**University of Veterinary Medicine Hannover**
**Institute of Virology**

# Biological characterization of Bovine Hepacivirus

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**Anna Lena Baron**
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Academic supervision: Prof. Dr. Paul Becher

1. Referee: Prof. Dr. Paul Becher

Institute of Virology, University of Veterinary Medicine Hannover

Prof. Dr. Martin Messerle

Institute of Virology, Hannover Medical School

2. Referee: Prof. Dr. Norbert Tautz

Institute of Virology and Cell Biology, University of Lübeck

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## List of Abbreviations

| | |
|---|---|
| A | adenosine |
| $(AAA)_n$ | poly-adenosine tail |
| aa | amino acid |
| Ago2 | argonaute 2 |
| AST | aspartate aminotransferase |
| ATP | adenosine triphosphate |
| AUG | translation initiation codon |
| BDV | border disease virus |
| BFH12 | bovine fetal hepatocytes |
| BHV | bat hepacivirus |
| BovHepV | bovine hepacivirus |
| BVDV | bovine viral diarrhea virus |
| C | capsid |
| CAT | cationic amino acid transporter |
| CD | cluster of differentiation |
| CSFV | classicl swine fever virus |
| E | envelope |
| EBV | Epstein-Barr virus |
| EHcV | equine hepacivirus |
| EIA | enzyme immune-based assay |
| elF | eukaryotic translation initiation factor |
| ELISA | enzyme-linked immunosorbent assay |
| ER | endoplasmatic reticulum |
| GBV-B | GB virus B |
| GDP | guanosine diphosphate |
| GLDH | glutamate dehydrogenase |
| GTP | guanosine triphosphate |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis c virus |
| HeLa | human epithelioid cervix carcinoma cell line |
| Hep3B | human liver cell line |
| HepG2 | human liver cell line |
| HEV | hepatitis Evirus |
| HIV | human immunodeficiency virus |
| Huh | human hepatocarcinoma cell line |
| Igf1R | insuline-like growth factor 1 receptor |
| IgG | immunoglobulin G |
| INF | interferon |
| IRES | internal ribosome entry site |
| IRF | interferon regulatory factor |
| LIPS | luciferase immunoprecipitation system |
| LNA | locked nucleic acid |
| M | membrane |
| MAVS | mitochondrial antiviral signaling protein |

| | |
|---|---|
| Met | methionine |
| mg | milligramm |
| miR | micro RNA |
| ml | milliliter |
| mRNA | messenger RNA |
| NF-κB | nuclear factor kappa light chain enhancer of activated B-cells |
| no. | number |
| NPHV | non-primate hepacivirus |
| NS | non-structural |
| NTP | nucleosidtriphosphate |
| NTR | non translated region |
| nts | nucleotides |
| ORF | open reading frame |
| PAB | poly(a) tail binding protein |
| peg | polyethylene glycol |
| qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
| RACE | rapid amplification of cDNA ends |
| RdRP | RNA dependent RNA polymerase |
| RHV | rodent hepacivirus |
| RISC | RNA-inducedsilencing complex |
| RNA | ribonucleic acid |
| S | ribosomal subunit |
| SL | stem-loop |
| ss | single-stranded |
| TMA | transcription-mediated amplification |
| tRNA | transfer RNA |
| U | uridyl |
| WHO | World Health Organization |
| WLSV | wenling shark protein |
| XRN | 5`-3` exoribonuclease |
| ɣ-GT | gamma glutamyl transferase |
| 7Me-$G_{ppp}N$ | guanylylated 5` RNA end (cap) |

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# 1. Introduction

## 1.1 Family *Flaviviridae*, especially genus *Hepacivirus*

### 1.1.1 Genus *Flavivirus*

The family *Flaviviridae* includes the genera *Flavivirus, Pestivirus, Pegivirus* and *Hepacivirus* (1). The *Flavivirus* genus consists of more than 70 viruses, many of which are arthropod-borne human pathogens including dengue, Japanese encephalitis, yellow fever and West Nile virus causing a variety of severe diseases (2). Geographically, the dengue virus is endemic in Africa, America and the Southeast Asia (3). The distribution of West Nile virus overlaps with the dengue virus regions, but extends to parts of Europe and Australia (4). Moreover, the Japanese encephalitis virus is restricted to Southeast Asia and overlaps with Yellow fever virus while this virus is also present in South America (3, 5).

The genus *Flavivirus* contains viruses with single-stranded, positive sense RNA genomes of about 9,200-11,000 nucleotides (nts). The 5` end of the flavivirus genome possesses a type I cap (m-7GpppAmp) and the 3` end lacks a terminal poly(A) tract (6, 7). The genomic RNA consists of a single open reading frame that codes for three structural (capsid (C), membrane (M) and envelope (E)) and seven non-structural proteins (NS1, -2A, -2B, -3, -4A, -4B and -5) (figure 1A).

The envelope protein E is a viral haemagglutinin that mediates receptor binding and pH-dependent fusion activity after receptor mediated endocytosis (8). NS1 plays a role in viral RNA replication (9, 10), whereas its secreted form regulates complement activation (11). NS2A is involved in virus assembly (12) and RNA replication (13) and has been shown to act as an interferon (INF) antagonist by inhibiting interferon signaling (14). NS2B together with the N-terminal one-third of NS1 forms the viral serine protease complex, which is involved in processing of the viral polyprotein (8) and forms a stable complex with NS3, which is a co-factor for the NS2B-NS3 serine protease (15). This protease cleaves the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions and generates the C-termini of the mature capsid protein (16) and NS4A (17). Furthermore, the C-terminal part of NS3 contains a RNA helicase domain that is involved in RNA replication (8), and RNA unwinding activity has been demonstrated for flavivirus NS3 proteins (18). Moreover, it shows RNA triphosphatase

activity, which is likely to be involved in dephosphorylation of the genomic 5` end before cap addition (19). A role for NS4A in replication is supported by the colocalization of this protein with the replication complexes (20). NS4B colocalizes with NS3 and viral double stranded RNA in the endoplasmatic reticulum (ER) derived membrane structures presumed to be sites of RNA replication (21, 22). Like NS2, NS4A and NS4B can interfere with the immune system by blocking type I interferon signaling, which leads to an attenuated immune response (14). The RNA dependent RNA polymerase NS5 (23) contains a methyltransferase activity that is involved in the modification of the viral cap structure (24). In addition, NS5 forms a complex with NS3 and can stimulate the nucleoside as well as RNA triphosphatase activities of NS3 (25, 26).

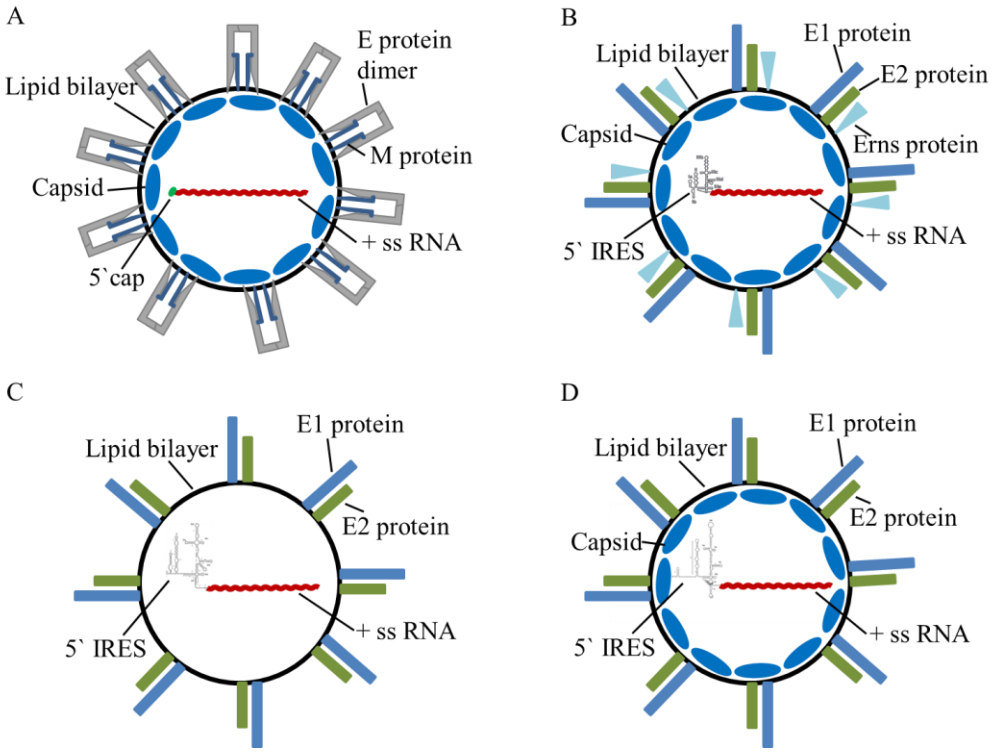


Figure 1: Viral architecture of the genus *Flavi*- (A), *Pesti*- (B), *Pegi*- (C) and *Hepaciviruses* (D) within the family *Flaviviridae.* ss: single-stranded. E: envelope. M: membrane. IRES: internal ribosome entry site.

### 1.1.2 Genus *Pestivirus*

The genus *Pestivirus* is divided into 11 species, A-K (27) and comprises viruses like bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV) and border disease virus (BVD) of sheep. These are animal pathogens of major economic importance for the livestock industry (28). Pestivirus infections can be subclinical or produce a range of clinical conditions like acute fatal disease, acute hemorrhagic syndrome, acute diarrhea and wasting disease (29). Low virulent strains can induce chronic infections and may be disseminated during a longer period than highly virulent strains (30).

The pestivirus genome consists of a single-stranded RNA of approximately 12,300 nucleotides in length (31) and the genome contains one open reading frame leading to translation of a single large polyprotein that is processed into individual proteins (32, 33). Similar to hepaciviruses, pestivirus genomes lack a 5` cap and a 3` poly(A) tract (33, 34) and the open reading frame encoding approximately 3900 amino acids (aa) is instead flanked by a 5` non-translated (NTR) of 372 to 385 nts and a 3` NTR of 185 to 273 nts (34–36). The 5` terminal stem-loop structure domains Ia and Ib in the BVDV genome are important for virus replication (37, 38). Following the open reading frame, the 3` NTR consists of a variable region followed by a conserved 3` terminal stem-loop preceded by a single-stranded region (39, 40). The cap-independent translation initiation is mediated by an internal ribosome entry site (IRES) structure that bears structural and functional similarity to that of HCV (41). The minimal IRES includes 5` NTR domains II and III and can be influenced by structured sequences downstream from the initiator AUG (42–44). As seen in HCV, the pestivirus IRES binds ribosomal 40S subunits without the need for translation initiation factors elF4A, elF4B and elF4F (45–47). The open reading frame contains four structural (C, $E^{rns}$, E1 and E2) and eight non-structural ($N^{pro}$, NS2, -3, -4A, -4B, -5A, -5B and p7) proteins (48–50) (figure 1B). Unlike other members of the *Flaviviridae*, the first pestivirus protein is a non-structural protein ($N^{pro}$), an autoprotease responsible for cleavage at the $N^{pro}$/C site (33, 51, 52). A host signal peptidase is believed to cleave at C/$E^{rns}$, E1/E2, E2/p7 and p7/NS2 sites (53–55). The mechanism of NS2-3 cleavage has only been elucidated by identification of a NS2 autoprotease (56). The remaining cleavages of the polyprotein, which generate NS4A, -4B, -5A and -5B, are catalyzed by the pestivirus NS3-4A serine protease (57–59). In addition to its autoprotease activity, $N^{pro}$ acts as an antagonist of interferon regulatory factor 3 (IRF-3) and

IFN production (60, 61). The $E^{rns}$ glycoprotein is associated with released virus via an unusual type of membrane anchor (62) and is also secreted from infected cells (55, 63, 64). An untypical feature of $E^{rns}$ is its ribonuclease activity (65). The envelope proteins E1 and E2 are integral membrane proteins (66) and it is suggested that E2 is a receptor or co-receptor for binding and entry (67). The formation of an E1-E2 heterodimer and binding of the complement activating glycoprotein CD46 as receptor is essential for the pestivirus bovine viral diarrhea virus (BVDV) cell entry (68–70). The protein p7 is required for infectious virus production (54) but not for RNA replication (71). As in the *Hepacivirus* member hepatitis C virus (HCV), pestivirus p7 can form an ion channel, suggesting that it could have a function in virus assembly and entry (72, 73). Moreover, the protein NS2 is a cysteine protease that is responsible for processing NS2-3 (56, 74). As for all members of the family *Flaviviridae*, pestivirus NS3 contains a N-terminal serine protease domain (58, 75, 76) and a C-terminal RNA helicase domain (77). Like HCV, the pestivirus NS3 serine protease requires NS4A as a cofactor (59) and possesses a RNA helicase (78) and a RNA-stimulated NTPase (79). NS5A is phosphorylated by a cellular serine or threonine kinase with properties similar to kinases that phosphorylate flavivirus NS5 and hepacivirus NS5A (80). Moreover, the crystal structure of the RNA dependent RNA polymerase (RdRP) NS5B protein is determined and consists of a right hand including the fingers, palm and thumb domains (81, 82). This polymerase is responsible for viral genome replication and synthesizes an intermediate negative RNA strand (82).

### 1.1.3 Genus *Pegivirus*

Viruses of the genus *Pegivirus* were originally called hepatitis G virus/GB virus type C (83) and are divided into 11 species, A-K (84). Apart from primates and humans, pegiviruses were identified in several mammalian animal species, including bats (85), horses (86), rodents (87) and pigs (88). Pegiviruses establish persistent infections without signs of clinical hepatitis or disease (89, 90) but have an immunomodulatory effect that may be beneficial for patients co-infected with HIV (91) and possible other infectious agents such as Ebola virus (92). High viral loads are found in circulating lymphocytes (93, 94) leading to the suggestion that the replication takes place primarily in the bone marrow (95).

Pegiviruses are characterized by a single-stranded, positive sense RNA genome ranging from 8,900-11,300 nucleotides in length. An IRES that directs translation of the polyprotein directly from viral genomic RNA (96), is present in the 5` NTR and the length varies between 300-550 nts. Most pegiviruses have a type I picornavirus-like IRES, whereas a hepacivirus structurally related type IV IRES can also be found (8). In contrast to hepaciviruses, micro RNA-122 (miR-122) binding sites (for more detailed explanation see 1.5) have been identified neither in human pegiviruses nor in members of other pegivirus species (84). The pegivirus genome exhibits a single open reading frame which is flanked by the structured 5` and 3` NTR and contains two structural (E1 and E2) and six non-structural proteins (NS2, -3, -4A, -4B, -5A and -5B) (figure 1C). Interestingly, equine and bat pegiviruses have an additional structural protein called protein X downstream of E2, the function of which is unknown (97, 98). Moreover, cellular proteases process the structural proteins, whereas the NS3-4A viral protease cleaves the non-structural proteins. In comparison to other members of the *Flaviviridae* a homologue to the core protein is lacking.

### 1.1.4 Genus *Hepacivirus*

The most prominent member of the genus *Hepacivirus* is the hepatitis C virus, which was identified in 1989 (99) and has a worldwide distribution with about 3 % of the human world population infected. Approximately 170 million people are chronically infected (8) and therefore at significant risk of developing severe liver diseases such as progressive fibrosis, cirrhosis and hepatocellular carcinoma (100, 101). Following acute infection, HCV establishes persistence in 60-80 % of individuals (102). While the origin of HCV is still unknown, the vast majority of emerging infectious diseases is caused by viral zoonoses (103). Especially in high-risk areas around the world humans are constantly exposed to diverse animal viruses through direct contact with domestic or wild animals (coronavirus, ebola virus) and via vector intermediates such as arthropods (dengue virus, zika virus) (104). The identification and characterization of animal derived viruses therefore warrant attention as these viruses may represent reservoirs of human pathogens as well as potential surrogates for the study of human homologues (105, 106).

The 5` NTR at the beginning of the genome is a 341 nts sequence element that folds into a complex structure consisting of four major domains and a pseudoknot. Additionally, as

mentioned above, the IRES structure enables cap-independent translation and consists of domains II to IV in the 5` NTR (31). The first 120 nts, which form domain I of the 5` NTR, serve as a minimal replication element, although nearly the entire 5` NTR is needed for efficient RNA replication (107). The result of the cap-independent translation is a product of one large open reading frame of 3,011 amino acids. Free 40S ribosomal subunits directly bind to the 5` NTR domains IIId-f (46, 108, 109) followed by the interaction with the initiation factor elF3 and probably the ternary complex elF2 GTP Met-tRNA to form the 48S intermediate complex, in which the initiation AUG codon is within the ribosomal P-site (109, 110). The IRES interacts with elF3 via domain IIIb (111) and thereby mimics 5` cap-binding complex elF4F (112). Following GTP hydrolysis and recruitment of the 60S ribosomal subunit the 48S intermediate is converted into a translationally active 80S complex (46, 109, 110). Furthermore, cellular factors participate in IRES function, like the La protein that stimulates IRES activity via binding near the initiator AUG and recruiting the 40S ribosomal subunit (113, 114).

The HCV 3` NTR consists of an approximately 40 nts long variable domain and a polyuridine/polypyrimidine (polyU/UC) tract followed by a highly conserved 98 nts long 3` X domain (115) that is important for RNA replication (116). The HCV genome is 9,600 nts in size and codes for a single polyprotein that is cleaved by cellular and viral proteases into 10 proteins (figure 1D). The polyprotein contains three structural proteins (C, E1 and E2) and seven non-structural proteins (the ion channel p7, NS2,-3,-4A,-4B,-5A and -5B) (117) and the core coding protein multimerizes and binds the HCV genome to form the viral nucleocapsid (118). The structural proteins are cleaved by host signal peptidases and the non-structural proteins by the virus encoded proteinases NS2-3 and NS3 (119). The glycoproteins E1 and E2 mediate HCV attachment and membrane fusion (120) and E2 binds to multiple putative receptors, including CD81 (121). It is not yet known whether p7 is virion associated (122), but it is essential for infectivity *in vivo* (123) though not necessary for RNA replication (124). The C-terminal domain of non-structural protein 2 contains the active site residues of a cysteine protease (125) and cleaves the NS2/3 junction (126), which is required for HCV replication (127). Otherwise the autoprotease activity of NS2 requires the expression of the NS3 N-terminal domain (125). Furthermore, NS2 can interact with cellular proteins and inhibits the cellular pro-apoptotic molecule CIDE-B (128). The multifunctional protein NS3

contains a N-terminal serine protease domain and a C-terminal RNA helicase/NTPase domain; both of these enzymes are critical for replication (31, 119). The serine protease domain of NS3 requires the interaction with NS4A for complete folding and enzyme activity (129). The NS3-NS4A serine protease domain is responsible for cleavage at NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B (130) and antagonizes innate antiviral defenses by blocking the activation of transcription factors IRF-3 and NF-κB (131). On the other hand the C-terminal domain of NS3 encodes an RNA helicase/NTPase to utilize the energy derived from NTP hydrolysis to translocate along and unwind double-stranded nucleic acids (132). Although the precise role of NS3 helicase is not yet known its activity has been shown to be essential for RNA replication and viral infectivity (127, 133). NS4A is the smallest of the non-structural proteins and is a co-factor of the serine protease NS3-NS4A (134). NS4B is an integral membrane protein (135), plays a critical role in organizing of the membrane bound replication complex (136) and encodes a GTPase activity that is critical for RNA replication (137). The phosphoprotein NS5A has an important role in RNA replication, which is localized to active replication complexes (138, 139). The last non-structural protein 5B is the RNA dependent RNA polymerase (RdRP) that synthesizes a complementary negative strand RNA by using the genomic positive strand RNA as template. As typically seen in all RNA dependent RNA polymerases the catalytic domain exhibits the classical fingers, palm and thumb subdomains. Furthermore, NS5B is able to conduct a template-directed RNA synthesis on its own, requiring only divalent metals (magnesium or manganese) as co-factor (140–142). A high HCV replication rate, such as $10^{12}$ virions per day, and the absence of proofreading activity of NS5B polymerase are the main factors that contribute to mutations in the viral genomes (143).

## 1.2 The bovine hepacivirus and other recently described animal hepaciviruses

### 1.2.1 Equine hepacivirus (EHcV)

So far, the genus *Hepacivirus* comprises viruses identified in humans, horses, rodents, bats, sharks, primates and cattle (1, 144). Until 2010 only two species, HCV and GB virus B, had been known within the genus *Hepacivirus* (145, 146), but in 2011 evidence for the existence of a wider hepaciviral host range was found. Using high-throughput sequencing to identify

viral causes of respiratory illness in dogs, Kapoor and colleagues discovered a novel hepacivirus species (147). However, there were some unusual observations. Firstly, nucleotide analyses revealed a 99.2 % sequence convergence of a partial NS3 coding sequence from animals of two independent outbreaks, which is atypical for most RNA viruses. Secondly, high viral loads were observed in nasal swabs, which is unusual as HCV exhibits strict hepatotropism leading to chronic hepatitis, cirrhosis and hepatocellular carcinoma in humans (99). Furthermore, antibodies against the NS3 helicase protein of this newly identified hepacivirus could not be detected in serum samples from dogs (148–150), while several sera from horses reacted highly positive in the same serological assay (148). Therefore, the investigators decided to reclassify the virus as non-primate hepacivirus (NPHV) (148) or equine hepacivirus (EHcV) (151). Two possibilities were suggested regarding the origin of canine hepacivirus in dogs. Canine hepacivirus infection in dogs could be the result of a recent and direct cross-species transmission or a false transmission event could have occurred by feeding of dogs with horse meat or the usage of veterinary products containing horse serum derived components (152, 153). EHcV, like HCV, shows acute and chronic stages of infection. However, liver analyses of infected horses showed no evidence of severe disease, even though viral negative strand RNA was only detected within hepatocytes in tissue samples (106). Additionally, experimental inoculation of horses with EHcV RNA resulted in persistent infection, mild hepatitis, delayed seroconversion, signs of hepatic inflammation and hepatocellular damage. These findings give an indication that EHcV is a hepatotropic virus, like HCV, but that it does not cause severe liver damage (152, 154).

The genome organization is characteristic for hepaciviruses and the single large open reading frame encodes three structural (C, E1and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (figure 2A) (147). The EHcV genome consists of 9,538 nts and encodes a 2,942 aa polyprotein (152). The structural proteins are cleaved by signal peptidases, whereas the non-structural proteins are processed into mature proteins by viral proteases (87). The EHcV 5` NTR is 384 nts in length and contains domains II to IIIf comparable to the HCV IRES. However, the EHcV IRES lacks domain IV and has only one miR-122 match site, whereas HCV contains a domain IV including translation initiation codon AUG and two miR-122 match sites (155). Moreover, EHcV domain I structure is much larger than predicted for HCV domain I (148, 152). The equine IRES domains II, III and the

pseudoknot are responsible for IRES activity, whereas the longer stem-loop I is not involved in the translation process (156, 157). Binding of miR-122, which is highly expressed in the liver, at two match sites in the HCV 5` NTR leads to an increased translation efficiency (158). The fact that the EHcV genome contains one miR-122 match site in the 5` NTR and shows the highest levels of viral RNA genomes in the liver similarly suggests a liver tropism for this virus (106, 148). The predicted 3` NTR structure of EHcV is similar to the respective structure of the HCV 3` NTR. This region comprises around 328 nts and includes a short poly(A) tract, a variable region followed by a poly(U/C) tract, a conserved intermediate region, a long poly(U) tract, and a conserved 3` X region (152).

Pfaender et al. performed a study to analyze a possible zoonotic potential of EHcV, but neither the results obtained with a serological assay nor qRT-PCR analyses for detection of viral RNA could support this hypothesis (159). In horses, EHcV infection can be persistent, although the chronicity rate appears to be somewhat lower compared to HCV (106). EHcV is the closest genetic relative to HCV (105, 160). A recent study showed that EHcV viral RNA as well as antibodies against the virus could be detected in donkey serum samples and it has been suggested that the close genetic relationship of donkeys and horses is involved in cross-species transmission (161, 162).

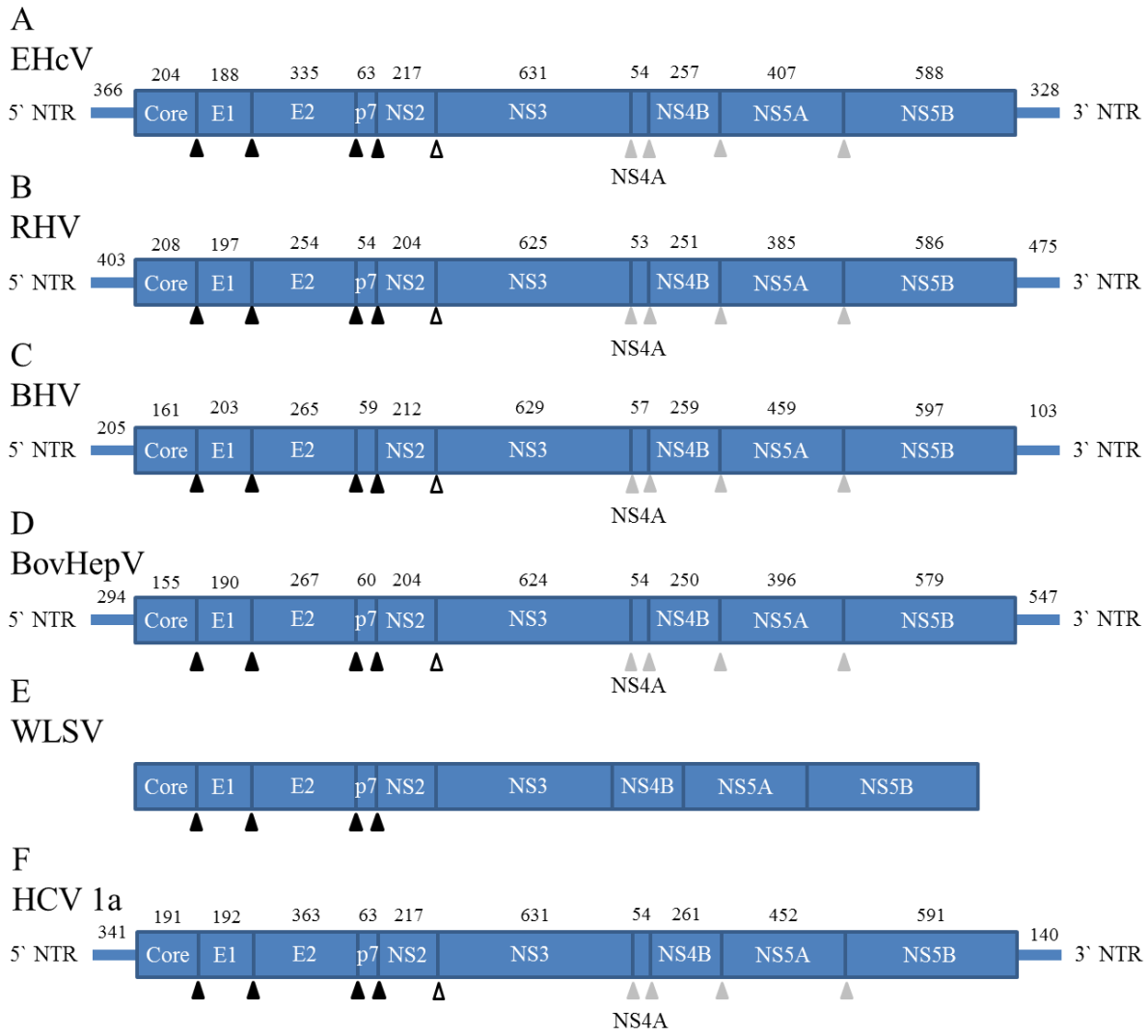


Figure 2: Predicted genome diagrams of animal hepaciviruses EHcV, RHV, BHV, BovHepV, WLSV and human hepacivirus HCV 1a. Numbers: number of amino acids in the individual viral proteins and lengths of the 5´and 3` NTR, respectively. Black triangles: proposed cellular peptidase cleavage sites. White triangles: NS2-3 protease cleavage site. Gray triangles: NS3-4A protease cleavage site. EHcV: equine hepacivirus. RHV: rodent hepacivirus. BHV: bat hepacivirus. BovHepV: bovine hepacivirus. WLSV: Wenling shark virus. HCV: Hepatitis C virus. NTR: non-translated region. C: core protein. E: envelope protein. NS: non-structural protein. GenBank accession numbers used for preparing this figure: EHcV (JQ434008), RHV (KC411807), BHV (KC796077), BovHepV (KP641123), WLSV (KR902729) and HCV 1a (M62321).

### 1.2.2 Rodent hepacivirus (RHV)

The rodent hepacivirus (RHV) was identified and first described in 2013 (87, 163). Kapoor and colleagues established the complete genome sequence of a rodent hepacivirus encompassing 8,879 nts from plasma samples of a deer mouse. This genome is predicted to encode a long polyprotein of 2,748 aa flanked by 5` and 3` NTRs. The 5` NTR consists of 403 nts and shows homology to other hepacivirus sequences (GBV-B, HCV, EHcV) in the 200 nts region upstream of the translation initiation codon, which includes domains IIIa to IIIe stem-loops and the pseudoknot IIIf. Moreover, RHV genomes contain a strain dependent number of miR-122 binding sites in the 5`NTR ranging from one to two (87, 163). The predicted polyprotein is significantly shorter than those of HCV-1a (3,011 aa) and EHcV (2,942 aa) and genetic analysis predicts three structural (C, E1 and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (figure 2B). The 3` NTR comprises 230 nts and has a structured region that is equivalent to the 3` variable region of HCV followed by a short poly(C) tract of around 10 nts and a 3` X region of 158 nts that forms four stem loop structures (87). A further group studied rodent hepacivirus in rats from New York and ascertained replication of this virus in liver tissue by detecting replicative intermediates. Viral RNA was most consistently found in liver and serum, which is an indication of liver tropism (164). Furthermore, Drexler and colleagues showed a low correlation of viral RNA and anti-RHV antibodies, suggesting that rodents may be able to clear hepacivirus infections (163). Recently, in 2018, rodent hepacivirus RNA could also be observed in the liver tissue and serum samples of rats in Vietnam (165).

### 1.2.3 Bat hepacivirus (BHV)

Also in 2013 a bat hepacivirus (BHV) was discovered by unbiased high-throughput sequencing. Like other members of the genus *Hepacivirus*, BHV has a positive-sense, single-stranded RNA genome with a single open reading frame (ORF) flanked by a 5` and 3` NTR (figure 2C). The genomic sequence comprises at least 8,916 nts and encodes polyproteins of 2,842-3,469 aa in length covering structural and non-structural proteins like other members in the genus *Hepacivirus*. Interestingly, some genomes contain a region of variable length upstream of predicted E1 and further studies are necessary to investigate if this variable

region is a part of E1 or codes for a separate protein. All analyzed bats were healthy, although high levels of viremia were detected, suggesting no pathogenic potential in the hosts (166).

### 1.2.4 Shark hepacivirus-like (WLSV)

In 2015, the first hepacivirus-like in the cold-blooded cartilage fish was identified in Proscyllium hebereri and was termed Wengling shark virus (WLSV). But phylogenetic analysis does not place this virus clearly within either the *Hepacivirus* or *Pegivirus* genera (84). The amino acid identity varies between 27.9 % and 29.3 % with other viruses in the genus *Hepacivirus* and as in human-, horse-, rodent- and cattle hepaciviruses the viral RNA could be found in the liver tissue. The genome consists of 9,653 nts, 3,086 aa and contains multiple target sites for host signalases in the N-terminal part of the polyprotein (144). The identified 5` NTR of 131 nts seems to be incomplete and there is no homology to equivalent regions in pegi- and hepaciviruses. In the 262 nts long 3` NTR sequence the typical hepacivirus poly(U/C) tract is missing, but the predicted 3` NTR secondary structure is comparable to other members of the family *Flaviviridae* (84). The open reading frame encodes for four structural proteins (C, E1, E2 and p7) as in the other hepacivirus members, but only five non-structural proteins (NS2, -3, -4B, -5A and -5B) whereas a sequence encoding NS4A could not be observed so far (figure 2E) (144).

### 1.2.5 Bovine hepacivirus (BovHepV)

In 2015 a HCV related virus was described in cattle from Africa (155), Germany (167) and in 2018 in China (168) and is termed bovine hepacivirus (BovHepV). Like other hepaciviruses BovHepV contains the 10 typical hepacivirus proteins: Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (figure 2D) (155, 167). For the African serum samples a full length genome of 8,879 and 8,891 nts was assumed, whereas serum samples of German cattle showed a length of 8,881 nts encoding a polyprotein of 2,779 aa (155, 167). This viral polyprotein contains fewer amino acids than HCV 1a (3.011 aa) or EHcV (2,942 to 2,946 aa) and is flanked by a 5` NTR (294 nts) (155, 167) and 3` NTR (247 nts) (167), respectively 235 and 250 nts (155). Compared to HCV, the BovHepV 5`NTR is shorter and lacks domain I, while the remaining IRES structure, including domains II, IIIa to IIIe, pseudoknot IIIf and domain IV containing the translation initiation codon, coincides with HCV. The complete

BovHepV 5` NTR (294 nts) is shorter than the EHcV 5` NTR (384 nts) and the large domain I in the EHcV IRES is missing. The second non-translated region, the 3` NTR, is predicted to form three highly ordered stem-loop structures (SLI-SLIII) and similar to HCV and contrary to EHcV no evidence for a poly(A)-rich region was found. Moreover, in contrast to HCV, BovHepV contains no poly(U) stretch that separates the viral polyprotein and the 3` NTR (155). In comparison to the predicted four stem-loops in the 3` NTR of the rodent hepacivirus, BovHepV 3` NTR has only three predicted stem-loops. In line with EHcV and RHV, BovHepV exhibits only one miR-122 match site, which is equivalent to the first match site for miR-122 in the HCV IRES. In addition, BovHepV is more closely related to GBV-B, bat and rodent hepaciviruses than to HCV or EHcV, but exhibits a large genetic distance to these species and occupies a separate phylogenetic branch in the tree of hepaciviruses (167). A Brazilian working group reconstructed a phylogenetic tree for the genus *Hepacivirus* including three Brazilian, two African and five German BovHepV sequences. The Brazilian sequences are separated from the African or German sequences that cluster closely together, though only short partial sequences of the 5` NTR were included (169).

Like other members of the genus *Hepacivirus*, the BovHepV genomic sequence contains one miR-122 match site, as mentioned above, the highest virus load was found in liver tissue (155, 167) and the mature miR-122 sequence is conserved among vertebrates (170, 171). Consequently, a liver tropism can be suggested like it was shown for HCV (172), EHcV (106) and RHV (163). Several EHcV infected animals show elevated concentrations of liver specific enzymes without impairment of liver function (106), whereas one working group associated EHcV infection in a horse with liver disease (173). In the case of the bovine hepacivirus liver specific enzymes and post-mortem liver tissue of infected and non-infected cows were analyzed. No significant differences were observed in liver enzyme concentrations and liver injury attributable to virus infections was not present (167). However, bovine hepacivirus infections can lead to persistent infection (167) (161) like it is a main characteristic of HCV (174). A Brazilian working group detected the bovine hepacivirus in 2017 and their results indicate that the 5` NTR bovine hepacivirus sequences from Germany, Africa and Brazil grouped together in the same branch, but the Brazilian sequences differed from European and African strains (169). Additionally, a Chinese working group processed the German, African and their two Chinese polyprotein sequences for phylogenetic analyses,

which grouped the sequences into two clades. One clade contains the German BovHepV sequences and a second clade composes the African and Chinese sequences (168).

## 1.3 Eukaryotic canonical cap-dependent and viral IRES-mediated translation initiation

### 1.3.1 Eukaryotic canonical cap-dependent translation initiation

The initiation process for the eukaryotic translation is the formation of the GTPase eukaryotic translation initiation factor 2 (elF)•GTP•Met-tRNA ternary complex including guanosine triphosphate (GTP) and methionine initiator transfer-RNA (Met-tRNA) (175), which then binds to the small (40S) ribosomal subunit to form the 43S complex (figure 3).

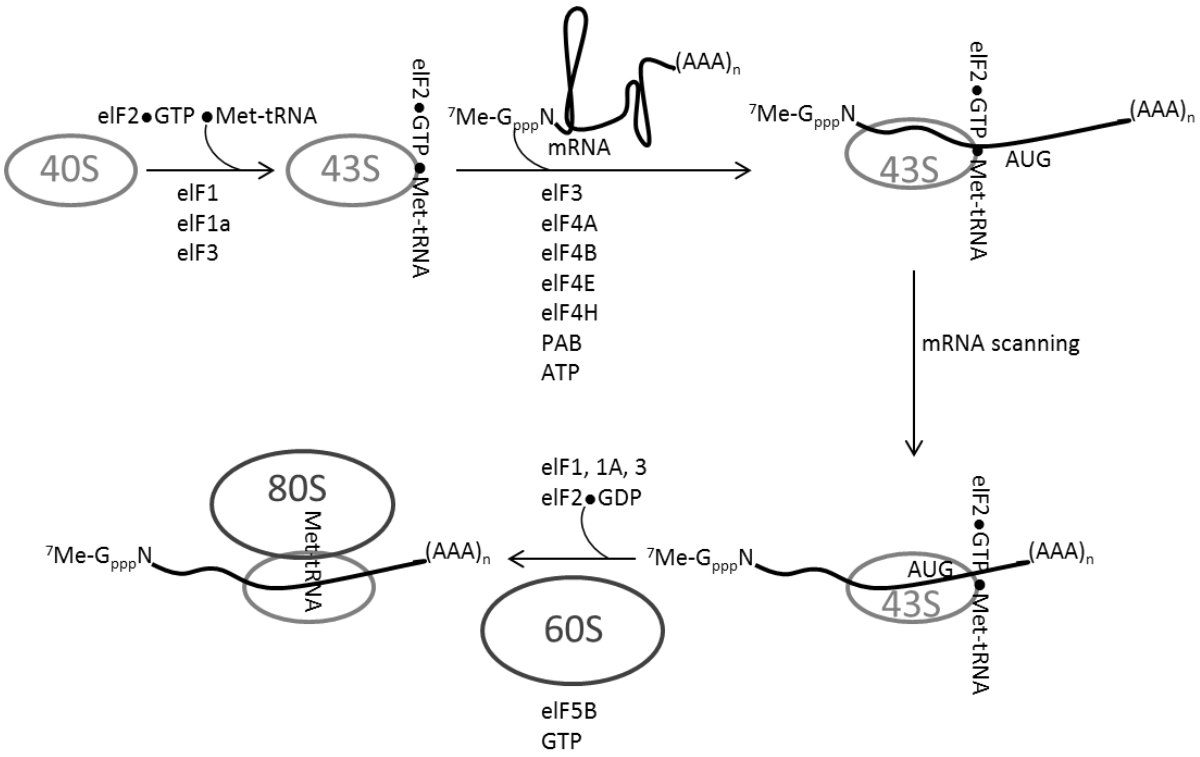


Figure 3: Schematic overview of the eukaryotic cap-dependent translation initiation. 40S: ribosomal subunit 40. elF: eukaryotic translation initiation factor. GTP: guanosine triphosphate. Met: methionine. tRNA: transfer-ribonucleic acid. 7Me-$G_{ppp}$N: guanylylated 5` RNA end (cap). mRNA: messenger RNA. PAB: poly(A) tail binding protein. ATP: adenosine

triphosphate. $(AAA)_n$: poly-adenosine tail. AUG: translation initiation codon. GDP: guanosine diphosphate.

This binding is mediated by elFs1, elF1A and elF3 whereas elF4E interacts with the 5`-cap of the mRNA and unwinds secondary structures in the 5` NTR. Moreover, binding of elF1, elF1A and elF3 to the recycled 40S subunit leads to the release of deacetylated P-site tRNA and dissociation of mRNA, while the factors remain bound to the 40S, priming it for the next round of initiation (176). The unwinding step is enabled by the ATP-dependent activity of elF4A, RNA-binding proteins elF4B and elF4H, whereas the elF4A RNA-dependent ATPase uses the energy of ATP hydrolysis to disrupt the RNA structure (177–179). The eukaryotic translation initiation factor elF4A together with elF3 and the 3`-poly(A) tail binding protein (PAB) loads the mRNA onto the 43S complex. Next, this complex starts ATP-dependently scanning the mRNA in the 5` to 3` direction looking for the translation initiation codon (AUG) (180). Elf1 might detect the formation of codon-anticodon interaction by interacting with the body of the initiator tRNA responding to conformational changes in it when the initiation codon is reached (181). When the 43S complex reaches the initiation codon, which is surrounded by favorable sequence context, like Kozak sequence, codon-anticodon base pairing takes place between the initiation codon and the initiator tRNA in the ternary complex. Moreover, elF1 might play a role in discriminating between AUGs in favorable and unfavorable contexts by destabilizing preinitiation complexes on incorrect AUGs (182). Afterwards, elF2 hydrolyses GTP by GTPase-activating protein elF5 whereby the elF2•GDP releases the initiation amino acid Met-tRNA into the 40S subunit P site and dissociates together with elF1, elF1A, elF3 and elF5 from the complex (183). Furthermore, elF5 stimulates the dissociation of elF1 and increases the stability of elF1A binding (184, 185). After these factors are gone, the second GTPase elF5B•GTP facilitates the joining of the large (60S) ribosomal subunit to the 40S•Met-tRNA•mRNA complex, which leads to GTP hydrolysis by elF5B and the dissociation of the low affinity for ribosome elF5B•GDP (186). Moreover, in addition to the 7-methylguanosine cap structure in the 5`-end, the mRNA has a 3`-poly(A) tail that is bound by poly(A)-binding protein (PAB). This protein interacts with elF4G which is thought to lead to the circulation of the mRNA, which stimulates translation.

This provides a quality control mechanism. If the mRNA is partially degraded and/or the 3`-end is lost, the RNA will be translated with low efficiency (187, 188).

### 1.3.2 Viral IRES-mediated translation initiation

There are some viruses that contain a cap-structure at the 5` NTR for viral translation, like double-stranded RNA rotavirus (189) or the single-stranded RNA vesicular stomatitis virus (190). However, one further strategy for the translation of the viral genome is an internal ribosome entry site (IRES) structure at the 5` NTR, instead of a cap-structure. Such 5` NTR structures can be observed in different genera belonging to the family *Picornaviridae* (191) and in the *Flaviviridae* genera *Pestivirus* and *Hepacivirus*.

The type I or type II IRES structure of picornaviruses directly recruits the translational machinery to the internal translation initiation codon AUG in the mRNA, so that a scanning process, as it is seen in the eukaryotic translation initiation, is not needed. During this procedure, the first contact of the cellular translation machinery is not at the exact beginning of the 5` NTR containing cap-structure (192). For this reason the IRES-driven translation does not require the cap-binding factor elF4E (193). Like in an efficient eukaryotic translation, cellular proteins, for example La human autoantigen or polypyrimidine tract binding protein (PTB), are necessary for the viral translation (194).

In the family *Flaviviridae*, pestiviruses and hepaciviruses contain IRES elements that are directly responsible for translation of the virus genome. The pestivirus IRES structure is most closely similar to the HCV IRES in the 5` NTR and both have the same genome translation initiation procedure (195) (figure 4).

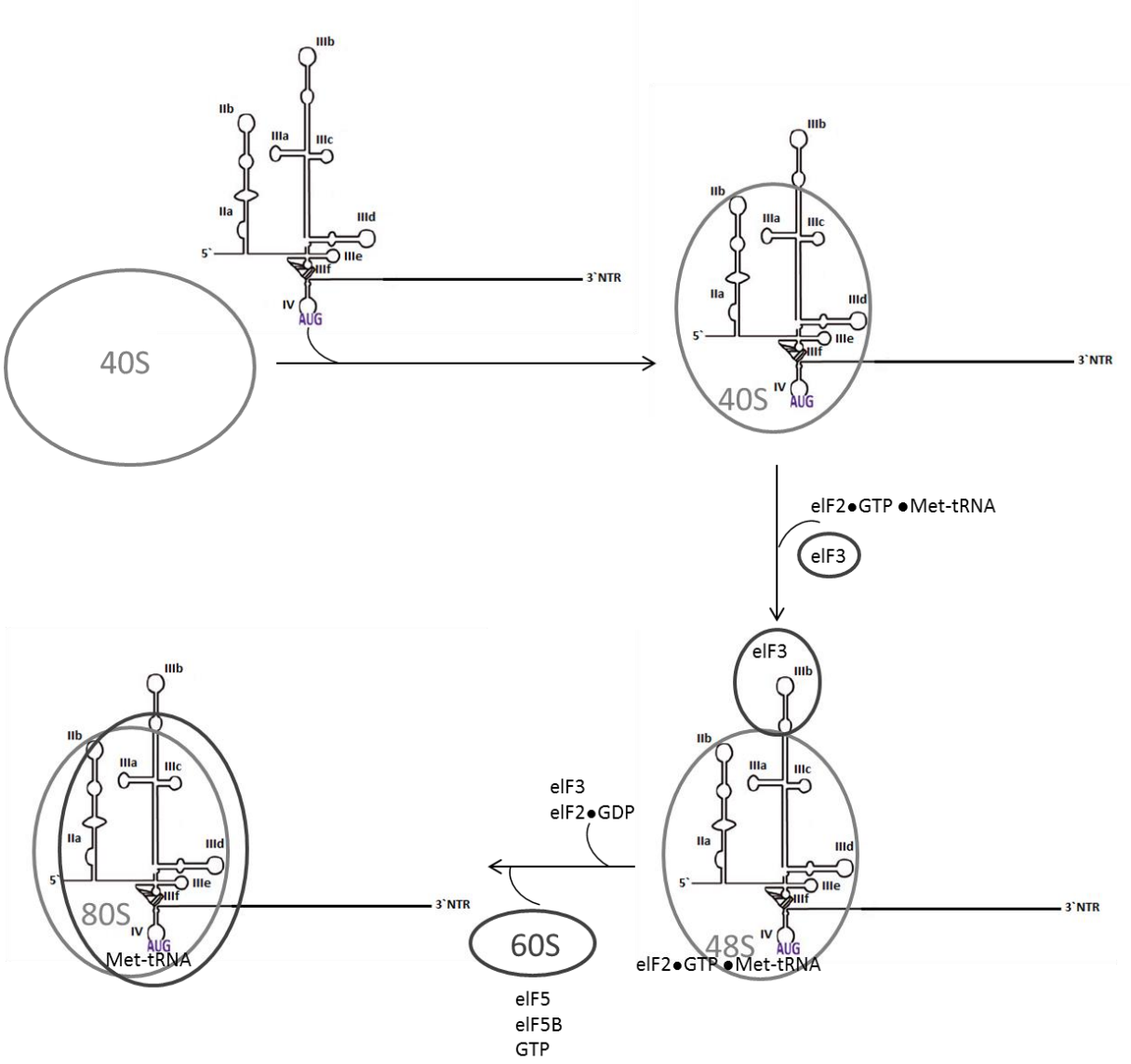


Figure 4: Schematic overview of the hepacivirus IRES-mediated translation initiation. 40S: ribosomal subunit 40. eIF: eukaryotic translation initiation factor. GTP: guanosine triphosphate. Met: methionine. tRNA: transfer-ribonucleic acid. mRNA: messenger ribonucleic acid. AUG: translation initiation codon. GDP: guanosine diphosphate.

In comparison to the eukaryotic cap-dependent translation, the 40S ribosomal unit is directly recruited to the initiation codon (AUG) present in domain IV, without the requirement of ribosome scanning and therefore elFs (46), by interacting with HCV IRES domain IIb, basal part of domain III (IIIdef), domain IIIc, and domain IV (196). Furthermore, by binding of

ribosomal 40 subunit, domain IV is unwound, due to its increased flexibility. This supports the correct positioning of the initiation codon and the subsequent binding of ternary complex elF2•GTP•Met-tRNA (197). In the next step elF3 together with the ternary complex interact with the apical part of IRES domain III (IIIab) and stabilize the pre-initiation translation assembly and form the 48S pre-initiation complex (110). Moreover, elF3 is essential for the formation of a translationally competent 80S ribosome on HCV mRNA (46). The domain II modulates the GTP hydrolysis of the ternary complex, mediated by elF5 and elF2-GDP is released after the establishment of AUG codon recognition by initiating Met-tRNA (198). The binding of 60S, which forms the translation active 80S ribosomal subunit, is dependent on GTP hydrolysis and interacts with domains II, IIIa and IIIabc (110).

## 1.4 Interactions between HCV 5`NTR and core coding sequences have an influence on the translation efficiency

### 1.4.1 Long-range interaction

A lot of investigations were performed on the influence of interactions between the HCV 5`NTR and core coding sequences on the translation efficiency. One long-range interaction seems to take place between nts 24-38 of the 5` NTR sequence and nts 428-442 located in the core coding region (figure 5; red boxes).

The binding of these complementary sequences leads to a decreased HCV genome translation (199–201). The first miR-122 match site (nts 22-28) overlaps with the complementary sequence in the 5` NTR, which is involved in the long range interaction. Hence, the binding of miR-122 inhibits this interaction resulting in the closed conformation which enhances the efficiency of translation and constitutes an integral regulatory component of the viral genome (202). Such complementary sequences can also be observed in the genome of BovHepV 5` NTR (nts 1-9) and partial core coding sequence (nts 397-404) at comparable positions to HCV. Mutations that disrupt such a complementary interaction enhance the HCV IRES dependent translation and compensatory mutations reduce the efficiency to a level comparable with the wild type (199). Besides this, Honda et al.´s results indicate that the nucleotide sequence but not the amino acid sequence of the HCV core protein determined translation efficiency by using frame shift mutations in the core coding sequence (203). Furthermore,

Tanaka et al. showed that the presence of the EHcV core coding region downstream of the pseudoknot has a negative influence on translation efficiency (156). Such long range interactions limiting viral protein levels might be implicated in the establishment of persistent HCV infections characterized by low viral loads in infected individuals (199). In addition, this interaction may play a role in the switch between translation and replication. For poliovirus such a switch was shown to be important for viral replication as continuous translation blocks replication complex movement (204, 205).

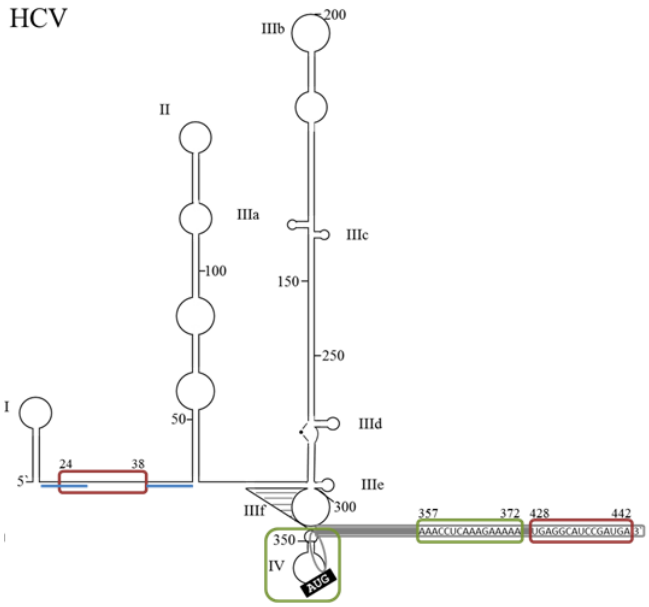


Figure 5: Predicted 5` NTR structure of HCV. HCV contains domains I-IV including pseudoknot IIIf and translation initiation codon AUG containing domain IV. Grey: partial core coding sequence. Red: complementary sequences in the 5` NTR and core coding sequence. Green: destabilizing effect of an adenosine-rich core sequence on domain IV. Blue: miR-122 match sites.

### 1.4.2 Destabilization of IRES domain IV

The translation initiation codon (AUG) is included in HCV domain IV that also comprises a few nucleotides of the core coding sequence. Mutations increasing the stability of domain IV lead to a decreased translation of HCV (206). This finding led to the suggestion for a folded and an unfolded single-stranded conformation of domain IV that modulates the efficiency of internal initiation of translation directed by the HCV IRES (199). Honda et al. assumed that the presence of this structure is not essential for IRES activity (206) and this stem-loop has to be melted for initial ribosome binding (207). Furthermore, there is an importance of the first 21 nts of the core coding sequence in IRES dependent translation (208, 209) and up to 32 nts of the core coding sequence are absolutely required for efficient HCV translation (206, 210). Upstream of the translation initiation codon AUG, an adenosine-rich region is located in the core protein coding sequence which has a destabilizing effect on domain IV, reduces the formation of the stable stem loop structure in domain IV and therefore enhances the translation efficiency (figure 5; green boxes) (207). For translation initiation the 40S ribosome subunit does not have to scan the 5` NTR but directly binds at the AUG codon (206, 211, 212). Because of this the AUG codon-containing domain IV has to disrupt in order to allow binding of ribosome subunit 40 to the AUG initiation codon (207). Additionally, Honda et al. showed that mutations that stabilize the stem-loop IV and do not hinder the passage of a scanning 40S ribosome subunit significantly inhibit IRES-mediated translation (206). It seems that an equilibrium between the formation of stem-loop IV and an open conformation is important for controlled binding of the ribosomal subunit and therefore for the efficiency of translation initiation (203). Moreover, it is suggested that the cellular La autoantigen interacts with the HCV RNA in the region of stem-loop IV and facilitates translation (213).

## 1.5 Micro RNAs (miRs), especially miR-122 and the role of miR-122 in HCV translation

Micro RNAs are small non-coding RNA molecules that are approximately 22 nucleotides in length. They control the expression of roughly one fourth of all cellular mRNAs by binding to sequences in the 3` NTRs (214, 215). Interestingly, micro RNAs are also encoded by different viruses and serve for the latency or immune evasion strategy, for example (216). Transcription of full-length miRNA genes by RNA polymerase II results in production of primary miRNA precursors (pri-miRNA), where the mature miRNA is included (217). The

precursor miRNA (pre-miRNA) of approximately 65 nts is obtained by a maturation process that involves nuclear cleavages by two RNase III enzymes and its transport into the cytoplasm (218, 219). Selection of the guide strand is determined at the RNA-induced silencing complex (RISC) loading step and is mainly dependent on the relative thermodynamic stability of the two ends of the miRNA duplex (220). The strand with the stable 5` terminus is selected as the guide strand to generate mature RISC, whereas the unstable strand is degraded by the RISC (221). Most target recognition by a miRNA involves imperfect complementarity, which nevertheless leads to translational inhibition of the target mRNA (222), whereas miRNAs targeting mRNAs with perfect complementarity lead to mRNA cleavage (223). MiRNAs typically inhibit gene expression by repressing mRNA translation and/or accelerating deadenylation and decay of target mRNAs (224). Interestingly, one miRNA has multiple target mRNAs and one mRNA is the target of multiple miRNAs. For example, the human miR-7 has about 90 cellular mRNA targets of different gene clusters, like histone 3 or ATPase mRNA. Furthermore, the human histone deacetylase 4 mRNA has target sites for nine miRNAs (225).

It was reported that miR-122 is specifically expressed in the liver where it constitutes 70 % of the total miRNA, which corresponds to above 50,000 copies per cell. Its sequence is strongly conserved across the vertebrate lineage (226–228). Interestingly, one study has shown that miR-122 is also present in human skin fibroblasts and the stability or activity may be controlled by the non-canonical poly(A) polymerase Gld2 (229). MiR-122 has a lot of cellular targets, for example the cationic amino acid transporter 1 (CAT-1) mRNA (227), and the CAT-1 mRNA has several miR-122 binding sites in the 3` NTR. The binding leads to translation repression and a change from mRNA to processing bodies (230). Sequestration of miR-122 results in a significant decrease in plasma cholesterol, low and high density lipoprotein levels and an increase in fatty acid oxidation and fat accumulation in the liver (231–235). Accordingly, it has been assumed that miR-122 might be a regulator of several important liver functions, including cholesterol biosynthesis, fatty acid oxidation, lipid metabolism, lipoprotein assembly, secretion and iron metabolism (231–234, 236). Studies in rodents and humans showed that miR-122 is downregulated in hepatocellular carcinoma (HCC) (237, 238). In addition, miR-122 target genes are increased in tumor tissues compared to non-tumor tissues from individuals with HCC (239). Several target genes of miR-122 that

are involved in hepatocarcinogenesis have been identified, such as serum response factor (SRF), insulin-like growth factor 1 receptor (Igf1R) or cyclin G1. Moreover, miR-122 knockout mice correspondingly develop steatohepatitis, fibrosis and hepatocellular carcinoma (240–243). Additionally, Girard et al., Xu et al. and Laudadio et al. showed a role of miR-122 on hepatocyte differentiation and liver development (244–246).

In contrast to the downregulating effects of miR-122 on cellular targets, upregulating effects on the HCV translation, replication and genome stabilization have been reported (247–249). In all HCV genotypes two sequence motifs in the 5` NTR viral genome (nts 22-29 and 38-43) were predicted to be perfect binding sites for miR-122 and nts 1-8 of miR-122 bind at the first match site, whereas nts 2-7 of an additional miR-122 molecule can interact with the second match site (figure 6) (250).

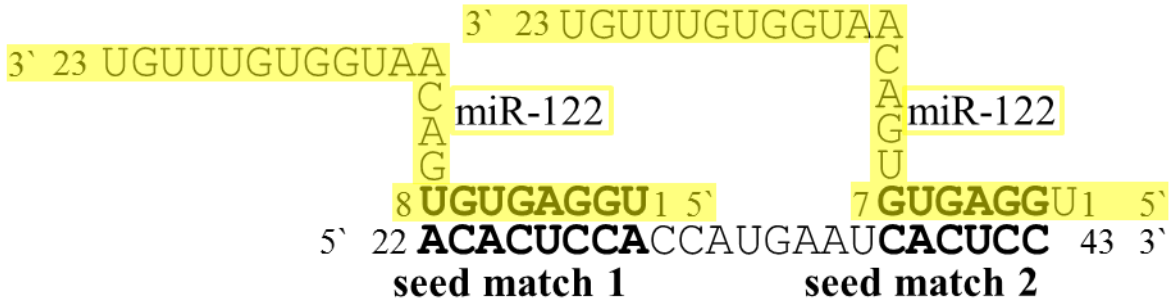


Figure 6: Binding of micro RNA miR-122 sequences (highlighted in yellow) to the two match sites in the 5` NTR of HCV.

Jopling and colleagues showed that the presence of miR-122 leads to accumulation of HCV RNA *in vitro* since sequestration of miR-122 by RNA oligonucleotides with exact complementarity to miR-122 impeded viral RNA accumulation. In their study, substitution mutations at position three and four in the binding site of the miR-122 sequence led to undetectable RNA amounts (251). Furthermore, to examine whether miR-122 affects viral RNA abundance directly by binding to the viral genome or indirectly by modulating the

expression of a host target mRNA, nucleotide sequences in both 5` NTR match sites were mutated (252, 253). This experiment did not permit accumulation of RNA and mutated miR-122 molecules predicted to bind to the mutated sequences in the 5` NTR match sites restored viral RNA abundance (252, 254). There are various models for the mechanism of miR-122 mediated viral RNA accumulation. The first possible explanation is the promotion of ribosome recruitment by miR-122. Henke and coworkers reported that miR-122 stimulates translation of chimeric mRNA containing HCV NTRs and full length viral RNA. Further studies in rabbit reticulocyte lysates showed enhanced association of the ternary 48S ribosomal complex in the presence of miR-122 (255). What is more, miR-122 is important for efficient production of infectious hepatitis C virus as Huh-7 cells infected with HCV strain HJ3-5 and transfected with anti-miR-122 displayed reduction of infectious particles by 65 %. Furthermore, no infectious particles could be detected in the supernatant of cells transfected with point mutated miR-122 match sites of HJ3-5 RNA and additionally, replication was rescued by transfection of a complementary point mutated miR-122 (254).

The 3` region of the miR-122 sequence that is not involved in target interaction is important for efficient translation and led to the suggestion that the 3` terminal nucleotides of miR-122 interact with proteins or other RNAs or alter structures in the IRES to perform translational enhancement (247). Another effect of miR-122 is to protect the HCV RNA against cellular exoribunucleases. For instance, Li et al. showed that genotype 1a HCV RNA was degraded by XRN1 and exosome complex in the absence of miR-122 (248). For genotype 1b and 2 it was shown that HCV RNA in infected cells was degraded by XRN2 and that sequestration of miR-122 in XRN1 depleted cells restored HCV RNA abundance, which demonstrates that miR-122 protects genotype 2 HCV from degradation (256). Ago2 was also shown to associate with miR-122-HCV 5` NTR complex, thus mediating the stability of HCV RNA leading to the protection of the viral genome from 5` exonuclease digestion by host mRNA decay machinery (257). Jopling and colleagues displayed a role of miR-122 to enhance replication as sequestering of miR-122 with exact complementarity oligonucleotides to the miR-122 sequence reduced the HCV genome amount by 80 % (252). The expression of miR-122 in conventional immortalized cell lines was investigated by northern blot and quantitative real time PCR but only Huh-7 and Huh-6 cells have a high expression level, whereas in Hep3B and HepG2 cells low or no detectable miR-122 expression were observed (252, 258).

Like in HCV, the GBV-B 5` terminus contains two miR-122 match sites and miR-122 interactions at each of these sites lead to viral accumulation in cell culture (259). Hepacivirus in horses, rodents, bats and Old World monkeys have one conserved miR-122 seed match sequence in the 5` NTR, while the complete bat 5` NTR is not yet available (85, 145, 146, 161, 164, 244). Furthermore, EHcV was suggested to be responsive to miR-122 in cell culture since sequestration of miR-122 decreased EHcV translation. Furthermore, Scheel et al. investigated the translation efficiency of a EHcV functional molecular clone with or without miR-122 sequestering locked nucleic acid (LNA) in Huh-7.5 cells and the translation was decreased in presence of LNA, analogous to the findings for HCV (152). However, the effects on EHcV viral RNA stability could not be investigated due to the lack of detectable EHcV replication in cell culture (152). Moreover, further microRNAs have influences on viral replication. Additionally, miR-122 possesses effects on further viruses like the hepatitis B virus (HBV) and hepatitis E virus (HEV), which show a liver tropism like HCV. In contrast to increasing the translation and replication of HCV, miR-122 leads to an inhibition of HBV replication and the miR-122 expression in the liver is significantly decreased in patients with HBV infection compared with healthy controls (261–263). HEV harbors one miR-122 match site and the presence of miR-122 facilitates replication (264).

Like EHcV, BovHepV contains only one miR-122 match site (nts 1-9) in the 5` NTR, which is comparable to the first one in HCV and can theoretically bind with the first eight nucleotides of the miR-122 sequence (167). Antisense inhibitors of miR-122 (miravirsen) have been used to treat humans chronically infected with HCV and led to a reduction in HCV RNA levels (265). Antisense targeting of miR-122 appears to have a high barrier to resistance and may be used in combination therapy, for example with ribavirin peg-IFN-alpha combination, to treat patients who do not respond to direct acting antivirals like sofosbuvir/velpatasvir or grazoprevir/elbasvir. However, miR-122 knockout studies in mice resulted in hepatosteatosis and hepatocellular carcinoma (240), warranting caution for the long term use of antagomirs against miR-122.

### 1.6 Diagnostic assays for the detection of hepaciviruses genomes and antibodies

The diagnosis and monitoring of HCV infection occurs by using direct tests for the detection of viral RNA and indirect serological assays to detect virus specific antibodies (266).

Quantitative and non-quantitative PCRs and transcription-mediated amplification (TMA) assays are used as direct tests for the identification of viral RNA (267). Commercial HCV RNA detection test systems are available from several companies (266) and the World Health Organization (WHO) has established an international standard for RNA units (268). Furthermore, an enzyme immune-based assay (EIA) detects and quantifies core antigen (266). Such commercial test systems are available only for HCV. The viral genome of EHcV was detected by quantitative reverse transcriptase PCR using various probes (147, 148, 159, 269, 270) or a SYBR green (151, 271) based PCR, in which the primers and/or probes are specific for the 5` NTR (148, 159, 269–271) or NS3 coding sequences (147, 151). Alternatively, gel based PCRs (148, 152) or nested PCRs (150, 272, 273) were used to identify EHcV 5` NTR (148, 152) or NS3 coding sequences (150, 272, 273). Besides the detection of viral hepacivirus RNA, the indirect system enzyme immunoassays (EIAs) identify mixtures of antibodies against different HCV epitopes of Core, NS3, NS4 and NS5 and are performed on microtiter plates or with microbeads (266). Moreover, in clinical use present antibodies are confirmed with immunoblot assays (274) and the HCV genotyping can be achieved by identification of type-specific antibodies with competitive EIAs (275).

Besides these EIAs, the recently developed luciferase immunoprecipitation system (LIPS) is an immunoprecipitation technology for identifying sera containing antigen specific antibodies and was first described in 2005 (276). This test system is based on fusing viral antigens to the enzyme renilla luciferase as a reporter and these fusion products are expressed in mammalian cells. With the help of protein A/G beads, which bind the Fc part of IgG antibodies, the light intensity is directly proportional to the amount of renilla luciferase and thus the antibody amount in the sample. The LIPS method is species independent as no secondary antibody is needed, offering an advantage over an indirect antibody ELISA. The LIPS assay has been used to detect antibodies against HIV, HBV, HCV, and EBV as well as autoantibodies in human autoimmune diseases (277–279). In 2012 Burbelo et al. investigated serum samples originating from dogs, rabbits, deer, cows and horses for antibodies against canine hepacivirus (now termed EHcV). Many horses and one cow were tested positive in this LIPS assay, which gave a hint that the virus origin was in horses (148). One year later, Bexfield et al. investigated 100 liver samples from dogs with chronic hepatitis of unknown cause and neither virus genome nor antibodies were detected (149). The first LIPS assay detecting EHcV NS3

helicase specific antibodies was performed by Pfaender et al. in 2015 and both virus genome and antibodies could be observed (106). The same working group evaluated a potential zoonotic transmission of EHcV by investigating samples from humans with and without exposure to horses. All samples were tested negative for EHcV antibodies and viral RNA (159). The possibility of vertical transmission was investigated and one of four mare foal pairs showed this kind of transmission (280). Nevertheless, so far, the LIPS method has only been developed for human, horse and donkey specific antibodies against species specific hepacivirus protein partitions.

### 1.7 Aim of the study

According to the World Health Organization, HCV infects approximately 3-4 million people each year and approximately 170 million individuals are infected with HCV and over 350,000 people die every year globally due to HCV related liver diseases (http://www.who.int/mediacentre/factsheets/fs164_apr2014/en/). An infection can lead to liver fibrosis, cirrhosis and hepatocellular carcinoma and so far no vaccine is available. Furthermore, no native animal models exist for the investigation of HCV *in vivo* to date. Despite the genomic homology between chimpanzees and humans, the natural course of infection differs since only few chimpanzees develop chronic HCV infection, no fibrosis and only one hepatocellular carcinoma case has been observed (281). Nonetheless, availability, cost and ethical constraints severely limit the use of primates for research. The National Institutes of Health of the United States Department of Health and Human Services decided to end the support for invasive research on chimpanzees (282). Alternative small animal models use rodents, but in general, rodents are naturally resistant to HCV infection and HCV has to be adapted to the rodent environment, humanization of rodents or xenotransplantation methods (283–285). Moreover, only the strain JFH1 can recapitulate the full viral cycle in the human hepatoma cell line Huh-7 *in vitro* (286), but genomes that acquired cell culture adaptive mutations were found to be highly attenuated in chimpanzees (287). For that reason it is of great interest to find other members of the genus *Hepacivirus* in different animals to compare the properties of these viruses and for the development of a possible animal model to study HCV *in vivo* in more detail regarding the viral life cycle, antiviral drugs and vaccine development.

The aim of this study was to characterize biological and functional properties of the bovine hepacivirus (BovHepV), a close relative to HCV. BovHepV was first described in 2015 and since then its presence in cows and the apparent liver tropism were observed. So far, a serological test system for the detection of BovHepV specific antibodies is not available and a possible transmission to other animal species or a zoonotic potential of BovHepV has not been investigated. Besides this, on a molecular level nothing is known about BovHepV, especially a potential IRES structure that enables cap-independent translation as shown for HCV and EHcV. Furthermore, the HCV IRES sequence shows interaction dependent effects on the translation efficiency. Therefore, goals of this study were the development of a serological test system to detect antibodies against BovHepV in serum samples and the establishment and validation of a quantitative real time PCR assay to identify animals infected with BovHepV. Furthermore, with the development and establishment of an antibody detecting luciferase immunoprecipitation system (LIPS) assay and a viral RNA identifying pan-hepaci PCR a possible inter-species viral transmission to non-bovine animals and a zoonotic potential could be investigated by analysis of porcine, equine, and human samples, respectively.

Apart from the serology and viral genome detection, further aims addressed the molecular details of IRES mediated translation. First, the IRES structure of BovHepV had to be predicted by statistical folding programs (*mfold* and *sfold*) and compared to the HCV IRES structure. Initially, the importance of individual IRES domains on translation initiation was studied. In addition, interactions of partial core coding sequences with the 5` NTR, as well as implications of presence or absence of miR-122 on viral translation were investigated using a dual luciferase system *in vitro*. In general, the aim was to characterize the bovine member of the genus *Hepacivirus* based on the knowledge about HCV and the recently discovered members in horses, rodents, bats and donkeys. This includes the distribution of the virus between cows and also to non-bovine animals and humans. Moreover, possible persistent infections that in the case of HCV can cause hepatitis, fibrosis and hepatocellular carcinoma are discussed in this thesis.

## 2. Identification of a Novel Hepacivirus in Domestic Cattle from Germany

Identification of a Novel Hepacivirus in Domestic Cattle from Germany

Christine Baechlein[a, b, *], Nicole Fischer[c, d, *], Adam Grundhoff [d, e], Malik Alawi[e, f], Daniela Indenbirken[e], Alexander Postel[a], Anna Lena Baron[a], Jennifer Offinger[g] , Kathrin Becker[h], Andreas Beineke[h], Juergen Rehage[i], Paul Becher[a, b, #]

Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Germany[a]; German Centre for Infection Research, Partner Site Hannover-Braunschweig, Hannover, Germany[b]; Institute for Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf, Hamburg, Germany[c]; German Centre for Infection Research, Partner Site Hamburg-Lübeck-Borstel, Hamburg, Germany[d]; Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Research Group Virus Genomics, Hamburg, Germany[e]; Bioinformatics Core, University Medical Center Hamburg-Eppendorf, Hamburg, Germany[f]; Veterinary Practice Legau, Germany[g]; Department of Pathology, University of Veterinary Medicine Hannover, Germany[h]; Clinic for Cattle, University of Veterinary Medicine Hannover, Germany[i]

Running head: Hepacivirus Infections of Domestic Cattle

* Both authors contributed equally

#Corresponding author: Paul Becher, paul.becher@tiho-hannover.de
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## 2.1 Abstract

Hepatitis C virus (HCV) continues to represent one of the most significant threats to human health. In recent years, HCV-related sequences have been found in bats, rodents, horses and dogs indicating a widespread distribution of hepaciviruses among animals. By applying unbiased high-throughput sequencing, a novel virus of the genus *Hepacivirus* was discovered in a bovine serum sample. De novo assembly yielded a near full length genome coding for a polyprotein of 2,779 amino acids. Phylogenetic analysis confirmed that the virus represents a novel species within the genus hepacivirus. Viral RNA screening determined 1.6% (n=5) of 320 individual animals and 3.2% (n=5) of 158 investigated cattle herds in Germany positive for bovine hepacivirus. Repeated RT-PCR analyses of animals from one dairy herd proved that a substantial percentage of cows were infected, with some of them being viremic for over six months. Clinical and postmortem examination revealed no signs of disease including liver damage. Interestingly, quantitative RT-PCR from different organs and tissues together with the presence of a miR-122 binding site in the viral genome strongly suggest a liver tropism for bovine hepacivirus, making this novel virus a promising animal model for HCV infections in humans.

## 2.2 Importance

Livestock animals act as important source for emerging pathogens. In particular, their large herd size and the existence of multiple ways of direct and food-borne infection routes emphasize their role as virus reservoirs. Apart from searching for novel viruses, detailed characterization of these pathogens is indispensable concerning risk analysis. Here, we describe the identification of a novel HCV-like virus in cattle. Beyond, determination of the prevalence and the course of infection in cattle herds provide valuable insights into the biology of this novel virus. The results presented here form a basis for future studies targeting viral pathogenesis of bovine hepaciviruses and their potential to establish zoonotic infection.

## 3. Further characterization of bovine hepacivirus: Antibody response, course of infection, and host tropism

**Further characterization of Bovine Hepacivirus: Antibody Response, Course of Infection, and Host Tropism**

**Running title:**

Characterizing bovine hepacivirus field infections

Christine Baechlein[1,2*], Anna Lena Baron[1*], Denise Meyer[1], Lara Gorriz-Martin[3], Vanessa Maria Pfankuche[4], Wolfgang Baumgärtner[4], Susanne Polywka[5], Sven Peine[6], Nicole Fischer[5,7], Jürgen Rehage[3], Paul Becher[1,2,†]

**1:** Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany; **2:** German Center for Infection Research, Partner Site Hannover-Braunschweig, Germany; **3:** Clinic for Cattle, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany; **4:** Department of Pathology, University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany; **5:** Institute for Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg Eppendorf, Martinistraße 52, 20246 Hamburg, Germany; **6:** Institute of Transfusion Medicine, University Medical Center Hamburg Eppendorf, Martinistraße 52, 20246 Hamburg, Germany; **7:** German Center for Infection Research, Partner Site Hamburg-Lübeck-Borstel-Riems, Germany

* equal contribution, CB and ALB should be considered joint first author

†corresponding author: paul.becher@tiho-hannover.de phone: +49-511-953-8840;

fax: +49-511-953-8898

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## 3.1 Abstract

Bovine hepacivirus (BovHepV) is a recently added member to the growing genus *Hepacivirus* within the family *Flaviviridae.* Animal hepaciviruses are rarely characterized so far. Apart from norway rat hepacivirus which represents a promising HCV surrogate model, only equine hepaciviruses have been studied to some extent. BovHepV has been initially identified in bovine samples and was shown to establish persistent infections in cattle. However, consequences of those chronic infections, humoral immune response and the possibility of an extended host spectrum have not been explored so far. Therefore, we here investigated i. the presence of anti-NS3-antibodies and viral RNA in cattle herds in Germany, ii. the course of infection in cattle, and iii. the host tropism including zoonotic potential of bovine hepaciviruses. Our results show that 19.9% of investigated bovine serum samples had antibodies against BovHepV. In 8.2% of investigated samples, viral RNA was detected. Subsequent genetic analysis revealed a novel genetic cluster of BovHepV variants. For twenty-five selected cattle in a BovHepV positive herd the presence of viral genomic RNA was monitored over one year in two to three months intervals by RT-PCR in order to discriminate acute versus persistent infection. In persistently infected animals, no serum antibodies were detected. Biochemical analyses could not establish a link between BovHepV infection and liver injury. Apart from a single sample of a pig providing a positive reaction in the antibody test, neither BovHepV-specific antibodies nor viral RNA were detected in porcine, equine or human samples implying a strict host specificity of BovHepV.

**Keywords:** hepacivirus, cattle, serology, phylogeny, persistent infection, host tropism

## 4. Mutational Analysis of the Bovine Hepacivirus Internal Ribosome Entry Site

**Mutational Analysis of the Bovine Hepacivirus Internal Ribosome Entry Site**

A.L. Baron[1], A. Schoeniger[2], P. Becher[1,3,*], C. Baechlein[1,3,*]

1: Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Germany

2: Institute of Biochemistry, Faculty of Veterinary Medicine, University of Leipzig, Germany

3: German Center for Infection Research, Partner-Site Hannover-Braunschweig

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*Corresponding authors:

christine.baechlein@tiho-hannover.de

paul.becher@tiho-hannover.de

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IRES elements of bovine Hepacivirus

**Key words:**

Bovine hepacivirus, 5` non-translated region, internal ribosome entry site, microRNA-122

## 4.1 Abstract

In recent years, hepatitis C virus (HCV)-related viruses were identified in several species including dogs, horses, bats and rodents. Additionally, a novel virus of the genus *Hepacivirus* has been discovered in bovine samples and was termed bovine hepacivirus (BovHepV). Prediction of the BovHepV IRES (internal ribosome entry site) structure revealed strong similarities to the HCV IRES structure comprising domains II, IIIabcde, pseudoknot IIIf and domain IV with the initiation codon AUG. Unlike HCV, only one microRNA-122 (miR-122) binding site could be identified in the BovHepV 5` NTR. In this study, we analyzed the necessity of BovHepV IRES domains to initiate translation and investigated possible interactions between the IRES and core coding sequences by using a dual luciferase reporter assay. Our results suggest that such long-range interactions within the viral genome can affect IRES-driven translation. Moreover, the significance of a possible miR-122 binding to the BovHepV IRES was investigated. When analyzing translation in human Huh-7 cells with high amounts of endogenous miR-122, introduction of point mutations to the miR-122 binding site resulted in reduced translation efficiency. Similar results were observed in HeLa cells after substitution of miR-122. Nevertheless, absence of pronounced effects in a bovine hepatocyte cell line, expressing hardly any miR-122 as well suggests additional functions of this host factor in virus replication.

## 4.2 Importance

Several members of the family *Flaviviridae* including HCV have adapted cap-independent translation strategies to overcome canonical eukaryotic translation pathways and use *cis*-acting RNA-elements, designated as viral internal ribosome entry sites (IRES) to initiate translation. Although novel hepaciviruses have been identified in different animal species, only limited information is available on their biology on molecular level. Therefore, our aim was a fundamental analysis of BovHepV IRES functions. The findings which show that functional IRES elements are also crucial for BovHepV translation expand our knowledge on molecular mechanism of hepacivirus propagation. In the course of that, we also studied possible effects of one major host factor implicated in HCV pathogenesis, miR-122. The

results of mutational analyses suggested that miR-122 enhances virus translation mediated by BovHepV IRES.

# 5. Discussion

## 5.1 NS3 helicase specific antibodies and viral RNA detection of a BovHepV positive tested cattle herd over a time period of 13 months

Besides the discovery of BovHepV in Germany (167) and Africa (155), presence of BovHepV has recently been described in calves from USA dairy herds (288), in cattle serum samples from Brazil dairy herds (169) and China (289). These findings demonstrate a wide distribution of BovHepV on several continents. Apart from the existence and prevalence of BovHepV the possibility of BovHepV causing persistent infections in cows, like HCV does in humans, was addressed in this thesis. For this, 25 cows in a German BovHepV positive herd were investigated over one year in two to three month intervals, which allowed a clear differentiation of acute and persistent infections under natural conditions. In a first study, it was suggested that the bovine hepacivirus is able to establish persistent infections (167). For further evaluation of the biological properties of BovHepV, including course of infection, antibody response, and host tropism, a qRT-PCR with primers and probe binding in a highly conserved region in the viral 5` NTR was established to detect viral RNA and a LIPS assay using the NS3 helicase domain as antigen was developed and used to analyze the animals' immune responses. With regard to the course of infection the animals could be allocated into three groups depending on the presence of BovHepV RNA in the serum.

The first group contained five animals that were tested positive at five consecutive sampling points and were thus classified as persistently infected. 10 cattle in group 2 were at least once positive on RNA level and the remaining 10 animals in group 3 were negative at all sampling points. Interestingly, all five animals in group 1 showed high RNA genome copy numbers between $10^4$ and more than $10^8$ copies per ml serum, but specific antibodies against NS3 helicase were not detectable. Especially animals 3, 6, 25 and 35 in group 1 showed

consistently high virus copies, which is typical for persistent infections. In addition, these four cows were tested negative for antibodies at each sampling point. One possible explanation for the antibody absence might be the production of antibodies against other viral proteins than NS3, like the envelope protein E2, as immune response. Another possibility could be a stressed immune system by different pathogens like viruses or bacteria weak antibody production occured. By using a third-generation anti-HCV kit (core, NS3, NS4 and NS5) or an enzyme immunoassay (E2) it was shown that immunocompromised HCV infected patients can exhibit detectable antibodies against the envelope protein 2 whereas antibodies against the core, NS3, NS4 and NS5 were below the detection limit and some patients were tested positive on HCV RNA and E2 antibodies (290).

Three cows [no. 19, 31 and 39] in group 2 showed a low RNA level at only one sampling point. The remaining animals of group 2 [no. 11, 12, 17, 21, 24, 26 and 40] were tested positive at least twice in succession. An immune response by production of antibodies against NS3 could be observed in five cattle [no. 17, 21, 24, 26 and 40]. In animals 11 and 12 BovHepV RNA was detectable in the serum at four and five sampling points respectively, but no antibodies were identified [no. 12] or the antibody level dropped from the first to the last time point of sampling [no. 11]. The remaining cows, which were PCR positive only once [no. 19, 31 and 39], showed an immune response, although BovHepV RNA was not detectable at an earlier time point [no. 19], showed an increased antibody response at the last sampling [no. 31] or showed no immune response over the whole range [no. 39]. Additionally, high antibody titers could be detected at the sampling dates after viral RNA was detected [no. 17, 21 and 24], which is typical for an acute infection and virus clearance, whereas cattle 26 and 40 displayed a reinfection after the clearance. Moreover, the antibody levels decreased under the detection level in the LIPS assay after virus elimination. Surprisingly, animal 11 had NS3 antibodies that dropped during the sampling periods while the RNA copies increased. Perhaps the infection was cleared at the first sampling time point in the presence of NS3 specific antibodies, whereas after re-infection antibodies against other BovHepV proteins, like envelope proteins, were produced by the immune system, but could not be detected by the NS3 specific LIPS assay. Furthermore, after the clearance at the fifth visit a re-infection occurred in this cow. Animal 12 showed relatively high copy numbers at visits four to seven but the immune response was absent with the exception of sampling time

point seven, whereas the immune system seemed to produce antibodies against BovHepV NS3. A possible reason could be a suppressed immune system caused by other infectious agents or negative environmental influences leading to delayed immunological reactions. The antibody production of animal 31 was missing despite infection with BovHepV, while in animal 39 it was initiated at late time points after genome detection. One explanation could be that a very low copy number of about $10^2$ copies per ml found in animal 39 might be insufficient to activate the immune system. Furthermore, animal 31 displayed a higher copy number than animal 39 (about $10^4$) but nevertheless lower than the other animals with a fast immune response. Perhaps the virus load was not high enough to stimulate the immune system immediately. Surprisingly, animal 19 exhibited a high antibody level at time point five although at earlier time points virus detection was negative. Reasons could be a cross reactivity with other related hepaciviruses or with an unknown virus or a short infection with low virus load between time point four and five. Group 3 was tested negative on genome level as well as on antibody level, which showed the absence of an active infection and a specific immunological response to BovHepV.

Burbelo et al. investigated herd independent horse serum samples at a single time point on presence of EHcV NS3 specific antibodies and viral genomes. Some horses cleared the EHcV infection because no EHcV viral RNA but antibodies were observed at this time point or the virus loud was under the detection level. Other horses displayed an infection with EHcV as the samples were tested positive on virus genome RNA and antibodies. All negative tested samples on EHcV virus genome were also negative for NS3 specific antibodies, which indicated that these animals had never been in contact with EHcV so far or that the infection occurred a long time ago (148). A second working group analyzed an equine cohort for the presence of viral RNA and antibodies for at least 6 months. As found in our study, some animals were acutely or persistently infected and some cleared the EHcV infection because no viral RNA but antibodies were still detectable, or the virus load was under the detection level (106). Moreover, Reichert et al. tested equine serum samples for viral RNA and immune response and, in accordance with our and other working groups studies, they found acute, persistent, as well as cleared infections and horses that were never infected with EHcV (271).

The hepatitis C virus is a blood-borne virus and transmission occurs mainly via parenteral route (291). Gather et al. could show that EHcV can be transmitted vertically in horses (280). Some authors envisaged the possibility that HCV evolved from a horse-to-human transmission event because EHcV is closely related to HCV (105, 160), while others suggested that HCV originated in relatively recent times from one or multiple cross-species transmission events from a still to be defined species (160, 292, 293). However, the strict species-specificity as well as the ability of HCV to persist lifelong in humans led to the alternative hypothesis according to which HCV-related viruses have been infecting humans and other primates throughout their evolutionary history (160, 294). Whereas the hepaciviruses of the so far known species are far clustered and no cross-transmission could be observed so far, it seems that the hepaciviruses are species specific (167). In our study it could only be speculated how the virus was introduced into the cattle herd and how it was transmitted between the animals, but mass vaccination without changing needles can be one possibility. So far, there is no evidence for a transmission via milk, feces, urine or on a respiratory route because such samples of BovHepV positive animals were tested negative in a previous study (167).

### 5.2 Analysis of interspecies transmission and zoonotic potential of BovHepV

The transfer of zoonotic viruses like rabies or influenza virus can lead to severe diseases in humans (295, 296). People who are in close contact with animals and animal products are at high risk for zoonotic infections. In the case of the bovine hepacivirus this includes particularly persons who come into contact with bovine blood, such as slaughterhouse staff, farmers and veterinarians and their technical staff. Moreover, there are humans with elevated liver values associated with liver diseases of unknown origin that were tested negative for hepatitis A, B and C virus. On these grounds it was important to clarify a zoonotic potential of BovHepV that might affect human liver integrity. A recent study showed that the bovine hepaciviral NS3/4A protease is able to cleave the cellular innate immune adaptor MAVS (mitochondrial antiviral signaling protein) *in vitro* and thereby circumvents the human host immune system (297). While it remains highly speculative that BovHepV has such a zoonotic potential, the propagation and virus spread might be promoted by the interaction with the

human immune system. Besides this, the possible transmission of BovHepV to other animal species was also part of the present study.

First, 282 individual once sampled bovine samples of different herds were investigated by using the LIPS assay on antibody (S/P value ≥ 0.2) and a real-time RT-PCR with primers binding in the 5` NTR on BovHepV RNA level. Animals that tested positive for viral RNA, NS3 specific antibodies, or both were detected. Therefore, all immune states could be observed: 68.1 % were non-infected (IgG antibody and viral RNA negative), 8.2 % were acutely or persistently infected or the antibody amount was under the detection limit (viral RNA positive), 3.9 % were re-infected or infected cows with a following immune response (IgG antibody and viral RNA positive) and 19.9 % cleared the infection or the viral RNA load was under the detection limit (IgG antibody positive and viral RNA negative). To investigate a possible interspecies transmission equine and porcine serum samples from animals that are in close contact with equestrians or from agricultural farms were analyzed by using the LIPS assay. 13 of the horse samples showed a weak reactivity in the bovine hepacivirus NS3 specific LIPS assay. For these samples, an additional pan-hepaci PCR was performed in which eleven samples had an expected fragment size. However, *sanger* sequencing revealed EHcV specific sequences which leads to the suggestion of a cross-reactivity between EHcV antibodies and the BovHepV NS3 helicase domain. Furthermore, Burbelo et al. investigated serum samples from dogs, rabbits, deer, cows and horses for presence of EHcV specific NS3 antibodies. 35 % of horse samples had a high reactivity against the equine NS3 antigen (148). Interestingly, one cow also reacted with this equine specific antigen which undergirds the suggestion that the NS3 helicase domains of the equine and bovine hepacivirus proteins are similar in structure and amino acid sequence, enabling binding of serum antibodies resulting in cross-reactions. 2 % of the porcine samples reacted positive in the LIPS assay and one serum sample possessed a high reactivity with the NS3 antigen, while all of them were tested negative in the pan-hepaci PCR. The detected antibodies may be directed against an unknown porcine hepacivirus or a hepacivirus related virus and the weakly reacting samples may show cross-reactivity to the bovine NS3 helicase domain. Although a porcine hepacivirus is not known so far, it can be speculated that this pig had cleared the infection and therefore was tested negative by pan-hepaci PCR or alternatively, the primers used in the PCR assay were not able to amplify a specific product. All investigated serum samples of healthy human blood

donors reacted neither in the LIPS assay nor in the pan-hepaci PCR. To study if BovHepV might possibly be involved in liver disorders, human serum samples from individuals with elevated liver values that are tested negative for hepatitis A-E and are thus of unknown etiology, were investigated in the pan-hepaci PCR. Like the results for the healthy blood donors BovHepV RNA could not be detected by pan-hepaci PCR. In future studies it will be important to examine humans who are highly exposed to cattle or cattle products, such as farmers, slaughterhouse staff, farm veterinarians or breeders. A similar study was performed by Pfaender et al. to investigate antibodies against EHcV in humans who are highly exposed to horses (159). There, they compared the high-risk group with a low-risk group on EHcV RNA and antibody level. All of them were negative for viral RNA, but weak sero-reactivities independent of the group could be observed. For these reasons it was concluded that a transmission of EHcV from horses to humans did not occur. To summarize, a BovHepV NS3 helicase specific LIPS assay could be successfully developed and was applied to study the possibility of transmission to other animal species and the zoonotic potential of BovHepV. The obtained results demonstrated that there is no evidence for an interspecies transmission or zoonotic potential. The development of an E2 specific LIPS or/and ELISA will be helpful for further characterization of bovine hepacivirus infections. Furthermore, a herd might be investigated over a longer time period than one year and in shorter intervals for a more detailed analyses of disease progression and possible liver tissue alterations in relation to liver enzymes like AST, ɣ-GT, GLDH or total bilirubin as HCV infection can be asymptomatic for a certain time (298).

### 5.3 Predicted BovHepV IRES secondary structure and importance of the different domains for efficient translation

The rapid amplification of cDNA ends (RACE) system was used to identify the complete 5` NTR sequence with a length of 294 nts, which is shorter than the 5’ NTR of HCV and EHcV. The identity of the 5`NTR is nearly 100 % amongst BovHepV sequences but compared to HCV only approximately 50 %. The IRES sequence was folded with the algorithms used by *mfold* and *sfold,* which are based on the calculation of free enthalpy. Both programs calculated identical 5` NTR structure including the IRES, a prerequisite for cap-independent translation of hepaciviruses. The BovHepV IRES domains II to IV could be identified with a highly similar structure of the loops when compared to HCV (figure 7).

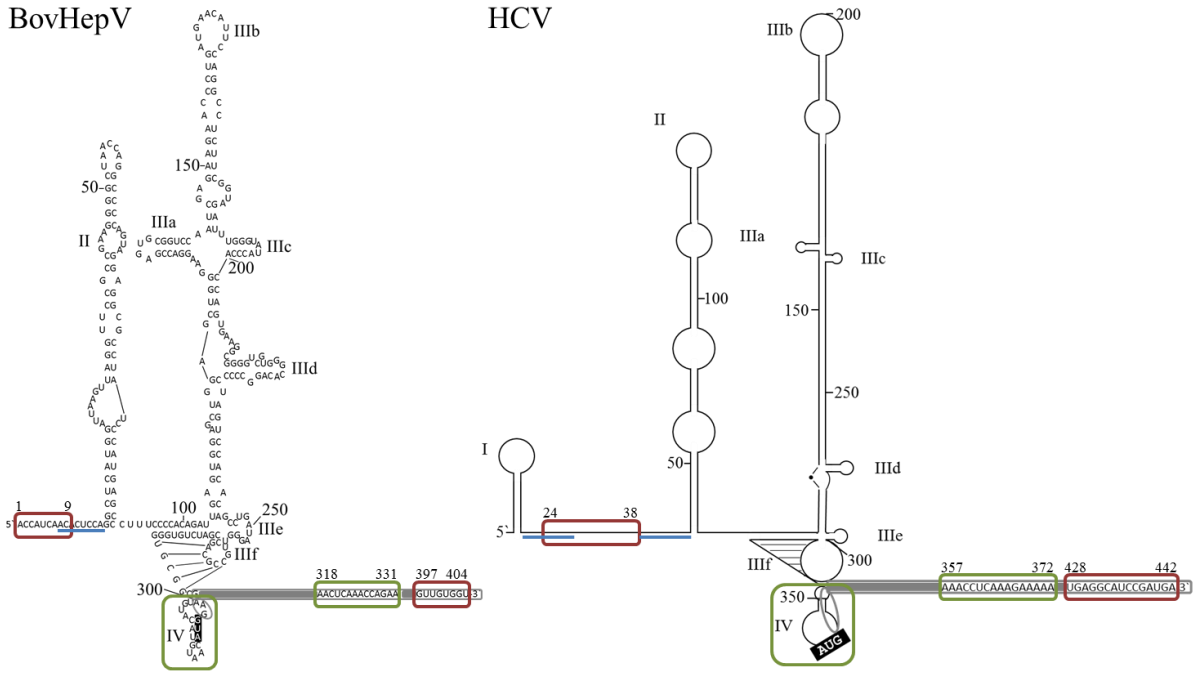


Figure 7: Predicted 5` NTR structure of BovHepV and HCV. HCV contains domains I-IV including pseudoknot IIIf and translation initiation codon AUG containing domain IV. Grey: partial core coding sequence. Red: complementary sequences in the 5` NTR and core coding sequence. Green: destabilizing effect of an adenosine-rich core sequence on domain IV. Blue: miR-122 match site/s.

The domain I of HCV, which is not necessary for virus translation but plays a critical role in replication, was missing in the BovHepV 5` NTR (107). A further basal loop is absent in BovHepV domain II, but apart from this the structure of the domain is very similar to HCV domain II. This finding suggests that this basal loop is not essential for the accumulation of the 40S ribosomal subunit at the IRES. Like HCV, the BovHepV IRES contains domain III including the subdomains IIIa to IIIf with high similarity and a pseudoknot is predicted by sequence analysis. Domain III is the largest structure and contains by far the largest part of the IRES sequence. It seems to be important that this structure is highly conserved for the binding of the ribosomal subunits (40S and 60S) and the eukaryotic translation initiation factor 3 (elF3). Moreover, the most highly conserved sequences can be observed in domain III,

especially in IIIa, and IIId, and the complete sequence of subdomain IIIe is identical to the sequence of HCV. Like HCV, BovHepV IRES domain III is predicted to forms a pseudoknot (IIIf), which is missing in the EHcV IRES structure (147, 148, 151, 157). Furthermore, the translation initiation codon (AUG) is included in domain IV. Seven nucleotides, including AUG, of the core coding sequence are necessary to form domain IV, which can also be observed in the HCV IRES structure. For efficient translation, domain IV has to be melted (206) because the translation efficiency is increased when domain IV is incomplete due to the lack of core coding sequences (detailed explanation in 5.4.2.). Tsukiyama-Kohara et al. and Wang et al. demonstrated efficient cap-independent translation of reporter proteins fused directly to the initiator codon AUG of HCV (299, 300). Unlike HCV, which contains two miR-122 match sites, the BovHepV 5` NTR comprises only one miR-122 match site that corresponds to the first one in HCV.

After establishment of the predicted 5` NTR structure the determination of domain dependent translation was investigated. For this, constructs were generated that contain the individual domains II to IV including the complete 5` NTR and the translation initiation codon AUG. When the complete virus IRES sequence was missing no translation could be observed, as is known for EHcV and HCV in the same experimental system (156). The results showed that all domains of the IRES are important for efficient translation. Furthermore, the construct 5` NTR to AUG has the highest efficiency in comparison to the other four constructs in all cell lines BFH12 (bovine fetal hepatocytes), Huh-7 (human hepatocarcinoma cells) and HeLa (human epithelioid cervix carcinoma cells) and increased the translation most in the bovine cell line BFH12. This result coincides with the finding for HCV that the nucleotide section around the translation initiation codon has to be single-stranded as mentioned before. In relation to the employed cell lines the most efficient translation could be observed in bovine cells, which constitute the natural system for the bovine hepacivirus 5` NTR followed by the human liver cell line Huh-7 and the HeLa cells, where the lowest translation was measured. Apparently the origin of the cells and tissues seem to play a role in translation efficiency. A recent publication in 2018 by Tanaka et al. studied the role of the EHcV 5` NTR in translation initiation and replication (156). First, they folded the EHcV 5` NTR structure to find that it contained domains I to III, that domain IV was missing, and that, in contrast to HCV and BovHepV, an additional domain upstream of domain I exists. Like HCV and BovHepV

domain III consists of the subdomains IIIa-IIIf, but the structure differs from that of HCV and BovHepV domain III, while BovHepV domain III structure is more similar to HCV than EHcV. Moreover, they analyzed the importance of individual domains for translation by deletion of domains II and III in the EHcV and HCV IRES. Comparable to our results, the translation intensity strongly dropped for both EHcV and HCV. By deletion of each domain III subdomains (IIIa-IIIf) the importance of the biggest domain of IRES structure was analyzed in more detail and the translation efficiency was strongly decreased for HCV and EHcV when any subdomain was missing. These results suggest that the highly conserved structure of domain III is necessary for efficient BovHepV and HCV translation. It is also known that the pseudoknot is important for HCV IRES activity (301) and Tanaka et al. disrupted the residue pair within the pseudoknot by mutations and mutated the sequence complementary to the mutations to restore the binding possibility. The translation was decreased with the mutations and increased in the case that putative binding was restored. It has been suggested that the stable pseudoknot positioned the subunit 40S at the site of the initiator AUG (206) so that these findings emphasize the presence of a pseudoknot in the BovHepV IRES structure. In summary, it can be stated that the BovHepV IRES structure which is needed for 5` cap-independent translation initiation, is very similar to the HCV IRES structure. Especially the ribosomal subunits and elF3 binding domain III seems to be highly conserved in the structure of the subdomains IIIa-IIIf and partial nucleotides of these subdomains. This suggests the necessity of domain III structure including the subdomains and conserved nucleotides for optimal binding of the translation complex. Furthermore, the complete IRES structure, including domains II-IV, is essential for translation initiation and disrupted domain IV, which is single-stranded, increased the translation in all three cell lines. The purpose of these two states observed for domain IV is a stabilization by binding of viral proteins during infection and is a mechanism for feedback regulation of translation that may be important for the persistence of BovHepV (206).

## 5.4 Impact of interactions between partial core coding sequences and partial IRES sequences on the BovHepV translation efficiency

### 5.4.1 Long-range interaction

A possible long-range interaction between complementary sequences in the BovHepV IRES and a core coding sequence, which is known for HCV (199–201), was also investigated for complementary BovHepV IRES and core coding sequences. In the presence of this BovHepV core coding sequence the translation was decreased in comparison to the construct with the incomplete domain IV and in the absence of the complementary BovHepV IRES sequence markedly increased. For the investigation of such a putative interaction by binding of these two complementary sequences in more detail the relevant sequences were mutated. The mutation referred to the sequence in the IRES, the core coding sequence or both sequences. Experiments with these three constructs yielded the same results: the translation levels were comparable to the case in which the complementary sequence to the core coding sequence in the IRES was absent. Moreover, the sequence in the IRES was complementarily mutated to the mutation in the core coding sequence to prove an effect on translation efficiency. The decreased translation efficiency was comparable to that observed for the native BovHepV sequences, where the effect of the complementary binding is independent of the nucleotides but only from the position in the BovHepV genome and the complementarity. Honda et al. also mutated a short sequence in the core coding sequence which led to decreased translation similar to our findings (200). One different result could be observed between the three cell lines in which the highly miR-122 expressing Huh-7 cells showed differences in translation of the mutated constructs. If the overlapping part of the miR-122 match site together with the complementary sequence in the 5` NTR was mutated, then the translation efficiency was decreased in comparison to the single mutation in the core coding sequence. This suggests additional effects of miR-122 on translation other than holding the BovHepV sequence in the open conformation and thus increasing translation. Moreover, it could be shown that the translation was decreased in the absence of miR-122 (302). Further functions of miR-122 affect stabilization of the genome, enhancement of HCV replication, the support of infectious particle production and the protection of non-capped RNA against exoribonucleases like Xrn1 (158, 252, 254, 303). Recently, in the beginning of 2018, Tanaka et al. investigated roles of the EHcV 5` NTR in translation initiation and viral replication. Among others they analyzed

the influence of core coding sequences on translation efficiency and could observe decreased translation, which suggests that this inhibitory interaction can also be found in NHPV (156). With regard to the utilized cell lines and their host origins, the bovine hepatocytes exhibited the highest activity followed by the human hepatocytes and the human cervix carcinoma cells. This suggests that the bovine hepacivirus IRES mediated translation is most efficient in the natural origin BFH12 cell line which contains translation machinery proteins of the authentic host origin.

### 5.4.2 Destabilization effect of an adenosine-rich part in the core protein coding sequence on BovHepV IRES domain IV

For HCV destabilization of HCV IRES domain IV by an adenosine-rich part in the core coding sequence could be observed. Due to this effect the ribosomal subdomain 40S can interact with the viral RNA and especially with the single-stranded translation initiation codon AUG (207). The study of a possible destabilizing effect of an adenosine-rich part in the core coding sequence on BovHepV domain IV showed decreased translation in the presence of such a sequence. The translation efficiency was comparable to the translation efficiency observed for the construct where the formation of domain IV was prevented (discussed in 5.3) and highly increased in comparison to the construct harboring and forming the complete domain IV. Honda et al. reported that mutations that enhance the stability of domain IV stem-loop adversely affect the rate of HCV translation and that domain IV is not essential for HCV translation (206). Further studies demonstrated efficient cap-independent translation of reporter proteins fused directly to the initiator AUG lacking the downstream HCV sequence that forms the 3` strand of the domain IV (299, 300). Destroying of domain IV is essential for the direct binding of ribosomal subunit 40S because it does not scan the 5` NTR (206). Furthermore, it has been proposed that the cellular La autoantigen interacts with the HCV RNA in this region of this stem-loop which facilitates translation (213). There might be an equilibrium between the open and closed conformation of domain IV and therefore between efficient and inefficient translation of HCV and BovHepV. This effect, like the long-range interaction, could play a role in the switch between translation and replication and in persistence of the virus in infected individuals. Moreover, reducing the expression of HCV

proteins to minimal levels would maximize the chance that the virus evades detection by the immune system and such a strategy could also apply for the bovine hepacivirus.

One positive tested cattle with a viral genome load of 2.92 x $10^5$ genome equivalents per mg liver tissue possessed no detectable viral RNA in different organs like brain, heart, lung, kidney, spleen, udder and different lymph nodes (167). Furthermore, *postmortem* liver tissue analysis showed mild lipidosis, while degenerative and inflammatory changes which can be frequently observed during virus infections, could not be detected. Moreover, the activities of the liver enzymes aspartate-aminotransferase (AST), ɣ-glutamyl transferase (ɣ-GT), glutamate dehydrogenase (GLDH) and total bilirubin were determined for BovHepV positive and negative cows, but significant differences could not be observed for infected and non-infected animals (167). These findings support the assumption that the suggested interaction between IRES and core protein coding complementary sequences may contribute to the switch between translation and replication, which may facilitate immune evasion and the establishment of persistent infection.

### 5.5 Role of cellular miR-122 on BovHepV translation

The micro RNA-122 is highly expressed in the human liver and the binding of miR-122 at both match sites in the HCV 5` NTR enhances the translation of the viral genome, for what reason HCV possesses liver tropism (255). In comparison to HCV, BovHepV has only one miR-122 match site in the 5` NTR that is equivalent to the first match site in HCV. Because BovHepV RNA is most abundant in the liver and the virus could be detected in liver cells by fluorescent *in situ* hybridization a liver tropism has been suggested (167). In comparison to BFH12 and HeLa cells, Huh-7 cells expressed by far the highest miR-122 amount. High miR-122 expression was also observed in native bovine liver tissue. Additionally, for human liver cell lines like HepG2 and Hep3B the absence of miR-122 expression has been described (252, 304). Furthermore, it has been suggested that the binding of miR-122 at the first match site in the HCV 5` NTR protects the non-capped viral RNA against degradation by host exoribonucleases like Xrn1, enhances viral replication, supports the production of infectious particles and shifts the thermodynamic equilibrium of closed and open conformation formed by the long-range interaction to the open form (158, 202, 252, 254, 303).

MiR-122 represents the majority of microRNA in hepatocytes and is expressed rarely in other tissues. Considering the observed BovHepV liver tropism, an influence of miR-122 on BovHepV translation efficiency was investigated. In a variety of experiments the miR-122 match site was excluded, completely or partially mutated and the cells were co-transfected with miR-122 duplexes, complementary micro RNA to the complete mutated sequence of the miR-122 match site, miR-122 inhibitory duplexes or a random micro RNA. It could be observed that the co-transfection of miR-122 increases the translation most efficiently in HeLa cells followed by BFH12 cells, but not in Huh-7 cells which express by far the highest miR-122 level followed by BFH12 and HeLa cells. Accordingly, a dose-dependent effect of miR-122 on translation was postulated. However, this effect seems to be limited because additional co-transfection of miR-122 showed no increasing effect on translation efficiency in Huh-7 cells. Diaz-Toledano et al. and Henke et al. also found a stimulating effect of miR-122 on HCV translation efficiency (202, 255). Moreover, Scheel et al. has recently reported that miR-122 has a stimulating effect on EHcV translation using a full length genome clone and co-transfected Huh-7.5 cells with or without miR-122 in a dual luciferase system in accordance to the bovine study. The studies of human, equine and bovine hepacivirus translation show the same stimulating effect of miR-122 contributing to the liver tropism of HCV, EHcV and BovHepV. When the miR-122 match site was deleted in the BovHepV 5` NTR in the presence of miR-122 the translation was strongest decreased in Huh-7 cells followed by HeLa and BFH12 cells. In comparison to native liver tissue the immortalized bovine liver cell line showed a much lower miR-122 expression, though an *in vitro* and an *in vivo* system cannot be directly compared. Moreover, there might be other cellular components that interact with the test system and therefore lead to the lower changes in comparison to the human liver cell line or the human non liver cell line. This possibility should be investigated in more detail in future studies. Honda et al. could show for HCV that the mutation of only two nucleotides in the miR-122 match site is sufficient to decrease the translation of HCV (247). Equivalent mutations were inserted in the BovHepV miR-122 match site and after co-transfection with miR-122 the translation efficiency was decreased in all three cell lines when compared to the parental construct supplemented with miR-122. The strongest effect was seen again in HeLa cells followed by Huh-7 cells and the lowest effect was observed in BFH12 cells. These results suggest that, as described before by Honda et al. and Roberts et al., only

two point mutations in the miR-122 match site are sufficient to decrease the translation for BovHepV in the presence of co-transfected miR-122. Moreover, for the investigation of whether the exact miR-122 match site nucleotide sequence is important for enhanced translation, the complete match site was mutated and co-transfected with a complementary micro RNA duplex. In comparison to the parental construct co-transfected with miR-122 duplexes, the translation efficiency was similar in BFH12 and Huh-7 cells and therefore could be restored. This result advises that the stimulating effect of the micro RNA binding at the match site in the bovine 5` NTR is independent of the nucleotide sequence. An enhancing effect could not be observed in the human non liver cell line, which suggested that other cellular factors might interact with the artificially produced micro RNA or a rapid degradation of the micro RNA by cellular exoribonucleases might occur. In addition, the inhibition of miR-122 and the resulting effect on translation was investigated by co-transfection with miR-122 and a miR-122 inhibitor. A diminished translation was seen in HeLa and less significant also in BFH12 cells compared to the parental constructs co-transfected with miR-122. This might be due to the fact that the miR-122 abundance in these two cell lines was too low to reveal a positive effect on translation. On the other hand the translation in the highly expressing Huh-7 cells was unchanged, which shows again the dose dependent impact of miR-122 and suggests that the high concentration of endogenously expressed miR-122 is sufficient for efficient translation. To investigate a possible effect on translation in the presence of an unspecific micro RNA the parental construct was co-transfected with a random micro RNA. In all three cell line the translation was unchanged in comparison to the parental construct without any miRs co-transfections.

To summarizing, by use of molecular methods it could be shown that the BovHepV 5` NTR forms an interaction with a sequence in the core coding region that decreases the translation efficiency of BovHepV. This could play a role in the switch of translation to replication and/or a strategy for persistence of BovHepV and to evade the host immune system by minimizing replication. Moreover, the destabilization of domain IV by an adenosine-rich part in the core coding sequence enhances the translation efficiency because domain IV has to be present as single strand for the binding of the ribosomal subunit at the translation initiation codon. In relation, the cellular miR-122 enhances the translation efficiency in a dose dependent manner and is highly expressed in Huh-7 cells and bovine liver tissue. Moreover,

the detection of hepaciviral genomes in bovine liver tissue suggests a liver tropism for BovHepV. Further studies in view of the role of miR-122 will focus on viral replication, stabilization and protection of the virus genome from degradation by cellular exoribonucleases like XrnI will be important in regard to HCV and to undergird the liver tropism of BovHepV.

## 6. Abstract

"Biological characterization of Bovine Hepacivirus" by Anna Lena Baron

The bovine hepacivirus (BovHepV) was initially described in 2015 and added to the genus *Hepacivirus* as cluster N within the family *Flaviviridae*. Besides HCV and BovHepV, hepaciviruses were also discovered in horses, bats, rodents and sharks (hepacivirus-like), while only the equine hepacivirus (EHcV) infecting horses has been studied in more detail with respect to the IRES and the role of microRNA-122 (miR-122). The BovHepV genome was initially identified in bovine serum samples and can cause persistent infections in cows. So far, the effects of these persistent infections and the response of the humoral immune system after acute or chronic infection have not been investigated. Moreover, the possibility of interspecies transmission including zoonotic potential is uncertain. In this study, a BovHepV positive herd was screened over a time period of one year in intervals of two to three months to detect acute versus persistent infections. Nearly 20 % of the individual bovine samples contained anti-NS3-antibodies and almost 8 % had detectable viral RNA. Phylogenetic analysis resulted in the identification of an additional genetic cluster in the genus *Hepacivirus*. Furthermore, the anti-NS3-antibody levels were negative at each time point in persistently infected cattle. No hint at liver injury could be observed. The analysis of samples from other animal species showed that only one porcine serum sample comprised antibodies reacting with BovHepV antigen, but BovHepV RNA was not detectable. All equine and human samples contained neither BovHepV-specific RNA nor antibodies.

On the other hand on a molecular level the predicted BovHepV IRES (internal ribosome entry site) structure revealed high accordance with the HCV IRES including domains II, IIIa-IIIe, the pseudoknot IIIf and the translation initiation codon (AUG) containing domain IV. Like HCV some nucleotides of the core coding sequence are required to form domain IV. Unlike HCV, domain I is missing and only one instead of two miR-122 match sites exists in the BovHepV 5` NTR. The necessity of individual IRES domains for efficient translation and the effects of interactions between BovHepV 5` non-translated region (NTR) and core coding sequences were investigated in this study. The results indicate that (i) all IRES domains are essential for efficient translation, (ii) a predicted long-range interaction between the 5` NTR

and a complementary sequence in the core coding region decreased the translation, and (iii) an adenosine-rich part in the core coding sequence seems to destabilize the AUG containing domain IV through which the translation is enhanced. Furthermore, the impact of a possible miR-122 binding at the 5` NTR on translation was analyzed. The employed human cell lines revealed an influence of miR-122 on translation, whereas in a bovine cell line a pronounced effect could not be observed. Therefore, additional functions of miR-122, for example on BovHepV replication, need to be investigated, as this micro RNA is highly expressed in the liver, contains the highest abundance of bovine hepacivirus RNA detected by RT-PCR and FISH (fluorescence *in sito* hybridization).

## 7. Zusammenfassung

"Biologische Charakterisierung des bovine Hepaciviruses" von Anna Lena Baron

Das bovine Hepacivirus (BovHepV) wurde erstmals im Jahr 2015 beschrieben und dem Genus *Hepacivirus* Gruppe N in der Familie der *Flaviviridae* zugeordnet. Neben HCV und BovHepV wurden weitere Hepaciviren in Pferden, Fledermäusen, Nagern und Haien (hepacivirusähnlich) entdeckt, wobei das equine Hepacivirus (EHcV) als einziges, in Bezug auf die IRES und die Rolle von miR-122 (mikroRNS-122), intensiver untersucht wurde. Das BovHepV Genom wurde zunächst in bovinem Serum identifiziert und kann zu persistenten Infektion bei Rindern führen. Bisher wurden die Effekte dieser persistenten Infektionen und die Reaktion des humoralen Immunsystems nach einer akuten oder chronischen Infektion nicht untersucht. Des Weiteren ist die mögliche Übertragung auf andere Spezies, welches ein zoonotisches Potential beinhaltet, nicht bekannt. In dieser Arbeit wurde eine BovHepV positive Herde über ein Jahr in Intervallen von zwei bis drei Monaten beprobt, um eine Aussage über akute im Vergleich zu persistente Infektionen treffen zu können. Nahezu 20 % der individuellen bovinen Proben enthielten anti-NS3-Antikörper und fast 8 % wiesen nachweisbare virale RNS auf. Phylogenetische Analysen führten zu der Identifizierung einer zusätzlichen genetischen Gruppe im Genus *Hepacivirus*. Zu jedem Zeitpunkt besaßen persistent infizierte Rinder keine anti-NS3-Antikörper und es gab keinen Hinweis auf eine Leberschädigung. Bei der Untersuchung von anderen Tierarten besaß nur eine porcine Serumprobe reagierende Antikörper mit dem BovHepV Antigen, wobei keine BovHepV RNS nachweisbar war. Zudem hatten die equinen und humanen Proben weder BovHepV spezifische RNA noch Antikörper.

Auf molekularer Ebene, hat die vorhergesagte BovHepV IRES (*internal ribosome entry site* entspricht interne Ribosomeneintrittstelle) Struktur eine hohe Ähnlichkeit mit der HCV IRES, was die Dömanen II, IIIa-IIIe, der Pseudoknoten IIIf und das in der Domäne IV vorhandene Translationsinitiationskodon (AUG) betrifft. Übereinstimmend mit HCV sind einige kapsidkodierende Nukleotide notwendig für die Ausbildung von Domäne IV. Im Gegensatz zu HCV fehlt Domäne I und es ist nur eine anstatt zwei miR-122 Bindungsstellen in der 5` NTR (nicht translatierenden Region) vorhanden. In dieser Arbeit wurde die

Notwendigkeit der individuellen IRES Domänen für eine effiziente Translation und die Effekte durch Interaktionen zwischen dem BovHepV 5` NTR und der kapsidkodierenden Sequenzen untersucht. Die Ergebnisse weisen darauf hin, dass (i) alle IRES Domänen für eine effiziente Translation essentiell sind, (ii) eine vorhergesagte lange Reichweiteinteraktion zwischen dem 5` NTR und einer komplimentären Sequenz in der kapsidkodierenden Region zu einer verminderten Translation führt und (iii) dass ein adenosinreicher Bereich in der kapsidkodierenden Sequenz die AUG beinhaltene Domäne IV zu destabilisieren scheint, wodurch die Translation gesteigert wird. Zusätzlich wurde eine Wirkung auf die Translation durch eine mögliche miR-122 Bindung im 5` NTR untersucht. Die humanen Zelllinien zeigten einen Einfluss auf die Translation durch eine mögliche Interaktion von miR-122, wohingegen in der bovinen Zelllinie kein ausgeprägter Effekt sichtbar war. Deswegen ist es notwendig weitere Funktionen der miR-122, zum Beispiel auf die BovHepV Replikation, zu untersuchen. In der Leber ist diese mikro RNS stark exprimiert und zudem ist die größte Menge an boviner Hepacivirus RNA, detektiert durch RT-PCR und FISH (*fluorescence in sito hybridzation* entspricht Fluoreszenz-*in sito*-hybridisierung) , in diesem Organ vorhanden .

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