus-strand RNA synthesis

The slow replication rate and high GC content of RV genome have hampered research in RV. The apparent similarity of RV to prototype alphaviruses has left RV investigators with few alternatives but to constantly refer to alphavirus models. In alphaviruses, the roles of NSPs in RNA replication and transcription have been studied in detail using a vaccinia virus transient expression system (Lemm and Rice, 1993a, 1993b; Lemm et al., 1994; Lemm et al., 1998). A model for temporal regulation of minus- and plus-strand RNA synthesis has been proposed for SIN (Fig. 4). In RV, the processing of RV NSPs is much simpler than that of SIN NSPs (Fig. 1). There is only a single cleavage in the p200, although RV protease possesses both cis- and trans-cleavage activity (Yao et al., 1998). It has been shown that uncleaved p200 functions only in minus-strand RNA synthesis, but not in plus-strand RNA synthesis (Liang et al., 2000). Therefore it is likely that uncleaved p200 forms the replication complex for negative-strand RNA synthesis, and that cleavage of p200 into p150 and p90 converts the complex to the capacity for efficient positive-strand RNA synthesis. Since RV appears to have a similar replication strategy to alphaviruses, the same approach used in the studies of SIN NSPs was undertaken in my study.

III-1.1. Expression of the RV NSPs by recombinant vaccinia viruses
To determine whether pi50 and/or p90 are required in positive-strand genomic and subgenomic RNA synthesis, I generated vaccinia recombinants expressing individual p200, p150, p90, p200/C1125S and p200/G1301S using the pTM3/T7 system (Moss et al., 1990). p200/C1152S is a p200 containing a mutation at the catalytic site (residue 1152, C→ S), whereas p200/G1301S is a mutant with a cleavage-site alteration at residue 1301 (G→ A) (Liang and Gillam, 2000). Expression of RV-specific proteins from the vaccinia virus recombinants was determined by immunoprecipitation (IP). BHK-21 cells coinfectected with vTF7-3 and various recombinants were labeled with [35S]methionine at 12 h p.i.. Cell lysates were immunoprecipitated with antibodies monospecific for RV p150 (NS1) and p90 (NS5). The precipitated proteins were analyzed by SDS-8% PAGE. As expected, the individual proteins expressed by the vaccinia virus recombinants with apparent molecular weights of 200 kDa, 150 kDa and 90 kDa were observed (Fig. 8). In vTM3/200-infected cells, three proteins with molecular weights of 200 kDa (p200), 150 kDa (p150) and 90 kDa (p90) were recognized by antiserum NS5 specific for p90 (Fig. 8, lane 4) and two proteins with molecular weights of 200 kDa and 150 kDa were recognized by NS1 specific for p150 (Fig. 8, lane 9). As reported previously by Yao et al. (1998), p150 was coprecipitated by NS5 antibody (Fig. 8, lane 3). In vTM3/150-infected cells, a protein with an apparent molecular weight of 150 kDa (p150) was recognized by both antisera NS1 and NS5 (Fig. 8, lanes 3 and 8). In vTM3/90-infected cells, a protein with a molecular weight of 90 kDa, migrating slightly above the protein band in the wt vaccinia virus infected cells, was only recognized by antiserum NS5 (Fig. 8, lanes 2 and 7). These RV-specific NSPs were not present in mock-infected cells (wt vaccinia virus).
(Fig. 8, lanes 1 and 6). Taken together, the data showed that these RV NSP recombinant proteins were expressed efficiently and correctly in the pTM3/T7 system.

Fig. 8. Expression of RV NSPs in cells infected with recombinant vaccinia viruses.

BHK cells were infected with individual vaccinia recombinant viruses. At 12 h p.i., the infected cells were pulse-labeled for 45 min. Cellular lysates were immunoprecipitated with antibodies specific to p90 (NS5) and p150 and p200 (NS1).
III-1.2. *Processing of RV NSPs in recombinant vaccinia viruses:*

Polyprotein processing is the strategy used by most positive-strand RNA viruses for viral replication. In SIN, RNA replication is highly regulated by NSP cleavage. The relationship between the NSP cleavage and viral RNA replication in RV is worthy of study.

The polyprotein-processing kinetics of RV NSPs was examined by the pulse-chase analysis of vTM3/200-infected cells. At 24 h p.i., vTM3/200-infected cells were labeled for 45 min with $[^{35}S]$methionine and chased with normal medium for periods up to 5 h. As shown in figure 9A, the intensity of p200 decreased substantially through the chase period (Fig. 9A, lanes 3 to 5). No residual fraction of p200 can be detected after 3 h (Fig. 9A, lane 5), whereas the intensity of p90 and p150 bands increased slightly or remained constantly during the 5 h chase time. This is consistent with the results reported by Yao *et al.* (1998). p150 and p90 bands can be easily observed even during a 45 min of pulse labeling (Fig. 9A, lane 2), suggesting that the cleavage of p200 is very efficient.

It has been shown previously that mutation at the protease cleavage site (G1301S) or protease active site (C1152S) completely abolishes p200 cleavage (Chen *et al.*, 1996; Liang and Gillam, 2000; Liang *et al.*, 2000; Yao *et al.*, 1998). I generated vaccinia virus recombinant expressing cleavage-defective and protease-inactive mutants (vTM3G1301S and vTM3C1152S) in order to assess the possible function of this polyprotein in RNA replication complex. A heavy band with molecular weight of 200 kDa was observed, up
to 1 h chase after 45 min labeling (Fig. 9B, lanes 2 to 5). In vTM3/200-infected cells, efficient p200 cleavage occurred during the 1 h chase period (Fig. 9B, lanes 6 and 7). The other protein species with molecular weights between 200 kDa and 150 kDa could be degraded products of NSPs or nonspecific host proteins coprecipitated with the NSPs. Interestingly, the protein with molecular weight of approximately 70 kDa (indicated by an arrowhead) and coprecipitated in all recombinants was similar with that observed by Yao et al. (1998). This protein may be a host protein coprecipitated by antiserum.

Fig. 9. Kinetics of RV NSP processing.

A. BHK cells were co-infected with vTF7-3 and vTM3/200 as described in Materials and Methods (lanes 2 to 5). B. BHK cells were co-infected with vTF7-3 and vTM3/1152 (lanes 2 and 3) or vTM3/1301 (lanes 4 and 5) or vTM3/200 (lanes 6 and 7), respectively. Cells were pulse-labeled with [35S]methionine for 45 min, and chased for 0, 1, 3, and 5 h after 24 h p.i., Cell lysates were immunoprecipitated with a mixture of antibodies monospecific to RV 150 (NS1) and p90 (NS5). Protein samples were analyzed by SDS-8% PAGE. RV-specific proteins are indicated on the right, and the molecular mass standards are indicated on the left. Lane 1: mock infection.
III-1.3. *Sensitivity and specificity of RNase Protection Assay (RPA)*

To evaluate the sensitivity and specificity of RPA for detection of positive-strand RV RNA, the negative-polarity RV RNA transcript was used as a negative control. *In vitro* RNA transcript of an infectious RV cDNA clone (pBRM33) with serial dilutions was subjected to RPA. Various amount of these transcripts were then hybridized with $5 \times 10^5$ cpm of $^{35}$S-labeled probe named pb18, containing 301-nt of the RV sequence and 27-nt of the pSPT18 vector sequence of reverse polarity. As shown in Fig. 10, the major protected fragment for plus-strand genomic RNA is 301-nt. This protected band was present in reaction mixtures containing 100 pg (lane 4), 1 ng (lane 3) and 10 ng (lane 2) of positive-strand RNA but was absent in reaction mixtures containing 20 ng of negative-polarity genomic RNA transcript (lane 5). These data indicate that this assay system is capable of specific detection of approximately $10^7$ molecules (appropriately 100 pg of transcript) without interference by the minus-polarity RNA in reaction mixture. To determine the amount of total cellular RNAs needed in the RPA assay, total cytoplasmic RNAs were extracted with TRIzol (GIBCO) reagent from BHK-21 cells transfected with RNA transcript synthesized from the infectious RV cDNA clone pBRM33 24 h after electroporation. The quantities of RNA were determined spectrophotometrically (Beckman). 10 µg, 5 µg and 1 µg (Fig. 10. lane 6-8) of total cellular RNAs were hybridized with $5 \times 10^5$ cpm of pb18 probe. As shown in Fig. 10, 1 µg of total cellular RNA was clearly detected as a protected band (Fig. 10. lane 8). Thus, I used 1-2 µg of total cellular RNA in the following RPA.
RPA reactions were carried out as described in Material and Methods. pBRM33 (positive-polarity) or pBRM33R (negative-polarity) *in vitro* transcripts were used as standard RNAs. They code for a full-length RV genome with either the forward or backward orientation. For sensitivity detection, the amount of 10 ng, 1 ng and 100 pg of pBRM33 was hybridized with 5×10^5 cpms of ^35S-labeled negative-polarity probe named pb18 (lanes 2 to 4). For specificity assay, 20 ng of negative-polarity transcript from pBRM33R were mixed with pb18 (lane 5). For determining the amount of total cellular RNAs to be used in the assay, 10 μg, 5 μg and 1 μg of total cytoplasmic RNAs (lanes 6 to 8) from pBRM33-infected cells were analyzed. The products of RPA reactions were resolved on a 5% polyacrylamide-7M urea gel, which was treated with Enhancer (DuPont), dried, and exposed to X-ray film. The positions of the 328-, 301-, and 188-nt are indicated on the right. Lane 1: 2×10^5 cpms of pb18.

III-1.4. *RV NSPs are self-replicons*

RV NSPs are believed to be involved in viral RNA replication (Frey, 1994; Liang and Gillam, 2000; Wolinsky, 1996). To address this question directly, I made a construct named pBRM33ΔStuI that had most of the structural proteins deleted but retained the complete NSF-ORF and 3' end poly (A) tail (deletions from nt 6959-9327). BHK cells were transfected with RNAs from either the infectious clone pBRM33 or the deletion construct pBM33ΔStuI and incubated at 37°C for up to 48 h after electroporation. Total cytoplasmic RNA was extracted from transfected BHK-21 cells at 0, 8, 24, and 48 h post-electroporation and subjected to RNase protection assay (RPA) for analysis of the
presence of positive-strand viral RNA. The protected bands were scanned to determine the relative amount of protected RV-specific genomic and subgenomic RNA at 24 and 48 h post-transfection.

As illustrated in Fig. 11, large quantities of input plus-strand RNA transcripts, which represent input virion-delivered genome, were seen at the beginning of transfection (lanes 3 and 7). By 8 h post-transfection, the input plus-strand RNAs were mostly degraded (lanes 4 and 8). Both protected bands, 301-nt, representing plus-strand genomic and 188-nt, representing subgenomic RNAs were clearly observed at 24 h post transfection (Fig. 11, lanes 5 and 9). Accumulation of both 188-nt and 301-nt protected fragments at 48 h post-transfection (lanes 6 and 10) indicated reinfection by RV. Quantation of the protected bands by image analysis showed that the molar ratio of subgenomic/genomic (SG/G) RNA at 24 h and 48 h post-transfection in these two constructs was similar (data not shown). This result showed that the deletion construct could replicate as efficiently as wt did. It is evident that RV RNA synthesis is related to the function of NSPs but not the SPs.
Fig. 11. RNA analysis of WT and deletion mutant pBRM33ΔStuI.

BHK cells were electroporated with RNAs transcribed \textit{in vitro} from a cDNA clone containing the wt sequence (lanes 3 to 6) or RV NSP-ORF (deletion from nt 6959-9327) (lanes 7 to 10). The total cellular RNAs were extracted with TRIzol reagent and subjected to an RPA using probe pb18, specific to plus-strand RNA synthesis, at 0, 8, 24 and 48 h post-electroporation. Lane 1: 2×10^3 cpm of pb18. Lane 2: BHK-21 cells were electroporated in the absence of RNA.

III-1.5. \textit{Complementation of virus replication by NSPs provided in trans}

In order to study the roles of NSPs in synthesizing distinct RV RNAs, complementation experiments were carried out. Genetic complementation assay of mutants with deficiencies in viral RNA replication is a powerful tool for studying the function of required genes and defining \textit{cis-} versus \textit{trans-}acting viral proteins. Two elements must be used in the assay: one is \textit{trans-}acting protein and the other is the appropriate template. In this study the \textit{trans-}acting proteins of RV NSPs were expressed either via a recombinant vaccinia virus or by co-transfection of RV NSPs helper genomes encoding NSPs (Fig. 12). The template used in the study included either protease-inactive or cleavage-defective mutant construct.
Mutated RV cDNA under control of SP6 promoter

In vitro transcription

HindIII linearized
SP6 RNA polymerase

I Mutated RNA transcripts (template)

II
cotransfection of BHK cells with helper RV RNA

Co-infection of BHK cells expressing RV NSPs from recombinant vaccinia viruses

Extraction of total cellular RNA
Analysis of accumulation of RV RNA by RPA

Fig. 12. Two methods were used to provide RV NSPs in trans

RNA template was obtained by in vitro transcription of the mutated RV cDNA construct. In Method I, RV helper RNA was co-transfected with mutated RV RNA. This helper genome provided all wild-type RV proteins. Its replication can be detected by RNase protection assay. In method II, expression of RV NSPs proteins was achieved with a recombinant vaccinia virus system.

Previous results show that full-length protease mutant pBRM33C1152S can synthesize minus-strand RNA but not plus-strand RNA (genomic or subgenomic RNA). No infectious virus was produced in the infected cells (Liang and Gillam, 2000). To determine which RV NSPs can rescue C1152S lethal mutation, I performed co-electroporation experiments using full-length protease mutant pBRM33C1152S and individual NSP constructs (Fig. 8). This was done with comparable amounts of two genome RNAs and their efficiency of transfection. As a control, the infectious clone pBRM33, full-length protease mutant pBRM33C1152S or individual RV NSP construct was electroporated into BHK-21 cells separately. Total cellular RNA was isolated and
subjected to RPA at the indicated times. The protected bands were scanned to determine the relative amount of protected RV-specific genomic (301-nt) and subgenomic RNA (188-nt) at 24 and 48 h post-transfection. As shown in Fig. 13, the input positive-strand RNAs were mostly degraded by 8 h post transfection, and accumulation of both protected 301-nt and 188-nt fragments was apparent for pBRM33 (lanes 5 and 6), pBRM33C1152S plus pBRM33G1301S (lanes 13 and 14), pBRM33C1152S plus p200 (lanes 17 and 18) and pBRM33C1152S plus p150 (lanes 21 and 22), but not for pBRM33C1152S (lanes 9 and 10), p200 (lanes 25 and 26) and p150 (lanes 29 and 30) at 24 and 48 h post-transfection. Positive-strand genomic RNA (G RNA) appeared as a 301-nt protected band, whereas subgenomic RNA (SG RNA) was observed as a 188-nt band. Further accumulation of both plus-strand SG and G RNAs at 48 h post-electroporation (lanes 14, 18 and 22) indicated the re-infection by rescued infectious viral particles. Image analysis showed that the molar ratio of SG/G RNAs of the rescued viruses was approximately 1.3:1, somewhat lower than the 2.7:1 ratio observed in pBRM33-transfected cells.
Fig. 13. Rescue of protease-inactive mutant by cotransfection of helper genome.

BHK cells were co-electroporated with *in vitro* transcripts from full-length protease-inactive mutant cDNA pBRM33C1152S together with RNA transcript of p200 (lanes 15 to 18); p150 (lanes 19 to 22) construct; or full-length cleavage-defective mutant pBRM33G1301S (lanes 11 to 14). Electroporation of pBRM33 (lanes 3 to 6), C1152S (lanes 7 to 10), p200 (lanes 23 to 26) or p150 (lanes 27 to 30) alone was used as positive and negative control. Total cytoplasmic RNAs were extracted with TRIzol and subjected to RPA at the indicated times. Lane 1: 2×10^3 cpmp of pb18. Lane 2: normal BHK-21 cells were electroporated in the absence of RNA.

There is a possibility that the low molar ratio of SG/G obtained in the rescued mutant is due to the low concentration of proteins that were expressed in helper genomes. To address this question, I used a second approach to provide high level expression of NSPs in *trans* by using recombinant vaccinia viruses expressing p200, p150 or p90 (Fig. 8). To analyze whether the expression of NSPs would rescue the replication of mutated RV RNA in *trans*, the cells were infected with recombinant vaccinia viruses 4 h after electroporation of full-length protease mutant pBM33C1152S. Total cytoplasmic RNAs were extracted with TRIzol reagent at 0, 8, 24 and 48 h after transfection and subjected to RPA. The quantities of SG/G molar ratio were examined as described above. Again, the protected genomic (301-nt) and subgenomic (188-nt) plus-strand RNAs were observed at 24 and 48 h after electroporation (Fig. 14, lanes 5, 6, 9, 10, 13, 14, 17, 18, 21 and 22).
As in co-transfection of helper genome, a lower molar ratio of SG/G (approximately 0.8) than with wt was obtained for rescued RNA. Thus over-expression of RV NSP-ORF could not change the pattern of lower molar ratio of SG/G shown in co-replication of a mutant construct together with the helper genomes.

Fig. 14. Rescue of protease-inactive and cleavage-defective mutants by recombinant vaccinia viruses.

BHk cells were infected with vTM3/200 (lanes 7 to 10) or vTM3/150 (lanes 11 to 14) 4 h after electroporation of full-length protease-inactive mutant pBRM33C1152S. BHK cells were infected with vTM3/200 (lanes 15 to 18), vTM3/150+vTM3/90 (lanes 19 to 22) 4 h after electroporation of full-length cleavage-defective mutant pBRM33G1301S. pBRM33 (lanes 3 to 6) was electroporated as a positive control. BHK cells were infected with vTM3/200 (lanes 23 to 26), vTM3/150 (lanes 27 to 30) or vTM3/90 (lanes 31-34) without electroporation of RNA template at the same time. Total cytoplasmic RNAs were extracted with TRIzol and subjected to RPA at the indicated times. Lane 1: 2×10^3 cpm of pb18. Lane 2: normal BHk-21 cells were electroporated in the absence of RNA.

III-1.6. p150 and p90 function in efficient plus-strand RNA synthesis.

Since the cleavage site mutant which provides uncleaved p200 can synthesize minus-strand RNA but not plus-strand RNA (genomic or subgenomic) (Liang and Gillam, 2000), I addressed the question whether p150 and p90 can function in efficient plus-strand RNA synthesis by providing NSP-ORF in trans using helper genomes or vaccinia
recombinant viruses. BHK-21 cells were co-transfected with p200 and full-length cleavage site mutant pBRM33G1301S. Total RNAs were extracted at 0, 8, 24 and 48 h after transfection and subjected to RPA. Again, most of the input RNA transcripts were degraded noticeably at 8 h post-electroporation (Fig. 15, lanes 3, 4, 7, 8, 11, 12, 15 and 16). Protected genomic (301-nt band) and subgenomic (188-nt band) RNAs were observed 24 h after electroporation, indicating that RNA replication occurred in rescued G1301S mutant (Fig. 15, lanes 5 and 17).

A concern about co-electroporation of three RNA genomes into one cell in co-transfection experiments is the low efficiency of transfection. Therefore, I chose to use vaccinia recombinants to express NSPs. Immunoprecipitation showed that p150 and p90 could be expressed at the same time without interfering with the expression of each other after 8 h infection (Fig. 8, lanes 5 and 10). Thus these recombinant vaccinia viruses (vTM3/150 plus vTM3/90) can be used to infect cells at the same time. BHK-21 cells were either infected with vTM3/200 or co-infected with vTM3/150 and vTM3/90 4 h after electroporation of RNA from full-length cleavage site mutant pBRM33G1301S. Total cytoplasmic RNAs were extracted and subjected to RPA at 0, 8, 24 and 48 h after electroporation. The results showed that the cleavage site mutant could be rescued by p200 expressed in cells (Fig. 14, lanes 15 to 18). This cleavage site mutant also could be rescued by providing p150 plus p90 (Fig. 14, lanes 19 to 22) but not by p150 or p90 alone (data not shown). Thus, I conclude that p150 and p90 can function in plus-strand RNA synthesis in this genetic complementation assay.
BHK cells were co-electroporated with \textit{in vitro} transcript from full-length cleavage-defective mutant cDNA pBRM33G1301S together with either RNA transcript of p200 (lanes 15 to 18) or p150 (lanes 11 to 14) construct. RNA transcript of pBRM33G1301S (lanes 7 to 10) or pBRM33 (lanes 3 to 6) was electroporated alone. Total cellular RNAs were extracted with TRizol reagent and subjected to RPA at the indicated times. Lane 1: 2×10^3 cpm of pb18. Lane 2: normal BHK-21 cells were electroporated in the absence of RNA.

### III-1.7. \textit{Inefficient complementation by rescue in trans}

In order to examine the effect of the low molar ratio of SG/G on virus replication, plaque assay and one-step growth curve assay were employed. BHK-21 cells were transfected by co-electroporation and assayed for infectious viral particles in the culture medium. The culture medium was replaced every 24 h by fresh medium. The harvested medium was titered for virus in Vero cells. Vero cells were incubated for six days. Results are shown in Fig. 16. The amount of rescued virus produced at day 1 post transfection was approximately 10^3 pfu/ml, whereas that of wt was approximately 10^4 pfu/ml. For wt, after day 1, the rate of virus production rose rapidly until four days post transfection and reached a peak at day 5, with the titer approaching 10^8 pfu/ml. For rescued viruses, the virus titer reached 8×10^6 pfu/ml at day 5. Taken together, these data indicated that
rescued viruses produced approximately tenfold less infectious virus particles into the culture medium than the wt, whereas the kinetics of virus production were similar to those of wt. This result confirmed the hypothesis that lower amount of SG RNA contributed to the lower production of viruses.

Table 3. Accumulation of viral RNAs in rescued viruses

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative RNA accumulation</th>
<th></th>
<th>SG/G</th>
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<tbody>
<tr>
<td></td>
<td>Subgenomic</td>
<td>genomic</td>
<td>molar ratio</td>
</tr>
<tr>
<td>wt</td>
<td>1.0</td>
<td>1.0</td>
<td>2.6, 2.8</td>
</tr>
<tr>
<td>1152+1301</td>
<td>0.6, 0.7</td>
<td>1.7, 1.7</td>
<td>0.9, 1.1</td>
</tr>
<tr>
<td>1152+P200</td>
<td>0.5, 0.5</td>
<td>1.0, 1.0</td>
<td>1.2, 1.4</td>
</tr>
<tr>
<td>1152+P150</td>
<td>0.5, 0.6</td>
<td>1.1, 1.1</td>
<td>1.2, 1.4</td>
</tr>
<tr>
<td>1301+P200</td>
<td>0.2, 0.2</td>
<td>0.4, 0.4</td>
<td>1.2, 1.4</td>
</tr>
<tr>
<td>1152+v200</td>
<td>0.1, 0.1</td>
<td>0.4, 0.4</td>
<td>0.7, 0.9</td>
</tr>
<tr>
<td>1152+v150</td>
<td>0.1, 0.1</td>
<td>0.4, 0.4</td>
<td>0.7, 0.9</td>
</tr>
<tr>
<td>1301+v200</td>
<td>0.1, 0.1</td>
<td>0.3, 0.3</td>
<td>0.6, 0.7</td>
</tr>
<tr>
<td>1301+(v150+v90)</td>
<td>0.1, 0.1</td>
<td>0.4, 0.4</td>
<td>0.7, 0.8</td>
</tr>
</tbody>
</table>

The amount of each sample was normalized with respect to the value obtained from wt, which is set as value of 1.0 (shown in the first group). The second group is the result from co-electroporation of two genomes. The results from RV-NSP-ORF provided in trans by recombinant vaccinia viruses in complementation assay to rescue two types of mutants were shown in the third group.
Fig. 16. One-step growth curves for the wt and rescued viruses

BHK-21 cells were co-electroporated with in vitro transcripts of protease-inactive full-length mutant pBRM33C1152S plus p200 or p150 construct or cleavage-defective full-length mutant pBRM33G1301S plus p200 construct. The culture medium was replaced every 24 h, and this medium was used for virus titer in Vero cells. Symbols: ♦, pBRM33; □, pBRM33C1152S+p200; Δ, pBRM33C1152S+p150; × pBRM33G1301S+p200.
III-1.8. Discussion

Replication and transcription of RV RNA is dependent on the viral NSPs that are the components of the viral replicase-transcriptase complex responsible for the synthesis of three viral RNAs: the genomic minus-strand RNA, the plus-strand 40S genomic RNA, and a 24S subgenomic RNA. In this study, I addressed the question of which NSPs are required for plus-strand genomic and subgenomic RNA synthesis.

Processing of NSPs in RNA viruses appears to be temporally regulated such that the ratio of polyproteins and cleavage products changes over the course of infection. Liang and Gillam (2000) have shown that p200 cleavage is essential for virus replication, and that the impaired virus replication in protease-inactive (pBRM33/C1152S) and cleavage-defective mutants (pBRM33/G1301S) is due to defective synthesis of positive-strand RNA but not of negative-strand RNA. In fact, the accumulation of negative-strand RNA in both mutants was found to be more than that of the wt (Liang and Gillam, 2000). Therefore, I speculate that the replication complex (p150/p90/host proteins) may act in synthesis of positive-strand genomic and subgenomic RNAs.

To examine the NSPs required for formation of active replication complexes in vivo, vaccinia virus recombinants expressing individual NSPs (p200, p150, p90), cleavage-defective (p200/G1301S) and protease-inactive (p200/C1152S) mutants were generated using the pTM3/T7 system (Moss et al., 1990). I found that the constructed vaccinia recombinants expressed authentic RV NSPs in high yield (Fig. 8). Using complementation experiments, I demonstrated that a protease-inactive mutant
(pBRM33/C1152S) could be rescued by *trans*-provided p200 or p150 translated from helper RNA genomes (Fig. 13) or by a vaccinia virus recombinant expressing p200 or p150 (Fig. 14), whereas cleavage-defective mutant (pBRM33/G1301S) was rescued by p200 provided in *trans* from a helper RNA genome (Fig. 15), or rescued by p200 or p150 plus p90 provided in *trans* from recombinant vaccinia virus. Successful rescue of protease-inactive mutant by *trans*-supplied p200 or p150 indicates that functional protease can be provided in *trans* to process p200 in pBRM33/C1152S mutant in virus replication. The requirement for both p150 and p90 in rescue of cleavage-defective mutant (pBRM33/G1301S) in RNA replication strongly supports the idea that a p150/p90 replication complex is involved in positive-strand RNA synthesis.

The low complementation efficiency obtained in the rescue experiment (Figs. 13 to 15) as well as the lower molar ratio of subgenomic/genomic RNA in the rescued mutant viruses (Table 3) suggests the *cis*-preferential replication of RV. The existence of *cis*-limited replication in positive-strand RNA virus has been reported. For example, in poliovirus, the co-replication of deletion variant RNAs depends on the presence of a translatable nonstructural cistron and mutations in the 2C nonstructural protein are poorly complementable in *trans* (Telerina *et al.*, 1995). The replication machinery of tobacco mosaic virus displays a *cis*-preference, such that the essential replication proteins made from a given genomic mRNA molecule assemble most efficiently into a replication complex on that same RNA molecule, facilitating the switch in the role of the RNA from mRNA to replicational template (Weiland and Dreher, 1993). It would be of interest to determine whether RV RNA replication is also *cis*-preferential by construction of
deletion constructs with deletions in either the "X" domain, protease domain, or helicase domain and carry out complementation experiments.

III-2. Mutational analysis of RV RdRp GDD motif

The biochemical and structural properties of RV protease (Chen et al., 1996; Liang et al., 2000) and helicase (Gros and Wangler, 1996) have been examined but those of methyltransferase and RdRp are poorly understood. Forng and Atreya (1999) demonstrated that the retinoblastoma protein binding motif LXCXE present in p90 (residues 1902 to 1906) is a functional motif required for RV replication (Fig. 17). Although these studies strongly suggest the idea that p90 is the putative RdRp of RV, there is no experimental evidence to demonstrate its catalytic activity. Since the C-terminal of p90 contains the highly conserved GDD motif that is very similar in all classes of polymerases (Table 1), it is possible to identify RdRp catalytic activity by testing whether the conserved GDD motif (at residues 1966 to 1968) of p90 itself is essential for virus replication. I therefore carried out oligonucleotide-directed mutagenesis to investigate the functional role of this motif on RV replication. The strategy of alanine scanning mutagenesis was employed.

Fig. 17. RNA-dependent RNA polymerase motifs in the C-terminal region of p90
Conserved amino acids for RdRp motif identified in a number of positive-strand RNA viruses are indicated as *. The retinoblastoma protein binding LXCXE motif of RV is underlined.


To examine the effect of the most conserved GDD motif on viral replication, a series of alanine mutations were created in the RV RdRp GDD motif. To avoid the possibility of disrupting the conformational alteration of the protein structure by deletions of the GDD amino acid sequence, point mutations were introduced by PCR-mediated mutagenesis. As described in Materials and Methods, the approximately 1.1 kb PCR fragments containing the desired mutations were re-inserted into the infectious clone pBRM33 between the unique FseI and BgIII sites (Fig. 5). The constructed mutant clones pBRM33G1966A, pBRM33D1967A and pBRM33D1968A are listed in Table 4.

### Table 4. Mutations created on RV RdRp GDD motif

<table>
<thead>
<tr>
<th>Mutation</th>
<th>positions of residue change</th>
<th>GDD motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBRM33 (wt)</td>
<td>NA</td>
<td>G D D</td>
</tr>
<tr>
<td>pBRM33G1966A</td>
<td>G1966A</td>
<td>A -- --</td>
</tr>
<tr>
<td>pBRM33D1967A</td>
<td>D1967A</td>
<td>-- A --</td>
</tr>
<tr>
<td>pBRM33D1968A</td>
<td>D1968A</td>
<td>-- -- A</td>
</tr>
</tbody>
</table>
In vitro transcripts of three full-length mutants and of infectious clone pBRM33 (as a control) were then transfected individually into BHK cells by electroporation. The synthesized structural proteins in transfected cells were detected by immunoprecipitation and the released virus particles were quantitated by plaque assay. The synthesis of RNA was determined by RPA.

III-2.2. The effect of mutations on protein synthesis

To evaluate the effect of mutations on RV replication, immunoprecipitation (IP) and pulse-chase experiments were employed. Synthesis and assembly of RV structural proteins into viral particles were analyzed by IP with human anti-RV serum. I found that protein species corresponding to RV E1 (58 kDa), E2 (37 to 45 kDa) and C (33 kDa) were detected intracellularly as well as in the culture medium for the wt at day 1 to day 5 post-transfection. For the mutants, only a minute amount of RV structural proteins was detected in pBRM33G1966A RNA transfected BHK cells after three days transfection (Fig. 18). No RV specific structural proteins could be detected in D1967A and D1968A mutants even five days after transfection (data not shown). Thus SPs were only expressed at a lower level in G1966A mutant. Both D1967A and D1968A mutants showed undetectable levels of expression. These results indicate that G1966A mutant might retain some RdRp enzymatic activity, whereas both G1967A and G1968A mutants are inactive in RdRp function.
Fig. 18. Synthesis and release of RV structural proteins following transfection of mutant RNAs

BHk cells were transfected with wt, G1966A, D1967A or D1968A RNAs by electroporation. At 3 days post-transfection, the transfected cells were pulse-labeled with $[^{35}\text{S}]$methionine for 3 h and then chased with unlabeled methionine for 16 h. The cell lysates and the corresponding chase media were immunoprecipitated with human anti-RV serum and analyzed by SDS-10% PAGE. Equal amount of the total immunoprecipitated proteins were loaded into each lane of the gel from chased medium (A) or cellular lysates (B), and the autoradiograph was exposed for 1 day. To detect low levels of protein synthesis in the mutants, SDS-10% PAGE was carried out using only 1/6 of the wt sample and the autoradiograph was exposed for 5 days (C). The positions of apparent molecular mass markers are shown on the right (in kilodaltons). The positions of migration of RV structural proteins El, E2 and C are shown on the left.

III-2.3. Growth curve and plaque assay

As described previously, after transfection of BHK cells with RNA transcripts by electroporation, viral particles released into the culture medium were quantitated by plaque assay in Vero cells. Virus titers for pBRM33G1966A (1.2x10^2 pfu/ml) were significantly lower than that for wt (7.2x10^7 pfu/ml) at day 5 post-transfection, whereas those for D1967A and D1968A were undetectable (Fig. 19).
Fig. 19. Effect of GDD mutations on RV replication

*In vitro* transcripts of wt and mutant cDNAs were transfected into BHK cells by electroporation. After five days, infectious virus in the supernatant was quantitated by plaque assay on Vero cells. The titer for wt was $7.2 \times 10^7$ pfu/ml, for mutants G1966A, D1967A and D1968A were $1.2 \times 10^3$ pfu/ml, 0, 0, respectively. The mutated nucleotide is underlined.

One of the possible reasons for the failure in detecting virus replication in D1967A and D1968A mutants could be the low level of viral production. To amplify the mutant viruses, Vero cells were infected with wt and mutant viruses harvested at day 5 post-transfection of BHK cells. Culture medium was harvested daily and replaced with fresh medium. The released virus in the harvested medium was determined by plaque assay. As shown in Table 5, the virus titer of G1966A virus was similar to that of wt. At day 1 post-infection, virus titers were $2.4 \times 10^3$ pfu/ml for mutant G1966A and $2.6 \times 10^3$ pfu/ml for wt. A significant increase in titer of passaged G1966A virus suggested the occurrence of revertants.
Table 5. Effect of virus passage on virus replication

<table>
<thead>
<tr>
<th>Time</th>
<th>wt</th>
<th>G1966A</th>
<th>D1967A</th>
<th>D1968A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>2.6×10³</td>
<td>2.4×10³</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 2</td>
<td>8.6×10⁴</td>
<td>9.2×10⁴</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 3</td>
<td>6.4×10⁶</td>
<td>9.2×10⁶</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 4</td>
<td>7.2×10⁷</td>
<td>6.4×10⁷</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 5</td>
<td>8.0×10⁷</td>
<td>6.8×10⁷</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Vero cells infected by a serially diluted virus stock were overlaid with 0.5% agarose in Eagle’s minimum essential medium (MEM) containing 5% of fetal calf serum (FCS), incubated at 35°C for six days, and stained with 5% neutral red diluted in MEM containing 5% FCS.

To investigate the possibility of reversion in G1966A, Vero cells were infected at a multiplicity of 0.1 pfu/ml with G1966A virus harvested at day 5 p.i. and virus replication was monitored by pulse-chase experiments. As shown in Fig. 20, at day 2 post-infection, low levels of virus were detected for both the wt and G1966A mutant. At days 4 and 5 post infection, similar high levels of viruses were released into the medium for both the wt and G1966A viruses (Fig. 20, lanes 4, 5, 8 and 9). Again, no virus replication was detected in Vero cells infected with undiluted culture medium harvested from Vero cells infected with culture medium from BHK cells transfected with D1967A and D1968A.
RNAs (Fig. 20, lanes 6, 7, 10 and 11). To isolate amounts of revertants sufficient for sequence analysis, culture medium harvested at day 5 post-infection was inoculated into a fresh monolayer of Vero cells and incubated for three days. After one more passage, viral RNA was isolated from virus particles and used for cDNA synthesis and subsequent PCR amplification. The sequence covering the region of the GDD motif was determined in three cDNA clones. In all three samples, the originally substituted alanine (1966A) had reverted to the glycine residue.

Fig. 20. Secretion of RV particles in Vero cells infected with wt or mutant virus

Monolayers of Vero cells (35 mm) were infected with wt or G1966A virus harvested from infected Vero cells at day 5 post-infection at a multiplicity of 0.1 pfu/cell. Other Vero cells were infected with passaged culture media from D1967A and D1968A mutants. Infected cells were labeled with $[^{35}\text{S}]$methionine for 3 h and chased with unlabeled methionine for 18 h. Labeled RV from media were immunoprecipitated with human anti-RV serum. The positions of apparent molecular mass markers are shown on the right (in kilodaltons). The positions of migration of RV structural proteins E1, E2 and C are shown on the left.
III-2.4. The effect of mutations on RNA replication

To further confirm virus replication and establish the relationship between virus replication and RNA synthesis, positive-strand genomic and subgenomic RNA synthesis were examined by RPA. At day 5 post-electroporation of BHK cells, no protected bands were observed for G1966A (Fig. 21B, lanes 2 to 4) D1967A, or D1968A mutants (data not shown), whereas protected 301-nt (genomic) and 188-nt (subgenomic) bands were apparent in the wt (Fig. 21A, lanes 3 to 5). As mentioned in III-2.2 and III-2.3, both structural protein synthesis and virus plaques could be detected for mutant G1966A, although the level was extremely low. I found that RPA was not sensitive enough to detect the limited amount of RNA synthesis in G1966A mutant.

Interestingly, RNA synthesis can be detected both in wt and G1966A mutant (Fig. 21) but not in D1967A and D1968A mutants after one passage in Vero cells. Protected 301-nt (genomic) and 188-nt (subgenomic) bands emerged at three days p.i., both in the wt (Fig. 21A, lane 7) and G1966A (Fig. 21B, lane 8). Further accumulation of both genomic (301-nt band) and subgenomic RNAs (188-nt band) at day 4 and day 5 (Fig. 21A, lanes 9 and 10; Fig. 21B, lanes 8 and 9) indicate the replication of viruses. Taken together, the results show that both structural proteins and RNA synthesis can be detected at a virus titer of $10^4$-$10^6$ pfu/ml in Vero cells. Presumably, $10^7$ molecules of RNA (that's the sensitivity for RPA) can form $10^4$-$10^6$ pfu/ml of virus particles in Vero cells.
Fig. 21. RNA analysis of wt and mutant

BHk cells were transfected with RNAs synthesized \textit{in vitro} from a cDNA clone containing the wt sequence (A) or G1966A (B) by electroporation. At the indicated times post-transfection, total cytoplasmic RNAs were isolated and subjected to an RPA using $[^{35}\text{S}]$-labeled probe pb18, which was loaded in lane 1. BHk cells electroporated in the absence of RNA served as a control (A, lane 2).

III-2.5. \textit{Discussion}

Using site-directed mutagenesis, three RdRp GDD motif mutants were generated, representing replacement of each conserved amino acid residue in turn by alanine. Cells exposed to these mutants were examined for infection. pBRM33G1966A mutant yielded only 0.00017\% of wt virus, whereas the two mutants pBRM33D1967A and pBRM33D1968A were lethal. These results suggest that mutation at the G residue allows retention of some of the enzymatic activity, whereas mutations at the first and second D residues are not tolerated.

In pursuing the aim of this work to study the functional relationship of the RV RdRp GDD motif and viral replication, single nucleotide mutations were created to avoid major
conformational changes of the RdRp enzymatic core domain. RNase Protection Assay (RPA), immunoprecipitation/pulse-chase and one-step growth curve/plaque assay were employed to detect positive-strand RNA synthesis, structural protein synthesis and to quantitate virus titer. Full-length GDD motif mutants were introduced into BHK cells by electroporation. At day 5 post-electroporation, the released virus was subjected to plaque assay. The result showed that G1966A gave a dramatically lower virus titer (1.2×10^5 pfu/ml) compared to the wt (7.2×10^7 pfu/ml). No plaques were observed with either D1967A or D1968A. No positive-strand RNA could be detected by RPA for any of the three mutants, whereas trace amount of SPs were detected for G1966A.

A reasonable explanation for failure to detect RNA synthesis with G1966A may be the relatively low sensitivity of RPA. The poor growth characteristic of G1966A (0.00017% of wt) may result from its being a poor RNA substrate and/or a defective protein generated by defective RNA template. The positive, single-strand RNA has a dual role as a template for replication and a template for translation. In order to accomplish viral replication after infection, replicase composed of RdRp and/or viral/cellular protein(s) must bind to promoter regions at the 3' and 5' ends of the genome and at the junction region between NSP-ORF and SP-ORF to initiate synthesis of minus-, plus- genomic and plus-subgenomic RNAs. The binding between promoter regions and RdRp involves in conformational alterations of these two molecules. The mutation of a single nucleotide within the GDD motif is unlikely to alter structure of the whole molecule. It is therefore reasonable to assume that the most likely reason for impaired viral replication in these
three mutants is the loss of enzymatic activity itself rather than the loss of the ability to bind to the promoter region.

A revertant from G1966A emerged after one passage of harvested virus from transfected BHK cells to Vero cells. At day 3 post-infection, detectable levels of both SPs and RNA were observed with large amounts of both SPs and RNA apparent at day 4 and day 5 post-infection (Figs. 19 and 20). A one-step growth curve showed that virus titer reached $10^6$ pfu/ml at day 3 and close to $10^8$ pfu/ml at day 5 post-infection (Table 5). Roughly, $10^4$-$10^6$ pfu/ml of viral particles is the threshold for detecting of SPs (by IP) or positive-strand RNA synthesis (by RPA).

The occurrence of a reversion of G1966A to wt after one passage suggests the importance of this residue for RdRp activity. Reversion is the common strategy for recovering the viability of mutated viruses with back mutation capable of overcoming the block imposed by the original mutation. The limited replication feature of pBRM33G1966A mutant may rely on leakage of RdRp activity. To survive, the only solution may be to change back to the original amino acid. The fact that the same nucleotide was changed back to wt (from GCC GAC GAT back to GGC GAC GAT) indicates that G1966A mutant altered the functional activity rather than the structural information.

No infectious virus plaques, SPs or RNA could be detected from either D1967A or D1968A mutant, in agreement with previous reports on poliovirus and hepatitis C virus, in which lethal mutants exhibited a strict requirement for both aspartate residues.
(Jablonski and Morrow, 1993; Lohmann et al., 1997; Longstaff et al., 1993; Sankar and Porter, 1992). As shown in Table 1, these two aspartate residues are significantly conserved not only for positive-strand RNA viruses but also for negative-strand, double-strand and some DNA viruses. Therefore my results support the hypothesis that the most important enzymatic function correlates with the most conserved sequence in the evolution of viruses.
IV. Summary and Perspective

The objective of my thesis project is to identify the functional roles of RV NSPs in virus replication with respect to individual NSPs required in plus-strand RNA synthesis, and to examine the effect of the conserved GDD motif within p90 on RV replication.

To determine whether p150 and/or p90 are required in positive-strand genomic and subgenomic RNA synthesis, I generated a panel of vaccinia virus recombinants expressing individual NSPs. Complementation experiments were carried out by providing individual RV NSPs in trans together with either pBRM33/C1152S or pBRM33/G1301S mutant constructs as the template. Rescue of protease-inactive mutant by either p200 or p150 indicates functional protease can be provided in trans. Rescue of cleavage-defective mutant by either p200 or p150 plus p90 suggests that p150/p90 replication complex functions in plus-strand RNA synthesis.

Site-directed mutagenesis was employed to investigate the effect of putative RV RdRp GDD motif on viral replication. Substitution of glycine by alanine (G1966A) resulted in impaired virus infectivity, reduced by 1.7×10^4-fold. Alteration of either aspartate residue completely abolished virus replication. A revertant was isolated from the passaged G1966A virus and its sequence confirmed. These results are consistent with the predication that the p90 is the RdRp of RV.
Despite the similarities in genome organization and structural protein expression strategies, RV is clearly very different from alphaviruses when we consider the relatively long latency period, slow replication and limited cytopathology exhibited by RV infection in cultured cells. Studies on RV NSPs will reveal novel insights into virus replication and transcription unique to RV. Understanding of RV replication will provide tools to investigate the molecular basis of rubella virulence, attenuation and pathogenesis.

RV replicates in the cytoplasm of host cells using its encoded replication enzymes. Methyltransferase, protease, helicase and RdRp offer promising targets for antiviral design and therapy. Information obtained from studies on RV replication enzymes will contribute to our understanding of factors regulating RV replication and the possibility of developing improved rubella vaccines or anti-RV drugs.
References


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