Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHAPTER 2

Recent Advances in Hantavirus Molecular Biology and Disease

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Contents

Abstract

Hantaviruses are emerging zoonotic pathogens that belong to the *Bunyaviridae* family. They have been classified as category A pathogens by CDC (centers for disease control and prevention). Hantaviruses pose a serious threat to human health because their infection causes two highly fatal diseases, hemorrhagic fever with

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renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). These pathogens are transmitted to humans through aerosolized excreta of their infected rodent hosts. Hantaviruses have a tripartite-segmented negative-sense RNA genome. The three genomic RNA segments, S, M, and L, encode a nucleocapsid protein (N), a precursor glycoprotein that is processed into two envelope glycoproteins (Gn and Gc) and the viral RNA-dependent RNA polymerase (RdRp), respectively. N protein is the major structural component of the virus, its main function is to protect and encapsidate the three genomic RNAs forming three viral ribonucleocapsids. Recent studies have proposed that N in conjunction with RdRp plays important roles in the transcription and replication of viral genome. In addition, N preferentially facilitates the translation of viral mRNA in cells. Glycoproteins, Gn and Gc, play major roles in viral attachment and entry to the host cells, virulence, and assembly and packaging of new virions in infected cells. RdRp functions as RNA replicase and transcriptase to replicate and transcribe the viral RNA and is also thought to have endonuclease activity. Currently, no antiviral therapy or vaccine is available for the treatment of hantavirus-associated diseases. Understanding the molecular details of hantavirus life cycle will help in the identification of targets for antiviral therapeutics and in the design of potential antiviral drug for the treatment of HFRS and HCPS. Due to the alarming fatality of hantavirus diseases, development of an effective vaccine against hantaviruses is a necessity.

I. INTRODUCTION

Two major outbreaks that occurred in the past led to the discovery of hantaviruses and directed the attention of researchers toward the hantavirus-associated diseases. The first outbreak occurred during the Korean War (1950–1953), with more than 3000 cases of illness reported among the United Nations troops. The disease was initially named ''Korean hemorrhagic fever'' and is now commonly referred to as hemorrhagic fever with renal syndrome (HFRS). The second outbreak, which occurred in the Four Corners region of the United States in 1993, triggered the attention of the World Health Organization toward a new highly lethal disease. The disease was initially referred to as four-corner disease and is now called hantavirus pulmonary syndrome (HPS) or hantavirus cardiopulmonary syndrome (HCPS). Hantaviruses cause serious human illness with a mortality rate that ranges from 15% (for HFRS) to 50% (for HCPS). Twenty-five years after the Korean War, the etiologic agent of this disease, the Hantaan virus (HTNV), was identified in the striped field mouse (*Apodemus agrarius*) (Lee *et al.*, 1981a). Further studies revealed that the newly identified virus belonged to the *Bunyaviridae* family. In 1981, a new genus termed as

''hantavirus'' was introduced into the *Bunyaviridae* family, which included the viruses that cause HFRS (Fig. 2.1). Further investigations revealed that, unlike other members of the *Bunyaviridae* family, hantaviruses did not have an arthropod vector and exclusively establish a persistent infection in the population of their specific rodent hosts. The landmark discovery of HTNV encouraged more efforts to identify the etiologic agents of HFRS-related disease in Asia, Europe, and the United States. These efforts led to the discovery of Haantan-like viruses in other rodents from Far East Russia, China, and South Korea. Dobrava virus (DOBV), a distinct hantavirus, was isolated from *Apodemus flavicollis*, *A. Agrarius*, and *Apodemus ponticus* in Europe (Avsic-Zupanc *et al.*, 1995, 2000; Golovljova *et al.*, 2000; Jakab *et al.*, 2007; Klempa *et al.*, 2005, 2008; Klingstrom *et al.*, 2006a). In 1980s, a rat-born Seoul virus (SEOV) was found to cause HFRS in urban areas of Asia (Chan *et al.*, 1987; Kim *et al.*, 1995). Around the same time period, Puumala virus (PUUV), the etiologic agent of nephropathia epidemica, a milder form of HFRS reported in 1930s in Europe, was identified in bank voles (*Myodes glareolus*) (Clement *et al.*, 2003). Despite these early efforts, the lack of

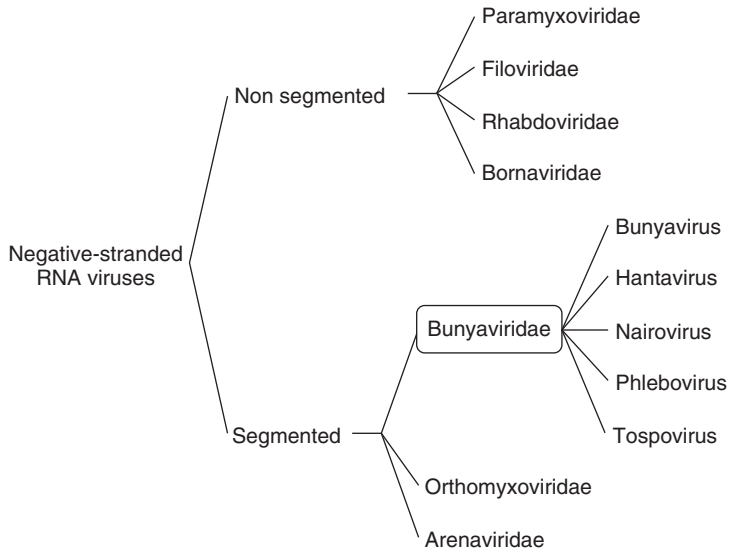

FIGURE 2.1 Classification of negative-stranded RNA viruses: negative-stranded RNA viruses are classified into two main groups: those with nonsegmented and segmented genomic RNA. The nonsegmented group includes four families: *Paramyxoviridae*, *Filoviridae*, *Rhabdoviridae*, and *Bornaviridae*. RNA viruses whose genomes are segmented have been further classified into three families: *Bunyaviridae*, *Orthomyxoviridae*, and *Arenaviridae*. Hantaviruses are classified with the family *Bunyaviridae*, which includes five genera: *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*.

advanced technology delayed the identification of etiologic agents of HFRS. However, the etiologic agent and rodent reservoir of HCPS, the Sin Nombre virus (SNV), were discovered within few weeks after the 1993s outbreak in the Four Corners region of the United States (Hjelle *et al.*, 1994; Nichol *et al.*, 1993). After this outbreak, other hantavirus strains causing HCPS were discovered and more than 2000 cases of HCPS have been reported throughout the Americas (Aquino *et al.*, 2003; Barclay and Rubinstein, 1997; Bayard *et al.*, 2004; Bohlman *et al.*, 2002; Chu *et al.*, 2006; Fulhorst *et al.*, 1997; Hjelle *et al.*, 1994, 1996; Jiang *et al.*, 1997; Johnson *et al.*, 1997; Jones *et al.*, 2008; Nichol *et al.*, 1993; Vincent *et al.*, 2000). Currently, the hantavirus genus includes more than 21 species and more than 30 genotypes (Avsic-Zupanc *et al.*, 2000; Chu *et al.*, 2006), although many more remain undiscovered due to the lack of advanced technology in other parts of the world including Africa, Middle East, and the Indian subcontinent. For example, recent discoveries of shrew-born hantavirus in several countries support the existence of other hantavirus species worldwide (Arai *et al.*, 2007; Kang *et al.*, 2009a). Annually, 150,000–200,000 cases of hantavirus infection are reported worldwide. Due to high emergence and significant mortality of hantavirus infections, a serious attention from research scientists and world health organizations is required to promote public awareness and accelerate efforts for the treatment of hantavirus-associated diseases. Here, we summarize the findings about the microbiology of hantaviruses with an emphasis on nucleocapsid protein, glycoproteins Gn, Gc, and RNA-dependent RNA polymerase (RdRp). A brief synopsis about different aspects of hantavirus-associated disease is also presented in this chapter.

II. HANTAVIRUS MOLECULAR BIOLOGY

A. Hantavirus structure

Hantavirus particles generally appear spherical or pleomorphic in electron micrographs (Fig. 2.2). They range in diameter from 80 to 120 nm (Goldsmith *et al.*, 1995; Martin *et al.*, 1985). Hantaviruses have tripartite single-stranded negative-sense RNA genome that encodes an RdRp (large or L segment), two glycoproteins (medium or M segment), and a nucleocapsid protein (N) (small or S segment). The negative-sense genomes serve as templates for producing positive-sense complementary RNA (cRNA) and messenger RNA (mRNA). The total size of the RNA genomes ranges from 11,845 nucleotides for HTNV to 12,317 nucleotides for SNV (Hooper and Schmaljohn, 2001; Jonsson *et al.*, 2010; Schmaljohn *et al.*, 1985, 1986). The viral RNA segments are coated with the nucleocapsid protein forming three helical ribonucleoprotein (RNP) complexes (Fig. 2.2). The viral RdRp is associated with these nucleocapsids (Dahlberg *et al.*, 1977; Obijeski *et al.*, 1976). The sequences at both the 3′ and 5′termini of each RNA segment are

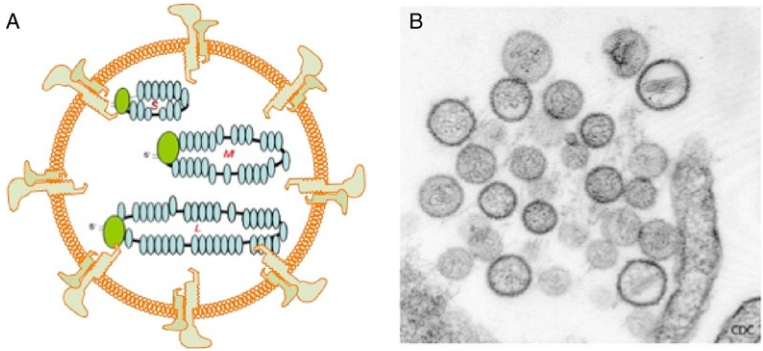

FIGURE 2.2 Hantavirus structure: (A) pictorial representation of hantavirus particle, showing three nucleocapsids enveloped in a lipid bilayer. Glycoproteins Gn and Gc buried in the lipid membrane are shown. (B) Thin-section electron micrograph of an SNV isolate from the outbreak of HCPS that occurred in the southwestern United States in 1993. Electron micrograph was obtained from the CDC website with permission. http://www.cdc.gov/ncidod/diseases/hanta/hps/noframes/hpsem.htm.

complementary forming ''panhandle'' structures that are specifically recognized by the N protein and were shown to be important for viral transcription and replication. Viral mRNAs contain untranslated regions (UTRs) at both their 5′ and 3′ends that flank the open reading frame (ORF) of the encoded viral protein. The 5′UTR of viral mRNA contains trinucleotide repeats (UAGUAGUAG), which were suggested to be involved in N-mediated translation initiation of viral mRNA. Similarly, a triplet repeat at the 3′UTR of vRNA has been proposed to play a role in the prime and realign mechanism of transcription initiation by the viral RdRp (Hewlett *et al.*, 1977; Mir and Panganiban, 2004, 2005, 2010; Pettersson and von Bonsdorff, 1975; Raju and Kolakofsky, 1989). Hantaviruses are enveloped with an outer lipid bilayer derived from the Golgi membranes (Fig. 2.2). The envelope carries two viral-encoded glycoproteins, Gn and Gc, in the form of heterodimers which assemble into higher order oligomers and appear as projections or spikes on the outer surface of the virion (Hepojoki *et al.*, 2010a; Huiskonen *et al.*, 2009, 2010; Overby *et al.*, 2008). The virion consists of $>50\%$ protein, 20–30% lipid, 7% carbohydrate, and 2% RNA (McCaughey and Hart, 2000; Obijeski and Murphy, 1977). Hantaviruses can be easily inactivated by treatment with lipid solvents or nonionic detergents, which destroy the viral envelope (Schmaljohn and Nichol, 2007).

B. Replication cycle of hantaviruses

Hantaviruses infect multiple cell lines including endothelial, epithelial, macrophages, dendritic, and lymphocytes. The replication cycle begins with the attachment of virus particles to host cell surface receptors

(Goldsmith *et al.*, 1995; Mackow and Gavrilovskaya, 2001; Markotic *et al.*, 2007; Raftery *et al.*, 2002; Spiropoulou, 2001). Several studies suggest that the attachment is mediated by the interaction of viral Gn protein with integrin receptors on the surface of host cells (Fig. 2.3). The β1 and β3 integrins are considered to be the receptors for apathogenic and pathogenic hantaviruses, respectively (Gavrilovskaya *et al.*, 1998, 1999; Larson *et al.*, 2005). However, it has been reported that hantaviruses can infect cells lacking integrin receptors, suggesting that integrins may not be the only receptors that hantaviruses employ to attach to host cells (Mou *et al.*, 2006). After attachment, cell entry is mediated via clatherin-coated pits, and virions are ultimately delivered to lysosomes (Fig. 2.3). Within the endolysosomal compartment, virions are uncoated and three viral capsids are released into the cytoplasm (Jin *et al.*, 2002). Viral RdRp initiates transcription and generates three mRNAs, one from each viral RNA segment S, M, and L. The translation of S and L segment-derived mRNAs occurs on free ribosomes, while M segment-derived mRNAs

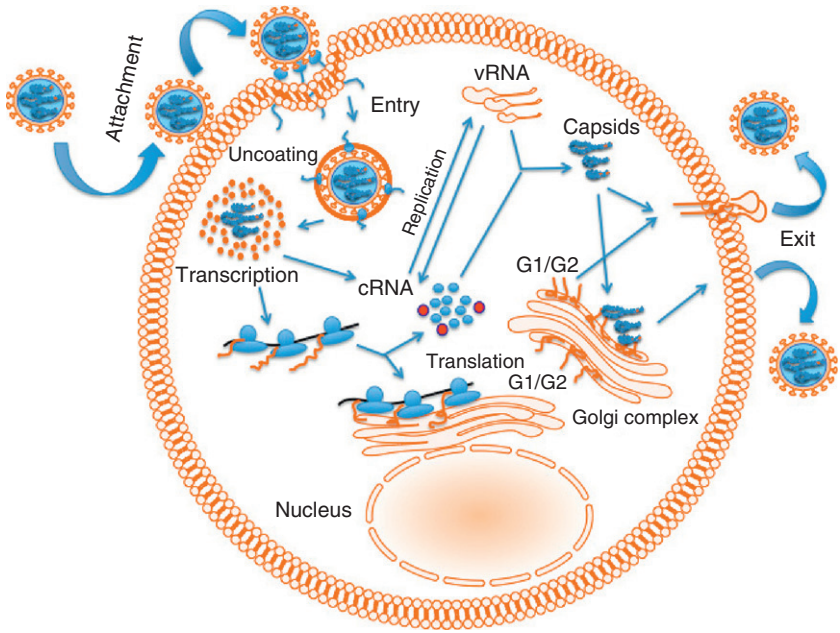

FIGURE 2.3 Hantavirus replication cycle: virus particle attaches to the integrin receptors on host cell surface. After entry, uncoating takes place, followed by mRNA synthesis by RdRp. The three mRNA molecules are translated by host cell translation machinery, generating viral proteins. Viral genome is synthesized by RdRp via a cRNA intermediate and is packaged into new virus particles that bud off the host cell.

are translated on the rough endoplasmic reticulum (ER). The glycoprotein precursor is intrinsically cleaved at a highly conserved amino acid motif, WAASA, generating two glycoproteins, Gn and Gc, respectively (Lober *et al*., 2001; Ruusala *et al*., 1992; Spiropoulou, 2001). After glycosylation in the ER, both Gn and Gc are transported to the Golgi complex, laying the foundations for final destinations where virions are maturated (Antic *et al*., 1992a; Plyusnin *et al*., 1997; Ravkov *et al*., 1998; Ruusala *et al*., 1992). After initial rounds of transcription, viral RdRp switches to the replication mode and generates three viral genomic RNAs, which are encapsidated by the viral N protein to form three nucleocapsids. Mature viral nucleocapsids are transported to specific destinations on the Golgi membrane that are studded with the Gn and Gc proteins. However, the molecular mechanisms governing the specific recognition and encapsidation of viral genome by N, transport of capsids to Golgi, and budding of nascent virions into and out of Golgi complex are still unknown. Mechanisms of incorporation of viral RdRp into virus particles are fascinating and need more attention. There are reports suggesting that the assembly and maturation of New World hantaviruses take place on the plasma membrane (Ravkov and Compans, 2001). The evidence supporting this pathway for assembly and maturation is based on observations that SNV and black Creek Canal virus (BCCV) particles were not observed in infected cells. However, localization of N protein from both old and new word hantaviruses on Golgi apparatus favors the possibility of their maturation on Golgi (Ramanathan and Jonsson, 2008; Ramanathan *et al*., 2007; Ravkov and Compans, 2001; Spiropoulou *et al*., 2003).

C. Nucleocapsid protein (N)

Hantavirus nucleocapsid protein (N) is the major viral structural component; its main function is to protect and encapsidate the viral RNA forming viral RNP complex. It is encoded by the S segment vRNA and is abundantly expressed in the cytoplasm of infected cells. Therefore, it is considered as the most predominant viral antigen in the serologic response to infection. Hantaviral N protein has a molecular mass of approximately 50 kDa and contains 429–433 amino acid residues (de Carvalho Nicacio *et al*., 2001; Plyusnin *et al*., 1997; Van Epps *et al*., 1999). It is highly conserved across hantaviruses and contains cross-reactive epitopes that encompass the first 100 amino acids at N-terminal (Elgh *et al*., 1996; Gott *et al*., 1997; Schmaljohn *et al*., 1986, 1987; Yamada *et al*., 1995). Further, serotype-specific conformational epitopes have been detected in about half of the C-termini of N proteins by serotype-specific monoclonal antibodies (MAbs) (Ruo *et al*., 1991; Yoshimatsu *et al*., 1996).

1. Biological functions of N protein

N protein is a multifunctional molecule that interacts with host cellular proteins and plays several important roles to facilitate hantavirus replication in infected cells.

a. Viral RNA encapsidation and assembly Several studies showed that N binds specifically to a region near the 5′terminus of vRNA, which is indicative of its function during encapsidation (Gott *et al.*, 1993; Jonsson *et al.*, 2001; Mir and Panganiban, 2005; Osborne and Elliott, 2000; Severson *et al.*, 1999). It was also reported that N differentially interacts with minus-sense vRNA, plus-sense cRNA, and mRNA. Cell culture-based experiments have demonstrated that encapsidation requires full-length vRNA or cRNA molecules, since no mRNA molecules were detected in viral nucleocapsids (Jin and Elliott, 1993). Moreover, SNV N protein was shown to play the role of RNA chaperone that facilitates transient dissociation of misfolded RNA structures and creates opportunities for the folding of RNA into biologically functional and thermodynamically stable higher order structures (Mir and Panganiban, 2005).

A three-dimensional (3D) structural model of N protein is still lacking. However, the functionally important regions have been identified in several mutagenesis studies. The RNA-binding domain of N protein was mapped to the central conserved region that extends from amino acid 175 to 217 (Li *et al.*, 2002). In addition, the lysine residues dispersed between positions 175 and 429 and three more residues, including E192, Y206, and S217 located in the RNA-binding domain, were also shown to be important for RNA binding (Severson *et al.*, 2005). Hantavirus N protein undergoes protein–protein interaction and forms stable trimers both *in vivo* and *in vitro*. It has been shown that trimeric N recognizes the vRNA panhandle with specificity and high affinity (Mir *et al.*, 2006). N-panhandle interaction has been proposed to mediate the selective encapsidation and packaging of vRNA genome during virus assembly (Alfadhli *et al.*, 2001, 2002; Kaukinen *et al.*, 2001, 2003, 2004; Mir and Panganiban, 2004). Using the MultiCoil prediction algorithm (Wolf *et al.*, 1997), coiled-coils spanning amino acid residues (1–34 and 38–80) have been predicted for the N protein of several hantaviruses, including HTNV, SEOV, SNV, Prospect Hill virus (PHV), and Tula virus (TULV) (Alfadhli *et al.*, 2001). Both N- and C-terminal regions have been implicated in mediating the homotypic interaction between N molecules, and putative coiled-coil motifs in the N-terminal region of N protein have been proposed to facilitate trimerization. A ''head-to-head, tail-to-tail'' model for trimerization was proposed, where three monomers of N protein are brought together via interaction of their N-terminal coiled-coil domains. This initial contact is followed by the interaction between

the C-terminal α-helices that form a shared hydrophobic space and thus consolidate the trimer formation (Alfadhli *et al.*, 2001, 2002; Kaukinen *et al.*, 2001, 2003, 2004).

b. Transcription and translation Apart from its role in packaging, N has been found to be involved in transcription and replication of viral genome in conjunction with viral RdRp or by interacting with template RNA during replication (Blakqori *et al.*, 2003; Bridgen and Elliott, 1996; Ikegami *et al.*, 2005; Kohl *et al.*, 2004; Pinschewer *et al.*, 2003). Viral mRNAs are translated by the host cell translation machinery. Recent studies have shown that SNV N protein binds to mRNA 5′ caps and 40S ribosomal subunit with high affinity and specificity (Fig. 2.4). These interesting findings have suggested that, in the host cell cytoplasm where cellular transcripts are competing for the same translation machinery, N protein facilitates the translation of hantaviral mRNAs by

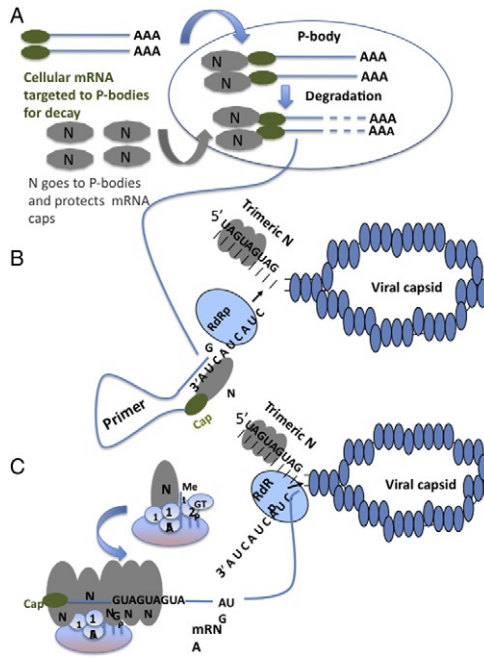

FIGURE 2.4 A model depicting the roles of N in transcription and translation initiation: (A) cellular mRNA targeted to P-bodies for decay. N moves to P-bodies and binds to mRNA caps. (B) N with a capped RNA primer loaded at the cap-binding site specifically binds the 3′terminus of vRNA and favors primer annealing. (C) Trimeric N binds the triplet repeat sequence of viral mRNA 5′UTR. N also binds 40S ribosomal subunit and loads it onto the 5′UTR.

preferentially loading ribosomes onto capped viral transcripts (Klempa *et al.*, 2008). Further studies have shown that the sequence GUAGUAG, in the 5′UTR of hantavirus mRNA, is sufficient for preferential N-mediated translation initiation (Mir and Panganiban, 2010). N-mediated translation initiation mechanism is different from the complex translation initiation strategy used by eukaryotic cells. It is likely that N initiates the translation of capped mRNA without the requirement of eIF4F complex, an amalgam of three initiation factors, eIF4E, eIF4G, and eIF4A (Panganiban and Mir, 2009).

In host cells, after the completion of translation, the mRNAs are deadenylated and decapped, followed by degradation by exonucleolytic digestion from both 5′ and 3′ends. In the 5′–3′ degradation pathway, the decapping enzyme DCP2 in coordination with several other proteins removes the 5′ mRNA cap, followed by degradation by exonuclease XRN1. This degradation mechanism takes place in discrete cytoplasmic foci called processing bodies (P-bodies) (Beckham and Parker, 2008). Interestingly, a detailed analysis by confocal imaging revealed that N resides in cellular P-bodies. Further studies revealed that cellular 5′-capped mRNA oligoribonucleotides are rescued by N in virus-infected cells and stored in P-bodies for the later use as primers by the viral RdRp during transcription initiation (Mir *et al.*, 2008). Moreover, it was reported that N protein has distinct cap and RNA-binding sites that independently interact with mRNA cap and viral genomic RNA, respectively. In addition, N can simultaneously bind to both mRNA cap and vRNA. N undergoes distinct conformational changes after binding to mRNA cap, vRNA, or both. The conformationally altered N with a capped primer loaded at the cap-binding site specifically binds the conserved 3′ nine nucleotides of vRNA (3′AUCAUCAUC) and assists the bound primer to anneal at the 3′terminus. The annealed primer is later elongated by the RdRp and a nascent viral mRNA is synthesized. Therefore, it was suggested that the cap-binding site of N, in conjunction with RdRp, plays key roles during the transcription and replication initiation of vRNA genome (Mir *et al.*, 2010). Taken together, these observations imply that N has a role in generating the capped RNA primer in P-bodies and also assists the capped primer to anneal to the 3′terminus of vRNA template during transcription initiation (Fig. 2.4).

c. Interaction with cellular proteins N protein performs some ambassadorial functions by interacting with some cellular proteins to facilitate viral dissemination in infected cells. For example, interaction of N with actin filaments has been reported to mediate the transport of newly synthesized viral RNPs to the plasma membrane (Ravkov *et al.*, 1998). It was also shown that N protein interacts with the Fas-mediated apoptosis enhancer Daxx, a death-domain adaptor protein, which transduces death

signals through the Jun N-terminal kinase (JNK) pathway (Yang *et al.*, 1997). In similar studies, interaction of N protein with Daxx and small ubiquitin-like modifiers (SUMO-1) pathway components in infected cells has been reported (Alfadhli *et al.*, 2002; Cho *et al.*, 2002; Kaukinen *et al.*, 2003; Lee *et al.*, 2003).

D. RNA-dependent RNA polymerase

The protein encoded by the largest ''L'' segment of hantaviruses is an RdRp. The RdRp is responsible for the replication and transcription of the viral genome. The negative-sense hantaviral RNAs (vRNAs) are copied by the L protein to produce positive-sense cRNA intermediates that serve as templates for genome replication and mRNA synthesis (Fig. 2.5).

1. Sequence homology and structural motifs

Sequences of several hantavirus genomes are now available. From the available sequencing data, it is evident that the size of the L RNA of hantaviruses (~6.5 kb) is similar to those of other members of the *Bunyaviridae* family, except for the *Tospovirus* (~8.9 kb) and the *Nairovirus* (~12 kb) genera. The reverse complement of the L segment of all the members of the *Bunyaviridae* family has a single large ORF with flanking 5′ and 3′UTRs. The predicted size of the protein encoded by this ORF is approximately 250 kDa, which has also been confirmed experimentally by few groups (Elliott *et al.*, 1984; Kukkonen *et al.*, 2004).

There are a total of four classes of nucleotide polymerases: DNA-dependent DNA polymerase (DdDp), DNA-dependent RNA polymerase (DdRp), RNA-dependent DNA polymerase (RdDp or reverse transcriptase), and RdRp. Amino acid sequence comparisons of all the nucleotide polymerases have revealed an interesting pattern of conserved motifs

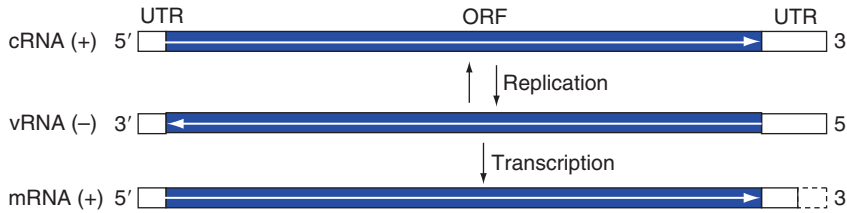

FIGURE 2.5 Schematic representation of reactions carried out by RdRp during the hantaviral life cycle: vRNA carried by the virus in the host cell is first transcribed to produce mRNA, which is translated into viral proteins. Later in the life cycle, vRNA is amplified for packaging through a cRNA intermediate. vRNA and cRNA are exact reverse complement copies of each other. mRNA in some cases has a shorter 3′UTR (depicted by dashed line).

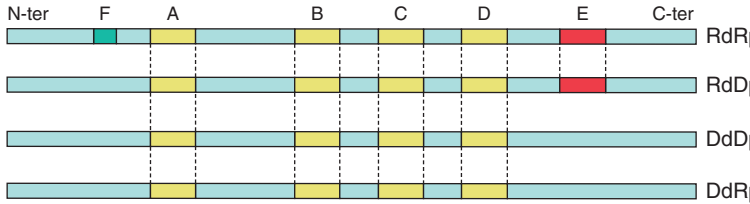

FIGURE 2.6 Conserved structural motifs among four classes of nucleotide polymerases: motifs A–D are found in all the polymerases. Motif E is found in polymerases that use RNA as a template. There is an extra motif F found only in RdRps.

found in different classes of polymerases (Fig. 2.6; Hansen *et al.*, 1997; Joyce and Steitz, 1995). Motifs A–D are shared by all polymerases. Motif E is found in those polymerases that recognize RNA as a template (reverse transcriptases and RdRPs). An extra motif F (also called as pre-motif A) is specifically found in RdRps (Bruenn, 2003; Hansen *et al.*, 1997; Kamer and Argos, 1984; Muller *et al.*, 1994; O'Reilly and Kao, 1998; Poch *et al.*, 1989; Toh *et al.*, 1985; Tordo *et al.*, 1988). The amino acid residues from these conserved motifs play critical roles in different aspects of the nucleotidyl transferase reaction of these enzymes, which include binding of metal ions, nucleoside triphosphates (NTPs), and the RNA/DNA template. Few additional conserved regions have been found in the amino acid sequences of RdRps of segmented negative strand RNA viruses (Aquino *et al.*, 2003; de Haan *et al.*, 1991; Kukkonen *et al.*, 1998; Muller *et al.*, 1994; Nemirov *et al.*, 2003; Stohwasser *et al.*, 1991); however, the functional importance of these motifs has not yet been validated.

Based on the organization of the conserved motifs in the amino acid sequence, the hantavirus RdRp is predicted to have a structure similar to other polymerases. The crystal structures of all polymerases show a common domain organization, which resembles a ''right hand,'' and it includes the subdomains denoted as ''finger,'' ''palm,'' and ''thumb'' (Kohlstaedt *et al.*, 1992). Among all known RdRps, an additional N-terminal domain is found that bridges the fingers and thumb domains to make a tunnel-like structure, which is otherwise open U-shaped in most of the other polymerases (Butcher *et al.*, 2001; Lesburg *et al.*, 1999; Ng *et al.*, 2008). The crystal structure of hepatitis C virus shows the palm subdomain to be constituted of β-strand of motifs A and C and α-helix of motif D. Motifs B and F, folded into an α-helix, form the fingers subdomain and the motif E, in form of an antiparallel β-sheet, is present between the palm and the thumb subdomains (Lesburg *et al.*, 1999). Conserved Arg and Lys residues in motif F and a highly conserved Asp in motif A play critical roles in binding of the RdRp with NTPs (Arnold and Cameron, 2004; Bressanelli *et al.*, 2002; Gohara *et al.*, 2000, 2004; Huang *et al.*, 1998; Joyce and Steitz, 1995).

Like other polymerases, the RdRps also show a dependence on binding of two divalent metal ions in the active site (Arnold *et al*., 1999; Doublie and Ellenberger, 1998; Rothwell and Waksman, 2005; Steitz *et al*., 1994). The conserved Asp residues in motifs A and C play a crucial role in binding of the two metal ions. Mutation of these Asp residues has been shown to inactivate or alter the activity of several RdRps (Arnold *et al*., 1999; Jablonski and Morrow, 1995; Vazquez *et al*., 2000). In a mutational analysis of Bunyamwera RdRp, replacement of the conserved aspartates of motifs A and C abolished the polymerase activity (Dunn *et al*., 1995).

In a large-scale mutational study carried out on RdRp of Lassa virus (family *Arenaviridae*), an RdRp domain (between residues 1040 and 1540) was identified and a functional element within RdRp was found, which was important for transcription but not replication of the genome (Hass *et al*., 2008).

RdRps of both positive-stranded RNA viruses such as poliovirus as well as negative-stranded RNA viruses including Sendai virus (Cevik *et al*., 2003; Smallwood *et al*., 2002), human parainfluenza type 3 virus (Smallwood and Moyer, 2004), measles virus (Cevik *et al*., 2004), lymphocytic choriomeningitis virus (LCMV) (Sanchez and de la Torre, 2005), and rift valley fever virus (RVFV) (Zamoto-Niikura *et al*., 2009) need oligomerization to exert their polymerization function.

a. Localization The site of RNA synthesis among the viruses of the *Bunyaviridae* family was believed to be the cytoplasm of the host cell. This was based on a study on La Cross virus in which the newly synthesized RNA was found to be in the cytoplasmic fraction where it was monitored by pulse-labeling followed by cell fractionation (Rossier *et al*., 1986). But based on later studies on localization of the two viral proteins essential for RNA synthesis, namely, L and N, it is now thought that RNA synthesis among bunyaviruses is membrane associated. Kukkonen *et al*. (2004) studied the localization of L-GFP fusion protein and found it to localize in the perinuclear region (Kukkonen *et al*., 2004). This is consistent with the fact that RNA synthesis of all positive-stranded viruses is membrane associated (Salonen *et al*., 2005). Further studies on other viruses of this family will be required to confirm if that is a general mechanism employed by these viruses.

b. Mechanism of action Similar to all the other negative-sense RNA viruses, hantavirus genomic RNA segments contain UTRs on both the 5′ and the 3′ends. The termini of the UTRs are partially complementary to each other due to which the segments fold to make a panhandle-like structure (Chizhikov *et al*., 1995; Kukkonen *et al*., 1998; Meyer and Schmaljohn, 2000; Padula *et al*., 2002). Several minireplicon systems in

which reporter genes (such as Renilla luciferase or chloramphenicol acetyltransferase) are flanked by the UTRs have been developed for members of the *Bunyaviridae* family (Blakqori *et al*., 2003; Dunn *et al*., 1995; Flick and Pettersson, 2001; Flick *et al*., 2003; Lopez *et al*., 1995). Studies based on these minireplicon systems have shown that 5′ and 3′UTRs serve as promoters for replication of the segments and transcription of the encoded reading frames. The UTRs are encapsidated by nucleocapsid protein and associate with RdRp both in the host cells and in the virion, and only these nucleocapsids are believed to be functional templates for mRNA synthesis and RNA replication by the viral RdRp.

c. mRNA synthesis The viral RdRp is responsible for the synthesis of viral mRNA. Several unique features involved in this process are detailed here. Sequence analysis of the 5′termini of mRNAs of several Bunyaviruses has revealed the presence of short (10–18 nucleotides) nontemplated 5′end sequences (Bishop *et al*., 1983; Garcin *et al*., 1995; Simons and Pettersson, 1991). These sequences were later found to be derived from the 5′ends of host mRNAs by a mechanism similar to ''cap-snatching'' originally described for influenza virus. In this mechanism, a viral endonuclease cleaves the 5′termini of the host mRNAs and uses them as primers to initiate the transcription of viral mRNAs (Bouloy *et al*., 1978; Krug, 1981; Krug *et al*., 1979; Plotch *et al*., 1981). The endonuclease activity required for cap-snatching is believed to reside in the viral polymerase itself (Patterson *et al*., 1984).

Based on the observation that all the mRNAs contain a G residue at −1 position, a prime and realign mechanism for mRNA synthesis in the *Bunyaviridae* family was proposed. In this mechanism, the G residue of the capped primer snatched from the host mRNA aligns with a C residue, upstream of the 3′terminus of the viral template. The primer is elongated few nucleotides by RdRp. The extended primer detaches and realigns to the 3′end of the terminus and then extends further to complete the mRNA synthesis (Garcin *et al*., 1995).

Termination of mRNA synthesis and polyadenylation of the synthesized mRNA has been shown to vary among S, M, and L segments (Hutchinson *et al*., 1996). Synthesis of S segment mRNA terminates downstream of a CCACCC motif found around 200 nucleotide downstream of the 5′end of the vRNA template. The S segment mRNA is not polyadenylated (Hutchinson *et al*., 1996). The synthesis of the L mRNA extends to the 5′terminus of the L vRNA and is also not polyadenylated (Hutchinson *et al*., 1996). However, the M segment of SNV was found to be polyadenylated and a potential polyadenylation–transcription termination signal was mapped and was found to be conserved among all hantaviruses (Hutchinson *et al*., 1996).

d. RNA replication Replication of the vRNA genome is carried out by the viral RdRp through an antigenomic RNA (cRNA) intermediate. Unlike mRNA, the cRNA is exact complement of the viral genomic segments without any cap structure and host-derived sequences (Bishop *et al*., 1983; Eshita *et al*., 1985; Garcin *et al*., 1995; Obijeski *et al*., 1980; Raju and Kolakofsky, 1987). The cRNA was shown to be encapsidated by the N protein (Patterson *et al*., 1984). The 5′terminus of the vRNA in hantaviruses was found to contain uridine monophosohate (pU) instead of triphosphate (pppU; Chizhikov *et al*., 1995; Garcin *et al*., 1995). This observation was explained by a prime and realign mechanism for cRNA and vRNA synthesis (Garcin *et al*., 1995). Briefly, after elongation by few nucleotides, the capped RNA primer with a terminal ''G'' residue slides back on the 3′ triplet repeat sequence of cRNA template, leaving a protruding GTP that is nonaligned with the 3′end. The protruding GTP is subsequently cleaved off by the polymerase leaving a pU residue at the 5′end (Garcin *et al*., 1995).

e. Error, evolution, and editing The promiscuous nature of polymerization by RdRps due to lack of proof reading ability is thought to be the primary source of evolution in RNA viruses. This gives the virus an ability to replicate in different hosts and become pathogenic to humans. But the virus has to maintain a close window of variation to avoid the ''error catastrophe'' as too much of these errors may lead to generation of nonviable genome and reduction of the overall viral fitness (Crotty and Andino, 2002). Drake *et al*. have estimated an error rate of approximately 1 mutation/replication/genome for hantaviruses (Drake, 1999). The mean rate of evolutionary change in hantaviruses has been approximated to be within the range of 10^2–10^4 substitutions/site/year (Ramsden *et al*., 2008). RNA viruses employ several mechanisms to generate useful variations and to edit deleterious ones, which include reassortment and recombination (Barr and Fearns, 2010). Natural reassortment has been detected among hantaviruses (Henderson *et al*., 1995; Li *et al*., 1995; Plyusnin *et al*., 1997), and several experimental reassortants have been generated in the laboratory by infecting different strains of the same hantavirus (Ebihara *et al*., 2000; Rodriguez *et al*., 1998). Recently, Handke *et al*. were able to generate *in vitro* reassortants between pathogenic PUUV and nonpathogenic PHV (Handke *et al*., 2010).

RNA recombination in viruses usually occurs by a ''copy choice'' mechanism, in which a replicating polymerase stops copying one RNA molecule and switches to another (Copper *et al*., 1974; Worobey and Holmes, 1999). A recombinant TULV was successfully generated through homologous recombination by infecting Vero E6 cells with one strain and by providing S cRNA of another TULV strain via plasmid-driven expression (Plyusnin *et al*., 2002). A recent study in Bunyamwera virus (BUNV)

has shown that recombination may also occur near the end of the templates to keep the ends intact (Walter and Barr, 2010).

E. Glycoproteins Gn and Gc

1. Expression, structural organization, and trafficking

The envelope glycoproteins are encoded by the viral M segment RNA. They are expressed as a glycoprotein precursor (GPC) polypeptide, which is cleaved by host signal peptidase in the ER lumen to generate N-terminal (G1 or Gn) and C-terminal (G2 or Gc) fragments (Fig. 2.7; Fazakerley and Ross, 1989; Spiropoulou *et al.*, 2003). Cleavage occurs after a highly conserved pentapeptide WAASA motif (Lober *et al.*, 2001). The GPC has neither been detected in infected cells nor in cells transfected with a plasmid encoding M segment mRNA, but was only detectable by *in vitro* translation of RNA transcripts. Therefore, it was suggested that the proteolytic processing of the GPC occurs during translation (Pensiero and Hay, 1992; Schmaljohn *et al.*, 1987; Ulmanen *et al.*, 1981).

Gn and Gc are type I transmembrane proteins modified by N-linked glycosylation, with the N-terminus exposed on the surface of the virion and the C-terminus anchored in the membrane (Shi and Elliott, 2004). They have high tendency to form disulfide bridges due to their high content of cysteine residues (4–7%) (Antic *et al.*, 1992a). SNV GPC is 1140 amino acids in length. The Gn of SNV consists of 652 residues (MW ≈ 75 kDa), with a predicted transmembrane domain and a cytoplasmic tail (CT) domain of 142 amino acids. Gc is 488 residues in length (MW ≈ 55 kDa) and has a predicted shorter CT of eight amino acids (Spiropoulou *et al.*, 2003). HTNV GPC has four hydrophobic domains. Domain I extends from position 1 to 17. Domains II (position 441–515) and

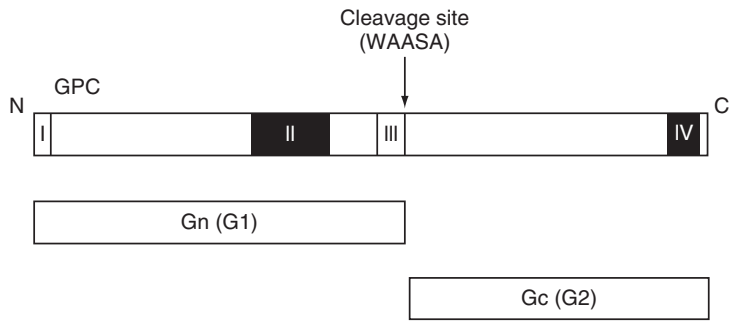

FIGURE 2.7 Schematic illustration of the hantavirus glycoprotein precursor (GPC) and its maturation: GPC is cotranslationally cleaved in the ER by host signal peptidase at a conserved WAASA motif, generating Gn (G1) and Gc (G2). The N-terminus, C-terminus, and the four hydrophobic regions (I–IV) are indicated.

IV (position 1097–1127) are the transmembrane domains of Gn and Gc, respectively (Spiropoulou *et al*., 1994). Domain II is very long and the actual membrane spanning portion is not well defined. Domain III extends from position 627 to 648 and ends with the highly conserved pentapeptide motif WAASA before the N-terminus of Gc (Spiropoulou, 2001).

Most enveloped viruses bud from the plasma membrane into the extracellular space. However, in the case of family *Bunyaviridae*, many members of this family mature by budding into the lumen of the Golgi complex. Progeny virions are then released by fusion of secretory vesicles with the plasma membrane (Kuismanen *et al*., 1982; Schmaljohn and Nichol, 2007). Additional evidence suggests that the assembly of New World hantaviruses (e.g., SNV and BCCV) may occur at the plasma membrane (Ravkov *et al*., 1997). The reason why some viruses have evolved to bud from the Golgi membranes is poorly understood, but it might be due to the presence of Golgi retention signals in viral glycoproteins, which allow them to accumulate in the Golgi complex after their maturation in the ER (Antic *et al*., 1992b; Shi and Elliott, 2002; Spiropoulou *et al*., 2003). There is no clear consensus on the exact location of these Golgi targeting signals in different members of family *Bunyaviridae*, and it appears that different viruses, even within a single genus, have their own strategies for Golgi targeting (Shi *et al*., 2004). For members of the *Bunyavirus* genus, this signal was mapped to the N-terminus of Gc (Lappin *et al*., 1994). The same signal was reported to be located in the transmembrane domain and CT of Gn for members of genus *Phlebovirus* (Andersson *et al*., 1997; Gerrard and Nichol, 2002; Matsuoka *et al*., 1994). It was also suggested that translocation of Gn and Gc from the ER to the Golgi complex depends on their interaction and heterodimerization because neither of them was able to leave the ER when expressed individually (Deyde *et al*., 2005; Ruusala *et al*., 1992; Shi and Elliott, 2002; Spiropoulou *et al*., 2003).

2. Roles in virus biology

Glycoproteins, Gn and Gc, play a significant role in the biology of hantaviruses, including virus entry to host cells, virulence, and assembly and packaging of new virions in infected cells.

a. Attachment and entry Hantaviruses enter the cells via receptor-mediated endocytosis. The acidic environment of the endosome facilitates fusion between the viral and cellular membranes, with a consequent release of the viral nucleocapsids into the cytoplasm (Jin *et al*., 2002). Viral glycoproteins mediate attachment of virus to endothelial cells via interaction with surface β3 integrin receptors (Arikawa *et al*., 1989; Gavrilovskaya *et al*., 1998, 1999).

b. Assembly and packaging Gn has a 142-residue C-terminal CT, which plays important roles in viral assembly and host–pathogen interactions. The lack of a matrix protein in hantaviruses, which plays an important role in the assembly and budding of many enveloped viruses, is thought to be substituted by a direct interaction between the nucleocapsid and envelope glycoproteins. Several lines of evidence seem to substantiate this model. The CT of Gn of Uukuniemi virus was shown by alanine scanning mutagenesis to mediate the packaging of a minigenome encoding a reporter gene into virus-like particles (VLPs; Overby *et al.*, 2007). Also, the CT domain of both Gn and Gc was shown to be indispensible for the replication of BUNV (Shi *et al.*, 2007). A highly conserved cysteine/histidine-rich region of the Gn tail was recently shown by NMR spectroscopy to form two classical zinc fingers (Estrada *et al.*, 2009). This suggested an involvement of this domain in nucleic acid binding or protein–protein interactions. However, it is not yet clear whether the CT interacts with the RNA genome or the protein component of the RNP during assembly. More recently, the N protein of PUUV was shown to coimmunoprecipitate with the glycoprotein complex. Mapping of the interaction sites revealed that the N protein has multiple binding sites in the CT of Gn and was also able to bind to the predicted CT of Gc (Hepojoki *et al.*, 2010b).

c. Virulence A clear association between viral glycoproteins and virulence was established by comparing the pathogenicity of virulent and attenuated or genetically reassorted strains of HTNV in an experimental newborn mouse model. Sequence comparison identified single amino acid changes in Gn and Gc as the genetic determinants responsible for the observed difference in viral virulence (Ebihara *et al.*, 2000; Isegawa *et al.*, 1994). Gn consists of an external domain, a transmembrane domain and a C-termianl CT (Spiropoulou, 2001). The CT of Gn appears to be a multifunctional virulence determinant that helps the virus to evade host innate responses and ensures productive infection in human endothelial cells. It contains immunoreceptor tyrosine-based activation motifs (ITAMs). These are cell-signaling elements involved in regulating the functions of immune and endothelial cells and their presence in Gn CT suggests a direct role in hantavirus pathogenesis (Geimonen *et al.*, 2003b). RIG-I (retinoic acid-inducible gene I) is an RNA helicase that triggers the cellular innate interferon (IFN) immune response upon detection of viral double-stranded RNA (Yoneyama *et al.*, 2004). The Gn CT of the pathogenic New York-1 virus (NY-1 V) was shown to inhibit the RIG-I-directed IFN responses (Alff *et al.*, 2006, 2008). To counteract this effect, it seems that cells have evolved a mechanism for downregulating Gn expression by targeting this protein for proteosomal degradation via polyubiquitination of the CT (Geimonen *et al.*, 2003a). These findings have created some

controversy in the literature. It was initially reported that the Gn CTs of pathogenic (NY-1 V, HTNV, and Andes virus ANDV), but not the nonpathogenic hantavirus PHV, are exclusively degraded via the ubiquitin–proteasome pathway in COS7 cells (Sen *et al*., 2007). However, this view was challenged by Wang *et al*., who reported that the Gn CTs of the nonpathogenic TULV and PHV were proteosomally degraded in HEK-293 and Vero E6 cells. Therefore, it was concluded that this degradation is not related to viral pathogenesis (Wang *et al*., 2009).

III. HANTAVIRUS DISEASE

A. Epidemiology

Unlike other members of the *Bunyaviridae* family, hantaviruses are not transmitted by biting insects. Human infection occurs accidentally via inhalation of contaminated rodent excreta (Hart and Bennett, 1999). Rodents are the main natural reservoir, where the virus can establish asymptomatic persistent infection. Within rodents, the disease is transmitted horizontally, mainly through aggressive behavior such as biting and scratching (Lee *et al*., 1981a). It is mainly a rural disease associated with the risk factors of farming, hunting, and camping because those activities bring humans into close contact with the rodent reservoirs (Simpson *et al*., 2010). The only hantavirus that causes disease in urban areas is SEOV because its host is the domestic rat (Mir, 2010). Human-to-human transmission is very rare and was reported only for ANDV in Argentina (Padula *et al*., 1998).

Approximately 150,000–200,000 cases of hantavirus infection are reported annually worldwide (Hart and Bennett, 1999). The geographic distribution of the disease reflects the distribution of the rodent host, hence the classification of hantaviruses into two main groups: Old World and New World (Jonsson *et al*., 2010). Occurrence of HFRS and HCPS depends on the type of virus causing the infection (Borges *et al*., 2006). HFRS is primarily considered a Eurasian disease, which was first reported in Korea in 1951 and is caused by Old World hantaviruses, for example, HTNV, SEOV, DOBV, and PUUV. More than 150,000 cases of HFRS are reported annually, half of which occur in China only (Peters *et al*., 1999). HCPS appears to be confined to the Americas and is associated with New World hantaviruses, for example, SNV, NY-1 V, and ANDV. It was first discovered in 1993 in the Four Corners region of the southwestern United States (Nichol *et al*., 1993). Approximately 300 cases of HCPS are reported each year in North and South America (Muranyi *et al*., 2005). SNV, which infects deer mouse, is the major cause of HCPS in North America (Fabbri and Maslow, 2001).

B. Reservoir hosts of hantaviruses

Rodents of the subfamily *Murinae* within family *Muridae* and the subfamilies *Arvicolinae*, *Neotominae*, and *Sigmodontinae* within family *Cricetidae* are considered to be the main natural reservoir hosts of hantaviruses. Old World hantaviruses, which cause HFRS, are carried by rodents of *Myodes*, *Rattus*, and *Apodemus* genera and New World hantaviruses, which cause HCPS are carried by the rodents of the subfamily *Sigmodontinae*. Recently, numerous novel hantaviruses from various sorcid and talpid insectivores have been discovered (Arai *et al.*, 2007; Kang *et al.*, 2009a,b; Song *et al.*, 2007a,b,c), but none of these viruses have been associated with any human disease.

An apparent coevolution of hantaviruses with their reservoir hosts is evident by the commonly observed close association of each hantavirus species with a certain rodent species. Moreover, phylogenetic analyses of hantavirus sequences and mitochondrial sequences from their rodent hosts suggest a long-standing coevolutionary history of hantaviruses with their rodent carriers (Jackson and Charleston, 2004; Plyusnin, 2002; Plyusnin and Morzunov, 2001). Depending on the population density, up to 50% of any given population of rodents are seropostive and considered as silent carriers for hantaviruses. Through experimental infections and field surveys, it has been shown that the transmission among rodents is exclusively horizontal and it occurs via inhalation of infected aerosols, through saliva or excreta, biting, and other aggressive behavioral interactions (Botten *et al.*, 2002; Glass *et al.*, 1988; Hinson *et al.*, 2004; Hutchinson *et al.*, 2000; Kariwa *et al.*, 1998; Lee *et al.*, 1981a,b; Root *et al.*, 2004). Unlike other rodent-borne viruses such as arenaviruses, there is no vertical transmission from the dam to its offspring, and maternal antibodies can protect offspring from infection for several months (Kallio *et al.*, 2006; Taruishi *et al.*, 2008). This view has been recently disputed by Calisher *et al.* (2009), and several scenarios have been presented for the transmission of hantaviruses from infected to uninfected hosts (Calisher *et al.*, 2009).

In general, infection of the rodent hosts by their respective hantaviruses is thought to be asymptomatic and no overt disease is produced. However, several recent studies have reported development of some disease symptoms in the reservoir hosts such as pulmonary edema and periportal hepatitis in white-footed mouse *Peromyscus leucopus* infected with NY-1 V (Lyubsky *et al.*, 1996) and SNV-infected deer mouse *Peromyscus maniculatus* (Netski *et al.*, 1999). Infection of hantavirus in the rodent host may also cause growth retardation as observed in *Rattus norvegicus* infected with SEOV (Childs *et al.*, 1989) and *P. maniculatus* infected with SNV (Kanerva *et al.*, 1998). Nevertheless, the general absence of an overt illness in rodent hosts despite a persistent and lifelong

infection is believed to be a smart way of survival for hantaviruses, by which they avoid killing their own hosts. It also highlights an amicable relationship between the virus and its host that was developed over hundreds of thousands of years of mutual interaction. The survival of rodent hosts in spite of the chronic viral infection is mediated by a combination of host's unique immunity against the virus and the viral genetic variability, which helps it evade the host's defense mechanisms (Schonrich *et al*., 2008).

Persistently infected rodents shed the virus in urine, feces, and saliva. Inhalation of virus-contaminated aerosols is the major route of transmission to humans. However, the general view that rodents are the only infection source for humans has been disputed by Zeier *et al*. (2005). It has been suggested that the close proximity of domestic animals such as cats, dogs, pigs, and cattle with rodents may cause some transmission events of hantaviruses to these animals. This species jump may cause the virus to evolve differently and result in more dangerous forms of the virus, which could be more pathogenic to humans (Zeier *et al*., 2005).

C. Diseases caused by hantaviruses in humans

In contrast to the asymptomatic infection of the rodent reservoir, hantavirus infection to humans results in two disease forms: HFRS and HCPS. In both syndromes, vascular endothelial cells show increased permeability and both are believed to result from host immune responses to infection, rather than damage caused by the viruses themselves (Khaiboullina and St. Jeor, 2002).

1. Hemorrhagic fever with renal syndrome

The incubation period of HFRS is about 3 weeks but can range from 10 days to 6 weeks (Jonsson *et al*., 2010; Kramski *et al*., 2009). The clinical course is classically subdivided into five overlapping phases: febrile, hypotensive, oliguric, diuretic, and convalescent (Schmaljohn and Hjelle, 1997). The febrile phase lasts for 3–5 days and is characterized by flu-like symptoms, thirst, nausea, and vomiting. The febrile phase is followed by a hypotensive phase, which lasts for few hours to 2 days, and is characterized by thrombocytopenia and petechial hemorrhage, retroperitoneal edema, and abdominal pain. The subsequent oliguric phase lasts for few days to two weeks. The mortality rate is 15%, with the majority of deaths occurring during the hypotensive and oliguric phases due to the complications of vascular leakage, renal failure, and acute shock. Patients who survive these complications usually progress into the diuretic phase and show improved renal function, with subsequent convalescence and recovery (Peters *et al*., 1999).

2. Hantavirus cardiopulmonary syndrome

The incubation period of HCPS ranges from 9 to 33 days (Young *et al.*, 2000). The clinical disease course of HCPS is usually divided into prodromal, cardiopulmonary, and diuretic or convalescent phases. The prodromal stage, which usually lasts 3–6 days, is characterized by flu-like symptoms such as fever, headache, chills, and muscle pain. Abdominal pain, nausea, vomiting, and dizziness may occur. This phase usually progresses rapidly to a severe respiratory disease characterized by nonproductive cough and dyspnea. In contrast to HFRS, the increased permeability and fluid leakage occur exclusively in the lungs instead of the kidneys. This bilateral pulmonary edema is usually visible in chest X-rays (Boroja *et al.*, 2002). Thrombocytopenia, hemoconcentration, and leukocytosis are the most prominent hematologic findings (Simpson *et al.*, 2010). Rapid deterioration, cardiac insufficiency, and respiratory failure caused by edema and hypotention, shock, and death may occur within 2–10 days after the onset of illness in almost 50% of cases. Patients who survive the acute phase recover within 5–7 days and enter the diuretic phase (Nolte *et al.*, 1995; Zaki *et al.*, 1995).

D. Pathogenesis

Hantavirus infection occurs via inhalation of infectious virus particles into the lungs. The inhaled viruses bind to β3 integrin receptors on the surface of pulmonary endothelial cells, macrophages, and dendritic cells (DCs), where virus replication mainly occurs (Gavrilovskaya *et al.*, 1998). Capillary leakage is the hallmark of hantavirus infection; however, the sequence of events from inhalation of infectious virus particles until pulmonary capillary leakage remains poorly understood. It was proposed that DCs play the role of a Trojan horse by helping the dissemination of virus throughout the body. Immature DCs located near the respiratory epithelial cells engulf inhaled virus particles, which can replicate in those cells without causing cell death. During their maturation, DCs migrate from the lungs to the lymph nodes where they present the engulfed antigen to immune cells (Raftery *et al.*, 2002).

The mechanisms by which pathogenic hantaviruses cause capillary leakage is an area of active research. Accumulating pieces of experimental evidences suggest that multiple immunopathologic mechanisms rather than direct viral-induced cytopathic effects are responsible for the disruption of vascular endothelium and the subsequent capillary leakage associated with hantavirus diseases (Borges *et al.*, 2006; Sundstrom *et al.*, 2001). In PUUV-infected patients, elevated levels of serum lactate dehydrogenase (LDH), indicative of cellular damage, correlated with high levels of serum perforin, granzyme B, and epithelial cell apoptosis markers. These

findings suggested that tissue damage is due to an immune reaction and that epithelial apoptosis contributed significantly to the damage (Klingstrom *et al*., 2006b).

Some changes in the endothelial cell barrier functions might be related to the use of β3 integrins as receptors. It was shown that the initial interaction of the infecting virus with β3 integrin receptors disturbs the integrin-directed migration of endothelial cells, which is essential for maintaining vascular integrity (Gavrilovskaya *et al*., 2002). The cellular immune response to infection involves strong stimulation of hantavirus-specific cytotoxic CD8 T lymphocytes (CTLs), which are released in large numbers into the blood. The severity of the disease was shown to correlate with the number of CTLs in the blood (Kilpatrick *et al*., 2004). This vast excess of activated CTLs is partly due to the lack of downregulation of T-cell function as evidenced by low serum levels of cytokines released by regulatory T cells, such as TGF-β, in HCPS patients (Chen and Yang, 1990; Mills, 2004). The intense antiviral immune response can contribute to the increased permeability of endothelial cells in two ways. First, the elevated levels of inflammatory mediators and cytokines, such as TNF-α, IL-6, and IL-10, released during the acute phase by activated T cells and infected endothelial cells increase the vascular permeability resulting in pulmonary edema (Linderholm *et al*., 1996). Second, it was shown in transwell permeability assays that hantavirus-specific cytotoxic T lymphocytes could directly lyse human endothelial cells infected with SNV (Hayasaka *et al*., 2007).

The exact mechanism of kidney failure in HFRS is unclear. Renal disease has been attributed to deposition of immune complexes and the presence of inflammatory cell infiltrations with subsequent tubular damage (McCaughey and Hart, 2000; Sironen *et al*., 2008).

E. Diagnosis

Diagnosis of hantavirus infection in the clinic relies on the establishment of a history of rodent exposure, symptoms suggestive of respiratory or renal involvement, blood exams showing severe thrombocytopenia, and positive serological tests. Virus isolation from clinical samples is difficult due to the presence of high levels of neutralizing antibodies (McCaughey and Hart, 2000). High titers of IgM and IgG antibodies against hantavirus N and Gn proteins are detectable in the sera of patients during the acute phase, enabling reliable serological confirmation of infection (Bostik *et al*., 2000; Elgh *et al*., 1997; Groen *et al*., 1994). SNV Gn antibodies are highly specific and do not cross-react with Gn antigens of other hantaviruses (Hjelle *et al*., 1994; Jenison *et al*., 1994). The most widely used serological tests for diagnosis of HCPS are IgM capture and IgG indirect ELISAs (Li *et al*., 2002). A rapid test for SNV and SEOV in the form of a strip

immunoblot assay has been developed, which relies on antigens derived from recombinant SNV N and Gn proteins and SEOV N protein. The assay was demonstrated to be highly sensitive and specific because it could identify all patients with acute SNV infection during the early course of the disease with no false-positive results (Hjelle *et al.*, 1997). Immunohistochemical detection of hantavirus antigens has been particularly useful in establishing retrospective diagnosis of HCPS from fixed tissue samples (Peters *et al.*, 1999). RT-PCR assays have also been used, but results should be always interpreted with caution due to the potential of cross-contamination and have to be always substantiated by positive results from immunodiagnostic assays (Moreli *et al.*, 2004).

F. Therapy

No specific antiviral therapy is currently available for treating hantavirus infections. Management of infected patients relies solely on supportive therapy in an intensive care unit until the virus is cleared by the immune system (Muranyi *et al.*, 2005). Ribavirin, a broad-spectrum nucleoside analogue antiviral drug, has been shown to possess, both *in vitro* and *in vivo*, inhibitory activity against hantaviruses (Huggins *et al.*, 1986; Medina *et al.*, 2007; Severson *et al.*, 2003). A clinical trial showed decreased virus titers, morbidity, and mortality rates in HFRS Chinese patients (Huggins *et al.*, 1991). Another trial on HFRS patients in Korea showed significant reduction in fatality and improved prognosis when given early in the course of the disease, as well as a reduction in the risk of renal insufficiency (Rusnak *et al.*, 2009). Intravenous injection of ribavirin was generally well tolerated by HCPS patients (Chapman *et al.*, 1999). However, a Placebo-controlled, double-blind trial of intravenous ribavirin in HCPS patients was ended prematurely due to low rate of enrollment (Mertz *et al.*, 2004). Therefore, ribavirin does not seem to have any clinical application in HCPS patients due to the lack of conclusive clinical data. Passive administration of neutralizing antibodies for PUUV has been shown to protect macaques against virus challenge; however, no human clinical trials were conducted (Klingstrom *et al.*, 2005).

G. Vaccines

Despite many efforts that have been invested in developing a safe effective vaccine to protect against hantavirus infection, currently, no FDA-approved vaccine is available in the United States (Ulrich *et al.*, 2002). A formalin-inactivated HTNV vaccine (Hantavax) produced from mouse brain-derived virus is licensed for use in Korea since the 1990s. Efficacy studies showed that 97% of human volunteers receiving this vaccine developed high titers of specific neutralizing antibodies after a booster

dose; however, the induced humoral immune response was short lived (Cho and Howard, 1999). Another formaline-inactivated vaccine produced from suspensions of Vero cells elicited a neutralizing antibody response in mice, which was fivefold higher than Hantavax (Choi *et al.*, 2003). However, it is unlikely that these vaccines can provide protection against all hantaviruses. The N, Gn, and Gc proteins of hantaviruses are highly immunogenic (Bharadwaj *et al.*, 2002). Therefore, different approaches were taken to develop recombinant vaccines using proteins expressed in baculovirus, vaccinia virus, and the yeast *Saccharomyces cerevisiae* (Chu *et al.*, 1995; Schmaljohn *et al.*, 1990; Yoshimatsu *et al.*, 1993). Recombinant N proteins of PUUV and DOBV expressed in yeast were shown to induce a protective immune response in rodent models (Dargeviciute *et al.*, 2002; Geldmacher *et al.*, 2004). Moreover, deer mice immunized with recombinant deer mouse cytomegalovirus (PCMV) expressing SNV Gn developed an antibody response to SNV (Rizvanov *et al.*, 2003). Although highly protective in mouse models, a recombinant vaccinia virus containing the S and M segments of HTNV proved to be inefficient in a phase II clinical trial. It elicited neutralizing antibodies in only 72% of the 142 participating volunteers (McClain *et al.*, 2000). Several other studies using naked DNA vaccines containing the M or S genome segments of SNV and SEOV have shown similar success in rodent models, but no clinical administration has been reported (Bharadwaj *et al.*, 1999; Hooper *et al.*, 1999).

IV. FUTURE PROSPECTS

Frequent emergence of zoonotic viruses is a serious concern to human health. For example, the recent emergence of H1N1 (swine flu) has created havoc in human lives, especially in pregnant women and young children who were predicted to be most susceptible for this new virus species. History witnesses the emergence and reemergence of numerous pathogens in the past century, with an estimated frequency of one new pathogen every 18 months. Many of these new pathogens were zoonotic RNA viruses, such as hantavirus, H1NI swine flu, Nipah virus, Hendra virus, Ebola virus, West Nile virus, and SARS (severe acute respiratory syndrome cornovirus; Jones *et al.*, 2008; Morens *et al.*, 2004). Despite their significant devastation to human lives, the ecology and natural history of these zoonotic viruses are still a mystery. The principles that govern their stable maintenance in their natural reservoirs, compulsions for switching their hosts, mechanisms of recombination and reassortment, molecular mechanisms of their transfer, survival and adaptation in the new host, are the interesting areas that remain poorly understood. The natural hosts of these zoonotic viruses continue to be the reservoirs for the generation of

new pathogenic strains and probable causes of future pandemics that pose serious threat to human lives.

Hantaviruses cause HFRS and HCPS when transmitted to humans but maintain a persistent infection in their rodent hosts. Coevolutionary existence of hantaviruses and their rodent hosts provides insights into mechanisms by which these zonotic viruses manage to exist in the environment for millions of years with a potential to infect humans upon transmission. However, understanding the mechanisms by which hantaviruses maintain a persistent infection in their rodent hosts without a disease may provide insights into the possible approaches that could be used for the treatment of hantavirus-associated disease in humans. Excessive proinflammatory cytokine (TNF-α) and CD8+ responses in humans are hypothesized to mediate the pathogenesis of human HFRS and HCPS, suggesting that anti-TNFα therapy might be helpful during the treatment.

Since HCPS is comparatively a rare disease, funding for the development of an HCPS vaccine is not a high priority for many countries. However, with the increasing number of HCPS cases, especially in South America, vaccine efforts for HCPS may receive better appreciation. Due to the requirements of high-containment laboratories for HCPS-causing viruses, plasmid DNA gene gun-based approaches will be preferred for the development of an HCPS vaccine.

Dissecting the molecular mechanisms of hantavirus replication in host cells will provide insights about the new potential targets for the design of antiviral therapeutic agents for the treatment of HFRS and HCPS. For example, the newly discovered translation initiation mechanism, operated by hantavirus nucleocapsid protein, demonstrates how a single viral protein lures the host cell translation machinery for the preferential translation of viral mRNAs in virus-infected cells where cellular transcripts are competing for the same translation apparatus (Mir and Panganiban, 2008). Shutting down this viral translation initiation strategy might inhibit virus replication in infected cells. In addition, viral RdRp uses capped RNA primers to initiate the transcription/replication of viral RNA genome. Capped RNA primers are generated from host cell mRNAs by a unique ''cap-snatching mechanism.'' This mechanism is well understood in influenza virus. However, recent studies suggest that hantaviral cap-snatching mechanism might be different from influenza virus (Mir *et al.*, 2008). In-depth understanding of cap-snatching mechanism will reveal novel targets that could be used for the design of antiviral agents for the treatment of diseases caused by a broad spectrum of viruses that use cap-snatching mechanism to initiate transcription/replication of viral genome.

Currently, the studies of hantavirus biology and pathogenesis are limited due to the lack of an animal model and a complete reverse genetic system for these viruses, although a minigenome system for HTNV has

been reported (Flick *et al.*, 2003). Reverse genetic system will generate hopes for screening chemical libraries to identify molecules with antiviral therapeutic potential, for the development of attenuated vaccine candidates, and for the investigation of molecular mechanisms involved in hantavirus replication, gene expression, as well as virus assembly and budding.

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