Chapter 6
Viral RNase Involvement in Strategies of Infection

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Contents

Abstract

The overwhelming majority of RNase activity is engaged in catabolic processes. Viruses have no metabolism of their own, but rely completely on host cellular energy and substrate provision to support the biochemical processes necessary for virus replication. It is therefore obvious that RNA hydrolysis does not represent an obligate step in the viral life cycle that would have to be governed by viral proteins.

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Accordingly, RNases are found only rarely in the viral proteomes and serve special functions. In this chapter, several virus-specific RNases will be described and their role in the viral life cycle discussed. The text will concentrate on RNases of members of the nidoviruses, herpesviruses, pestiviruses, and several viruses with segmented negative-strand RNA genome including influenza virus. These enzymes are involved in specific steps of viral gene expression, viral genome replication, shutoff of host cellular gene expression, and interference with the host's immune response to virus infection.

6.1 RNases with Special Tasks in Viral Genome Replication and Gene Expression

6.1.1 Endonucleases of Viruses with Segmented Negative-Strand RNA Genomes

A group of negative-strand RNA viruses comprising the members of the families *Orthomyxoviridae*, *Arenaviridae,* and *Bunyaviridae* use a special type of RNA endonuclease for one step during production of their mRNAs. The genome segments of these viruses are associated with viral proteins giving rise to so-called ribonucleoprotein complexes (RNP) (Buchmeier 2007; Palese and Shaw 2007; Schmaljohn and Nichol 2007). These are replicated by a replicase complex with a core unit composed of 1–3 viral polypeptides. This replicase is also responsible for transcription of the viral mRNAs. In contrast to the *de novo* initiation of RNA synthesis during replication, transcription occurs as a primer-dependent process. 5′ terminal segments of 1–15 nucleotides are cleaved from host cellular mRNAs by a viral endonuclease and serve as transcription primers carrying a 5′ cap structure. Data on the endonucleases of the above-mentioned viruses are described in the following sections.

6.1.1.1 Influenza Virus

The influenza A virus of the family *Orthomyxoviridae* represents one of the best studied viruses. Influenza A virus particles contain a genome of eight RNA segments with negative polarity (Palese and Shaw 2007). The three largest segments 1, 2, and 3 encode the polypeptides PB2, PB1, and PA, respectively, which constitute the viral RNA–dependent RNA polymerase (RdRP) responsible for genome replication and mRNA transcription (Fig. 6.1). The RdRP is not only an RNA polymerase but is also responsible for polyadenylation of viral mRNA and the endonucleolytic generation of the transcription primers mentioned above. Initially, the endonuclease was thought to reside in PB2 (Shi et al. 1995), but recent results

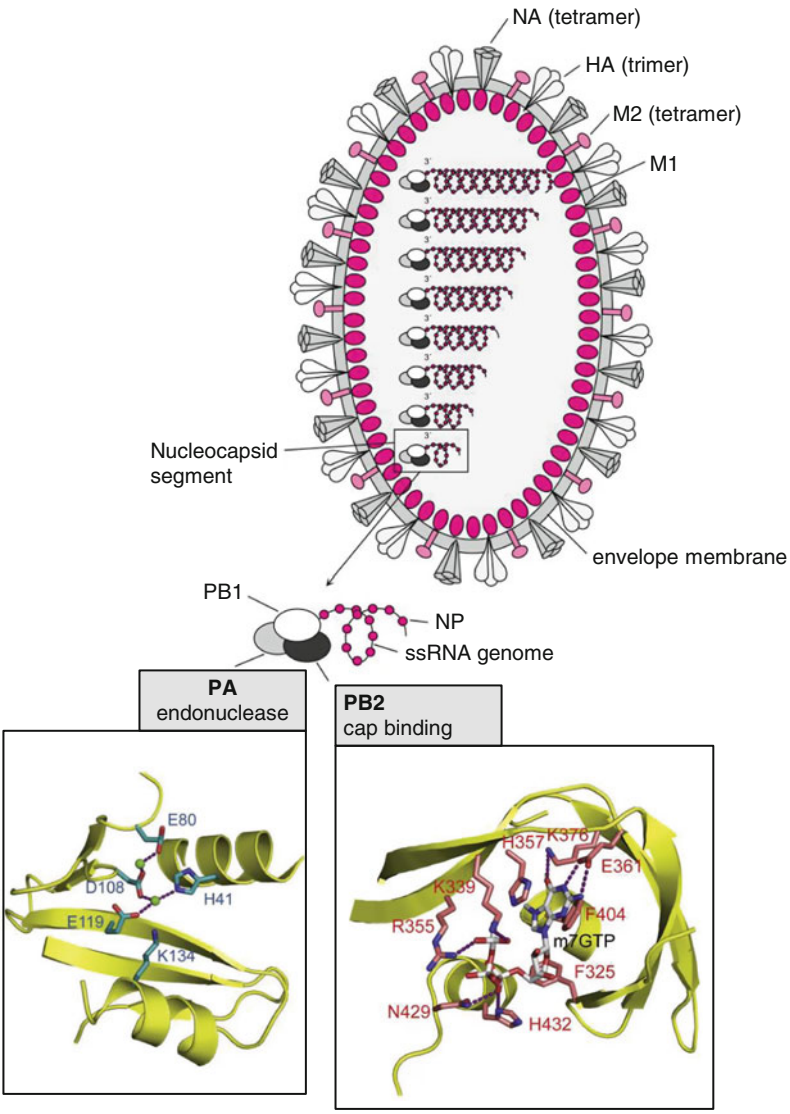

Fig. 6.1 On top, a schematic representation of an influenza A virus particle is shown with the envelope proteins neuraminidase (*NA*), hemagglutinin (*HA*), *M1* and *M2*, and the nucleocapsid segments that are each composed of one of the eight single-stranded genome segments of negative polarity, a certain number of nucleoprotein (*NP*) molecules and a polymerase complex encompassing polypeptides *PB1*, *PA,* and *PB2* (presentation of the scheme with kind permission from Springer Science and Business Media: Molekulare Virologie, chapter 16, 2010, page 357, Modrow et al. (2010), Fig. 16.6). As indicated, the PA protein contains the endonuclease domain, the active site of which is shown as a structure model at the bottom (*left part*). Helices and strands surrounding the active-site cleft are shown together with several residues important for coordination of the two catalytically active divalent metal cations (*green spheres*) shown as sticks (amino acids given in the one letter code). At the bottom on the right site, a structure model of the PB2

provided evidence for PA harboring the enzymatic domain (Dias et al. 2009; Yuan et al. 2009).

PA, a polypeptide of more than 700 amino acids, was expressed in insect cells in soluble form. Trypsin cleaves PA into N- and C-terminal domains of 25 and 55 kDa, respectively. The function of the latter is still unclear except for the fact that it mediates contacts with PB1 via a small C-terminal region (Ruigrok et al. 2010). The N-terminal ca. 250 amino acids of PA (PA-Nter) harbor the endonuclease activity of the influenza virus polymerase complex (Dias et al. 2009; Yuan et al. 2009). This fragment has been expressed in *E. coli* and subsequently crystallized. The overall structure shows an α/β architecture with a core of five mixed β strands forming a twisted plane surrounded by seven α-helices. Structure similarity searches gave no indications for entries with highly similar folds in the databases (Dias et al. 2009). The most similar structure identified in these searches was that of an archaeal Holliday junction resolvase (Hjc), obvious from superposition of helix α3 and strands β1–β5 encompassing a structural motif typically found in different nucleases including resolvases and type II restriction endonucleases (Knizewski et al. 2007; Kosinski et al. 2005). The motif conserved between Hjc and PA-Nter includes two acidic residues (Asp-108 and Glu-119 in the latter) that are known to be important for metal ion binding in the former enzyme. Residues Asp-108 and Glu-119 together with His-41 and Lys-134 of PA-Nter also align with catalytically important residues in the restriction endonuclease EcoRV (Glu -5, Asp-74, Asp-90 and Lys-92). Moreover, His-41, Asp-108, Glu-119, and Lys-134 together with Glu-80, a third acidic residue in the active site, are conserved among influenza viruses.

Members of the PD-(D/E)XK family of enzymes are known to contain up to three coordinately bound divalent metal ions that are involved in the catalytic process (Knizewski et al. 2007; Kosinski et al. 2005). Metal ions were also found in the influenza virus endonuclease. Within a negatively charged cavity surrounded by helices α2–α5 and strand β3, Yuan and coworkers identified an Mg^{2+} ion (Yuan et al. 2009). Dias et al. reported the presence of Mn^{2+} at the same position. In addition, the latter group detected a second Mn^{2+} about 4 Å away suggesting a two-metal based catalytic mechanism. A preference for Mn^{2+} over other divalent cations was found in stability and functional assays (Dias et al. 2009; Doan et al. 1999).

Mutagenesis analyses supported the model described above. Yuan and coworkers mutated the residues H-41, E-80, L-106, P-107, D-108, and E-119 proposed to be important for metal ion coordination (Fig. 6.1) (Yuan et al. 2009). In addition, K-134 was also included in these experiments because this residue is located close to the proposed active site of the endonuclease, and exchanges at this position had

Fig. 6.1 (continued) region binding the 5′ cap structure is shown. Again, important residues engaged in binding the m^7GTP group of the cap via stacking of ring structures are shown as sticks. The PB2 moiety of the polymerase confers the specificity for the endonucleolytic cleavage of capped substrate RNAs. (The schemes of the structures are reprinted from publication Ruigrok et al. 2010, with permission from Elsevier)

been shown before to specifically block transcription but not replication of the viral RNA (Hara et al. 2006). Polymerases with mutations E80A, D108A, E119A, and K134A retained significant genome replication efficiency, but were severely hampered in mRNA synthesis. The abrogation of transcription activity was indeed a consequence of blocked endonucleolytic activity of the polymerase since the mutants were able to perform transcription in the presence of ApG as a substitute for the cap-primer or after addition of a capped primer itself. In contrast, the L106A and P107A mutants retained some transcription activity but lost the ability to perform genome replication, while the H41A mutant was unable to perform RNA synthesis in general. The latter effects could not be circumvented in ApG-primed transcription assays. Finally, a direct endonuclease cleavage assay proved that all three exchanges affecting the acidic residues and the K134A mutation blocked the endonucleolytic activity (Yuan et al. 2009).

Taken together the data proved that the endonucleolytic activity in the influenza virus RNA polymerase resides in the N-terminal region of the PA polypeptide and that the respective enzyme belongs to the PD-(D/E)XK family of nucleases with the characteristic motif occurring at 107-PDLYDYK. Special features of the PA-Nter with regard to the other family members are the close vicinity of the two acidic residues in the conserved motif, an unusual position of the presumably catalytically important lysine (Lys-134), the histidine residue in the active site and the fact that RNA is the substrate instead of DNA.

A somewhat surprising result of the structure analysis was the fact that it did not provide information on how the RNA substrate could access the active site of the enzyme as it does not contain obvious determinants for RNA binding (Dias et al. 2009; Yuan et al. 2009). In fact, the endonuclease domain has a negatively charged surface with an even more acidic cavity containing the active site. The contact to the substrate RNA is presumably made by a number of positively charged residues that are located on the rim around the active site cavity. These residues are conserved and should help to position the substrate for cleavage.

As mentioned above, within the PD-(D/E)XK family of nucleases, the influenza virus endonuclease is special for its substrate representing RNA. It has been shown that the endonuclease cleaves also single-stranded DNA with only slightly reduced activity (Klumpp et al. 2000), but the natural substrate of this enzyme consists of a 5′ terminally capped RNA. The structural data do not provide any evidence how the substrate specificity for a 5′ capped RNA could be achieved. Experimental evidence has been provided for PB2 representing the cap-binding domain of the polymerase (Guilligay et al. 2008). A structure of the PB2 residues 320–483 crystallized with m^7GTP allowed identification of the residues responsible for binding the methylated guanine which are absolutely conserved among influenza A viruses. These residues include His-357 and Phe-404, which sandwich between their ring systems the purine of the methylated base, and Glu-361 and Lys-376 that specifically recognize the guanine base (Fig. 6.1). The mode of cap binding is very similar to what has been described for the nuclear cap-binding complex and the cap-binding translation initiation factor eIF4E found in the cytoplasm (Guilligay et al. 2008; Fechter et al. 2003; Li et al. 2001).

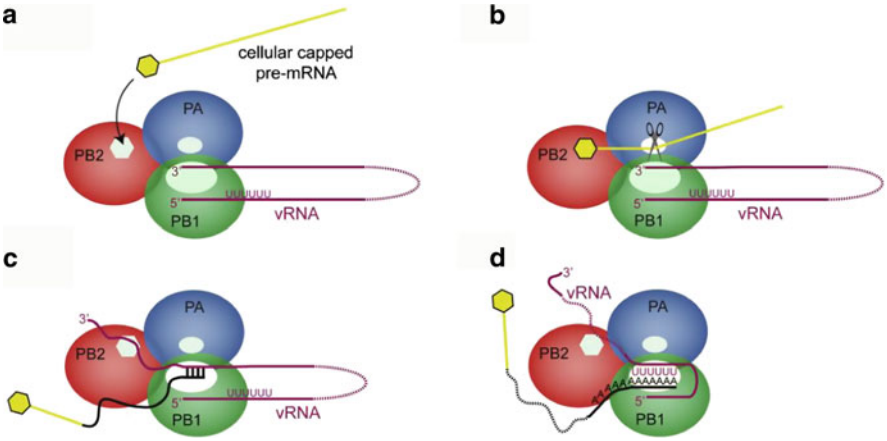

Fig. 6.2 Schematic diagram showing steps in cap-dependent transcription by influenza virus polymerase. (**a**) Binding of host pre-mRNAs (*yellow*) by the cap-binding domain located in the PB2 subunit. (**b**) Cleavage of the host mRNA 10–13 nucleotides downstream of the 5′ cap by the endonuclease located in the PA subunit. (**c**) Elongation of the chimeric viral mRNA by the nucleotidyl-transferase site in the PB1 subunit using the vRNA as template. (**d**) Polyadenylation of the viral mRNA by polymerase stuttering at the oligo-U sequence near the 5′ end of the vRNA. (The scheme is reprinted from the publication Ruigrok et al. 2010, with permission from Elsevier)

The function of the RNA endonuclease in the influenza virus life cycle is a special way to provide the viral mRNAs with a 5′ cap structure via the so-called cap-snatching mechanisms (Fig. 6.2). The requirement for a 5′ cap is a consequence of the mechanisms underlying translation initiation in eukaryotes (Sonenberg and Hinnebusch 2009). The cap structure represents the key element by which the translation system recognizes an mRNA to be translated. A so-called cap-binding complex assembles at the 5′ terminal cap structure in the initial phase of the translation initiation process. Thus, an RNA without a cap structure is usually not accepted as a substrate for translation.

Influenza viruses replicate in the nucleus of the infected cells, where also cellular mRNA synthesis and maturation takes place. However, because of the RNA nature of the viral genome, these viruses cannot employ the cellular machinery for the production of their mRNAs. To circumvent this problem, the viral RNP that includes the RNA polymerase binds capped cellular RNAs and cleaves these RNAs close to the 5′ end. The cleavage product is a short RNA fragment with a 5′ terminal cap and a free 3′ hydroxyl that is used as a primer for the synthesis of viral mRNA.

6.1.1.2 Arena- and Bunyaviruses

In addition to the orthomyxoviruses, members of virus families *Arenaviridae* and *Bunyaviridae* also use a “cap-snatching” mechanism during synthesis of their

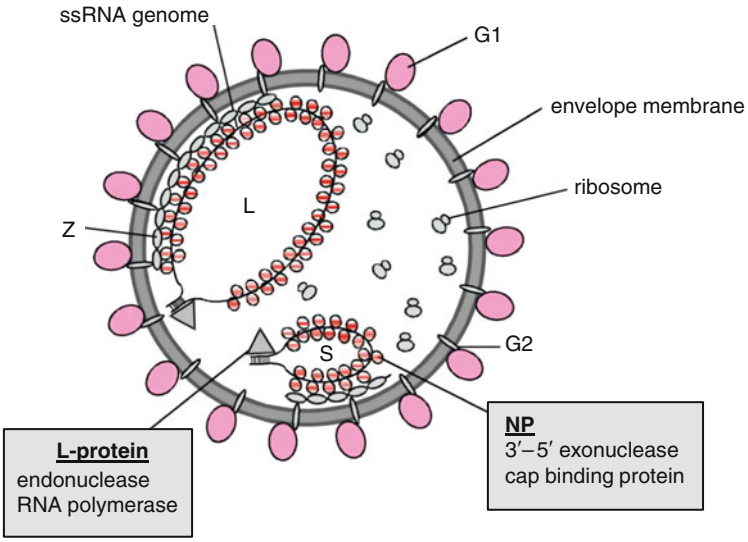

Fig. 6.3 Schematic representation of an arenavirus particle. The different viral proteins and the two segments of single-stranded (ss) genomic RNA are marked. Highlighted are the locations of the two proteins with RNase, namely, the L-protein with the endonuclease engaged in cap snatching and the NP harboring the 3′–5′ exonuclease, involved in blocking the innate immune response to virus infection of cells. (Presentation of the scheme with kind permission from Springer Science and Business Media: Molekulare Virologie, chapter 16, 2010, page 327, Modrow et al. (2010), Fig. 16.1)

mRNAs. Arena- and bunyaviruses are also enveloped viruses with segmented RNA genomes consisting of two or three segments, respectively (Fig. 6.3). Both families are classified as negative-strand RNA viruses even though the arenavirus genome segments both have an ambisense orientation (Buchmeier 2007; Schmaljohn and Nichol 2007).

In contrast to the orthomyxoviruses, the RNA-dependent RNA polymerases of arena- and bunyaviruses, known as the L-protein, consist of only one polypeptide that encompasses all the necessary activities including the endonuclease providing the capped primers for mRNA transcription (Buchmeier 2007; Schmaljohn and Nichol 2007).

In bunyaviruses, a quite heterogeneous group of mostly animal viruses comprising more than 300 species, the L-protein ranges in size from 240 to 460 kDa. It contains six motifs (preA, A-E) typical for RdRp in negative-strand RNA viruses, but specific functions could not be assigned to individual domains of the protein because of a lack of conserved sequence motifs (Schmaljohn and Nichol 2007). The endonucleolytic RNase activity crucial for cap snatching in bunyaviruses resides in the N-terminal ca. 180 amino acids of the L-protein (Reguera et al. 2010). The bunyavirus endonuclease has a very similar α-β topology as PA-Nter of influenza A virus even though the lengths of the helices are significantly different. This similarity is especially striking in the core structure with the active site even though

there is basically no homology between the two amino acid sequences. A core region of 55 residues contains the motif characteristic for the PD-(D/E)xK family of endonucleases. Within this region, a one-to-one mapping between the ligands coordinating the two metal ions was found (Reguera et al. 2010). For site 1, His-34, Asp-79, Asp-92, and the carbonyl oxygen of Tyr-93 in the bunyavirus LaCross virus correspond to His-41, Asp-108, Glu-119, and Ile-120 in PA-Nter. For site 2, Asp-52 and Asp-79 in the bunyavirus sequence correspond to Glu-80 and Asp-108 in influenza virus PA N-ter. This high degree of similarity should reflect functional equivalence, and indeed the bunyavirus endonuclease can be blocked by the same inhibitor, 2, 4-dioxo-4-phenylbutanoic acid (DBPA), as the influenza A virus enzyme. Important differences between the two structures concern the substrate-binding pocket. In PA-Nter, helix 2 together with the following loop are found in a position enabling them to restrict substrate access to the active site, whereas in the bunyavirus enzyme a wider channel is found that should allow binding and cleavage of larger, more structured substrates. Indeed, the bunyavirus enzyme is more active with a largely double-stranded RNA substrate (Reguera et al. 2010).

Mutagenesis analyses showed that the presence of divalent metal ions, which preferably represent Mn^{2+}, is crucial both for thermal stability of the protein and catalysis. There is good evidence that thermal stability is already obtained after binding of the ion in site 1, whereas enzymatic activity depends on the presence of ions at both sites. Similarly, as described above for the influenza A virus enzyme, mutations could discriminate between the endonucleolytic/transcriptional activity and the genome-replicating polymerase (Reguera et al. 2010).

The arenavirus L-protein is composed of approximately 2,200 amino acids and encompasses several conserved domains (Lopez et al. 2001) including one that contains the typical RdRp signature sequence motifs (Lukashevich et al. 1997; Vieth et al. 2004). Recently, the first part of an arenavirus L protein, namely, the N-terminal region of the lymphocytic choriomeningitis virus (LCMV), has been crystallized (Morin et al. 2010). The structure analysis of this fragment, denoted NL1, showed again a central fold composed of four mixed β-strands surrounded by seven α-helices. Also in this protein, the β-strands form a twisted plane creating a negatively charged cavity for binding of divalent cations. Even though the fifth beta strand is missing in NL1, a structure-based superimposition of the endonuclease-active sites from LCMV and influenza A virus revealed a striking similarity of these regions which included the positioning of the residues important for metal ion binding in PA-Nter. As with the bunyavirus enzyme, also the LCMV endonuclease shows some differences with regard to the influenza virus PA-Nter. The analyses did not reveal structural matches for the influenza virus His-41 or Lys-134 in the LCMV protein which is especially important since His-41 was proposed to play a direct role in the catalytic process.

Biochemical analyses revealed that the arenavirus endonuclease is most likely also an Mn^{2+}-dependent enzyme, since it was at least ~90-fold more active in the presence of Mn^{2+} than with other divalent cations. Mutagenesis experiments proved

that the endonucleolytic activity of the L protein was essential for arenavirus mRNA transcription but not for genome replication (Morin et al. 2010). RNA binding was shown by gel shift assays and cleavage reactions with synthetic RNA oligonucletides revealed a specificity for substrates containing uracil. RNA with adenosine in single-stranded regions is also cleaved, but with lower efficiency. The cleavage product was found to be unusually short, comprising only 1–4 nucleotides (Buchmeier 2007).

The function of the arena- and bunyavirus endonucleases in the cap-snatching process is equivalent to that of the influenza virus enzyme. So far, there is no idea how the bunyavirus RNase identifies the main feature of its substrate, namely, the 5′-terminal cap structure. The functional experiments with the arenavirus enzyme showed that the presence of a 5′ cap is not required for enzymatic activity in vitro. In fact, cleavage in close vicinity of a cap structure seemed to be not even preferred in these assays (Morin et al. 2010). It nevertheless has to be postulated that such substrate discriminating binding sites exist. Moreover, the activity of the endonucleases has to be regulated, for example, by allosteric effects in a way that it is activated only after binding of a cellular substrate RNA to prevent cleavage or decapping of nascent viral mRNAs during transcription. Recently, the cap-binding site in arenaviruses was demonstrated to reside in the N-terminal part of the arenavirus nucleoprotein (NP) (Qi et al. 2010). The respective protein domain adopts a completely new fold not related to other cap-binding proteins.

Qi et al. (2010) also reported that the C-terminal part of the lassavirus NP contains a second RNase. This enzyme represents a 3′–5′ exonuclease and was identified because it adopts a structure strikingly similar to known 3′–5′ exonucleases/exoribonucleases in humans and bacteria. The enzymatic activity of this protein domain was demonstrated by biochemical analyses that also revealed its dependency on divalent cations. Again Mn^{2+} was found to represent the preferred ion. One cation was identified in the structure, but the authors proposed a two cation–dependent catalytic mechanism in analogy to the homologous enzymes (Qi et al. 2010).

The lassavirus NP exonuclease efficiently degrades DNA and RNA substrates including different double-stranded RNAs (Qi et al. 2010). The latter is presumably important for the function of the exonuclease during the viral life cycle. It was already known before that NP is engaged in blocking the induction of type 1 interferon (IFN-1) expression in the arenavirus infected cell thereby impairing the innate immune response of the host. Qi and coworkers (2010) showed that the repression of the interferon response is dependent on the exonuclease activity in NP, whereas cap snatching and transcription is also detected when the exonuclease is inactivated by mutation. NP interacts with RIG-I and MDA5, two RNA helicases that serve as detectors of so-called pathogen-associated molecular patterns (PAMP) in intracellular interferon induction pathways (Zhou et al. 2010). Thus, it can be hypothesized that at least one function of the NP exonuclease is to associate with these helicases and degrade PAMP-containing RNAs that have been bound by them.

6.1.2 RNases of Viruses Belonging to the Order Nidovirales

Members of the order *Nidovirales* form a phylogenetically compact cluster of plus-strand (+) RNA viruses that diverged profoundly from other RNA viruses. In line with this, the ribonucleases encoded by nidoviruses (Bhardwaj et al. 2004; Ivanov et al. 2004; Minskaia et al. 2006) are not closely related to any of the other viral ribonucleases discussed in this chapter. The order *Nidovirales* currently includes three families, *Coronaviridae* (subfamilies *Coronavirinae* and *Torovirinae*), *Roniviridae,* and *Arteriviridae*. Nidovirus RNA replication, modification, and processing involves a complex set of enzymes, including polymerase, primase, helicase, ADP-ribose 1″-phosphatase, methyltransferase, and ribonuclease activities (Snijder et al. 2003; Ziebuhr and Snijder 2007; Ziebuhr 2008). These enzymes are expressed as part of large polyproteins, pp1a (450 kDa) and pp1ab (750 kDa), that are either encoded by ORF1a alone (pp1a) or ORFs 1a and 1b together (pp1ab). Expression of the latter involves a programmed (–1) ribosomal frameshift occurring just upstream of the ORF1a stop codon (Brierley et al. 1987). The polyproteins are co- and posttranslationally cleaved by viral proteases to produce mature processing products called nonstructural proteins (nsp) 1–16 (in coronaviruses) and nsp1–nsp12 (in arteriviruses) (Ziebuhr et al. 2000; Ziebuhr 2008).

6.1.2.1 The Nidovirus Exoribonuclease, a Putative Proofreading Enzyme

Members of the *Coronaviridae* and *Roniviridae* encode in ORF1b an exoribonuclease domain called ExoN which, in coronaviruses, occupies the N-proximal two-thirds of nsp14 (Minskaia et al. 2006; Snijder et al. 2003), while the C-terminal domain of this protein harbors a cap- (Gppp-RNA-)specific N7-methyl transferase (N7-MT) activity (Bouvet et al. 2010; Chen et al. 2009). ExoN is related to the DEDD superfamily of metal-dependent exonucleases (Snijder et al. 2003; Zuo and Deutscher 2001) whose members contain four invariant acidic residues (three Asp and one Glu) that are part of three conserved motifs. The DEDD family also includes enzymes involved in DNA proofreading, such as dnaQ, the ε subunit of *E. coli* DNA polymerase III (Echols et al. 1983; Scheuermann et al. 1983). In contrast to their cellular homologs, nidovirus ExoN domains contain a putative zinc-binding domain that is located between motifs I and II (Snijder et al. 2003). In roniviruses, a second putative zinc-binding domain was identified between motifs II and III (Sittidilokratna et al. 2008; Snijder et al. 2003). Bacterially expressed SARS-coronavirus (SARS-CoV) nsp14 was shown to possess 3′–5′ exonuclease activity that, in contrast to many cellular DEDD exonucleases, acts exclusively on RNA (Minskaia et al. 2006). Ribonucleolytic activity was found to require Mg^{2+} or Mn^{2+} or low concentrations of Zn^{2+} as a cofactor, while Ca^{2+} or high concentrations of Zn^{2+} inhibited activity. Substitutions of any of the four strictly conserved active-site residues (D-E-D-D) by Ala were shown to abolish

or significantly reduce nucleolytic activity, supporting previous predictions on the critical role of these residues in catalysis (Minskaia et al. 2006).

The observation that ExoN is only conserved in "large nidoviruses" featuring genome sizes of about 30 kb (*Coronaviridae*, *Roniviridae*) but not in the much smaller *Arteriviridae* (genome sizes between 13 and 16 kb) and the phylogenetic relatedness of ExoN with cellular exonucleases acting as proofreading enzymes in DNA replication led to the speculation that ExoN may be a critical factor in the evolution of large RNA virus genomes (Gorbalenya et al. 2006), most likely by increasing RNA replication fidelity, thus keeping the mutation frequency below a postulated critical threshold (Biebricher and Eigen 2005; Crotty et al. 2001; Gorbalenya et al. 2006).

Initial studies using human coronavirus 229E (HCoV-229E) ExoN active-site mutants generated by reverse genetics revealed a critical importance of ExoN in viral RNA synthesis (Minskaia et al. 2006). Accumulation of viral RNA was severely reduced, and viable virus progeny could not be obtained in these experiments. By contrast, viable ExoN active-site mutants could be obtained for SARS-CoV and murine hepatitis virus A59 (MHV-A59), although RNA synthesis and virus reproduction was again found to be significantly reduced (Eckerle et al. 2007, 2010). Consistent with the proposed role of ExoN in proofreading, mutation frequencies (determined for viable MHV-A59 and SARS-CoV ExoN mutants) were reported to be significantly (more than 15-fold) increased in ExoN mutants when compared to wild-type virus(es) (Eckerle et al. 2007, 2010). SARS-CoV ExoN mutants also displayed a higher diversity within the virus population. Interestingly, the extent of genome diversity remained essentially unchanged when ExoN mutants were passaged in cell culture, suggesting counterselection of deleterious mutations. A higher-than-average replication fidelity was calculated for (wild-type) SARS-CoV and MHV (9.0×10^{-7} and 2.5×10^{-6} substitutions, respectively, per nucleotide per replication cycle) when compared to other RNA viruses, while replication fidelities of SARS-CoV and MHV ExoN mutants were similar to those reported for other RNA viruses (Eckerle et al. 2010). As calculations were done on infectious viruses in this study, mutation frequencies likely represent an underestimate of the actual number of mutations occurring during viral replication. Overall, however, the data support the idea that ExoN has a role in keeping replication fidelity at a (high) level typical for small DNA (rather than RNA) viruses (Cuevas et al. 2009). Although a direct proof for ExoN acting as a proofreading exoribonuclease remains to be obtained, the available information would support this hypothesis (Eckerle et al. 2007, 2010).

6.1.2.2 The Nidovirus Endoribonuclease, NendoU

Nidoviral endonucleases derive their name, NendoU, from *N*idovirus *endo*ribonuclease specific for *U*(ridylate). NendoU domains are related to a family of cellular enzymes prototyped by the *Xenopus laevis* endoribonuclease XendoU (Snijder et al. 2003), an endoribonuclease involved in the processing of small

nucleolar (sno)RNAs (Laneve et al. 2003). NendoU is conserved across members of the *Nidovirales* but not in other RNA viruses, making the domain a genetic marker of nidoviruses (den Boon et al. 1991; Ivanov et al. 2004). NendoU resides in nsp15 in coronaviruses and nsp11 in arteriviruses. NendoU activities have been characterized for both coronavirus and arterivirus homologs (Bhardwaj et al. 2004; Cao et al. 2008; Ivanov et al. 2004; Kang et al. 2007; Nedialkova et al. 2009). The enzymes were reported to (1) cleave downstream of uridylate (and, in some cases and less efficiently, after cytidylate), (2) release products with 2′,3′-cyclic phosphodiester ends, and (3) cleave ssRNA more efficiently than dsRNA (Bhardwaj et al. 2004, 2006; Ivanov et al. 2004; Nedialkova et al. 2009).

Structural and biochemical studies revealed that coronavirus NendoUs (nsp15) and their cellular homolog XendoU possess a novel fold that is not found in other ribonucleases (Renzi et al. 2006; Ricagno et al. 2006). Coronavirus NendoUs form hexamers comprised of dimers of trimers (Bhardwaj et al. 2006, 2008; Ricagno et al. 2006; Xu et al. 2006). The monomers have an α + β structure comprised of three domains, with the nidovirus-wide conserved domain (den Boon et al. 1991) representing the C-terminal subdomain of the protein (Fig. 6.4). The NendoU hexamer forms a three petal-shaped surface that surrounds a small, predominantly negatively charged central channel with an inner diameter of ~15 Å. The two trimers interact head to head, mainly involving the N-terminal domains, while the C-termini are located at the surface where they form six independent active sites. Inter-monomer interactions are largely mediated by residues of the N-terminal and central domains (Ricagno et al. 2006; Xu et al. 2006).

Despite limited sequence and structural similarity, the active-site residues (His-234, His-242, and Lys-289 of SARS-CoV nsp15) can be superimposed with equivalent residues of the catalytic center of bovine RNase A (His-12, His-119 and Lys-41) (Ricagno et al. 2006), a well-characterized enzyme belonging to a different family of pyrimidine-specific ribonucleases. The proposed coronavirus NendoU catalytic His and Lys residues cluster inside a positively charged groove of the C-terminal domain (Fig. 6.4) (Ricagno et al. 2006; Xu et al. 2006) and mutagenesis data confirmed their critical role in nuclease activity (Bhardwaj et al. 2008; Ivanov et al. 2004; Kang et al. 2007). Additional active-site residues were implicated in binding of the substrate phosphate. These involve the side chain of a highly conserved Thr (Thr-340 in SARS-CoV nsp15, Fig. 6.4) and the main chain amide of a conserved Gly residue (Gly-247 in SARS-CoV nsp15, Fig. 6.4) (Bhardwaj et al. 2008; Ivanov et al. 2004; Kang et al. 2007; Xu et al. 2006). Although the NendoU reaction mechanism has not been analyzed in detail, it is generally thought to be similar to that of RNase A. This is supported by (1) similar spatial positions of the putative catalytic His and Lys residue(s) in the structure (see above), (2) the production of 2′,3′ cyclic phosphate-containing reaction products (Bhardwaj et al. 2008; Ivanov et al. 2004), and (3) the production of 3′-hydroxyl ends after extended reaction times using recombinant forms of NendoU.

Similarities between the active sites of RNase A and nidovirus NendoUs extend to residues presumably involved in substrate binding and, more specifically, pyrimidine specificity. Thus, in the RNase A structure, Thr-45 and Phe-120, which are

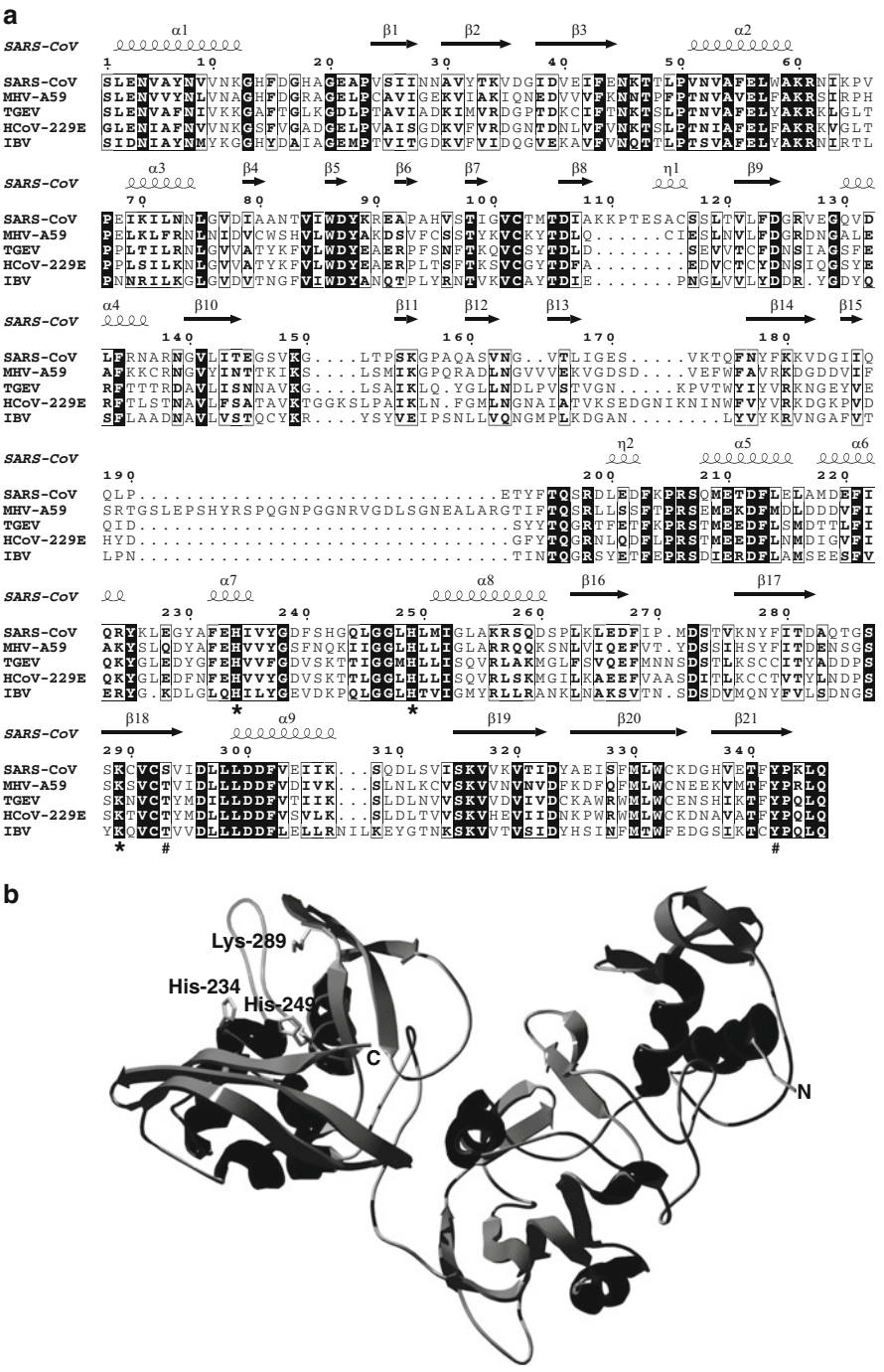

Fig. 6.4 Coronavirus endoribonucleases. (**a**) Multiple sequence alignment of coronavirus nsp15 (NendoU) domains, representing the three coronavirus genera, *Alphacoronavirus* (HCoV-229E, TGEV), *Betacoronavirus* (SARS-CoV, MHV-A59), and *Gammacoronavirus* (IBV). Sequences

known to contribute to pyrimidine specificity by forming hydrogen bonds (Thr-45) or stacking interactions (Phe-120) with the pyrimidine base (Raines 1998) occupy positions that are similar to those of two Ser/Thr and Tyr residues conserved in coronavirus and arterivirus NendoUs (Ser-293 and Tyr-342 in SARS-CoV nsp15; Fig. 6.4). Molecular modeling of uridine 3′-monophosphate binding to the SARS-CoV NendoU active site and mutagenesis data provided further evidence for a role of Ser-293 and Tyr-342 in substrate binding and specificity (Bhardwaj et al. 2008; Nedialkova et al. 2009; Ricagno et al. 2006). Thus, substitutions with Ala of Ser-293 in SARS-CoV nsp15 and Ser-228 in EAV nsp11 resulted in enzymes that had essentially lost their preference for uridine over cytidine. By contrast, specificity and activity were restored when the same Ser residues were substituted with Thr (Bhardwaj et al. 2008; Nedialkova et al. 2009). In addition, Pro-343 and Leu-345 (SARS-CoV nsp15 numbering) were implicated in uridylate specificity, again supported by mutagenesis data (Bhardwaj et al. 2008).

Thus far, the role of metal ions in NendoU activity has not been resolved conclusively. Whereas the nucleolytic activities of the cellular NendoU homologs, XendoU and human placental protein (PP11), and coronavirus NendoUs depend on (or are stimulated by) Mn^{2+} ions (Bhardwaj et al. 2004, 2008; Ivanov et al. 2004; Laneve et al. 2003, 2008), arterivirus NendoU activities do not appear to require metal ions (Nedialkova et al. 2009). At low concentrations of Mn^{2+}, the activities of bacterially expressed equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV) NendoUs were only slightly enhanced and, at higher concentrations, activities were found to be inhibited. The observed critical (or, at least, supportive) role of metal ions in most NendoU/XendoU homologs characterized to date is inconsistent with the proposed RNase A-like (metal-independent) reaction mechanism and structural studies did not reveal metal-binding sites in these enzymes (Renzi et al. 2006; Ricagno et al. 2006), thus questioning the requirement for Mn^{2+} ions in nuclease activity. Mn^{2+} ions were found to affect the intrinsic tryptophan fluorescence of SARS-CoV nsp15, suggesting that metal ion binding can induce conformational changes in the protein which were suggested to affect RNA binding (Bhardwaj et al. 2006; Guarino et al. 2005). Surprisingly, Mn^{2+} ions stimulated the RNA-binding activity of SARS-CoV nsp15 but not that of XendoU (Bhardwaj et al. 2006; Gioia et al. 2005).

Fig. 6.4 (continued) were aligned using ClustalW 2.0 (Larkin et al. 2007) and rendered with ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Abbreviations of virus names and accession numbers are as follows: *SARS-CoV* Severe acute respiratory syndrome coronavirus [AY291315], *MHV-A59* Murine hepatitis virus A59 [NC_001846], *TGEV* Transmissible gastroenteritis virus [Z34093], *HCoV-229E* Human coronavirus 229E [NC_002645], *IBV* Avian infectious bronchitits virus [NC_001451]. Secondary structure elements of SARS-CoV nsp15 (PDB 2H85) are shown above the sequence. Catalytic residues are indicated by *asterisks* and key residues involved in uridylate specificity are indicated by *hashes* (see text for details). (**b**) Ribbon representation of a SARS-CoV nsp15 (NendoU) monomer (pdb 2H85). The catalytic residues, His234, His249, and Lys289, are shown in *ball-and-stick* representation, and amino and carboxyl termini are indicated (*N*, *C*)

Biochemical studies suggest that the hexameric structure described above is the fully active form of coronavirus NendoUs (Bhardwaj et al. 2006; Guarino et al. 2005; Xu et al. 2006). This is supported by the conservation and functional relevance of residues predicted by structural and biochemical studies to stabilize intersubunit interactions in the trimer and hexamer. Also, a structure analysis of a truncated form of SARS-CoV nsp15, which lacked 28 N-terminal and 11 C-terminal residues and was monomeric, revealed that two loops of the catalytic domain were displaced compared to their location in the hexamer, causing major changes in the active site geometry and loss of activity (Joseph et al. 2007). On the basis of these observations, hexamerization has been suggested to act as an allosteric switch required to activate these enzymes. If confirmed, this regulatory mechanism would not apply to EAV nsp11 and XendoU, both of which were reported to be monomeric in solution (Nedialkova et al. 2009; Renzi et al. 2006).

The biological significance of NendoU activities in the nidovirus life cycle has not been elucidated. Substitution of coronavirus NendoU active-site residues by reverse genetics resulted in a slight reduction of both genomic and subgenomic (sg) RNA synthesis in MHV (Kang et al. 2007) and a slight reduction in virus reproduction. No significant differences in plaque size were observed for NendoU mutants compared to wild-type MHV. In striking contrast, NendoU active-site substitutions caused profound defects in arterivirus reproduction, with virus titers being reduced by up to five log in a few cases. Several substitutions in the EAV NendoU resulted in a selective reduction of sgRNA synthesis compared to genome replication. Substitutions that, based on the available structure information for coronavirus NendoU, are predicted to cause major structural changes in NendoU (and, possibly, the polyprotein) abolished RNA synthesis in EAV and HCoV-229E completely (Ivanov et al. 2004; Posthuma et al. 2006). Taken together, the data suggest that NendoU domains may have nonidentical roles and/or substrates in different nidovirus families/subfamilies/genera, possibly reflecting adaptation to specific hosts and/or ecological niches (Lei et al. 2009).

6.2 Viral RNase Influencing Host Cellular Metabolism and the Host Immune Response: The Herpesvirus *vhs* Protein

Herpesviruses constitute a family of enveloped viruses with large double-stranded DNA genomes of more than 100 kb that encode a large number of viral proteins (Pellet and Roizman 2007). The genomic DNA is linear and contains both unique regions and long repeats. In viral particles, it is packaged into an icosahedral capsid that again is surrounded by a host cell–derived lipid bilayer into which the viral envelope proteins are inserted. The space between envelope and capsid is filled by the so-called tegument, a mixture containing a variety of viral proteins (Fig. 6.5). During membrane fusion in the course of infection, the tegument is delivered to the cytoplasm of the newly infected cell together with the viral capsid. In addition to,

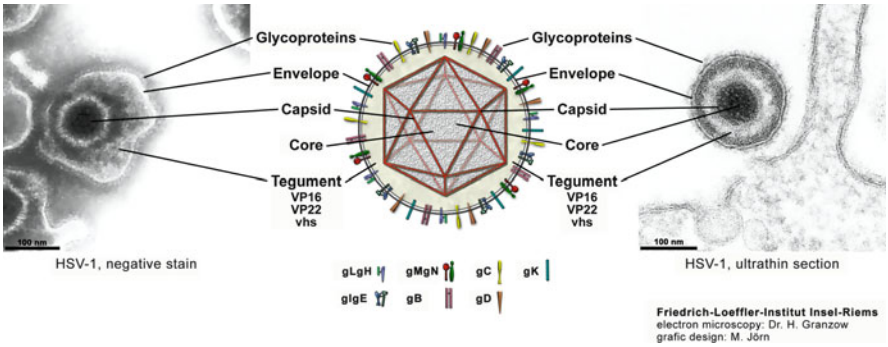

Fig. 6.5 Schematic representation of a herpes simplex virus particle. Basic elements including the envelope membrane, capsid, core, genome and tegument, and the different viral envelope glycoproteins are marked. As indicated, the tegument contains the *vhs* RNase and VP16 and VP22, two proteins involved in regulation of *vhs* activity. (The figure was kindly provided by H. Granzow and M. Jörn, electron microscopy Friedrich-Loeffler-Institut, Insel Riems)

for example, proteins necessary for activation of viral gene expression, tegument of viruses belonging to the genus *Alphaherpesvirus* contains the "virus host shutoff" (*vhs*) protein, a factor involved in shutting down host cellular gene expression (Roizman et al. 2007).

In herpes simplex virus 1 (HSV-1), *vhs* is a polypeptide of ca. 58 kDa that is encoded by the open reading frame UL41 (Roizman et al. 2007). It triggers a rapid shutoff of host cellular protein synthesis. This is achieved by disruption of preexisting polyribosomes and degradation of mRNAs. Since *vhs* is introduced into the infected cell as part of the tegument, viral gene expression is not necessary for shutoff (Smiley 2004).

The observed effects of *vhs* on cellular RNA metabolism could easily be explained if *vhs* activated RNA degradation or represented an RNase itself. In fact, extracts of HSV-1-infected cells and, even more importantly, extracts from partially purified virions were shown to contain an RNase activity (Smiley 2004). RNA hydrolysis could be prevented by *vhs*-specific antibodies and different mutations affecting *vhs*. Further evidence for *vhs* representing an RNase came from studies in which *vhs* was expressed in the absence of other viral proteins and tested in vitro or in yeast cells (Elgadi et al. 1999; Zelus et al. 1996). In addition, a complex of *vhs* with eukaryotic translation initiation factor eIF4H (see also below), partially purified from recombinant *E. coli*, showed RNase activity (Everly et al. 2002). Finally, the RNase activity of *vhs* was demonstrated with recombinant protein that had been expressed in *E. coli* and purified to homogeneity (Taddeo and Roizman 2006; Taddeo et al. 2006, 2010).

Similar to the RNases of the segmented negative-strand RNA viruses described above, the *vhs* RNAse seems not to belong to one of the known RNase families. Structural data are not available so far, but sequence comparison studies revealed a relationship to a family of cellular nucleases that are involved in DNA repair and

replication (Doherty et al. 1996; Everly and Read 1997; Everly et al. 2002). One of these cellular homologs is FEN-1, a nuclease engaged in the removal of RNA primers used for synthesis of Okazaki fragments during eukaryotic DNA replication. This similarity with cellular nucleases is obviously also important on a functional level, since mutations affecting conserved residues known to be important for enzymatic activity of the cellular proteins also abrogate *vhs*-induced repression of gene expression (Everly et al. 2002).

Isolated *vhs* has been shown to hydrolyze RNA with broad substrate specificity. It requires single-stranded RNA and cleaves 3′ of C and U residues, a substrate specificity equivalent to that of RNase A (Taddeo and Roizman 2006). However, when expressed within a living cell, this unusual enzyme selectively targets cellular and viral mRNAs while sparing other ribonucleic acids. This substrate specificity under physiological conditions seems to be not mediated by direct recognition of one of the mRNA specific modifications 5′ cap or 3′ poly(A) tail. Instead, *vhs* interacts with cellular proteins that represent components of the translation initiation complex, such as initiation factor eIF4H was demonstrated (Feng et al. 2001). Since eIF4H associates with eIF4A, a component of the cap-binding complex, *vhs* could be attached to the 5′ ends of mRNAs via an initiation factor bridge. In agreement with this hypothesis, it was reported for different mRNAs that *vhs* degradation starts close to the 5′ end (Elgadi et al. 1999; Karr and Read 1999). Along those lines, it also has to be mentioned that *vhs* cleaves the cap-less picornavirus RNAs immediately downstream of the so-called internal ribosomal binding site (IRES), whereas other cap-less mRNAs like cellular IRES-containing mRNAs are not degraded (Elgadi and Smiley 1999). Picornavirus IRES recruit most of the canonical initiation factors to the viral RNAs, whereas the cellular IRES elements function without many of these factors. Thus, even though mere tethering of *vhs* to the cap-binding complex is not sufficient for mRNA degradation (Page and Read 2010), it can be hypothesized that the *vhs* specificity for mRNAs is mediated by components of the host cellular translation initiation system.

Recent work has shown that the model of an initiation factor-mediated specific degradation of mRNAs is presumably oversimplified. Several mRNAs were found to be cleaved at a site close to the 3′ end despite the presence of a 5′ cap structure. According to these data, at least three classes of mRNAs can be distinguished based on their fate after infection (Corcoran et al. 2006; Smiley 2004; Taddeo et al. 2010). The mRNAs of housekeeping genes such as GAPDH or β-actin are rapidly degraded. At least some inducible genes generating mRNAs with AU-rich elements (AREs) in their 3′ nontranslated regions are deadenylated and cleaved endonucleolytically close to their 3′ end giving rise to 5′ fragments that persist for hours before 3′–5′ degradation occurs. Several stress-inducible genes coding for proteins with regulatory functions like IEX-1, c-fos, or the α subunit of the inhibitor of nuclear factor κB (IκBα) belong to this group. A third class of mRNAs, which are derived from inducible genes encoding, for example, tristetraprolin (TTP) is not degraded at all, so that the encoded proteins accumulate in the infected cells. Since TTP binds both to *vhs* and AREs of mRNAs (Esclatine et al. 2004), the *vhs*-induced cleavage

of the latter was hypothesized to be mediated by TTP (Chen et al. 2001; Lykke-Andersen and Wagner 2005).

The RNase activity of *vhs* seems to be essentially modulated by at least three viral proteins. Two of these proteins, VP16 and VP22, are also present in the viral tegument (Fig. 6.5), and therefore already delivered to the newly infected cells. VP16 alone is able to associate with *vhs,* whereas VP22 cannot bind *vhs* but interacts with it only via VP16. The interaction of *vhs* with these two proteins is believed to interfere with the RNase activity and to be necessary for an appropriate level of viral gene expression in the early phase of the infection. However, it is not clear whether this effect is achieved alone by limiting mRNA degradation. Instead, there is evidence that these two proteins also function through enabling mRNA translation (Taddeo et al. 2007). In addition, a third protein termed ICP27 was shown to be also involved in modulation of *vhs* activity (Corcoran et al. 2006; Taddeo et al. 2010). ICP27 is an immediate early protein of HSV with different functions in regulation of viral and cellular gene expression. It stimulates transcription and translation of viral mRNA and contributes to HSV host shutoff by downregulation of host transcription and blocking of nuclear export of host cellular mRNAs. Moreover, ICP27 seems to be important for the specificity of *vhs* activity, but the exact role of ICP27 is still a matter of debate. It seems to interact with *vhs* bound to cap-and poly(A)-binding proteins. It is also essential for the synthesis of new *vhs* in the late phase of the viral replication cycle. The absence of ICP27 leads to degradation of mRNAs with AREs late after infection, which was proposed to result from a stabilizing effect of ICP27 on these RNAs (Corcoran et al. 2006) or the absence of the protective effect of *vhs* itself because of the absence of the newly synthesized *vhs* (Taddeo et al. 2010).

The *vhs* function is dispensable for alphaherpesvirus replication, and *vhs* homologs are absent from beta- and gamma-herpesviruses (Smiley et al. 2001; Smiley 2004). Nevertheless, *vhs* is found in all alphaherpesviruses so that it likely plays an important role in the biology of these viruses. In fact, *vhs*-negative HSV is strongly attenuated. In accordance with its name, a primary function of *vhs* is certainly the shutoff of host cellular gene expression. At least the concentration of most stably expressed cellular mRNAs is lowered dramatically in the early phase of the replication cycle. Moreover, the function of the translational apparatus is altered so that the translation of most of the residual mRNAs is severely impaired. As a consequence, higher capacities of the translation system are available for the synthesis of viral proteins. However, it is obvious that *vhs* also degrades viral mRNAs so that maximization of viral gene expression seems to be not the final aim. In fact, *vhs* also ensures the rapid turnover of viral mRNAs, a process that is needed to regulate the progression of the replication cycle from the immediate early to the early and then to the late phase.

In addition to gross effects on viral and cellular mRNA metabolism, *vhs* plays also a major role in alphaherpesvirus evasion of host immunity. It is involved in the loss of major histocompatibility complex (MHC) class I and class II from the surface of infected cells (Ambagala et al. 2000; Gopinath et al. 2002; Hinkley et al. 2000; Koppers-Lalic et al. 2001; Tigges et al. 1996). Moreover, the production

of proinflammatory chemokines and cytokines is hampered by HSV infection (Suzutani et al. 2000). Recently, *vhs* has been shown to functionally inactivate human monocyte–derived dendritic cells (Samady et al. 2003). Collectively, *vhs* activity dampens the innate and both arms of the adaptive immune system. The effects of *vhs* on the host immune system are regarded as the major cause for the attenuation of *vhs* deletion mutants. *Vhs* deletion mutants have been shown to induce very robust immune responses in experimentally infected animals and, consequently, deletion of *vhs* is regarded as one possible step during the establishment of novel vaccines against alphaherpesviruses. It also has to be mentioned that its ability to help block the host immune response seems to be the major reason for conservation of *vhs* among alphaherpesviruses since the ability of *vhs* to induce host shutoff varies considerably from HSV-2 (very strong shutoff) to HSV-1 (strong shutoff), pseudorabies virus (moderate shutoff) and equine herpesvirus (no detectable host shutoff) (Smiley 2004).

Taken together, the alphaherpesvirus *vhs* protein represents a new type of RNase with multiple functions in the viral life cycle and virus–host interaction. These different functions and the adaptation of *vhs* activity to changing demands in the course of the viral life cycle are achieved by a complex regulatory network established by different viral proteins controlling the activity of tegument-associated *vhs* early in infection and expression of new *vhs* during the late phase of the cycle.

6.3 Viral RNase Important for the Interaction Between Virus and Host Organism: The Pestivirus E^{rns} RNase

Pestiviruses represent a group of pathogens that are responsible for economically important diseases of farm animals (Lindenbach et al. 2007). Pestiviruses are enveloped positive-strand RNA viruses that are classified as one genus in the family *Flaviviridae* because of their general genome organization and strategy of gene expression. The pestiviral genome encodes a single polyprotein of roughly 4,000 amino acids that is cleaved into 12 mature viral proteins by cellular and viral proteases. Four of these proteins are found in the virus particle, a capsid protein C and three glycosylated proteins embedded into the viral envelope membrane (Fig. 6.6). The presence of three envelope proteins represents a special feature of pestiviruses, whereas the closely related human hepatitis C viruses (HCV) have only two envelope proteins. With regard to location in the polyprotein, biochemical properties, and presumed function, the glycoproteins E1 and E2 of pestiviruses are thought to be equivalent to E1 and E2 of HCV, respectively. Thus, the so-called E^{rns} protein (envelope, ribonuclease secreted), formerly termed E0, located in the polyprotein between C and E1 seems to represent the additional acquirement of pestiviruses.

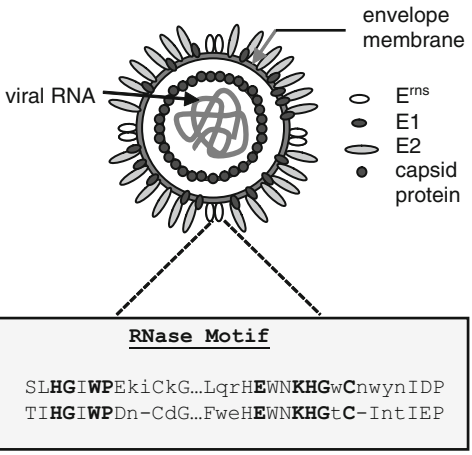

Fig. 6.6 Schematic representation of a pestivirus particle. Basic elements like envelope membrane, single-stranded positive-sense RNA genome, and the viral structural proteins are marked. Below the virion, a blowup of the part of the E^{rns} sequence containing the RNase motif is shown in comparison with the corresponding sequence from T2 RNase

E^{rns} exhibits several unusual features. It is highly glycosylated with more than 50% carbohydrate in its mature form, and usually forms disulfide-linked homodimers (Hulst and Moormann 2001; Thiel et al. 1991). The C-terminal part of E^{rns} folds into a long amphipathic helix that associates with lipid bilayers in an in-plane configuration and thereby mediates membrane anchoring of the protein (Tews and Meyers 2007). This unusual form of membrane anchoring is believed to be responsible for the fact that part of the E^{rns} protein synthesized within a cell is secreted into the cell-free supernatant.

The most unusual feature of E^{rns}, however, is its enzymatic function. E^{rns} represents the only known viral surface protein that exhibits RNase activity (Hulst et al. 1994; Schneider et al. 1993). E^{rns} was identified as ribonuclease because of two short stretches within the N-terminal half of the protein (Box A **LH_{30}GIWP** and Box B **HEWNKH$_{79}$GWC**) that displayed homology to ribonucleases of the T2 family (Fig. 6.6). Ribonucleases of the T2 family are transferase-type RNases and are classified by their similarity to RNase T2 from *Aspergillus oryzae* (Luhtala and Parker 2010). T2 RNases are widespread among bacteria, protozoa, plants, insects, vertebrates, and viruses. T2 RNases are endonucleases that typically have their enzymatic optima at acidic pH (pH 4–5). This acidic activity of T2 RNases correlates with their localization in lysosomal or vacuolar compartments. Most T2 RNases have little substrate specificity and cleave phosphodiester bonds at all four bases (Luhtala and Parker 2010). Biochemical characterization of the E^{rns} activity revealed that various heteropolymeric RNA molecules such as ribosomal RNA or pestivirus genomic RNA were susceptible to E^{rns} activity (Hulst et al. 1994; Schneider et al. 1993; Windisch et al. 1996). Of single-stranded homopolymeric RNA molecules, only polyrU was cleaved while polyrA, polyrG, and polyrC were resistant to E^{rns} degradation (Schneider et al. 1993; Windisch et al. 1996). Addition of the complementary homopolymer (polyrA) to the polyrU resulted in massively reduced degradation indicating the inability of E^{rns} to accept double-stranded RNA as substrate. In conflict with this

observation is experimental evidence that suggests that E^{rns} also degrades dsRNA such as poly IC (Iqbal et al. 2004) or double-stranded transcripts (Magkouras et al. 2008; Mätzener et al. 2009). E^{rns} is insensitive to chelating agents such as EDTA or EGTA, and can be inhibited with Mn^{2+} and Zn^{2+} ions. Zn^{2+} leads at 15 μM concentration to a 50% reduction of E^{rns} activity likely because of the interaction with histidine residues His-30 and His-79 that were shown to be essential for RNase activity (Meyers et al. 1999).

As the function of E^{rns} is enigmatic, the exact substrate specificity was analyzed by Hausmann et al. (Hausmann et al. 2004). Using radiolabeled substrates, the preference for uridine residues was confirmed for heteropolymeric substrates. Interestingly, the cleavage occurs at an Np/U site in which N can be formed by any nucleotide. While most T2 RNases have a specificity determined by the B1 site (Irie and Ohgi 2001), the determinant of the B2 position is not a unique property of E^{rns} among the T2 RNases. RNase MC from the pumpkin *Momorica charantia* also has a specificity for Np/U (Irie et al. 1993).

Labeling of the transcribed substrate RNAs that contained a single uridine residue with uridine triphosphate (^{32}P alpha UTP) and separation of the cleavage product on a denaturing polyacrylamide gel revealed that the labeled phosphate was transfered to the nucleotide in the B1 position (the 3′ end of the 5′ cleavage product), while the nucleotide at the B2 position was dephosphorylated. This fits to the general mechanism of T2 RNases using transesterification and hydrolysis (Irie and Ohgi 2001). A 2′–3′ cyclophosphate is an intermediate structure that in most T2 RNases is hydrolyzed to a 3′ phosphate. Experiments by Windisch suggest that E^{rns} is unable to modify 2′–3′ cyclophosphate mononucleotides.

Initial determination of kinetic parameters was reported by Windisch et al. (Windisch et al. 1996). Using polyrU as substrate and incubation times of 15–20 min a K_m of about 872.5 10^{-6} M was determined. In a different approach using radioactively labeled single-stranded substrates with a single cleavage site each (GpU, CpU, ApU, and UpU), turnover was assayed for up to 60 s after mixing with the enzyme. These analyses revealed affinity constants of 83.8–258.7 10^{-9} M in the order UpU > GpU > CpU > ApU and that were 10^3- to 10^4-fold lower than described by Windisch. This is indicative for a high affinity of E^{rns} for the substrate. New analyses using highly purified E^{rns} and radiolabeled RNA oligonucleotides of 23 nt length revealed even higher affinities to the substrate (K_m 2.17 10^{-9} M) and a K_{cat} of 11.5. (Rümenapf, unpublished).

T2 RNases are endonucleases that cleave polymeric RNA molecules internally. In the case of E^{rns}, NpU sites represent the preferred substrate and the size of the cleavage products depends on the distribution and accessibility of uridine residues. Analysis of the cleavage products of E^{rns} digested RNA molecules with known cleavage sites gave a different picture. Depending on the enzyme concentrations, the expected cleavage products were completely degraded to mononucleotides or shorter molecules forming a “ladder” of 5′ coterminal fragments. This led to the assumption that the endonuclease E^{rns} possesses an additional exonucleolytic activity. This exonucleolytic activity of E^{rns} is only apparent with substrates that contain at least one uridine residue, and an endonucleolytic cleavage is required

before exonucleolytic degradation ensues. Interestingly, a comparable activity has not been reported for other T2 RNases, and it is currently not understood how a uridine-specific endonuclease switches to a substrate-independent exonuclease. A hypothetical 3D structure modeled on the basis of an RNase Rh template was published (Langedijk et al. 2002), but this model does not allow conclusions with regard to the molecular basis of substrate recognition of E^{rns}. However, crystal structure analysis of E^{rns} is in progress (T. Krey, personal communication) so that deeper elucidation of this interesting enzyme will hopefully be possible soon.

The identification of a sequence in the genome of an RNA virus which codes for an RNase raised the question how the viral genome is protected from this dangerous enzymatic activity. Experimental work showed that highly purified E^{rns} protein was able to degrade viral genomic RNA isolated from virions demonstrating the absence of a modification preventing cleavage of the viral RNA (Windisch et al. 1996). Protection of the viral genome could also be achieved by separating the genomic RNA and the RNase through membranes. Since mature E^{rns} is highly glycosylated and contains four intramolecular disulfide bonds, folding of the protein into its enzymatically active form could well be dependent on its translocation into the endoplasmatic reticulum (ER). As a matter of fact, prevention of translocation by expression of E^{rns} without signal sequence resulted in an inactive form of the protein exhibiting no RNase activity (Meyers, unpublished results). Since membrane topology of viral envelope proteins and genome is conserved during budding and membrane fusion in the course of infection, it can be concluded that active RNase and viral genome are always separated by membranes which prevents degradation of the viral genome by its own RNase.

The results and conclusions described above are also important for the question about the function of the E^{rns} RNase. Since the active RNase is obviously never present within the cytoplasm of the infected cell, the enzymatic activity cannot have any function in the viral replication cycle. Indeed, mutation of predicted active-site residues of the RNase led to inactivation of the enzyme but allowed recovery of viable viruses with growth characteristics similar to wild-type viruses (Hulst et al. 1998; Meyer et al. 2002; Meyers et al. 1999). Thus, the ability to express an active E^{rns} RNase seems to offer no significant advantage for virus replication in tissue culture cells. However, the RNase motifs and therefore most likely also the enzymatic activity of the protein have been conserved during evolution of pestiviruses indicating an important function of the RNase.

Animal studies provided first hints pointing at a putative function of the E^{rns} RNase. Viruses with inactivated RNase were shown to be attenuated in their natural hosts (Meyer et al. 2002; Meyers et al. 1999). Importantly, the initial reaction of the animals infected with the RNase-negative mutants was very similar to what was seen upon infection with a wild-type virus. However, around day 7–10 post infection, the animals were apparently able to control the RNase-negative virus and recovered whereas the amount of wild-type virus increased dramatically resulting in very severe symptoms of disease. Despite the early control of the RNase-negative virus, the animals infected with the mutant virus showed a potent antiviral immune response able to protect from a stringent challenge infection

(Meyer et al. 2002; Meyers et al. 1999). Based on these data, it can be hypothesized that the function of the pestivirus RNase is to somehow interfere with the immune system of the host, thereby delaying the immunological control of the infection.

The immune response to virus infection can be divided into the two functionally and temporally differentiated systems called the innate and the adaptive immune response. The latter type of response relies on a powerful, antigen-specific system designed for final clearance of the invading pathogen. Since, however, the development of an adaptive immune response takes a rather long time, a first line of defense is necessary to prevent uncontrolled amplification of the pathogen. This first line of defense is established by the innate immune system relying on the identification of pathogen-associated molecular patterns (PAMPs) (Janeway 1989). In RNA viruses, PAMPs are molecular structures like double-stranded RNA or cytoplasmic RNA with a 5′ triphosphate structure which are not found in eukaryotic cells, and therefore specific for the pathogens (Hengel et al. 2005; Takeda and Akira 2004). Binding of such a molecular structure by a PAMP-specific receptor triggers a cascade of reactions among which the synthesis and secretion of type I interferon represents one of the earliest and most important steps. Secretion of IFN-1 induces an antiviral state in the infected as well as in neighboring cells characterized by a whole set of events aiming at repression of pathogen replication. This first line of defense represents a serious barrier for viruses so that most, if not all of them, have evolved mechanisms counteracting the innate immune system especially at the level of the IFN-1 response (Hengel et al. 2005).

Because of the importance of the interferon response for the innate immune system and the fact that several types of RNA molecules represent PAMPs, it was proposed that the E^{rns} RNase could be involved in blocking the interferon system. The high affinity to single-stranded RNA and the postulated exonuclease activity of E^{rns} support the assumption that E^{rns} is involved in degrading viral RNA molecules that could stimulate endosomal (Toll like receptors 3, 7, 8) or cytoplasmic innate immunity sensors (MDA5 and RIG-I). The low k_m facilitates interaction of minute concentrations of E^{rns} with target RNA molecules, that is, in the endosomal compartment. Since endonucleolytic cleavage results in a molar increase of breakdown products that still are sensed as pathogen-associated molecular patterns (PAMPs), it is probably beneficial for the survival of pestiviruses to fully degrade these fragments by the exonucleolytic activity of E^{rns}.

Tissue culture experiments showed that supplementation of culture supernatant with E^{rns} blocked the interferon response triggered by addition of double-stranded RNA (Poly-IC) (Iqbal et al. 2004; Magkouras et al. 2008; Mätzener et al. 2009). This was definitely an effect initiated extracellularly since E^{rns} could not block the induction of interferon after transfection of Poly-IC. In vitro experiments showed that E^{rns} was indeed able to bind and degrade dsRNA, even though the requirements necessary for hydrolysis of dsRNA were different in two labs (Iqbal et al. 2004; Magkouras et al. 2008; Mätzener et al. 2009). Thus, the hypothesis was put forward that secreted E^{rns} was responsible for blocking an interferon response to extracellular dsRNA. This conclusion is in agreement with the published results, but has to be questioned in some points mainly because the origin of dsRNA in the extracellular

milieu in an infected animal is obscure. This dsRNA should directly result from virus infection but since the overwhelming amount of pestiviruses are not cytopathic (Meyers and Thiel 1996; Thiel et al. 1996), lysis of considerable numbers of infected cells containing viral dsRNA is unlikely. It is therefore not clear where the dsRNA substrate to be degraded by the E^{rns} RNase should come from. It also has to be stressed that E^{rns} is much more active on single-stranded RNA and could therefore be regarded as a predominantly ssRNA-specific RNase. If dsRNA was the main substrate of this RNase, one could expect that evolution would have taken a different way.

Pestiviruses express one further protein for which repression of the interferon response of an infected cell has been published. This protein named N^{pro} is a nonstructural protein with protease activity that induces proteasomal degradation of interferon regulatory factor 3 (IRF3) within the infected cell (Bauhofer et al. 2007; Chen et al. 2007; Hilton et al. 2006; La Rocca et al. 2005; Ruggli et al. 2005, 2009; Seago et al. 2007). In contrast to the E^{rns} RNase, N^{pro} is able to block the expression of type 1 IFN upon transfection of dsRNA, supporting the idea that N^{pro} is responsible for prevention of an innate immune response triggered from inside of the infected cell. Thus, if the E^{rns} RNase was engaged in blocking a process leading to IFN-1 expression, this process should most likely take place outside of the infected cell.

A formal proof for the involvement of the E^{rns} RNase in the prevention of innate immunological control of virus infection was obtained in a special animal model. Pestiviruses are known to establish long-lasting persistent infections when introduced into the fetus in a pregnant host animal (Thiel et al. 1996). This process is best understood for the pestivirus bovine viral diarrhea virus (BVDV) (Fig. 6.7). Intrauterine infection of a fetus in the first trimester (day 40–120) of gestation by noncytopathic BVDV may lead to viral persistence accompanied by an acquired immunotolerance with high specificity for the infecting virus strain (Fig. 6.7). It is generally believed that self-reactive elements of the adaptive immune system, including the ones directed against the persisting virus, are inactivated in this developmental stage. Thus, the developing individuals do not produce an adaptive immune response against the persisting virus strain for their lifetime (Thiel et al. 1996). Furthermore, the virus is protected against the immune response of the mother cow by the barrier established by the bovine placenta that cannot be crossed by antibodies. To maintain a persistent infection for years, BVDV has to deal also with the innate immune system of the host. As a matter of fact, persistently infected fetuses or calves do not mount an IFN-1 response despite massive virus replication resulting in large amounts of PAMPs (Charleston et al. 2001). The absence of innate immune reactions is regarded as an important contribution to viral persistence.

When fetuses were infected with an RNase-negative BVDV mutant, a significant IFN-1 response was observed in contrast to parallel experiments with the corresponding wild-type virus (Meyers et al. 2007). From these experiments, it can be concluded that the E^{rns} RNase is involved in blocking the IFN-1 response to pestivirus infection in vivo. Similar results were also obtained for N^{pro} deletion mutants. Most importantly, the combination of the N^{pro} deletion and the

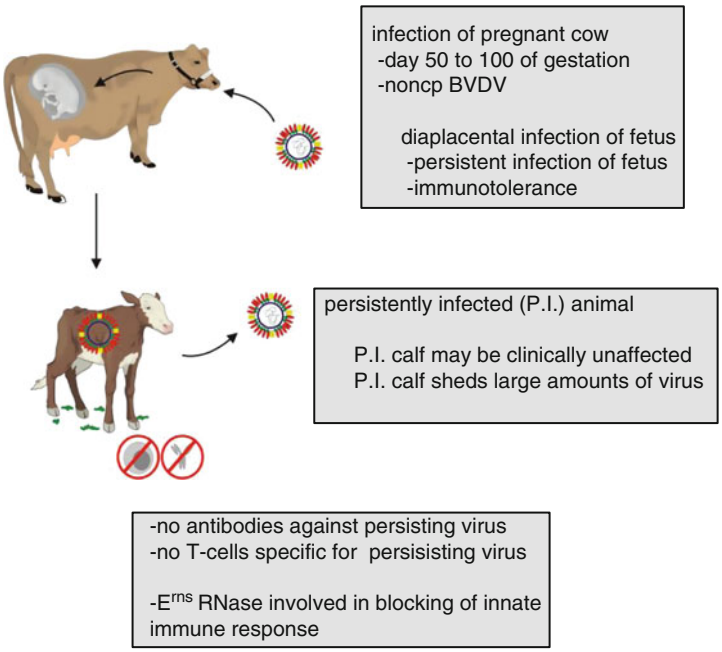

Fig. 6.7 The cartoon shows the principle pathway to virus-specific acquired immunotolerance and establishment of persistent infection by BVDV

RNase-inactivating mutation resulted in a virus that provoked an overboosting IFN-1 response in the fetus that finally led to its abortion (Meyers et al. 2007).

Taken together the experimental evidence available so far shows that the E^{rns} RNase is one of the pestiviral factors responsible for inhibition of the innate immune response. These viral factors are especially important for the establishment and maintenance of persistent pestivirus infections. Since persistently infected animals play a crucial role in the strategy that keeps pestiviruses within their host populations, the E^{rns} RNase is of major importance for these viruses and was therefore conserved during virus evolution.

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