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Development of an intra- and intergenotypic HCV cell culture method to phenotype and assess antiviral susceptibilities and resistance development of HCV NS3 protease genes from HCV genotypes 1-6

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To my family and loved ones

Abstract

The development of specific antiviral drugs directly targeting the hepatitis C virus (HCV) is clinically important, as the current standard interferon/ribavirin combination treatment is only partially effective, expensive and often associated with severe side effects. Inhibitors of the NS3 protease (PI) therefore represent a promising alternative or additional therapy. To date, the development and *in vitro* evaluation of PIs is restricted to the genotype 1/2 based replicon and the genotype 2a full length viral cell culture system. However, proteases of the different HCV genotypes vary substantially in their amino acid sequence and secondary structure and require separate evaluation of their efficacy before they go into clinical trials.

To address this issue, a panel of intra- and intergenotypic recombinants based on the recombinant infectious clone Jc1 (pFK JFH1/J6/C-846) was developed in this work. The viability of these recombinants was assessed in the Huh7.5 cell culture system, where replicating viruses were detected by HCV-NS5A immunostaining. Intergenotypic recombinants containing genotype 1a, 1b, 3a, 4a and 6a derived proteases were replication defective, whereas the recombinant with genotype 5a derived protease replicated efficiently after acquiring cell culture adaptive mutations. The replacement of not only the NS3 protease gene region, but also its cofactor NS4A, allowed the generation of replication competent intra- and intergenotypic recombinants for all 6 major genotypes. Replacing the NS3 protease of the recombinants with that of patient-derived proteases also generated replicating recombinants, greatly expanding the panel of intergenotypic recombinants available for phenotyping and PI evaluation. However, intra- and intergenotypic recombinants showed substantial differences in their replication kinetics, which may be influenced by naturally occurring polymorphism between genotypes and the differential requirement of adaptive/attenuating cell culture mutations. Genotype 1a recombinants replicated very poorly, which may be due to incompatibilities between the type 1a NS3/4A protease and the type 2a backbone.

50 % inhibitory concentrations (IC_{50}) of different PIs were measured using Foci Forming Units/ml (FFU/ml) reductions and replication inhibition assays. The different recombinants showed consistent, genotype-associated differences in their susceptibility to the PI BILN 2061, with genotypes 2a, 3a and 5a derived recombinants showing approximately 100-fold lower susceptibility than genotype 1b, 4a and 6a derived recombinants. These observations are consistent with major differences in response rates found in recent treatment trials of genotype 1, 2 and 3 infected patients. Differences in susceptibility were also observed for VX-950, with genotype 1b, 2a and 6a derived recombinants being twice as susceptible than genotype 3a, 4a and 5a derived recombinants. Passaging the intra- and intergenotypic recombinants under increasing concentrations of PI allowed the identification of PI resistance mutations. Resistance mutations to BILN 2061 mapped to the previously identified positions 156 and 168 within the NS3 protease, with a great diversity of amino acid substitutions observed within each genotype. Reintroduction of the identified resistance mutations into the original recombinant viruses conferred increased resistance towards BILN 2061 and some mutations also affected replication kinetics of the recombinants. The developed system will be of major value for the phenotypic characterisation of naturally occurring and treatment induced resistance mutations within all 6 major HCV genotypes towards different PIs. This will allow treatment response predictions for newly developed PIs before they enter clinical trials and the development of individually tailored antiviral treatment regimes.

Declaration of originality

I hereby declare that the research recorded in this thesis and the thesis itself was composed and originated entirely by myself at the Centre for Infectious Diseases at the Royal Dick School of Veterinary Studies at the University of Edinburgh.

Ingrid Imhof

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Abbreviations

2’5’OAS	2’5’ Oligoadenylate Synthetase
3D	3-Dimensional
A	Adenosine
AIDS	Acquired Immune Deficiency Syndrome
ALT	Alanine Aminotransferase
ASP	Aspartate Aminotransferase
AP	Antarctic Phosphatase
APC	Antigen Presenting Cell
ApoE	Apolipoprotein E
ARFP	Alternate Reading Frame Protein
BMI	Body Mass Index
bp	base pair
BSA	Bovine Serum Albumin
C	Cytosine
CARD	Caspase Recruitment Domain
Cardif	CARD adaptor IFN-β
CARMV	Carnation Mottle Virus
cDNA	complementary DNA
CMV	Cytomegalovirus
CREs	Cis-acting Replication Elements
CypA/B	Cyclophilin A/B
CysA	Cyclosporine A
DCs	Dendritic Cells
DEPC	Diethylpyrocarbonate
DEPC-PBS	DEPC-treated PBS
CD81	Cluster of Differentiation 81
CLDN1	Claudin-1

DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin
DM	Diabetes Melltius
D-MEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dNTP	deoxy-Nucleoside Triphosphate
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EB	Elution Buffer
EBV	Ebstein-Barr Virus
EC_{50}	50 % Effective Concentration
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EHMs	Extrahepatic Manifestions
eIF	eukaryotic Initiation Factor
ELISPOT	Enzyme-Linked Immunospot
EoTR	End of Treatment Response
ER	Endoplasmic Reticulum
EtBr	Ethidium Bromide
EVR	Early Virological Response
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFU	Focus Forming Units
G	Guanosine
GAGs	Glycosaminoglycans
GAS	Gamma Activated Sequence
GBV-A	GB Virus A
GBV-B	GB Virus B
GBV-C	GB Virus C

GTP	Guanosine Triphosphate
HAART	Highly Active Antiviral Retrotherapy
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HCVcc	cell culture produced HCV
HCVpp	HCV pseudoparticles
HDL	High Density Lipoprotein
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
H_2O	Water
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSPG	Heparan Sulfate Proteoglycan
Huh	Human hepatoma
HVR	Hyper Variable Region
IC_{50}	Half maximal Inhibitory Concentration
IDU	Injecting Drug User
ISDR	Interferon Sensitivity-Determining Region
IFN	Interferon
IFNAR	IFN-α/β Receptors
IMP	Inosine Monophosphate
IMPDH	IMP Dehydrogenase
IPS-1	IFN Promoter Stimulator
IR	Insulin Resistance
IRES	Internal Ribosome Entry Site
IRF	IFN Regulatory Factor
ISDR	Internal Ribosome-Entry Site
ISG	IFN Stimulated Gene
ISGF3	IFN Stimulated Gene Factor 3
kb	kilo base pairs

LB	Luria-Bertani
LD	Lipid Droplet
LDL	Low-Density Lipoprotein
L-SIGN	Liver/lymph Node-Specific Intercellular Adhesion Molecule-3-Grabbing Integrin
MAVS	Mitochondrial Antiviral Signaling Protein
MC	Mixed Cryoglobulinaemia
MDA5	Melanoma Differentiation Antigen 5
$MgCl_2$	Magnesium Chloride
$MgSO4_4$	Magnesium Sulfate
MHC	Major Histocompatibility Complex
M.O.I	Multiplicity of Infection
MSM	Men who have Sex with Men
nAb	neutralizing Antibody
NANBH	Non-A, non-B Viral Hepatitis
NF-κB	Nuclear Factor κB
NHL	Non-Hodgkin Lymphoma
NNI	Non-Nucleoside Inhibitor
nt	nucleotide
OD	Optical Density
ORF	Open Reading Frame
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1 Receptor
PePHD	PKR-eIF2α Phosphorylation Homology Domain
PI	Protease Inhibitor
PKR	dsRNA-activated Protein Kinase R
PNR	Primary Non-Responder
QALY	Quality-Adjusted Life Year
QC	QuickChange® site directed mutagenesis

REMs	Replication Enhancing Mutations
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic Acid Inducible Gene-I
RNA	Ribonucleic Acid
rpm	rounds per minute
RT	Reverse-Transcription
RVR	Rapid Virological Response
SC	SuperScript^{TM} III One-Step RT-PCR
SCID	Severe Combined Immunodeficiency
shRNA	short hairpin RNA
siRNA	small interfering RNA
SN	Supernatant
SOC	Super-Optimal Broth with Catabolite Repression
SOCS	Suppressors of Cytokine Signalling
SR-BI	Scavenger Receptor Class B Type I
ssRNA	single-stranded RNA
STAT	Signal Transducers and Activators of Transduction
STAT-C	Specifically Targeted Antiviral Therapy for Hepatitis C
SVR	Sustained Virological Response
T	Thymine
TAE	Tris-Acetate-EDTA
TAH	Transfusion-Associated Hepatitis
$TCID_{50}$	50 % Tissue Culture Infective Dose
TLR	Toll-Like Receptor
TMD	Transmembrane Domain
TNE	Tris-Sodium Chloride-EDTA
TRIF	Toll-IL-1 Receptor Domain-containing Adaptor Inducing IFN-β
VLDL	Very Low Density Lipoprotein
VPU	Viral Protein U
U	Units
U	Uracil

uPA	urokinase Plasminogen Activator
UTR	Untranslated Region
UV	Ultra-Violet
WT	Wild-Type

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Chapter 1
Introduction

1.1 History of Hepatitis C Virus

Viral hepatitis is the main cause of liver cancer and the most common reason for liver transplantations. It is a major public health issue and prevalent around the world. The problem of non-A, non-B viral hepatitis (NANBH) became evident in 1975 after serological tests for hepatitis A virus (HAV) and hepatitis B virus (HBV) became available. Until then HAV and HBV were considered to be the major aetiological agents of transfusion-associated hepatitis (TAH). HAV is a positive-stranded RNA virus in the genus *Hepatovirus* in the family *Picornaviridae*. Primary transmission route is the faecal-oral route and it is a major concern in developing countries with poor sanitation (Melnick (1982); Matthews (1979)). HBV is a hepatotropic double-stranded DNA (dsDNA) virus with a circular genome classified within the genus *Orthohepadnavirus*, which is a member of the family *Hepadnaviridae*. It causes inflammation of the liver, which can lead to cirrhosis and hepatocellular carcinoma and has also been associated with increased risk of pancreatic cancer recently (Hassan *et al.* (2008)).

Serological testing for HAV and HBV in TAH showed that most cases did not have serological markers of either of these viruses (Feinstone *et al.* (1975)). In this clinical survey on 22 patients, 9 patients had an antibody response to cytomegalovirus (CMV), also known to cause liver damage. However, it was difficult to relate this seroconversion to their hepatitis. Furthermore, all patients had pre-existing antibodies to Epstein-Barr virus (EBV), another virus associated with liver damage. This lead to the hypothesis that at least some of these serologically-negative TAH were caused by other infectious agents not yet identified. Later it was shown that as many as 10 % of transfusions resulted in NANBH and that the incidence of hepatitis was associated to alanine aminotransferase (ALT) levels in the donor blood (Berman *et al.* (1979);

Aach *et al.* (1981)). NANBH was later demonstrated to cause persistent liver damage in the majority of cases, which could lead to liver cirrhosis in up to 20 % of cases (Hoofnagle & Alter (1985)). It took another 14 years before the aetiological agent of NANBH was identified. The search was hampered by the inability to culture the agent in organ or cell culture. But the development of the chimpanzee model, in which acute and chronic NANBH could be induced by experimental infection with NANBH patient blood proved to be a considerable step forward (Tabor *et al.* (1978); Alter *et al.* (1978); Hollinger *et al.* (1978)). A serological study following up cardiovascular patients, who had received donor blood, provided evidence that NANBH could be transmissible between humans. The existence of an additional virus(es), named hepatitis type C was suggested (Prince *et al.* (1974)). The ability of this virus to pass through 80 nm membranes, the apparent lack of nucleic acid homology with HBV and the sensitivity to chloroform led to suggestions that the aetiological agent of NANBH was a small, enveloped RNA virus (Feinstone *et al.* (1983); Bradley *et al.* (1983, 1985)).

In 1989, a random-primed complementary DNA (cDNA) library was constructed from plasma containing the so far uncharacterised NANBH agent. Screening of expressed recombinant proteins from the cDNA library with serum from a patient, diagnosed with NANBH, revealed a cDNA clone that was shown to encode an antigen associated specifically with NANBH infections. Using Southern blot analysis it was shown that this clone, named 5-1-1, was derived from an RNA molecule present in NANBH infections and not from chimpanzee or human DNA. Clone 5-1-1 and subsequent overlapping clones derived from the same cDNA library hybridised to a large, single-stranded (ss) RNA molecule of about 10,000 nucleotides that was present only in NANBH-infected samples. In addition, it could be shown that an antigen directly derived from the RNA molecule itself was immunoreactive in NANBH-infected chimpanzees but not in those infected with HAV or HBV, indicating that it was positive-stranded like the *Flaviviridae* genomes. Other proof of the aetiological origin of clone 5-1-1 provided the fact that the majority of NANBH patients in a small cohort had circulating antibodies specific for the gene products of clone 5-1-1 (Choo *et al.* (1989)). Based on these findings the aetiological agent of NANBH was named hepatitis C virus (HCV).

The development of a first-generation blood screening test was based on antigen c100-3, produced in recombinant yeast from 5-1-1 and adjacent HCV clones. Using c100-3, an enzyme immunoassay for circulating antibodies against c100-3 was developed. This assay showed that circulating HCV antibodies were traceable in most infectious blood donors and chronically infected NANBH patients. It could also be demonstrated that HCV was the major global cause of parentally-transmitted NANBH (Kuo *et al.* (1989)).

Many applications were now possible, the most important being the development of a blood test to protect the blood supply, as well as diagnostics and new, more effective drugs for the treatment of HCV patients. In 1990 the first generation blood test became available and universal screening of donor blood was initiated in most Western countries. The first generation assay detecting antibodies against HCV led to a 70 % decrease in HCV infection incidence rates down to 1.5 %. A more sensitive second generation assay, introduced in 1992, nearly extinguished the HCV transmission rate (Alter & Houghton (2000)). Today the main risk for HCV infection is intravenous drug use.

1.2 The Molecular Virology of HCV

1.2.1 Genome structure and classification

Worldwide about 170 million individuals are estimated to be infected with HCV (WHO (1999); Shepard *et al.* (2005)). Following the discovery of the aetiological agent of hepatitis C in 1989 (clone 5-1-1), several other clones were reconstructed from overlapping cDNA clones (Choo *et al.* (1991); Takamizawa *et al.* (1991); Okamoto *et al.* (1991); Kato *et al.* (1990)). Sequence comparison between these strains showed only 80-90 % homology, reflecting the diverse nature of an RNA virus and the existence of several subtypes. From comparative analysis of these genomes, the structure and organisation of the virus was determined. It was demonstrated that the virus has an RNA genome of approximately 9,500 nucleotides (nt) with a single open reading frame

(ORF) encoding for a 3,010 to 3,033 amino acid long single polyprotein. The ORF is flanked by a well conserved 5' untranslated region (5'UTR), postulated to be 341 nt long, and a more variable 3'UTR. The polyprotein itself could be processed by cellular proteases and viral proteases as proposed for other flaviviruses. This generated the structural proteins core (C), E1 and E2 (envelope proteins) and p7, as well as the downstream encoded non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B.

Comparative sequence analysis of the HCV genome and encoded polyprotein showed that HCV has a basic genetic organisation and polyprotein structure similar to those of pestiviruses and flaviviruses (Choo *et al.* (1991)). Computer analysis revealed that HCV possessed statistically significant similarity to 2 sequences in the protein data bank, the NS3 protein of dengue type 2 and the putative replicase of carnation mottle virus (CARMV), a member of the *Carmovirus* family. This was unexpected as CARMV is a plant virus. It was suggested that this finding adds support to the hypothesis that there is an evolutionary relationship between animal and plant viruses (Miller & Purcell (1990)).

Since then HCV has been classified as a member of the *Flaviviridae* family (Fig. 1.1). Members of this family are small, enveloped viruses and contain a positive-sense, ssRNA genome, which encodes a single polyprotein (Thiel (2005)). The family of *Flaviviridae* is divided into the 3 genera of *Flavivirus*, *Hepacivirus* and *Pestivirus*, but also includes the unassigned viruses Hepatitis G Virus (HGV)/GB Virus C (GBV-C) and GBV-A. HCV and GBV-B are the only members of the genus *Hepacivirus*. GBV-A and GBV-B were discovered in tamarins infected with the sera of a patient (initials GB) with mild hepatitis and are closely related to HCV (Deinhardt *et al.* (1967); Simons *et al.* (1995)). GVB-B is the phylogenetically closest relative to HCV and GVB-B infected tamarins develop an acute infection that evolves to chronicity similar to HCV infection (Bukh *et al.* (1999); Martin *et al.* (2003)). GBV-C, formerly known as HGV, is closely related to GBV-A and GBV-B and shares about 25-30 % sequence similarity with HCV (Leary *et al.* (1996)). GBV-C infects humans, but has not been associated with any disease. However, it is believed that GBV-C can prolong HIV disease progression (Shankar *et al.* (2008)).

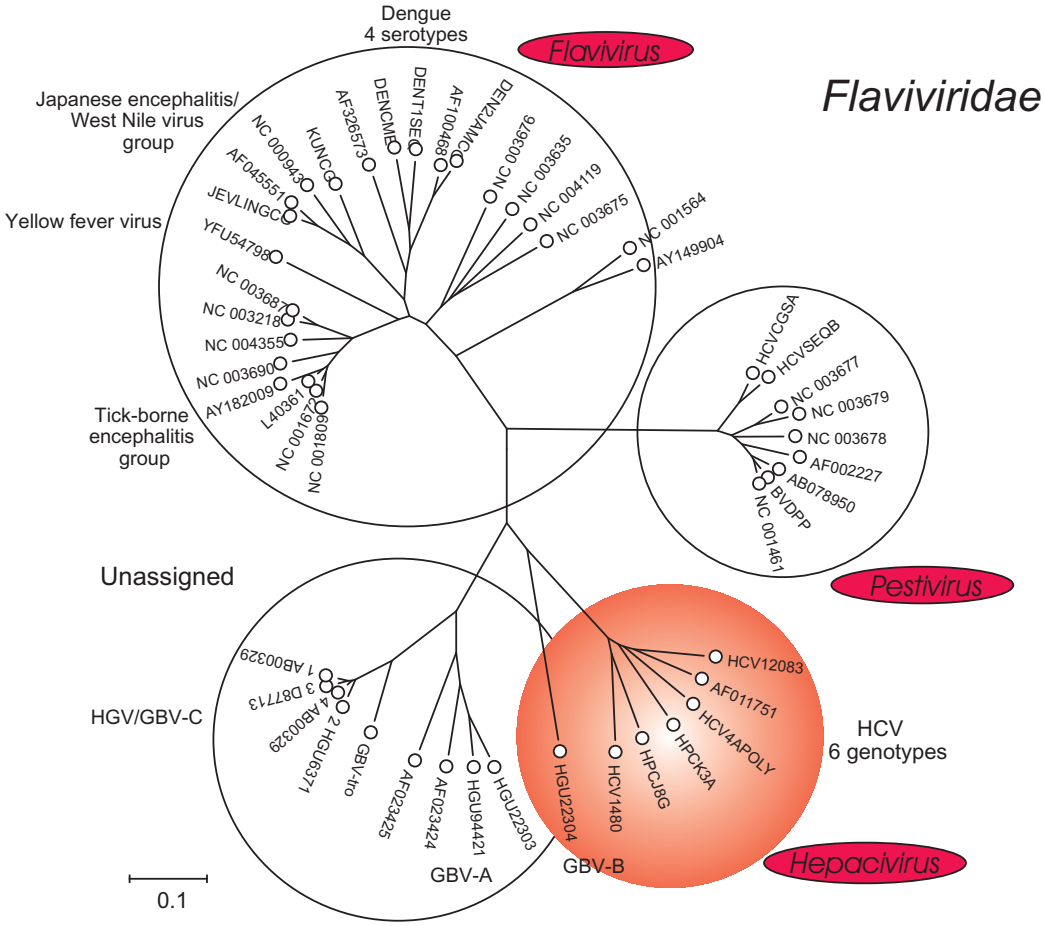

Figure 1.1: ***Phylogenetic tree of the Flaviviridae Family.*** *The Flaviviridae are a family of viruses divided into the 3 genera Flavivirus, Hepacivirus and Pestivirus. It also includes the unassigned viruses HGV/GBV-C and GBV-A. HCV and GBV-B are the only members of the genus Hepacivirus. (Reproduced with permission of P. Simmonds.)*

1.2.2 HCV structure

HCV genome organisation

The 9.6 kilo base pairs (kb) long HCV genome is translated into one long polyprotein that undergoes co-translational and post-translational proteolytic processing in the cytoplasm or endoplasmic reticulum (ER) of the infected cell. Host and viral peptidases process the polyprotein from the N-terminal region into at least 10 structural and non-structural proteins: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Some reports have suggested the existence of an 11^{th} protein, translated from an alternative ORF overlapping the core gene at nucleotide +1 (Walewski *et al.* (2001); Xu *et al.* (2001); McMullan *et al.* (2007)). The structural proteins comprise the N-terminal third of the polyprotein and the non-structural proteins are located within the C-terminal "two thirds" (Fig. 1.2).

An important characteristic of the HCV genome is its high degree of genetic variability, with different regions varying widely in their mutation rate (Martell *et al.* (1992); Pawlotsky (2006)). The most variable are the regions E1 and E2, whereas the 5'UTR and the terminal segment of the 3'UTR show the highest degree of sequence conservation among different HCV variants. The 5'UTR, which is around 341 nt long, contains an internal ribosomal entry site (IRES). The IRES, which contains the 4 highly structured domains I-IV, is essential for the cap-independent translation of the viral RNA (Bukh *et al.* (1992); Brown *et al.* (1992); Honda *et al.* (1999); Tsukiyama *et al.* (1992); Wang *et al.* (1993)). These structures are mostly conserved among HCV and related viruses (Brown *et al.* (1992); Honda *et al.* (1999)). As seen in other viruses with IRES-mediated expression, the HCV 5'UTR contains critical elements for translation, as well as cis-acting replication elements (CREs). CREs are defined stem-loop structures located in the 5'UTR, core, NS5B (stemloop 5BSL3.2) and the 3'UTR that operate in *cis* and are required for genome replication. Disruption of their structure blocks RNA replication. In addition, long-range interactions (kissing-loop) between the elements regulating replication have been described (Friebe *et al.* (2001); Kim *et al.* (2003); You *et al.* (2004); Diviney *et al.* (2008); Romero-López & Berzal-Herranz (2009)).

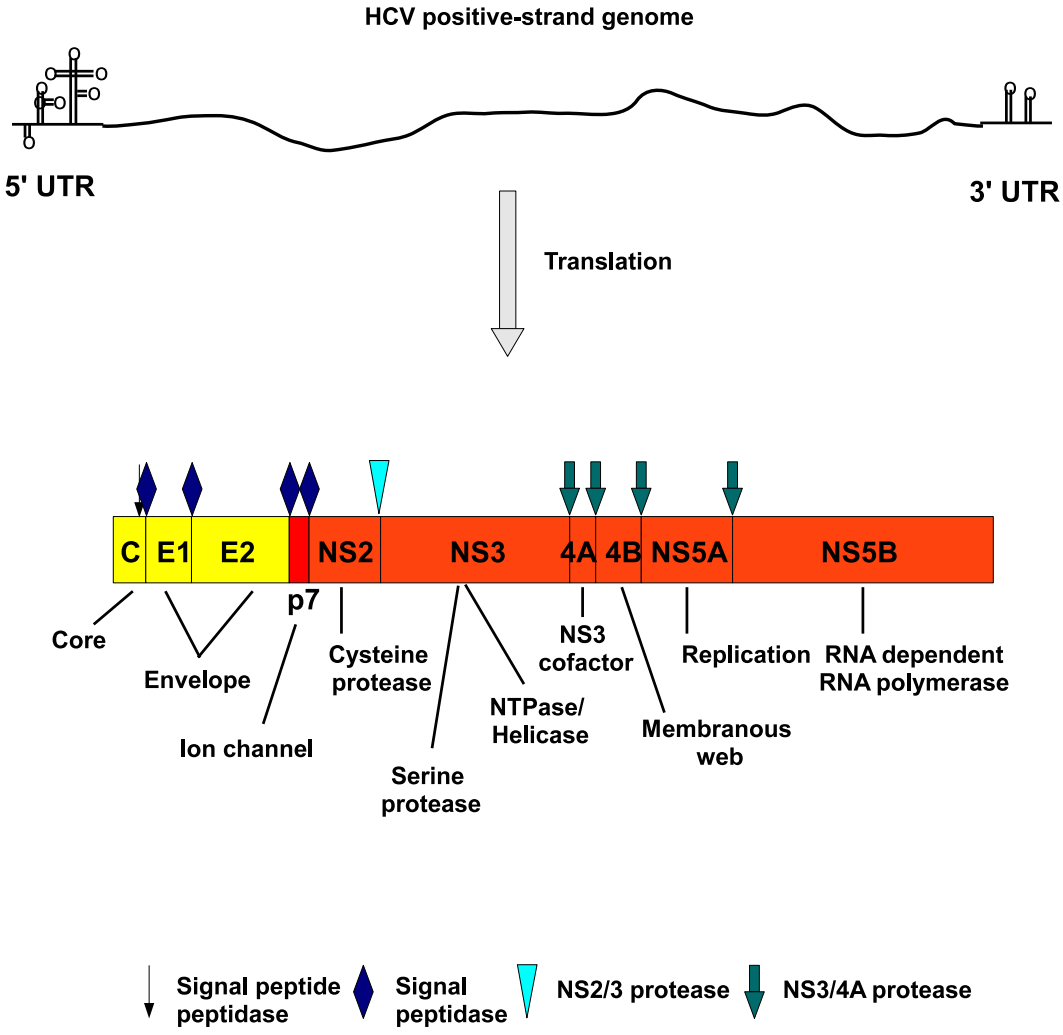

Figure 1.2: ***Genomic organisation and gene products of HCV.*** *The single-stranded positive sense RNA strand is translated into one single polypeptide, which is then processed by cellular and viral peptidases/proteases. Depicted in yellow are the structural genes and in red the non-structural genes.*

It has been shown that (i) the sequences upstream of the IRES are essential for viral RNA replication, (ii) the first 125 nt of the HCV 5'UTR are sufficient for RNA replication, but for efficient RNA replication the complete 5'UTR is necessary and (iii) stem-loop II of the IRES is crucial for replication (Friebe *et al.* (2001)). Furthermore, it has been demonstrated that the 5'UTR is capable of binding to a liver-specific microRNA, miR122, resulting in enhanced HCV RNA replication (Jopling *et al.* (2005)).

The 3'UTR varies in length from 200-235 nt and includes a short variable region, a poly(U/UC) tract with average length of 80 nt and a nearly invariant 98 nt long X-tail region (Kolykhalov *et al.* (1996); Tanaka *et al.* (1995, 1996)). The 3'X-tail forms 3 stable stem-loop structures, also called "clover-leaf", that are highly conserved among all genotypes and are essential for RNA replication. The 3'X region and the 52 nt upstream of the poly(U/UC) tract are crucial for RNA replication, while the rest of the 3'UTR plays a role in the enhancement of replication (Yi & Lemon (2003); Friebe & Bartenschlager (2002)).

Features of the viral proteins

Core

The core protein is an RNA binding protein that forms the nucleocapsid and is synthesised as the most N-terminal component of the polyprotein. The newly synthesised polyprotein has an internal signal sequence between core and the envelope protein E1 that targets it to the ER, where E1 is translocated into the ER. Cleavage by the ER signal peptidase at position 191 liberates the N-terminal end of E1 into the ER and leaves core, in a immature form, attached to the ER membrane (Santolini *et al.* (1994); Yasui *et al.* (1998)). The signal peptide is then further cleaved by a signal peptide peptidase and core is released from the ER membrane into the cytosol (McLauchlan *et al.* (2002); Hussy *et al.* (1996); Santolini *et al.* (1994)). This now mature form of core is transferred from the ER membrane to the surface of lipid droplets (LDs), where it interacts with NS5A, which is also attached to LDs (McLauchlan *et al.* (2002); Barba *et al.* (1997)). The core protein has also been located in the outer mitochondrial membrane, where it has been associated with oxidative stress (Schwer *et al.* (2004); Okuda *et al.* (2002); Boudreau *et al.* (2009)). Core is a multifunctional protein probably essen-

tial for viral replication, maturation and pathogenesis. It has, for example, also been shown to induce hepatic steatosis in transgenic mice (Moriya *et al.* (1997)). Besides its involvement in the formation of the HCV virion, core also has numerous regulatory functions, including modulation of signalling pathways, cellular and viral gene expression, cell transformation, apoptosis and lipid metabolism (McLauchlan (2000); Suzuki & Suzuki (2006)).

E1 and E2 envelope proteins

E1 and E2 are the glycosylated envelope proteins essential for virus entry and hence good targets for specific antiviral inhibitors and neutralising antibodies (Helle *et al.* (2006); Goffard *et al.* (2005)). These type-I trans-membrane proteins are N-glycosylated in their large N-terminal ectodomains and are anchored into membranes by the hydrophobic C-terminal transmembrane domains (TMDs) (Op *et al.* (2001)). E1 and E2 can form 2 types of complexes, properly folded heterodimers which are stabilised by noncovalent interactions and misfolded disulfide-like aggregates (Dubuisson *et al.* (1994)). The exact mechanism of cell entry is not clear, although several putative cell surface receptors of HCV or recombinant E2 protein have been identified. Using soluble E2, different potential HCV receptor(s) have been identified: Cluster of Differentiation 81 (CD81) tetraspanin (Pileri *et al.* (1998)), scavenger receptor class B type I (SR-BI) (Scarselli *et al.* (2002)), claudin-1 (Evans *et al.* (2007)), heparan sulphate (Barth *et al.* (2003)), and the mannose binding lectins DC-SIGN and L-SIGN (Lozach *et al.* (2003); Pohlmann *et al.* (2003); Gardner *et al.* (2003)). DC-SIGN stands for dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin and LC-SIGN for liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin. Additionally, the low-density lipoprotein (LDL) receptor has been suggested to play a role in HCV entry, because HCV associates with LDL and very low-density lipoprotein (VLDL) in serum (Lozach *et al.* (2003); Monazahian *et al.* (1999)). Of these putative HCV receptors, CD81 and SR-BI have been shown to play a direct role in HCV entry (Cocquerel *et al.* (2006)). Other receptors, not expressed on hepatocytes, such as DC-SIGN and L-SIGN may play a role in the establishment of a persistent infection. DC-SIGN, expressed on dendritic cells (DCs), and L-SIGN, expressed on endothelial cells in liver sinusoids, capture HCV particles and may thereby

facilitate infection of nearby hepatocytes (Cormier *et al.* (2004)). Further details to HCV cell entry are described in section 1.2.3.

E1 and E2 are also the gene regions with the highest genetic diversity in the HCV genome. E2 contains hypervariable domains (e.g., HVR1) which are the most variable regions of the HCV genome (Hijikata *et al.* (1991); Weiner *et al.* (1991); Kato *et al.* (1992)). A hyperimmune rabbit serum, raised against a synthetic peptide corresponding to HVR1 of HCV isolate H77, was able to protect from infection with H77 in cell culture. However, the serum was not able to protect from an infection with H90 (a genetically divergent isolate from the same patient), demonstrating that neutralisation of HCV was at least partly mediated by isolate-specific antibodies recognising HVR1 (Shimizu *et al.* (1996)). The huge variability of HCV within HVR1 is likely to play an important role in HCV persistence. It allows HCV to escape from the pressure exerted by the host immune response. This was also demonstrated in a patient with agammaglobulinaemia, who, during a follow up of 2.5 years, did not show any variability within the HVRs (Kumar *et al.* (1994)). *In vitro* experiments later showed that cross-genotype neutralisation is possible, indicating that it might be possible to identify and raise cross-neutralising antibodies, which is important for the development of active and passive immunisation strategies (Scheel *et al.* (2008)).

p7 protein

p7 is a small hydrophobic polypeptide that spans the ER double membrane and is essential for the production of infectious virions *in vivo* (Sakai *et al.* (2003); Brohm *et al.* (2009)). It has been classified as a viroporin, as it is a small channel-forming viral membrane protein that affects the infectivity of the virus and can be blocked by amantadine (Gonzalez & Carrasco (2003); Griffin *et al.* (2003)). The family of viroporins includes proteins like influenza M2 and human immunodeficiency virus type-1 (HIV-1) viral protein u (vpu), which alter membrane permeability and facilitate the release of viral particles. The ion channels formed by p7 may play an important role in the life-cycle of HCV and drugs that block them may affect virus replication (StGelais *et al.* (2009)).

NS2 protein

NS2 is a transmembrane protein with a highly hydrophobic N-terminus, which forms 3-4 transmembrane helices within the ER membrane and a zinc-stimulated cysteine protease that cleaves the NS2-NS3 junction, for which it requires the N-terminal part of NS3 as a cofactor (Schregel *et al.* (2009)). It has also been suggested to play an important role in viral assembly and release (Pietschmann *et al.* (2006)). Recently it has been shown that NS2 also is involved in the inhibition of cyclophilin A (CypA), a NS5B modulator, mediated by CypA inhibitors such as cyclosporine A (CysA) and DEBIO-025 (Ciesek *et al.* (2009)). Furthermore, several crossover sites for natural or artificial infectious intergenotypic HCV chimaeras have been mapped within NS2 (Kalinina *et al.* (2002); Pietschmann *et al.* (2006)).

NS3/4A complex

NS3 is a multi-functional protein with a N-terminal serine protease and a C-terminal RNA helicase/NTPase domain. Composed of 2 beta-barrel domains, the protease has a chymotrypsin-like fold, with His57, Asp81 and Ser139 being the catalytic triad. Its activity is enhanced by the NS4A cofactor, a 54-residue amphipathic peptide with a hydrophilic N-terminus and a hydrophobic C-terminus (Kim *et al.* (1996); Failla *et al.* (1994)). The N-terminal 21 amino acids of NS4A form a transmembrane α-helix, which is required for the integral membrane association of the NS3/4A complex (Brass *et al.* (2008)). NS4A associates non-covalently with the NS3 protein, which has no transmembrane domains, and thereby anchors it to ER/ER-like membranes (Wolk *et al.* (2000)). However, NS3 has been reported to have an amphipathic α_0-helix, which interacts in-plane with the membrane surface, providing additional association with the membrane (Brass *et al.* (2008)). The NS3/4A complex is essential for viral polyprotein processing and RNA replication (Failla *et al.* (1994)). It cleaves the polyprotein downstream of NS3, which generates the essential components for the formation of the replication complex and was therefore one of the first targets for the development of new anti-HCV molecules (Lindenbach & Rice (2005); De Francesco & Migliaccio (2005)). It is responsible for the proteolytic cleavage at 4 junctions of the HCV polyprotein precursor: NS3/NS4A (self cleavage), NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. NS4A is essential for processing at the NS3/NS4A, NS4A/NS4B and

NS4B/NS5A sites and enhances cleavage efficiency between NS5A and NS5B. Amino acids 21-32 of NS4A are sufficient for its cofactor activity (Bartenschlager *et al.* (1993, 1995); Failla *et al.* (1995); Lin *et al.* (1995a); Tanji *et al.* (1995); Tomei *et al.* (1996)). The NS3 protease also cleaves in *trans* within the NS3 helicase domain, with the NS3 helicase and NS4A as cofactors.
However, the amino acid residues of NS4A involved in the internal cleavage of NS3 are different from those required for the cofactor activity of the NS3 serine protease (Shoji *et al.* (1999); Yang *et al.* (2000); Pan *et al.* (2009); Kou *et al.* (2007)). The NS3/4A protease has an unusually shallow substrate-binding pocket, which requires substrate binding with high affinity and avidity. This poses a big problem in the design of efficient NS3 inhibitors (Kim *et al.* (1996); De Francesco *et al.* (1998)).

NS3/4A is furthermore involved in the blocking of the host cell innate immune response (Foy *et al.* (2003)). NS3/4A has been shown to interfere with the double-stranded RNA (dsRNA) signalling pathway, thereby suppressing the interferon (IFN) induction in HCV replicating cells. dsRNA, a replication product generated by most viruses, is recognised by Toll-like receptor 3 (TLR3) and the cytosolic RNA helicase retinoic acid-inducible gene I (RIG-I). The protease interferes with the cellular RIG-I pathway through cleavage of the adaptor protein of the IFN regulatory factor-3 (IRF-3) and the IFN promoter stimulator protein IPS-1 (also cardif/VISA/MAVS) (Meylan *et al.* (2005)). IPS-1 is thereby dislodged from the mitochondrial membrane and the signalling to the antiviral immune response is disrupted (Li *et al.* (2005c)). NS3/4A also cleaves TRIF, which links the TLR-3 response to IRF-3 and NF-κB activation (Li *et al.* (2005b)). TRIF stands for Toll-IL-1 receptor domain-containing adaptor inducing IFN-β. The process of IFN disruption is discussed in more detail in section 1.6.2.

The NS3 C-terminus encodes a DexH/D-box RNA helicase responsible for RNA unwinding and can be structurally divided into NTPase domain, RNA binding domain and a helical domain. Members of the DexH/D-box helicase superfamily 2 unwind RNA-RNA substrates in a 3'- to -5' direction (Tai *et al.* (1996); Cho *et al.* (1998)). Structural analysis has suggested that the NS3 helicase exists as a dimer with the NT-

Pase of one helicase building an interface with the RNA binding domain of the other (Cho *et al.* (1998)). The helicase plays a role in the initiation of RNA replication together with NS5A and NS5B (Zhong *et al.* (2005); Murayama *et al.* (2007a)). It joins unwinding of dsRNA, or of ssRNA regions with extensive secondary structures, to ATP hydrolysis. Unwinding of dsRNA is done in a highly coordinated cycle of fast ripping and local pausing with regular spacing along the duplex substrate (Serebrov & Pyle (2004); Levin *et al.* (2005)). The helicase activity is furthermore positively modulated by the NS3 protease domain and NS4A (Frick *et al.* (2004)). Although attractive as an antiviral target, the development of specific inhibitors lags behind those of protease and polymerase inhibitors (Kwong *et al.* (2005)).

NS4B protein

NS4B is a relatively poorly characterised integral membrane protein, which is predicted to contain at least 4 transmembrane segments, a cytosolic N-terminal part and a cytosolic C-terminal part containing an amphipathic α-helix mediating membrane association (Lundin *et al.* (2003); Gouttenoire *et al.* (2009)). The amphipathic α-helix is also involved in the induction of the membranous web, a specific membrane alteration that serves as a scaffold for the RNA replication complex (Egger *et al.* (2002)). NS4B has been reported to be palmitoylated at two C-terminal cysteine residues and to form oligomers (Yu *et al.* (2006a)). Similar to other HCV non-structural proteins, NS4B has been reported to play a role in virus assembly and release (Jones *et al.* (2009)). Furthermore, NS4B has been demonstrated to have an NTPase activity and to play a role in HCV pathogenesis (Thompson *et al.* (2009); Gouttenoire *et al.* (2010)).

NS5A protein

The NS5A protein is a membrane anchored zinc-metalloprotein that is observed in basally (56 kDa) and hyperphosphorylated (58 kDa) forms (Tellinghuisen *et al.* (2004, 2005)). NS5A hyperphosphorylation inhibits HCV replication and in accordance with that, cell culture adaptive mutations are often identified at the phosphorylation site (Tellinghuisen *et al.* (2005); Evans *et al.* (2004); Blight *et al.* (2000); Krieger *et al.* (2001)). NS5A has been shown to interact with other HCV nonstructural proteins, namely the RNA-dependent RNA polymerase (RdRp) NS5B, an interaction which is

essential for HCV RNA replication (Shimakami *et al.* (2004); Shirota *et al.* (2002)). It has been demonstrated that NS5A binds to 3'-ends of HCV plus and minus strand RNAs with high affinity and that this interaction is important for genome replication (Huang *et al.* (2005)). Recently it was found that domain III of NS5A is implicated in both RNA replication and assembly of hepatitis C virus particles in JFH1-infected cells (Hughes *et al.* (2009)). NS5A also plays a crucial role in evading the host immune response through inhibition of the IFN-induced dsRNA activated protein kinase R (PKR), an important component of the IFN pathway (Gale *et al.* (1998)). The importance of this interaction has been revealed in a very recent study, which showed that mutations within the IFN sensitivity-determining region (ISDR) are associated with rapid viral response during peg-IFN/ribavirin therapy (Enomoto & Maekawa (2010)). Together with core, NS5A has been reported to associate with LDs (Brass *et al.* (2002); Shi *et al.* (2002)). NS5A specifically interacts with Apolipoprotein E (ApoE), an interaction suggested to be important for viral assembly and release of infectious viral particles (Benga *et al.* (2010)).

NS5B protein

NS5B is the RNA-dependent RNA polymerase (RdRp) with a glycine - aspartate - aspartate (GDD) motif that produces catalytic activity. The C-terminal 21-amino acid region forms an α-helical transmembrane domain, which is responsible for post-translational targeting to the ER membrane. Membrane association of NS5B is indispensable for HCV RNA replication, but disruption of the insertion sequence, which abolished RNA replication, did not affect membrane association. This indicates that the C-terminal domain might be involved in intramembrane protein-protein interactions (Moradpour *et al.* (2004b); Schmidt-Mende *et al.* (2001)). The crystal structure of NS5B shows a typical polymerase structure resembling a right hand with palm, finger and thumb subdomain. The palm domain contains the fully encircled active site. Finger and thumb interact with each other to create a tunnel through which a ssRNA is directed to the active site (Ago *et al.* (1999); Bressanelli *et al.* (1999); Lesburg *et al.* (1999)). NS5B is structurally distinct from human RNA and DNA polymerases and a crucial player in HCV RNA replication, making it an important target for antiviral drug development (Walker & Hong (2002); Di Bisceglie *et al.* (2002)).

1.2.3 Life cycle

Attachment and entry

The HCV infectious particle is composed of a nucleocapsid or ribonucleoprotein complex containing the HCV genome. A phospholipid bilayer, into which E1 and E2 envelope proteins are anchored, surrounds this inner structure. During virus assembly, the infectious particle associates with VLDL and is (co-) secreted with VLDL. However, the exact mechanism of this association remains to be elucidated (Gastaminza *et al.* (2008)). Studies carried out with HCV pseudoparticles (HCVpp) provided the first evidence that the E1-E2 heterodimer is involved in virus entry (Bartosch *et al.* (2003b); Drummer *et al.* (2003)). The E2 glycoprotein probably is one of the major players in the interaction between the virus and its cellular receptors CD81 and SR-BI/claudin-1/occludin. CD81, proven to be necessary for HCV entry, was the first (co)-receptor shown to interact with HCV E2 (Flint *et al.* (1999); Cocquerel *et al.* (2003)). It has been suggested to act as a post-binding entry molecule. In fact, studies with antibodies against CD81 have shown that HCV virus infection is only inhibited after virus attachment (Koutsoudakis *et al.* (2006)). SR-BI has been identified as another receptor for HCV (Scarselli *et al.* (2002)). Binding of SR-BI to E2 HVR1 has been shown to be very selective, as neither mouse SR-BI nor closely related human SR-CD36 were able to bind E2 (Scarselli *et al.* (2002)). In addition, SR-BI plays an important role in the high density lipoproteins (HDL) and VLDL metabolism (Van Eck *et al.* (2008)). Similar to CD81, SR-BI acts as a post-binding receptor. Antibodies against SR-BI also significantly reduced the infectivity of HCVpp (Bartosch *et al.* (2003a)).

In 2007 a new protein involved in HCV entry was discovered, claudin-1 (CLDN1) a tight junction component. The expression of CLDN1 conferred susceptibility to HCVpp infection in non-hepatic cell lines and antibodies against CLDN1 blocked HCV infection. Kinetics of that inhibition indicate that CLDN1 acts late in the entry process, after virus binding and interaction with the co-receptor CD81 (Evans *et al.* (2007)). Two other members of the claudin family have also been implicated in the HCV entry process, CLDN6 and CLDN9. Like CLDN1, CLDN6 and CLDN9 are expressed in the liver, but unlike CLDN1 they are also expressed on peripheral blood

mononuclear cells, which are another possible site of HCV replication (Zheng *et al.* (2007); Meertens *et al.* (2008)). Several human cell lines, such as HeLa and HepH, co-expressing CD81 and SR-BI still remained HCV resistant when overexpressing CLDN1, suggesting that additional factors are required for HCV entry (Evans *et al.* (2007)).

The pallet of receptors involved in HCV entry has been expanded by the discovery of another tight junction protein, occludin. Downregulation of CLDN1 and occludin by small interfering (siRNA) and short hairpin (shRNA) RNA interference inhibited HCVpp and cell culture produced HCV (HCVcc) cell entry (Liu *et al.* (2009)). Recent studies in mouse cells have identified occludin as an essential entry factor for HCV. It was demonstrated that mouse cells expressing CD81 and occludin, together with mouse CLDN1 and SR-BI, are capable of supporting HCV replication (Ploss *et al.* (2009)). Interestingly, it has been observed that HCV alters the localisation of tight junction proteins (Benedicto *et al.* (2008)). Additionally, CLDN1 and occludin expression levels were downregulated after infection, rendering infected cells refractory to HCVpp superinfection (Liu *et al.* (2009)). Thus, it appears that HCV infection leads to a global reduction of tight junction proteins in HCV infected cells. As tight junctions are critical in the maintenance of polarity in hepatocytes and their essential functions, alteration of their expression level by HCV might be a possible explanation for symptoms associated with HCV infection, such as cholestatic disorders.

Other factors involved in HCV entry are glycosaminoglycans (GAGs). These linear polysaccharides act as binding sites for many viruses and have been suggested to be low affinity receptors involved in the initial binding of the virus. An interaction between E2 and surface heparan sulfate proteoglycans (HSPGs) has been demonstrated to be essential for viral binding to the cell and might present the initial step in the interaction between HCV and the cell surface (Barth *et al.* (2003)).

Lectins are another class of molecules involved in binding and cell entry for many viruses. DC-SIGN and L-SIGN are C-type (calcium-dependent) lectins containing a carbohydrate recognition domain, enabling them to recognise and bind virus carbohydrates. Both bind sE2 and HCVpp, as well as natural viruses from sera of in-

fected individuals through high mannose-type oligosaccharides (Lozach *et al.* (2003); Pohlmann *et al.* (2003); Gardner *et al.* (2003)). However, L-SIGN and DC-SIGN are not expressed on hepatocytes, excluding their role as receptors for HCV entry. A possible role of L-SIGN and DC-SIGN involves the capture and transfer of HCV to hepatocytes (Cormier *et al.* (2004); Lozach *et al.* (2003)).

The LDL receptor is another candidate receptor. It was found that HCV particles associate with lipoproteins in serum and that their infectivity correlates with HCV internalisation. The LDL receptor has been demonstrated to mediate HCV internalisation by binding to virion-associated LDL particles (Agnello *et al.* (1999)). However, the function of the LDL receptor in HCV entry remains controversial as the role of LDL-R in the *in vitro* HCVcc infection model has not been demonstrated yet. It has been suggested that the binding is mediated by lipoproteins rather than viral components. In the currently proposed model for cell entry, virus binding and internalisation is triggered by the interaction between HCV-associated lipoproteins (mainly VLDL) and the lipoprotein receptors SR-BI and/or LDL-R and/or GAGs. Following interaction of the virus with the SR-BI-CD81 complex, the virus is transferred by CD81 to tight junctions, where it interacts with CLDN1 and occludin. From the tight junctions the virus then enters the cell via clathrin-mediated endocytosis and fusion, which is mediated by the envelope glycoproteins and allows the virus to escape the lipoprotein degradation pathway (Burlone & Budkowska (2009)).

Translation

Unlike cellular capped mRNA molecules, which are translated via cap-dependent mechanism, the uncapped RNA molecules of viruses, such as picornaviruses and flaviviruses, are translated via a cap-independent IRES-mediated process (Tsukiyama *et al.* (1992); Wang *et al.* (1993)). The IRES-mediated translation is initiated by direct binding of the 40S ribosomal subunit to IRES, followed by eukaryotic initiation factor-3 (eIF-3), eIF-2: Met-tRNA:GTP and 60S subunit to subsequently form the 80S complex, after which the first peptide is synthesised (Otto & Puglisi (2004)).

Polyprotein processing

As described in section 1.2.2, the HCV polyprotein is processed by cellular proteases at the junctions core/E1, E1/E2, E2/p7 and p7/NS2, followed by intramolecular cleavage at NS2/NS3 by the NS2/3 protease and downstream cleavage between NS3/NS4A/ NS4B/NS5A/NS5B by the NS3/4A protease. Processing at the NS3/NS4A junction is intramolecular and those at other sites are intermolecular.

RNA replication

RNA replication occurs via minus strand RNA intermediate at a site of specific membrane alteration, named the membranous web (Lohmann *et al.* (1999)). This was first identified in the human hepatoma cell line Huh7 containing the subgenomic HCV replicons (Gosert *et al.* (2003)). As mentioned above the membranous web is induced by NS4B and is probably ER derived (Egger *et al.* (2002)). HCV replication is influenced by lipid metabolism. In cell culture it is inhibited by polyunsaturated fatty acids or the inhibition of fatty acid synthesis. It is stimulated by saturated and mono-unsaturated fatty acids. Furthermore, it has been demonstrated that HCV RNA replication depends on the geranylgeranylation of one or several host proteins (Ye *et al.* (2003); Kapadia & Chisari (2005)). An additional host factor involved in RNA replication is CypB, which was shown to interact with NS5B stimulating its RNA binding activity (Watashi *et al.* (2005); Heck *et al.* (2009)). CysA is a natural inhibitor of CypB and non-immunosuppressive CysA analogues are currently being developed as antivirals against HCV (Paeshuyse *et al.* (2006); Gallay (2009)).

Secretion

The late stages of HCV replication still remain obscure. NS2, which has been shown to be important in assembly and release of HCV particles, and possibly other nonstructural proteins are involved in these processes (Pietschmann *et al.* (2006)). Virions are thought to bud from ER, or ER-like membranes, and exit the cell through the secretory pathway. Furthermore, it has been shown that the production and release of HCV in Huh7 cells depends on the assembly and secretion of VLDL, providing a link between the HCV secretion and lipid metabolism (Huang *et al.* (2007)). Evidence of an asso-

ciation between HCV secretion and lipid metabolism has also come from observations that the density of intracellular virus particles produced *in vitro* is much higher than those of secreted HCVcc. Association of viral particles with VLDL during secretion would lower their density (Gastaminza *et al.* (2006, 2008)). Low-density, i.e., VLDL-associated, HCV particles are efficiently secreted from the infected cells, whereas high-density (immature) HCV particles are degraded actively in a proteasome-independent manner. These observations are in agreement with the presence of very-low-density, infectious virus particles in patient sera and the involvement of lipoprotein receptors in cell entry. It has been suggested that by hitch hiking the VLDL pathway of assembly, maturation, degradation and the secretory machinery of the cell, HCV obtains its hepatocyte tropism and through mimicry its tendency to persist (Gastaminza *et al.* (2008)).

1.2.4 HCV genotypes

HCV isolates are classified into genotypes and subtypes (Simmonds *et al.* (2005)). There are 6 major genotypes that differ from each other by 31-33 % at the nucleotide level (Fig. 1.3). Recently, a 7^{th} genotype (7a) was discovered in Canadian and Belgian patients, who are thought to have been infected in Central Africa (Murphy *et al.* (2007)). The different genotypes are associated with different clinical outcomes and geographical distributions, also see section 1.3.

The approximately equidistant genetic groups can be further divided up into a varying number of more closely related, genetically (and epidemiologically) distinct subtypes that differ from each other by 20-25 %. Due to the lack of proof-reading by the NS5B polymerase and the very high replication rate of up to 10^{12} new virions per day, many new variants of a HCV genome are created every day (Lam *et al.* (1997); Neumann *et al.* (1998)). Within an individual a HCV subtype circulates in the form of so-called "quasispecies", a population of genetically heterogeneous HCV genomes (Farci *et al.* (2000)). Currently over 80,000 HCV sequences are deposited on 3 HCV sequence databases: the European HCV database (euHCVdb (2010)), the Los Alamos national laboratory HCV database (Kuiken *et al.* (2005)) and the Japanese

hepatitis virus database (jaHCVdb (2010)). These provide several tools for analysing HCV sequences, protein structures and CD4+ and CD8+ T-cell epitopes (Kuiken *et al.* (2006)).

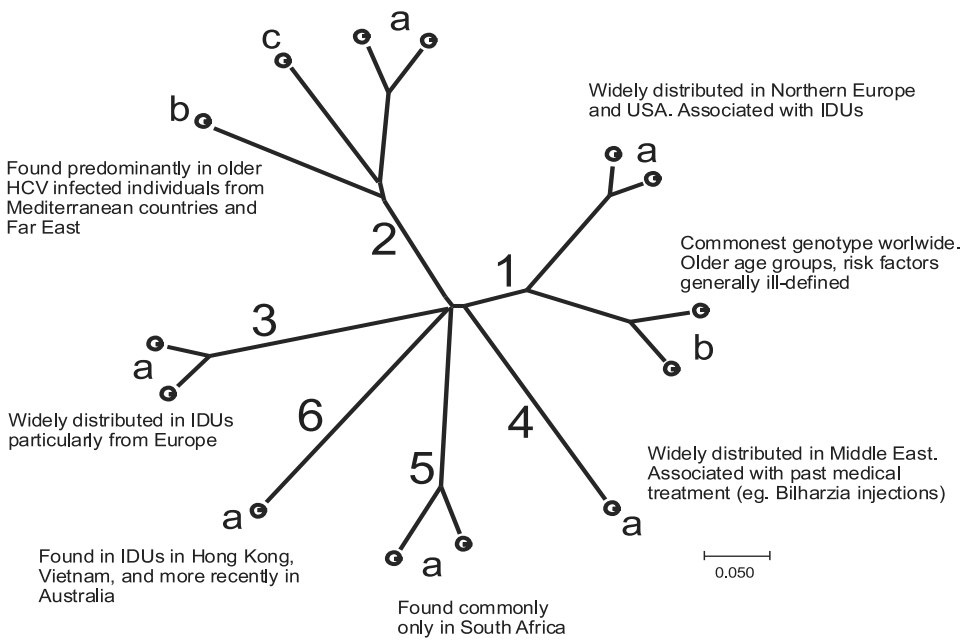

Figure 1.3: *Evolutionary tree of the principal genotypes of HCV found in industrialised countries. Phylogenetic analysis is based on complete genome sequences of HCV genotypes found in the main identified risk groups for HCV infection (injecting drug users (IDUs), recipients of unscreened blood or blood products, other parenteral exposures). (Reproduced with permission of P. Simmonds (Kuiken & Simmonds (2009))).*

1.3 Epidemiology and Global Distribution

1.3.1 Prevalence and incidence

The estimated global prevalence of HCV infection is 2-3 %, representing 170 million people (WHO (1999); Shepard *et al.* (2005)). However, regional estimates vary widely with less than 1.0 % in Northern Europe to over 2.9 % in Northern Africa and Asia (Shepard *et al.* (2005)). The United Kingdom and the Scandinavian countries have been reported to have among the lowest prevalence rates (0.01-0.1 %), whereas the highest prevalence has been reported from Egypt (10-20 %) (Bird *et al.* (2001b,a); Frank *et al.* (2000)). The prevalence of infection in Egypt increases continuously with age, and high rates of infection are observed in all age groups (Abdel-Aziz *et al.* (2000)). Over 90 % of all infections in Egypt are of genotype 4, indicating an increased risk in the distant past (Ray *et al.* (2000)). It has been suggested that a campaign of parenteral antischistosomal therapy, where medical equipment was not sterilised properly, played a major role in the spread of HCV in Egypt (Frank *et al.* (2000)). Populous countries in the developed world with relatively low rates of HCV seroprevalence include Germany (0.6 %) (Palitzsch *et al.* (1999)), France (1.1 %) (Desenclos (2000)) and Australia (1.1 %) (Law (1999)). Slightly higher, but still low incidence rates have been reported in the United States (1.8 %) (Alter *et al.* (1999)) and Japan (0.1-2 %) (Chung *et al.* (2010)). Incidence rates mostly peaked around 2000 but are now decreasing in developed countries (Chung *et al.* (2010)). Relatively high incidence rates were reported from Pakistan (5 %) (Waheed *et al.* (2009)). Worldwide an estimated 27 % of cirrhosis and 25 % of hepatocellular carcinoma (HCC) are attributed to HCV infection (Perz *et al.* (2006)).

1.3.2 Modes of transmission

The most efficient transmission of HCV is through large or repeated direct percutaneous exposure to blood, mainly through transfusion or transplantation from infectious donors or injecting drug use. In 1985 heat-inactivation of plasma products led to a substantial reduction in infection with enveloped viruses. Prior to 1990, when the

first-generation blood test became available and made it possible to screen for HCV, most HCV transmissions were due to transfusion of blood and non-inactivated blood products, such as factor VIII and IX concentrates (Alter & Houghton (2000)). For example, in a study of haemophiliacs who had received non-virus-inactivated concentrates, 80 % were positive for HCV post-treatment (Jarvis *et al.* (1996)). Since then, transfusion-associated HCV infection has virtually been eliminated in countries routinely screening for HCV in donors (Schreiber *et al.* (1996)). However, screening is far less common in poorer countries and receipt of blood transfusions remains an important source for infection in developing countries (Hladik *et al.* (2006)).

A major role in the spread of HCV also play unsafe therapeutic injections performed by both professionals and non-professionals. In 2000 the WHO estimated that yearly 2 million new HCV infections are acquired from contaminated health care injections, accounting for up to 40 % of all HCV infections worldwide (Hauri *et al.* (2004)).
In the United States and Australia, the predominant mode of transmission in the last 40 years has been injecting drug use. It now also accounts for most newly acquired infections in many other countries, including those in Northern, Western and Southern Europe. Although infection rates among injecting drug users (IDUs) have declined dramatically since the late 1980s in developed countries (down from 80 % to 10-30 % (Des *et al.* (2003); Hope *et al.* (2001))), they remain extremely high (up to 70 %) in second world countries, such as Bulgaria and Vietnam (Vassilev *et al.* (2006); Quan *et al.* (2009)).

HCV is far less efficiently transmitted by mucosal exposures to blood or serum-derived fluids (e.g., sex with an infected partner, birth to an infected mother) or by single small dose percutaneous exposures (e.g., accidental needle sticks). An Italian study has shown that the vertical transmission rate was overall very low with 2.7 % and was restricted to infants born to HCV viremic mothers, but could increase up to 5.4 % in HIV co-infected women (Ferrero *et al.* (2003)). Sexual transmission is possible, but the risk depends on the type of sexual relationship. Persons in long-term monogamous relationships are generally less likely to be infected (0-0.6 % per year) than persons who often change their sex partner (0.4-1.8 % per year). These differences might be

due to differences in sexual practices or differences in exposure rates to nonsexual sources of HCV such as toothbrushes and shared razors (Terrault (2002)). Occupational transmissions of HCV infection, mostly contaminated needle stick injuries, are largely confined to health care workers and the average incidence rate is 1.8 %, with transmissions associated with hollow-bore needles and deep injuries (Puro *et al.* (1995); Yazdanpanah *et al.* (2005)).

Numerous other biologically plausible modes of transmission include: cosmetic procedures (tattooing, body-piercing), religious or cultural practices such as ritual scarification, circumcision, acupuncture and cupping and intranasal drug use. Adequate studies are missing for most regions of the world as sufficient data is not available. However, a study among American students has shown that there was no increased risk for HCV or HBV infection in low-risk adults based solely on history of cosmetic procedures or snorting drugs (Hwang *et al.* (2006)). Nevertheless, in developing countries with low sanitary standards, where medical equipment is often not properly sterilised, transmission through cosmetic procedures or religious/cultural practices are more likely to occur, as discussed above in the case of parenteral antischistosomal therapy in Egypt (Frank *et al.* (2000)).

1.3.3 HCV geographical distribution

As previously described, HCV can be divided into 6 major genotypes which have different geographical distributions, also see section 1.2.4 (Simmonds *et al.* (2005)). In the early 90s the most predominant genotypes in European blood donors and HCV patients were genotypes 1b and 2 followed by 3 (Touzet *et al.* (2000)). Increased safety in blood transfusion has changed the main risk of HCV transmission to IDUs and with that the genotype distribution from genotypes 1b and 2 to genotypes 1a, 3a and 4a. The predominant genotype in Southern Europe still is genotype 1b, followed by 1a, 3a and 2, whereas type 1a and 3a followed by 2 predominate in Northern Europe. Genotype 4 is equally distributed throughout Europe except for the United Kingdom and Norway (Esteban *et al.* (2008)). Genotype 5a, once believed to be restricted to South Africa, has recently been reported to have been endemic in isolated areas of Central

France and West Belgium for a long time (Henquell *et al.* (2004)). In Japan and other Far Eastern countries genotype 1b, followed by 2a and 2b are responsible for most infections (Hara *et al.* (1996); Yun *et al.* (2008)). The predominant genotypes in the United States are 1a and 1b with genotype 2a, 2b, 3a and 4a occurring less frequently (Shiboski & Padian (1996)).

Compared with Western countries and Japan, where a few genotype subtypes are predominant, in Africa, South East Asia and India highly divergent subtypes circulate. Infections in West Africa are predominantly by genotype 2 (Jeannel *et al.* (1998); Ruggieri *et al.* (1996); Candotti *et al.* (2003)), whilst genotype 1 and 4 are predominant in Central African countries such as the Democratic Republic of Congo and Gabon (Fretz *et al.* (1995); Xu *et al.* (1994); Ndjomou *et al.* (2003)). Similar genetic diversity can be found within genotypes 3 and 6 in South and Eastern Asia (Tokita *et al.* (1994a,b)).

These observations have led to the hypothesis that HCV has been present in human populations for a long time in parts of Africa and Asia, compared to industrialised countries where HCV is less diverse and believed to have been introduced more recently (Ndjomou *et al.* (2003); Simmonds (2001); Simmonds *et al.* (2005)). The rapid spread of relatively new viruses in industrialised countries from areas of endemic infection has mainly been due to blood transfusions, use of unsterilised medical equipment and most recently sharing injection equipment among IDUs and unsafe sex practices among HIV positive men who have sex with men (MSM) (Cochrane *et al.* (2002); Pybus *et al.* (2001); van de Laar *et al.* (2009)).

1.4 Experimental Models for HCV Research

1.4.1 Cell-based *in vitro* HCV systems

The first subgenomic replicon system to study the non-structural genes of HCV became available in 1999. A genotype 1b subgenomic replicon had been created by replacing the structural genes and p7 of the consensus genome of Con1b with that

of a neomycin resistance gene (Lohmann *et al.* (1999)). To initiate translation of the non-structural genes, they engineered a second IRES in front of the HCV genes and selected with neomycin for highly replicating replicons in specific cell lines. The generated subgenomic replicon replicates autonomously in Huh7 cells and can be propagated in cell culture for many years (Pietschmann *et al.* (2001)). This system allowed for the first time to study the replication of the HCV genome *in vitro* and has proven very valuable in studying antivirals targeting the NS3/4A protease or the NS5B polymerase. Since then, the replicon system has been further modified to include different reporter systems, such as the firefly luciferase or fluorescent proteins, which enable high-throughput screening for replication inhibitor efficacies (Krieger *et al.* (2001); Moradpour *et al.* (2004a)). The apparent disadvantage of this system is the lack of the structural genes and therefore the inability to secrete infectious viral particles. Even though later replicons were created that expressed all viral proteins, they remained unable to secrete infectious viral particles (Blight *et al.* (2002); Pietschmann *et al.* (2002)). This discrepancy might be explained by a recent report which showed that cell culture adaptive mutations or replication enhancing mutations (REM) interfere with viral assembly and secretion (Pietschmann *et al.* (2009)).

A big breakthrough came with the establishment of the consensus sequence of the genotype 2a clone JFH1. JFH1 was isolated from a Japanese patient with fulminant hepatitis C, and a subgenomic replicon containing the JFH1 derived structural genes replicated, without acquiring REMs, much more efficiently in Huh7 cells than the Con1b replicon (Kato *et al.* (2003)). *In vitro* transcribed full-length RNA from pJFH1 replicated very efficiently in Huh7 cells and also produced infectious virus, unlike the replicons. The cell culture generated HCV was also infectious in chimpanzees (Wakita *et al.* (2005)). An intragenotypic recombinant containing the structural genes plus part of NS2 from another genotype 2a isolate, pJ6CF, and the remaining genes from the JFH1 subtype, replicated even more efficiently in Huh7-Lunet cells (subclone of Huh7 cells) (Lindenbach *et al.* (2005); Pietschmann *et al.* (2006)). Transfection of *in vitro* transcribed RNA from JFH1 or different intragenotypic pJ6CF/JFH1 chimaeras replicated very efficiently in Huh7 and Huh7.5.1 cells (highly permissive subclone of Huh7 cells). Also, the secreted viral particles were infectious in cultured cells,

chimaeric mice and chimpanzees and could be blocked by anti-CD81 and anti-E2 antibodies (Lindenbach *et al.* (2005); Wakita *et al.* (2005); Zhong *et al.* (2005)). This system, for the first time, allowed the *in vitro* investigation of the whole viral lifecycle of HCV. Since then, several intergenotypic recombinants, replacing the core-E1-E2-p7-NS2 region with that of genotype 1-7, have been generated (Pietschmann *et al.* (2006); Gottwein *et al.* (2007); Yi *et al.* (2007); Jensen *et al.* (2008); Scheel *et al.* (2008); Gottwein *et al.* (2009)). However, as most of the nonstructural genes are JFH1-derived, these recombinants most likely do not represent the genotype specific replication characteristics. Nevertheless, these recombinants provide an important tool for the study of the entry process and allow the investigation of vaccines and entry inhibitors for all 7 genotypes.

Another cell culture system has been developed using retroviral or lentiviral cores. Retroviruses incorporate heterologous glycoproteins into their envelope when they bud from cells. This mechanism was used to incorporate the envelope proteins E1 and E2 into the envelopes of these very efficiently replicating vectors, creating so-called HCV pseudoparticles (HCVpp) (Bartosch *et al.* (2003b)). These particles are highly infectious and can mimic the HCV entry process, providing an important tool for the study of the viral entry process. Several different entry receptors, such as CD81, were identified using the HCVpp system, also see section 1.2.3 (Zhang *et al.* (2004)). The advantage of the HCVpp system is that it can be used to investigate the HCV entry process in many different cell lines, as it is independent of replication.

The developments of the HCVpp, replicon and infectious HCV cell culture system have allowed a great leap forward in HCV research. The study of the different parts of the viral lifecycle, as well as the investigation of vaccines and efficacies of antivirals are now possible. However, the major limitations are that the infectious cell culture system is currently restricted to Huh7-derived cell lines and the JFH1 isolate. In order to better represent and understand the huge diversity of HCV genotypes, subtypes and quasispecies within a patient, the expansion of the infectious cell culture system to the other genotypes is crucial.

1.4.2 HCV animal models

The human is the only natural host of HCV, but HCV infected chimpanzees also develop hepatitis, even though in a milder form. The chimpanzee is the only animal model in which both, acute and chronic infection can be followed (Alter *et al.* (1978); Hollinger *et al.* (1978); Tabor *et al.* (1978); Shimizu *et al.* (1997); Yanagi *et al.* (1997, 1998); Dash *et al.* (2001); Nascimbeni *et al.* (2003)). However, chimpanzees are rare animals and studies with chimpanzees are ethically problematic and expensive. Therefore alternative animal models were searched. Other primates, such as cynomolgus monkey, rhesus monkey, green monkey, Japanese monkey and doguera baboon and woodchucks were inoculated with HCV, but none supported HCV replication (Abe *et al.* (1993)).

Several different small animals have been developed as experimental HCV animal models. Because HCV only replicates in very few specific cells, mice or rat models have to be engrafted with human liver cells. The first was the severe combined immunodeficiency (SCID)-mouse. SCID-mice are animals that were treated with a very high dose of irradiation. All the stem cells are thereby destroyed and the mice are reconstituted with new bone marrow, excluding functional T and B lymphocytes. Human liver cells infected with HCV can then be grafted under the kidney capsula and are not rejected, as the mouse lacks functional T and B lymphocytes. These immunodeficient mice with human liver transplants show detectable HCV viremia up to 50 days post-transplantation (Galun *et al.* (1995)). The chimaeric human liver uPA/SCID mouse model is presently the physiologically closest small animal model to human HCV infection. The Alb-uPA transgenic mouse overexpresses the urokinase plasminogen activator (uPA) transgene, which results in increased hepatocyte death. The deceased hepatocytes can be replaced with new human hepatocytes, which establish themselves in the chimaeric mouse liver and can be infected *de novo* with HCV-positive human serum. HCV replication can be observed in the human hepatocyte portion of the liver and is supported for months (Mercer *et al.* (2001)).

Furthermore, a rat model has been developed, where human hepatocytes were engrafted into spleens of foetal rats before immune maturation, generating an immuno-

competent animal model. The HCV-inoculated rats supported HCV replication, but HCV replication rates were quite low (Wu *et al.* (2005)).

Another animal being investigated as a possible HCV model is the tupaia belangeri, a tree shrew. Tupaias are non-primates permissive for HCV infection and can develop chronic infection (Xie *et al.* (1998); Xu *et al.* (2007)). Tamarin, a new world primate, cannot be infected with HCV, but with GBV-B, a flavivirus phylogenetically closely related to HCV. Infected animals develop an acute infection, which can evolve to chronicity similar to HCV infection, providing a model to study protective immunity and evaluate antivirals (Bukh *et al.* (1999); Martin *et al.* (2003)).

The chimpanzee has played a pivotal role in the early days of HCV research and has contributed substantially to the current understanding of basic and clinical aspects of HCV and HCV infection. However, the considerable cost and ethical constraints have limited the use of this animal model. To date the most useful animal model is the uPA/SCID mouse model, which has helped to understand viral entry, replication and therapy. Difficulties associated with the transplantation of human hepatocytes into the mice, which results in very low efficiencies when generating these mice, make them relatively expensive. The future development of animal models will be towards an immunocompetent small animal model that would allow the study of the immune response, coming close to the chimpanzee model.

1.5 Disease Associations

1.5.1 Acute hepatitis

Diagnosis

Acute hepatitis is defined as the presence of clinical signs or symptoms of hepatitis for a period of 6 months or fewer after the presumed time of HCV exposure (Blackard *et al.* (2008)). However, acute HCV infection is mostly asymptomatic, which makes diagnosis very difficult, meaning that cases are underreported (Berman *et al.* (1979);

Orland *et al.* (2001)). Serum HCV RNA levels can be detected 1-3 weeks post-infection and after a 2-12 week long incubation period symptoms may appear (Farci *et al.* (1991)). If symptoms occur, they are generally very mild and frequently mistaken for the symptoms of a common cold; symptoms often reported include fatigue, abdominal pain, nausea, vomiting, anorexia (decreased appetite), dyspepsia (indigestion) and jaundice (Santantonio *et al.* (2003); Blackard *et al.* (2008)). After 4-12 weeks the first signs of liver injury become apparent with an increase in ALT (Heathcote *et al.* (2003)). Seroconversion of anti-HCV antibody may become apparent 4-12 weeks post-infection and is currently used for diagnosis of acute HCV infection together with detection of HCV RNA (Mondelli *et al.* (2005); Pawlotsky (2002)). Further criteria for diagnosing HCV are significantly elevated ALT levels (10-20 times above the upper limit of normal), suspected HCV exposure or elevated levels of reactive proteins in immunoblot assays and exclusion of all other possible causes for acute liver damage (Mondelli *et al.* (2005)). The incubation time of 4-12 weeks before antibodies can be detected is called "window period" and serological testing of donor blood will not detect contaminated blood, therefore RT-PCR techniques to detect HCV RNA are used as well.

Spontaneous resolution of acute hepatitis C

On average 26 % of all patients (range 20-67 %) are able to spontaneously resolve an acute hepatitis and clear HCV from their system, which is most likely to happen 3 months after the onset of disease and is more likely in females (Micallef *et al.* (2006)). If the patient has not cleared the virus 6 months after onset of disease, it is very likely a chronic disease will develop. The clinical course of the disease can be influenced by many different factors, such as HCV genotype, human leukocyte antigens (HLAs), co-infection with HIV, gender, race and advanced age (Kenny-Walsh (1999); Schnuriger *et al.* (2009)). For example, a clinical survey in a German prison has shown that Caucasian men with an acute infection with genotype 3 were more likely to clear the infection than those infected with genotype 1 (Lehmann *et al.* (2004)). Very recent studies have reported that genetic variation in the IL-28B gene, which encodes the type III IFN-λ3, is associated with spontaneous HCV clearance. It was demon-

strated that a single C/C polymorphism 3 kb upstream of the IL-28B gene strongly enhanced spontaneous resolution in European and African individuals and was also associated with better IFN/ribavirin treatment response. This led to the suggestion that the gene product is likely to be involved in the innate immune control of HCV (Ge *et al.* (2009); Tanaka *et al.* (2009); Thomas *et al.* (2009); Suppiah *et al.* (2009)). Several studies have reported that viral clearance is dependent on a strong and multispecific cellular immune response (Diepolder *et al.* (1995); Gerlach *et al.* (1999); Gruner *et al.* (2000); Thimme *et al.* (2001); Grakoui *et al.* (2003); Lucas *et al.* (2007)), also see section 1.6.2.

1.5.2 Chronic hepatitis

The majority of HCV infected individuals are not able to clear an acute infection and will develop a chronic infection. The progression of chronic hepatitis is characterised by persistence of HCV RNA in the serum. Serum ALT levels though, are only elevated in two thirds of all patients (Zoulim *et al.* (2003)). Disease development differs from individual to individual, but is usually asymptomatic initially. However, over the course of 20 to 30 years (range 5 to 50 years) the infection may develop to fibrosis, leading to cirrhosis and ultimately to HCC. HCC is a major health issue in the developed world and it is strongly associated with chronic hepatitis B and C, which account for about 80 % of all HCC cases (Thomas & Zhu (2005)). Chronic infection is characterised by inflammatory lesions in the liver and besides the progression towards fibrosis, intrahepatic lipid accumulations, called steatosis, may occur as well (Moradpour & Blum (2005)). Hepatic steatosis has been associated with genotype 3 and also with increased fibrosis progression (Rubbia-Brandt *et al.* (2000); Hui *et al.* (2002); Castera *et al.* (2004); Cross *et al.* (2009)). Other factors that can increase the rate of progression towards fibrosis include alcohol intake (Jamal *et al.* (2005)), coinfection with HBV and/or HIV, male sex, diabetes mellitus (DM) (El Serag *et al.* (2004)), obesity (Chen *et al.* (2008)), advanced age at the time of infection and duration of infection (Niederau *et al.* (1998); Zoulim *et al.* (2003); Pradat *et al.* (2007)). Some studies have associated genotype 1b with increased risk of HCC development

(Silini *et al.* (1996); Bruno *et al.* (1997, 2007); Raimondi *et al.* (2009)), but these findings have not been supported by others (Niederau *et al.* (1998); Serfaty *et al.* (1998); Fattovich *et al.* (2001)). Very recent reports from Pakistan have suggested that genotype 3a might be associated with an increasing incidence of HCC as well (Khan *et al.* (2009)). Follow up of individuals who had received IFN therapy has shown that successful clearance of chronic HCV infection reduced the incidence of HCC and the overall liver-related mortality, demonstrating the involvement of HCV in this cancer (Kasahara *et al.* (1998)).

The exact mechanisms underlying the development of HCC in chronic HCV infection are still unclear (McGivern & Lemon (2009)). HCV is the only RNA virus inducing cancer with a predominantly cytoplasmic life cycle, suggesting an indirect role of HCV in the development of hepatocarcinogenesis. Not only has HCV been shown to induce chronic inflammation, steatosis, fibrosis and oxidative DNA damage, but also direct oncogenic effects and upregulation of mitogenesis have been reported for some HCV proteins (Koike (2007)). Proteins with reported associations with hepatocyte transformation are core, E1/E2, NS2, NS3 protease, NS4A, NS4B and NS5A (Bartosch *et al.* (2009)). For example, studies with transgenic mice have shown that core induces intracellular oxidative stress in the liver in the absence of inflammation, indicating a possible role of core in the development of HCC in HCV infection (Moriya *et al.* (1998, 2001)). Elsewhere it was reported that core interacts with p53, a tumour suppressor (Lu *et al.* (1999)).

1.5.3 HCV and extrahepatic manifestations

Several extrahepatic manifestations (EHMs) have been reported to occur during natural HCV infection. Up to 70 % of patients infected with HCV will develop at least one EHM during the course of the disease (Cacoub *et al.* (1999)). Most often EHMs observed in HCV patients involve joints, muscles and skin. Mixed cryoglobulinemia (MC) is the most known and studied, though anti-nuclear antibodies and anti-smooth muscle antibodies are observed as well. MC is characterised by the deposition of circulating immunocomplexes in small and medium-sized blood vessels, which leads to

inflammatory destruction of blood vessels, also named systemic vasculitis. In individuals with HCV, MC can be found in 20-50 % of cases, depending on the study (Lunel *et al.* (1994); Wong *et al.* (1996); Pawlotsky *et al.* (1995)).

Another frequently reported disease associated with HCV infection is non-Hodgkin lymphoma (NHL) (Dammacco *et al.* (1998); Mele *et al.* (2003)). One possible explanation for the underlying mechanism might be the long-term HCV infection, resulting in clonal B cell expansion of immunoglobulin (cryoglobulin)-secreting lymphocytes. Together with genetic and environmental factors this might result in mutations within the oncogenes and therefore their activation, resulting in NHL (Galossi *et al.* (2007)). Dermatological manifestations reported in association with HCV infection are porphyria cutanea tarda and lichen planus (Fargion *et al.* (1992); Pilli *et al.* (2002)). Several endocrinological manifestations have also been associated with HCV infection, including thyroid disease and DM (Huang *et al.* (1999); Mason *et al.* (1999); Knobler *et al.* (2000)). DM has been recognised to influence the course of HCV infection on the stage of insulin resistance (IR), a condition leading to type 2 DM (Hui *et al.* (2003); Leandro *et al.* (2006)). HCV infected individuals may develop IR independently of HCV, but several studies have supported the hypothesis that HCV contributes to pathogenesis of IR. IR not only seems to accelerate the progression of chronic hepatitis C, but has also been implicated in influencing the response towards antiviral therapy (Negro (2006)). Finally, rheumatological manifestations, such as rheumatoid arthritis and arthralgias (joint pain) are also commonly observed in the course of chronic HCV infection (Rivera *et al.* (1999); Buskila (2009)).

1.6 Immune Response to HCV Infection

1.6.1 Adaptive immunity to hepatitis C virus

Studies of the host response to HCV infection have been hampered due to the often asymptomatic disease progression of HCV during acute infection. These infections often go unnoticed and the only immunocompetent animal model susceptible to HCV

infection is the chimpanzee. As there are major differences between HCV infection in human and chimpanzees and because it is ethically and practically difficult to work with chimpanzees, most studies on host's immune responses rely on patient cohorts.

The humoral response to HCV infection

Early studies of the immune response in humans and chimpanzees have suggested that antibody response alone was not sufficient to clear HCV infection in most cases. Different studies have shown that a strong, multispecific and persistent cytotoxic T lymphocyte response during the acute phase is crucial for clearing a HCV infection (Lechner *et al.* (2000a,b); Thimme *et al.* (2002); Logvinoff *et al.* (2004)). The role of antibodies in acute clearance of an infection is unclear. Studies in previously infected and recovered chimpanzees have demonstrated a stronger T-cell response after rechallenge with HCV, which helped to clear the infection, whereas there were conflicting results on whether there was an increased antibody response (Bassett *et al.* (2001); Major *et al.* (2002)). A survey among IDUs in Australia even suggested that HCV infection is more likely following prior infection and clearance, implying no increased immunity against future infections (Aitken *et al.* (2008)). Two other studies have reported that neutralising antibodies are induced during the early phase of infection in individuals who control or resolve the viral infection (Lavillette *et al.* (2005); Pestka *et al.* (2007)). In haemodialysis patients with nosocomial acquired HCV infection, decreased viremia and HCV replication control was associated with a strong response of neutralising antibodies, whereas individuals who were not able to clear the infection lacked a neutralising response (Lavillette *et al.* (2005)). In a similar case, pregnant women who acquired HCV in an accidental single-source outbreak were followed up. Women who were able to clear the infection had a rapid induction of high-titre and cross-neutralising antibodies in the acute phase, while those who developed chronic infection, completely lacked or had a reduced capacity to neutralise the virus during acute infection (Pestka *et al.* (2007)). In summary these results suggest that a strong, early and broad neutralising antibody response may help to resolve HCV in the acute phase of an infection, while a delayed antibody response may contribute to the development of chronic infection.

HCV has evolved many mechanisms to escape from the host immune response, one of them is viral escape from antibody-mediated neutralisation (Zeisel *et al.* (2008)). This may occur through several mechanisms: (i) high variability of the HCV genome and limited induction of cross-neutralising antibodies, (ii) the association of HCV with serum factors such as VLDL and LDL, which might cover HCV epitopes, (iii) the interaction of HCV glycoproteins with HDL, also possibly masking epitopes, (iv) the covering of neutralising epitopes by glycosylation of certain amino acids on E1 and E2 and (v) the direct transfer of the virus from cell to cell (Zeisel *et al.* (2008)). A very recent clinical survey has investigated the induction of neutralising antibodies and viral escape from the neutralising response *in vivo* in a cohort of young IDUs in China (Dowd *et al.* (2009)). The survey found that during acute HCV infection older HCV variants were neutralised by antibodies from the same individual before the neutralisation of newer HCV variants. This indicates that neutralising antibodies drive the envelope sequence to change over time. The so-called antibody driven sequence evolution might in some individuals determine the outcome of chronic infection. Another escape mechanism has recently been described, whereby non-neutralising antibodies bind to neutralising antibodies and disrupt their neutralisation of the virus. This mechanism might help the HCV virus to persist, even if plenty of neutralising antibodies are present (Zhang *et al.* (2007, 2009)).

T cell responses to HCV infection

As mentioned above, T-cells play an important role in the immune response during acute HCV infection. Most studies have been carried out in experimentally infected chimpanzees or in individuals accidentally infected with a contaminated source, like needle sticks. Many surveys, conducted in humans and chimpanzees, have shown that the spontaneous clearance of the infection during acute phase is associated with a vigorous CD4+ and CD8+ T cell response, which targets multiple HCV regions and produces IFN-λ (Lechner *et al.* (2000a); Thimme *et al.* (2002); Wedemeyer *et al.* (2002); Bowen & Walker (2005)). One survey followed up 5 health care workers who were exposed to HCV through accidental needle stick injuries (Wedemeyer *et al.* (2002)). The only patient who cleared the acute HCV infection showed an early, strong and

sustained CD4+ and CD8+ T cell response. Another study, which followed individuals who had spontaneously eradicated HCV infection, reported that persistent CD4+ and CD8+ T cell responses targeting HCV epitopes last up to 35 years post-infection (Wertheimer *et al.* (2003)). Additionally, it has been suggested that not only the number of active CD8+ cells, but also their breadth is important in spontaneous clearance of HCV (Wertheimer *et al.* (2003)). A recent study confirmed these findings, by showing that proliferating CTLs producing IFN-γ alone did not ensure recovery, but that the presence or absence of CD4+ T cell help (HCV-specific interleukin-2 production) was crucial for the priming of CTLs (Smyk-Pearson *et al.* (2008)).
The onset and duration of the cellular immune response might also be crucial in the outcome of chronic infection. This was demonstrated in a prospective survey of 20 subjects with acute infection, where the number of Th1 cytokine-producing CD4+ T cells was higher in the first months in acute resolvers compared to those who failed to clear the virus (Aberle *et al.* (2006)). Once established, cellular immunity appears to persist for many years after clearance of infection (Wertheimer *et al.* (2003); Folgori *et al.* (2006); Chang *et al.* (2001)).

Contrary to acute resolving HCV infections, persistent infections are characterised by a weak and only monospecific CD4+ T cell response and even a strong CD8+ T cell response in the acute phase of infection may not be able to prevent progression towards chronicity (Cox *et al.* (2005b); Urbani *et al.* (2006a)). Virus specific CD4+ and CD8+ T cell responses are still detectable in the chronic phase, but the HCV specific CD4+ and CD8+ T cells display defects in function and maturation (Wedemeyer *et al.* (2002); Spangenberg *et al.* (2005)). Urbani *et al.* reported that the CD8+ T cell response is generally weak and narrow, not only in chronically evolving, but also in self-limited infections (Urbani *et al.* (2006a)). In patients with chronic outcome, CD4+ T cells were severely impaired as well, being weak and of narrow specificity. In resolvers on the other hand, the CD4+ T cell dependent Th1 response, which is necessary for CD8+ T cell activation, was found to be very strong and broad. After an initial stunned phenotype, CD8+ T cells of resolvers were able to mature and become active, whereas they did not mature in patients with chronic outcome. This indicates that a functional CD4+ T cell response, and its promotion of CD8+ T cell maturation, is crucial for

resolving an infection (Urbani *et al.* (2006a)).

Another possible mechanism involved in T cell failure of HCV-specific CD4+ and CD8+ T cells in chronic infection is the downregulation of virus-specific T-cell response by signalling through the programmed death 1 receptor (PD-1). PD-1 is an inhibitory receptor on T cells and down-regulates their activation. It has been shown to be markedly up-regulated on the surface of exhausted virus-specific CD8+ T cells in mice and on HIV-specific CD8+ T cells in HIV-infected individuals naïve to anti-HIV treatment (Barber *et al.* (2006); Day *et al.* (2006)). Several recent studies have reported elevated levels of PD-1 in HCV-specific CD8+ T cells with an exhausted phenotype in patients with persistent HCV. Blocking of the PD-1/PD-L1 (PD-1 Ligand) interaction led to an enhanced proliferative phenotype, indicating a possible relationship between PD-1 expression and T-cell exhaustion (Radziewicz *et al.* (2007); Urbani *et al.* (2006b); Bowen *et al.* (2008); Rutebemberwa *et al.* (2008)).

Furthermore, an increased frequency of CD4+ CD25+ FoxP3+ regulatory T cells (Treg) has been observed in patients with chronic HCV infection (Sugimoto *et al.* (2003); Cabrera *et al.* (2004); Boettler *et al.* (2005); Rushbrook *et al.* (2005)). Treg cells have a suppressive function and are involved in the control of auto-immunity and immune responses (Shevach (2009)). Boettler *et al.* have shown in *in vitro* depletion studies that peptide specific proliferation and IFN-γ production of HCV-specific CD8+ T cells were inhibited by Treg cells, that this inhibition was dose-dependent and required a direct cell to cell contact (Boettler *et al.* (2005)). However, other studies do not support these results. A recent study in chimpanzees found no difference in Treg frequencies and the extent of their suppression between resolvers and those who developed chronic infection. But they did report a difference between Tregs of HCV-recovered and HCV-infected chimpanzees compared to naïve chimpanzees, in that they had an increased IL-2 responsiveness and lower T-cell receptor content, indicating a history of *in vivo* proliferation (Manigold *et al.* (2006)). Evidence against a role of Tregs in promoting the development of chronic infection was reported in another study (Smyk-Pearson *et al.* (2008)). In this prospective study of 27 acutely infected subjects no significant difference in the proportion of Treg cells in the peripheral blood at base-

line between resolvers and those who developed chronic infection could be observed. As in the chimpanzee studies, differences were observed compared to healthy control groups; the frequency of Treg cells was higher than for the control group, but did not vary over time. Thus, further studies are required to define the role of Tregs in the outcome of HCV infection.

Yet another important mechanism of T cell response failure is viral escape from CD8+ T cells. Several studies have demonstrated that CTL exerts positive selection pressure against the HCV quasispecies and that the outcome of the infection depends on mutations within class I major histocompatibility complex (MHC) restricted epitopes (Chang *et al.* (1997); Weiner *et al.* (1995); Erickson *et al.* (2001); Cox *et al.* (2005a); Tester *et al.* (2005)). Findings from these studies revealed 2 important mechanisms of sequence evolution in chronic HCV infection: viral escape from CD8+ T cells and optimisation of replicative fitness. Specific mutations within HLA class I have been shown to be associated with persistence. These epitopes are targeted by CD8+ T cells during acute immune selection pressure and the virus evades that pressure by substituting amino acids in the targeted area (Timm *et al.* (2004); Ray *et al.* (2005)). A clinical survey of an Irish cohort of women, accidentally infected with HCV, showed that women with HLA class I alleles A3, B27 and Cw*01 were more likely to spontaneously resolve the infection than women with an HLA class I allele B8, suggesting that the host genetic background plays an important role in the outcome of an infection (Neumann-Haefelin *et al.* (2006)).

Most likely, the clearance of HCV is a combined event of cellular and neutralising immune response. In a rare study, both humoral and cellular immune responses were analysed in a patient with chronic HCV infection (von Hahn *et al.* (2007)). The patient was followed up for 26 years and the development of autologous neutralising antibodies, the HCV-glycoprotein-specific T-cell response and their influence on viral sequence evolution during chronic infection analysed. Von Hahn *et al.* reported that during chronic HCV infection the virus undergoes selected evolution under the pressure of humoral and cellular immune response, leading to the continuous generation of escape variants. These data underscore the above discussed findings that impairment in

both neutralising antibody response and cellular antiviral immunity lead to viral escape from the host's immune surveillance and the development of chronic infection.

1.6.2 Innate immunity to hepatitis C virus

HCV persists in more than 70 % of individuals, due to numerous very efficient mechanisms evolved by HCV to evade the host immune response. The cytokines type I IFNs play an important role in innate immunity. HCV interferes with the IFN system on many different levels: with the induction of IFN-β in infected cells, with IFN-α/β signalling through the Jak-STAT pathway and with IFN induced proteins with antiviral properties.

Induction of type I IFNs

More than 50 years ago Isaacs and Lindenmann discovered that the immune system produced a substance in response to a viral infection that acted as an anti-viral agent, the IFN (Isaac & Lindemann (1957)). Since then over 10 mammalian IFN species and numerous subspecies have been identified (Pestka (2007)). Today IFNs are classified into three groups: type I, type II and type III IFNs. Type I IFNs include all IFN-αs, IFN-β, IFN-ϵ, IFN-κ, IFN-ω and IFN-ν; IFN-γ is the only member of type II IFNs and type III IFNs include IFN-λ2 (IL-28A), IFN-λ3 (IL-28B) and IFN-λ1 (IL29) (Pestka *et al.* (2004)). As discussed in section 1.5.1, mutations near the IL-28B gene are associated with increased response rates to treatment with peg-IFN-α/ribavirin, suggesting an important role of type III IFNs in the control of HCV (Ge *et al.* (2009); Tanaka *et al.* (2009); Thomas *et al.* (2009); Suppiah *et al.* (2009)). Upon infection with a virus, the cell responds with the production of IFN-αs and IFN-β. As viruses are composed of proteins and lipids mostly derived from the host, the cellular receptors have evolved to recognise the presence of viral nucleic acid. Two important pathways have evolved to detect viral genomes and induce the production of type I IFNs. Firstly, the TLR-dependent pathway detects many different pathogen-associated molecular patterns (PAMPs), some of which are unmethylated CpG DNA of bacteria and viruses (TLR-9) (Bauer *et al.* (2001)), dsRNA (TLR-3) (Alexopoulou *et al.*

(2001)) and ss viral RNA (TLR-7,-8) (Diebold *et al.* (2004); Heil *et al.* (2004)). The cytosolic pathway on the other hand is triggered by viral RNA binding to the RNA helicases RIG-I and the melanoma differentiation antigen 5 (MDA5) (Yoneyama *et al.* (2004); Sumpter *et al.* (2005)). Unlike TLRs recognising bacterial components, which are expressed on the cell surface, TLR-3, TLR-7 and TLR-9 are localised in intracellular compartments, such as endosomes. TLR-3 signals through the adaptor protein TRIF, which results in translocation of IRF3 and NFκB to the nucleus and leads to IFN-β production. TRL-7 and TLR-9 on the other hand signal through MyD88, which leads to the translocation of IRF-7 and NFκB into the nucleus, resulting in IFN-α production (Kawai *et al.* (2004); Hoshino *et al.* (2006)).

In the cytosolic pathway RIG-I and MDA5 recognise viral 5'triphosphate RNA and dsRNA, which leads to a conformational change of these sensors (Schlee *et al.* (2009); Schmidt *et al.* (2009)). This results in the binding of the downstream adaptor protein MAVS. The adaptor protein MAVS has been identified by 4 different groups at the same time and is therefore also called IPS-1, cardif or VISA (Meylan *et al.* (2005); Kawai *et al.* (2005); Seth *et al.* (2005); Xu *et al.* (2005)). The signal is then propagated through other mediators down to IRF3 and NFκB, which bind to the IFN-β promoter and induce gene transcription. Secreted IFN-β is then bound by neighbouring and the secreting cells, which induces the Jak-STAT pathway leading to IRF7, IFN-αs and IFN-stimulated genes (ISGs) gene transcription. This positive feed-back loop ensures a fast and strong induction of an antiviral state in the infected and neighbouring cells (Rehermann (2009)).

HCV interference with the innate immune response

As described above, infection with HCV activates the RIG-I and TLR-3 pathway, which induces transcription of IFN-β. However, HCV has evolved mechanisms to evade the action of IFN-β. The HCV NS3/4A protease has been reported to cleave and inactivate MAVS (cardif, IPS-1 or VISA) thereby interfering with the RIG-I pathway (Meylan *et al.* (2005)). It has also been shown that the NS3/4A protease blocks the phosphorylation of IRF-3, a downstream protein of MAVS, thereby disrupting the RIG-I pathway further down (Foy *et al.* (2003)). Treatment of cells with an active

site protease inhibitor (PI) prevented this blockage and restored intracellular antiviral defence (Foy *et al.* (2005)). Moreover, it has been reported that the NS3/4A protease also specifically breaks down TRIF, thus disrupting the TLR3 downstream signalling and antiviral defence induction (Li *et al.* (2005b)). Targeting the NS3/4A protease with antivirals will therefore not only prevent viral replication but also restore innate immunity.

Induction of IFN stimulated genes by HCV

Interestingly, HCV still induces endogenous IFN transcription even though it blocks several proteins of the IFN system, indicating the inhibition of the IFN pathway is incomplete. Studies in chimpanzees have shown that an acute infection leads to rapid activation of the endogenous IFN system in the liver (Bigger *et al.* (2001)). Using DNA microarray technology, the changes in liver gene expression were analysed in an animal with an acute-resolving HCV infection. Upregulation of numerous ISGs expression levels were observed as early as 2 days post-infection, suggesting incomplete inhibition of IFN-β induction. Follow-up of the IFN response in chimpanzees indicated no fading away of IFN levels after the initial upregulation of IFNs as the virus starts to persist (Bigger *et al.* (2004)). Also using DNA microarray analyses, gene transcription regulation of chronically infected animals were compared with that of uninfected controls. Similarly to acute-resolving HCV infections, ISGs showed higher transcriptional activity, indicating an ongoing IFN response towards HCV. Genotype-associated differences were observed as well. A genotype 3 infected animal showed upregulated expression in some genes potentially involved in steatosis, but an overall diminished ISG expression level when compared to genotype 1 infections. Comparing gene expression levels in humans lead to similar results. Chen *et al.* identified 18 genes whose expression profile was markedly different between all IFN/ribavirin treatment non-responders and all responders (Chen *et al.* (2005)). Many of those genes were IFN sensitive and the upregulation of a subset of 8 genes allowed a quite accurate prediction of response to therapy. Interestingly, non-responders show an already high expression level of ISGs before therapy and treatment with peg-IFN-α does not induce expression of ISGs above pre-treatment levels (Sarasin-Filipowicz *et al.* (2008)). This

phenotype of a preactivated IFN signalling pathway is more emphasised in patients infected with genotype 1 and 4, compared with genotype 2 and 3, possibly explaining the difference in response to therapy between these 2 groups. It was concluded that the endogenous IFN system is not only ineffectively activated, but may also be in an insensitive state that aggravates the response to therapy. Similar results have been reported in another study, where the expression profile of liver mRNAs was analysed using real-time quantitative PCR (RT-qPCR) (Asselah *et al.* (2008)). They demonstrated that non-responders and patients who achieve a sustained virological response have different liver gene expression profiles before treatment and that the changes during treatment were mostly associated with ISGs. Two gene signatures with predictive response were identified in genes IF127 and CXCL9.

HCV interference with IFN signalling through the Jak-STAT pathway

Type I IFNs bind to IFN-α/β receptors (IFNAR), which are constituted of 2 subunits. Each subunit binds a member of the Janus kinase (Jak) family; one to the tyrosine kinase 2 (TYK2), the other to JAK1. Binding of type I IFNs to IFNAR leads to cross-phosphorylation between those 2 kinases, initiating a downstream signalling cascade to activate signal transducer and activator of transcription 1 (STAT1), STAT2 and STAT3. STAT1 and STAT2 then combine with a third transcription factor, IRF9, building the IFN stimulated gene factor 3 (ISGF3), which binds to the IFN response elements and initiates transcription of ISGs. Alternatively, gamma activated sequence (GAS) elements can be activated if STAT1 combines with STAT3. The STATs induced gene expression leads to the transformation of the cell into an antiviral state (Darnell *et al.* (1994); Darnell (1997)).

Suppressors of cytokine signalling (SOCS) are proteins that inhibit, as the name implies, the cytokine response through the Jak-STAT pathway (Krebs & Hilton (2001)). SOCS1 and SOCS3 are induced by type I IFN (Song & Shuai (1998)). The importance of these negative regulators has been demonstrated in SOCS1-deficient mice, which developed severe inflammatory disease due to IFN-γ hypersensitivity, but were very resistant to viral infections, likely a result of increased type I IFN signalling (Alexander *et al.* (1999); Fenner *et al.* (2006)). Overexpressing the HCV core protein

has been shown to inhibit IFN-α-induced tyrosine phosphorylation and STAT1 activation in hepatic cells. This inhibition is most likely due to induction of SOCS3-mRNA expression by core (Bode *et al.* (2003)). Elsewhere it has been reported that core induces upregulation of SOCS3 in HepG2 cells, which was associated with changes in the glucose metabolism (Kawaguchi *et al.* (2004)). In addition, it has been shown that the expression of HCV proteins in Huh7 cells leads to selective STAT1 degradation in a proteasome-dependent way. As HCV core protein was found to bind STAT1, it was suggested to be associated with the STAT1 degradation (Lin *et al.* (2005b)). Another group using HCV protein expression in Huh7 cells, reported that high-level expression of HCV core protein inhibited the IFN-α induced accumulation of STAT1 in the nucleus (Melen *et al.* (2004)). STAT3 levels have also been shown to be reduced in livers of HCV infected patients and Huh7 cells containing the full-length HCV replicon (Larrea *et al.* (2006)). It was concluded that HCV replication impairs the Jak-STAT signalling pathway and might thereby improve viral replication and favour liver disease progression.

The type I IFN induced antiviral state in a cell results in upregulation of many different genes. One of them is PKR, which phosphorylates eIF2α when a cell is in an antiviral state. This inhibits translation of most cellular and viral mRNAs, thereby preventing viral replication (Roberts *et al.* (1976); Farrell *et al.* (1978)). The HCV protein NS5A has been shown to prevent PKR activation in cell culture by directly interacting with the protein kinase catalytic domain. This presents yet another mechanism how HCV manages to avoid the antiviral effects of IFN (Gale *et al.* (1997)). A survey among genotype 1b infected individuals has reported a correlation between mutations within the ISDR of HCV NS5A, which is involved in PKR binding, and response to IFN (Enomoto *et al.* (1996)). This observation was supported by another study with genotype 1b infected individuals in Australia and a very recent one in Tunisia (MacQuillan *et al.* (2004); Bouzgarrou *et al.* (2009)). However, similar correlations were not observed in analogous studies carried out in Europe and the United States (Zeuzem *et al.* (1997); Gerotto *et al.* (2000); Murphy *et al.* (2002)). A meta-analysis in 2004 (Pascu *et al.* (2004)) and a more recent study (Murayama *et al.* (2007b)) have concluded that a correlation between the number of mutations within ISDR and the re-

sponse rates to IFN-ribavirin combination therapy is dependent on genotype subtype and not geographical region. In individuals infected with the J-type HCV 1b, ISDR can serve as a predictable marker for response to IFN-ribavirin combination therapy, but not in individuals infected with other genotype 1b strains and other genotypes.

Another protein reported to interact with PKR is the HCV E2 envelope protein. HCV E2 was demonstrated to bind PKR through a 12-amino acid sequence that resembles the PKR auto-phosphorylation site and the eIF2α phosphorylation site, named the PKR-eIF2α phosphorylation homology domain (PePHD) (Taylor *et al.* (1999)). E2 was shown to block PKR in transfected cells and yeast, promoting protein synthesis and cell growth. The authors argue that together with the inhibitory effect of NS5A on PKR, E2-PKR blocking may be a possible cause for the development of resistant infections. The promoted protein synthesis, which allows the cell to grow again, may explain HCV-associated HCC. The investigators compared the sequences of PePHD domains of different genotypes and found that those of genotypes 1a and 1b were more closely related to the sequence of PKR and eIF2α than those of genotypes 2a, 2b and 3a. Genotype 1a and 1b show higher resistance to IFN treatment compared with genotype 2a, 2b and 3a. This might explain the better response rates in individuals infected with the latter genotypes. However, a clinical study comparing sequences of this 12-amino acid motif between different genotypes and their response to IFN therapy did not find a similar association, suggesting that other factors might influence the response to IFN-α to a greater extent (Abid *et al.* (2000)).

HCV is very successful in establishing persistent infections. To achieve this, the virus has evolved different mechanisms that interfere with the host innate immune system. HCV not only interferes with the induction of IFN-β in infected cells, but also disrupts IFN-α signalling through the Jak-STAT pathway and directly inhibits IFN induced effector mechanisms, such as the PKR-mediated inhibition of translation. Together with the above discussed abilities of the virus to evade the adaptive immune response, disruption of the IFN response allows HCV to effectively persist in the host.

1.7 Current Treatment Options

1.7.1 IFN-ribavirin combination treatment

Some individuals spontaneously cure a HCV infection, but the majority go on to develop a chronic infection if no diagnosis is made during acute infection and treatment initiated. If acute hepatitis C is diagnosed, the chances of treatment response are quite high, with treatment success rates being highest if treatment commences within 12 weeks of diagnosis (Jaeckel *et al.* (2001); Gerlach *et al.* (2003); Corey *et al.* (2009)). Chronic hepatitis C (CHC) on the other hand is resistant to treatment in almost 50 % of individuals. Initially therapy only consisted of IFN-α, which resulted in about 15-20 % of individuals with chronic hepatitis achieving sustained virological response (SVR) (Lin *et al.* (1995b); Poynard *et al.* (1996); Carithers & Emerson (1997)). SVR is defined as the absence of any detectable HCV-RNA 6 months after end of treatment. Treatment outcome was further improved with the introduction of the nucleoside analogue ribavirin and the pegylation of IFN-α (McHutchison *et al.* (1998); Poynard *et al.* (1998)). The addition of a polyethyleneglycol molecule to IFN increases its half-life, thereby creating more favourable pharmacokinetics and allowing a more comfortable once-weekly dosing.

The exact mechanism underlying HCV replication inhibition through ribavirin is unclear. Ribavirin is a guanosine nucleotide that is incorporated by the HCV polymerase and can pair with cytosine or uracil. It has been shown for poliovirus that ribavirin can induce lethal mutagenesis by increasing the viral error rate, leading to error catastrophe and repression of the viral fitness of the population (Crotty *et al.* (2000, 2001)). A similar study on the HCV genome has shown that passaging the HCV replicon under ribavirin leads to mutations within specific regions of the genome, supporting a mechanism of error catastrophe induced by ribavirin (Contreras *et al.* (2002)). Furthermore, it has been proposed that ribavirin incorporation by the HCV polymerase blocks RNA elongation during RNA synthesis, directly inhibiting HCV RNA replication (Maag *et al.* (2001)). Ribavirin may also affect the immune response to HCV. Activation of isolated T-cells *in vitro* led to an increased type 1 and a suppressed type

2 cytokine response upon ribavirin treatment (Tam *et al.* (1999)). The authors proposed that the promotion of a type 1 cytokine-mediated immune response may be in part responsible for the additive effect of ribavirin in the IFN-α/ribavirin combination treatment. Another possible effect of ribavirin might be the inhibition of the inosine monophosphate dehydrogenase (IMPDH), which converts inosine monophosphate (IMP) to guanosine triphosphate (GTP). This leads to depletion of GTP and reduced RNA synthesis (Lau *et al.* (2002)). Ribavirin is given twice daily, depending on body weight.

The combination treatment of peg-IFN-α and ribavirin constitutes the standard of care since 2001 and results in SVR rates of 46-55 % (Manns *et al.* (2001); Fried *et al.* (2002); Hadziyannis *et al.* (2004)). To determine treatment duration, the HCV genotype and the initial response to therapy defined by HCV RNA viral loads in serum at baseline and after 4 and 12 weeks of therapy have to be considered. Treatment responses are categorised depending on the viral load after end of treatment, as outlined in Table 1.1.

Table 1.1: ***Definition of treatment response.*** *Adapted from Sarasin-Filipowicz* et al. *(2008).*

Rapid virological response (RVR)	Negative HCV-RNA 4 weeks from treatment onset
Early virological response (EVR)	> 2 $\log_{10}$ IU/ml reduction in HCV-RNA at week 12
Complete EVR (cEVR)	Negative HCV-RNA 12 weeks from treatment onset
Primary non-responders (PNR)	< 2 $\log_{10}$ IU/ml decrease in viral titre after 12 weeks
End of treatment response (EoTR)	Undetectable serum HCV-RNA after end of treatment
End of treatment non-response (EoNR)	Detectable serum HCV-RNA after end of treatment
Sustained virological response (SVR)	No detectable HCV-RNA 6 months post-treatment
Relapse	Detectable HCV-RNA after having achieved EoTR

Ideally a patient undergoing therapy achieves a response at the end of the treatment (EoTR) and maintains negative serum-HCV levels after the treatment has ended. However, not all patients who respond to therapy are able to achieve a SVR and HCV-RNA is detectable again 6 months post-treatment; these patients are classified as relapsers. When deciding on the duration of the therapy, the genotype plays an important role. Patients infected with genotype 1 have been shown to benefit from a longer treatment duration of 48 weeks (McHutchison *et al.* (1998)). Individuals infected with geno-

type 4 also benefit from a longer 48 week treatment, whereas genotype 2 and 3 infected individuals show better response rates and 24 weeks of treatment are recommended (Hasan *et al.* (2004); Fried *et al.* (2002); Hadziyannis *et al.* (2004)). However, side effects and the very high cost associated with IFN-α/ribavirin treatment has led to recent suggestions that individuals showing RVR should only be treated for 12-16 weeks, if infected with genotype 2 and 3, and 24 weeks if infected with genotype 1 (Mangia *et al.* (2005); Mangia (2007)). Different clinical studies have shown that about 50-60 % of individuals infected with genotype 1, 20 % infected with genotype 2 or 3 and 30-40 % infected with genotype 4 do not respond to the current standard treatment (Manns *et al.* (2001); Fried *et al.* (2002); Hadziyannis *et al.* (2004); Hasan *et al.* (2004); McHutchison *et al.* (2009a)). In patients who do not achieve an EVR after 12 weeks of treatment, therapy is discontinued. African-Americans and individuals with steatosis generally achieve lower SVR rates (Reddy *et al.* (1999); Muir *et al.* (2004); Poynard *et al.* (2003)). Other factors associated with lower SVR rates are male gender and age (Hayashi *et al.* (1998)). Low pre-treatment serum HCV RNA levels are associated with higher SVR rates (Martinot Peignoux *et al.* (1995)), whereas high body fat mass (body mass index (BMI) $>$ 30) (Hickman *et al.* (2002); McCullough (2003)) and high alcohol intake will reduce the efficacy of IFN-treatment (Okazaki *et al.* (1994); Ohnishi *et al.* (1996)). The rate of fibrosis progression and insulin resistance have also been associated with decreased SVR rates (Myers *et al.* (2003); Nasta *et al.* (2008)). As discussed in section 1.6.1, different HLA types have been associated with SVR rates as well.

Individuals with genotype 5 and 6 are generally underrepresented in clinical studies and only very few data are available on their response to treatment. A recent study, analysing retrospectively individuals who received IFN-α/ribavirin combination treatment and were diagnosed with genotype 6, concluded that individuals infected with genotype 6 should be treated with a full course of 48 weeks of treatment (Nguyen *et al.* (2008)). No specific recommendations on dose and treatment duration are currently available for genotype 5 infected individuals, as there is not sufficient data available.

IFN-α/ribavirin combination treatment is frequently associated with severe side-effects and 10-14 % of patients discontinue their therapy due to adverse events (Manns *et al.* (2001)). Side-effects often reported include influenza-like symptoms, such as fatigue, headache, nausea, pyrexia (fever), rigors and myalgia (muscle pain); skin disorders, such as pruritus (itch), alopecia (hair loss) and dermatitis; arthralgia (joint pain); digestive dysfunction; neutropenia (abnormally low levels of neutrophils); thrombocytopenia (low levels of platelets in blood); thyroid dysfunction and depression (Fried *et al.* (2002)). Addition of ribavirin in combination treatment can lead to extra side effects such as haemolysis, resulting in anaemia in 30 % of patients. Surprisingly, a decline in haemoglobin during the early phase of treatment has been found to be associated with better treatment outcome in genotype 1 infected individuals (Sulkowski *et al.* (2009a)).

Risks and benefits of IFN-α/ribavirin combination treatment have to be evaluated for each patient individually, as the course of infection can proceed more slowly in some individuals and occurrence of side-effects may differ. Furthermore, treatment costs can differ considerably as well. Treatment costs for individuals infected with genotype 1, with mild or moderate disease, and patients under the age of 40, is below £20,000 per quality-adjusted life year (QALY). For genotype 1 infected individuals, with cirrhosis, aged 50 and older, treatment costs can rise to over £60,000 per QALY (Grishchenko *et al.* (2009)).

Taking these factors into consideration, therapy has been recommended for individuals with persistently elevated transaminase levels (ALT and ASP) (Martinot-Peignoux *et al.* (2001); Persico *et al.* (2000)), detectable serum HCV-RNA (sign of viral replication) and progressed fibrosis (metavir fibrosis stage F $\geq$ 2, where 4 is the maximum, as determined by liver histology) (Wong & Koff (2000); Levine *et al.* (2006); Ghany *et al.* (2009)). However, more recent studies have shown that high-dose IFN-α/ribavirin combination therapy is effective, safe and well tolerated in patients with normal ALT/ ASP levels as well (Zeuzem *et al.* (2004); Yu *et al.* (2006b)). This has led to the suggestion that individuals with normal ATL/ASP levels might benefit from IFN-α/ ribavirin treatment as well and that liver biopsies should be taken to determine whether

treatment should be initiated or not.

1.8 Antivirals

While the current standard treatment is reasonably effective for individuals infected with genotype 2 and 3, there is still a high number of patients without response and others with reemerging HCV-RNA after end of treatment. Due to the partly very low response rates and the frequently occurring side effects, the development of new, highly specific small molecules targeting the HCV virus is highly desired. The so called specifically targeted antiviral therapy for HCV (STAT-C) targets viral proteins, mostly by small molecules. Highly active antiviral retrotherapy (HAART), using the combined effect of several small molecule inhibitors to target the virus, is already part of the current standard treatment for HIV infection, but even though the causative agent of hepatitis C has only been identified shortly after that of acquired immune deficiency syndrome (AIDS), no specific antivirals for HCV are approved by the American Food and Drug Administration (FDA) as to date (Dunning & Nelson (2009)). Of the 10 viral HCV proteins, NS2, NS3/4A and NS5B have enzymatic functions and present possible targets for antiviral therapy. NS3/4A, a serine protease, also plays a role in RNA replication and assembly, as does the HCV polymerase NS5B. These 2 proteins have therefore been 2 particularly promising targets in antiviral drug development, as their inhibition prevents viral replication. Nevertheless, antiviral strategies targeting viral entry, translation and protein-protein interactions are pursued as well. After showing efficacy in cell culture and not inducing any severe cytotoxic effects in cell culture and small animal models, the evaluation of antiviral drugs is carried out in 4 clinical phases, which are explained in Table 1.2.

Table 1.2: ***Definition of clinical trial phases.*** *Adapted from ClinTrials (2010).*

Phase I	Safety, dose range and side-effects evaluation in a small group (20-80 people)
Phase II	Testing of efficacy and safety in a larger group (100-300 people)
Phase III	Confirmation of efficacy, monitoring of side-effects and comparison to commonly used treatments in large groups (1,000-3,000 people)
Phase IV	Monitoring of drug risks and benefits post-marketing and adjustment of optimal use

1.8.1 NS3/4A protease inhibitors

The development of NS3/4A PIs was greatly spurred when the crystal structure of the NS3 N-terminal protease domain and the full-length NS3 protein, complexed with a NS4A peptide, was solved (Kim *et al.* (1996); Yao *et al.* (1999)). The crystal structure revealed an unusually broad and shallow substrate binding site, which has complicated the development of specifically binding small molecules using rational structure-based drug design. However, it was observed that the carboxy-terminal NS3 residues are located within the active site, presenting a "product inhibition" mechanism, where cleaved substrate peptides occupy the active site. This finding has been very helpful for drug design and peptidomimetic compounds dominate drug development against the NS3/4A protease today (Steinkuhler *et al.* (1998)). The first of these peptidomimetic compounds to enter clinical trials was the macrocyclic inhibitor BILN 2061 (ciluprevir). This small, orally available molecule inhibitor resulted in an impressive reduction of HCV RNA levels after only 2 days in genotype 1 infected individuals. On average patients showed a 2 to 3 $\log_{10}$ reduction in HCV RNA levels (copies/ml) with some patients even reaching undetectable levels within 24-28 hours after administration (Lamarre *et al.* (2003)). Specifically developed for genotype 1 proteases, BILN 2061 had a nearly 2 $\log_{10}$ weaker binding affinity for genotype 2 and 3 proteases (Thibeault *et al.* (2004)) and in *in vivo* studies BILN 2061 showed as expected lower efficacy in patients infected with genotype 2 and 3 (Reiser *et al.* (2005)). Unfortunately, the development of BILN 2061 had to be halted due to cardiotoxicity in laboratory animals (Vanwolleghem *et al.* (2007)).

Because of the genetic variability of HCV proteins of different genotypes, the structure of protease and polymerase enzymatic sites differs substantially and potentially limits the effectiveness of certain classes of inhibitors (Holland-Staley *et al.* (2002)). The case of BILN 2061 demonstrated early on that it is crucial to investigate drug efficacies in all genotypes. Reduced effectiveness of antivirals on certain genotypes also potentially facilitates the development of resistance mutations, section 1.8.4.

Newer macrocyclic inhibitors currently in clinical trials include ITMN-191 and TMC-435 (Table 1.3). In phase Ib clinical trials, ITMN-191 was able to reduce HCV RNA levels by about 3.5 $\log_{10}$ IU/ml after 14 days of treatment (Bradford *et al.* (2008); Forestier *et al.* (2008)). Biochemical inhibition studies have shown that ITMN-191 is about 10-fold more potent against proteases from genotype 4, 5 and 6 compared to proteases from genotype 2a and 3a. However, the biochemical potency is still in the nanomolar range for all genotypes and peg-IFN-α-2a showed an additive effect, making it a very potent inhibitor (Seiwert *et al.* (2008)). In biochemical assays, TMC-435 showed potent inhibition of HCV NS3/4A proteases from all genotypes except for genotype 3 proteases (Tsantrizos (2009)). In phase I and II clinical trials with genotype 1 infected individuals, TCM-435 has demonstrated significant reductions in HCV-RNA levels without any severe side-effects (Reesink *et al.* (2009)). TMC-435 is currently evaluated in phase IIa clinical trials in combination with peg-IFN-α and ribavirin.

The currently most advanced PIs are 2 linear ketoamide peptidomimetic inhibitors, VX-950 (telaprevir) and SCH 503034 (boceprevir). They have advanced into phase IIb/III clinical trials. In monotherapy, VX-950 was able to reduce HCV RNA levels 4.4 $\log_{10}$ IU/ml on average after 14 days of treatment and was well tolerated (Reesink *et al.* (2006)). In 2 recent phase II clinical trials it could be demonstrated that the addition of the STAT-C agent VX-950 to the current standard of care could increase SVR rates, although discontinuation rates were higher due to VX-950 induced side-effects (Hezode *et al.* (2009); McHutchison *et al.* (2009b)). Tibotec conducted a similar phase II study on previously untreated genotype 1 infected patients in Europe. Up to 85 % of patients, taking 3 times daily VX-950 plus peg-IFN-α-2a and ribavirin, achieved a SVR (Marcellin *et al.* (2009). Vertex is currently conducting a phase III clinical trial that includes evaluation of 24-week and 48-week VX-950-based regimens in genotype 1 treatment-naïve HCV patients. An 8-12 week period of peg-IFN-α/ribavirin/VX-950 triple-combination therapy is followed by 12-16 weeks of peg-IFN-α/ribavirin dual-combination treatment and patients follow-up for 48 weeks. *In vitro* studies have reported similar efficacies for VX-950 against genotype 1a, 1b and 2a (Paulson *et al.* (2009)). In an ongoing phase IIa clinical trial, VX-950 has demon-

strated substantial activity in genotype 2 infected patients, but only limited efficacy in genotype 3 infected individuals (Foster *et al.* (2009)). Highlighting again how influential the structural differences between the proteases of the different genotypes are on drug efficacies and that it is important to take these into consideration during drug design.

SCH 503034 (boceprevir), the other linear ketoamide peptidomimetic, reduced replicon RNA levels over 4 $\log_{10}$ during continued exposure in cell lines (Malcolm *et al.* (2006)). In a phase II clinical trial with genotype 1 infected individuals, addition of SCH 503034 to the current standard care of treatment resulted in increased SVR rates compared to the control group (Schering Plough (2009)). Schering-Plough is currently running a phase III clinical trial testing SCH 503034/IFN/ribavirin combination therapy on genotype 1 infected individuals, which is expected to be completed in mid-2010. BI 201335, the new PI from Boehringer Ingelheim, demonstrated a rapid and potent antiviral activity in a very recent phase II clinical trial, where genotype 1 infected patients were given BI 201335 in combination with the current standard care of treatment (Sulkowski *et al.* (2009b)).

As the NS3/4A protease also interferes with the IFN response, an inhibition of this enzyme might potentially also rescue the endogenous IFN response. However, a recent study showed that the concentration of the PI TMC-435 required for the rescue of the virus-imposed inhibition of the IFN signalling, was significantly higher than that necessary for the inhibition of viral replication (Liang *et al.* (2008)). Thus, PIs are unlikely to provide any additional benefit when compared with inhibitors targeting other HCV proteins.

Table 1.3: ***HCV NS3 PIs and phase of clinical development.***

Compound	Phase of development	Company
SCH 503034 (Boceprevir)	Phase III	Schering-Plough
VX-950 (Telaprevir)	Phase III	Vertex
BI 201335	Phase II	Boehringer Ingelheim
BMS-650032	Phase II	Bristol-Myers Squibb
ITMN-191 (RG7227/R05190591)	Phase II[1]	Intermune/Roche
ITMN-121	Phase II	Roche
MK-7009	Phase IIa	Merck
TMC-435	Phase IIa	Medivir/Tibotec
SCH 900518 (Narlaprevir)	Phase II	Schering-Plough
ABT-450 HCV	Phase I	Abbott and Enanta
ACH-1625	Phase I	Achillion
BMS-791325	Phase I	Bristol-Myers Squibb
BMS-824393	Phase I	Bristol-Myers Squibb
PHX1766	Phase Ia	Phenomix
VX-500	Phase Ib	Vertex
VX-813	Phase I	Vertex
VX-985	Phase I	Vertex
AVL-181	Preclinical	Avila Therapeutics
EA-058	Preclinical	Abbott and Enanta
EA-063	Preclinical	Abbott and Enanta
SCH 567312	Preclinical	Schering-Plough
ACH-806/GS9132	Discontinued	Gilead/Achillion
BILN 2061	Discontinued	Boehringer Ingelheim
SCH6	Discontinued	Schering-Plough

[1] *Phase I in combination with the nucleoside analogue polymerase inhibitor RO5024048.*
Information presented in this table has been gathered from public sources, accuracy is not guaranteed. Sources include company websites and the following public websites:
http://www.hcvdrugs.com
http://www.hivandhepatitis.com/hiv_hcv_co_inf_articles.html
http://www.hcvadvocate.org/hepatitis/hepC/HCVDrugs.html
http://clinicaltrials.gov

1.8.2 NS5B polymerase inhibitors

The other important target in HCV specific antiviral drug development is the RdRp NS5B. Similar to NS3 PI development, developing NS5B inhibitors has also been greatly propelled forward by solving its 3-dimensional structure. Crystallographic structure analysis has revealed a typical right-handed polymerase conformation with palm, thumb and finger domains. The finger and thumb domains interact closely with each other, providing a fully encircled active site (Lesburg *et al.* (1999)). More recent structure analyses have disclosed that NS5B can exist in an open or closed conformation (Biswal *et al.* (2005); Chinnaswamy *et al.* (2008)). It was proposed that during *de novo* initiation NS5B consists in a closed conformation, which is opened up during elongation due to steric clashes induced by the growing polypeptide.

To date many small molecule inhibitors of NS5B have been identified and some have entered clinical trials (Table 1.4). They can be classified into 2 groups: nucleoside analogs that bind to the active site and allosteric, non-nucleoside inhibitors (NNIs) that bind sites at variable distances from the active site of the polymerase. Nucleoside inhibitors bind to the active site and inhibit transcription initiation and elongation of RNA synthesis, whereas NNIs binding to allosteric sites only seems to inhibit transcription initiation by blocking the open conformation (Biswal *et al.* (2005)). Nucleoside inhibitors of HCV RdRp include 2'-C-methyl, 2'-O-methyl, and 4'-substituted nucleoside analogs, working on a chain terminating mechanism (Carroll *et al.* (2003); Klumpp *et al.* (2006)). There are currently 3 nucleoside analogues in phase II development; IDX-184, PSI-7977 and R7128.

Table 1.4: ***HCV NS5B polymerase inhibitors and phase of clinical development.***

Compound	Phase of development	Company
Nucleoside inhibitors		
IDX-184	Phase II	Idenix
PSI-7977 (Prodrug of PSI-7851)	Phase IIa	Pharmasset
R7128 (Prodrug of PSI-6130)	Phase IIb	Roche/Pharmasset
RO5024048	Phase I[1]	Roche
INX08189	Preclinical	Inhibitex
PSI-938	Preclinical	Pharmasset
R1626	Discontinued	Roche
Non-nucleoside inhibitors		
ABT-333	Phase II	Abbott
ANA598	Phase II	Anadys
GS 9190	Phase II	Gilead
IDX-375	Phase II	Idenix
PF-868554 (Filibuvir)	Phase II	Pfizer
VCH-759	Phase II	Vertox/ViroChem
VCH-222	Phase Ib/IIa	Vertox/ViroChem
A-837093	Phase I	Abbott
ABT-072	Phase I	Abbott
BI 207127	Phase I	Boehringer Ingelheim
MK-3281	Phase I	Merck
PF-4878691	Phase I	Pfizer
VCH-916	Phase Ib	Vertox/ViroChem
A-782759	Preclinical	Abbott
A-848837	Preclinical[2]	Abbott
GL59728	Preclinical	Genelabs/Novartis
GL60667	Preclinical	Genelabs/Novartis
GSK625433	Discontinued	GlaxoSmithKline
HCV-796	Discontinued	ViroPharma/Wyeth
JTK 003	Discontinued	Akros Pharma
NM107/NM283	Discontinued	Idenix Pharmaceuticals
R803	Discontinued	Rigel Pharmaceuticals
XTL-2125	Discontinued	XTL Pharmaceuticals

[1] *In combination with the PI RO5190591/ITMN-191.*
[2] *Efficacious in the HCV-infected chimpanzee (Molla* et al. *(2007)).*
Information presented in this table has been gathered from public sources, accuracy is not guaranteed. Sources include company websites and the following public websites:
http://www.hcvdrugs.com
http://www.hivandhepatitis.com/hiv_hcv_co_inf_articles.html
http://www.hcvadvocate.org/hepatitis/hepC/HCVDrugs.html
http://clinicaltrials.gov

Among the NNIs there are 7 inhibitors in phase II clinical trials now: ABT-333, ANA598, GS 9190, IDX-375, PF-868554, VCH-222 and VCH-759. NS5B inhibitors generally lower HCV RNA levels between 2 to 3 $\log_{10}$ IU/ml, slightly less than PIs (Cretton-Scott *et al.* (2008); Lalezari *et al.* (2008); Cooper *et al.* (2009)). However, in combination therapy with standard of care, R7128, a nucleoside analogue, was able to lower HCV RNA levels up to 5 $\log_{10}$ IU/ml (Le Pogam *et al.* (2009)). As with PIs, genotype associated differences in the enzyme structure can play a major role in the susceptibility of polymerases to polymerase inhibitors. For example, *in vitro* studies with ABT-333 have shown that it is a potent inhibitor of genotype 1b and 1a polymerases, but nearly ineffective against genotype 2a, 2b, 3a and 4a polymerases, demonstrating again the importance of genotype associated differences in the enzyme structure (Maring *et al.* (2009)).

1.8.3 Other HCV inhibitors

The exact role of the HCV NS3 helicase in the HCV viral life cycle is not clear yet, but it is essential for viral replication and therefore another interesting target for antivirals. To date though, no specific small molecules that are free of cytotoxicity have been identified. As the helicase is similar to human DEAD-box helicases it is difficult to find compounds that discriminate between the two, leading to non-toxic drugs (Du *et al.* (2002)). The NS2 protease is another possible target for STAT-C. A recent study has reported that the cyclophilin inhibitor CysA acts on HCV replication through NS2. It was the first anti-HCV drug shown to act through NS2, demonstrating a possible role of this protein as an antiviral target (Ciesek *et al.* (2009)).

Antiviral targeting is also possible against non-enzymatic proteins, as has been shown with NS5A. Currently 3 anti-NS5A inhibitors are in phase II clinical trials (Table 1.5), but the exact mechanism underlying their inhibition is unclear. In a proof-of-concept study, a single dose of the NS5A inhibitor BMS-790052 was able to reduce HCV RNA levels 3.6 $\log_{10}$ IU/ml with no rebound during a 6 day follow-up period. BMS-790052 also proved highly effective not only against genotype 1a, 1b and 2a, but also against genotype 3a, 4a and 5a in the replicon system. This high efficacy across genotypes

clearly is an important advantage over other antivirals currently being evaluated, which are mostly only effective in genotype 1 and 2 (Nettles *et al.* (2008)).

Another possible non-enzymatic target is the NS4B protein. Clemizole hydrochloride, a generic oral antihistamine, is the first molecule targeting NS4B being evaluated in clinical trials. In *in vitro* studies it has demonstrated 50 % effective concentration (EC_{50}) values in the low micromolar range and is now being evaluated in a phase I clinical trial (Einav *et al.* (2008)). Furthermore, p7, the putative ion channel, provides another possible target. Finally, the envelope protein E2 has also been targeted, with the entry inhibitors ITX-4520 and ITX-5061.

Table 1.5: ***Other HCV inhibitors and phase of clinical development.***

Compound	Phase of development	Company
Entry inhibitors		
ITX-5061	Phase IIa announced	iTherX
ITX-4520	Phase I	iTherX
SP-30	Preclinical/ Phase I announced	Samaritan Pharmaceuticals
NS4A inhibitors		
ACH-1095	Preparing to enter Phase I	Achillion
NS4B inhibitors		
Clemizole hydrochloride	Phase I	Eiger BioPharmaceuticals
NS5A inhibitors		
A-832	Phase II	AstraZeneca
AZD-2836 (A-831)	Phase II	AstraZeneca
BMS-790052	Phase II	Bristol-Myers Squibb
AZD-7295 (A-689)	Phase I	AstraZeneca

Information presented in this table has been gathered from public sources, accuracy is not guaranteed. Sources include company websites and the following public websites:
http://www.hcvdrugs.com
http://www.hivandhepatitis.com/hiv_hcv_co_inf_articles.html
http://www.hcvadvocate.org/hepatitis/hepC/HCVDrugs.html
http://clinicaltrials.gov

1.8.4 Development of antiviral resistance

A major issue with STAT-C is the preexistence of drug-resistant genetic variants among the hugely diverse pool of quasispecies and the short time frame within which new drug-resistant genetic variants can be generated by the error-prone RdRp. The impact of a drug-resistant variant depends on its replicative fitness and on the degree of drug resistance it confers. Depending on the bio-availability and potency of a compound, the drug-resistant variant will be able to evade the drug pressure, continue to replicate and spread. Even though the replicon and full-length HCV systems do not represent the huge diversity of quasispecies within an infected individual, it was still possible to select for resistance mutations developing *in vitro* under different protease and polymerase inhibitors. Importantly, some of these resistance mutations identified *in vitro* have been observed in clinical trials and worryingly also in treatment naïve patients (Sarrazin *et al.* (2007a); Bartels *et al.* (2008); Colson *et al.* (2008)).

As all NS3/4A PIs bind to the active site of the protease, it is no surprise that the identified resistance mutations partly locate to the same positions within the NS3 protease. Resistance mutations at R155K/Q, A156T and D168V/A have been reported in BILN 2061 resistance development and confer 357-fold (A156T), 24-fold (R155Q) and 144-fold (D168V) increases in the EC_{50} value of BILN 2061 (a macrocyclic inhibitor) (Lu *et al.* (2004)). ITMN-191, another macrocyclic PI, also induced resistance mutations at position D168A/V/E as well as A156S/V, F43S, Q41R and S138T. Unlike BILN 2061, where all mutations associated with decreased sensitivity located to the NS3 protease, for ITMN-191 one mutation (S489L) was identified within the helicase domain and one (V23A) within the NS4A cofactor (Seiwert *et al.* (2007b)). VX-950, which is a linear ketoamide peptidomimetic, shows a different drug resistance profile. The dominant resistance mutation under VX-950, A156S, still remains susceptible to BILN 2061 and the dominant BILN 2061 resistance mutations, D168V/A, remain susceptible to VX-950, supporting combination treatments. However, the resistance mutations A156V/T confer cross-resistance to both PIs, so do R155 substitutions (Lin *et al.* (2005a)). The decreased susceptibility of these mutations towards PIs is possibly diminished by their impaired replicative fitness, which was shown to be the case in the

replicon system (Lin *et al.* (2005a)).

As discussed in section 1.8.2, polymerase inhibitors bind to different sites in the enzyme leading to very different resistance profiles. While the nucleoside analogues bind to the catalytic domain, the NNIs bind to sites with variable distance to the active site. The substantial differences in the resistance profiles could explain the relatively low cross-resistance between polymerase inhibitors. As the NNIs bind outside of the catalytic domain, functional constraints are lower and resistance mutations can arise more easily than with nucleoside analogues. The 3-dimensional structure comprising the active site is very important and one change of amino acid can lead to a functionally impaired polymerase. R1626 was one of the first nucleoside polymerase inhibitors to enter clinical trials. In a phase Ib study, 14 days of treatment reduced the HCV RNA levels up to 3.6 $\log_{10}$ IU/ml in the highest dosed arm. Importantly, no resistance mutations were observed after 14 days of treatment with R1626, demonstrating a clear advantage of nucleoside inhibitors over PIs and NNIs, where resistance can develop within 2 weeks (Roberts *et al.* (2006, 2008)). In comparison, viral resistant variants emerged in peg-IFN/VX-950 combination treatment within 14 days of treatment (Reesink *et al.* (2006)). Similarly, in monotherapy with the NNI HCV-796, antiviral resistance variants were selected within the first week of treatment (Villano *et al.* (2006)). Unfortunately, further studies with R1626 had come to a halt, as dangerous levels of neutropenia have been observed. However, R7128, another nucleoside analogue, has also proven to have a high resistance barrier, with no resistance mutations appearing after 4 weeks of therapy (Le Pogam *et al.* (2009)). Studies in the replicon system have identified the NS5B polymerase amino acid substitution S282T, which confers a 3 to 4-fold decrease in susceptibility to R7128 *in vitro* (Ali *et al.* (2008)). However, a decrease in sensitivity was only observed when the S282T mutation was present in a very high proportion of the quasispecies population in a patient (above 90 %) (Le Pogam *et al.* (2009)).

Many mutations conferring resistance to STAT-C agents also significantly reduce viral fitness. This loss in viral replication competence can often be attributed to impaired enzymatic function. For example, A156T, which confers high-level resistance to

many PIs, also greatly reduces the replication capacity of the virus (Lin *et al.* (2005a); Tong *et al.* (2006); Yi *et al.* (2006a); Cubero *et al.* (2008)). However, not all resistance mutations induce a loss in replication fitness, as shown in *in vitro* studies with the replicon and the HCV cell culture system (Lin *et al.* (2005a); He *et al.* (2008)). Results from *in vitro* studies though, show some inconsistencies and do not always coincide with clinical results (Le Pogam *et al.* (2009)). Studies with VX-950 have shown that mutations conferring high level resistance were associated with lower levels of viremia after the end of treatment, whereas resistant mutations conferring low level resistance and having less impact on viral fitness were dominant. 3-7 months after dosing, high level resistant variants had disappeared, the frequency of low level resistant variants had decreased and wild type virus became dominant again. This demonstrates the necessity of combination treatment of standard of care with STAT-C to avoid the development of resistant mutations (Sarrazin *et al.* (2007a)). Importantly, it has been shown that second-site compensatory mutations may partly rescue defects in the viral fitness induced by a high level resistant mutation, enabling the high level resistant variant to spread and dominate within a population (McCown *et al.* (2009)).

1.8.5 Prevention of antiviral resistance

As the development of resistance mutations in monotherapy with current drugs is almost certain, combination treatment with other STAT-C agents and/or IFN and ribavirin is inevitable. Ideally, drugs targeting different parts of the virus/viral lifecycle are combined with each other and/or other agents targeting host proteins or supporting the host immune system. Thereby the emergence of cross-resistance can be prevented. This strategy is currently successfully used in HIV therapy and consists of at least 3 different drugs from at least 2 different “classes” of drugs. Typically therapies are based on 2 nucleoside analogue reverse transcriptase inhibitors, plus either a PI or a non-nucleoside reverse transcriptase inhibitor. Future HCV treatment regimes will most likely consist of similar triple combination regimes with STAT-C agents from different “classes”.

In vitro studies have shown that compounds targeting different viral enzymes, such as a PI and a polymerase inhibitor, generally do not select for cross-resistance mutants and can be used in combination treatment. Nevertheless, it is possible to select for cross-resistance mutations in combination therapy, as has been shown in the replicon system. Culturing the subgenomic 1b replicon under the PI BILN 2061 and A-782759, a polymerase inhibitor, selected not only for inhibitor specific, but also for cross-resistance mutations. However, the replicons resistant to both compounds were severely impaired in their replication kinetics and significantly lower in frequency, demonstrating again the inverse relationship between impaired viral fitness and drug susceptibility. Nevertheless, the inhibitors showed a synergistic effect and could reduce cellular replicon RNA levels over 7 $\log_{10}$ (Mo *et al.* (2005); Koev *et al.* (2007)). Combinations of BILN 2061 or SCH 503034 with NNIs (A848837 and A837093) also showed synergistic effects and were able to cure the replicon from the cells after long term treatment, whereas monotherapy led to the development of resistance mutations (Koev *et al.* (2006)). These studies provide encouraging results for future STAT-C inhibitor combination therapy. Besides protease and polymerase inhibitor combinations, it might also be possible to combine different NNIs, as they show lower cross-resistance as discussed above. However, nucleoside polymerase inhibitors are likely to play an important role in future combination treatments as they have a high barrier for resistance development.

Because resistance mutations against STAT-C agents develop so quickly, peg-IFN and probably also ribavirin, will remain part of combination therapy for the near future. Current clinical trials usually consist of a 8-12 week triple combination therapy of a STAT-C agent plus peg-IFN and ribavirin, which is followed by 12 weeks of peg-IFN/ribavirin combination treatment. With this treatment regime, an initial rapid decline of viral load is achieved by the STAT-C agent and peg-IFN/ribavirin. The standard care of treatment (peg-IFN/ribavirin) is then continued to suppress any viral rebound. The ultimate goal though, is to develop STAT-C agent combinations that will allow a rapid clearing of the virus and achieve a sustained virological response without the need of peg-IFN and ribavirin. A step towards this goal has been taken by Roche, who have recently completed a phase I clinical trial evaluating the efficacy and

safety of combination treatment with a HCV nucleoside analogue polymerase inhibitor (RO5024048) and an HCV PI (RO5190591/ITMN-191).

1.8.6 Targeting host enzymes

Compared to STAT-C antivirals, targeting host enzymes essential for viral replication provides the advantage of an intrinsically much higher barrier to resistance, as the virus is not directly targeted. The first molecules identified in this class were the cyclophilin inhibitors (Table 1.6). As described in section 1.2, cyclophilin B (CypB) enhances the RNA binding activity of the RdRp NS5B and can be inhibited by CysA. DEBIO-025, a CysA analogue, has advanced to phase II clinical trials, where it significantly reduced RNA levels in combination with peg-IFN-α-2a (Flisiak *et al.* (2009)). Even though DEBIO-025 targets a cellular protein, it has been reported to exert selective pressure on the HCV genome, which led to the emergence of resistance mutations in the replicon system (Robida *et al.* (2007)).

Another interesting target is the cellular micro-RNA miR-122. miR-122 has been shown to interact directly with the 5'UTR, thereby facilitating the replication of RNA (Jopling *et al.* (2005)). Therapies are currently being developed trying to sequester miR-122 using nucleic acid technology. A very recent study with SPC3649, a nucleic acid homologue to miR-122, has reported promising results in chimpanzees. Animals treated with SPC3649 showed a long lasting suppression of viral RNA without HCV rebound and no evidence of viral resistance or side-effects. As both binding sites of miR-122 are conserved in all HCV genotypes and subtypes, it is likely that this kind of treatment will be genotype-independent (Lanford *et al.* (2010)). Moreover, other possible targets include the 4 cellular receptors necessary for viral attachment and entry (Ploss *et al.* (2009)) or the geranylgeranylation of host proteins required for RNA replication (Ye *et al.* (2003); Kapadia & Chisari (2005)). The important issue with targeting host cell proteins though is cellular toxicity of compounds, which has to be carefully considered when administering these drugs in longterm. Furthermore, several phase I and II clinical trials are currently evaluating the effectiveness of immunodulators, TLR agonists (selectively stimulate IFN production), A3AR agonists (tumour

growth inhibitors), caspase and pancaspase inhibitors (anti-apoptotic), thiazolides (interfere with virus maturation) and anti-inflammatory agents.

Table 1.6: ***HCV inhibitors targeting host enzymes and phase of development.***

Compound	Phase of development	Company
Cyclophilin inhibitors		
DEBIO 025	Phase II (IIb announced)	Debiopharm
NIM811	Phase II	Novartis
SCY-635	Phase Ib	Scynexis
microRNA inhibitors		
SPC3649	Phase I	Santaris Pharma

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http://clinicaltrials.gov

Even though major achievements have been accomplished in the development of specific antiviral drugs targeting HCV in the last decade, still no STAT-C drug has been approved. This is mostly due to the highly replicative nature of HCV infection and the error-prone RdRp NS5B, which provide an enormous challenge to drug development. In the near future, peg-IFN will therefore most likely remain part of the standard care of treatment. Hopefully though, multiple, direct-acting STAT-C agents that can be used in combination to achieve a sufficiently high resistance barrier, will soon become available and render peg-IFN unnecessary, providing a less-toxic and simpler treatment regime.

Chapter 2
Materials and Methods

2.1 Sources of HCV Clones

pJFH1 and pJFH1-GND (AB047639) used in recombination construction were provided by T. Wakita (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) (Wakita *et al.* (2005)) and pFK JFH1/J6/C-846_dg (Jc1) by R. Bartenschlager (Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany) (Pietschmann *et al.* (2006)). pJ6CF (AF177036) and pH77* (differs from pH77 (AF011751) at M1205T) were provided by J. Bukh (NIH, Hepatitis Viruses Section, National Institute of Health, Bethesda, Maryland) (Yanagi *et al.* (1997, 1999)) and pHCV3a-Gla (p3a) by E.A. McCruden (Division of Virology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK) (Shaw *et al.* (2003)). HC-J4 (p1b, D10750) was described by Okamoto (Okamoto *et al.* (1992)). ED43* (p4a), differs from ED43 (Y11604) in 4 amino acids (T1048A, T1064I, I1160T, R1176A); EUH1480* (p5a) differs from EUH1480 (Y13184) by 9 amino acids (L1045V, F1061V, I1072T, L1081V, K1117T, G1118R, R1122P, I1694V, T1695I) and EUHK2* (p6a) differs from EUHK2 (Y12083) in 6 amino acids (I1196V, K1094R, F1087S, D1065A, V1070L, P1085A). Plasmids p4a, p5a and p6a were provided by Richard Elliot (Centre for Biomolecular Sciences, University of St Andrews, St Andrews, UK). Differences between clones and prototype sequences within the analysed NS3/4A region, were present in received clones and likely arose during cloning. Huh7, Huh7.5 and polyclonal sheep anti-NS5A serum were a gift from M. Harris (Institute of Molecular and Cellular Biology, University of Leeds, UK). Sequences of HCV isolates used for sequence diversity analysis were retrieved from the HCV sequence database (Kuiken *et al.* (2005)) and the NCBI GenBank. 570 NS3 and NS4A sequences of genotype 1a; 459 NS3 sequences of genotype 1b; 242 NS3 and 180 NS4A sequences of genotype 3a; 39 NS3 sequences of genotype 4a and 15 NS3 sequences of genotype 6a were analysed.

2.2 Cell Culture

2.2.1 Cell lines

Huh7 are human cells established from hepatoma tissue taken from a Japanese patient with well-differentiated hepatocellular carcinoma. They have a very low expression of the exogenous dsRNA sensor Toll-like receptor 3 and show an enhanced permissiveness for the replication of HCV RNA (Lohmann *et al.* (1999); Nakabayashi *et al.* (1982); Li *et al.* (2005a)). The Huh7.5 cell line is a highly permissive subclone from the Huh7 cell line, with a defect in RIG-I signalling (Blight *et al.* (2002); Sumpter *et al.* (2005)).

Huh7 and Huh7.5 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 4.5 g/l glucose, 2 mM L-glutamine, 10 % heat-inactivated fetal calf serum (FCS, Harlan Sera-Lab), MEM non-essential amino acids (Invitrogen), 20 mM hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and incubated at 37°C, 5 % CO_2 and 100 % relative humidity.

2.2.2 Harvesting and reseeding of cells

Adherent cells at 70-80 % confluency were removed from the flasks by trypsinisation. Following the removal of growth medium and washing of the cells with phosphate buffer saline (PBS), trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05 % trypsin, 0.53 mM EDTA, Invitrogen) was added and left at 37°C until cells detached. An equal volume of media was added to deactivate the trypsin. The cells were then concentrated at the bottom of an universal tube by centrifugation at 6,200 $\times$ g for 5 minutes. Supernatant was taken off, cells resupended in fresh medium and reseeded into a fresh tissue culture flask at a ratio of 1:3.

2.2.3 Freezing and thawing of cells

Cells were frozen if they were healthy and had not been passaged more than 2-3 times. It was crucial that cells had not been grown to more than 70-80 % confluency, as they lose their susceptibility to HCV. After washing and trypsinisation, cells were pelleted, resuspended in 10 ml fresh medium and counted using a haemocytometer. Once counted, cells were pelleted again and resuspended in freezing media at a concentration of 2-5 $\times$ 10^6 cells per ml. Freezing media consisted of 10 % dimethyl sulfoxide (DMSO) in 10 % FCS-DMEM. 1 ml of this cell suspension was then slowly frozen in 1.5 ml Nunc cryovials within a freezing box containing isopropanol, at -80°C. The slow freezing of the cells is crucial to allow water to escape from the cells, preventing the damaging of cells. Isopropanol reduces the cooling to approximately 1°C/minute. The following day cells were transferred to liquid nitrogen, storing them below -130°C and thereby preventing the formation of damaging ice crystals.

Cells were thawed up again in a 37°C water bath. It was crucial to keep the cells on dry ice while transporting and then thawing them as quickly as possible. Before the cryovials were incubated at 37°C, their cap was loosened slightly to allow gases that might have built up to escape. Cells in freezing media were then diluted in 10 ml fresh media and pelleted at 6,200 $\times$ g for 5 minutes. After pouring off the supernatant, the pellet was resuspended in fresh media and cells were seeded into a fresh T25 flask. The next day they were transferred to a T80 flask.

2.3 Extraction of Viral RNA

2.3.1 RNA extraction from patient plasma

HCV RNA was isolated form patient plasma using the QIAGEN RNeasy kit according to the manufacturer's guidelines. 150 μl patient plasma was mixed with 350 μl RLT buffer containing 1 % β-mercaptoethanol. To this 500 μl 70 % ethanol was added, mixed by pippeting, then transferred to an RNeasy mini spin column. The column in a collection tube was subjected to 15 seconds centrifugation at 8,000 $\times$ g. The

flow through was disposed of and the RNA bound to the silica membrane was washed by adding 700 μl RW1 buffer to the membrane and centrifuging at 8,000 $\times$ g for 15 seconds. Two further washes were carried out with 500 μl RPE buffer, centrifuging at 8,000 $\times$ g for 15 seconds, then for 2 minutes. The column was transferred to a new collection tube and spun for one minute at 8,000 $\times$ g to dry the column and remove excess ethanol which would affect the purity of the RNA. The column was then placed into an Eppendorf tube, 30 μl RNase free water added to the membrane and incubated for one minute. To elute the RNA, the column was spun at 8,000 $\times$ g for one minute. The elution was repeated using the 30 μl eluate. Purified samples were stored at -80°C.

2.3.2 RNA extraction from virally infected cells

To extract RNA from virally infected cells, cells were detached from tissue culture wells by trypsination and pelleted by centrifugation at 6,200 $\times$ g for 5 minutes. About 1×10^6 infected cells were lysed using 350 μl RLT buffer containing 1 % β-mercapto-ethanol. The cell lysate was directly pipetted onto a QIAshedder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at full speed. One volume of 70 % ethanol was added to the homogenised lysate and mixed well by pipetting. Up to 700 μl of the sample was transferred to an RNeasy mini spin column and purification proceeded as described above.

2.3.3 RNA extraction from cell culture supernatant

Alternatively, the QIAamp viral RNA kit from QIAGEN was used to extract viral RNA from cell culture supernatant. 140 μl cell culture supernatant was added to 560 μl AVL buffer containing carrier RNA at a concentration of 1 μg/μl. The carrier RNA helps the binding of viral nucleic acid to the QIAamp mini membrane and reduces the chance of degradation of the viral RNA by RNases, which might have escaped degradation by AVL. After pulse-vortexing for 15 seconds, the sample was incubated at room temperature for 10 minutes. The tube was briefly centrifuged to remove drops from inside the lid before 560 μl ethanol (96-100 %) was added. The sample was again

mixed by pulse-vortexing for 15 seconds and the tube briefly centrifuged, before 630 μl of the sample was applied to a QIAamp mini column. The column was centrifuged at 8,000 $\times$ g for one minute then placed into a new collection tube. The rest of the sample was applied to the QIAamp mini column and the spin step repeated. The column was then washed by applying 500 μl of AW1 buffer to the centre of the column, followed by centrifugation at 8,000 $\times$ g for one minute. A second wash step was performed by adding 500 μl AW2 buffer to the column and centrifugation at full speed for 2 minutes. The QIAamp mini column was placed into a new collection tube and centrifuged at full speed for one minute to remove residual buffers. To elute the RNA, the column was placed into a clean Eppendorf tube and 40 μl AVE buffer added. Following incubation for one minute, the column was centrifuged at 8,000 $\times$ g for one minute. To increase the concentration of the viral RNA, the eluate was passed through the column a second time. Purified samples were stored at -80°C.

2.4 First-strand cDNA Synthesis by Reverse Transcription

Reverse transcription was performed in a 20 μl reaction volume which contained the following: 500 ng total or 5 μl viral RNA and DNase/RNase-free water to a final volume of 8 μl. The mixture was heated for 10 minutes at 70°C and then chilled on ice for 5 minutes. The tubes were kept on ice and 4 μl of 25 mM $MgCl_2$, 2 μl reverse transcription 10X buffer, 2 μl of 10 mM dNTPs, 0.5 μl recombinant RNasin ribonuclease inhibitor, 2.8 μl of 0.1 mM random primers and 15 Units (U) of AMV reverse transcriptase were added. The reaction mixture was first incubated for 10 minutes at room temperature, then for 50 minutes at 42°C. To inactivate the reverse transcriptase, the reaction mixture was incubated for 5 minutes at 95°C. The generated cDNA was stored at -20°C.

2.5 Plasmid Dilution Series

To test primers and optimise the PCR reaction, plasmid dilution series were used. Parameters varied for optimisation included annealing temperature, $MgCl_2$ and DMSO concentrations and elongation time. Plasmid pH77* (genotype 1a) and pHCV3a-Gla (genotype 3a) were diluted in tris-sodium chloride-EDTA (TNE) buffer, containing 250 pg/ml herring DNA. Final dilutions contained 4.4×10^9 DNA molecules per μl for dilution 1, then 10-fold dilutions were prepared down to 4.4 molecules of DNA per μl in dilution 10.

2.6 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed to amplify nucleic acids for downstream applications. All PCR and RT-PCR reactions were performed using a TECHNE Flexigene thermocycler. Depending on the downstream application, 3 different thermostable polymerases were used. Fragments used for cloning were amplified using the high-fidelity VentR® polymerase (New England BioLabs (NEB)). All primers were made up to a concentration of 10 OD/ml (OD, optical density), sequences are shown in Appendix A. PCR reactions were set up in a 50 μl reaction volume. Reagents used for the Vent PCR are listed in Table 2.1 and the thermal cycling carried out in Table 2.2.

Table 2.1: *Reagents used for Vent PCR.*

Reagent	Quantity
10X ThermoPol reaction buffer	5 μl
10 mM dNTPs	1 μl
Sense primer (10 OD/ml)	0.5 μl
Anti-sense primer (10 OD/ml)	0.5 μl
Template DNA	5 μl
VentR DNA polymerase (2 U/μl)	0.5 μl
DNase/RNase-free water	to 50 μl

Table 2.2: *Vent PCR protocol.*

# Cycles	Temperature	Time
1	95°C	2 minutes
35	95°C	30 seconds
	55°C	30 seconds
	72°C	90 seconds
1	72°C	5 minutes

The 10X reaction buffer contained: 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ and 0.1 % Triton X-100 at pH 8.8. A non-template (deionized (d) H_2O) control was used in every PCR run to test possible contamination of the reagents. PCR

products were analysed by agarose gel electrophoresis (0.7-2 % agarose gel, depending on the size of the product).

Fragments which could not be amplified successfully with VentR polymerase were amplified using the high-fidelity KOD polymerase (Novagen). PCR reactions were set up in a 50 μl reaction volume. Reagents used in the KOD PCR are listed in Table 2.3 and the thermal cycling carried out in Table 2.4.

Table 2.3: *Reagents used for KOD PCR.*

Reagent	Quantity
10X KOD Buffer	5 μl
25 mM $MgSO_4$	3 μl
2 mM dNTPs	5 μl
Sense primer (10 OD/ml)	1.5 μl
Anti-sense primer (10 OD/ml)	1.5 μl
Template DNA	5 μl
KOD Hot Start polymerase (1 U/μl)	1 μl
DNase/RNase-free water	to 50 μl

Table 2.4: *KOD PCR protocol.*

# Cycles	Temperature	Time
1	95°C	2 minutes
35	95°C	20 seconds
	55°C	10 seconds
	72°C	10 seconds/kb

kb, kilo base pair

The enzyme buffer contained: 50 mM tris-HCl, 1 mM DTT, 0.1 mM EDTA, 50 % glycerol, 0.001 % Nonidet P-40 and 0.001 % Tween-20 at pH 8.

For diagnostic PCR and for sequencing of the amplified fragments standard GoTaq DNA polymerase (Promega) was used. PCR reactions were set up in a 30 μl reaction volume. Reagents used in the GoTaq PCR are listed in Table 2.5 and the thermal cycling carried out in Table 2.6.

Table 2.5: *Reagents used for GoTaq PCR.*

Reagent	Quantity
5X GoTaq Mix (7.5 mM $MgCl_2$)	6 μl
10 mM dNTPs	0.6 μl
Sense primer (10 OD/ml)	0.3 μl
Anti-sense primer (10 OD/ml)	0.3 μl
Template DNA	3 μl
GoTaq DNA polymerase (5 U/μl)	0.2 μl
DNase/RNase-free water	to 30 μl

Table 2.6: *GoTaq PCR protocol.*

# Cycles	Temperature	Time
1	95°C	2 minutes
35	95°C	30 seconds
	55°C	30 seconds
	72°C	90 seconds
1	72°C	5 minutes

2.7 One Step RT-PCR and Amplification of Viral RNA

The reverse transcriptase-polymerase chain reaction (RT-PCR) followed by a second round, nested, PCR reaction was used to amplify the N-terminal protease of the NS3 gene and the NS4A gene of HCV subtypes 1a, 1b, 3a, 4a and 6a, from clinical strains. For the RT-PCR step, the Access Reverse Transcriptase PCR kit (Promega) or the SuperScript™ III One-Step RT-PCR System with Platinum®Taq DNA polymerase was used. The Promega Access kit PCR reactions were set up in a 50 μl reaction volume. Reagents used in the Access RT-PCR are listed in Table 2.7 and the thermal cycling carried out in Table 2.8.

Table 2.7: *Reagents used for Access RT-PCR.*

Reagent	Quantity
AMV/T*fl* 5X Reaction Buffer	10 μl
2 mM $MgSO_4$	2 μl
10 mM dNTPs	1 μl
Sense primer (10 OD/ml)	1 μl
Anti-sense primer (10 OD/ml)	1 μl
Extracted viral RNA	10 μl
AMV RT polymerase (5 U/μl)	1 μl
T*fl* DNA polymerase (5 U/μl)	1 μl
DNase/RNase-free water	to 50 μl

Table 2.8: *Access RT-PCR protocol.*

# Cycles	Temperature	Time
RT step	48°C	45 minutes
1	94°C	2 minutes
35	94°C	36 seconds
	55°C	30 seconds
	68°C	2 minutes
1	68°C	5 minutes

For the second step PCR reaction, the first round PCR product was amplified using primers that anneal inside the primary reaction, performing nested PCR.

Alternatively, the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA polymerase (SC) was used. The outer primers were used to initiate the RT step to generate a cDNA and the following first round PCR product. SC RT-PCR reactions were set up in a 25 μl reaction volume. Reagents used in the SC RT-PCR are listed in Table 2.9 and the thermal cycling carried out in Table 2.10. The 2X reaction buffer contained: 0.4 mM of each dNTP and 3.2 mM $MgSO_4$.

If the RT-PCR amplification was not successful, a second round, nested PCR was performed using primers that anneal inside the first amplification product. For this, oligonucleotide primers flanking the NS3 protease gene or the NS4A gene were de-

Table 2.9: *Reagents used for SC RT-PCR.*

Reagent	Quantity
2X Reaction buffer	12.5 μl
Sense primer (10 OD/ml)	0.25 μl
Anti-sense primer (10 OD/ml)	0.25 μl
Extracted viral RNA	5 μl
Superscript^{TM} III RT/Platinum$^{®}$Taq mix	1 μl
DNase/RNase-free water	to 25 μl

Table 2.10: *SC RT-PCR protocol.*

# Cycles	Temperature	Time
RT step	43°C	60 minutes
20	53°C	1 minute
	55°C	1 minute
1	70°C	15 minutes
1	94°C	2 minutes
35	94°C	30 seconds
	54°C	30 seconds
	68°C	90 seconds
1	68°C	5 minutes

signed (Table A.1, A.2 and A.4, Appendix A). Second round primers were designed to introduce restriction sites to the NS3 fragment or NS4A for future cloning purposes. *Not*I was added to the 5'NS3 sense primer and *Spe*I to the 3'NS3 anti-sense primer. Alternatively, a *BstB*I restriction site was added to the 5'NS3 sense primer and a *Bgl*II site to the 3'NS3 anti-sense primer (Table A.5, Appendix A). A *Sap*I restriction site was added to the 5'NS4A sense primer and a *Mlu*I site to the 5'NS4A anti-sense primer (Table A.6, Appendix A). Second round PCR was performed as described above. To test whether HCV RNA isolation was successful, primers which cover the core region were used as a control (Table A.7, Appendix A). PCR products were used for sequencing without any further purification.

2.8 Agarose Gel Electrophoresis

Analysis of DNA and RNA was performed by agarose gel electrophoresis. Depending on the size of the DNA or RNA 0.7-2 % agarose gels were prepared using 1X tris-acetate-EDTA (TAE) buffer with 0.5 μl ethidium bromide (EtBr). Samples were mixed with 10X loading buffer and loaded into the wells of the agarose gel. To separate different fragments of the nucleic acid, an electric current of 150 V was applied for 30-60 minutes, depending on the size of the fragments. As a size marker a DNA ladder of 10 kb or 1,000 bp (base pair) was used. Nucleic acid fragments were visualised using an ultraviolet (UV) transilluminator. EtBr intercalates into the DNA and exhibits

fluorescence under UV light, thereby allowing the visualisation of the DNA/RNA.

2.9 Sequencing

Sequencing of PCR products and purified plasmids was performed using the ABI BigDye terminator technology. To sequence the NS3 protease regions, primers annealing outside the NS3 protease gene region where designed, which can be found in Table A.11 and A.13, Appendix A. The primers used for sequencing of NS4A and the rest of the HCV genome can be found in Table A.9, A.10 and A.12, Appendix A. The sequencing reactions were set up in a 20 μl reaction volume. Reagents used in the sequencing reaction are listed in Table 2.11 and the sequencing protocol carried out in Table 2.12.

Table 2.11: *Reagents used for sequencing.*

Reagent	Quantity
PCR product	1-4 μl
or plasmid DNA	300-500 μg
BIG Dye	1 μl
Primer (10 OD/ml)	1 μl
dH_2O	to 20 μl

Table 2.12: *Sequencing reaction protocol.*

# Cycles	Temperature	Time
25	95°C	30 seconds
	50°C	20 seconds
	60°C	4 minutes
1	68°C	5 minutes

Sequencing reactions were sent to the departmental sequencing facility and results were returned in the AB1 file (Chromas) and SEQ file format. Sequence analysis was performed using the Simmonics2005_v1.8 software.

2.10 Restriction Digest

Digestion reactions were set up as follows: 1-10 μg DNA, 5 μl 10X restriction enzyme buffer 1, 2, 3 or 4 (NEB), 0.5 μl 100X bovine serum albumin (BSA), 0.3-1 μl restriction enzyme (1 U/μg DNA) and nuclease-free water to a final volume of 50 μl. The reaction mixture was incubated for 2-4 hours at 37°C or 65°C and analysed by gel electrophoresis.

2.11 Dephosphorylation

To prevent recircularisation of digested vectors in ligation reaction, the 5' phosphate of the vector was removed using Antarctic Phosphatase (AP) (Invitrogen). Phosphatase-treated vectors lacking the 5' phosphoryl termini required by the ligase, cannot self-ligate and vector background is thereby reduced. Dephosphorylation reactions were set up as follows: 1-10 μg DNA, 5 μl 10X restriction enzyme buffer 1, 2, 3, 4 (NEB) or 10X AP reaction buffer (NEB), 0.3-2 μl AP (1 U/μg DNA) and DNase/RNase-free water to a final volume of 50 μl. The reaction mixture was incubated for 30 minutes at 37°C and the enzyme heat inactivated through incubation for 5 minutes at 65°C.

2.12 Purification of PCR Products

To purify single banded, digested PCR products up to 10 kb, the QIAquick PCR purification kit was used. In brief, 5 volumes of buffer PB were added to one volume of the digestion reaction and mixed by pippeting. To bind, the DNA sample was applied to the QIAquick column and the column was centrifuged for one minute at 17,900 $\times$ g. Flow-through was discarded and 750 μl of buffer PE was added followed by one minute centrifugation at 17,900 $\times$ g. To remove any residual ethanol, an additional centrifugation step of one minute at 17,900 x g was performed. After adding 30 μl elution buffer (EB, 10 mM Tris-HCl at pH 8.5), the column was incubated for one minute, before DNA was eluted by centrifugation at 17,900 $\times$ g for one minute. Samples were stored at -20°C for further experiments.

2.13 Gel Purification

Digested plasmids or amplified PCR products with multiple bands were separated by gel electrophoresis and DNA visualised under long wavelength UV light. The appropriate band on the gel was excised using a sterile blade, weighed and purified using the QIAGEN gel extraction kit for fragments of up to 10 kb. Three volumes of buffer QG

were added and the sample incubated at 50°C until completely dissolved. One volume of isopropanol was added and the sample mixed before it was applied to a QIAquick column and centrifuged for one minute at 17,900 $\times$ g. The low pH conditions allowed the DNA to bind to the silica membrane. Flow-through was discarded and the column washed with 500 μl QG buffer to remove any traces of agarose. A further wash step was carried out by applying 750 μl buffer PE and centrifuging for one minute at 17,900 $\times$ g. To ensure complete removal of residual ethanol, the QIAquick column was centrifuged for an additional minute at 17,900 $\times$ g. DNA was then eluted under basic and low-salt conditions. To increase the yield of DNA, 30 μl of EB was added to the centre of the column and incubated for one minute, before it was centrifuged for one minute at 17,900 $\times$ g to elute the sample.

For fragments larger than 10 kb the QIAEX II gel extraction kit was used. The appropriate band on the gel was excised using a sterile blade, weighed and 3 volumes of buffer QX1 and 2 volumes of H_2O added. For every 10 μg DNA, 30 μl QIAEX II resins were added, mixed by flicking the tube and incubating for 10 minute at 50°C to solubilise the agarose and bind the DNA. To keep the QIAEX II in suspension, the mixture was mixed by flicking and inverting the tube every 2 minutes. The sample was centrifuged at 17,900 x g for 30 seconds, the supernatant taken off and the pellet washed with 500 μl buffer QX1. Two further washes were carried out with 500 μl buffer PE. All wash steps consisted of resuspension of the sample in the wash buffer and centrifugation at 17,900 x g for 30 seconds. The pellet was air-dried for 15 to 30 minutes or until it became white. To elute the DNA, 20 μl buffer EB was added and the pellet resuspended by flicking and inverting the tube. After 10 minutes incubation at 50°C the solution was centrifuged at 17,900 $\times$ g for 30 seconds. The supernatant containing the DNA was taken off and transferred to a clean microcentrifuge tube. To increase the yield, a second elution step was performed. Samples were stored at -20°C for further experiments.

2.14 DNA Precipitation and Purification

To precipitate and purify DNA a phenol/chloroform extraction, followed by ethanol precipitation was used. Samples smaller than 200 μl were diluted in water to a final volume of 200 μl and an equal volume of phenol:chloroform:isoamyl-alcohol:25:24:1 added. Following vortexing for 30 seconds, samples were centrifuged at 17,900 $\times$ g for 5 minutes. The upper aqueous phase containing the DNA was removed and transferred to a new microcentrifuge tube. 200 μl chloroform was added and the mixture vortexed for 30 seconds. After centrifugation at 17,900 $\times$ g for 5 minutes, the upper aqueous phase containing the DNA was transferred to a new microcentrifuge tube and DNA precipitated by adding 1 μl glycogen, 0.1 volume of 5 M NaCl and 2 volumes of ethanol (96-100 %). Following incubation on ice for 30 minutes, the precipitated DNA was pelleted by centrifugation at 17,900 $\times$ g for 30 minutes at 4°C. Supernatant was carefully removed and the pellet washed by resuspension in 400 μl 70 % ethanol and centrifugation at 17,900 $\times$ g for 15 minutes at 4°C. Supernatant was carefully removed and the pellet redissolved in DNase/RNase-free water.

2.15 Bacterial Techniques

2.15.1 Bacterial cultures

Different strains of *E.coli* were used. Transformed bacteria were grown on Luria-Bertani (LB) plates with 1.5 % agarose or in LB medium, supplemented with appropriate antibiotics (100 μg/ml ampicillin or 50 μg/ml kanamycin). LB agar was poured into Petri dishes with a diameter of 10 cm. Bacteria were plated on the LB agar surface using a glass spreader and incubated inverted at 30°C for approximately 16 hours. All incubations for bacteria were carried out at 30°C to reduce spontaneous mutations within the plasmid. Single colonies were then picked to inoculate 5 ml of LB broth, which was incubated at 30°C for approximately 16 hours with constant shaking at 200 rounds per minute (rpm) in an orbital shaker. 500 μl of the cell culture was mixed with 500 μl glycerol to generate a glycerol stock of the corresponding colony for longterm

storage at -20°C. The remaining culture was used for plasmid extraction using mini or maxiprep kits, see section 2.15.3 and 2.15.4. All bacterial procedures were performed under a flame ensuring aseptic conditions.

2.15.2 Transformation of chemically competent cells

Transformation of NEB 5-α *E.Coli* (subcloning efficiency). NEB 5-α *E.Coli* were used for the transformation of intact plasmids dissolved in dH_2O or EB. 1-10 ng of plasmid DNA was used to transform the bacterial cells according to the protocol provided by the supplier of the competent cells (NEB). In brief, half a vial (25 μl) of competent cells was thawed on ice, plasmid DNA added, mixed gently and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for 30 seconds and 250 μl prewarmed super-optimal broth with catabolite repression (SOC) medium added. Vials were incubated at 37°C for one hour with constant shaking at 200 rpm in an orbital shaker. 50-100 μl of the transformed bacteria were then plated on LB agar plates containing the appropriate antibiotic and incubated overnight at 30°C.

Transformation of NEB 10-β competent *E.Coli* (high efficiency). Alternatively, highly efficient NEB 10-β competent *E.Coli* were used for the transformation of ligation products. The same protocol was followed as described above for the NEB 5-α. After the heat shock 500 μl instead of 250 μl SOC medium was added to the cells.

Transformation of TOP10 One Shot® chemically competent *E.Coli* (high efficiency). For TOPO cloning reactions, see section 2.16.1, TOP10 One Shot® chemically competent *E.Coli* (Invitrogen) were used for the transformation of ligation products. The same protocol was followed as described above for NEB 5-α cells.

Transformation of XL1-Blue supercompetent cells. Plasmids modified by site directed mutagenesis were transformed into XL1-Blue supercompetent cells (Stratagene). Protocol see section 2.16.3.

2.15.3 Small scale plasmid DNA preparation (mini prep)

To isolate small amounts of plasmid DNA for diagnostic purposes (sequencing and restriction digestions) the QIAprep spin miniprep kit was used. 5 ml of LB medium supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin was inoculated with a single bacterial colony picked from an LB agar plate. Liquid cultures were incubated overnight at 30°C and continuously shaken at 200 rpm. Bacteria were then pelleted by centrifuging at 6,200 $\times$ g for 3 minutes. Pelleted bacterial cells were resuspended in 250 μl buffer P1 and transferred to a microcentrifuge tube. 250 μl lysis buffer P2 was added and gently mixed by inverting the tube 4-6 times. Following the addition of 350 μl buffer N3 the tube was again mixed gently, then centrifuged at 17,900 $\times$ g for 10 minutes. The supernatant was transferred to a QIAprep spin column and the column centrifuged for one minute at 17,900 x g to allow the DNA to bind to the silica-gel membrane. The column was washed by adding 500 μl buffer PB followed by centrifugation at 17,900 $\times$ g for one minute. A further wash step was carried out by adding 750 μl buffer PE and centrifuging at 17,900 $\times$ g for one minute. To remove residual wash buffer, the column was centrifuged for an additional minute. To elute DNA, 50 μl buffer EB was added, the column allowed to stand for one minute and centrifuged at 17,900 $\times$ g for one minute. The quality and purity of plasmid DNA was assessed by spectrophotometry and test digests, followed by gel electrophoresis on a 1.5 % agarose gel. Alternatively, enzymes were heat inactivated (65°C for 20 minutes) and plasmid DNA subjected to further treatments. Depending on the amount of plasmid DNA, reaction mixtures were scaled up. Plasmid DNA was stored at -20°C.

2.15.4 Large scale plasmid DNA preparation (maxi prep)

For the extraction of large amounts of high quality plasmid DNA the QIAGEN plasmid maxi kit was used. 5 ml of LB medium containing the appropriate antibiotic, 100 μg/ml ampicillin or 50 μg/ml kanamycin, were inoculated with a single bacterial colony picked from a LB agar plate. The starter culture was incubated for 8 hours at 30°C in an orbital shaker at 200 rpm. 500 μl of the starter culture was used to inoculate 250 ml LB medium supplemented with the appropriate antibiotic and incubated

for another 12-16 hours at 30°C and continuously shaken at 200 rpm. Bacteria were pelleted by centrifugation at 6,000 × g for 15 minutes at 4°C. Following the removal of LB medium, pelleted bacterial cells were resuspended in 10 ml buffer P1. 10 ml of buffer P2 was added, thoroughly mixed and lysis allowed to proceed for no more than 5 minutes. After adding 10 ml of chilled buffer P3 to the lysate, the mixture was thoroughly mixed, then immediately poured into the barrel of a QIAfilter cartridge and incubated at room temperature for 10 minutes. The cell lysate was then filtered into a QIAGEN-tip previously equilibrated with 10 ml buffer QBT and allowed to enter the resin by gravity. The QIAGEN-tip was washed twice with 30 ml buffer QC and DNA eluted with 15 ml buffer QF. DNA was precipitated by the addition of 10.5 ml isopropanol and pelleted by centrifugation at 5,000 × g for 60 minutes at 4°C. The pellet containing the DNA was washed with 5 ml 70 % ethanol and centrifuged at 5,000 × g for 60 minutes. The supernatant was carefully decanted and the pellet air-dried for approximately 10 minutes before it was resuspended in 300 μl DNase/RNase-free water and stored at -20°C.

2.16 Molecular Cloning

2.16.1 TOPO cloning

For amplification, further cloning and to check insert sequence, NS3 fragments amplified by PCR were cloned into pCR®-blunt II-TOPO vector. The plasmid vector is supplied linearised with Vaccinia virus DNA topoisomerase I covalently bound to the 3'end of each DNA strand. Blunt-end PCR products can directly be inserted and no DNA ligase is necessary. DNA fragments were amplified using primers described in Appendix A and Vent Polymerase, giving 95 % blunt-ended PCR products. TOPO cloning was performed according to the manufacturer's user manual. The cloning reaction contained the following: 4 μl PCR product, 1 μl salt solution and 1 μl TOPO-vector. The reaction mixture was gently mixed, then incubated for 30 minutes at room temperature. 2 μl of the TOPO cloning reaction was used to transform TOP10 One Shot® competent *E.coli* as described in section 2.15.2. Transformed bacteria were

spread on a plate containing 50 μg/ml kanamycin and incubated over night at 30°C. The next day colonies were picked, grown up and plasmids isolated as described in section 2.15.3. The quality and purity of plasmid DNA was assessed by spectrophotometry and test digests followed by gel electrophoresis on a 1.5 % agarose gel. Insert integrity was analysed by sequencing using M13 forward and M13 reverse primers (sequences can be found in Table A.11, Appendix A). Alternatively, a TOPO vector containing a 100 bp insert was digested with restriction enzymes targeting the restriction sites of the multiple cloning site and dephosphorylated. The linearised and dephosphorylated vector was purified from the insert and enzymes by gel purification and used in ligation reactions with inserts digested with the same restriction enzymes.

2.16.2 Colony PCR

To quickly identify colonies containing the correct insert, colony PCR was performed. Primers annealing within the insert of interest were used for a one-step PCR. The PCR mixture was prepared and added to the PCR tubes. Using a pipette tip individual colonies were picked, a fresh LB agar plate touched and the rest immersed in the PCR mix. The PCR reaction was performed as described in section 2.6. PCR products were analysed on an agarose gel and sequenced.

2.16.3 Site directed mutagenesis

To introduce specific mutations into a desired plasmid, the QuickChange® site directed mutagenesis kit (Stratagene) (QC) was used. The high fidelity *PfuTurbo* DNA polymerase** and 2 primers containing the desired mutation/s are used to amplify a mutated plasmid from supercoiled dsDNA. The parental plasmid is then digested with the endonuclease *DpnI*, which specifically targets methylated and hemimethylated DNA. The mutated, nicked and non-methylated DNA remains intact and can be transformed into XL1-Blue supercompetent cells, which then repair the nicks in the mutated plasmid.

Primer design. For each desired mutation 2 primers annealing to the same sequence on opposite strands and containing the same desired mutation were designed using Simmonics2005_v1.8 software and NetPrimer (PREMIER Biosoft International). A maximum of 4 mutations per primer were introduced. A list of all primers used to introduce mutations can be found in Table A.8, Appendix A. Primers were designed to be between 25 and 45 bp in length and to have a melting temperature (T_m) above 78°C. The desired mutation was designed to be in the middle of the primer. The minimum of the GC content was 40 % and the termini contained desirably one or two G or C bases.

Mutant strand synthesis reaction. The reaction was set up in thin-walled tubes to allow ideal contact with the temperature cycler's heat block. The mutagenesis PCR reactions were set up in a 25 μl reaction volume. Reagents used in the QC PCR are listed in Table 2.13 and the thermal cycling carried out in Table 2.14.

Table 2.13: *Reagents used for QC PCR.*

Reagent	Quantity
10X Reaction buffer	2.5 μl
Plasmid DNA	15 ng
Sense primer (5 OD/ml)	0.4 μl
Anti-sense primer (5 OD/ml)	0.4 μl
10 mM dNTP mix	0.5 μl
DNase/RNase-free water	to 25 μl
PfuTurbo DNA polymerase (2.5 U/μl)	0.5 μl

Table 2.14: *QC PCR protocol.*

# Cycles	Temperature	Time
1	95°C	30 seconds
18	95°C	30 seconds
	55°C	1 minute
	68°C	1 minute/kb[1]

[1] *Total length of plasmid*

Following temperature cycling the reaction mixture was placed on ice for 2 minutes. To digest the parental supercoiled (non-mutated) dsDNA, 0.5 μl *Dpn* I was added and the reaction incubated at 37°C for one hour.

Transformation of XL1-Blue supercompetent cells. 25 μl of XL1-Blue cells were thawed on ice and 1 μl of the *Dpn* I treated DNA added and gently mixed. Following 30 minutes incubation on ice, the cells were heat pulsed for 45 seconds at 42°C and placed back on ice for 2 minutes. 500 μl pre-warmed SOC medium was added and the vial incubated at 37°C for one hour and constantly shaken at 200 rpm in an orbital shaker. 50-100 μl of the transformed bacteria were plated on LB agar plates containing the appropriate antibiotic and incubated overnight at 30°C. Individual colonies were

picked, grown up in LB and plasmid DNA isolated as described in section 2.15.3. More than 95 % of all colonies analysed contained the desired mutation.

2.16.4 Construction of pJFH1 and Jc1-based intra- and intergenotypic recombinants

Nucleotide positions used for numbering are referred to according to their position in the H77 reference strain, AF009606. pJFH1 contains the full-length HCV genome of genotype 2a isolated from an Japanese patient with fulminant hepatitis (Wakita *et al.* (2005)). pJFH1-GND is the replication deficient negative control, encoding a GDD to GND mutation in the NS5B RdRp polymerase (Kato *et al.* (2003)). Jc1 (pFK-JFH1/J6/C-846 dg) is a recombinant containing the structural genes and part of NS2 from the genotype 2a isolate pJ6CF, and the remaining non-structural genes from the genotype 2a isolate JFH1 with the cross-over point within NS2 (Pietschmann *et al.* (2006)). The general cloning protocol consisted of digestion of the insert and the vector with equivalent restriction enzymes. If the 2 restriction enzymes did not exhibit at least 75 % activity in the same buffer, sequential digests were performed. After the first digest, DNA was precipitated with ethanol, redissolved in EB and the second digest set up. To prevent self ligation of cut vector, the vector was dephosphorylated using AP. Digested insert and vector were purified by PCR and gel extraction purification as described in section 2.12 and 2.13. Both insert and linearised vector were run on a agarose gel to verify their size. Subsequently, a ligation reaction using a 1:10 molar ratio of vector to insert DNA was set up. The ligation reaction had a final volume of 10 μl and contained 300 ng DNA, 1 μl 10X ligase buffer, 0.5 μl T4 DNA ligase and dH_2O to a final volume of 10 μl. To calculate the amounts of insert and vector required for each reaction equation 2.1 was used

$$\frac{m_v \times l_v}{l_v} \times 0.1 = m_i \tag{2.1}$$

where m_v is the vector mass (ng), m_i the insert mass (ng), l_v the vector length (kb)

and l_i the insert length (kb). The reactions were incubated overnight at 4°C, followed by incubation for further 4-5 hours at 16°C. To estimate the extent of vector religation and as a negative control, cut vector without insert was subjected to the same ligation reaction. 2 μl of the ligation reaction was then used to transform highly efficient NEB 10-β competent *E.Coli* as described in section 2.15.2.

Replacement of the NS3 protease gene in pJFH1 using naturally occurring restriction sites ($\mathbf{F}_n\mathbf{x}_n$). The NS3 protease gene was amplified by PCR from plasmid or cDNA generated from patient plasma using corresponding genotype specific primers containing the desired restriction sites. Primer sequences can be found in Table A.1 and A.2, Appendix A; primers -IS contain *Not*I and -IA *Spe*I. For the amplification of the protease the naturally occurring restriction sites *Not*I (nt position 2945) and *Spe*I (nt position 4094) were used. The 1149 bp long fragment includes the N-terminal 475 bp of NS2, the entire NS3 protease and the C-terminal 131 bp of the NS3 helicase. PCR products were digested using *Not*I and *Spe*I in a double digest in NEB buffer 2. Digested inserts were run on a gel to confirm their size, cleaned up and ligated into pJFH1. pJFH1 was likewise digested with *Not*I and *Spe*I, dephosphorylated and cleaned up.

Replacement of the NS3 protease gene using genetically engineered restriction sites (Fx/Jx). To construct F1a, F1b, F2a, F3a, F4a, F5a and F6a a *Bst*BI restriction enzyme site was generated at the junction of NS2 and NS3 of the pJFH1 plasmid and a *Bgl*II restriction enzyme site at the junction of the NS3 protease and NS3 helicase domain. To reduce the possibility of PCR errors, the NS3 gene was subcloned into Zero Blunt® TOPO using the naturally occurring restriction sites *Not*I and *Spe*I. QuickChange® site directed mutagenesis, see section 2.16.3, was used to introduce 2 silent point mutations to generate a *Bgl*II (C3398T and C3401G) and one to create a *Bst*BI site (G3993A). The entire length of the subcloned fragment was sequenced to verify each of the introduced base changes and to make sure no further base changes were introduced. The NS3 protease region was amplified from pH77* (genotype 1a) with primer 1aBstBI and 1aBglII, introducing a *BstB*I and a *Bgl*II restriction site. The same strategy was used to amplify the NS3 protease from genotype 1b-6a prototype

plasmids using the corresponding genotype specific primers. Primers were designed to include JFH1 (type 2a) sequence for the NS2 and NS3 helicase region and the corresponding intra- or intergenotypic sequence for the NS3 protease region (nt position 3420-3963). Their sequence can be found in Table A.5, Appendix A. PCR products were digested with *Bst*BI and *Bgl*II, gel purified and ligated into pJFH1. pJFH1 has similarly been digested with *Bst*BI and *Bgl*II, dephosphorylated using AP and gel purified. Each introduced NS3 protease gene was verified by DNA sequence analysis. To generate the corresponding Jc1-based recombinants (Jx), pJFH1-based recombinant plasmids (Fx) were digested with *Not*I and *Sfi*I, gel purified and ligated into Jc1 which had likewise been digested with *Not*I and *Sfi*I and dephosphorylated.

Replacement of the NS3 protease and NS4A cofactor gene using genetically engineered restriction sites (Fxx/Jxx). To generate F1a1a, F1b1b, F2a2a, F3a3a, F4a4a, F5a5a and F6a6a, 2 new restriction sites were introduced into pJFH1. To reduce the possibility of PCR errors, a 4381 bp long JFH1 fragment including the NS4A cofactor was subcloned into Zero Blunt® TOPO using the naturally occurring restriction sites *Nsi*I (nt position 5286) and *Xba*I (nt position 9667). A *Blp*I restriction site was generated by introducing one silent point mutation (C5297G) at the NS3 helicase and NS4A junction and a *Mlu*I site at the NS4A and NS4B junction by introducing 4 point mutations (T5478A, T5480G, A5481C, G5483T), were one was non-silent. The NS4A region was amplified from pH77* (genotype 1a) with primer 1aSapI and 1aMluI, introducing a *Sap*I (5'-end) and a *Mlu*I (3'-end) restriction site. The same strategy was used to amplify NS4A from genotype 1b-6a prototype plasmids using the corresponding genotype specific primers. Primers were designed to include JFH1 sequence up to the start of the NS4A gene (nt position 5313) and from the end of the NS4A gene (nt position 5474) to the restriction site. Primer sequences can be found in Table A.6, Appendix A. Products were digested with *Sap*I and *Mlu*I, gel purified and ligated into the TOPO vector containing the JFH1 insert digested with *Blp*I and *Mlu*I and dephosphorylated. A5478 was mutated back to T by site directed mutagenesis to recreate native JFH1 amino acid sequence (Ala to Thr) outside of the NS4A region. To generate Fxx and Jxx, TOPO vector including the modified insert was digested with *Not*I and *Sfi*I, gel purified and ligated into pJHF1 and Jc1, respectively, digested with *Nsi*I and *Sfi*I

and dephosphorylated.

Construction of adapted genomes. RNA was extracted from infectious supernatant using the QIAmp viral RNA mini kit. If no infectious supernatant was generated, RNA was alternatively extracted from cell pellets using QIAGEN shredder columns followed by a QIAGEN RNeasy kit and a PCR product generated using Superscript III RT-PCR. The primers JFH-s and JFH-as were used to amplify a 1.2 kb long fragment encompassing the HCV NS3 protease, JFH-5230 and JFH-5536 for a 300 bp long fragment encompassing the NS4A cofactor. Their sequences can be found in Table A.12 and A.13, Appendix A. The PCR-product was then subjected to bulk sequence determination or clonal sequence analysis. To generate J2a2a$_{\text{-C3538G}}$ (J2a2a$_{\text{-T1066S}}$), J3a3a-6$_{\text{-A3364G,A3478G,A4005T}}$ (J3a3a-6$_{\text{-Q1008,N1046S,T1222S}}$), J5a5a$_{\text{-C3416G,T3968C,A4081T}}$ (J5a5a$_{\text{-Q1247L}}$), J6a6a$_{\text{-A3458G,G3459T}}$ (J6a6a$_{\text{-V1040L}}$) and Jxx plus resistance mutation, the fragment encompassing the HCV NS3 protease was digested with *Spe*I and *Not*I and cloned into the corresponding Jxx recombinant plasmids. To generate J3a3a-3$_{\text{-C5328G,T5329C}}$ (J3a3a-3$_{\text{-L1663A}}$), J3a3a-8$_{\text{-C5328G,T5329C}}$ (J3a3a-8$_{\text{-L1663A}}$) and Jxx plus resistance mutations, point mutations were introduced by site directed mutagenesis and cloning. Modified fragments were verified by sequencing.

2.17 RNA Transcription

Plasmid templates were linearised by *Xba*I (for pJFH1 and pJFH1 recombinants) or *Mlu*I (for Jc1 and Jc1 recombinants) digestion at 37°C for 4 hours. After blunting with Mung Bean Nuclease (NEB) at 30°C for 30 minutes, DNA was cleaned by phenol/chloroform extraction followed by ethanol precipitation. RNA was synthesised from 1 μg DNA template using the T7 RNA polymerase (Promega), incubating the reaction mixture at 37°C for one hour. Following treatment with RNase-free DNase for 15 minutes at 37°C, to remove template DNA, RNA was cleaned up using the RNeasy kit. RNA concentrations and the integrity of the RNA were analysed by non-denaturing agarose gel electrophoresis. RNA concentrations were determined using spectrophotometry and 10 μg aliquots were stored at -80°C until use.

2.18 RNA Transfection into Huh7 and Huh7.5 Cells

RNA was transfected into Huh7 or Huh7.5 cells by electroporation. Huh7/Huh7.5 cells were washed with PBS and detached with trypsin. Cells were pelleted by centrifugation (6,200 $\times$ g for 7 minutes at 4°C), then resuspended in 10 ml chilled diethylpyrocarbonate (DEPC)-treated PBS and counted with a hemocytometer. Counted cells were washed 3 times with 10 ml chilled DEPC-treated PBS (6,200 $\times$ g for 7 minutes at 4°C), then chilled on ice for at least 5 minutes. 10 μg of RNA was mixed with 5×10^6 cells suspended in 400 μl of chilled DEPC-treated PBS and transferred to an electroporation cuvette (0.4 cm gap width, Bio-Rad). Electroporation consisted of one square wave pulse for 25 ms of current delivered by the Bio-Rad Gene Pulser Xcell electroporation device, set at 150 V. Transfected cells were immediately resuspended in 4.5 ml 50:50 mix of conditioned and fresh complete growth medium (containing 10 % FCS), then transferred into T25 flasks containing 10 ml complete growth medium or seeded into 24-well plates containing coverslips for NS5A immunostaining. Cells were incubated in the Cat III lab at 37°C, 5 % CO_2 and 10 % relative humidity. Reaching 70-80 % confluency (every 3-4 days) cells were passaged by trypsination and reseeding with a 1:3 to 1:4 split ratio into fresh culture vessels or 24-well plates for NS5A immunostaining. Virus containing supernatant was collected, cleared of cell debris by centrifugation at 6,200 $\times$ g for 4 minutes and stored at 4°C overnight or at -80°C for long term. For supernatant infectivity assays, collected supernatants were additionally filtered through a 0.2 μm filter.

2.19 Immunostaining

Viral replication was assessed by anti-NS5A immunostaining. Polyclonal antibodies have been raised in sheep against the non-structural protein NS5A. This was carried out by M. Harris in Leeds. The polyclonal sheep anti-NS5A serum was used to detect NS5A protein in Huh7 and Huh7.5 cells. Electroporated cells or split cells during passaging experiments were seeded into 24-well plates containing cover slips and immunostained for NS5A when they were subconfluent. Following fixation of cells in

4 % paraformaldehyde for 20 minutes, plates were decontaminated on the surface with 5 % trigene and taken out of the Cat III lab. Subsequently, cells were washed 3 times with PBS by adding 1 ml of PBS to each well and shaken for 5 minutes. Cells were then permeabilised by adding 0.8 ml 0.01 % Triton-X 100 in PBS per well and incubated for 7 minutes. Following 2 further washes with PBS, cells were incubated with sheep anti-NS5A serum diluted 1:5,000 in 400 μl 10 % FCS PBS per well for one hour. After cells were washed 2 times with PBS, bound NS5A-specific antibody was detected by one hour incubation with Alexa Fluor 488 donkey anti-sheep IgG (Invitrogen), diluted 1:1,000 in 400 μl 10 % FCS PBS per well. Cells were further washed once with PBS and once with dH_2O before NS5A-positive cells were detected using a fluorescence microscope (Zeiss Axioskop 2 plus). For visualisation, coverslips were removed from plates and mounted onto glasslides using 5 μl Mowiol mounting medium. To determine percentage of positive cells and titres, 3 images per coverslip were taken from 2 coverslips per sample and the percentage of HCV-positive cells was determined using the AxioVision 4.8 software. Low titre samples were measured by counting foci forming units per millilitre (FFU/ml) or well, which can be single cells stained for NS5A or groups of stained cells. Alternatively, the percentage of HCV-positive cells of the total number of cells was determined, where 0 % is no cells infected and 100 % all cells infected. Jc1 served as a positive and JFH1-GND as a negative control. Staining of 96-well plates was carried out similarly, with 100 μl wash volumes and a volume of 50 μl for antibody dilutions.

2.20 Determination of 50 % Tissue Culture Infectious Dose in HCV Culture

Naïve Huh7.5 cells were seeded in 96-well plates the previous day at a concentration of 6×10^3 cells per well. The sample was serially diluted in complete growth media and inoculated onto the cells at 10-fold dilutions with 6 replicates at each dilution. After 3 days incubation, cells were stained for NS5A as described in section 2.19. Viral titres were determined by calculating tissue culture infectious dose at which 50 % of

the wells were positive for viral antigen ($TCID_{50}$). Wells were scored positive if at least one positive cell was detected. $TCID_{50}$ was calculated according to the method of Reed and Muench (Reed & Muench (1938)).

2.21 Drug Inhibition Studies

Susceptibility towards the PIs BILN 2061 and VX-950. The HCV-specific PI BILN 2061 was resuspended at 5 mM in DMSO and VX-950 at 20 mM in DMSO. Naïve Huh7.5 cells were seeded in 96-well plates the previous day at a concentration of 6×10^3 cells per well. The next day cells were infected for 8 hours with the corresponding infectious supernatant at a multiplicity of infection (MOI) of 0.015, washed with PBS and the media replaced with complete growth medium containing 0.1 % DMSO, as a carrier control, with or without the PI. Concentrations increased from 0.1 nM to 10,000 nM in 10-fold increments. After 3 days incubation, cultures were stained for NS5A as described in section 2.19. Each concentration was assayed in triplicate. Percentage of inhibition in supernatant infectivity was calculated from reduction in FFU/ml after antiviral addition compared to FFU/ml in virus control without antiviral addition.

Alternatively, 10 μg synthetic RNA was electroporated into Huh7.5 cells as described in section 2.18, and seeded into 24-well plates containing cover slips. The next day media was replaced with complete growth medium containing 0.1 % DMSO with or without increasing concentrations of the PI. After 3 days incubation, cultures were stained for NS5A as described in section 2.19. Antiviral efficacy was measured as inhibition of RNA replication by staining for NS5A and determining the percentage of HCV-positive cells. Each concentration was assayed in triplicate. For J2a2a 1 μg instead of 10 μg was used to achieve an infection frequency of around 40 %. For J6a6a cells were first passaged for 24 days to allow 40 % of the culture to be infected before the PI was added. Dose-response curves were fitted to a standard 4 parameter logistic model, also called the Hill-Slope model (equation 2.2), to obtain half maximal inhibitory concentrations (IC_{50}s)

$$y = yo + \frac{a}{1 + (\frac{x}{xo})^b} \tag{2.2}$$

where y is the percent activity/inhibition; x the corresponding PI concentration; yo percent activity/inhibition at the bottom of the fitted curve; a the test substance activity, b the slope of the fitted curve and xo the calculated relative IC_{50} value. The IC_{50} is defined as the concentration giving a response half way between the fitted top and bottom of the curve.

2.22 *In Vitro* Selection of PI Resistant Recombinant Viruses

Synthetic RNA of the recombinant viruses J1b1b, J2a2a$_{\text{-T1066S}}$, J3a3a, J4a4a-19, J5a5a-$_{\text{-Q1247L}}$ and J6a6a were electroporated into Huh7.5 cells as described in section 2.18 and seeded into 12-well plates. Three (J1b1b and J4a4a-19) or 4 (J2a2a$_{\text{-T1066S}}$, J3a3a and J5a5a$_{\text{-Q1247L}}$) days post-electroporation the PI was added and the cells serially passaged under increasing PI concentrations. J6a6a was first passaged for 35 days before antiviral addition. Alternatively, J6a6a$_{\text{-V1040L}}$ was used for selection experiments. During the course of selection, infected cells were split when 70-80 % confluency was reached. Fresh medium and PI were added every 3 or 4 days, regardless of whether the cell culture was split or not. Whenever the cell culture was split, cell pellets were collected, total cellular RNA extracted and subjected to RT-PCR. To determine the frequency of PI resistance mutations, 1.2 kb long RT-PCR products containing the NS3 protease of HCV RNA were ligated into the TOPO cloning vector, individual bacterial colonies subjected to colony PCR and sequenced. Each passaging experiment was carried out in duplicate and with a no antiviral control parallel passage.

A list of all primers can be found in Appendix A.
Common solutions and buffers are described in Appendix B.

Chapter 3
Variability of the HCV NS3 Protease and NS4A Cofactor Gene in Genotype 1a and 3a

3.1 Introduction

The ssRNA genome of HCV displays a high genetic diversity. This diversity is due to the error prone RdRp, the lack of a 5'-3'exonuclease to repair the induced errors and the rapid replication rate of HCV. An estimated 10^{12} virions are produced per day in an infected individual (Neumann *et al.* (1998)). Along the genome, HCV displays different degrees of genetic variability. The 5'UTR, 3'UTR and core show the highest degree of sequence conservation across genotypes, whereas the non-structural genes NS2, NS3, NS5A and NS5B are relatively variable. The envelope proteins E1 and E2, together with NS4 and the C-terminus of NS5A, display a high degree of inter- and intragenotypic diversity (Pawlotsky (2006)). The difference in sequence variability within the different genome regions reflects the functional constraints of some proteins (e.g., NS5B) and the immune pressure acting on others (e.g., E2). The intergenotypic variability of the HCV genome has important implications in treatment efficacies. As discussed in section 1.7.1, patients infected with genotype 1 and 4 respond more poorly to IFN-α/ribavirin combination treatment than patients infected with genotype 2 and 3. Genotype specific differences in efficacies have also been found to have implications in treatment with STAT-C antivirals. For example, genotype 1 infected patients showed higher response rates to the PI BILN 2061 than did genotype 2 and 3 infected individuals (Lamarre *et al.* (2003); Reiser *et al.* (2005)). The considerable intragenotypic genetic diversity of HCV is likely to further influence treatment response rates within a genotype. To obtain the highest degree of treatment response rates within each individual, an understanding of the intragenotypic diversity is crucial. A detailed map of

not only inter- but also intragenotypic variability will help the targeted development of new STAT-C compounds, which ideally should be equally effective in all individuals.

While the genetic diversity of HCV has been largely explored in the envelope and core domain, the NS3 protease region has not been investigated to the same extent. Even though the NS3 protease domain is considered one of the less variable proteins, different studies have reported variability in the nucleotide and amino acid sequence (Holland-Staley *et al.* (2002); Lodrini *et al.* (2003); Vallet *et al.* (2005); Pawlotsky (2006); Winters *et al.* (2006); Akhavan *et al.* (2009)). Holland-Staley *et al.* reported significant variability at both the nucleotide and amino acid sequence, with 11 % amino acid variability in genotype 1a and 9.9 % in genotype 1b variants. The analysis was further extended by Vallet *et al.*, who analysed the natural polymorphism on the quasispecies level. They showed that the substitution rates were evenly distributed throughout the NS3 protease gene and that the proportion of synonymous substitutions was significantly higher than that of non-synonymous substitutions (Vallet *et al.* (2005)). This indicates that mutations within the NS3 protease gene region are predominantly due to random genetic drift rather than positive selection pressure. Another study (Lodrini *et al.* (2003)) reported that the domains involved in the protease enzymatic function were highly conserved within genotype 1a and 1b variants, but showed some variability in genotype 3 variants.

Together these studies suggest that the NS3 protease gene is flexible enough to tolerate substitutions despite the various functional and structural constraints. NS4A has been shown to be highly diverse, with only 50-60 % amino acid sequence similarity within the antigenic region (amino acid 21-39) between genotypes 1, 2 and 3 (Simmonds *et al.* (1993)). Elsewhere it was reported that the N-terminal predicted membrane segment (amino acid 1-20) and the C-terminal acidic domain (amino acid 40-54) are highly conserved across genotypes (Brass *et al.* (2008)). Although data is available on NS4A variability, it has not been systematically analysed for intragenotypic variability.

To analyse the natural genetic diversity within the NS3 protease and NS4A gene region, a nested PCR method was developed in this study. Primers reading into the NS3 protease and NS4A gene were used to directly amplify the NS3 protease and NS4A genes from clinical isolates and subjected to bulk sequencing. NS3 protease and NS4A sequences were aligned and a consensus sequence generated using the Simmonics2005_v1.8 software. Nucleotide and amino acid sequence divergence were calculated with Simmonics and synonymous and non-synonymous nucleotide substitutions relative to the consensus sequence identified. Finally, molecular modelling was used to estimate the potential effect of genetic variability on enzyme structure.

3.2 Results

3.2.1 Patients

For the study of the NS3 protease gene diversity, the plasma of 29 genotype 1a infected patients (3 women and 26 men) with a mean age of 43 years and of 32 genotype 3a infected patients (7 women and 25 men) with a mean age of 39 years were analysed. For the NS4A gene, plasma was analysed from 28 genotype 1a infected patients (9 women and 19 men) with a mean age of 41 and of 31 genotype 3a infected patients (5 women and 26 men) with a mean age of 39 years. The overall mean age was 40 years with a range from 19-64 years.

3.2.2 Development of nested PCR reaction

The NS3 protease gene and the NS4A cofactor gene were amplified from plasma from patients infected with genotype 1a or 3a. Primers flanking the NS3 protease and the NS4A cofactor gene region were designed to include specific restriction sites for further cloning purposes. Primers-1a/3a-NS3-OS and -OA were used for the RT-PCR step, while primers NS3-1a/3a-IS and -IA were used for the second round nested PCR. Second round -IS primers included a *Not*I restriction site, -IA a *Spe*I restriction site. NS3-1a/3a-OMS, -MS, -MA and -OMA align in the middle of the protease gene and

were used for sequencing. Sequences of primers can be found in Table A.1 and A.2, Appendix A. To optimise PCR reactions, DMSO and $MgCl_2$ concentrations were adjusted and primer sets optimised using serial dilutions of plasmids pH77* for genotype 1a and pHCV3a-Gla for genotype 3a primers. DMSO enhances the specificity of an assay by lowering the effective T_m of the primers through reduction of complex secondary structures. Lowering the $MgCl_2$ concentration can further raise the specificity of the PCR reaction, as the Mg2+-ion binds tightly to the phosphate sugar backbone of nucleotides and nucleic acids, thereby lowering the T_m of the primer/template complex. Adjusting the annealing temperature also influences the specificity of the PCR reaction. The higher the annealing temperature, the better the specificity, but yield might be reduced. Table 3.1 gives an overview of the investigated parameter combinations. Amplification of the NS3 protease and the NS4A gene from patient plasma resulted in a single band PCR product, which was subjected to sequencing. Figure 3.1 shows the PCR product from patients plasma infected with genotype 3a, which results in a 823 bp fragment for the NS3 protease gene and a 644 bp product for NS4A. PCR amplification was successful in 40-70 % of reactions. The same PCR products were generated for genotype 1a.

3.2.3 Investigation of nucleotide and amino acid sequence divergence within the NS3 protease and NS4A gene region

NS3 protease amino acids were numbered from 1 (1027 absolute numbering) to 181 (1207) and standard 3 letter code used. Amino acid numbering for NS4A was from 1 (1658 absolute numbering) to 54 (1711) with lower case 4 (e.g., $Ile25_4$) to distinguish NS4A numbering from NS3 protease numbering. The entire NS3 protease sequence of 543 nucleotides, encoding 181 amino acids and the entire NS4A cofactor sequence of 162 nucleotides, encoding 54 amino acids were aligned. In total 29 NS3 protease variants from genotype 1a and 32 variants from genotype 3a, as well as 28 NS4A isolates of genotype 1a and 31 variants of genotype 3a were analysed.

Table 3.1: ***PCR parameter optimisation.*** *To optimise the PCR reaction, the DMSO concentration, the $MgCl_2$ concentration and the annealing temperature were adjusted. Final settings used are in bold.*

Agent	Variation						
DMSO	1.0 %	1.5 %	**2.0 %**	2.5 %	5.0 %	7.5 %	10.0 %
$MgCl_2$	1.0 mM	1.5 mM	2.0 mM	2.5 mM	**3.0 mM**	3.5 mM	
Temperature	50°C	52°C	52.5°C	53°C	**55°C**	57.5°C	60°C

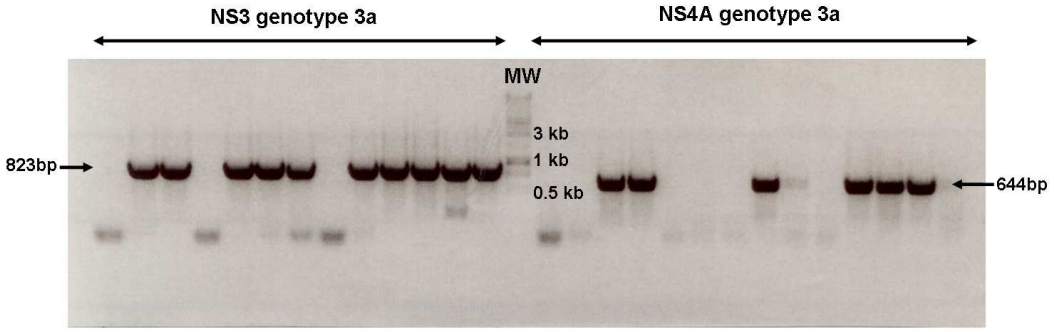

Figure 3.1: ***Amplification of the NS3 protease and NS4A gene of genotype 3a using nested PCR.*** *Primers flanking the NS3 protease and NS4A gene region were designed to introduce restriction sites on both ends for future cloning purposes. Amplification of the NS3 protease gene of genotype 3a resulted in a 823 bp product, left-hand side. Amplification of the NS4A gene of genotype 3a resulted in a 644 bp product, right-hand side. MW, molecular weight marker.*

A consensus sequence was generated for the NS3 protease and NS4A gene, respectively, by incorporating all the sequences belonging to the same genotype. Figure 3.2 shows the amino acid alignment of genotype 1a NS3 protease sequences to the 1a consensus sequence and Fig. 3.3 that of genotype 3a NS3 protease sequences to the 3a consensus sequence. The NS4A amino acid alignment of genotype 1a and 3a variants to their respective consensus sequence is depicted in Fig. 3.4. The subtype 1a consensus sequence differed from that of the 1a prototype strain H (NCBI accession number M62321) at 2 positions (Ile48Phe and Ser91Ala) within the NS3 protease; the subtype 3a consensus sequence differed to that of the prototype strain pHCV3a-Gla at 3 positions (Ile48Val, Thr91Ala and Thr179Ala) within the NS3 protease. Ten sporadic amino acid changes were found in the NS3 protease sequences of genotype 1a

variants, 15 in that of genotype 3a variants. A mean nucleotide and amino acid sequence divergence between the variants of the same genotype was calculated using the Simmonics2005_v1.8 software. Analysis of the genotype 1a NS3 protease sequences showed a mean sequence divergence of 7.6 % on the nucleotide level, and 2.5 % on the amino acid level between the 29 analysed variants. Out of 181 amino acids, substitutions were identified at 25 positions (13.8 %). Between the 32 genotype 3a NS3 protease sequences a mean sequence divergence of 6.9 % on the nucleotide level and 2.7 % on the amino acid level was calculated. Out of 181 amino acids, substitutions were identified at 29 positions (16 %). No insertions or deletions were detected.

The consensus sequence of the genotype 1a NS4A variants differed to the HCV H strain prototype sequence at one amino acid position ($Val29Ile_4$); the consensus sequence of genotype 3a NS4A was identical to the prototype pHCV3a-Gla amino acid sequence. Five sporadic amino acid substitutions were found in genotype 1a variants and all 6 substitutions identified in genotype 3a variants were sporadic. Within the NS4A gene a mean sequence divergence of 7.4 % on the nucleotide level and 3.7 % on the amino acid level between the 28 analysed genotype 1a variants was identified. For genotype 3a NS4A genes the calculated mean sequence divergence was 5.3 % on the nucleotide level and 0.7 % on the amino acid level between the 31 analysed variants. Overall 8 out of 54 (14.8 %) amino acids within genotype 1a variants and 6 out of 54 (11 %) within genotype 3a variants were substituted compared to the consensus sequence. Table 3.2 summarises the genetic variability within the NS3 protease and NS4A nucleotide and amino acid sequence in genotype 1a and 3a variants.

Types of amino acid substitutions

The amino acid sequences of clinical variants were compared to the consensus amino acid sequence and amino acid substitutions relative to the consensus sequence identified. In Fig 3.2 and 3.3, conservative as well as non-conservative amino acid substitutions away from the consensus sequence, together with the functional domains of the NS3 protease are indicated. The most frequent non-conservative amino acid substitutions were represented by non-polar to polar substitutions, followed by a change from amino acids with a neutral side chain to amino acids with a charged side chain,

e.g., the non-polar amino acid Ala was often replaced by the polar amino acid Thr. A common change from an amino acid with a neutral side chain to one with a positively charged side chain was a Gln to Lys substitution. Table 3.3 gives an overview of all the physico-chemical types of substitutions in the NS3 protease sequences of genotype 1a and 3a variants.

None of the genotype 1a or 3a variants showed any substitutions in the catalytic triad, constituted of His57, Asp81 and Ser139. No variant was identified that contained any of the described resistance mutations for PIs at position 36, 41, 43, 54, 109, 155, 156, 168 and 170 (Tong *et al.* (2006); Bartels *et al.* (2008); Cubero *et al.* (2008); Kuntzen *et al.* (2008); He *et al.* (2008)). Furthermore, no variant had an amino acid substitution within the zinc binding site Cys97, Cys99, Cys145 and His149, the S1 substrate binding pocket Ile/Leu132, Leu135, Phe154 and Ala157 or the S2 substrate binding pocket Arg155, Arg123, Arg/Lys161, Arg/Gln165 and Asp/Gln168. Gly137, which is part of the oxyanion hole, was not substituted either. Val29 and Ile/Leu64, which are involved in the contact with the NS4A amino acid $Ile25_4$, are substituted in several genotype 1a variants but in no genotype 3a variants. One genotype 1a variant and 9 genotype 3a variants had an Ala to Thr change at position 7 within the NS4A binding site, which is highly conserved among the remaining residues (amino acid 1-22). Apart from highly polymorphic sites at position 40 and 174 in genotype 1a and position 7, 98 and 176 in genotype 3a, amino acid substitutions were evenly distributed throughout the protease gene. The diversity of the NS3 protease gene was further evaluated by determination of the type of nucleotide substitution. The proportion of nucleotide sites with substitutions were divided into synonymous (no amino acid change) and non-synonymous (resulting in amino acid change). Within genotype 1a variants the proportion of synonymous nucleotide substitutions was 25.1 %, that of non-synonymous substitutions 1.3 %. For genotype 3a variants the proportion of synonymous nucleotide substitution was 22.2 % and 1.3 % for non-synonymous substitutions. In both cases the proportion of synonymous substitutions is significantly higher than the proportion of non-synonymous substitutions, suggesting that the substitutions away from the consensus sequence arose due to random genetic drift rather than positive selection pressure.

Most amino acid substitutions away from the consensus sequence in NS4A were conservative, with only one non-conservative amino acid substitution within a genotype 3a variant and a polymorphic site at position 46 including non-conservative amino acid changes within the genotype 1a variants (Fig. 3.4). The overall diversity of NS4A within a genotype is rather low when compared to the high heterogeneity between genotypes within the antigenic region (Simmonds *et al.* (1993)). The proportion of synonymous nucleotide changes was significantly higher than the proportion of non-synonymous substitutions within genotype 1a variants (23.5 % versus 2.1 %) as well as in genotype 3a variants (20.9 % versus 0.4 %). This suggests a natural sequence divergence arisen through random genetic drift as described above for the NS3 protease gene.

Table 3.2: ***Nucleotide and amino acid sequence divergence within the NS3 protease and NS4A gene.*** *The nucleotide (nt) and amino acid (aa) sequence divergence between the NS3 protease sequences from different clinical variants was analysed using p-value calculations and are shown in column 2 and 3. 29 genotype 1a and 32 genotype 3a sequences were analysed. Likewise the nt and aa sequence divergence between the NS4A sequences of 28 genotype 1a and 31 genotype 3a variants were analysed. The proportion of synonymous nt substitutions and non-synonymous nt substitutions are shown in the forth and fifth column.*

Gene	nt sequence divergence	aa sequence divergence	Synonymous nt substitutions	Non-synonymous nt substitutions
1a NS3 protease	7.6 %	2.5 %	25.1 %	1.3 %
3a NS3 protease	6.9 %	2.7 %	22.1 %	1.3 %
1a NS4A	7.4 %	3.7 %	23.5 %	2.1 %
3a NS4A	5.3 %	0.7 %	20.9 %	0.4 %

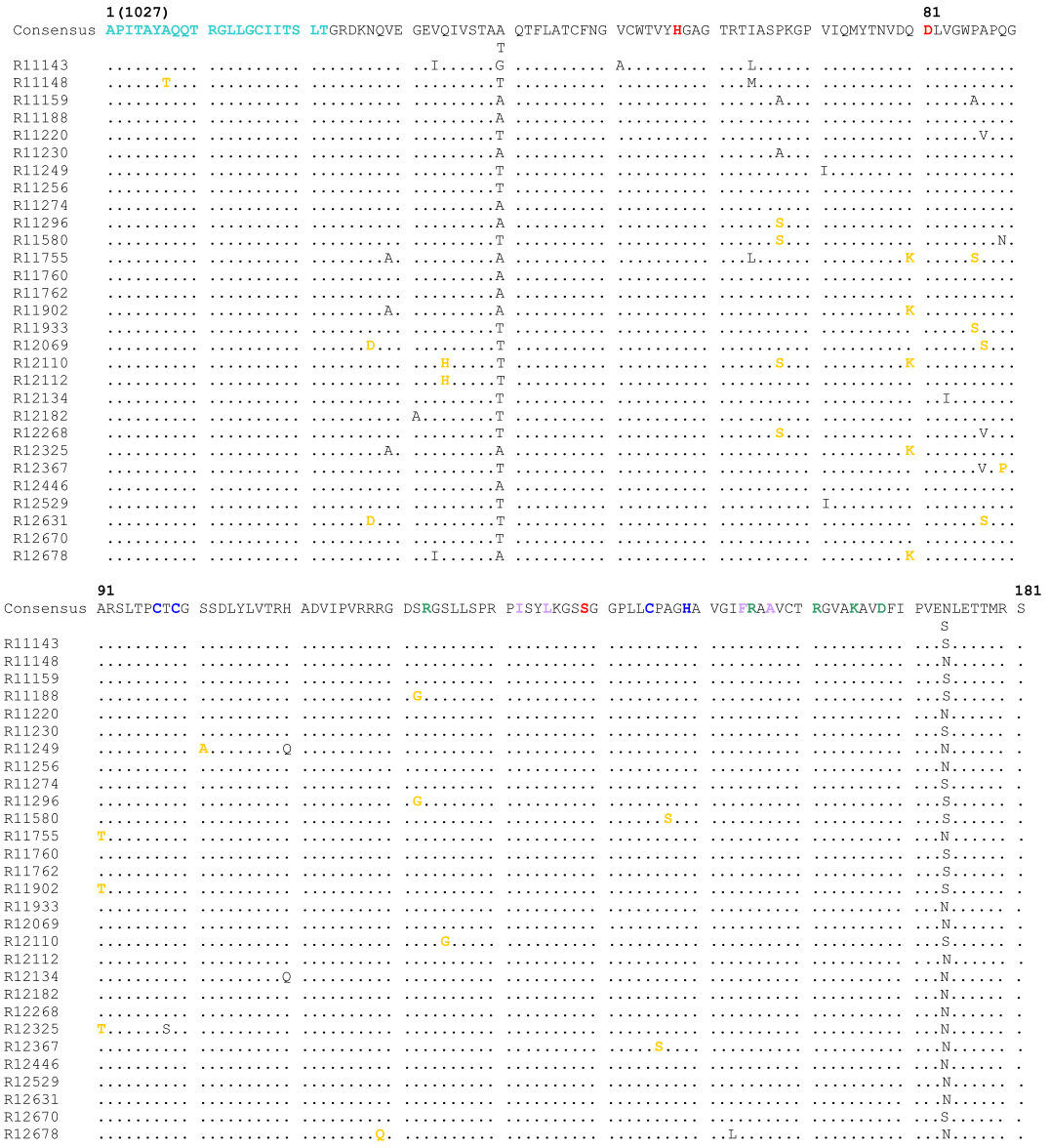

Figure 3.2: ***Amino acid alignment of full-length NS3 protease sequences (residues 1 (1027) to 181 (1207)), from 29 variants of genotype 1a to the consensus sequence.*** *Amino acid residues are indicated by standard single-letter codes. The consensus sequence is listed on the top line and points indicate residues identical to the consensus sequence. Colour code: Red: catalytic triad His57, Asp81 and Ser139; Dark blue: zinc binding residues Cys97, Cys99, Cys145 and His149; Light blue: NS4A binding site; Purple: S1 binding pocket Ile132, Leu135, Phe154 and Ala157; Green: S2 binding pocket Arg123, Arg155, Arg161, Lys165 and Asp168; Black: conservative substitutions; Orange: non-conservative substitutions.*

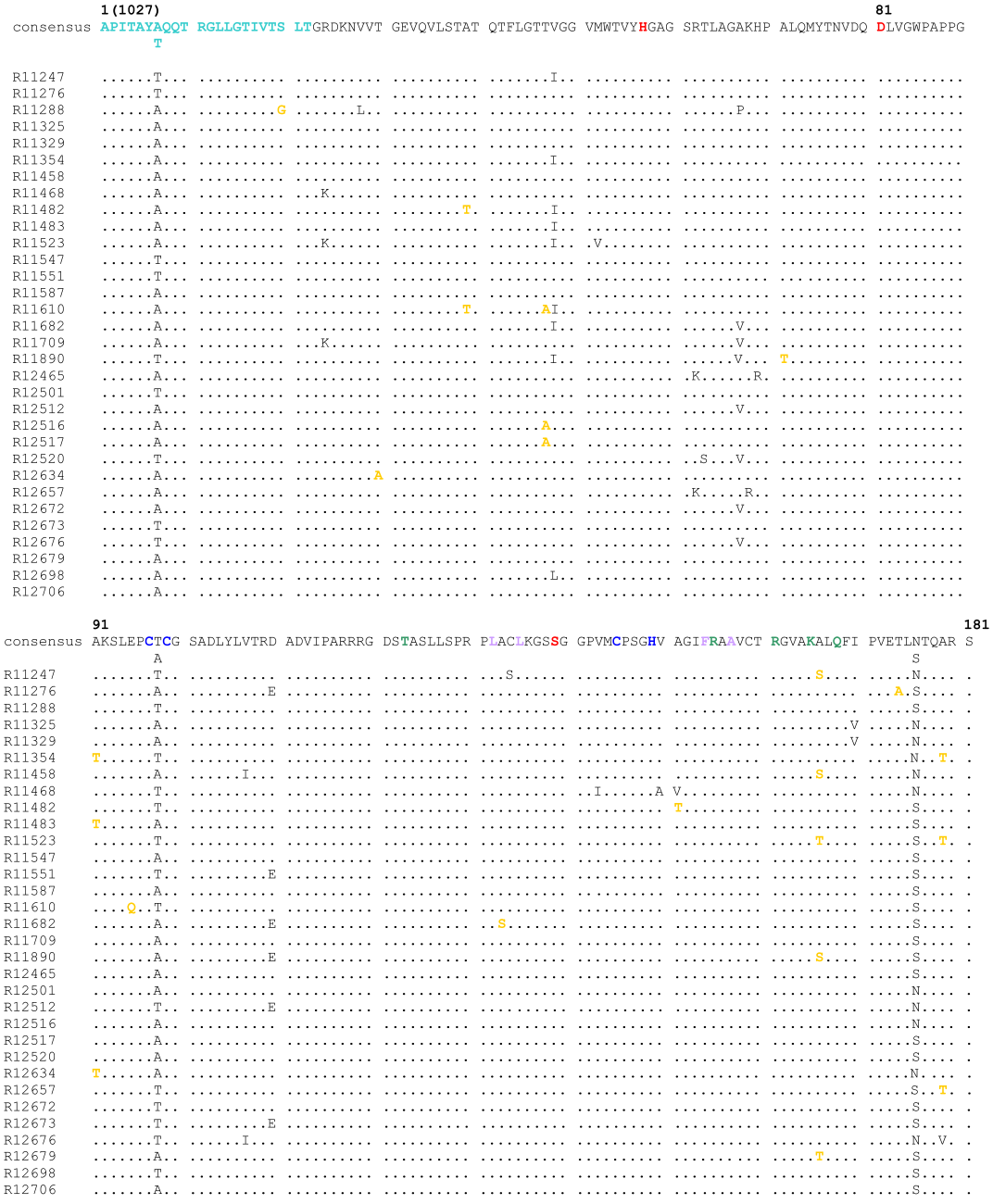

Figure 3.3: ***Amino acid alignment of full-length NS3 protease sequences (residues 1 (1027) to 181 (1207)), from 32 variants of genotype 3a to the consensus sequence.*** *Amino acid residues are indicated by standard single-letter codes. The consensus sequence is listed on the top line and points indicate residues identical to the consensus sequence. Colour code: Red: catalytic triad His57, Asp81 and Ser139; Dark blue: zinc binding residues Cys97, Cys99, Cys145 and His149; Light blue: NS4A binding site; Purple: S1 binding pocket Leu132, Leu135, Phe154 and Ala157; Green: S2 binding pocket Thr123, Arg155, Arg161, Lys165 and Gln168; Black: conservative substitutions; Orange: non-conservative substitutions.*

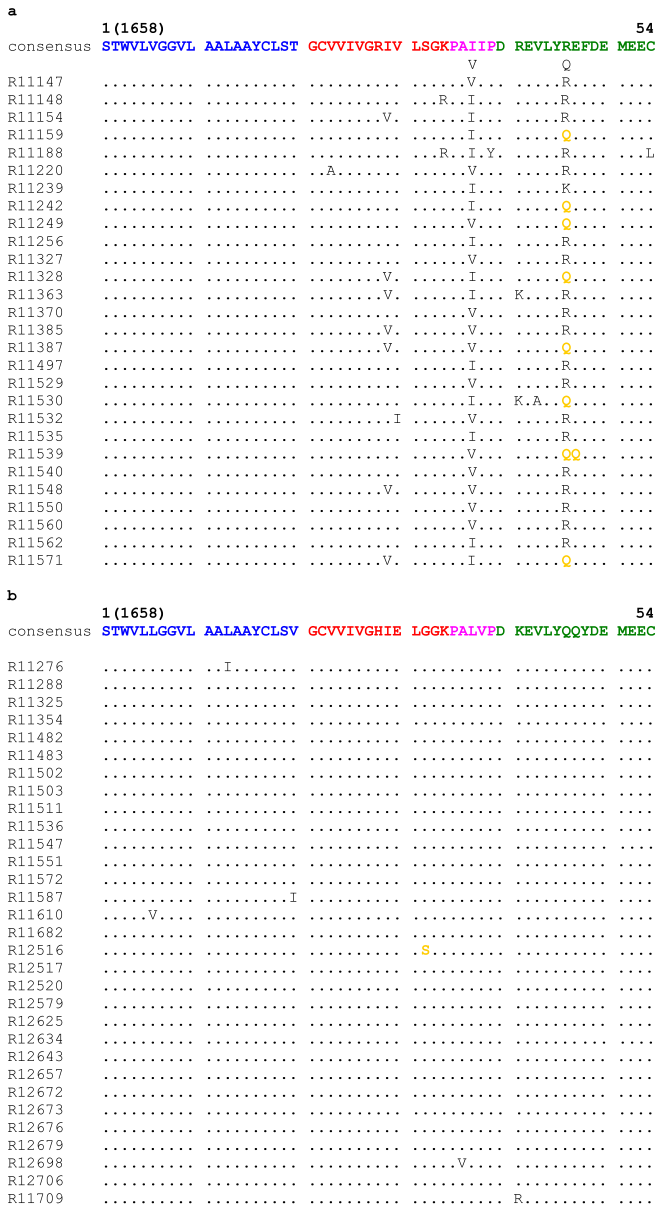

Figure 3.4: ***Amino acid alignment of full-length NS4A sequences (residues 1 (1658) to 54 (1711)), from 28 genotype 1a variants (a) and 31 genotype 3a variants (b) to their consensus sequence.*** *Amino acid residues are indicated by standard single-letter codes. The consensus sequence is listed on the top line and points indicate residues identical to the consensus sequence. Blue: N-terminal membrane segment (highly conserved); Red: amino acids essential for cofactor activity; Pink: kink segment; Green: C-terminal acidic domain (highly conserved); Black: conservative substitutions; Orange: non-conservative substitutions.*

Table 3.3: ***Physico-chemical types of substitutions in the NS3 protease of genotype 1a and 3a variants aligned to their consensus sequence****. Amino acid residues are indicated by standard single-letter code. Substitutions away from the consensus sequence were sorted according to their physico-chemical properties and listed if they occurred in at least 2 variants.*

Type of substitutions	1a NS3 protease	3a NS3 protease	1a NS4A	3a NS4A
Hydrophobic to hydrophobic	V29A, G31A, V33I, A40G, I64L, P67A, V71I, A87V	V48I, A67V, V107I, I170V	$I29V_4$, $I37V_4$	
Polar neutral to polar neutral	Q89N, H110Q, N174S	N176S		
Polar basic to polar basic		R24K, R62K	$K34R_4$, $R41K_4$	
Polar acidic to polar acidic		D110E		
Hydrophobic to neutral polar	A7T, P67S, P86S, A87S, A91T, A147S	A7T, A39T, A91T A165S/T, A179T		
Polar neutral to non-polar hydrophobic	S122G			
Polar basic to polar neutral			$R46Q_4$	
Polar neutral to polar charged	N27D, Q33H, Q80K			
Hydrophobic to polar charged				

3.2.4 3-Dimensional (3D) modelling of amino acid substitutions

To estimate the influence of the identified conservative and non-conservative amino acid substitutions on the structure or function of the NS3 protease, they were modelled onto the 3D structure of the genotype 1a NS3 protease from the HCV H strain (Kim *et al.* (1996)). Using the UCSF Chimera software, functional domains and amino acid positions where substitutions were identified, were outlined. Figure 3.5 shows the 3D structure of the HCV H strain protease complexed with the NS4A peptide and polymorphic sites identified within genotype 1a variants highlighted. Figure 3.6 shows the equivalent for genotype 3a variants. As the 3D model of genotype 1a protease was used, amino acid residues do not always correspond to the type 3a residues. However, spatial arrangement will be similar. Most of the non-conservative amino acid substitutions within the genotype 1a variants mapped to surface loops of the protease and pointed away from the remaining protein residues. However, Ser122 is located between Asp168 and Arg155, which are part of the S2 substrate binding pocket and involved in the binding of the PI BILN 2061 (Courcambeck *et al.* (2006)). Two variants have a Gly122 substitution, a change from a polar amino acid with a hydroxyl group to a non-polar smaller amino acid without a hydroxyl group. Gly122, without a hydroxyl group, might possibly interact differently with Arg155 and Asp168, having an influence on the structure of the S2 binding pocket.

The majority of non-conservative amino acid substitutions within the genotype 3a variants also map to surface loops, except for Ala166, which points towards Arg123 of the S2 substrate binding pocket. Three genotype 3a variants have a Ser166 and 2 have a Thr166 substitution, 2 amino acids which are polar and bulkier than the non-polar amino acid Ala, possibly pushing Arg123 out of his position and affecting the S2 substrate binding pocket. Two further polymorphic residues are Thr47 and Ala151. Both are located on interior β-strands. At position 47, 3 variants have a Thr to Ala substitution and at position 151, one variant has an Ala to Thr substitution. A change from a bulkier and polar amino acid, such as Thr, to a smaller and non-hydrophobic amino acid, such as Ala, possibly has an influence on the 3D structure of the protein.

Overall, the amino acid substitutions within the genotype 1a and 3a variants are either conservative, which can be found on β-strands, α-helices and loop regions, or non-conservative, which mostly locate to the surface loops where they do not influence the structure of the protease. Furthermore, all sites of substitution are located away from the active site and other functional domains, except for a few substitutions possibly affecting the S2 substrate binding pocket. Modelling the positions of substitutions onto the 3D structure of the NS3 protease allows speculations about their possible influence on protein structure and function. The observed natural sequence diversity most likely arose through random genetic drift rather than positive selection pressure, as non-conservative amino acid changes locate mostly to the surface area where they are less likely to have an influence on the functional domains.

3.2.5 Summary of results

In summary, these results show that the NS3 protease of genotype 1a and 3a is sufficiently flexible to tolerate substitutions, despite the functional constraints provided mainly by the catalytic domain, but also its substrate binding pockets and the cofactor binding site as well as the zinc binding site. In concordance with the functional constraints, polymorphic sites are mainly found away from the functionally important domains. Substitutions away from the consensus sequence are evenly distributed throughout the gene and synonymous nucleotide substitutions are far more common than non-synonymous substitutions, supporting a mechanism of natural genetic polymorphism induced through random genetic drift. The NS4A gene tolerates substitutions within the cofactor activity region and the kink segment, but is more conserved within the functionally constraint N-terminal membrane segment. Even though NS4A is highly diverse between genotypes, it is fairly conserved within genotypes.

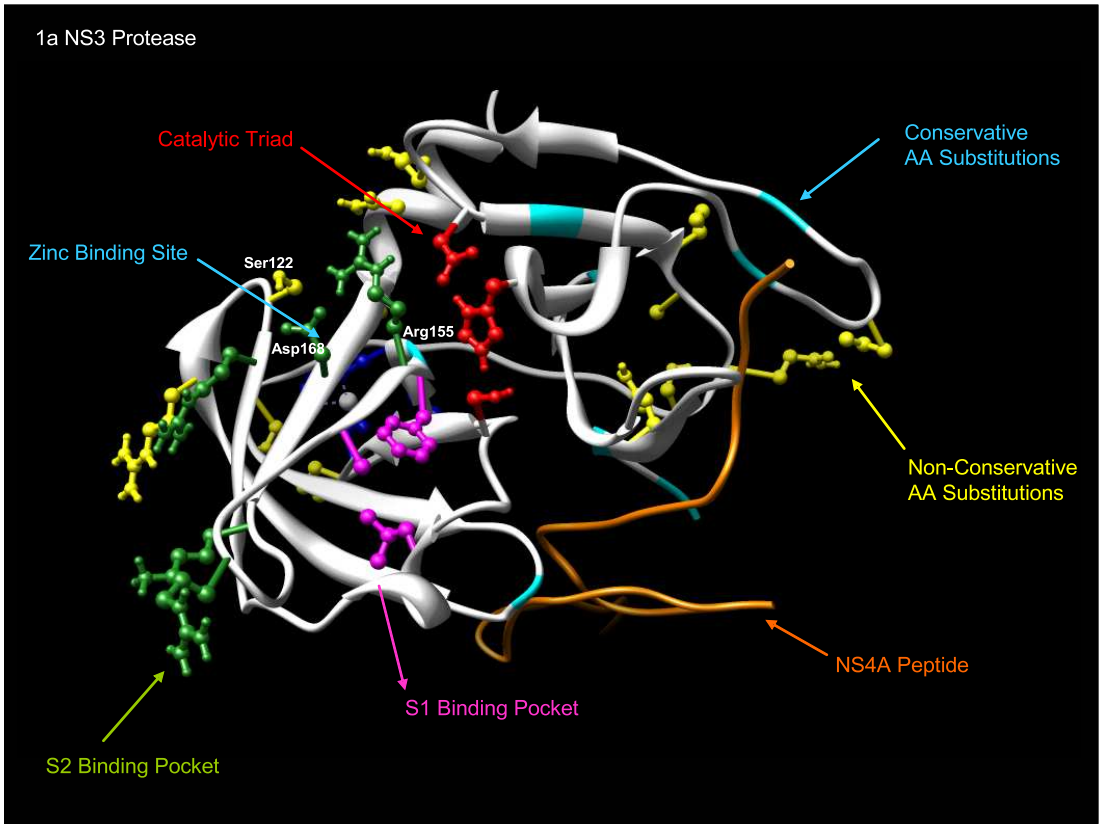

Figure 3.5: ***3D analysis of sequence diversity in genotype 1a variants.*** *Amino acid positions where substitutions away from the consensus sequence have been identified, were mapped onto the crystal structure of the genotype 1a HCV H strain NS3 protease. The NS3 protease domain (white) is shown complexed with a synthetic NS4A cofactor peptide (orange). Colour code: Light blue: sites of conservative amino acid substitutions; Yellow: sites of non-conservative amino acid substitutions; Red: catalytic triad His57, Asp81 and Ser139; Dark blue: zinc binding residues Cys97, Cys99, Cys145 and His149; Purple: S1 binding pocket Ile132, Leu135, Phe154 and Ala157; Green: S2 binding pocket Arg123, Arg155, Arg161, Lys165 and Asp168.*

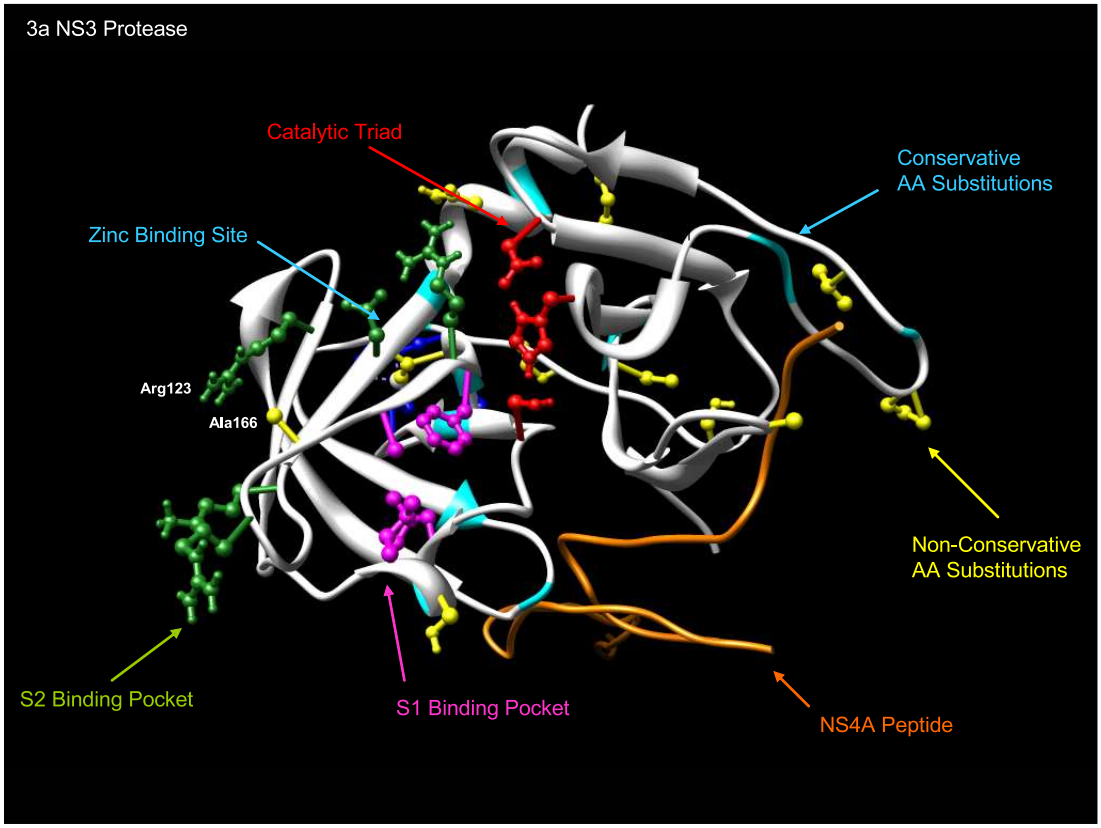

Figure 3.6: ***3D analysis of sequence diversity in genotype 3a variants.*** *Amino acid positions where substitutions away from the consensus sequence have been identified, were mapped onto the crystal structure of the genotype 1a HCV H strain NS3 protease. As the genotype 1a protease is used as a model, amino acids depicted correspond to genotype 1a residues. The NS3 protease domain (white) is shown complexed with a synthetic NS4A cofactor peptide (orange). Colour code: Light blue: sites of conservative amino acid substitutions; Yellow: sites of non-conservative amino acid substitutions; Red: catalytic triad His57, Asp81 and Ser139; Dark blue: zinc binding residues Cys97, Cys99, Cys145 and His149; Purple: S1 binding pocket Leu132 (Ile in the model), Leu135, Phe154 and Ala157; Green: S2 binding pocket Thr123 (Arg in the model), Arg155, Arg161, Lys165 and Gln168 (Asp in the model).*

3.3 Discussion

The HCV RNA genome displays high genetic diversity, which is not evenly distributed throughout the genome (Choo *et al.* (1991)). The NS3 protease has previously been identified as one of the less variable gene regions of the HCV genome (Pawlotsky (2006)). Due to its role as a target for specifically targeted antiviral therapy a detailed understanding of the NS3 protease diversity is crucial. As discussed in section 1.8.1 intergenotypic diversity plays a major role in the efficacies of protease and polymerase inhibitors. The intragenotypic diversity most likely will have further influence on how patients within the same genotype respond to treatment and a detailed understanding is important to facilitate drug development. The aim of the results presented in this chapter was to investigate the genetic diversity of the HCV NS3 protease and its cofactor NS4A in the absence of any specific antiviral pressure and to possibly identify pre-existing resistance mutations. The NS3 protease diversity has been studied previously by different groups (Holland-Staley *et al.* (2002); Lodrini *et al.* (2003); Vallet *et al.* (2005); Pawlotsky (2006); Winters *et al.* (2006); Akhavan *et al.* (2009)). This study will add to the current knowledge of NS3 protease sequence diversity, especially for genotype 3a, which has not been studied extensively. The work focused on genotype 1a and 3a, as these are the predominant genotypes in Northern Europe and are less well described than genotype 1b, on which research mostly focuses.

Even though the NS3 protease is one of the less diverse genes in the HCV genome, variability was found among the studied HCV variants on the nucleotide (7.6 % sequence divergence between variants of genotype 1a and 6.9 % for genotype 3a) and amino acid level (2.5 % for genotype 1a and 2.7 % for genotype 3a). The genetic diversity between genotype 1a variants and genotype 3a variants was found to be fairly similar with amino acid sequence divergences of 2.5 % and 2.7 %, respectively. However, within the NS4A cofactor amino acid sequence, divergence was found to be higher in genotype 1a variants compared to genotype 3a variants (3.7 % versus 0.7 %). A previous study (Lodrini *et al.* (2003)) has reported a slightly higher value for genotype 1a variants, with 4.5 % nucleotide and 4 % amino acid sequence divergence and a lower value for genotype 3a variants, with a nucleotide sequence divergence of 3.7 % and

an amino acid sequence divergence of 1.6 %. Even though the results presented here could not identify a significant difference between genotype 1a and 3a NS3 protease diversity, a difference was found within the NS4A gene. A lower amino acid sequence diversity was found within genotype 3a variants, as reported by Lodrini *et al.* for the NS3 protease. However, the overall diversity is low within both genotypes and sample sets differ in number, making a comparison difficult. Furthermore, Lodrini *et al.* do not distinguish between subtypes in the genotype 3 group.

Holland-Staley *et al.* have reported slightly higher values for NS3 protease diversity, with a nucleotide sequence divergence of 30.2 % and an amino acid sequence divergence of 11 % for genotype 1a variants (Holland-Staley *et al.* (2002)). Significantly higher values were reported by Vallet *et al.*, who found an amino acid sequence divergence of 22.1 % (nucleotide sequence divergence 41.2 %) within genotype 1a variants (Vallet *et al.* (2005)). Whereas the 2 first studies also directly sequenced the predominant variant in each plasma sample, as in this study, Vallet *et al.* sequenced a mean of 17 variants per clinical isolate. Clonal analysis allows the detection of the depth of the quasispecies and will include the possibly highly variable sequences of less dominant viral variants, increasing the overall genetic diversity detected. The higher diversity in the Vallet and Holland-Staley study might also be attributed to the age of the analysed patients. The mean age of patient in these 2 studies was 50 and 52 years, whereas patients in this study were on average 10 years younger, with a mean age of 40 years. It is possible that the patients studied here had a shorter time of infection, which might influence the degree of genomic diversity (Casino *et al.* (1999); Farci *et al.* (2000)). As discussed in section 1.6.1, viral proteins are under the pressure of the immune system and will evade it through mutational escape, generating a diverse pool of viral variants over time. Positive selection pressure though, is limited by the functional constraints of the protease.

Like most RNA viruses, HCV has a high mutation rate and if it is random, theoretically each site will contain an equal number of mutations. However, this is not the case as e.g., has been shown for E1 HVR1 and also NS3 (Holland-Staley *et al.* (2002); Penin *et al.* (2001)). As demonstrated by these studies, substitutions are random, al-

though some regions of the genome are more permissive to mutations than others because proteins like the NS3 protease are functionally constrained. Kim *et al.* have reported that the NS4A binding domain within the N-terminal region of the NS3 protease is flexible (Kim *et al.* (1996)). They demonstrated that the binding of NS4A induces a structural rearrangement of the NS3 protease catalytic domain towards a more favorable conformation. This suggests that some mutations are possible within this region. The NS4A binding domain of the NS3 protease was fairly conserved within the genotype 1a and 3a variants analysed, with only one polymorphic site at position 7. Ala7 is the dominant amino acid, although Thr is tolerated as well. Only one nucleotide substitution is necessary to change from Ala to Thr, making this substitution fairly accessible. Ala7 is part of a hydrophobic pocket formed by the NS3 protease residues Ala5, Ala7, Val33, Ile/Val35, Leu44, Val107, Ala111 and the NS4A residue $Leu31_4$. This interaction is part of the extensive hydrophobic interaction between NS4A and NS3 (Kim *et al.* (1996)). Although Thr has a hydrophilic hydroxyl group, it might still replace Ala with its hydrophobic methyl group. The methyl group is likely to be positioned within the hydrophobic pocket and the hydroxyl group away from it. It is important that the hydrophobic pocket maintains its hydrophobic character to not disrupt the interaction between NS3 and NS4A. The addition of a hydroxyl group though, might affect the 3D conformation. Other residues of the hydrophobic pocket with polymorphism are Val33 and Val107. Two genotype 1a variants show a Ile33 substitution and 2 genotype 3a variants a Ile107 substitution. Both substitutions keep the hydrophobic character and are unlikely to have a major effect on the protease structure or function.

$Ile25_4$ and $Gly27_4$ of NS4A are highly conserved across genotypes and together with $Val23_4$, $Ile/Leu29_4$ and $Leu31_4$ they make significant contact with the NS3 protease ($Ile/Leu29_4$; first amino acid is the one occurring in genotype 1a, the second the one occurring in genotype 3a) (Kim *et al.* (1996)). The 4 NS3 protease residues Pro88, Ile64, Val29 and Val/Leu36 are involved in the contact with NS4A $Ile25_4$ (Fig. 3.7). All 4 positions are conserved within genotype 1a and genotype 3a variants except for 6 genotype 1a variants that have a conservative amino acid substitution (2x Leu64, 1x Met64 and 3x Ala29). These substitutions away from the consensus sequence are un-

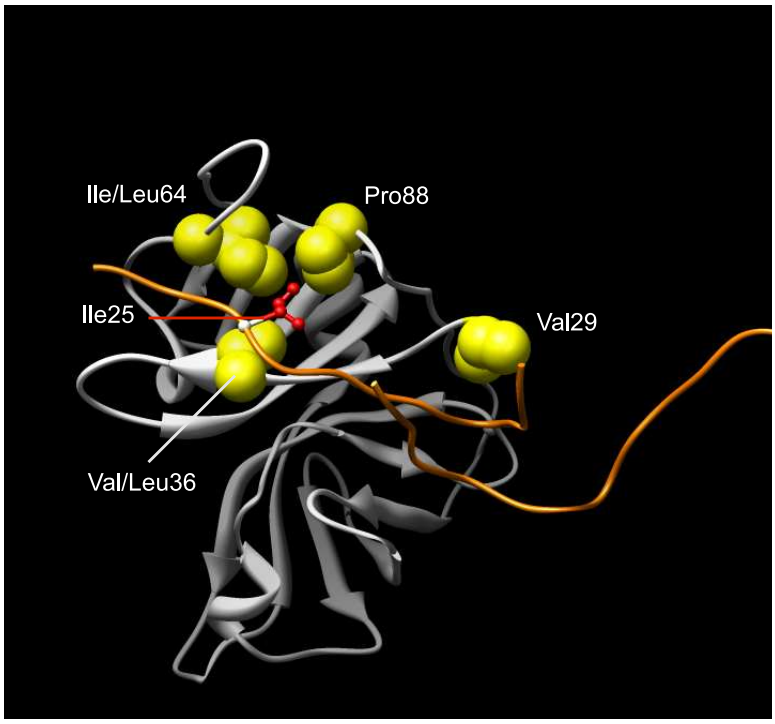

Figure 3.7: ***NS3/4A interaction.*** *The highly conserved $Ile25_4$ of NS4A (red, ball and stick) interacts with the 4 hydrophobic NS3 residues Pro88, Ile64, Val29 and Val36 (yellow spheres). The NS3 protease is depicted in white as β-sheets and α-helices and the NS4A peptide in orange. Conservative substitutions away from the consensus sequence are tolerated as long as the hydrophobic character is maintained.*

likely to have an effect on the interaction between NS3 and NS4A, as they all are nonpolar neutral to nonpolar neutral changes and amino acids are about the same size as well. However, it demonstrates that it is important to keep the hydrophobic character.

Analysis of all variants revealed mostly synonymous nucleotide substitutions within genotype 1a and 3a variants. About 20-25 % of all nucleotide positions had synonymous substitutions and only about 1-2 % were non-synonymous and resulted in amino acid changes. The same was true for NS4A. The far greater proportion of synonymous versus non-synonymous nucleotide substitutions indicates that the natural sequence divergence is a product of random genetic drift and not due to positive selection pressure. Functional constraints within the NS3/4A protease make mutations within the catalytic, substrate binding and zinc domains highly unlikely as shown by others (Holland-Staley *et al.* (2002); Vallet *et al.* (2005)). None of the variants analysed had

a mutation within the catalytic domain comprised of the 3 amino acids His57, Asp81 and Ser139. However, mutations within the catalytic domain have been described by others. One study e.g., found 2 sporadic mutations affecting the catalytic Ser129 (Ser139Pro) and Asp81 (Asp81Gly) (Vallet *et al.* (2005)). These changes most likely are lethal as they present non-conservative amino acid changes and variants would circulate in very low numbers, which could only be picked up by clonal analysis. As no clonal analysis was performed in this study, it was unlikely to detect any variants with lethal mutations. Gly137, together with Ser139, creates an oxyanion hole. This helps to stabilise the transition state during the hydrolysis of peptides, thereby lowering the activation energy. This favoured energetic state is important for efficient catalysis (Kim *et al.* (1996)). Expectantly, residue Gly137 and Ser139 were conserved across all variants analysed.

NS3 has a very flat substrate binding site and requires peptide-binding spanning the S6 to S4' binding site. The Schechter and Berger nomenclature describes the subsites of a protease (Schechter & Berger (1967)). The amino acid residues of a polypeptide binding to the active site of the protease are called P (for peptide) and the subsites on the protease they are binding to are called S (for subsites). Depending on the size of the peptide, substrate residues around the cleavage site can be numbered up to P8. The subsites on the protease are accordingly numbered, e.g., S3, S2, S1, S1', S2', S3' etc., where S1 and S1' are located adjacent to the scissile bond. The S1 substrate binding pocket of the NS3 protease is lined by the hydrophobic amino acids Ile132, Leu135, Phe154, and Ala157. Arg155, Arg/Thr123, Arg161, Lys165, and Asp/Gln168, which are mainly charged residues, line the S2-S6 binding pocket (Kim *et al.* (1996)). None of the variants analysed showed any substitutions at the S1 or S2 substrate binding pocket, which is consistent with findings by others (Vallet *et al.* (2005)). This suggests that the integrity of the substrate binding pockets is important, and that preserving polarity and hydrophobicity alone are not sufficient.

The NS3 protease contains a zinc ion that is tetrahedrally coordinated by the 4 residues Cys97, Cys99, Cys145, and His149 and a water molecule (Kim *et al.* (1996)). The 3 Cys residues are particularly crucial for protease activity. Mutational studies showed that replacement of any of these with an Ala led to significantly reduced protease activity (Hijikata *et al.* (1993)). Substituting His149 to Ala had a less dramatic effect, which is consistent with the indirect interaction of His149 with the zinc ion. As the zinc ion is located 20 Å away from the catalytic site, it has been suggested to play a structural role (Kim *et al.* (1996)). Folding analysis in *E.coli* have revealed that the NS3 protease does not fold properly in the absence of zinc, supporting this hypothesis (De Francesco *et al.* (1996)). Consistent with this crucial role of zinc and its coordination, no mutation within the zinc binding site was found in any variant.

None of the variants contained any of the reported resistance mutations for PIs at position 36, 41, 43, 54, 109, 155, 156, 168 and 170 (Tong *et al.* (2006); Bartels *et al.* (2008); Cubero *et al.* (2008); Kuntzen *et al.* (2008); He *et al.* (2008)). As described in section 1.8.4, viral variants containing resistance mutations are often impaired in their replication kinetics and will not be the main strain circulating in a pool of quasispecies. As no clonal analysis was performed in this study, it was unlikely to detect any variants with PI resistance mutations.

Outside the NS3 protease active site and substrate binding pockets, polymorphisms have been identified at several positions. Conservative amino acid substitutions were found on interior and surface β-strands, as well as surface loops and generally only 2-3 variants differed in their sequence from the consensus sequence, suggesting that these substitutions are sporadic. Most conservative substitutions involved changes from one hydrophobic to another hydrophobic amino acid. Within genotype 1a variants polar neutral to polar neutral substitutions were observed several times as well. A highly polymorphic site is amino acid position 40, which lies on a surface loop and faces away from the protease, allowing amino acids of different sizes to replace each other. Furthermore, only one nucleotide change is necessary for a Thr to Ala change, whereas 2 nucleotide changes are necessary for a change to Gly. This explains the Thr/Ala polymorphism within genotype 1a variants and why there is only one variant with a

Gly. Why no polymorphism was found at this site within genotype 3a is unclear. The genotype 1a polymorphic site 174 is part of a surface α-helix, allowing certain flexibility of the amino acid. However, Asn174 points towards Gln80 on the opponent loop. This restricts the size of amino acid 174, explaining the conservative Asn174Ser polymorphism. Both amino acids are polar and are about the same size. Also, only one nucleotide change is necessary to change form Asn to Ser. Interestingly, the opponent amino acid Gln80 can be substituted with Lys, a non-conservative change from a polar neutral to a polar positive amino acid. An Asn174Ser change though, was not associated with a Gln80Lys change. As no data is available on the fitness of the individual variants, it cannot be estimated whether a specific combination of amino acids at position 174 and 80 are favourable.
Within genotype 3a variants, highly polymorphic sites were found at position 7, 98 and 176. Ala7 is part of the NS4A binding domain and has been discussed above. Thr98 is part of a surface loop pointing away from the protease allowing flexibility with amino acid substitution. A simple adenosine to guanosine nucleotide substitution results in a change of Thr to Ala, explaining the Thr to Ala polymorphism. Asn176 is part of a surface α-helix facing away from the protease, suggesting that a certain flexibility of the amino acid residue at this position will be allowed. As the Asn176Ser substitution is a conservative amino acid change, it is unlikely to have a major impact on protease structure and function.

The most common non-conservative amino acid changes involved substitutions of a hydrophobic amino acid with a neutral polar one, followed by substitutions of neutral side chains with charged side chains. In general non-conservative amino acid substitutions away from the consensus sequence mapped to surface loops. Within genotype 1a variants though, 2 variants had a Ser122Gly change. Ser122 is located on a loop, but points towards Asp168 and Arg155, which are part of the S2 binding pocket. Both are involved in the resistance mechanism against the PI BILN 2061 (Courcambeck *et al.* (2006)). Arg155, together with Ala156, directly binds to the inhibitor, whereas Arg168 indirectly affects inhibitor binding. The hydroxyl group of Ser122 could possibly build a hydrogen bond with the guanidinium group of Arg155. The non-polar amino acid Gly does not have a hydroxyl group to build a hydrogen bond. This disrupted interac-

tion might have an influence on the 3D structure and affect substrate binding as well as inhibitor binding. More detailed structural modelling though is necessary to get conclusive prediction on this amino acid polymorphism.

Within genotype 3a variants, the polymorphic site Ala166 is located on a β-strand and points towards Thr123, which is part of the S2 binding pocket. Ser166 was found in 3 genotype 3a variants and Thr166 in 2 variants. Ala is a small hydrophobic amino acid, whereas Ser and Thr are both polar and bulkier. Substitution of Ala with Ser/Thr is therefore likely to push away the opponent Thr123 and change the S2 binding pocket environment. As the binding of PIs is influenced by the 3D structure of the binding site, the efficacy of some PIs might be reduced in variants containing Ser166/Thr166.

Thr47 and Ala151 are 2 further polymorphic sites located on interior β-strands. Thr47 points towards Asp110, which is also polymorphic (Asp110Glu). Ala47 was found in 3 genotype 3a variants. Substitution of Thr47 with a larger amino acid would displace Asp110 and modulate the 3D structure. However, Ala is smaller than Thr and it is unclear what kind of effect a substitution would have. Val151 and Thr151 were found in one genotype 3a variant each. Ala151 points towards Arg118 on the opponent β-strand and a replacement with the slightly bulkier amino acids Val and Thr might displace Arg118 and have an effect on the 3D structure and the functional domains.

The genetic diversity of NS4A was slightly higher within genotype 1a variants compared to genotype 3a variants. Like NS3 protease sequence diversity, NS4A nucleotide sequence diversity was mostly synonymous. Around 23 % of nucleotide positions within genotype 1a variants had synonymous substitutions and 21 % within genotype 3a variants. The proportion of non-synonymous substitutions away from the consensus sequence was higher in genotype 1a variants compared to genotype 3a variants (2.1 % versus 0.4 %), but overall low and most likely due to genetic drift. The N-terminal membrane segment, which is highly conserved across genotypes, is also highly conserved within genotype 1a and 3a variants (Brass *et al.* (2008)). Only 3 variants of genotype 3a had a substitution away from the consensus sequences, which were conservative and sporadic and unlikely to affect the membrane topology of NS4A. Some variability was found within the region essential for cofactor activity. As discussed

above, $Val23_4$, $Ile25_4$, $Gly27_4$ and $Ile29_4$ are crucial for the interaction with the NS3 protease and expectantly only conservative amino acid substitutions were observed. Whereas an $Ile29_4Val$ polymorphism was found in genotype 1a variants, genotype 3a variants were highly conserved in this area. $Ile25_4$ interacting with the NS3 hydrophobic pocket is conserved within both genotypes. Some diversity was also found within the kink region and the C-terminal domain, but except for an Arg46Gln substitution, all amino acid substitutions were conservative. These results suggest that the NS4A gene is highly conserved within a genotype, but flexible enough to allow some mutations, especially within the genotype 1a kink and C-terminal region.

3.4 Conclusion

This study describes the polymorphisms within the NS3 protease and NS4A cofactor gene of genotype 1a and 3a clinical variants. It shows that the NS3/4A protease is flexible enough to tolerate substitutions despite the different functional constraints. Even though no mutations were found within the catalytic site, the S1 and S2 binding pocket or the zinc binding domain, natural polymorphism observed outside of the functional domains of the NS3 protease could potentially have an effect on clinical resistance to HCV PIs. Amino acid substitutions from smaller to bulkier and from neutral to charged residues or vice versa influence the interaction between individual amino acids and potentially alter the 3D structure of the protease, thereby affecting functional sites. 3D modelling was used to estimate which non-conservative substitutions would influence the 3D structure of the NS3 protease and have an effect on the functional domains. As discussed in section 1.8, PIs show different efficacies for different genotypes. Because of the genetic variability of HCV proteins encoded by different genotypes, the molecular structure of protease and polymerase enzymatic sites differs and potentially limits the effectiveness of certain classes of inhibitors (Holland-Staley *et al.* (2002)). The case of BILN 2061 demonstrated very early on that structural differences within genotypes have a big impact on drug efficacies. The intragenotypic diversity potentially has a similar influence on protease efficacies in individuals infected with the same genotype. As shown in chapter 4 and 5, NS3 proteases from clinical variants of the same

genotype subtype behave differently in intergenotypic 2a recombinants. Differences in their susceptibility to the PI BILN 2061 could be demonstrated as well.

The information provided here will be very valuable for the design of new PIs. Considering sites of potentially influential polymorphism, inhibitors could be designed to target sites which are conserved across and within genotypes and subtypes. This will enable the development of inhibitors that are more likely to be effective in all genotypes and subtypes. Furthermore, individuals with known amino acid polymorphism at target sites could be excluded from treatments with potentially ineffective drugs, saving them from antivirals with often severe side effects. However, clonal analysis will have to be performed in order to obtain information on all the circulating viral variants within a patient.

Chapter 4
Construction of Intra- and Intergenotypic Recombinants

4.1 Introduction

Research into antiviral drugs and vaccines has been hampered by the lack of a full viral life cycle cell culture system. Only recently a full-length HCV cell culture system in which infectious virus can be generated in Huh7 cells from transfection of complete HCV genomic RNA sequences has been described (Lindenbach *et al.* (2005); Wakita *et al.* (2005)). JFH1 was isolated from a patient with fulminant hepatitis C and a subgenomic replicon containing the JFH1 derived non-structural genes replicated very efficiently in Huh7 cells (Kato *et al.* (2003)). Surprisingly, *in vitro* transcribed full-length RNA from pJFH1 replicated very efficiently in Huh7 cells as well and the cell culture generated HCV was also infectious in chimpanzees (Wakita *et al.* (2005)). It was the first full-length HCV RNA to replicate efficiently in cell culture and proved to be an important milestone in HCV research. The big advantage of full-length virus replication is that it also produces infectious virus, unlike the replicons, thereby providing insight into the whole viral lifecycle. For the first time this allowed the analysis of viral proteins in the context of the entire viral lifecycle. Unfortunately, as to date, the genotype 2a isolate JFH1 has remained the only isolate to efficiently replicate in cell culture. However, the JFH1-system has been expanded to other genotypes by replacing parts of the JFH1 genes with those of other genotypes. Viable JFH1-based intergenotypic recombinants containing genotype specific structural proteins (core, E1 and E2), p7 and NS2 have been developed for all 7 genotypes (Pietschmann *et al.* (2006); Gottwein *et al.* (2007); Yi *et al.* (2007); Jensen *et al.* (2008); Scheel *et al.* (2008); Gottwein *et al.* (2009)), which allow the study of vaccines and entry inhibitors for all genotypes. However, full-length HCV cell culture systems allowing the study

of the NS3 protease are currently only available for genotype 2a (JFH1 and J6CF) (Lindenbach *et al.* (2005); Wakita *et al.* (2005); Murayama *et al.* (2007a)) and 1a (H77), which needs adaptive mutations to replicate efficiently (Yi *et al.* (2006b)). The limited number of replication competent full-length reference sequences limits the assessment of how genetic variation between the different genotypes and within subtypes influences susceptibility to antiviral therapy and development of resistance.

The use of chimaeric replicon vectors, where protease genes of different genotypes were inserted into the genotype 1a/1b/2a standard replicons, has been demonstrated in several reports (Binder *et al.* (2007); Qi *et al.* (2009)). These intergenotypic replicons have greatly expanded the ability of tools to assess genetic variation within genotypes, subtypes and quasispecies. Before, replication competent replicons were restricted to laboratory optimised strains which did not represent the immense genetic diversity of HCV. Furthermore, they demonstrated that the NS3 protease is functional in a heterologous background. However, replacing the helicase as well greatly reduced replicon fitness, indicating that the helicase is highly specific in recognising and interacting with other parts of the RNA complex (Qi *et al.* (2009)). Other approaches involve the release of reporter molecules upon NS3/4A cleavage (Ludmerer *et al.* (2008)). However, none of these methods release infectious virus that would allow the whole replication cycle of HCV to be analysed.

The aim of the current study was to develop effective cell culture systems for the 6 major genotypes of HCV. This will allow the comparison of the susceptibility of each to different PIs, such as BILN 2061 (chapter 5). Furthermore, through passaging in subinhibitory concentrations of the drug, the ability and mechanism of antiviral resistance development between genotypes can be compared (chapter 6).

Recently, the full-length replication competent clone Jc1 (pFK JFH1/J6/C-846) has been developed. This clone comprises the J6CF core, the envelope and the p7 coding sequences and a portion of the NS2 gene, with the remainder of the polyprotein derived from JFH1 (Lindenbach *et al.* (2005); Pietschmann *et al.* (2006)). It replicates autonomously and yields high infectious titres in the Huh7.5 cells. It was therefore chosen as a backbone for the construction of the intra- and intergenotypic recombinants in

the current study. Intra- and intergenotypic recombinants were first constructed within the JFH1 backbone and then cloned into the Jc1 backbone. In an initial approach, naturally occurring restriction sites outside the NS3 protease were used for the construction of recombinants. As this approach was not successful, specific restriction sites at the exact boundaries of the NS3 protease were designed. This was only partly successful, which led to the strategy of including the NS4A cofactor in the recombinant construction. Homologous NS4A allowed the rescue of replication defective intergenotypic recombinants for most variants. The development of these replication competent intergenotypic recombinants will improve the ability to predict clinical doses, efficacies and development of drug resistance mutations to different PIs in a diverse range of HCV variants circulating worldwide.

4.2 Results

4.2.1 Design and construction of intra- and intergenotypic recombinants containing heterologous NS3 protease

Using naturally occurring restriction sites

To establish full-length cell culture systems allowing the analysis and characterisation of the NS3 protease gene of all 6 major genotypes, intergenotypic recombinants based on the genotype 2a clones JFH1 and Jc1 were constructed. Intergenotypic recombinants containing the NS3 protease gene of heterologous genotypes were created through ligation of protease domain sequences from different genotypes into the Jc1 and JFH1 backbone sequence (Fig. 4.1). JFH1 contains 2 naturally occurring unique restriction sites, *Not*I within NS2 and *Spe*I within the NS3 helicase. Primers flanked with these restriction sites were used to amplify the corresponding fragment from genotype 1a (pH77*) and 3a (pHCV3a-Gla) prototype plasmids or patient plasma. The 1149 bp fragment was digested in a double digest, cleaned and cloned into the likewise digested JFH1 backbone. The resulting intergenotypic recombinants contain the full-length NS3 protease plus the 3' 464 nucleotides of NS2 as well as the 5' 142 nu-

cleotides of the NS3 helicase and were named F_nx_n, where x stands for the genotype the NS2-3 fragment is derived from (Fig. 4.2). RNA transcribed from the recombinant plasmids and the replication defective pJFH1-GND as well as Jc1 and JFH1 was electroporated into the HCV permissive Huh7 cells and virus replication assessed by NS5A immunostaining (Fig. 4.3). Initially Huh7 cells were used for assessment of viral replication, but they were replaced with the far more permissive Huh7 subclone Huh7.5, which has a defect in RIG-I signalling (Blight *et al.* (2002); Sumpter *et al.* (2005)). HCV variants replicate to higher levels in these cells. To create the corresponding Jc1 based recombinants (J_nx_n), the *Spe*I restriction site in the plasmid backbone of Jc1 first had to be mutated, to render the *Spe*I restriction site within the NS3 helicase unique. This was done by PCR using primers including point mutations and religation of the modified insert back into Jc1.

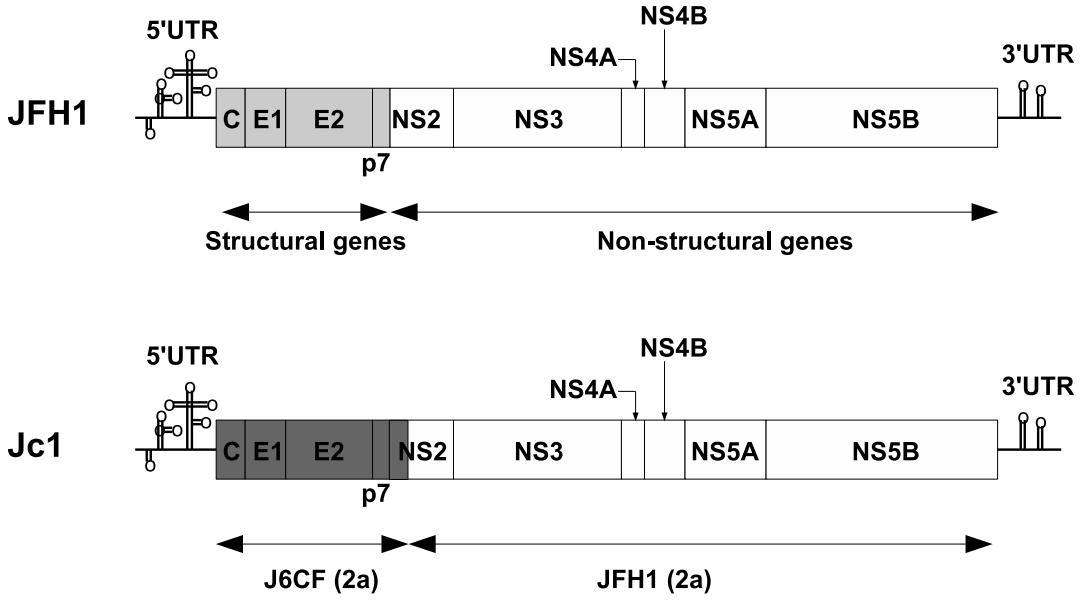

Figure 4.1: ***Genome map of JFH1 and Jc1****. Genome map of JFH1 and Jc1 used in recombinant construction. The structural genes are depicted in grey; non-structural genes in white. The intragenotypic recombinant Jc1 contains the non-structural genes and part of NS2 of the genotype 2a isolate J6CF (dark grey) and the remaining non-structural genes from JFH1.*

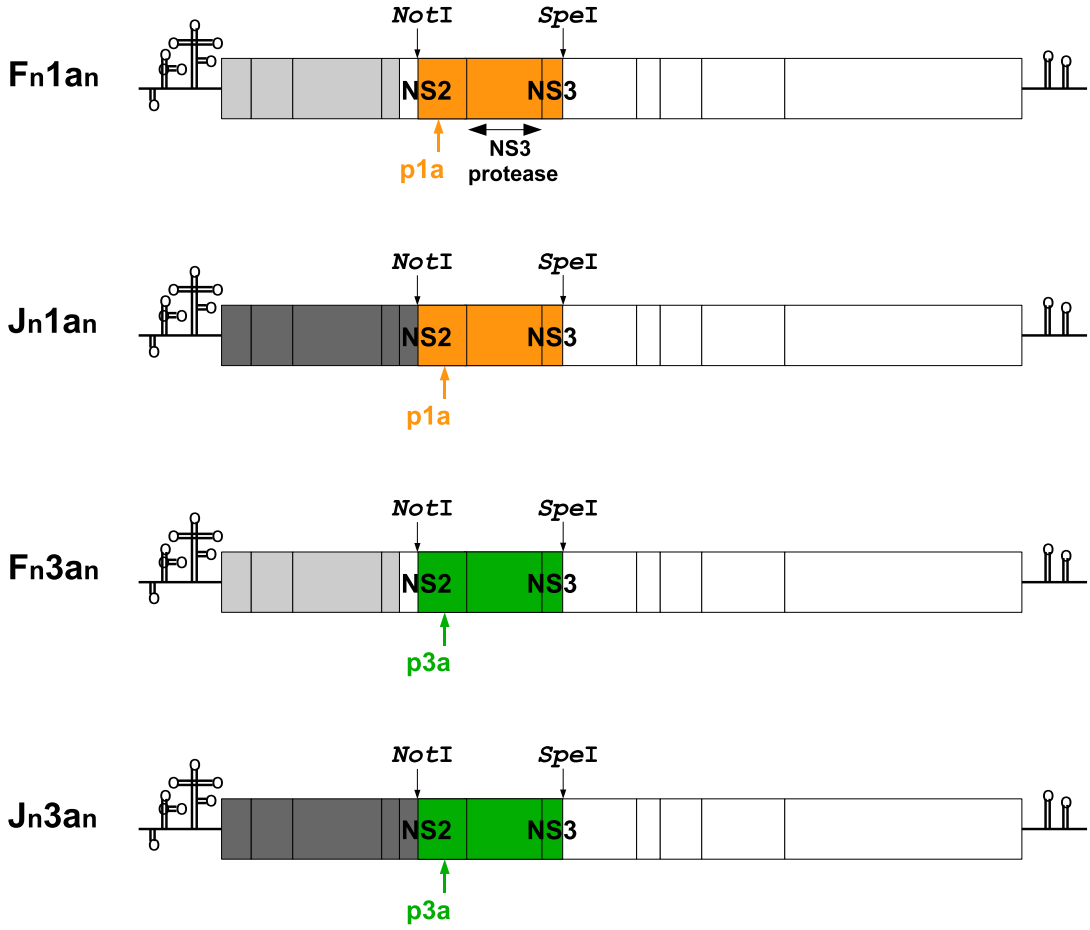

Figure 4.2: ***F_nx_n* and *J_nx_n* recombinants**. *Genome map of cDNA clones: JFH1 backbone, light grey and white; Jc1 backbone, dark grey and white. The fragment between the restriction sites NotI and SpeI was replaced with the corresponding intergenotypic gene (replaced region, coloured).*

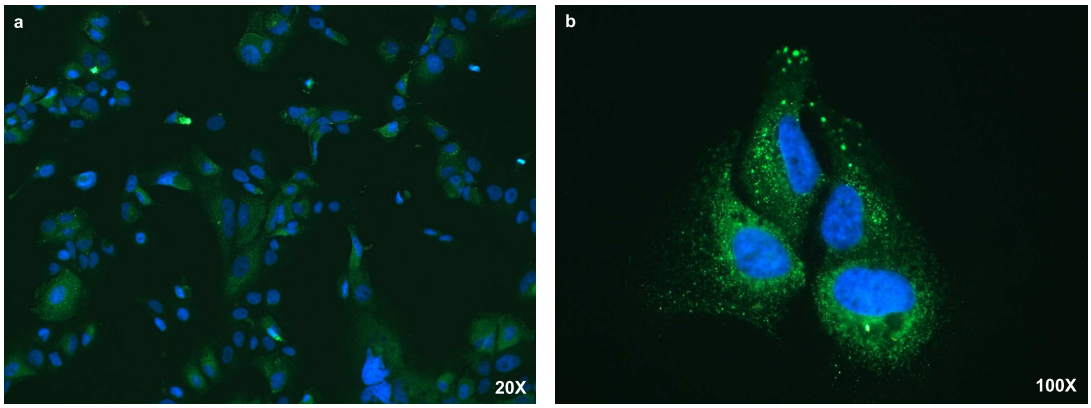

Figure 4.3: ***Anti-NS5A immunostaining***. *RNA transcripts from JFH1, Jc1, JFH1/Jc1 recombinants (here J2a) or the replication defective JFH1-GND were electroporated into Huh7 or Huh7.5 cells (here Huh7.5) and replication was assessed by immunostaining against the HCV non-structural protein NS5A. Cells were counterstained with the nucleic acid probe 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) (blue) and sheep anti-NS5A serum (green).* ***a*** *20X magnification;* ***b*** *100X magnification.*

Replication assessment in Huh7 cells

JHF1 and Jc1 replicated in Huh7 cells with infectivity titres of 5×10^3 FFU/ml and 2.9×10^4 FFU/ml, respectively. In contrast, Huh7 cells transfected with RNA transcripts from pJ6CF, the replication defective pJFH1-GND and mock transfected cells were negative for anti-NS5A staining. For all further experiments carried out in this study, Jc1 was used as a positive and JFH1-GND as a negative control, respectively.
The JFH1 recombinants F_n1a_n and F_n3a_n were severely impaired or defective in their replication kinetics and transfected cells were negative for NS5A. Even though Jc1 replicates to higher levels in Huh7 cells than JFH1, using Jc1 as a backbone to create the intergenotypic recombinants J_n1a_n and J_n3a_n did not result in improved replication kinetics. Cells transfected with J_n1a_n and J_n3a_n RNA were NS5A negative as well.

Replication assessment in Huh7.5 cells

Alternatively, the replication kinetics of parental and recombinant variants were assessed in Huh7.5 cells. 24 hours post-electroporation about 80 % of all cells transfected with JFH1 and Jc1 RNA were positive for NS5A, indicating replication (Fig. 4.4). In contrast, JFH-GND replication was not detected. Whereas Jc1 continues to

infect 70-80 % of all cells, JFH1 infectivity is slightly reduced during passaging. In all graphs of HCV RNA passaging in cell culture, data points are plotted with lines connecting them for better visualisation. During the course of the experiment the cell cultures were continuously split and variants have to spread between cells to not be diluted out. Serial dilutions of cell culture supernatants were used to infect naïve Huh7.5 cells and to determine the tissue culture infective dose ($TCID_{50}$). The $TCID_{50}$ for Jc1 ($10^{4.5}$ $TCID_{50}$/ml) was expectantly higher than that of JFH1 ($10^{3.6}$ $TCID_{50}$/ml). Due to the better replication kinetics and higher infectivity of Jc1, it was used for further recombinant construction. Neither of the 4 recombinants (F_n1a_n, J_n1a_n, F_n3a_n or F_n3a_n) replicated in the Huh7.5 cell culture to detectable levels.

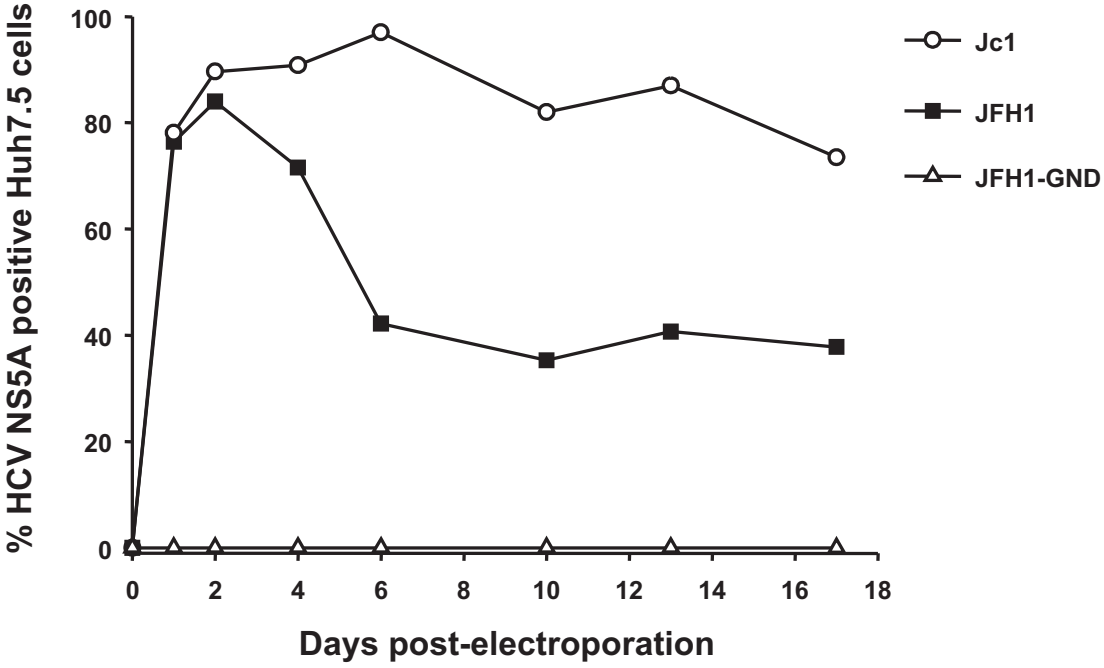

Figure 4.4: ***Replication kinetics of JFH1, JFH-GND and Jc1 full-length RNA transcripts in Huh7.5 cells****. 10 μg of RNA was electroporated into Huh7.5 cells and cells incubated for 24 hours. The cells were then stained for NS5A or alternatively passaged and assessed for NS5A positive cells every 3 to 4 days. The y-axis records the percentage of HCV NS5A-positive cells as scored by fluorescence microscopy.*

Introducing artificial restriction sites

The defective replication kinetics of the F_nx_n and J_nx_n recombinants might be due to the partial inclusion of gene segments from NS2 and the NS3 helicase. This creates intergenotypic NS2 and NS3 helicase proteins. The considerable differences between genotypes possibly disrupt the correct folding of these proteins and affect their function. The cloning strategy was therefore changed to only swap the protease domain and leave the entire NS2 and NS3 helicase of backbone identity. To generate recombinants containing only the NS3 protease gene of heterologous genotypes, 2 unique restriction sites, a *BstB*I site at the 5'end and a *Bgl*II site at the 3'end of the NS3 protease were introduced, creating Jc1-BB. The unique restriction sites were identified using Vector NTI scan. Jc1-BB containing the introduced restriction sites showed similar replication kinetics to the parental strain Jc1 (Fig. 4.5). Protease genes from genotypes 1a (H77*), 1b (HC-J4), 2a (J6CF), 3a (HCV3a-Gla), 4a (ED43*), 5a (EUH1480*) and 6a (EUHK2*) were amplified by PCR and cloned into Jc1-BB. The primers were designed to include JFH1 sequence up to the start of the NS3 protease gene (nt position 3420) and the corresponding genotype sequence to the 3'end of the NS3 protease (nt position 3963). From the 3'end of the protease to the restriction site, the primer sequence was designed to include JFH1 sequence again. Because p4a (ED43*) contained a naturally occurring *Bgl*II restriction site within the protease domain, this restriction site first had to be mutated by site directed mutagenesis. Recombinants containing heterologous proteases were termed Jx, where x identifies the genotype of the protease domain (Fig. 4.6).

RNA transcribed from the recombinant plasmids and the replication defective pJFH1-GND as well as Jc1 was electroporated into the highly permissive Huh7.5 cells and virus replication assessed by NS5A immunostaining. Besides J2a, which showed similar replication kinetics to that of the parental strain Jc1, only J5a replicated to detectable levels and it took 25 days to spread to 80 % of the cell culture (Fig. 4.7). As the cell culture was split through the course of the experiment, the virus would have had to spread to neighbouring cells to achieve this infection frequency during passaging, indicating that the J5a recombinant is able to spread between cells.

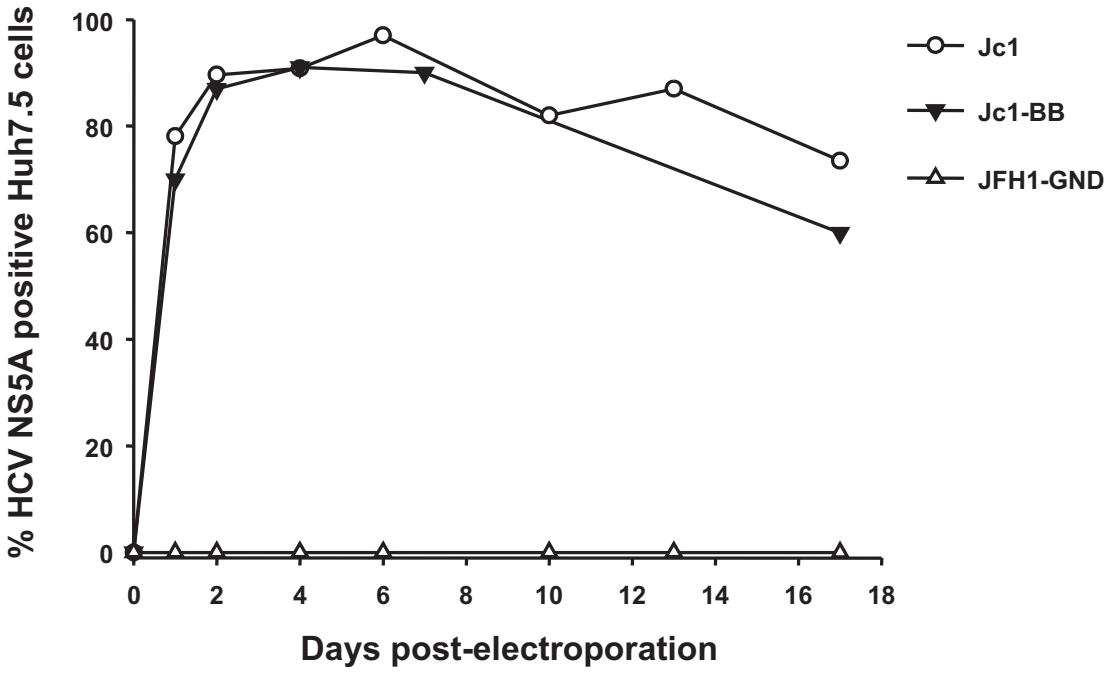

Figure 4.5: ***Replication kinetics of Jc1, Jc1-BB and JFH-GND full-length RNA transcripts in Huh7.5 cells****. 10 μg of RNA was electroporated into Huh7.5 cells and cells incubated for 24 hours. The cells were then stained for NS5A or alternatively passaged and assessed for NS5A positive cells every 3 to 4 days.*

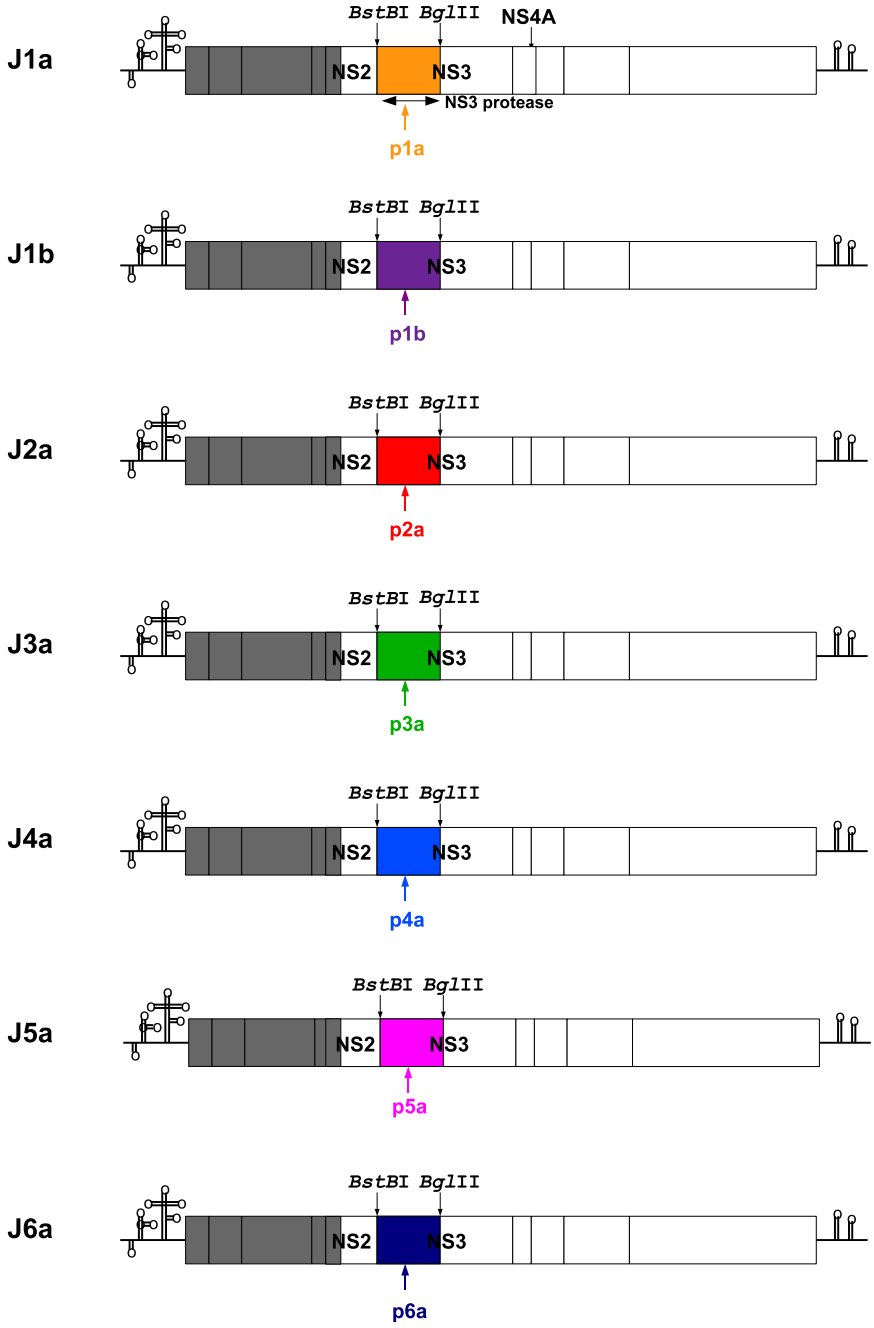

Figure 4.6: ***Genome map of Jx recombinants.*** *Genome map of cDNA clones (pJ6CF, dark grey; pJFH1, white). The NS3 protease gene of Jc1 was replaced with the corresponding intra- or intergenotypic gene (replaced region, coloured) using the genetically engineered restriction sites BstBI and BglII.*

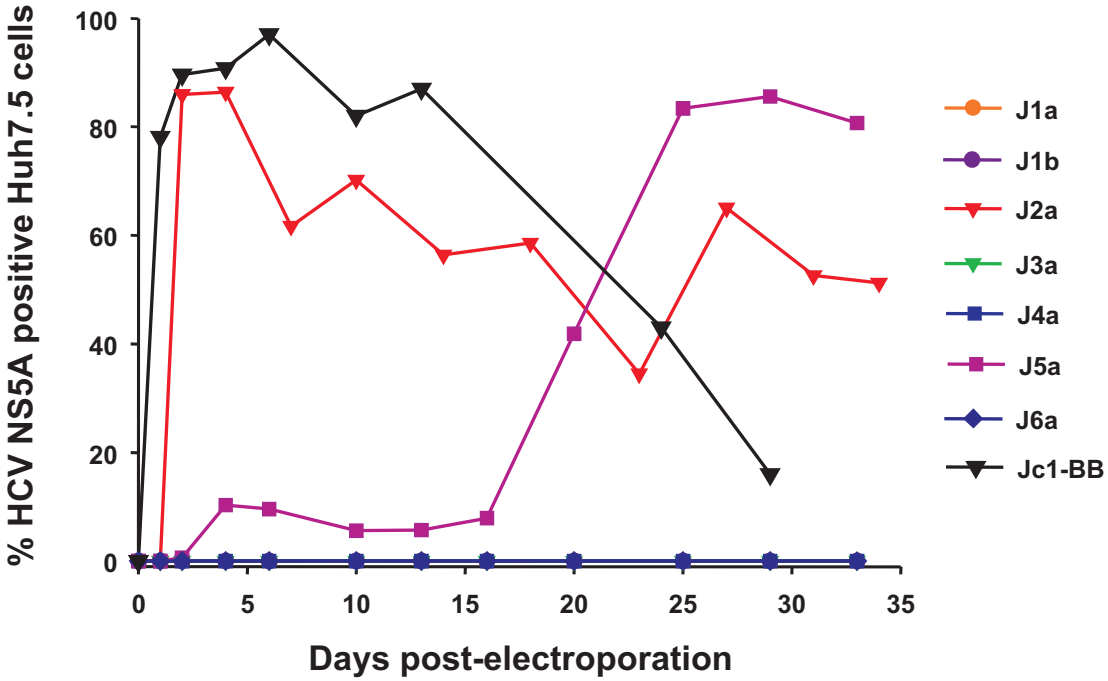

Figure 4.7: ***Jx recombinants and their viability in Huh7.5 cells.*** *RNA transcripts from J1a, J1b, J2a, J3a, J4a, J5a and J6a were electroporated into Huh7.5 cells and replication was assessed by immunostaining against NS5A. Transcripts from the J1a, J1b, J3a, J4a and J6a clones showed no detectable replication (all values lying on the x-axis line).*

4.2.2 Design and construction of intra- and intergenotypic recombinants containing heterologous NS3 protease and NS4A

Since the NS4A cofactor is an indispensable part of the NS3 protease function and highly variable in sequence between genotypes, it was investigated whether inclusion of the homologous NS4A gene in the intra- and intergenotypic recombinants improved their replication ability (Bartenschlager *et al.* (1995); Failla *et al.* (1995); Tanji *et al.* (1995)). Two unique restriction sites were introduced at the 5'end (*Blp*I) and at the 3'end (*Mlu*I) of NS4A and the corresponding NS4A genes from genotypes 1a, 1b, 2a (pJ6CF), 3a, 4a, 5a and 6a were amplified by PCR using primers with *Sap*I and *Mlu*I restriction sites. Introducing a *Blp*I restriction site into pJFH1 results in one non-synonymous nt change on the 3'end of the restriction site. Due to that an alternative restriction site, with compatible ends but different recognition sequence was introduced to the 5'end of the inserted fragment. *Sap*I produces NNN..3' overhangs and the recognition sequence is entirely located on the 5'site of the cutting site. This allowed the design of a recognition site for *Sap*I that changed the vector sequence back to that of JFH1 once the insert was religated into the vector. The obvious disadvantage of this approach is that the restriction site is destroyed and fragments cannot be cut out anymore with the same restriction enzymes. The primers were designed to include JFH1 sequence up to the start of the NS4A gene (nt 5313), then the corresponding genotype sequence to the 3'end of NS4A (nt 5474) and JFH1 sequence again to the restriction site. As the introduction of the *Mlu*I restriction site was not synonymous and no alternative compatible restriction enzymes could be found, it was reversed to the JFH1 amino acid sequence after NS4A gene insertion by site directed mutagenesis. The entire mutagenesis reaction was carried out on fragments subcloned into the TOPO vector. JFH1 fragments including the intra- or intergenotypic NS4A gene were then recloned into JFH1 and Jc1, respectively. The corresponding intra- and intergenotypic recombinants containing the NS3 protease and NS4A gene sequence of hetero- or homologous genotypes in the JFH1 background were termed Fxx, where xx stands for the corresponding genotype in the protease and NS4A regions, respectively (Fig. 4.8). Recombinants with Jc1 as a background were named Jxx (Fig. 4.9).

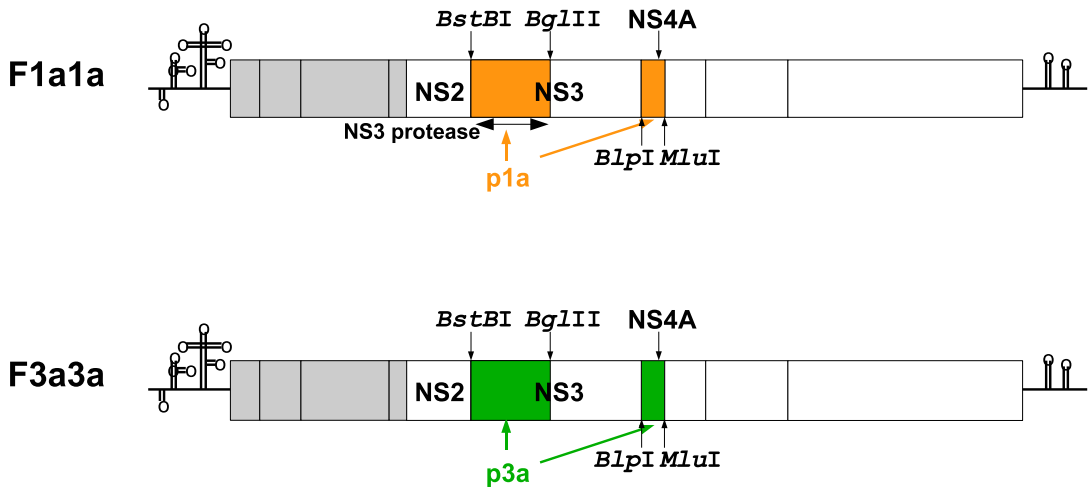

Figure 4.8: ***Genome map of Fxx recombinants.*** *Genome map of cDNA clones (pJFH1, light grey and white). The NS3 protease gene of JFH1 was replaced with the corresponding intergenotypic gene (replaced region, coloured) using the genetically engineered restriction sites BstBI and BglII. The NS4A gene of JFH1 was replaced with the corresponding intergenotypic gene using the genetically engineered restriction sites BlpI and MluI.*

Comparing Fxx and Jxx replication kinetics in Huh7 cells

Initially recombinant replication was assessed in Huh7 cells. Because the replicative fitness of Fxx and Jxx were too low to determine percentage positive NS5A cells, foci forming units (FFU) per well were determined. Recombinants were passaged in Huh7 cells and seeded into 24-well plates for anti-NS5A staining. Jc1 and JFH1 RNA transcripts replicated in 60-70 % of all cells and were omitted in the comparison of Fxx with Jxx recombinants (Fig. 4.10). F1a1a and J1a1a replication levels were below the detection limit and no NS5A positive cells were detected during the passaging experiment. Both, F3a3a and J3a3a replicated to detectable levels within Huh7 cells (Fig. 4.10). As expected from the better replication kinetics of Jc1, the Jc1 recombinant J3a3a replicated to higher levels than the JFH1 based recombinant F3a3a. After peaking on day 7, FFU/well levels start to decrease for both recombinants. Because the Jc1 based recombinant replicated more efficiently, for the following experiments only Jxx recombinants were assessed.

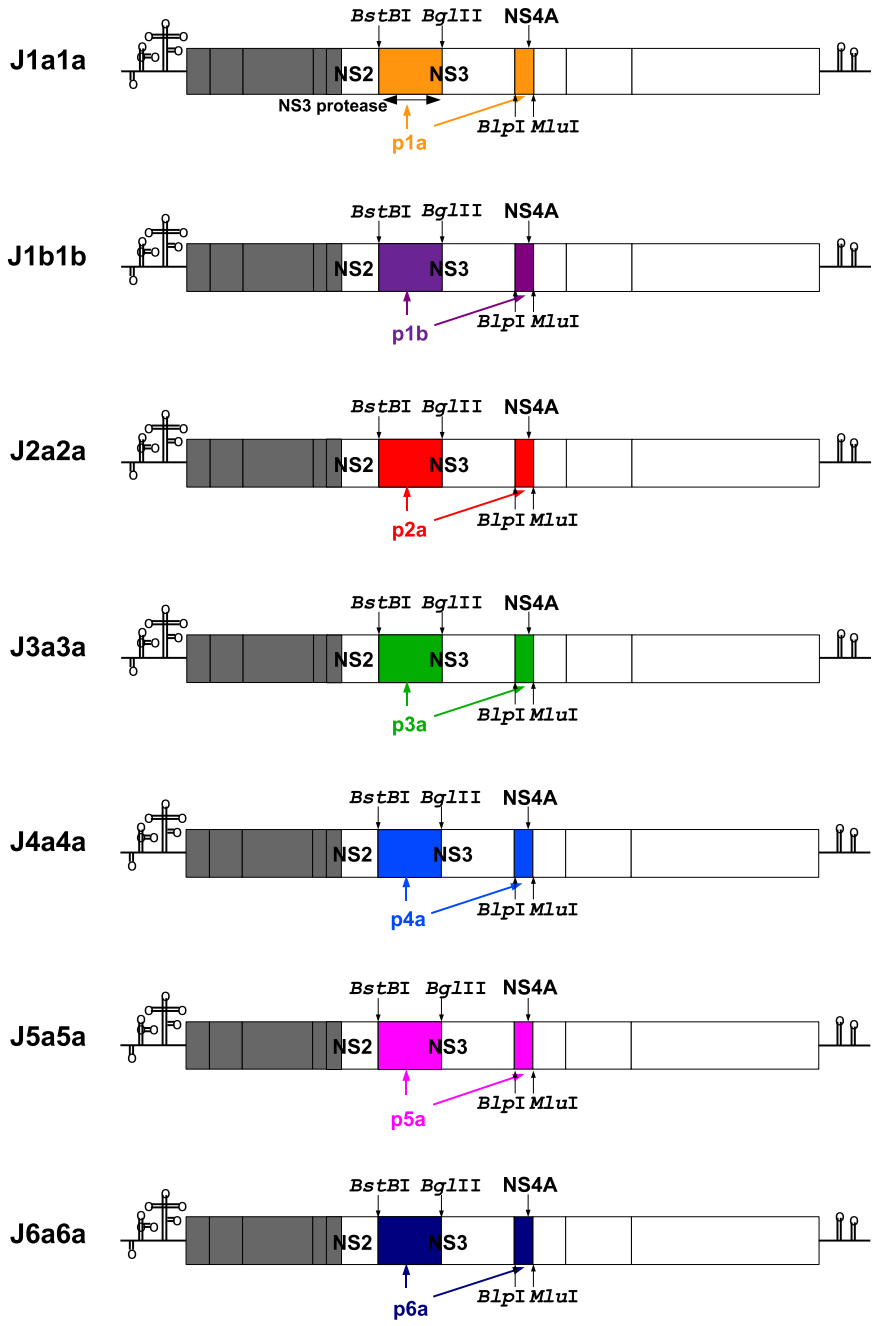

Figure 4.9: ***Genome map of Jxx recombinants.*** *Genome map of cDNA clones (pJ6CF, dark grey; pJFH1, white). The NS3 protease gene of Jc1 was replaced with the corresponding intra- or intergenotypic gene (replaced region, coloured) using the genetically engineered restriction sites BstBI and BglII. The NS4A gene of Jc1 was replaced with the corresponding intra- or intergenotypic gene using the genetically engineered restriction sites BlpI/SapI and MluI.*

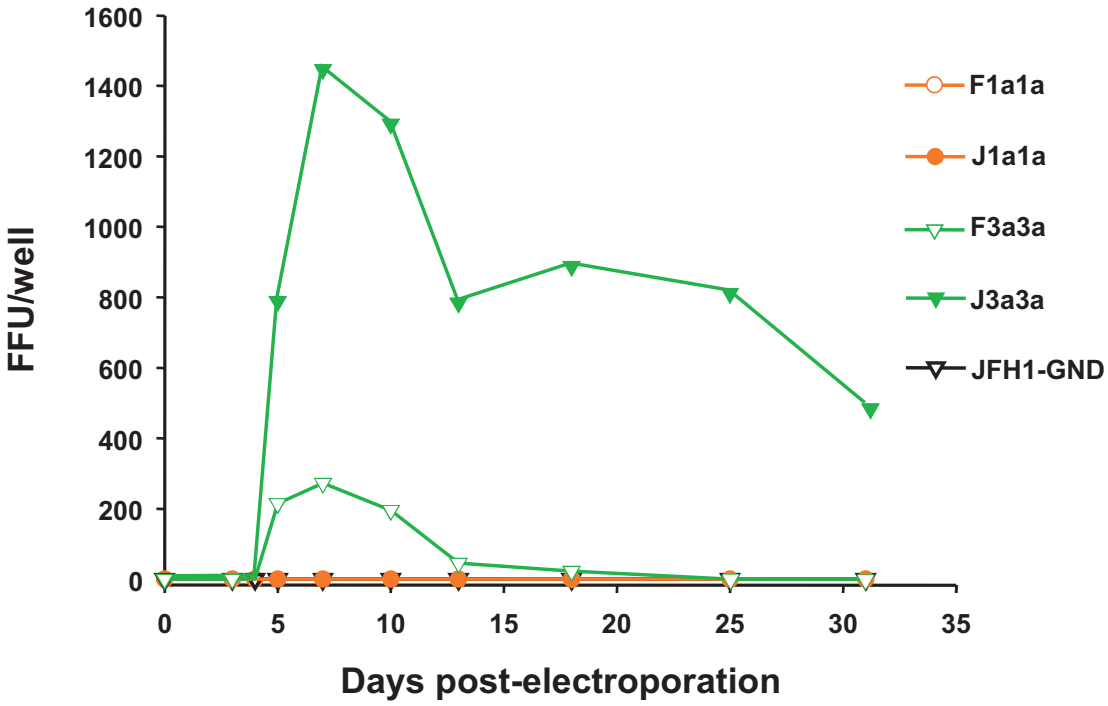

Figure 4.10: ***Recombinants F1a1a, J1a1a, F3a3a and J3a3a and their viability in Huh7 cells.*** *RNA transcripts from F1a1a, J1a1a, F3a3a, J3a3a and JFH1-GND were electroporated into Huh7 cells and replication was assessed by immunostaining against NS5A. The y-axis records the number of foci forming units (FFU) per well in 24-well plates. Transcripts from the F1a1a, J1a1a and JFH1-GND clones showed no detectable replication (all values lying on the x-axis line).*

Comparing replication kinetics of Jxx recombinants in Huh7.5 cells

As the replication kinetics of the J3a3a recombinant was very low in the Huh7 cells, all remaining experiments were carried out with Huh7.5 cells. Replication competent recombinants could be created with genotype 1b, 2a, 3a, 5a and 6a (Fig. 4.11, 4.12 and 4.13). Four days after transfection, J1b1b was detected in 50 % of the cell culture, but was then cleared from the cell culture likely reflecting an inability to infect Huh7.5 cells *de novo* beyond the transfection stage (Fig. 4.11 top). In marked contrast, J2a2a and J5a5a replication was detected in almost all cells 2 days after transfection, comparable to Jc1 (Fig. 4.12). The immediate spread of these recombinants and Jc1 was accompanied by increased cell death, followed by proliferation of HCV-NS5A negative Huh7.5 cells as described in previous studies (Zhong *et al.* (2006); Gottwein *et al.* (2007); Mateu *et al.* (2008); Scheel *et al.* (2008)). However, both start to re-spread in the cell culture after this initial decrease in infected cell frequency, indicating sequence changes that reduce the cytopathic effect of the virus and promote its survival in cell culture. Theses changes were termed as "attenuating", to make clear the difference with conventional "adaptive" mutations that enhance replication ability.

Replicating J3a3a and J6a6a viruses were found in 80 % of the cell culture after an eclipse phase of 6 and 33 days, respectively (Fig. 4.13). Compared to other Jxx recombinants, where the percentage of NS5A positive cells was reduced again after an eclipse phase, J3a3a still infected 80 % of the cell culture 70 days post-electroporation, indicating continuous spread to uninfected cells. Supernatant infectivity was measured at the peak of the infection by determining $TCID_{50}$s (Table 4.1). The highest infectivity titre was measured for J2a2a with a $TCID_{50}$/ml of $10^{4.2}$. The $TCID_{50}$ measurement for J1b1b was below detection limit ($TCID_{50}$/ml <10), indicating that this recombinant does not secrete any infectious virus into the supernatant or only at levels below the detection limit. J1a1a and J4a4a were not viable or their replication was not efficient enough to be detected with our assay. However, replacement of the NS3 protease gene in J4a4a with that of patient-derived protease genes generated the replication competent recombinants J4a4a-7, -8, -19, also see section 4.2.3 (Fig. 4.11 bottom and 4.17 bottom). J4a4a-7 and J4a4a-19 showed a similar replication profile to J1b1b,

with NS5A-positive cells initially detected in 50 % of the cell culture but then cleared out as the culture was passaged. As with J1b1b, no detectable infectious virus was secreted into the supernatant ($TCID_{50}$/ml $<$10). J1a1a and J4a4a both differ to the prototype sequence H77 and ED43 by one (H77-Met1205Thr) and 4 (ED43-Thr1048Ala, Thr1064Ile, Ile1160Thr and Arg1176Ala) amino acids, respectively, in the protease gene, whereas both are identical to H77 and ED43, respectively, in the NS4A gene (Table C.2 and C.4, Appendix C). Which amino acid polymorphism potentially influences the impaired replication kinetics of J1a1a and J4a4a is discussed in section 4.3.

Table 4.1: *Supernatant infectivity titres determined by $TCID_{50}$ assay.*

Viral variant	Days post-electroporation	$TCID_{50}$/ml
JFH1	5	$10^{3.6}$
Jc1	5	$10^{4.5}$
JFH1-GND	5	< 10
J1a1a	5	< 10
J1b1b	5	< 10
J2a2a	5	$10^{4.2}$
J3a3a	15	$10^{3.4}$
J4a4a-19	5	< 10
J5a5a	5	$10^{1.9}$
J6a6a	33	$10^{2.6}$

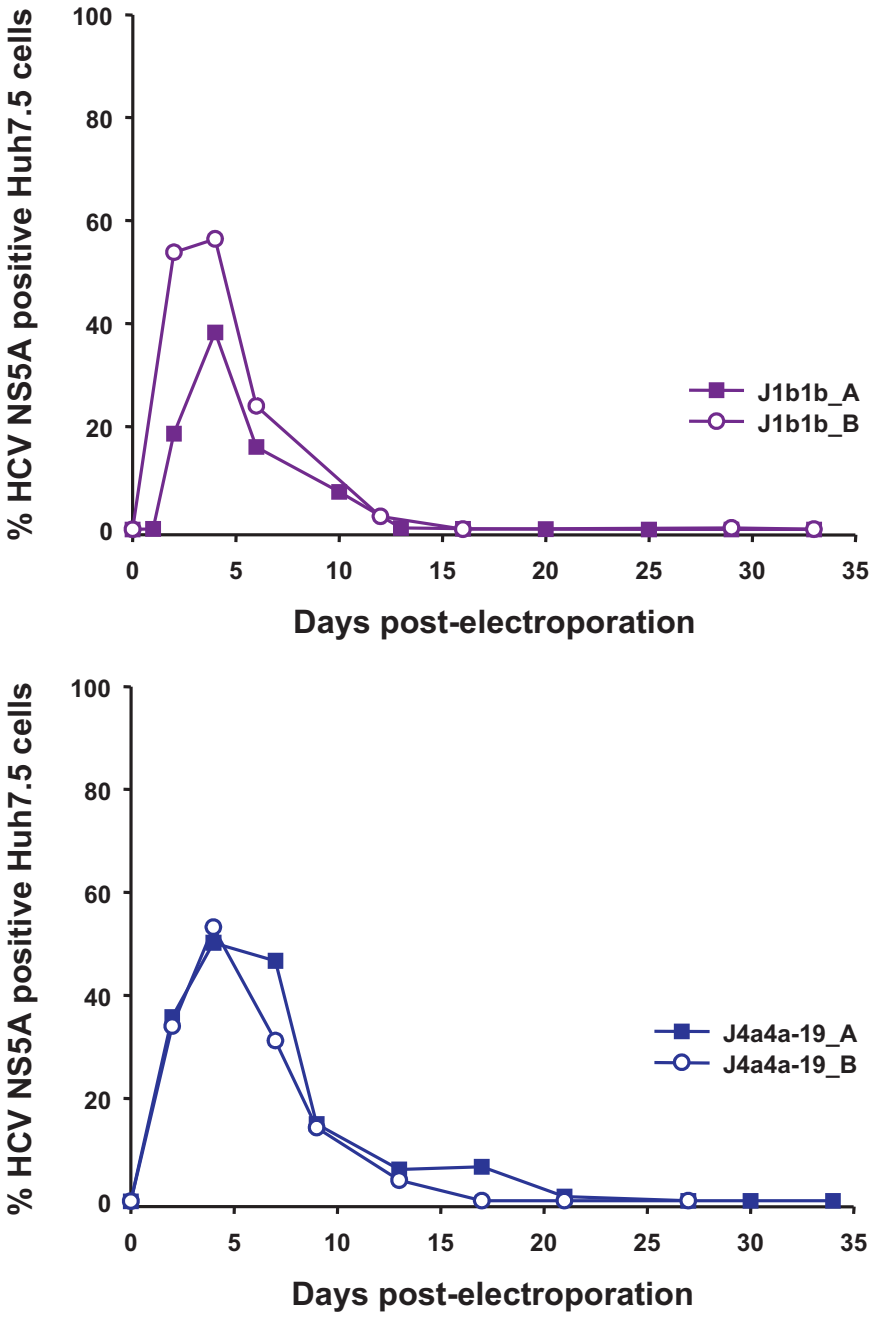

Figure 4.11: ***Recombinants J1b1b and J4a4a and their viability in Huh7.5 cells.*** *Results from 2 independent experiments are presented (Jxx_A, Jxx_B). RNA transcripts from J1b1b (top) and J4a4a-19 (bottom) were electroporated into Huh7.5 cells and replication was assessed by immunostaining against NS5A.*

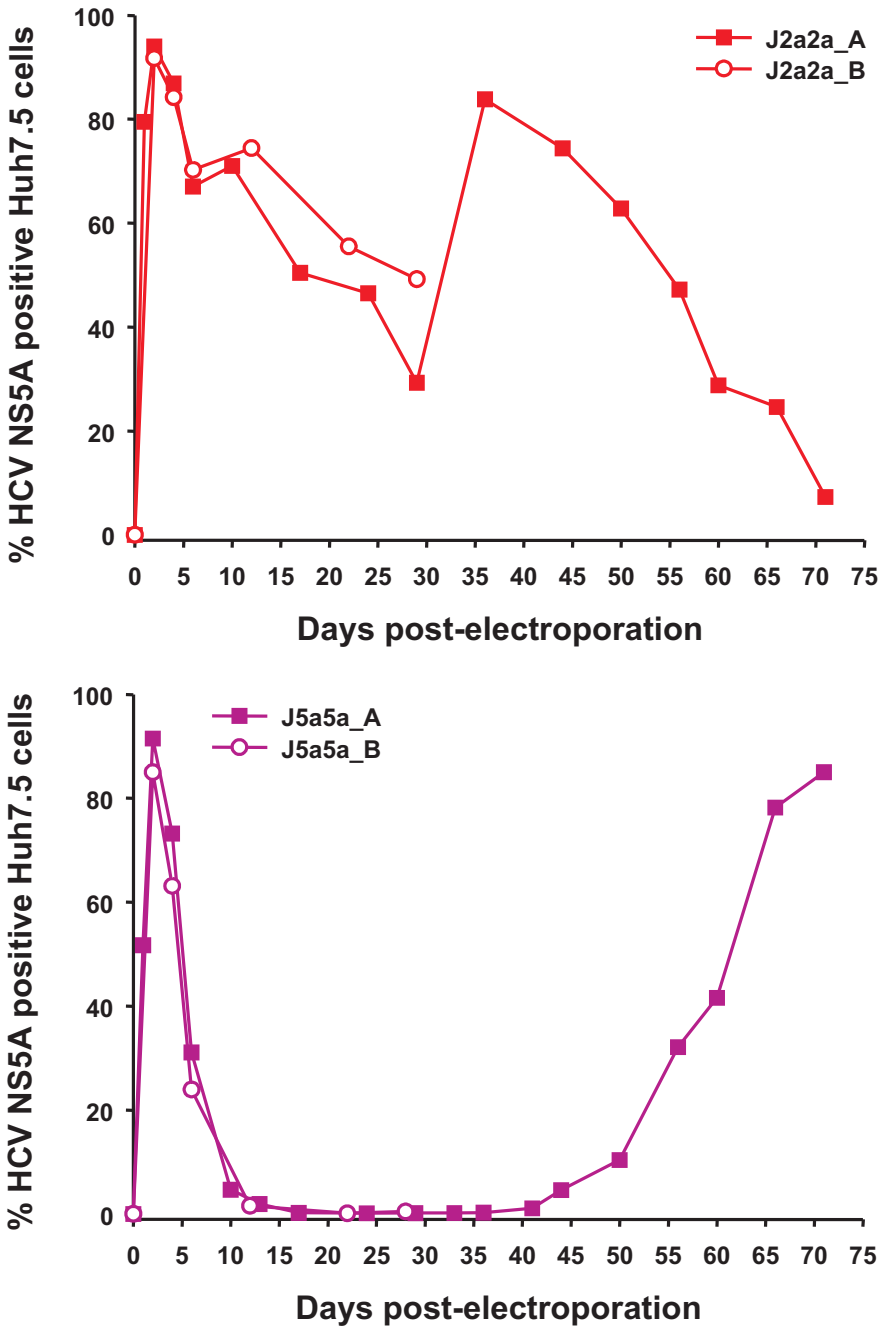

Figure 4.12: ***Recombinants J2a2a and J5a5a and their viability in Huh7.5 cells.*** *Results from 2 independent experiments are presented (Jxx_A, Jxx_B). RNA transcripts from J2a2a (top) and J5a5a (bottom) were electroporated into Huh7.5 cells and replication was assessed by immunostaining against NS5A.*

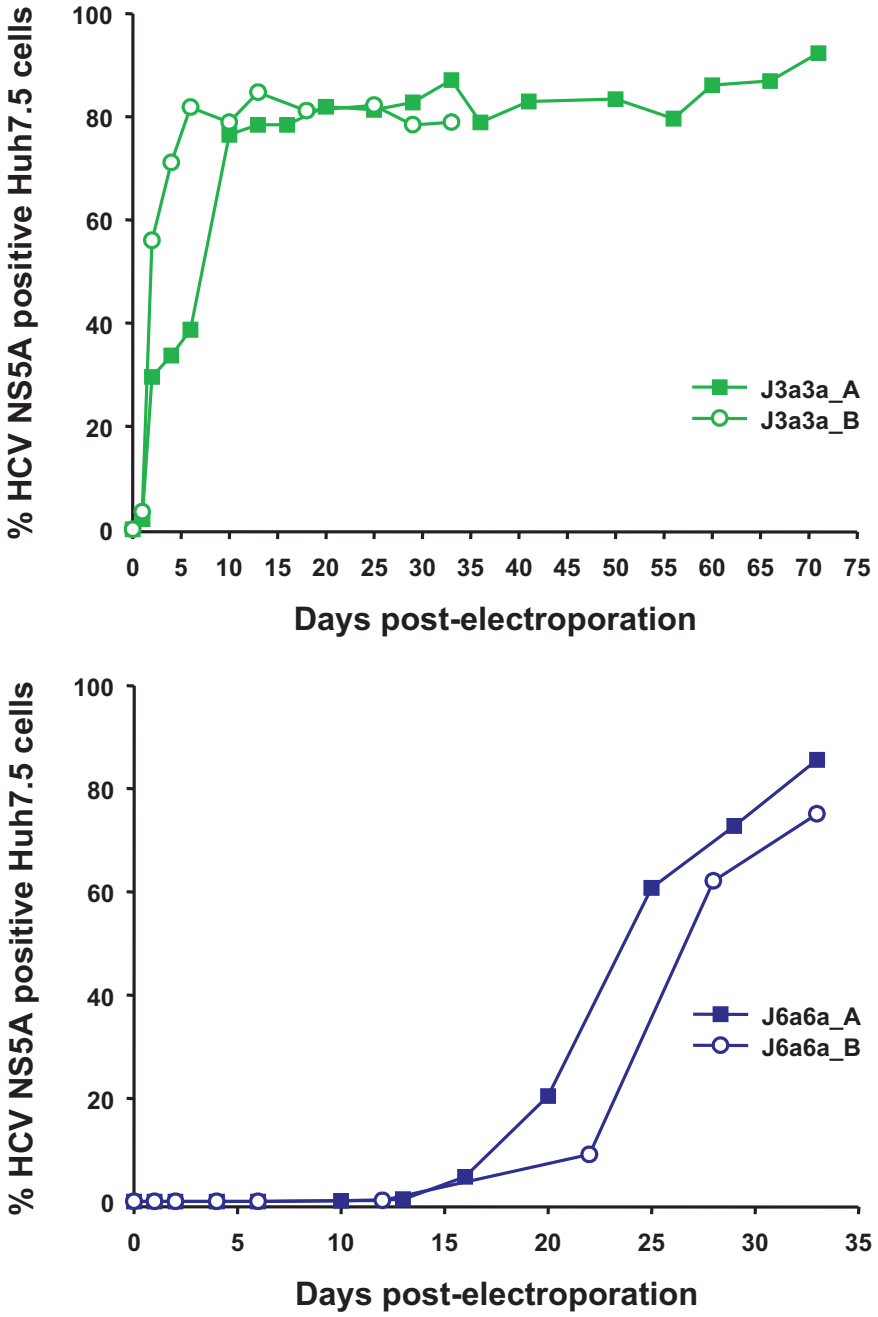

Figure 4.13: ***Recombinants J3a3a and J6a6a and their viability in Huh7.5 cells.*** *Results from 2 independent experiments are presented (Jxx_A, Jxx_B). RNA transcripts from J3a3a (top) and J6a6a (bottom) were electroporated into Huh7.5 cells and replication was assessed by immunostaining against NS5A.*

Investigation of the replication defect of the J1a1a recombinant

To investigate whether the defect in replication in the J1a1a recombinant was due to the genotype 1a NS3 protease, the genotype 1a NS4A cofactor or incompatibilities between these and the genotype 2a backbone, both proteins were replaced with that of genotype 3a. The J1a1a NS3 protease or the NS4A cofactor was replaced with that of genotype 3a, which are compatible with the genotype 2a backbone, to create J1a3a and J3a1a. J1a3a contains the NS3 protease of genotype 1a and the NS4A cofactor of genotype 3a. J3a1a contains the genotype 3a NS3 protease and the NS4A cofactor from genotype 1a (Fig. 4.14). Neither of the 2 recombinants replicated to detectable levels in Huh7.5 cells, indicating that incompatibility issues of NS3 protease or NS4A alone is not responsible for the defect in replication. Replacement of the NS3 protease gene in J1a1a with that of patient-derived protease genes partly rescued the replication defective phenotype of J1a1a, as described in section 4.2.3.

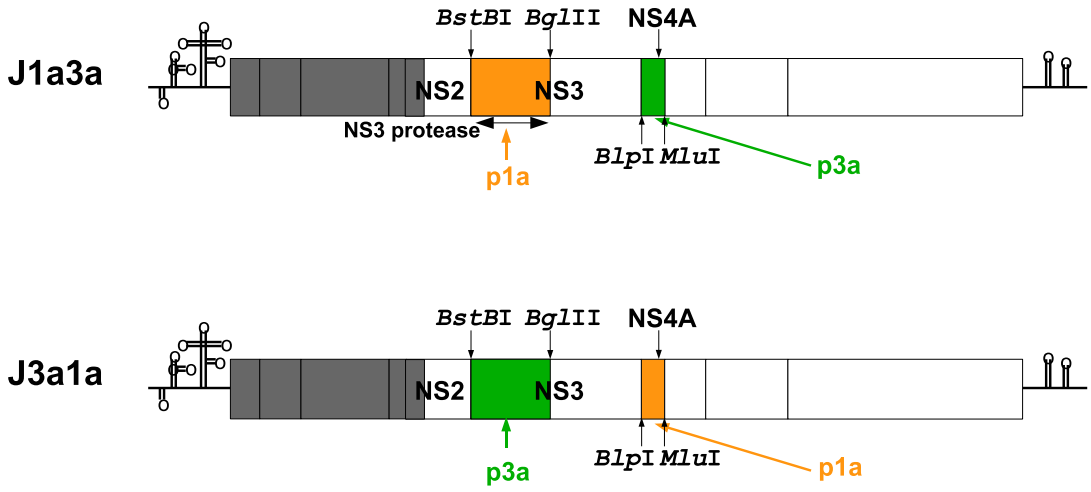

Figure 4.14: ***Genome map of the recombinants J1a3a and J3a1a.*** *Genome map of cDNA clones (pJ6CF, dark grey; pJFH1, white). The NS3 protease gene of JFH1 was replaced with the corresponding intergenotypic gene (replaced region, coloured) using the genetically engineered restriction sites BstBI and BglII. The NS4A gene of JFH1 was replaced with another corresponding intergenotypic gene using the genetically engineered restriction sites BlpI and MluI.*

Role of Huh7.5 cells in recombinant replication kinetics

Huh7.5 cells lose their susceptibility to support HCV replication when they are left to grow to confluency. During passaging of Huh7.5 cells it was therefore made sure they never reached more than 70-80 % confluency. Nevertheless, HCV RNA replicated to lower levels in cells that have been passaged for several weeks before they were transfected compared to fresh cells grown up from liquid nitrogen (Fig. 4.15). A large number of Huh7.5 aliquots was therefore prepared, stored in liquid nitrogen and for each transfection a fresh batch of cells grown up.

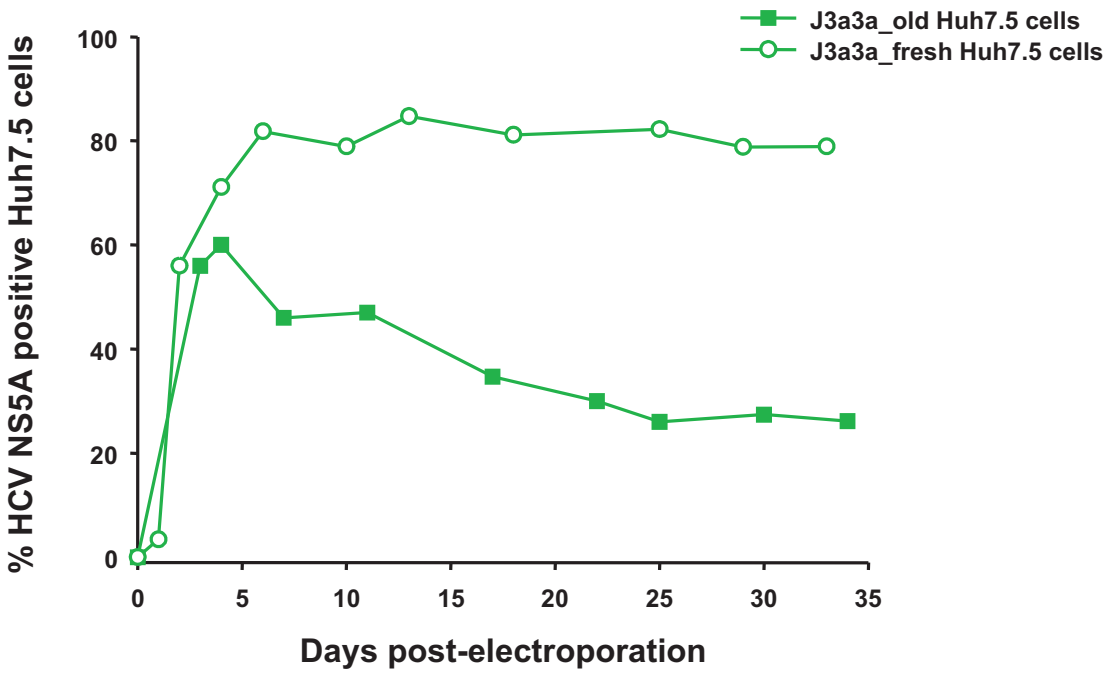

Figure 4.15: ***Passaging of J3a3a in fresh and passaged Huh7.5 cells.*** *J3a3a replicates more efficiently in fresh Huh7.5 cells than Huh7.5 cells that have been passaged several times and have reached high confluency.*

4.2.3 Replication kinetics of J1a1a, J1b1b, J3a3a, J4a4a and J6a6a containing NS3 protease genes derived from HCV-infected patient plasma

This part investigates whether the differing replication kinetics of recombinants constructed from different genotypes were a consistent genotype-associated property or whether it originated from naturally occurring variability between HCV variants within a genotype. For this purpose recombinants from protease genes amplified from multiple patients infected with genotypes 1a, 1b, 3a, 4a or 6a that showed markedly different replication kinetics and abilities to generate infectious virus (Fig. 4.11, 4.12 and 4.13; Table 4.1), were constructed. For each genotype, recombinants were created using PCR-amplified protease sequences from epidemiologically unlinked patients infected with genotypes 1a, 1b, 3a, 4a or 6a, which were then cloned into the corresponding intergenotypic recombinant. From the 32 genotype 3a variants analysed in chapter 3, the protease genes of 4 were used to replace the protease domain of J3a3a. Clones containing amino acid polymorphisms not found in any other sequence in the Los Alamos HCV sequence database, GenBank or the consensus sequences in our dataset (Fig. 3.3) were discarded, ensuring representation of naturally occurring polymorphism, except for J3a3a-6. J3a3a-6 has an Asn at position 1046, which is a polymorphic site with Ser/Gly/Arg. Asn did not occur in the analysed dataset, but Ser1046Asn is a conservative amino acid change and might occur naturally. Clone J3a3a-6 possibly represents a non-dominant viral variant of patient R11354 it was amplified from. Recombinants with patient-derived NS3 protease genes were named Jxx-number of patient. All 4 constructs (J3a3a-3, -6, -8, -11) yielded replicating virus but with different replication kinetics (Fig. 4.16 top). J3a3a-11 (R11276) replication was detected in most cells on day 9, slightly earlier than with J3a3a. J3a3a-8 (R11288) and J3a3a-3 (R11482) spread occurred after an eclipse phase of 28 days and that of J3a3a-6 (R11354) after 37 days.

It was unclear whether the differences in the replication kinetics of the J3a3a recombinants represented different degrees of compatibility between the patient-derived protease sequences and the NS4A cofactor sequence derived from the reference strain or whether there were specific compatibility problems with the genotype 2a backbone

sequence. To investigate this, we compared the NS4A sequences of the prototype (HCV3a-Gla) with those of the 4 subjects (Table C.3, Appendix C). All J3a3a recombinants' NS4A cofactor sequences were identical at the amino acid level, except for a Leu1670Ile amino acid substitution in the J3a3a-11 recombinant. Leu1670 is dominant among genotype 3a variants, although 14 sequences with Val and 3 with Phe were identified among the 180 variants analysed. These observations indicate that reduced compatibility between the NS3 protease and the prototype HCV3a-Gla NS4A is most likely not a determinant for the observed differences in the replication kinetics of the J3a3a recombinants. Differences in replication kinetics between the different J3a3a recombinants are therefore most likely due to the protease itself.

By the same methods, J1a1a recombinants with patient-derived proteases were created. From the 29 genotype 1a variants analysed in chapter 3, the protease genes of 9 were used to replace the protease domain of J1a1a. 7 recombinants replicated to detectable levels, J1a1a-1 (R11188), -2 (R11159), -3 (R11148), -4 (R11143), -5 (R11296), -6 (R11256) and -8 (R11249). J1a1a-7 (R11256) and J1a1a-9 (R11220) did not produce any detectable virus (Fig. 4.18). However, none spread to more than 0.08 % of the cell culture and all cells were NS5A-negative after 30 days. Except for J1a1a-1, all J1a1a recombinants contained naturally occurring amino acid polymorphisms represented among sequences of the 570 variants available from the databases and the 31 variants analysed in chapter 3 (Table C.2, Appendix C). In contrast, J1a1a-1, which was the recombinant showing the best replication kinetics, contained one amino acid substitution (Glu1056Gly) that does not occur in any other variant analysed. To investigate whether the H77*-NS4A cofactor was responsible for the severally impaired replication fitness of the J1a1a recombinants, NS4A amino acid sequences of subjects whose proteases were used in the recombinants, were compared with that of H77*. The membrane segment and the NS3 cofactor region showed amino acid identity (Table C.2, Appendix C). Some polymorphisms occurred in the C-terminal domain of the NS4A cofactor, which though, is not involved in the direct interaction with the NS3 protease (Brass *et al.* (2008)). Differences in replication kinetics between the different J1a1a recombinants are therefore most likely due to the protease itself. Whether the overall poor replication kinetics of the J1a1a recombinants can be attributed to

the reduced enzymatic function of the 1a proteases or their incompatibility with the remaining type 2a sequence remains to be investigated.

The generation of J4a4a patient-derived protease recombinants resulted in a replicating virus in 3 (J4a4a-7, -8, -19) out of 4 (J4a4a-7, -8, -9, -10) recombinant viruses (Fig. 4.17 bottom). A total of 40-50 % of all cells were positive for NS5A 4 days post-electroporation with J4a4a-7 and J4a4a-19 RNA, whereas J4a4a-8 only replicated to very low levels. J4a4a-ED43* (Ala1048 and Ile1064), J4a4a-7 (Thr1039 and Arg1056), J4a4a-10 (Pro1169) and J4a4a-19 (Ile1153) all show amino acid polymorphisms which do not exist in any other genotype 4a variant analysed (n = 39) (Table C.4, Appendix C). Only recombinant J4a4a-8 shows an amino acid polymorphism that also exists within the 4a variants from the database. It is unclear which amino acid polymorphism contributes to the impaired replication phenotype in J4a4a-ED43* and J4a4a-10.

The generation of J1b1b patient-derived protease recombinants resulted in replicating virus for both of the generated recombinants J1b1b-2 and J1b1b-12, with both showing similar replication kinetics to the prototype recombinant J1b1b (Fig. 4.17 top). All amino acid polymorphisms in the 2 recombinants are naturally occurring (Table C.1, Appendix C). The same was true for J6a6a patient-derived protease recombinants (Fig. 4.16 bottom). J6a6a-4 immediately infected 70-80 % of the cell culture, whereas J6a6a-8 took 30 days to spread to the rest of the cell culture, similar to the prototype recombinant J6a6a. All amino acid polymorphisms in the 2 recombinants are naturally occurring (Table C.5, Appendix C).

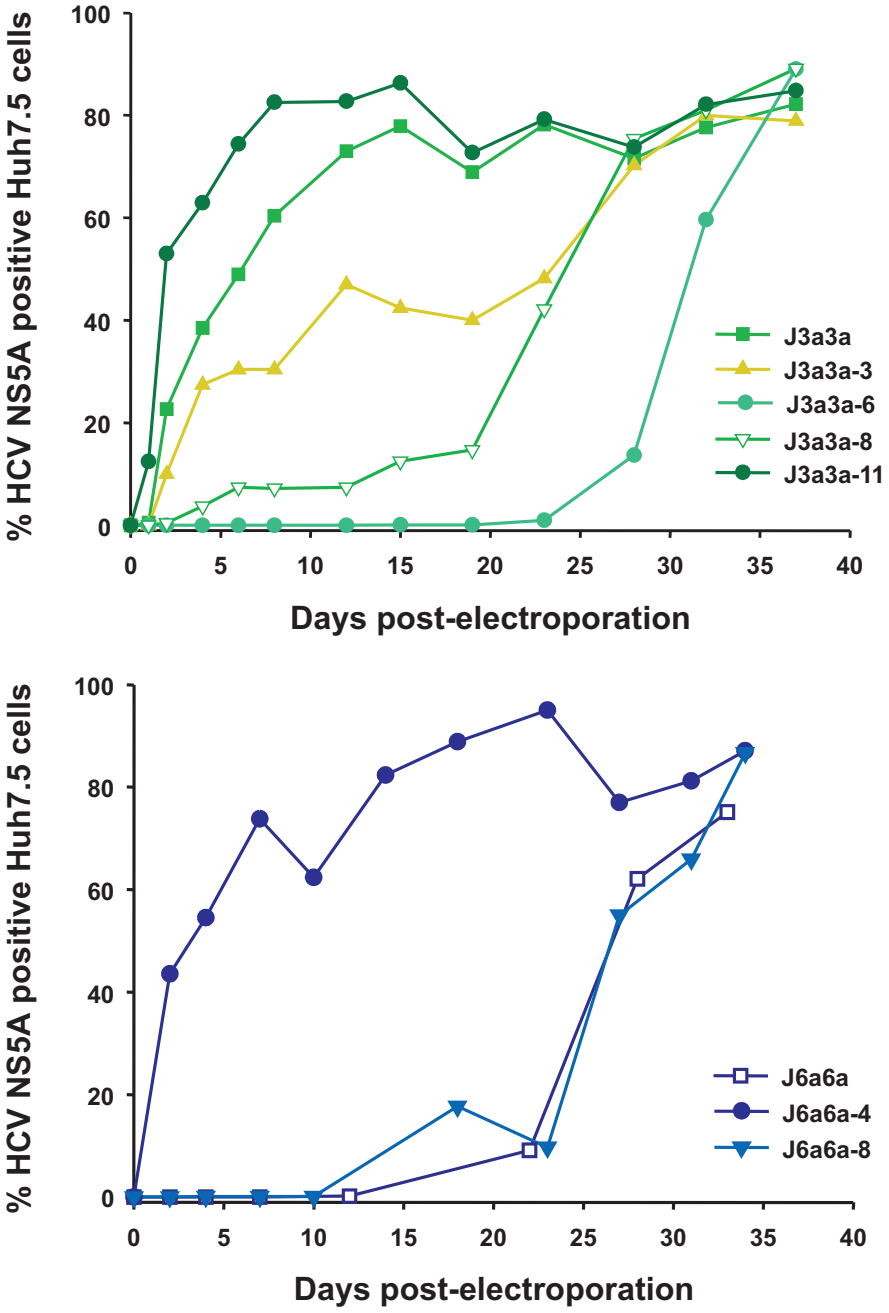

Figure 4.16: ***J3a3a and J6a6a patient-derived protease recombinants and their viability in Huh7.5 cells.*** *The protease region of J3a3a (top) and J6a6a (bottom) was replaced with that of 2 or 4 patient-derived protease genes and their replication capacity assessed in the Huh7.5 cells.*

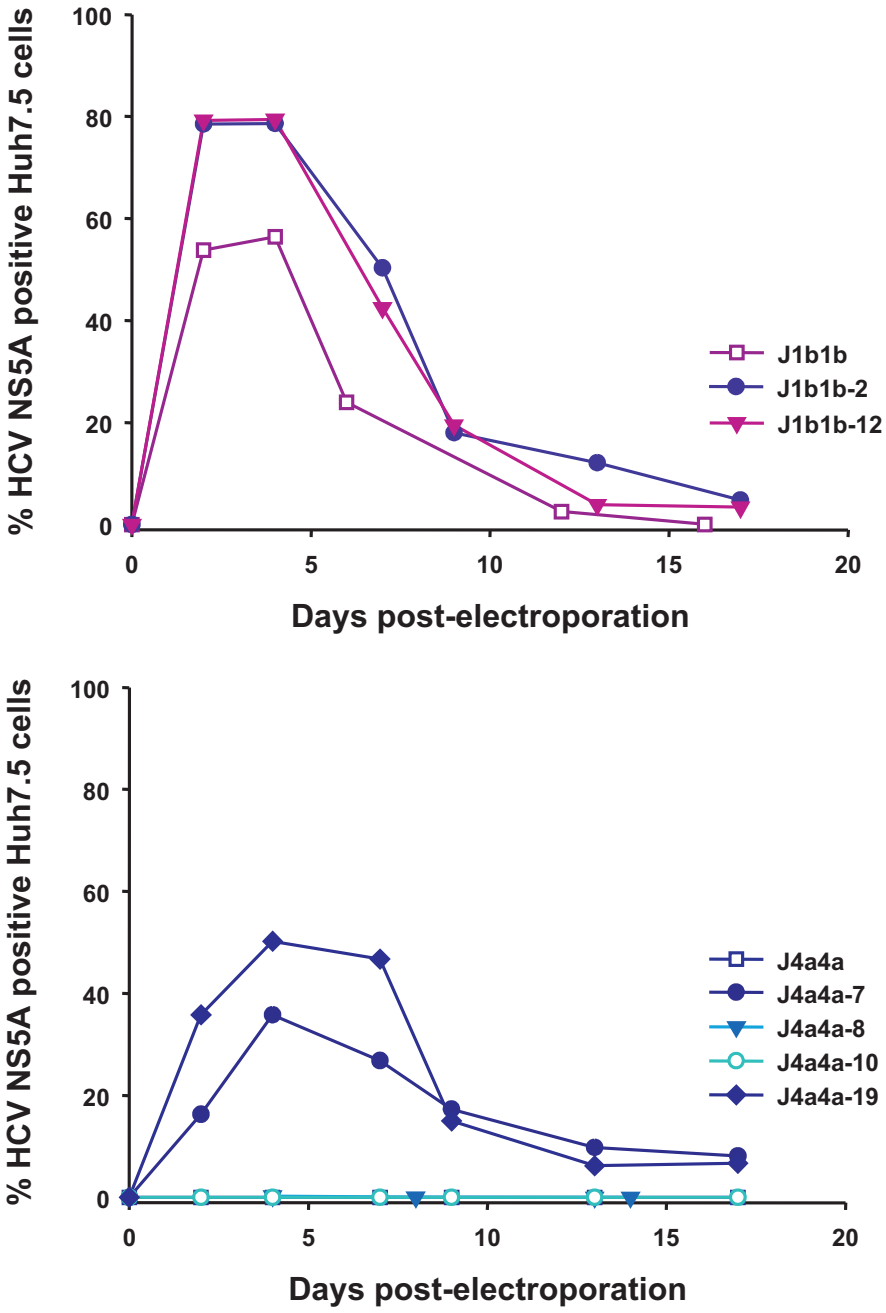

Figure 4.17: ***J1b1b and J4a4a patient-derived protease recombinants and their viability in Huh7.5 cells.*** *The protease region of J1b1b (top) and J4a4a (bottom) was replaced with that of 2 or 4 patient-derived protease genes and their replication capacity assessed in the Huh7.5 cells.*

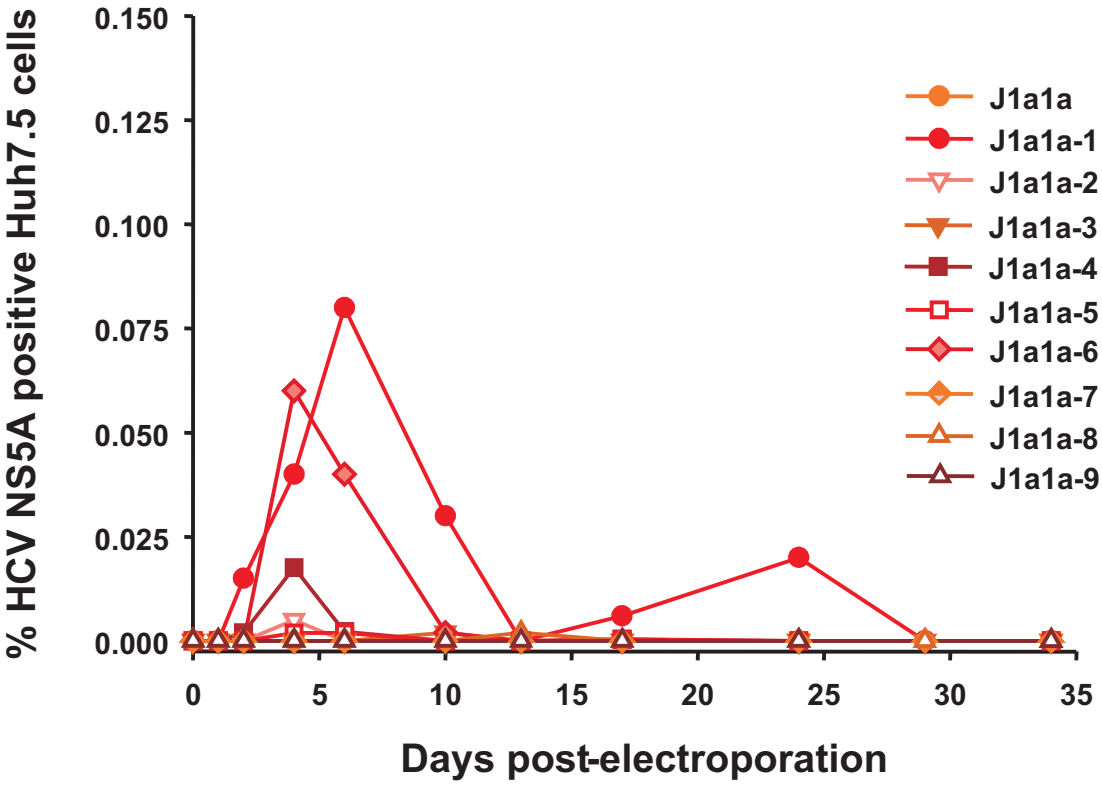

Figure 4.18: ***J1a1a patient-derived protease recombinants and their viability in Huh7.5 cells.*** *The protease region of J1a1a was replaced with that of 9 patient-derived protease genes and their replication capacity assessed in the Huh7.5 cells. The replication levels of the J1a1a recombinants were very low and the scale adjusted accordingly.*

4.2.4 Identification of adaptive and attenuating mutations in recovered Jx and Jxx viruses

J5a, J6a6a and the patient-derived recombinants J3a3a-3, J3a3a-6 and J3a3a-8 only spread in the cell culture after an eclipse phase. To investigate whether the delay in replication reflected a requirement for adaptive mutations to allow efficient replication, the nt sequence from the NS3 protease and NS4A gene regions amplified from cell culture supernatants after the recombinant had spread to 80 % of the cell culture was determined (Table 4.2). The gene region from nt position 2863 to 4178, which included the 3'end of NS2 and the 5'end of the NS3 helicase and nt position 5230 to 5536, including NS4A, were sequenced at peak infectivity of the individual viruses. In the recovered genome of passaged J3a3a-6, mutations were detected within NS2 (A3364G), the NS3 protease (A3478G) and the NS3 helicase domain (A4005T) in all 5 clones analysed. These led to a Gln1008Arg amino acid change in NS2, a Asn1046Ser amino acid change in the NS3 protease domain and a Thr1222Ser amino acid change in the NS3 helicase domain. In the recovered genomes of passaged J3a3a-8 and J3a3a-3, no substitutions were detected except in NS4A, where all 5 clones analysed had the C5328G and T5329C substitutions among others. The resulting Leu1663Arg amino acid substitution only became dominant in the J3a3a recombinant after 43 days passaging in the cell culture.

J5a had 2 substitutions within the NS3 protease domain (A3649G and C3854G), leading to the amino acid substitutions Asn1103Ser and Cys1171Trp. Within the type 2a NS4A, one nt substitution (G5430A) led to the amino acid change Asp1679Asn. The J6a6a recombinant virus had 2 substitutions (A3558G and G3439T), resulting in a Val1040Leu amino acid change within the N-terminal part of the NS3 protease. This amino acid is highly conserved among all genotypes, only allowing Leu, Val or Ile (Brass *et al.* (2008)). Both J2a2a and J5a5a replicated to high levels in cell culture, accompanied by increased cell death. After an initial clearance of the virus, both spread in cell culture again, indicating the acquisition of attenuating mutations (Fig. 4.12). A C3538G nt change within the protease domain of J2a2a leads to a Thr1066Ser amino acid change. Within J5a5a, 3 nt changes (C3416G, T3968C and A4081T), leading to

a Gln1247Leu amino acid change, were identified (Table 4.2).

Table 4.2: ***Mutations of Jx and Jxx recombinants during passaging in Huh7.5 cells.*** *Nt positions are numbered according to the H77 (AF009606) reference position.*

Viral variant	NS2	NS3 protease	NS3 helicase	NS4A
J5a		A3649G + C3854G		G5430A
J2a2a		C3538G		
J3a3a				C5328G + T5329C
J3a3a-3				
Clone 1,4,5				C5328G + T5329C
Clone 2				C5328G + T5329C + T5365C
Clone 3				C5328G + T5329C + T5447C
Clone 6				C5328G + T5329C + T5358C
J3a3a-6				
Clone 1-4	A3364G	A3478G	A4005T	
Clone 5	A3364G	A3478G	A4005T + A3362G	
J3a3a-8				
Clone 1,3,5				C5328G + T5329C
Clone 2				C5328G + T5329C + T5389C + A5434G
Clone 4				C5328G + T5329C + A5472G
J5a5a		C3416G	T3968C + A4081T	
J6a6a		A3458G + G3459T		

4.2.5 Recombinant adapted/attenuated viruses efficiently infect Huh7.5 cells

To identify whether the substitutions occurring in NS4A accounted for the differences in replication kinetics of the J3a3a-3 and -8 recombinant viruses, mutations were introduced into the original plasmids and their viability tested in Huh7.5 cells. Both J3a3a-3$_{\text{-L1663A}}$ and J3a3a-8$_{\text{-L1663A}}$ spread directly within the cell culture and after 11 and 17 days, respectively, almost all cells were infected, with replication kinetics similar to that of J3a3a and J3a3a-11 (Fig. 4.19). The Leu1663Ala amino acid change at the 5'end of NS4A was therefore sufficient to restore efficient replication. In contrast, J3a3a-6$_{\text{-Q1008R,N1046S,T1222S}}$ infected 4 % of Huh7.5 cells after only 3 days compared to the original J3a3a-6, which took 20 days before any Huh7.5-positive cells could be detected (Fig. 4.20 top). The mutant failed to subsequently spread to the rest of the cell culture, indicating the likely presence of mutations outside of the sequenced NS3 protease and NS4A gene region in the original adapted clone.

Reintroducing the Val1040Leu amino acid change into J6a6a generated a recombinant (J6a6a$_{\text{-V1040L}}$) which showed improved replication kinetics as well (Fig. 4.20 bottom). At 2 days post-electroporation, J6a6a$_{\text{-V1040L}}$ replicated in 80 % of the cell culture and continued to infect most of the cell culture 14 days post-electroporation. J2a2a and J5a5a both replicated to high levels but replication was accompanied by increased cell death. Reintroducing the identified mutations (Table 4.2) generated 2 attenuated viruses, J2a2a$_{\text{-T1066S}}$ and J5a5a$_{\text{-Q1247L}}$ (Fig. 4.21). Both J2a2a$_{\text{-T1066S}}$ and J5a5a$_{\text{-Q1247L}}$ were immediately able to infect about 80 % of the cell culture and continued to infect the majority of cells 14 days post-electroporation. Decreased cell death was observed as well.

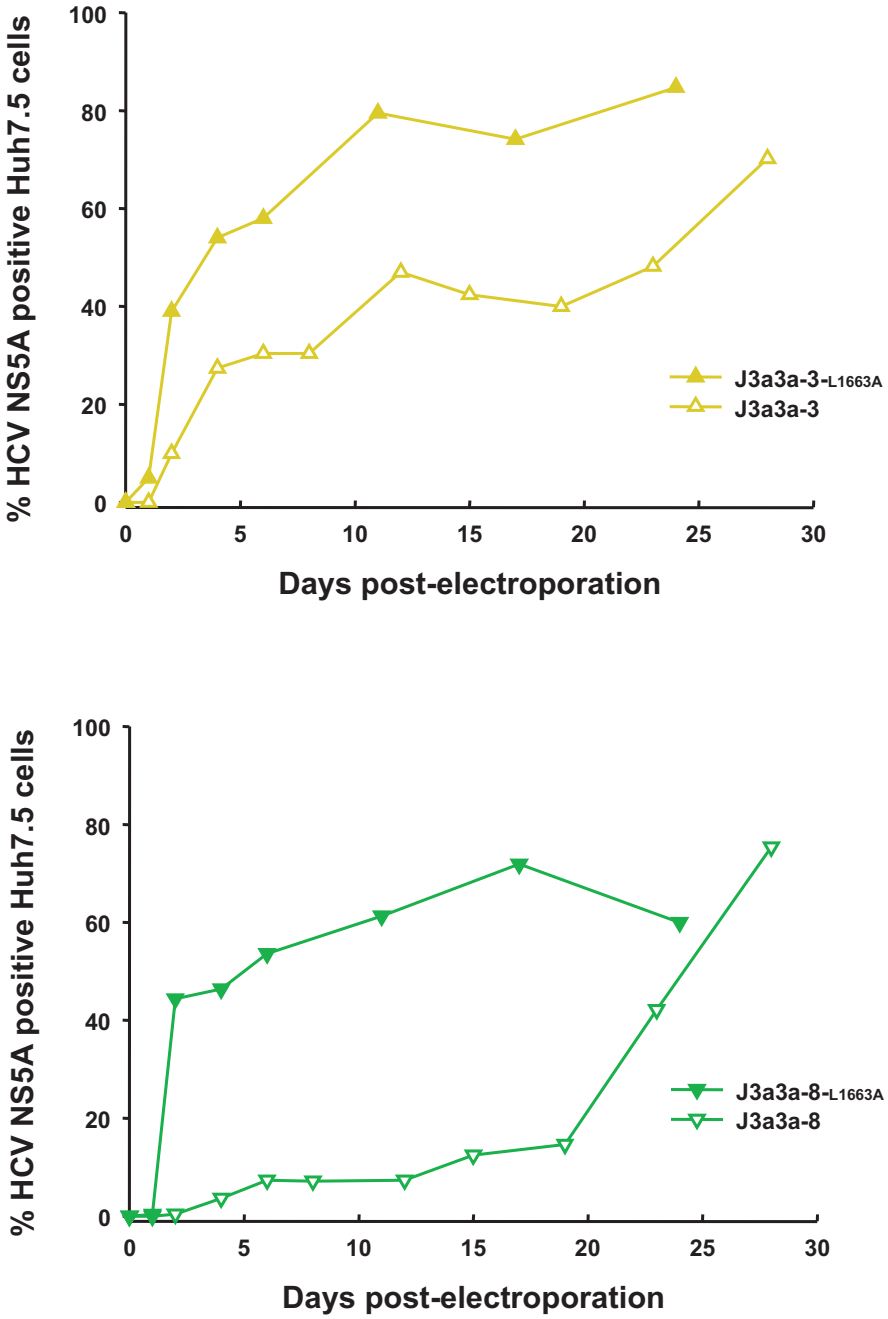

Figure 4.19: ***Recombinants J3a3a-3 and J3a3a-8 including adaptive mutations and their viability in Huh7.5 cells.*** *Viability of J3a3a-3$_{-L1663A}$ and J3a3a-8$_{-L1663A}$ were assessed by anti-NS5A immunostaining.*

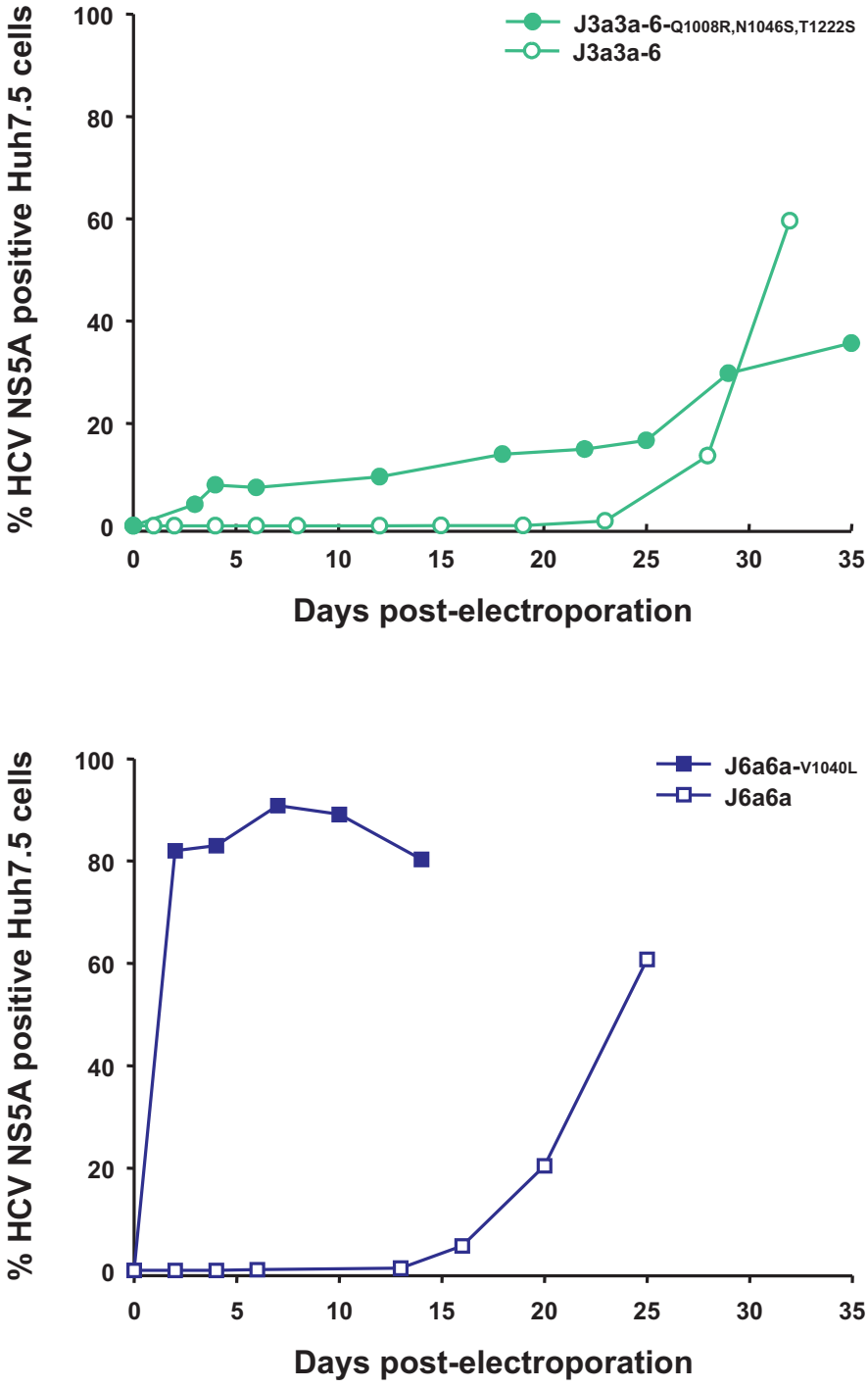

Figure 4.20: ***Recombinants J3a3a-6 and J6a6a including adaptive mutations and their viability in Huh7.5 cells.*** *Viability of J3a3a-6*$_{-Q1008R,N1046S,T1222S}$ *and J6a6a*$_{-V1040L}$ *were assessed by anti-NS5A immunostaining.*

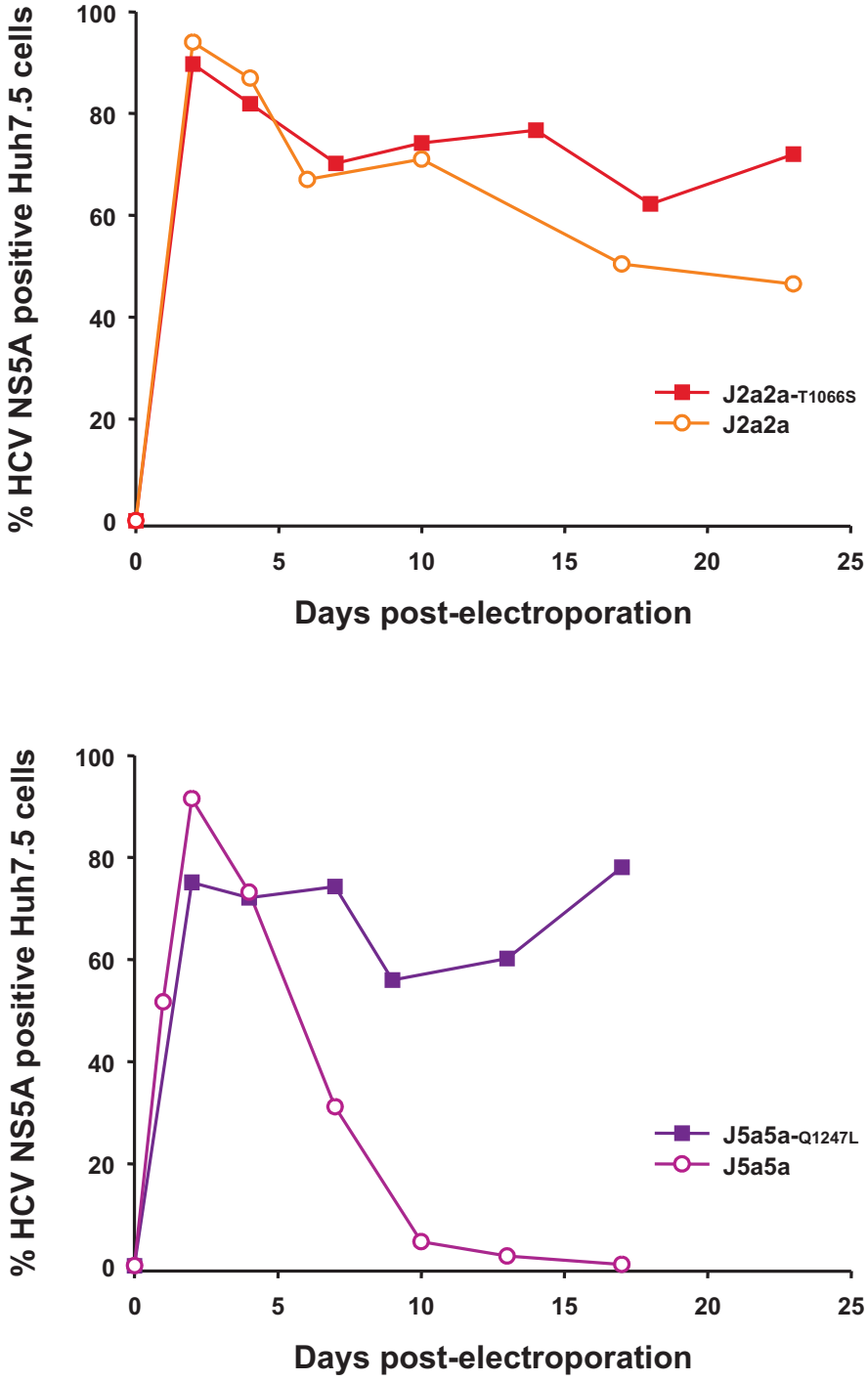

Figure 4.21: ***Recombinants J2a2a and J5a5a including attenuating mutations and their viability in Huh7.5 cells.*** *Viability of J2a2a$_{\text{-T1066S}}$ and J5a5a$_{\text{-Q1247L}}$ were assessed by anti-NS5A immunostaining.*

Genetic stability of J3a3a

To investigate whether recombinant virus secreted into the supernatant of the cell culture infects naïve cells, $TCID_{50}$ measurements were performed (Table 4.1). Using the J3a3a recombinant it was also investigated how this infectious viral recombinant spreads within a cell culture of naïve Huh7.5 cells. J3a3a cell culture derived supernatant was passed through a 0.2 μm filter to remove all cells, diluted 1 in 5 and used to infect naïve Huh7.5 cells. Supernatants collected from the J3a3a passaging experiment (Fig. 4.13 top, J3a3a_A) on day 15 and day 30 were compared (Fig. 4.22). Day 30 supernatant spread faster than day 15 supernatant, indicating that the infectivity of cell culture supernatant has increased during the initial cell culture passaging experiment. After 24 days of passaging, the entire genome of day 30 supernatant recovered virus was sequenced. Besides the before described cell culture adaptive mutation (Leu1663Ala), no additional mutations were identified in the consensus sequence, indicating stable replication of J3a3a in Huh7.5 cells.

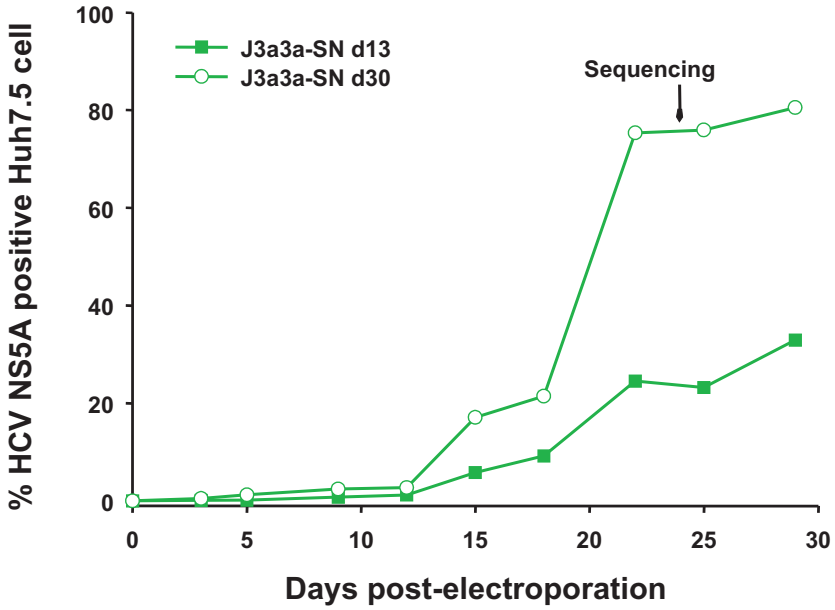

Figure 4.22: ***Passaging of J3a3a supernatant (SN) in naïve Huh7.5 cells.*** *Supernatant collected on day 13 and day 30 from the J3a3a passaging experiment (Fig. 4.13 top, J3a3a_A) was used to infect naïve Huh7.5 cells and spread within the cell culture followed by anti-NS5A immunostaining. The arrow indicates the day of supernatant collection for whole genome sequencing.*

4.2.6 Summary of results

A panel of intra- and intergenotypic recombinants based on the full-length replication-competent clone Jc1 were constructed and their replication assessed in the Huh7.5 cell culture system. Using naturally occurring restriction sites to replace a fragment including the NS3 protease plus parts of NS2 and the NS3 helicase with that of Jc1, resulted in replication defective recombinants. The introduction of unique restriction sites on the boundaries of the NS3 protease led to a replication competent intergenotypic recombinant for genotype 5a. Including the NS4A cofactor gene in the recombinant construction rescued the intergenotypic recombinants of genotype 1b, 3a and 6a. For genotype 1a and 4a including the homologous NS4A was not sufficient to restore replication. However, additional replacement of the NS3 protease with that of patient-derived ones improved replication kinetics to detectable levels. Intra- and intergenotypic recombinants varied widely in their replication kinetics. Some required adaptive, others attenuating cell culture mutations. Reintroducing these into the original viruses resulted in stably replicating intra- and intergenotypic recombinants. The generated intergenotypic recombinants provide an effective cell culture system for the assessment of antiviral susceptibilities and resistance development of NS3 protease genes for the 6 major genotypes of HCV.

4.3 Discussion

The genetic variability of HCV proteins of different genotypes influences the molecular structure of protease and polymerase enzymatic sites and potentially limits the effectiveness of antiviral therapy targeting viral replication proteins (Holland-Staley *et al.* (2002)). As discussed in section 1.8.1, the PI BILN 2061 shows a nearly 2 log weaker binding affinity to genotype 2 and 3 proteases than to type 1 proteases (Thibeault *et al.* (2004)), a difference that translates to much weaker antiviral efficacy of BILN 2061 among patients with HCV genotype 2 or 3 infection (Reiser *et al.* (2005)) than those with genotype 1 (Lamarre *et al.* (2003)). The reduced effectiveness of antiviral drugs on certain genotypes also potentially facilitates the development of resistance muta-

tions. Another PI, VX-950, showed similar efficacy in genotype 1a, 1b and 2a, but lower efficacy in genotype 3a (Perni *et al.* (2006); Reesink *et al.* (2006)).

Current drug discovery and optimisation is mostly dependent on laboratory-optimised standard replicons. However, the standard replicon system only allows the evaluation of a limited number of laboratory strains that do not reflect the great genetic inter- and intragenotypic diversity of HCV. Different replicon-based vector approaches have to date been limited to the investigation of the activity of compounds against different HCV variants from genotype 1 to 4 (Binder *et al.* (2007); Qi *et al.* (2009)). However, the replicon based system does not include structural genes and therefore does not represent the full viral life cycle of HCV. This chapter describes the development of a full-length HCV cell culture system, allowing the study of the NS3 protease of the 6 major genotypes. Recombinant viruses were constructed from Jc1, an intragenotypic genotype 2a recombinant, which replicates efficiently in Huh7.5 cells (Pietschmann *et al.* (2006)). The resulting recombinant viruses encode the NS3 protease or the NS3 protease and NS4A gene from genotype 1a, 1b, 2a, 3a, 4a, 5a or 6a prototype strains or different clinical variants, respectively.

At the beginning only Huh7 cells and pJFH1, the first full-length HCV RNA to replicate efficiently in Huh7 cells, were available and recombinants were constructed with pJFH1 as a backbone (Wakita *et al.* (2005)). Initially, naturally occurring restriction sites were used to swap a fragment of JFH1 including the NS3 protease with the equivalent from genotype 1a or 3a prototype plasmids. The fragment also contained part of the NS2 protein and the NS3 helicase domain, resulting in intergenotypic NS2 and NS3 helicase proteins. The replication kinetics of the resulting recombinants, F_n1a_n and F_n3a_n, were assessed in the Huh7 cell culture system by immunostaining for the non-structural protein NS5A. Neither of the recombinants produced detectable virus, suggesting that the recombinants were either replication incompetent or the replication fitness was too low to be detected by immunostaining. The generated intergenotypic NS2 and NS3 helicase proteins are possibly not functional. The 3D structure of a protein is important for its function and changing amino acids within a protein will most likely alter its 3D structure and thereby affect the function. The replication defect of

the recombinants might also be due to incompatibilities between the genotype 1a/3a proteins and the genotype 2a backbone. The genotype 2a sequence of the NS3 protease differs considerably to that of genotype 1a and 3a (Fig. 4.23). Furthermore, the NS3 protease closely interacts with its cofactor NS4A and genotype 1a/3a proteases might work less efficiently with a genotype 2a NS4A cofactor, as discussed below.

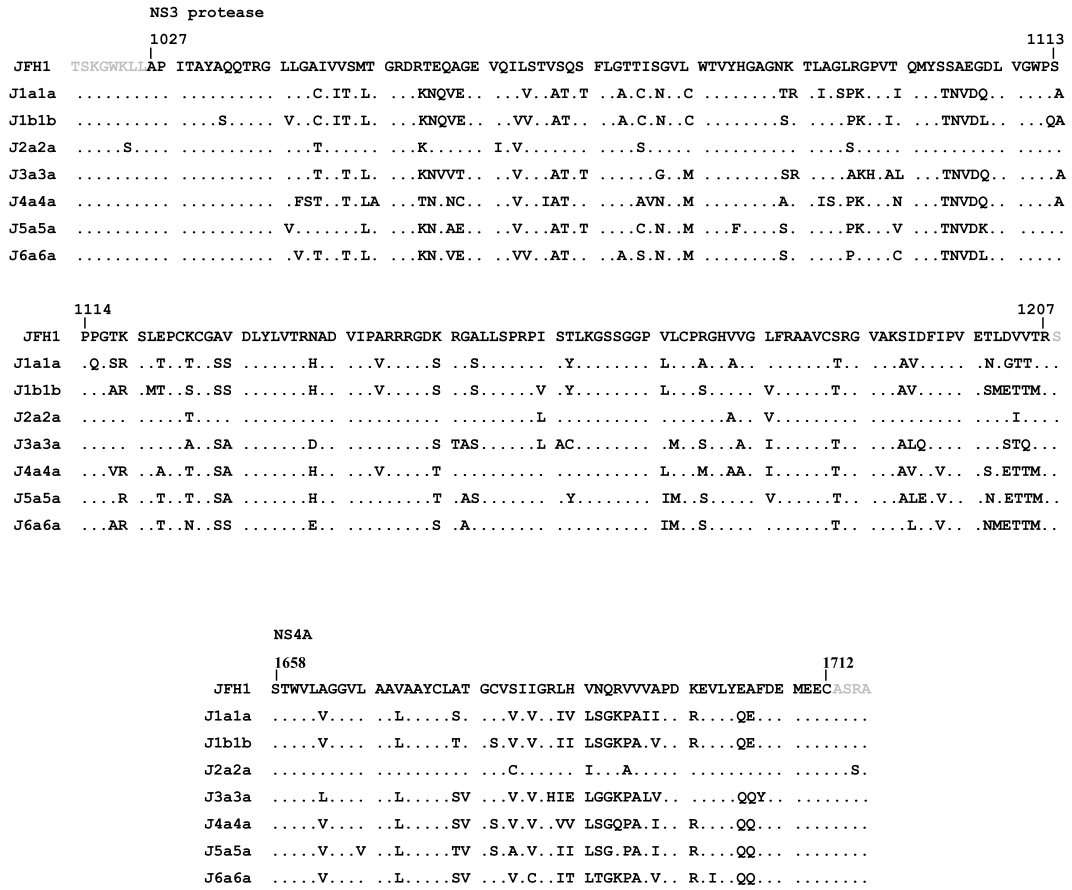

Figure 4.23: ***Comparison of genotype 1a, 1b, 2a, 3a, 4a, 5a and 6a NS3 protease and NS4A cofactor sequences.*** *Top, alignment of NS3 protease residues (in black) from recombinants J1a1a, J1b1b, J2a2a, J3a3a, J4a4a, J5a5a and J6a6a to JFH1 (NCBI accession number AB047639). Bottom, alignment of NS4A protease cofactor residues (in black) from recombinants J1a1a, J1b1b, J2a2a, J3a3a, J4a4a, J5a5a and J6a6a to JFH1. Dots indicate amino acid sequence identity.*

Because recombinants based on the JFH1 clone were not replicating to detectable levels in the Huh7 cell culture system, further recombinants were constructed based on the Jc1 backbone. Jc1 is an intragenotypic genotype 2a recombinant, which replicates efficiently in Huh7.5 cells and yields infectious titres 100 to 1,000-fold higher than JFH1 (Pietschmann *et al.* (2006)). Furthermore, Huh7 cells were replaced with the far more permissive Huh7 subclone Huh7.5, in which HCV variants replicate to higher levels (Blight *et al.* (2002); Sumpter *et al.* (2005)).

To reduce a possible influence of intergenotypic NS2 and NS3 helicase proteins on recombinant viability, 2 unique restriction sites were introduced at the boundaries of the NS3 protease. This allowed the construction of intra- and intergenotypic recombinants including the NS3 protease alone from another genotype. However, the attempt to replace the NS3 protease alone was not successful in creating replication competent intergenotypic viruses, except for genotype 5a (J5a) (Fig. 4.7), which only spread within the cell culture after acquiring adaptive mutations (Table 4.2). Two adaptive mutations were identified within the NS3 protease (Asn1103Ser and Cys1171Thr) and one within NS4A (Asp1697Asn). Asn1103 is the dominant amino acid within all genotypes except those of type 2a, where Ser is dominant. This suggests that the Asn1103Ser amino acid change towards type 2a consensus sequence is a rescue for the incompatibility between the type 5a NS3 protease and the remaining type 2a sequence. Cys1171 is part of the zinc binding site Cys1123, Cys1125, Cys1171 and His1175, playing a structural role (De Francesco *et al.* (1996); Kim *et al.* (1996)). It has been reported that these 3 Cys are necessary within NS3 (Tedbury & Harris (2007)), though one genotype 1b variant with Trp1171 and one genotype 3a variant with Tyr1171 can be found among GenBank variants. It cannot be excluded that those arose due to Taq errors or represent non-viable variants, but as the consensus sequence of a viable variant was sequenced here, it is unlikely that Cys1171Thr is a Taq error. As Thr is bulkier and bigger than Cys, this amino acid change might result in changes in the 3D structure of the protease to better interact with the remaining type 2a proteins. Asp1697Asn is part of the C-terminal acidic domain of NS4A, which has been shown to fold into an α-helix in a pH-dependent manner. Using Ala substitutions it has been demonstrated that Asp1697, among other residues within the C-terminus, is crucial in RNA repli-

cation (Lindenbach *et al.* (2007)). Also, Asp1697 is dominant among all genotypes, supporting a crucial role of Asp at this site. However, Asn1697 is tolerated as well, as some genotype 1 sequences within GenBank have an Asn1697. The Asp1697Asn amino acid change might therefore play a structural role as well. Introducing the individual adaptive mutations into the original J5a recombinant would be necessary to get conclusive results on the influence of the individual mutations on the replicative fitness of J5a.

Jc1 including the 2 unique restriction sites (Jc1-BB) and the intragenotypic recombinant J2a replicated similarly to the parental strain (Fig. 4.5 and 4.7), indicating that the introduction of new restriction sites and fragment swapping has not a major affect on the replication ability of Jc1. Since the NS3 protease forms a stable complex with NS4A (Bartenschlager *et al.* (1995)) and is required for its *trans* cleavage activity and membrane anchoring (Failla *et al.* (1994, 1995); Tanji *et al.* (1995)), it was reasoned that the poor replication ability of most Jx constructs may have originated through incompatibilities between these 2 protease components. Due to their close interaction and the substantial sequence variability in the NS4A contact zone of genotype 2a and the other genotypes (Fig. 4.23), it might be expected that cofactors of different genotypes might attenuate protease activity. However, it has been shown that the NS3 protease domain can functionally cross-interact with the NS4A cofactor from another genotype (Franco *et al.* (2008); Wright-Minogue *et al.* (2000)) and NS3 protease genes from different genotypes cloned into 1b/2a reference strain replicons do produce replication competent replicons. Attempts by other groups to include full-length NS3/4A genes of other genotypes, including the helicase, in the 1b or 2a chimaeric replicon system generally failed to create efficiently replicating replicons, although in the latter case this may reflect a further compatibility restriction for the helicase to be of the same genotype as the RNA polymerase encoded by NS5B (Binder *et al.* (2007); Qi *et al.* (2009)).

The NS4A of genotype 2 has been shown to be much less efficient in heterologous combinations than those of other genotypes (Wright-Minogue *et al.* (2000)), which might explain why most of our recombinant viruses containing the type 2a cofactor

did not replicate efficiently enough for detection. NS4A of genotype 1a, 1b, 3a, 4a, 5a and 6a are more similar to each other on the amino acid sequence level than they are to genotype 2a (Fig. 4.23). Why though the genotype 5a protease forms a viable complex with the 2a backbone and the others do not remains unclear.

To resolve this compatibility issue, the corresponding homologous genotype specific NS4A cofactor was included in the construction of the recombinant viruses (Fig. 4.8 and 4.9). This led to viable recombinants for genotypes 1b, 2a, 3a, 5a and 6a (Fig. 4.11, 4.12 and 4.13). Preliminary experiments in Huh7 cells, comparing the JFH1 based recombinant F3a3a with the Jc1 based recombinant J3a3a, showed as expected that recombinants based on the Jc1 backbone replicated more efficiently (Fig. 4.10). J1a1a and J4a4a were impaired in their replication, but replacement of the NS3 protease gene in J4a4a with that of patients allowed the generation of replication competent 4a recombinants (Fig. 4.11 bottom and 4.17 bottom). As expected, J2a2a replicated most efficiently and immediately spread within the cell culture (Fig. 4.12 top) and J5a5a also spread to most cells within 2 days (Fig. 4.12 bottom), both comparable with the parental Jc1. Similarly, the spread of Jc1 and these 2 recombinant viruses was also accompanied by increased cell death, followed by proliferation of HCV-NS5A negative Huh7.5 cells, as described in previous studies (Zhong *et al.* (2006); Gottwein *et al.* (2007); Mateu *et al.* (2008); Scheel *et al.* (2008)). Reintroduction of attenuating mutations into J2a2a and J5a5a (Table 4.2) allowed the generation of 2 recombinants which immediately and continuously infected the cell culture and were associated with decreased cell death (Fig. 4.21). The Thr1066Ser amino acid change within the NS3 protease of J2a2a is a change towards the JFH1 sequence, which has Ser1066. Thr1066 is dominant among genotype 2 sequences, but Ser1066 occurs as well and is therefore naturally occurring. The attenuating effect of Thr1066Ser on J2a2a is in accordance with the lower replication kinetics of JFH1 compared to Jc1. For J5a5a an attenuating amino acid change was found within the NS3 helicase domain, Gln1247Leu. Gln1247 is dominant across genotypes, indicating a crucial role of Gln at this position. A non-conservative amino acid change away from the consensus sequence of HCV explains the attenuating effect of Gln1247Leu.

In contrast, the observed clearance of J1b1b and J4a4a-19 after transfection is the likely consequence of their failure to generate any infectious virus (Fig. 4.11). Unlike the other recombinant viruses, where spread is reduced after an eclipse phase, J3a3a continuously infected about 80 % of the cell culture, with high frequencies of infected cells even after 70 days of passaging in cell culture (Fig. 4.13 top). As it has been shown that the catalytic efficiencies of genotype 1a, 3a and 4a proteases are similar (Franco *et al.* (2008)), the inability of genotype 1a and some genotype 4a recombinants to replicate efficiently likely is a consequence of the incompatibility between the NS3 protease/NS4A and the remaining type 2a sequence.

J1a1a and J4a4a both differ from the prototype sequences H77 and ED43 by one (H77-Met1205Thr) and 4 (ED43-Thr1048Ala, Thr1064Ile, Ile1160Thr and Arg1176Ala) amino acids, respectively, in the protease gene, whereas both are homologous to H77 and ED43, respectively, in the NS4A gene (Table C.2 and C.4, Appendix C). Met1205 is predominant among genotype 1a variants, although one of the 570 available sequences of other genotype 1a variants contained a Thr residue at this site, one a Val and one an Ile. Four of 459 genotype 1b variants in the HCV database contained the Thr residue at this position as well, largely discounting this substitution as a cause of the poor replication ability of the J1a1a clone. In contrast, the Thr1048Ala mutation in genotype 4a is, with one exception in a genotype 3a variant (Ala1048), absent among protease gene sequences of all genotypes including all available genotype 4a sequences (n = 39). The presence of an Ala at this site is therefore certainly consistent with the poor/impaired replication of J4a4a. The same is true for Thr1064, which is, with the exception of one variant, conserved among the analysed variants of genotype 1a, 1b, 3a, 4a and 6a. However, in genotype 2 variants a Thr/Ser polymorphism can be found at this position, suggesting that mutation at this site is tolerated. Nevertheless, Thr1064Ser is a conservative amino acid change, whereas Thr1064Ile is non-conservative and possibly affecting the fitness of J4a4a. Thr1160 and Ala1176 are the predominant amino acids at these positions within the genotype 4a variants analysed, making these substitutions unlikely candidates for the poor replication kinetics of J4a4a.

The J6a6a recombinant only spread within the cell culture after an eclipse phase, indicating adaptive mutations (Fig. 4.13 bottom). The identified mutation within the NS3 protease (Val1040Leu) is a change at a position with a highly conserved Leu/Val/Phe/Met polymorphism and reintroducing it into the original recombinant rescued its impaired replication kinetic (Fig. 4.20 bottom). Val1040 is part of a highly conserved NS3 amino acid segment (residue 1036-1050) that forms an α-helix. The hydrophobic residues at positions 1039, 1040, 1043, 1044 and 1047 are conserved among all HCV genotypes and form the very strong hydrophobic side of the helix on the protein surface (Brass *et al.* (2008)). Val1040Leu is a conservative amino acid change maintaining the hydrophobic character, but changing towards the more dominant amino acid at a polymorphic site, explaining the big impact Val1040Leu has on the replication fitness of J6a6a.

Even though the NS3 protease is considered one of the more conserved proteins encoded by the HCV genome, different genotypes do show substantial amino acid sequence variability that potentially influences its structure and function (Holland-Staley *et al.* (2002); Lodrini *et al.* (2003); Vallet *et al.* (2005); Winters *et al.* (2006)). Furthermore, it has been demonstrated that catalytic efficiencies within a subtype can vary widely, especially within genotype 1b, whereas genotype 3a proteases showed the most homogenous range of activities (Franco *et al.* (2008)). Cloning of patient-derived NS3 protease genes of genotype 1 into the 1b replicon construct has shown 2- to 7-fold differences in the replication capacities of the constructs (Qi *et al.* (2009)). To investigate the influence of these intrasubtype sequence differences on recombinant viability, NS3 protease gene sequences from study patient plasma samples were directly amplified and cloned into the corresponding J1a1a, J1b1b, J3a3a, J4a4a and J6a6a recombinant viruses.

All 4 J3a3a recombinants constructed from the original J3a3a and patient-derived protease genes generated viable recombinants (Fig. 4.16 top). The 2 recombinants J3a3a-3 and -8, which showed diminished replication kinetics, could be rescued by introducing an adaptive Leu1663Ala amino acid change within the membrane segment of NS4A (Fig. 4.19). The Leu1663Ala amino acid change presumably is crucial for efficient and

continuous spread of the J3a3a recombinants as it was observed within the J3a3a recombinant as well after 43 days in cell culture (Table 4.2). Furthermore, Leu1663Ala is an amino acid change towards the consensus genotype 2a sequence, suggesting adaptation to the Jc1 backbone sequence. Position 1663 is part of the membrane segment and the amino acid repertoire of 26 reference positions is Val/Ala/Leu, with Val being the most frequent one (Brass *et al.* (2008)). The Leu1663Ala is therefore an amino acid change towards the more frequently occurring amino acid, explaining its positive effect on the replicative fitness of J3a3a-3 and -8. Passaging J3a3a supernatant in naïve Huh7.5 cells did not reveal any further mutations, indicating that J3a3a stably replicates in Huh7.5 cells. Three adaptive mutations were identified in virus recovered from the J3a3a-6 cell culture. Gln1008Arg (NS2 protein) and Thr1222Ser (NS3 helicase domain) are both amino acid changes from genotype 2 to genotype 3 sequence. This suggests that these residues are interacting with the NS3 protease and the substitutions counteract incompatibilities between the genotype 3a protease and the type 2a NS2 and NS3 helicase proteins. Ser1046Asn is an amino acid change back towards consensus genotype 3 sequence. This suggests that the chosen clone represents a quasispecies with reduced fitness and the mutation towards consensus sequence rescues it. Reintroduction of these into the original J3a3a-6 resulted in a virus that immediately was detectable in Huh7.5 cells but did not spread any faster than the wild type after an initial spread to 10 % of the cell culture. This suggests that there are other determinants for efficient spread of this recombinant (4.20 top).

Similarly, NS3 proteases from patients infected with genotype 1b, 4a or 6a were cloned into the corresponding J1b1b, J4a4a and J6a6a recombinants. Both J1b1b recombinants with patient derived protease genes replicated to the same extent as the prototype J1b1b, indicating that patient-derived sequences can be easily swapped and analysed (Fig. 4.17 top). Replacement of the NS3 protease gene of the J4a4a prototype recombinant with those amplified from genotype 4a-infected patients reversed the impaired replication phenotype in 3 (J4a4a-7, -8, -19) out of 4 cases (Fig. 4.17 bottom). J4a4a-7 and J4a4a-19 replicated similarly to J1b1b, whereas J4a4a-8 replication kinetics were very low and those of J4a4a and J4a4a-10 were below detection limit. To which amino acid polymorphism this discrepancy is due is unclear, as not only J4a4a and J4a4a-10,

but also J4a4a-19 and -7 have an amino acid polymorphism that does not occur in any other sequence of the analysed dataset. Replacing the J6a6a NS3 protease with that of patient-derived genes resulted in replicating recombinants in both cases (J6a6a-4 and -8) (Fig. 4.16 bottom). J6a6a-4 immediately spread within the cell culture and maintained a continuous infection, whereas J6a6a-8 showed a replication profile similar to that of prototype J6a6a. As discussed above, Val1040Leu has been identified to be a crucial adaptive change for J6a6a. J6a6a-8 also contains a Val at position 1040 whereas J6a6a-4 contains an Ile, a bulkier amino acid similar to Leu which may underlie its improved replication kinetics.

In a similar manner, NS3 proteases from patients infected with genotype 1a were cloned into the corresponding J1a1a recombinants. The replication kinetics of the resulting recombinants were very low or below detection limit and none of the viruses were able to acquire adaptive mutations; all Huh7.5 cell cultures were NS5A-negative after 30 days (Fig. 4.18). It has been reported that the *in vitro* catalytic efficiency of the genotype 1a NS3 protease is similar to those of 3a and 4a, both of whose recombinants were replication competent *in vitro* (Franco *et al.* (2008)). It is therefore unlikely that the J1a1a recombinants reproduce less efficiently than the J3a3as and J4a4a-7 and J4a4a-19 because of differences in the enzymatic activity of the proteases. This suggests that genotype 1a NS3 protease/NS4A are less compatible with the genotype 2a backbone than genotype 1b, 3a, 4a, 5a and 6a.

To investigate why J5a was replication competent and the other Jxs not and why J5a5a, J3a3a and J6a6a replicated more efficiently than J1a1a, J1b1b and J4a4a, their nt and amino acid sequence within the NS3 protease and NS4A gene were compared to that of JFH1 (Table 4.3). No significant difference between the sequence divergence of the different Jxxs to JFH1 could be identified, suggesting that the differences in replication kinetic are due to specific individual amino acids rather than the overall sequence.

Table 4.3: ***Nucleotide and amino acid sequence divergence within the NS3 protease and the NS4A gene between JFH1 and Jxx.*** *The nt and amino acid sequence of the NS3 protease and the NS4A cofactor gene of each Jxx were compared with that of JFH1. Using p-value calculations the sequence divergence between JFH1 and Jxx was determined (JFH vs. Jxx, JFH1 sequence compared to the corresponding Jxx sequence).*

JFH1 vs. Jxx	Nucleotide sequence divergence (%)	Amino acid sequence divergence (%)
NS3		
JFH1 vs. J1a1a	34.6	28.0
JFH1 vs. J1b1b	33.9	30.0
JFH1 vs. J2a2a	10.5	7.0
JFH1 vs. J3a3a	35.5	27.0
JFH1 vs. J4a4a	35.7	29.0
JFH1 vs. J5a5a	34.8	26.0
JFH1 vs. J6a6a	34.4	24.0
NS4A		
JFH1 vs. J1a1a	35.7	32.1
JFH1 vs. J1b1b	35.7	32.1
JFH1 vs. J2a2a	12.5	5.4
JFH1 vs. J3a3a	41.7	35.7
JFH1 vs. J4a4a	42.9	33.9
JFH1 vs. J5a5a	40.5	33.9
JFH1 vs. J6a6a	38.7	33.9

4.4 Conclusion

This chapter describes the development of a full-length HCV cell culture system, which allows the investigation of protease gene function in all 6 major genotypes, a much greater range than the genotype 2a and 1a full-length replication competent clones described previously (Lindenbach *et al.* (2005); Wakita *et al.* (2005); Yi *et al.* (2006b); Murayama *et al.* (2007a)). Replacing the NS3 protease alone only generated a replication competent intergenotypic recombinant for genotype 5a. Including the NS4A gene in the recombinant construction resulted in replication competent intra- and intergenotypic recombinants representing all 6 major genotypes. Through introduction of cell culture adaptive/attenuating mutations, stably replicating recombinants could be created for genotype 2a, 3a, 5a and 6a. The developed system represents a powerful tool to study the NS3 protease within the full viral life cycle and adds to the currently available JFH1-based systems for the study of the non-structural genes (Pietschmann *et al.* (2006); Gottwein *et al.* (2007); Yi *et al.* (2007); Jensen *et al.* (2008); Scheel *et al.* (2008); Gottwein *et al.* (2009)). This system allows the assessment of antiviral susceptibilities and resistance development of HCV NS3 protease genes from genotypes 1 to 6, which will be described in chapter 5 and 6. The ease with which protease gene sequences directly amplified from clinical specimens can be inserted in the expression vector of the appropriate genotype, allows quick assessment of the huge diversity within HCV genotypes, subtypes and quasispecies variants.

Chapter 5
Susceptibility of Different HCV Genotypes to PIs

5.1 Introduction

The HCV-NS3 serine protease is essential for viral replication and therefore an attractive target for HCV-specific antiviral therapy (Pawlotsky & McHutchison (2004)). As discussed in section 1.8.1 and chapter 3, the genetic variability of HCV proteins encoded by different genotypes results in substantial differences in the molecular structure of protease and polymerase enzymatic sites. Because antiviral drugs are often specifically designed for the protease of one genotype, they can show limited efficacy in other genotypes (Holland-Staley *et al.* (2002)). One of the first PIs developed was BILN 2061, which is a small, orally bioavailable molecule identified in a substrate-based approach (Fig. 5.1 left) (Lamarre *et al.* (2003)). BILN 2061 was specifically developed for genotype 1 enzymes and was the first PI to enter clinical trials. Genotype 1 infected patients showed an impressive reduction in HCV RNA levels after only 2 days, with some patients even reaching undetectable levels within 24-28 hours after administration (Lamarre *et al.* (2003)). Due to structural differences in the protease protein, BILN 2061 showed weaker binding affinity for genotype 2 and 3 proteases (Thibeault *et al.* (2004)) and as expected lower efficacy in patients infected with genotype 2 and 3 (Reiser *et al.* (2005)). However, K_i values for nongenotype 1 NS3/4A proteins are still below 100 nM, making it a potent and competitive inhibitor for all genotypes. Even though further development of BILN 2061 had to be halted due to cardiotoxicity in laboratory animals, it is a good PI for proof of concept studies (Vanwolleghem *et al.* (2007)).

VX-950 is another PI with potent activity against genotype 1 and 2 NS3/4A proteins (Fig. 5.1 right) (Reesink *et al.* (2006)), but lower efficacy against genotype 3a proteases (Perni *et al.* (2006); Paulson *et al.* (2009); Foster *et al.* (2009)). The case of BILN 2061, and VX-950 later, demonstrated very early on that it is crucial to investigate drug efficacies in all 6 genotypes. Incomplete eradication of the virus due to reduced efficacy of antiviral drugs on certain genotypes also potentially facilitates the development of resistance mutations, also see section 1.8.4.

This chapter describes the assessment of antiviral susceptibilities of HCV NS3 protease genes from HCV genotypes 1 to 6. Using the cell culture system described in chapter 4, BILN 2061 and VX-950 efficacies on the NS3/4A protease of all 6 major genotypes were assessed and compared. Intragenotypic differences in susceptibility to BILN 2061 between patient-derived proteases were additionally investigated.

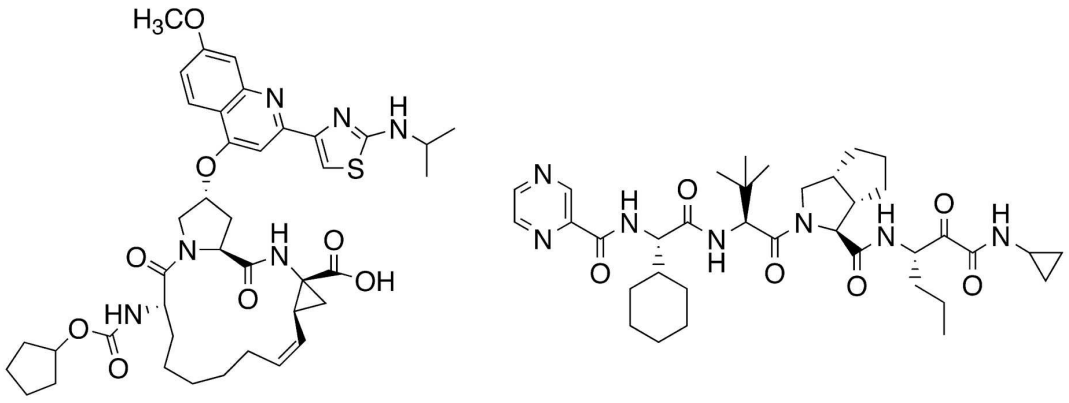

Figure 5.1: ***HCV protease inhibitors***. *Chemical structure of the HCV PIs BILN 2061 (left) and VX-950 (right).*

5.2 Results

5.2.1 Susceptibility of Jxx recombinants to BILN 2061

Effect of BILN 2061 on supernatant infectivity

Intra- and intergenotypic recombinants derived from genotype 2a, 3a, 5a and 6a that produced infectious virus were evaluated for PI sensitivity. This section describes how the activity of BILN 2061 is affected by the sequence differences of enzymes from various genotypes (Fig. 4.23). Huh7.5 cells were infected with virus containing supernatant (MOI of 0.015), then the reduction in FFU/ml upon BILN 2061 treatment assessed (Fig. 5.2a-d). Supernatant infectivities of all recombinant viruses were inhibited, but to different extents. J2a2a, J3a3a and J5a5a showed similar dose-responses (IC_{50} = 210 nM, 80 nM and 110 nM, respectively), whereas J6a6a (IC_{50} = 2 nM) was 40- to 100-fold more susceptible to BILN 2061. To visually compare the reduction in supernatant infectivity between the different genotypes, plots from Fig. 5.2 were also plotted against each other (Fig. 5.3).

Effect of BILN 2061 on viral replication

Since no infectious virus could be generated for J1b1b and J4a4a-19, PI susceptibility was also assessed after synthetic RNA had been electroporated into fresh Huh7.5 cells (Fig. 5.4 and 5.5). After 24 hours, PI was added and the reduction in the frequency of NS5A positive cells was assessed at 96 hours post-electroporation. J1b1b, J4a4a-19 and J6a6a showed 100- to 1,000-fold greater susceptibility (IC_{50} = 3 nM, 2 nM and 1 nM, respectively) than J2a2a, J3a3a and J5a5a (IC_{50} = 720 nM, 105 nM and 480 nM, respectively). To visually compare the reduction in viral replication between the different genotypes, plots from Fig 5.4 and 5.5 were also plotted against each other (Fig. 5.6).

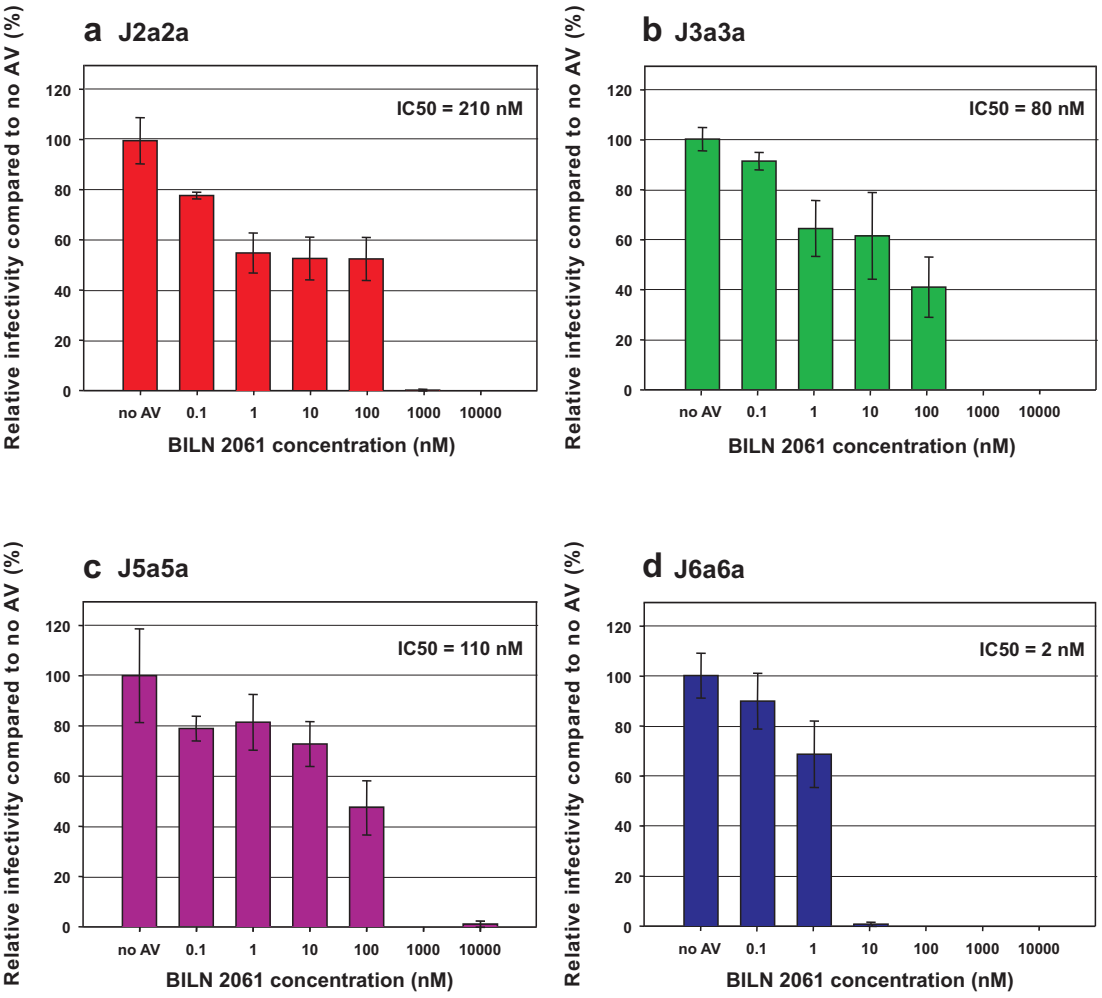

Figure 5.2: ***Antiviral inhibition of Jxxs; reduction in supernatant infectivity.*** *After 8 hours inoculation with (a) J2a2a, (b) J3a3a, (c) J5a5a and (d) J6a6a (MOI 0.015), Huh7.5 cells were washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061. Inhibition was calculated at 72 hours post-infection as reduction in supernatant infectivity (FFU/ml; mean ±SEM; n = 3) after antiviral addition compared to infectivity of the control without antiviral.*

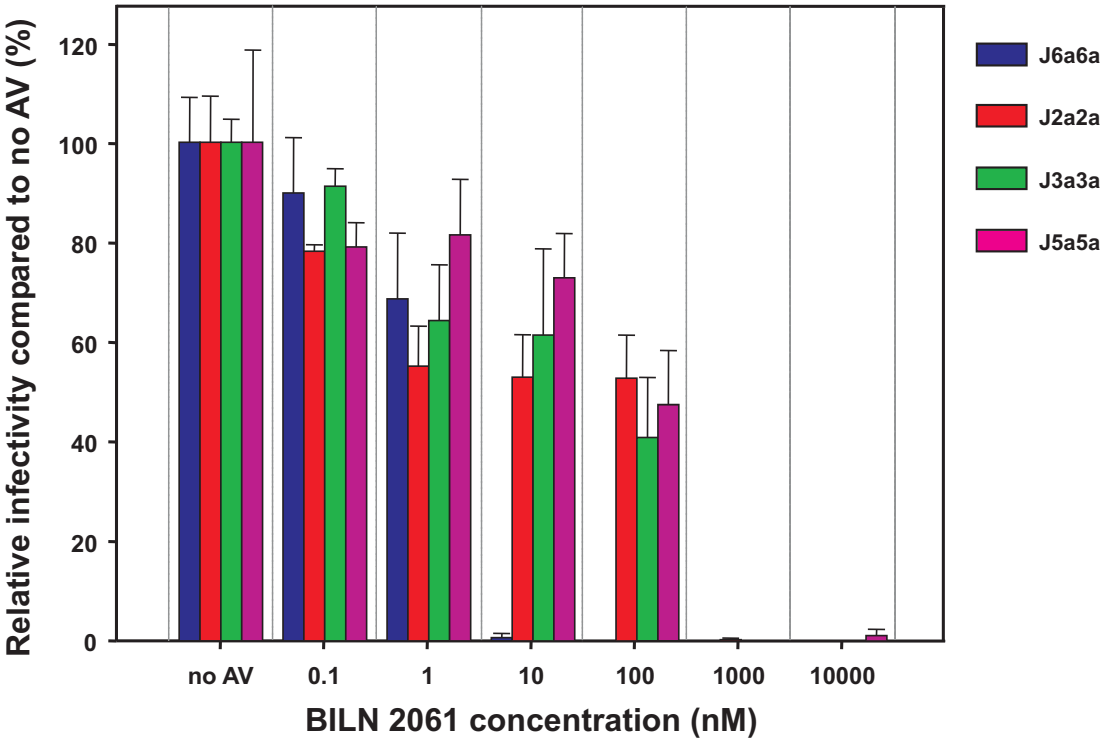

Figure 5.3: ***Antiviral inhibition of Jxxs; comparing reduction in supernatant infectivity.*** *To compare the reduction in supernatant infectivity between the different genotypes, plots from Fig. 5.2 were plotted against each other.*

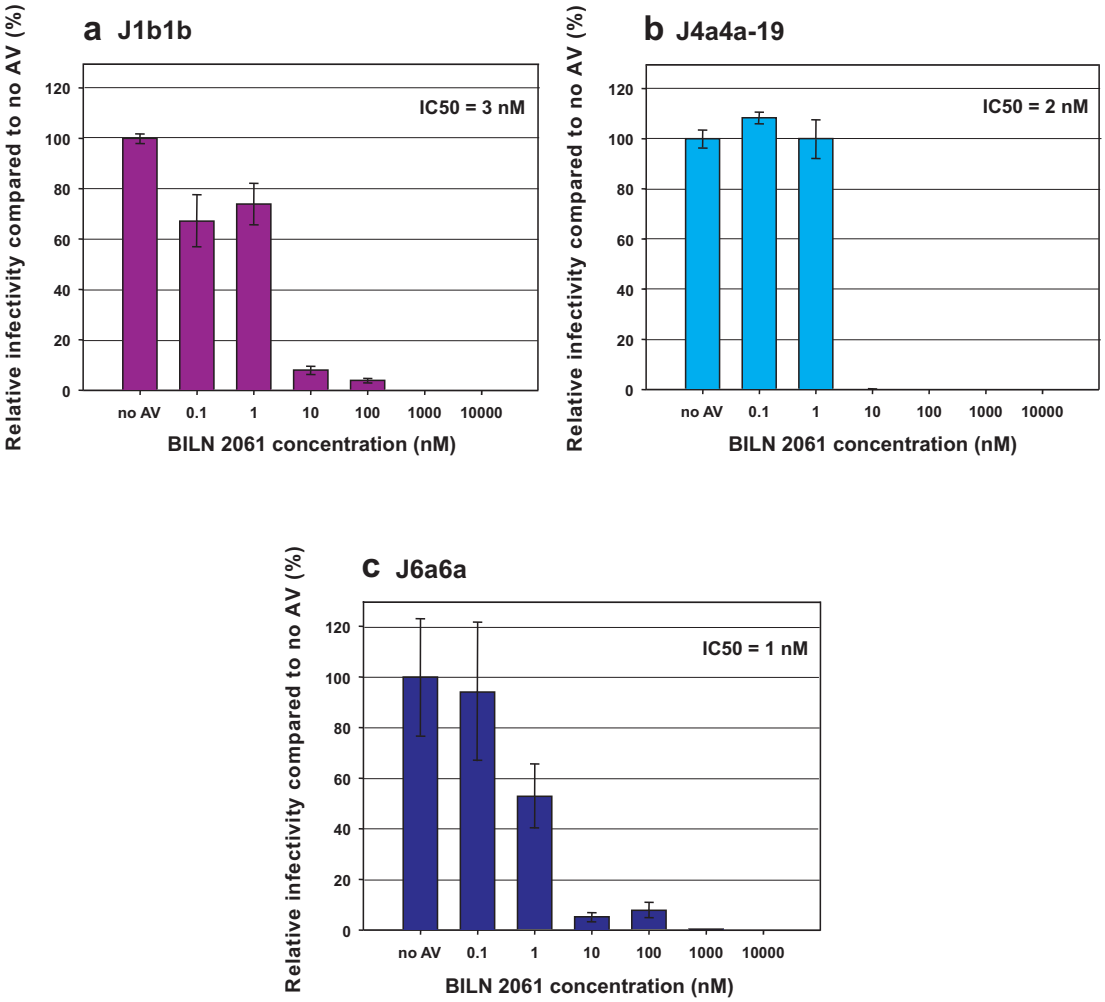

Figure 5.4: ***Antiviral inhibition of (a) J1b1b, (b) J4a4a-19 and (c) J6a6a; reduction in viral replication****. 1-10 μg RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral.*

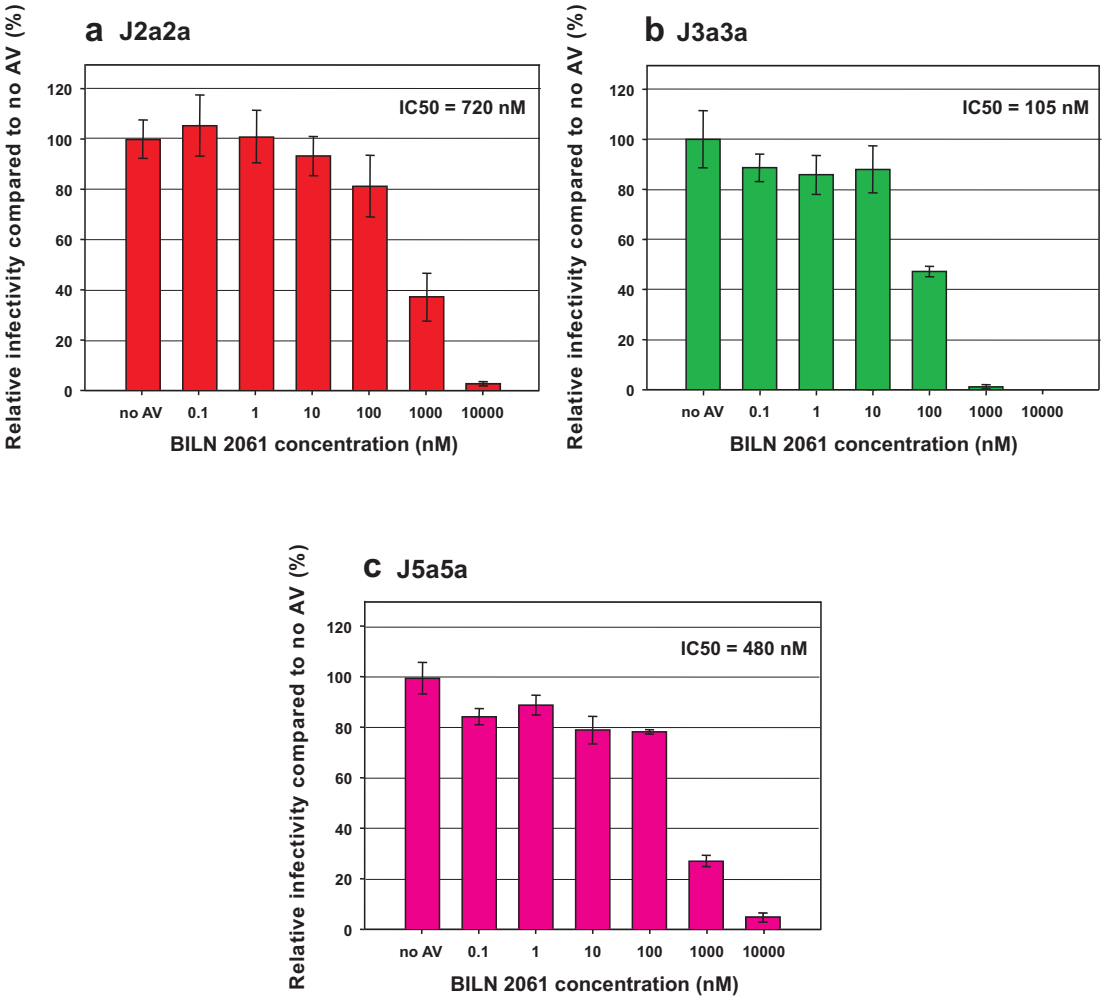

Figure 5.5: ***Antiviral inhibition of (a) J2a2a, (b) J3a3a and (c) J5a5a; reduction in viral replication****. 1-10 μg RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean ±SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral.*

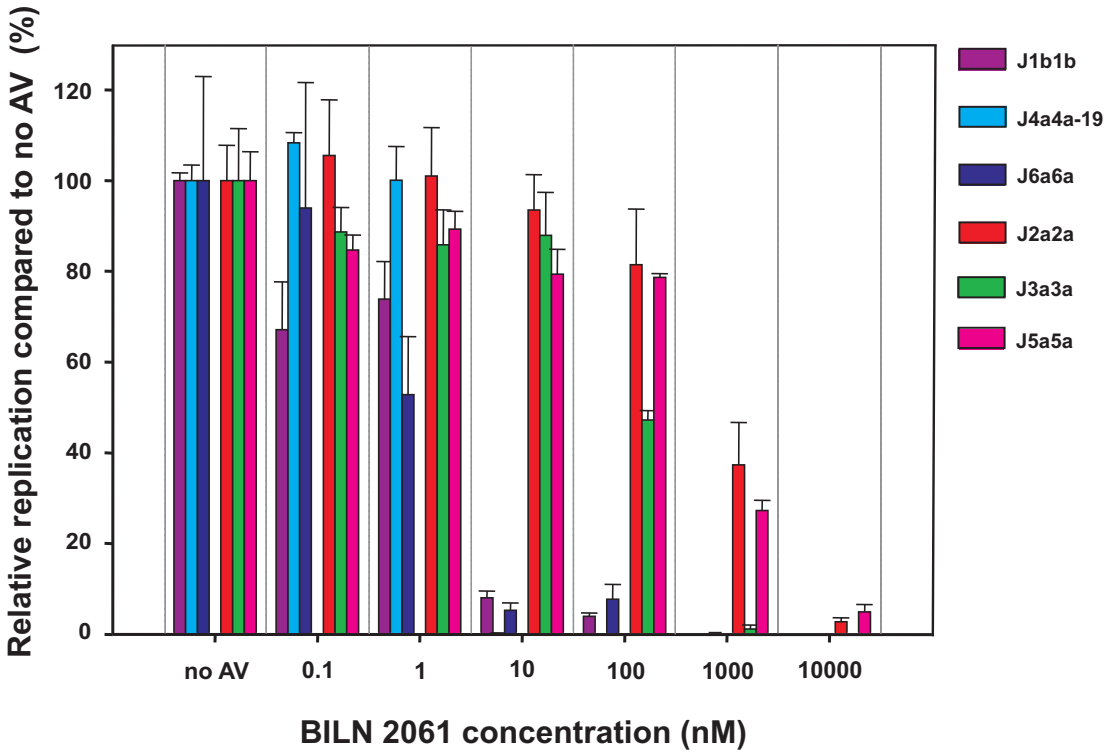

Figure 5.6: ***Antiviral inhibition of Jxxs; comparing reduction in viral replication.*** *To compare the reduction in viral replication between the different genotypes, plots from Fig. 5.4 and 5.5 were plotted against each other.*

Effect of naturally occurring sequence variability on BILN 2061 efficacy

To investigate whether naturally occurring sequence variability within a genotype led to differences in antiviral susceptibilities, the J3a3a recombinants with patient-derived proteases including adaptive mutations (J3a3a-3$_{\text{-L1663A}}$, J3a3a-8$_{\text{-L1663A}}$ and J3a3a-11) were subjected to BILN 2061 treatment as described above. The J3a3a recombinant generated from the HCV3a-Gla prototype sequence showed an IC_{50} value of 130 nM, comparable to previous assays (Fig. 5.5), but 2- to 3-fold lower than IC_{50}s of the patient-derived sequences (310 nM for J3a3a-11, 300 nM for J3a3a-8$_{\text{-L1663A}}$ and 240 nM for J3a3a-3$_{\text{-L1663A}}$) (Fig. 5.7). Although requiring further evaluation of more replicates' antiviral dilutions to establish formal statistical significance, these small differences in apparent susceptibility suggest that some of the naturally occurring sequence variability within a subtype or genotype might have a direct and potentially clinically significant effect on response to antiviral therapy.

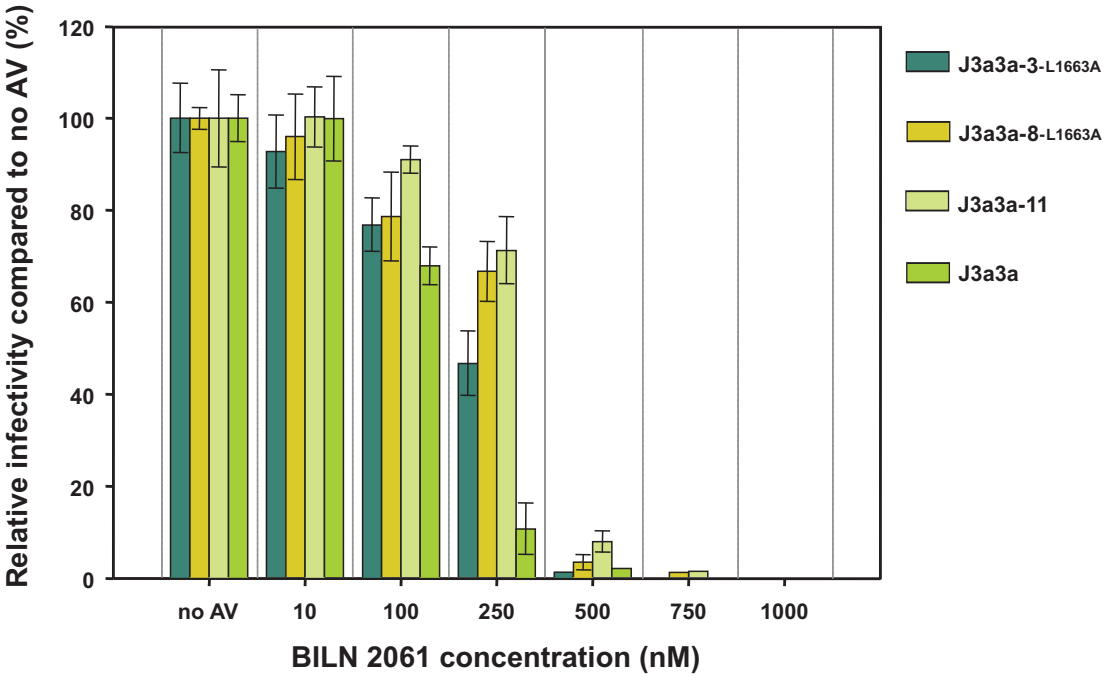

Figure 5.7: ***Antiviral inhibition of J3a3a-recombinants with patient-derived proteases; reduction in viral replication.*** *1-10 μg of RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral.*

5.2.2 Susceptibility of Jxx recombinants to VX-950

Effect of VX-950 on viral replication

Initially VX-950 susceptibility was assessed for the intergenotypic recombinants J1b1b, J3a3a and J4a4a-19. Following electroporation of synthetic RNA into Huh7.5 cells, VX-950 was added and the reduction in the frequency of NS5A positive cells was assessed at 96 hours post-electroporation. J1b1b (IC_{50} = 870 nM) showed about 2-fold greater susceptibility to VX-950 than J3a3a (IC_{50} = 1,520 nM) and about 3-fold greater susceptibility than J4a4a-19 (IC_{50} = 2,330 nM) (Fig. 5.8a-c). VX-950 susceptibility testing for genotype 2a, 5a and 6a was carried out with the cell culture attenuated/adapted recombinants. J5a5a$_{\text{-Q1247L}}$ (IC_{50} = 1,230 nM) showed similar susceptibility to VX-950 as J3a3a, whereas J2a2a$_{\text{-T1066S}}$ and J6a6a$_{\text{-V1040L}}$ showed similar susceptibility to J1b1b (IC_{50} = 1,020 nM and 650 nM, respectively) (Fig. 5.9a-c). To visually compare the reduction in viral replication between the different genotypes, plots from Fig. 5.8 and 5.9 were additionally plotted against each other (Fig. 5.10). Further dilutions of VX-950 concentrations have to be assessed between 500 nM and 10,000 nM to get more accurate results on the IC_{50} values of J2a2a, J5a5a and J6a6a.

5.2.3 Summary of results

The J6a6a recombinant was about a 100-fold more susceptible to BILN 2061 than J2a2a, J3a3a and J5a5a, whether assessed by reduction in supernatant infectivity or viral replication. Genotype 1b and 4a based recombinants showed similar susceptibility to BILN 2061 as genotype 6a. Subtle differences in susceptibility to BILN 2061 were also found between J3a3a recombinants with patient-derived proteases. Likewise, genotype specific differences in susceptibility were observed upon VX-950 treatment. J1b1b, J2a2a$_{\text{-T1066S}}$ and J6a6a$_{\text{-V1040L}}$ were most susceptible to VX-950, whereas J3a3a and J5a5a$_{\text{-Q1247L}}$ showed intermediate susceptibility and J4a4a-19 proved to be the most resistant recombinant. Overall BILN 2016 was about a 100-fold more potent on Jxx recombinants than VX-950.

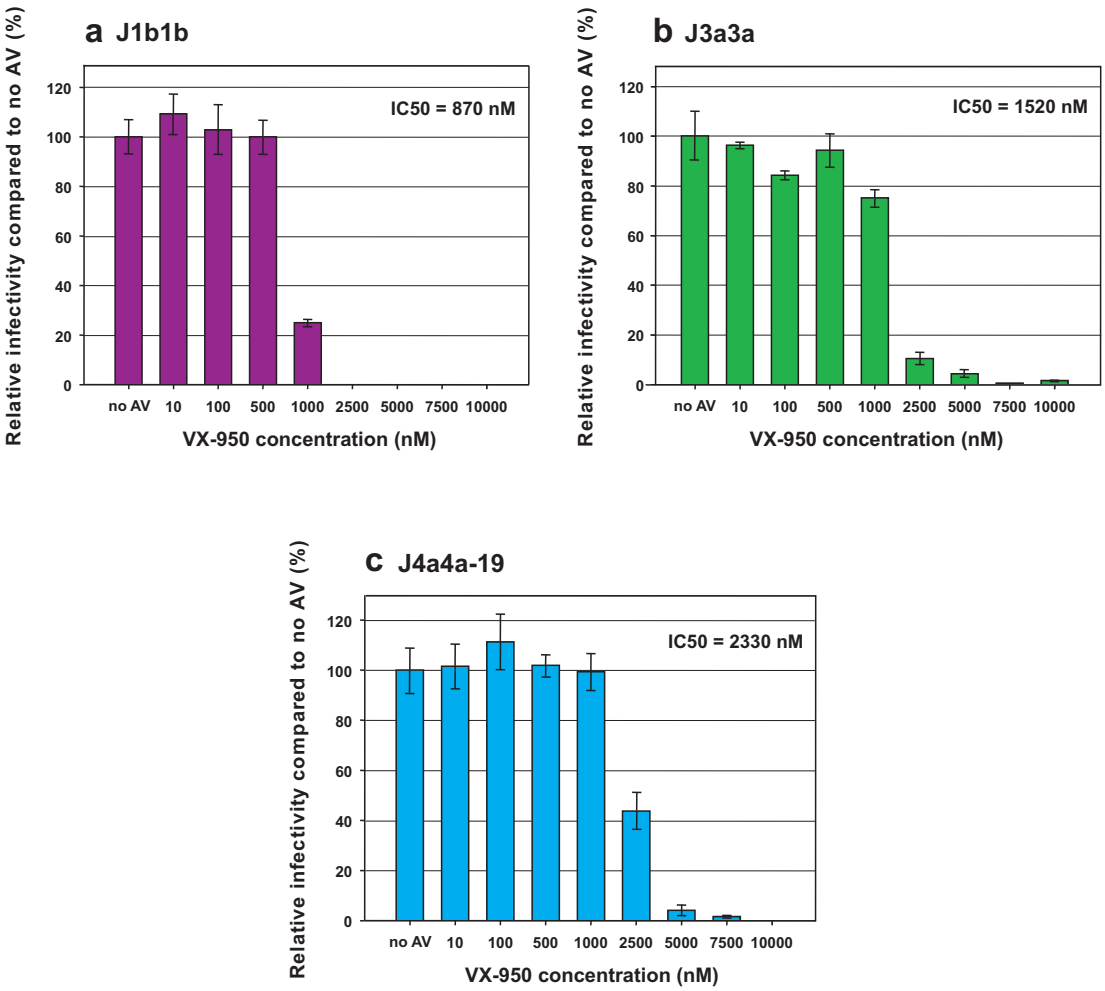

Figure 5.8: ***Antiviral inhibition of (a) J1b1b, (b) J3a3a and (c) J4a4a-19; reduction in viral replication***. *1-10 μg RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of VX-950 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3), and calculated as the ratio of NS5A-positive cells in VX-950-treated cells to those of the control without antiviral.*

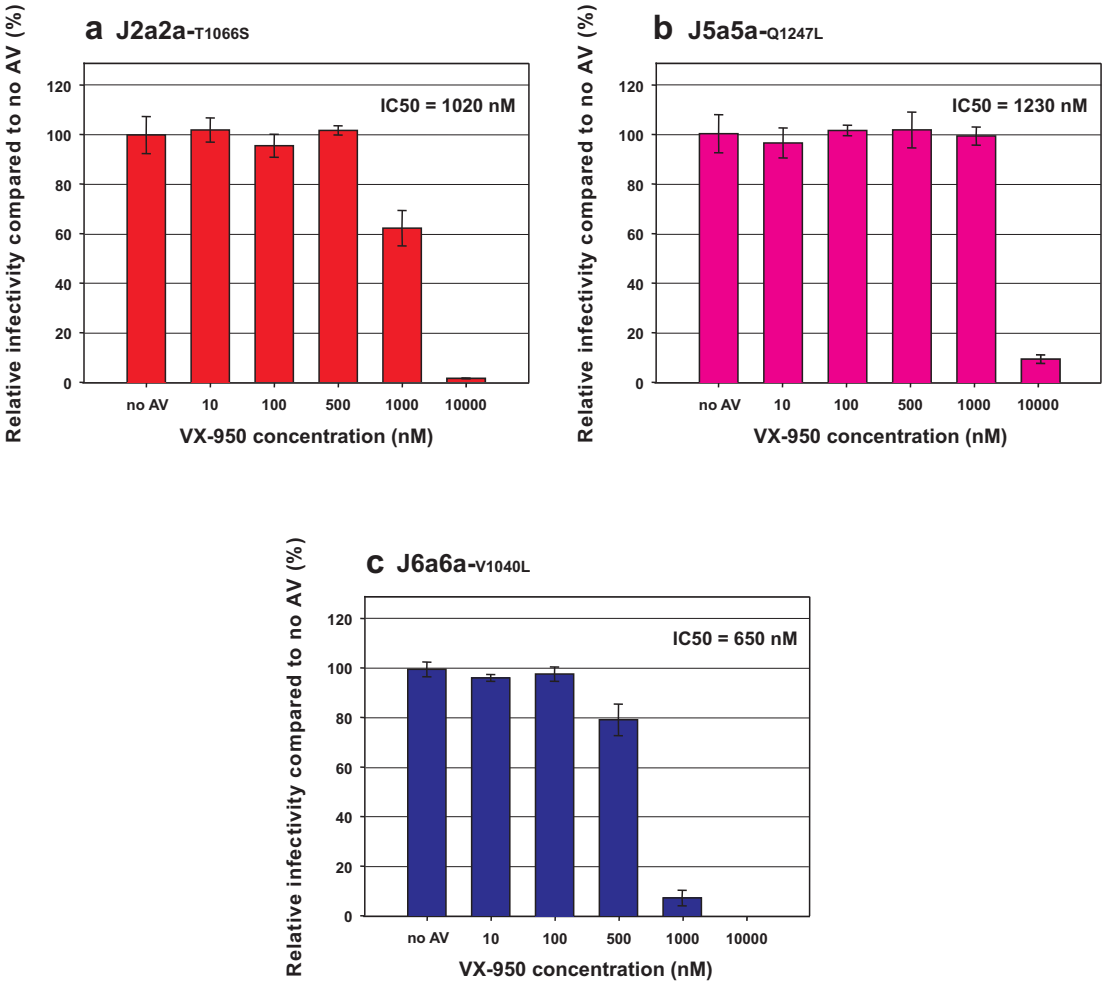

Figure 5.9: ***Antiviral inhibition of (a) J2a2a$_{-T1066S}$, (b) J5a5a$_{-Q1247L}$ and (c) J6a6a$_{-V1040L}$; reduction in viral replication.*** *1-10 μg RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of VX-950 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3), and calculated as the ratio of NS5A-positive cells in VX-950-treated cells to those of the control without antiviral.*

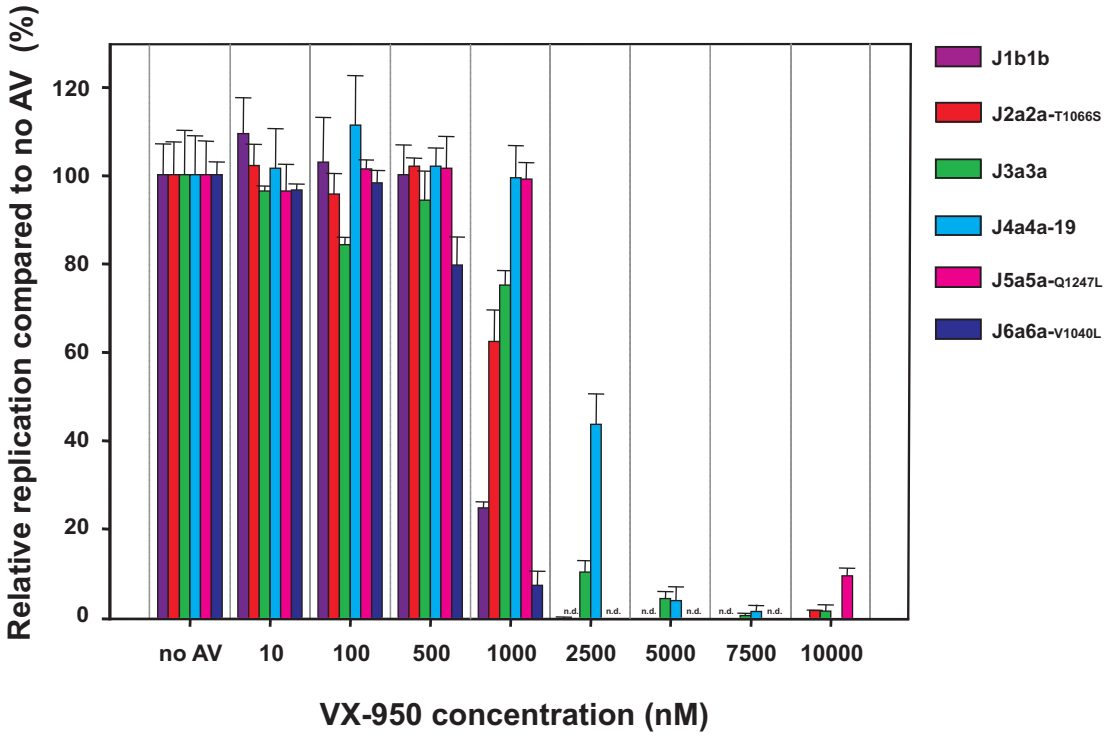

Figure 5.10: ***Antiviral inhibition of J2a2a, J5a5a and J6a6a; comparing reduction in viral replication***. *To compare the reduction in viral replication between the different genotypes, plots from Fig. 5.8 and 5.9 were plotted against each other.*

5.3 Discussion

The PI BILN 2061 has been shown to rapidly reduce HCV RNA plasma levels in patients infected with genotype 1 (Lamarre *et al.* (2003)). The drug development of this PI has been targeted to genotype 1 proteases, because genotype 1 infected patients respond poorly to the current standard care of treatment and also represent the majority of HCV patients in the developed world. However, other genotypes, such as type 2 and 3, account for many infections around the world as well, whereas genotype 4-6 are mainly found in Asia and Africa, also see section 1.3.3. Due to intergenotypic genetic differences, PIs that are highly optimised to bind the 3D structure of proteases of one genotype do not efficiently bind to proteases of other genotypes. In *in vitro* sensitivity studies, BILN 2061 showed decreased affinity for the NS3/4A protease of genotype 2 and 3 (Thibeault *et al.* (2004)). As expected, patients infected with HCV genotype 2 and 3 were less susceptible to BILN 2061 treatment (Reiser *et al.* (2005)). It is therefore crucial to assess the antiviral efficacy of PIs on different genotypes before they go into clinical trials.

In this chapter, the antiviral susceptibility of NS3/4A proteases of all 6 major genotypes towards BILN 2061 and VX-950 were assessed. Even though BILN 2061 development had been halted due to cardiotoxicity in laboratory animals, it is a good PI to test the practicality of the cell culture system described in chapter 4 and to estimate IC_{50}s of PIs towards the 6 major genotypes (Vanwolleghem *et al.* (2007)). Using the intra- and intergenotypic recombinants described in chapter 4, J1b1b was shown to be more susceptible to BILN 2061 than J2a2a and J3a3a, as has been demonstrated previously *in vitro* and *in vivo* (Reiser *et al.* (2005); Thibeault *et al.* (2004)). The *in vitro* affinity of BILN 2061 to genotype 1 proteases has been described to be about 100-fold higher than for those of genotype 2 and 3, which reflects the observed 35- to 240-fold difference in susceptibility between J1b1b and J3a3a and J2a2a, respectively (Thibeault *et al.* (2004)).

Interestingly, J4a4a-19 and J6a6a were equally susceptible to BILN 2061 as J1b1b and J5a5a similarly to J2a2a and J3a3a. Experiments using expressed proteins showed similar IC_{50} values for genotype 1a, 1b, 4a, 5a and 6a, whereas a lower susceptibility of J5a5a towards BILN 2061 was observed in this study (Seiwert *et al.* (2008); Massariol *et al.* (2009)). In the study by Massariol, the NS3 protein from genotype 1b and 2a replicon as well as from clinical strains of genotype 4-6 were expressed and assessed for their cleavage efficiency at the NS5A-NS5B junction. The reported IC_{50} values for genotype 2a and 5a are about 20- to 100-fold lower than those described here. However, a comparison is difficult as purified proteases outside the context of the remaining virus were analysed, whereas this study assessed protease activity within the full viral lifecycle (Massariol *et al.* (2009)). Furthermore, only the cleavage efficiency at the NS5A-NS5B junction was analysed. The study by Seiwert *et al.*, which analysed biochemical potencies of purified proteins as well, also reported 10- to 50-fold lower IC_{50} values for genotype 3a and 5a, respectively (Seiwert *et al.* (2008)). The discrepancy between these studies and the results described here, most certainly are due to differences in the assay itself. The IC_{50} values for genotype 1b compared to 3a and 2a (35- and 240-fold differences) reported here, reflect closely the *in vivo* described efficacies of BILN 2061 on genotype 1 compared to 2 and 3 (10- to 100-fold difference in HCV RNA level reduction) (Lamarre *et al.* (2003); Reiser (2003)). The system described here therefore reflects the efficacy of PIs in patients infected with the different viral strains more accurately than protein based kinetic studies. Although BILN 2061 is no longer in development, the observed similar IC_{50} values for genotype 1b, 4a and 6a suggest that future PIs of that class developed for genotype 1 may be effective not only against genotype 1, but also against genotype 4a and 6a proteases.

Equivalent differences in susceptibility to BILN 2061 sensitivity were observed whether determined by supernatant infectivity reduction or inhibition of replication (Fig. 5.3 and 5.6). Differences in susceptibility extended even to variants within a genotype, with IC_{50} values ranging from around 130 nM to 310 nM in a panel of patient-derived and reference genotype 3a protease sequences (Fig. 5.7). This 2- to 3-fold difference may influence the effectiveness of antiviral therapy as well.

Even though BILN 2061 has been specifically developed for genotype 1 protease enzymes, the residues that are in direct contact with the inhibitor are well conserved, suggesting that this class of PI should be active against all genotypes and subtypes (Thibeault *et al.* (2004)). X-ray crystallography of the NS3/4A protease complexed with a macrocyclic tripeptide inhibitor, identified 19 residues within 5 Å of the inhibitor, of which 13 were conserved among genotype 1, 2 and 3 variants (Thibeault *et al.* (2004)). Positions 78, 79 and 80 of the NS3 protease differed between genotype 1 and genotype 2 sequences and position 123 and 168 between genotype 1 and 3 sequences. Position 132 was replaced in both genotype 2 and 3 when compared to type 1. Mutational analysis has suggested that substitutions at these 5 residues are responsible for 80 % of the binding energy difference for BILN 2061 binding to proteases of different genotypes (Thibeault *et al.* (2004)).

The Jxx recombinants described in chapter 4 all have Val78 and Asp79 except for J2a2a, discounting those residues for the differences between J1b1b, J4a4a-19, J6a6a and J5a5a recombinant susceptibility (Fig. 4.23) (J4a4a and J4a4a-19 are identical on the amino acid level in all positions discussed here). J5a5a is the only recombinant with a charged amino acid at position 80, possibly partly accounting for its decreased susceptibility. Substitutions at position 123 and 132 within genotype 3 variants have also been suggested to play a role in BILN 2061 binding differences (Thibeault *et al.* (2004)). However, amino acid residue 123 is identical among J1b1b, J2a2a, J4a4a-19, J5a5a and J6a6a recombinants and the polymorphism at position 132 is conservative. These 2 residues therefore do not explain the difference in susceptibility between J5a5a and J1b1b, J4a4a-19 and J6a6a. Furthermore, residue 122 has been described to play a role in inhibitor binding (Thibeault *et al.* (2004)). J2a2a is the only recombinant with a charged residue (Lys) at position 122, whereas all the other recombinants have polar neutral amino acids (Thr/Ser), suggesting residue 122 plays a role in type 2 specific binding. Finally, the genotype 3a residue Gln168 has been identified as the major determinant for the reduction in affinity of BILN 2061 binding to genotype 3a proteases. Although residue 168 does not directly interact with the inhibitor, it has been shown to be a crucial determinant of inhibitor binding, as the development of resistance mutations have been mapped to this position (section 1.8.4). Asp168

is conserved among genotype 1, 2, 4 and 6 and interacts with Arg123 and Arg155 that are connected to BILN 2061. The Gln168 substitution in J3a3a results in abrogation of salt bridges to residues 123 and 155, which are therefore less stabilised and the binding affinity to BILN 2061 is reduced. The Glu168 substitution within the J5a5a recombinant maintains the negative charge and the salt bridges are not affected. However, the Glu side chain is longer than that of Asp, possibly pushing the 2 BILN 2061 binding residues Arg123 and Arg155 out of their optimal interaction position (Courcambeck *et al.* (2006)). J4a4a-19 and J6a6a have identical amino acids to genotype 1 at the described positions, providing one possible explanation for their similarity in susceptibility towards BILN 2061. Interestingly, genotype 1b, 4a and 6a, which showed the highest susceptibility to BILN 2061, are also the genotypes showing lower response rates to the current IFN-α/ribavirin combination treatment. This could suggest that genotype 6a is closely related to genotype 1 and 4, which have been shown to be marginally closer related to each other than to the other genotypes (Simmonds *et al.* (2005)).

VX-950 is currently the most advanced PI in clinical trials. A previous *in vitro* study has reported about 2-fold higher susceptibility of the genotype 1a/1b replicon to VX-950 when compared to the genotype 2a replicon (Paulson *et al.* (2009)). Biochemical cleavage analysis with purified proteases showed that genotype 3a is about 10- to 40-fold and genotype 4 about 5- to 10-fold less susceptible to VX-950 than the other genotypes (Seiwert *et al.* (2008)). In an ongoing phase IIa clinical trial VX-950 has demonstrated substantial activity in genotype 2 infected patients, but only limited efficacy in genotype 3 infected individuals, for whom treatment as a result was stopped (Foster *et al.* (2009)). As demonstrated by these studies, VX-950 shows considerable differences in potency against different genotypes like BILN 2061, which highlights again how important it is to evaluate PIs on all genotypes before clinical assessment. Susceptibility testings of Jxxs upon VX-950 treatment have shown highest susceptibility for the J1b1b, J6a6a$_{\text{-V1040L}}$ and J2a2a$_{\text{-T1066S}}$ recombinants. The measured IC_{50} values (600-1,000 nM) are slightly higher than those reported previously (Seiwert *et al.* (2008); McCown *et al.* (2009); Paulson *et al.* (2009)). For the J1b1b recombinant the IC_{50} value described here (870 nM) is similar to the IC_{50} value reported from the

replicon system (625 nM) (Paulson *et al.* (2009)). However, IC_{50} values measured in biochemical cleavage studies are 5- to 15-fold lower for all genotypes except for genotype 3a, for which they are almost identical (Seiwert *et al.* (2008)). Comparisons are difficult though, due to the considerable differences in the assays. Furthermore, that study does not specify which subtypes of genotype 4, 5 and 6 were used. In agreement with that study though, the genotype 6a recombinant shows the highest susceptibility towards VX-950, followed by genotype 1 and 2. However, the low susceptibility of J3a3a and J5a5a$_{\text{-Q1247L}}$ and the very low susceptibility of J4a4a-19 are in disagreement with that study (Seiwert *et al.* (2008)). A clinical trial using VX-950 monotherapy has been conducted with genotype 4 infected patients, but no published results could be found online. From the here described preliminary results it would be expected that genotype 4a infected patients do not respond to VX-950 therapy, whereas genotype 6a infected patients should show good response rates. Genotype 5a infected patients would be expected to show only a minimal response, similar to genotype 3 infected individuals (Foster *et al.* (2009)).

Residues identified during resistance development studies in the Con1b replicon (NS3 protease residues Val36, Thr54, Arg155, Ala156 and Val170) (He *et al.* (2008)), are identical to Con1b amino acid sequence in all Jxxs, except at position 36 and 170. The recombinants J1b1b and J6a6a$_{\text{-V1040L}}$, which were most susceptible to VX-950, have Val36, whereas J2a2a$_{\text{-T1066S}}$, J3a3a, J4a4a-19 and J5a5a$_{\text{-Q1247L}}$ have Leu36. The Val36Leu substitution has been described to show some resistance to VX-950, providing a possible explanation for the reduced susceptibility of J2a2a$_{\text{-T1066S}}$, J3a3a, J4a4a-19 and J5a5a$_{\text{-Q1247L}}$ (Zhou *et al.* (2008)). However, J2a2a$_{\text{-T1066S}}$ still is more susceptible than J3a3a, J4a4a-19 and J5a5a$_{\text{-Q1247L}}$, indicating that further residues play a role in the variability of VX-950 binding. Residue 170 is a polymorphic site with amino acid Val/Leu occurring across genotypes. As all the recombinants described here have Val/Leu170, it is unlikely that this position plays a role in the variability of susceptibilities between Jxxs.

5.4 Conclusion

The intra- and intergenotypic recombinants developed and described in chapter 4 were used in this chapter to demonstrate that different NS3 proteins react differently to the same PI. Previously published results on differences between genotypes upon their sensitivity towards the PIs BILN 2061 and VX-950 have been confirmed, underscoring the usefulness of the developed cell culture system. Furthermore, data is presented on inhibitor sensitivity of genotype 4, 5 and 6, which have not been studied in the full-length viral lifecycle yet. Any PI identified in high-throughput screening can be evaluated for its efficacy on different genotypes and treatments designed according to the outcome. Antiviral susceptibilities can be tested through assessment of reduction in both supernatant infectivity and replication kinetics. This and the fact that the whole viral lifecycle can be studied is a major advantage to the replicon system. Protease genes from patients naïve to treatment can also be easily assessed for their sensitivity towards PIs, providing a valuable tool for individually tailored treatment options.

Chapter 6
Identification and Phenotyping of Resistance Mutations against HCV PIs

6.1 Introduction

A major issue in HCV treatment is the rapid selection of drug-resistant genetic variants. Viral variants with drug-resistant phenotypes have been observed in patients experiencing viral rebound during therapy with STAT-C agents (Sarrazin *et al.* (2007a)). Due to the huge pool of genetically different variants circulating in an individual, the so-called quasispecies, resistant variants may already be present in a patient. For example, the resistance mutation Ala156Thr has been found to be present in close to 1 % of NS3 sequences within the liver quasispecies of a treatment-naïve chronic patient (Cubero *et al.* (2008)). Other reports have found pre-existing protease-resistant variants in treatment-naïve patients with a frequency of 0.2-2.8 % (Bartels *et al.* (2008); Colson *et al.* (2008); Kuntzen *et al.* (2008)). Furthermore, resistance mutations were also identified in the replicon system, even though it does not represent the huge diversity of a quasispecies pool within an infected individual (Trozzi *et al.* (2003); Lu *et al.* (2004); Lin *et al.* (2004); Tong *et al.* (2008)). This shows that antivirals not only rapidly select for pre-existing resistant variants, but that resistance mutations can also be rapidly induced due to the high replication and error rate of the RdRp.

Resistance mutations often have a major impact on the fitness of a viral variant, because a mutation affecting inhibitor binding is also likely to impair enzymatic function. Accordingly, compensatory mutations have been identified within resistant variants (Yi *et al.* (2006a)). For effective treatment with STAT-C agents, it is therefore crucial to eradicate a viral population before resistance mutations can arise. As the development of resistance mutations in monotherapy with current drugs is almost cer-

tain, combination treatment with other STAT-C agents and/or IFN-α and ribavirin is inevitable.

To screen patients for possible pre-existing PI resistant variants, which would make them unresponsive towards that PI, resistance mutations have to be identified *in vitro*. Furthermore, the identification of the resistance profile of different PIs will allow the design of combination treatment of drugs that do not select for the same resistant variants.

In this chapter, the *in vitro* selection of Jxx recombinants with decreased BILN 2061 and VX-950 susceptibility is described. In addition, the influence of the identified resistance mutations on the phenotype and PI susceptibility of the corresponding Jxx recombinant was investigated. Because all NS3/4A PIs bind to the same active site of the protease, their resistance profile is often very similar. For example, residue 156 of the NS3 protease has been described as a resistance locus for several PIs and mutations at residue 168 have been reported in BILN 2061 and ITMN-191 resistance (Lin *et al.* (2004); Lu *et al.* (2004); Tong *et al.* (2006); Seiwert *et al.* (2006)). As BILN 2061 and ITMN-191 are both macrocyclic inhibitors, their resistance profile is very similar and identification of resistance mutations under BILN 2061 will help understand resistance development for macrocyclic inhibitors in general. The results on resistance development in nongenotype 1 viruses presented in this chapter add to the current knowledge of resistance mutations which are mostly based on the genotype 1b replicon and *in vivo* analysis. Table 6.1 summarises the resistance mutations that have been described in the literature as to date.

Table 6.1: ***Resistance mutations described in the literature***. *Mutations that confer high level resistance are in bold. (Lin* et al. *(2004); Lu* et al. *(2004); Lin* et al. *(2005a); Mo* et al. *(2005); Seiwert* et al. *(2007a); Sarrazin* et al. *(2007a,b); He* et al. *(2008); McCown* et al. *(2008); Susser* et al. *(2008); Tong* et al. *(2008); Welsch* et al. *(2008); Thompson & McHutchison (2009)).*

	V36	V36+T54	Q41	F43	T54	S138
VX-950	M/A	V36M+T54A	R	C	A	
SCH503034	M/A		R	C(S)	A	
ITMN-191			R	S		T
BILN 2061						

	R155	R155+V36	A156	A156+V36	D168	V170
VX-950	K/Q/T	**R155K+V36M**	S/V/**T**	**A156T+V36M**		A
SCH503034	K/Q/T	R155K+V36M	S/**T**	n.d.		A
ITMN-191	**K**/Q/T	R155K+V36M	S/V/T	n.d.	V/A/E	
BILN 2061	K/Q/T	**R155K+V36M**	**T/V**	n.d.	**V/A**	

n.d., not described in literature but association with antiviral resistance has been suggested.

6.2 Results

6.2.1 *In vitro* selection of BILN 2061-resistant recombinant viruses

To select for resistance mutations, intra- and intergenotypic recombinants described in chapter 4 were passaged in initially subinhibitory but increasing concentrations of BILN 2061 beyond the IC_{50}s determined for each genotype. For simplicity the NS3 protease residues 1027 to 1207 (absolute numbering) are numbered 1 (1027) to 181 (1207) in this chapter.

To establish the protocol for the passaging experiment, the most stably replicating recombinant J3a3a was passaged under increasing concentrations of BILN 2061, starting with 0.3 $\times$ IC_{50}. The PI concentration was then increased every 4 or 5 days to reach about 70 $\times$ IC_{50}. Every 4 or 5 days RNA was extracted from the supernatant and the NS3 protease gene sequenced. Mutations at position 168 of the NS3 protease gene started to appear at a concentration of 3 $\times$ IC_{50}. In a second approach, the PI was directly added at a concentration of 3.3 $\times$ IC_{50} and increased to 70 $\times$ IC_{50} after 7 days. Substitutions at residue 168 of the NS3 protease gene were identified at day 10 of the passaging experiment. The following passaging experiments were therefore started at PI concentrations of 10 $\times$ IC_{50}. Because the J6a6a cell culture adaptive mutation (V1040L) had not been identified at the start of the passaging experiment, J6a6a was first passaged in Huh7.5 cells for 35 days before PI was added and PI addition started from 1 $\times$ IC_{50}. For genotype 2a and 5a the original recombinants J2a2a and J5a5a were initially used for the passaging experiment, starting with PI concentrations of 10 $\times$ IC_{50}. The RT-PCR on RNA extracted from day 12 supernatant was unsuccessful, because RNA levels were relatively low. To increase the amount of RNA for further experiments, cell pellets were collected for RNA extractions. Furthermore, the cell culture attenuated recombinants J2a2a$_{\text{-T1066S}}$ and J5a5a$_{\text{-Q1247L}}$ were constructed and used for resistance development studies in genotype 2a and 5a. In addition, PI concentrations for type 2a and 5a were kept at 1 $\times$ IC_{50}. Table 6.2 gives the individual schedules for each passaging experiment.

Table 6.2: ***Schedule for inducing antiviral (AV) resistance upon BILN 2061 treatment.*** *PE, post-electroporation.*

Clone	Day PE of AV addition	AV concentration	Days of AV pressure
J1b1b	3	1 day 23 nM, 7 days 230 nM	8
J2a2a$_{\text{-T1066S}}$	4	8 days 500 nM, 14 days 1,000 nM	22
J3a3a	2	7 days 350 nM 14 days 7,000 nM	21
J4a4a-19	3	3 days 10 nM 10 days 100 nM	13
J5a5a$_{\text{-Q1247L}}$	4	21 days 500 nM	21
J6a6a	35	10 days 1 nM 18 days 10 nM	28

Recombinants were passaged for 3 weeks and each passaging experiment was carried out in duplicate and without antiviral addition as a control. At the end of passaging, RNA was extracted and subjected to RT-PCR to amplify the coding region of the HCV NS3 protease domain. To delineate the identity and frequency of substitutions, the RT-PCR product from each genotype passage was subcloned into the TOPO vector, and 10 individual colonies of 2 replicates and the control were subjected to sequencing. As J1b1b and J4a4a-19 do not stably replicate in Huh7.5 cells, clonal analysis was performed on day 8. Initially the primers JFH1-s and -as (Table A.13, Appendix A) were used to amplify the protease gene and their restriction sites used for cloning the fragment into TOPO. Using this approach, fragments including resistance mutations could be directly cloned into the corresponding Jxx recombinants and their phenotype assessed *in vitro*, section 6.2.3. However, in J4a4a-19 clonal analysis only one clone contained a mutation at a resistance locus. The RT-PCR protocol was therefore optimised to be more sensitive by using primers without additional restriction sites and that would generate a shorter PCR product (JFH-3265 and xBglIIas, Table A.12 and A.5, Appendix A). With this approach, RNA could be extracted and successfully am-

plified from the J4a4a-19 cell culture on day 13. Clonal analysis identified resistance mutations at either position 156 or 168 in all 10 clones analysed of each duplicate. Figures 6.1 to 6.6 show the alignment of 10 clones from each passaging experiment. The NS4A cofactor was not analysed because no resistance loci have been described within the NS4A gene in the literature. All 6 genotypes showed substitutions at position 168, although this position differed in both the identity of the wild type encoded amino acid and the substitutions that arose during passaging (Table 6.3). In contrast, this site remained invariant in each control passage experiment performed in parallel without addition of BILN 2061. Genotypes 1b, 2a, 3a and 4a showed complete replacement of the wild type codon in both replicate passages, while replacement frequencies of genotype 5a and 6a ranged from 70-90 %. Genotypes 1b and 4a additionally showed a further substitution of the Ala residue at position 156 to Val and Gly in a proportion of clones (Table 6.3). Wherease double mutants occurred within genotype 1b clones, genotype 4a clones contained either a mutation at position 156 or 168. One of the 2 replicates from the genotype 2a recombinant showed a change at position 195 in half of the clones analysed. All other substitutions recorded among clones in either BILN 2061 or control passages occurred infrequently (0-3 among each set of 10 clones), at variable positions and were equally frequent among BILN 2061-passaged and control virus populations. The Val14Leu substitution in genotype 6a clones corresponds to the Val1040Leu cell culture adaptive mutation described in chapter 4.

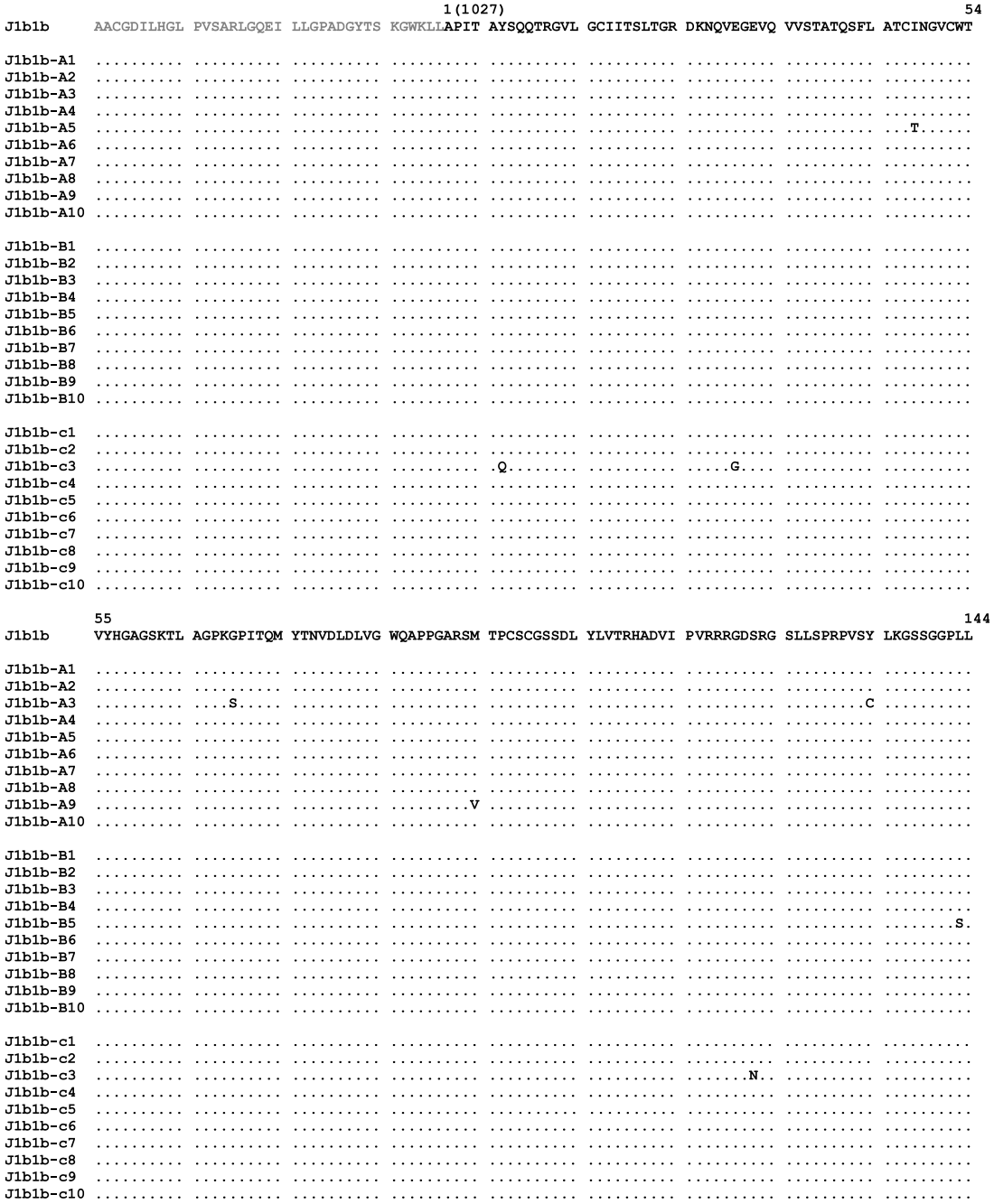

Figure 6.1: ***Acquisition of mutations in J1b1b-NS3 during passaging under BILN 2061.*** *NS3 residue 1 (1027) to 144 (1170), continued on next page.*

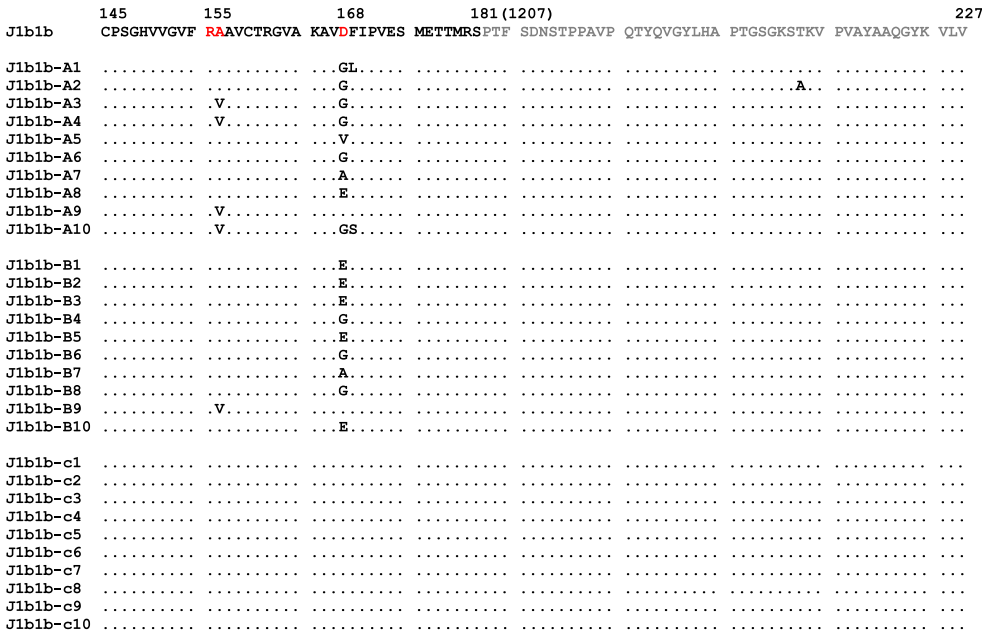

Figure 6.1: ***Acquisition of mutations in J1b1b-NS3 during passaging under BILN 2061.*** *NS3 residue 145 (1171) to 227 (1253). The J1b1b recombinant was passaged in Huh7.5 cells under increasing concentrations of BILN 2061 (23-230 nM). On day 8 of the passaging experiment viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J1b1b-A1 to -A10, J1b1b-B1 to -B10). As a control J1b1b was passaged without BILN 2061 and 10 clones analysed (J1b1b-c1 to -c10). Previously described residues where resistance mutations can arise under BILN 2061 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end and the NS3 helicase 5'end are indicated in grey.*

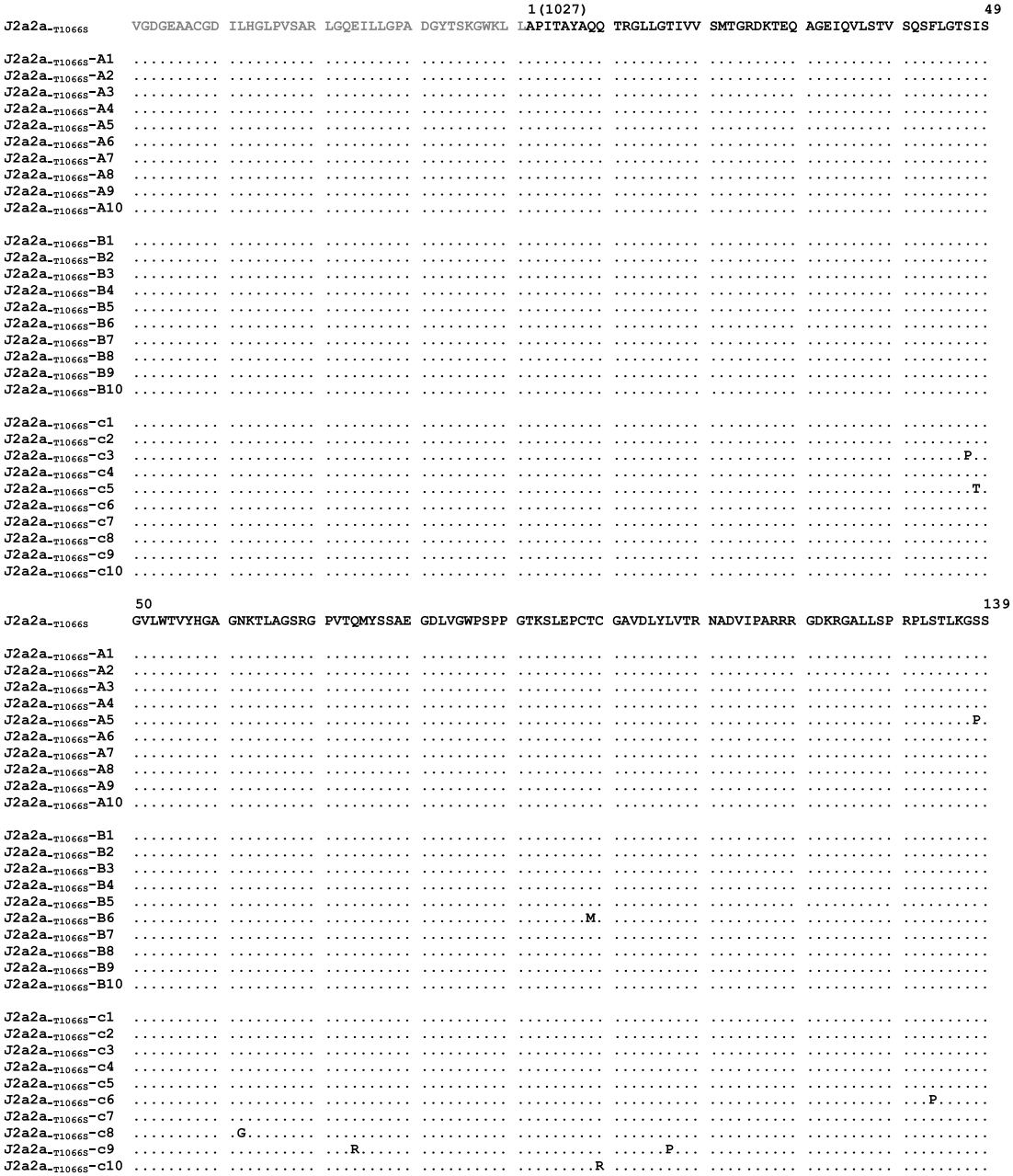

Figure 6.2: ***Acquisition of mutations in J2a2a$_{\text{-T1066S}}$-NS3 during passaging under BILN 2061.*** *NS3 residue 1 (1027) to 139 (1165), continued on next page.*

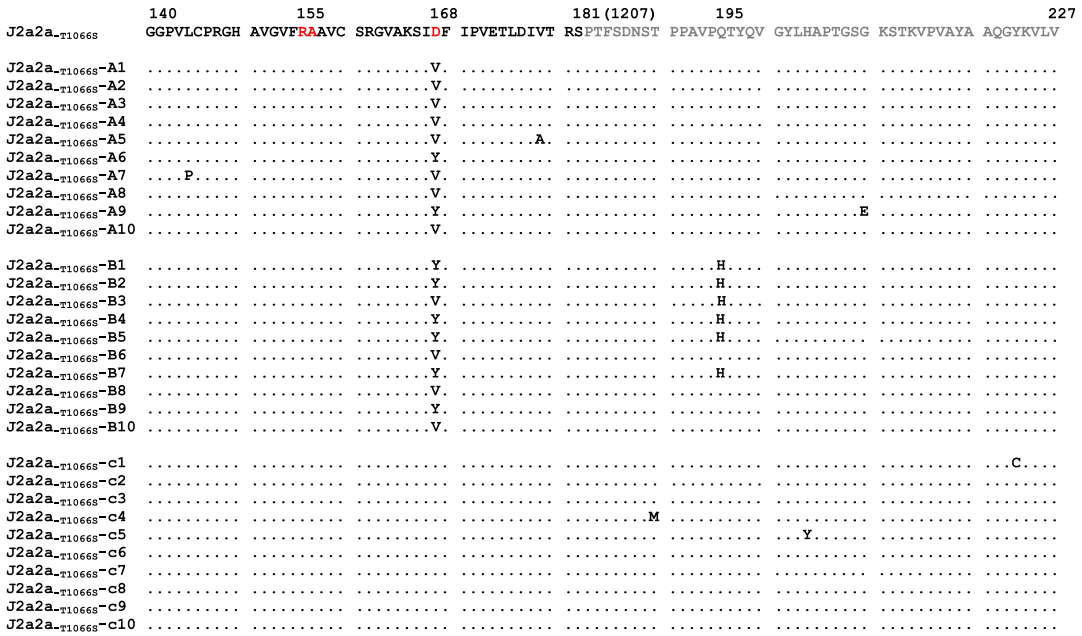

Figure 6.2: ***Acquisition of mutations in J2a2a$_{\text{-T1066S}}$-NS3 during passaging under BILN 2061.*** *NS3 residue 140 (1166) to 227 (1253). The J2a2a$_{\text{-T1066S}}$ recombinant was passaged in Huh7.5 cells under increasing concentrations of BILN 2061 (500-1,000 nM). On day 22 of the passaging experiment viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J2a2a$_{\text{-T1066S}}$-A1 to -A10, J2a2a$_{\text{-T1066S}}$-B1 to -B10). As a control J2a2a$_{\text{-T1066S}}$ was passaged without BILN 2061 and 10 clones analysed (J2a2a$_{\text{-T1066S}}$-c1 to -c10). Previously described residues where resistance mutations can arise under BILN 2061 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end and the NS3 helicase 5'end are indicated in grey.*

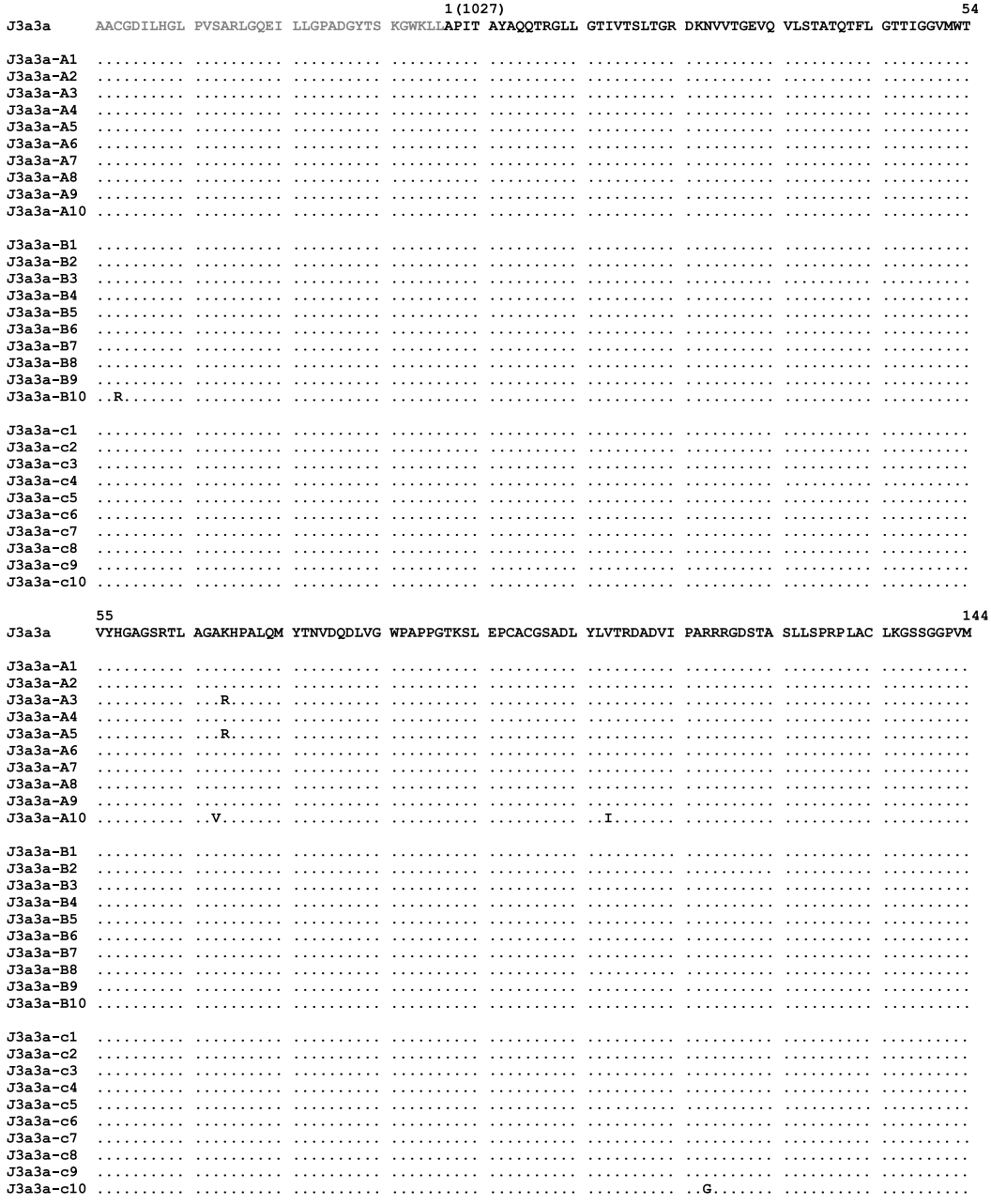

Figure 6.3: ***Acquisition of mutations in J3a3a-NS3 during passaging under BILN 2061.*** *NS3 residue 1 (1027) to 144 (1170), continued on next page.*

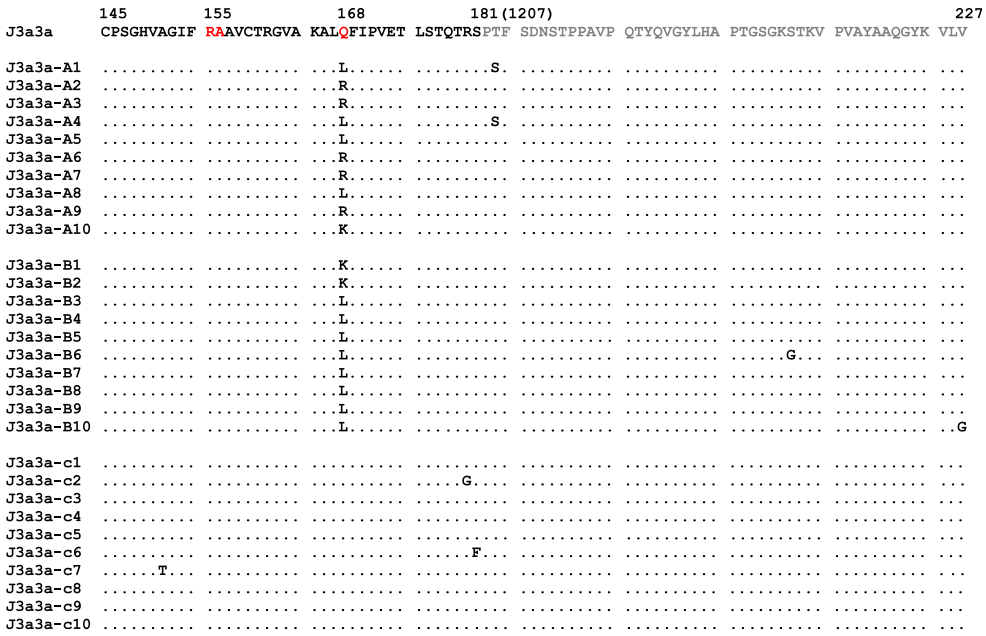

Figure 6.3: ***Acquisition of mutations in J3a3a-NS3 during passaging under BILN 2061.*** *NS3 residue 145 (1171) to 227 (1253). The J3a3a recombinant was passaged in Huh7.5 cells under increasing concentrations of BILN 2061 (35-7,000 nM). On day 21 of the passaging experiment viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J3a3a-A1 to -A10, J3a3a-B1 to -B10). As a control J3a3a was passaged without BILN 2061 and 10 clones analysed (J3a3a-c1 to -c10). Previously described residues where resistance mutations can arise under BILN 2061 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end and the NS3 helicase 5'end are indicated in grey.*

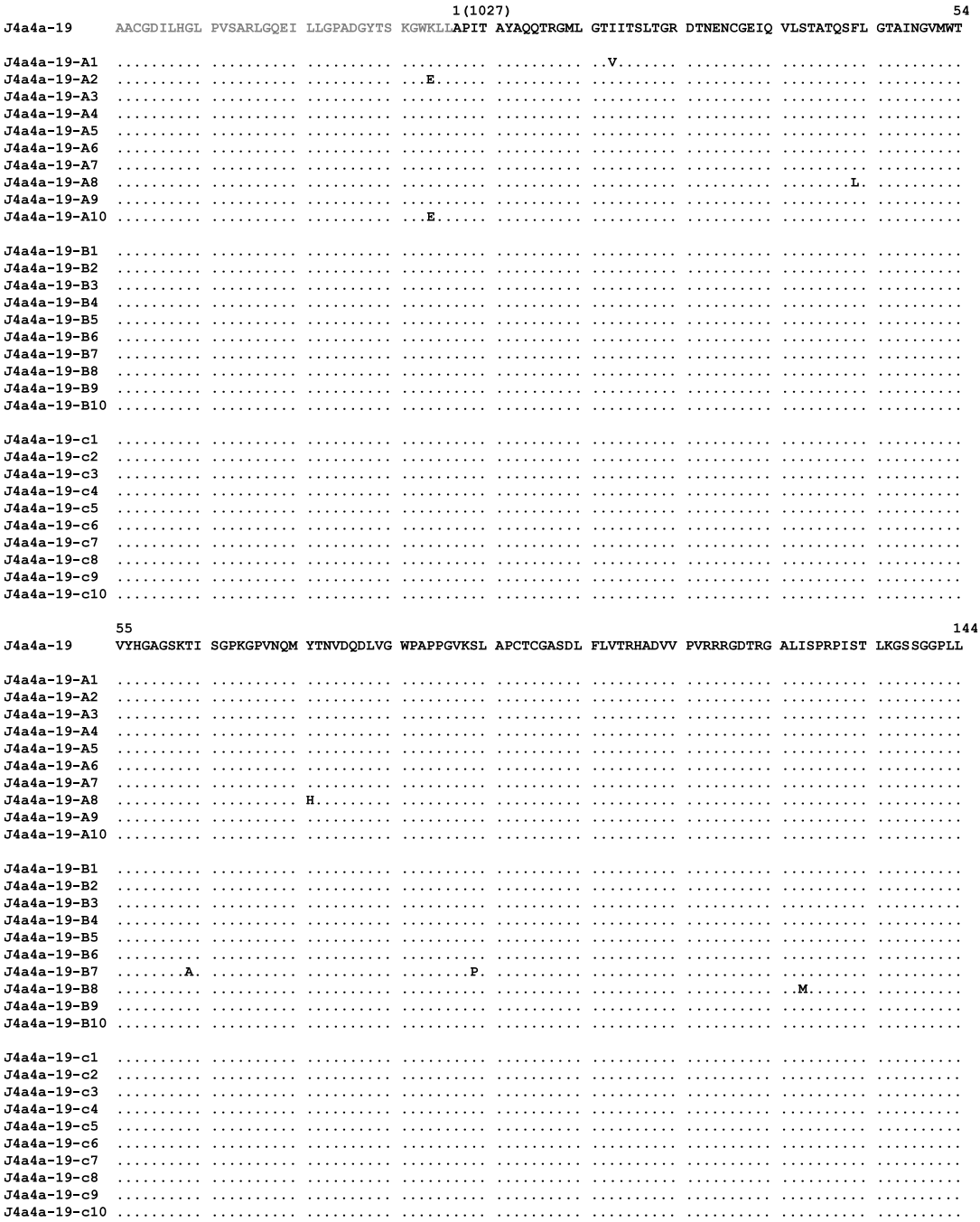

Figure 6.4: ***Acquisition of mutations in J4a4a-19-NS3 during passaging under BILN 2061.*** *NS3 residue 1 (1027) to 144 (1170), continued on next page.*

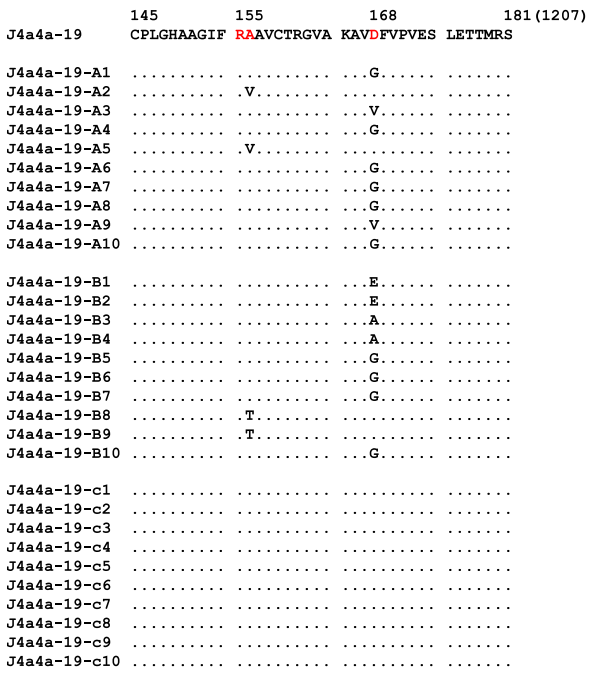

Figure 6.4: ***Acquisition of mutations in J4a4a-NS3 during passaging under BILN 2061.*** *NS3 residue 145 (1171) to 181 (1207). The J4a4a-19 recombinant was passaged in Huh7.5 cells under increasing concentrations of BILN 2061 (10-100 nM). On day 13 of the passaging experiment viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J4a4a-19-A1 to -A10, J4a4a-19-B1 to -B10). As a control J4a4a-19 was passaged without BILN 2061 and 10 clones analysed (J4a4a-19-c1 to -c10). Previously described residues where resistance mutations can arise under BILN 2061 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end is indicated in grey.*

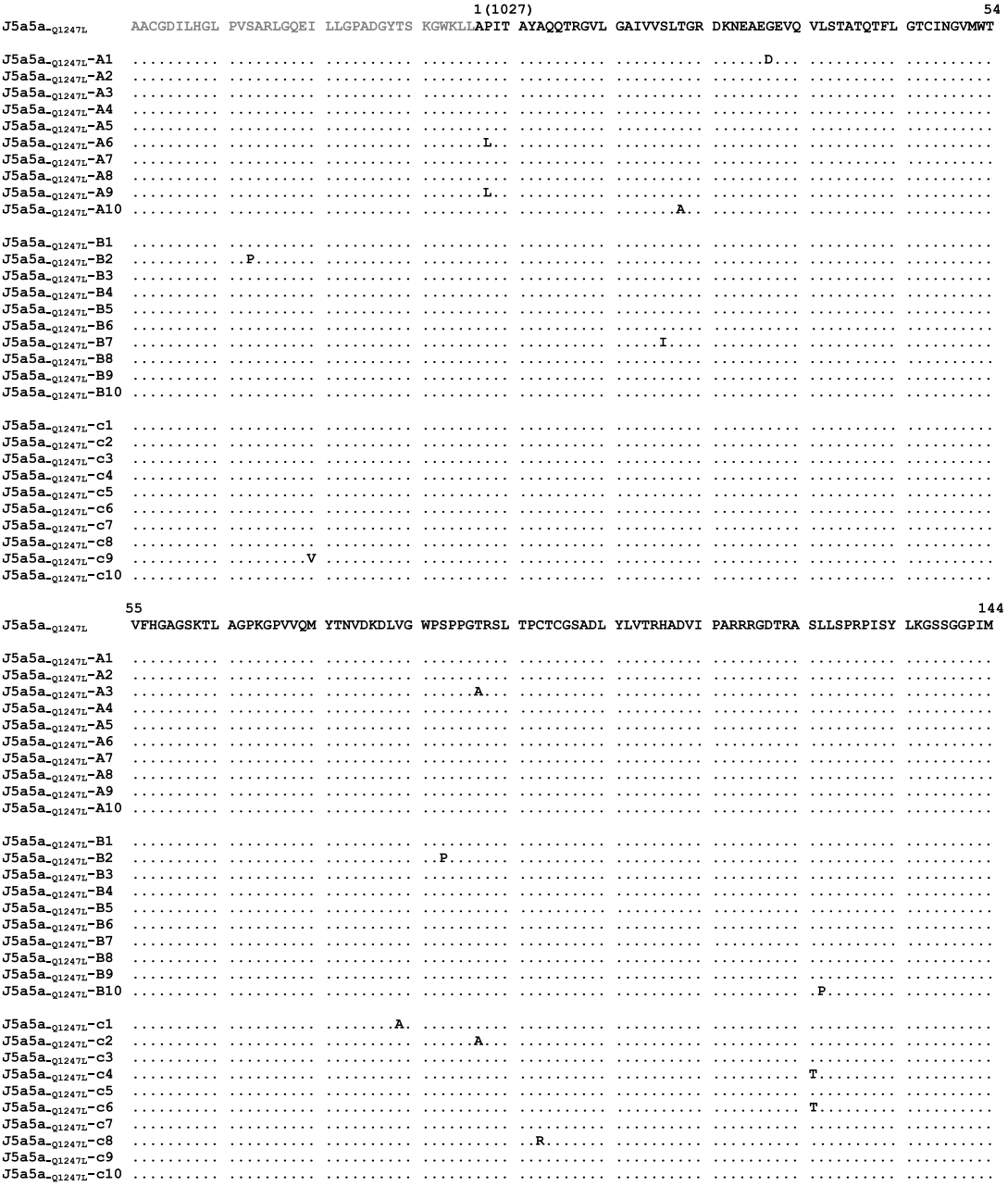

Figure 6.5: ***Acquisition of mutations in J5a5a$_{\text{-Q1247L}}$-NS3 during passaging under BILN 2061.*** *NS3 residue 1 (1027) to 144 (1170), continued on next page.*

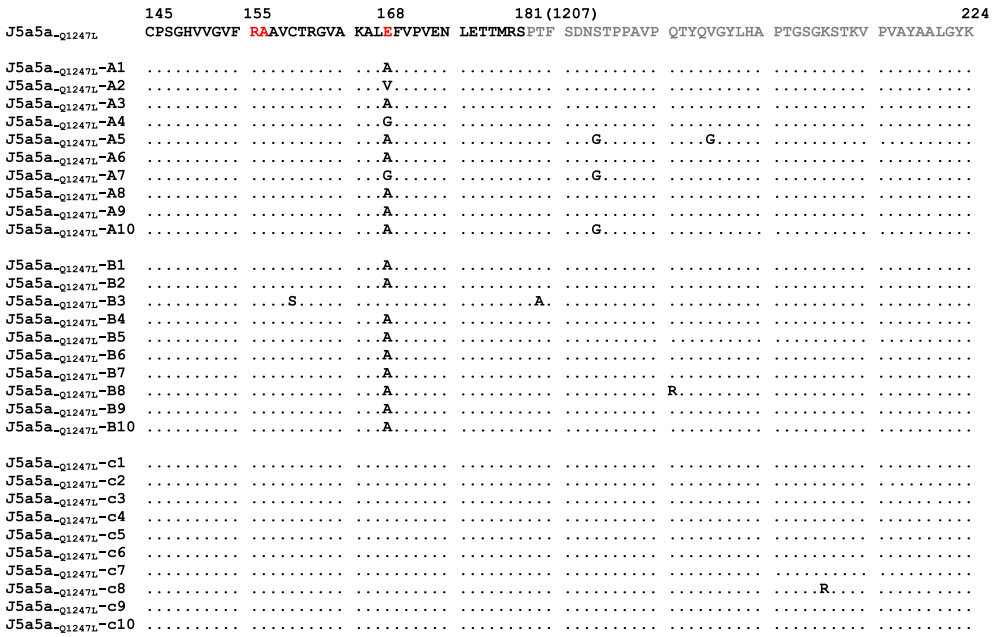

Figure 6.5: ***Acquisition of mutations in J5a5a$_{-Q1247L}$-NS3 during passaging under BILN 2061.*** *NS3 residue 145 (1171) to 224 (1250). The J5a5a$_{-Q1247L}$ recombinant was passaged in Huh7.5 cells under BILN 2061 (500 nM). On day 21 of the passaging experiment viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J5a5a$_{-Q1247L}$-A1 to -A10, J5a5a$_{-Q1247L}$-B1 to -B10). As a control J5a5a$_{-Q1247L}$ was passaged without BILN 2061 and 10 clones analysed (J5a5a$_{-Q1247L}$-c1 to -c10). Previously described residues where resistance mutations can arise under BILN 2061 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end and the NS3 helicase 5'end are indicated in grey.*

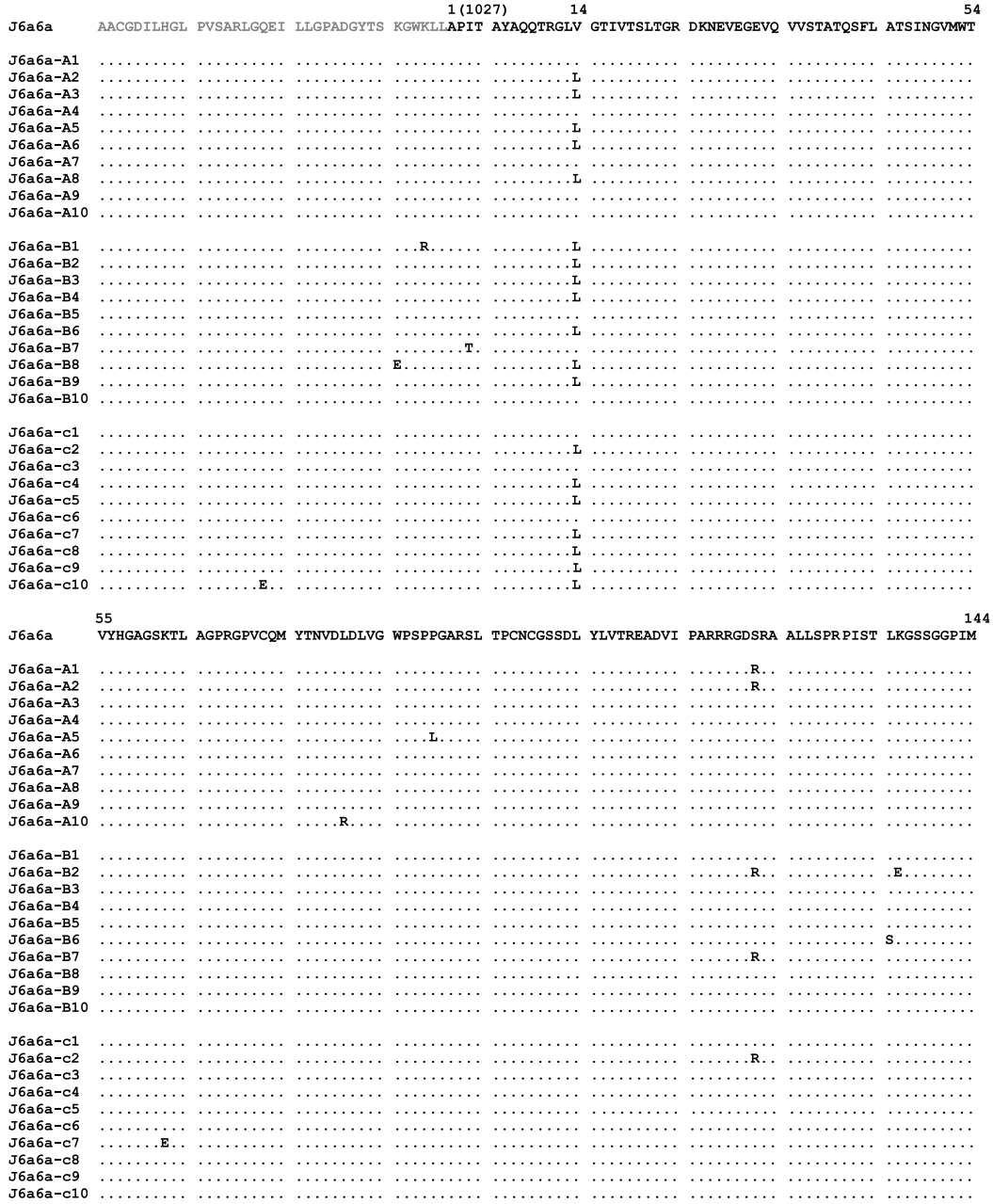

Figure 6.6: ***Acquisition of mutations in J6a6a-NS3 during passaging under BILN 2061.*** *NS3 residue 1 (1027) to 144 (1170), continued on next page.*

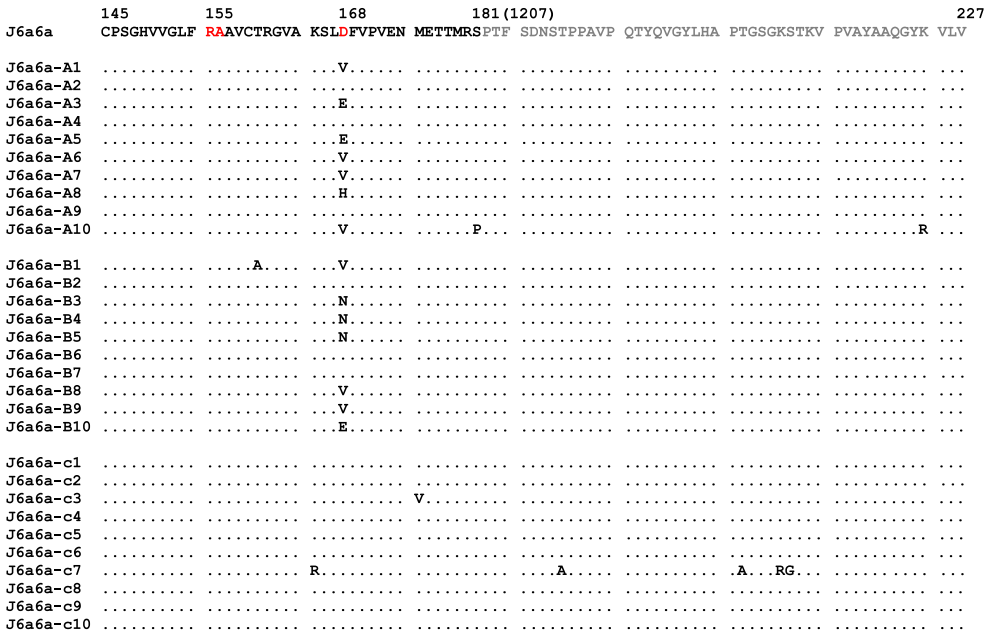

Figure 6.6: ***Acquisition of mutations in J6a6a-NS3 during passaging under BILN 2061.*** *NS3 residue 145 (1171) to 224 (1250). The J6a6a recombinant was passaged in Huh7.5 cells under increasing concentrations of BILN 2061 (1-10 nM). On day 28 of the passaging experiment viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J6a6a-A1 to -A10, J6a6a-B1 to -B10). As a control J6a6a was passaged without BILN 2061 and 10 clones analysed (J6a6a-c1 to -c10). Previously described residues where resistance mutations can arise under BILN 2061 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end and the NS3 helicase 5'end are indicated in grey.*

Table 6.3: ***Summary of mutations acquired within the Jxx NS3 protease gene during passaging under BILN 2061.***

Viral variant	BILN 2061	Day	Position	WT	% remaining WT	(mutations[2])	
	(nM)[1]				*Replicate 1*	*Replicate 2*	Control
J1b1b	230	8	168	D	0 (7G, 1A, 1E, 1V)	10 (3G, 1A, 5E)	100
			156	A	60 (4V)	90 (1V)	100
J2a2a$_{T1066S}$	1,000	22	168	D	0 (8V, 2Y)	0 (4V, 6Y)	100
			195	Q	100	40 (6H)	100
J3a3a	7,000	21	168	Q	0 (4L, 5R, 1K)	0 (8L, 2K)	100
J4a4a-19	100	13	168	D	20 (6G, 2V)	20 (4G, 2A, 2E)	100
			156	A	80 (2V)	80 (2T)	100
J5a5a$_{Q1247L}$	500	21	168	E	0 (7A, 1V, 2G)	10 (9A)	100
J6a6a	10	28	168	D	30 (4V, 2E, 1H)	30 (3V, 1E, 3N)	100

[1] *Final passaging concentration of BILN 2061.*
[2] *Proportion of clones retaining original amino acid in 2 replicate passaging experiments (10 clones analysed in each) and in a control passaged without BILN 2061 (10 clones analysed). Mutations were included in this table if they occurred in at least 4 clones, or at a position of previously identified resistance mutations. WT, wild type.*

6.2.2 *In vitro* selection of VX-950-resistant recombinant viruses

The development of antiviral resistance in the Jxx recombinants upon VX-950 treatment was investigated as described in section 6.2.1 for BILN 2061. As resistance development was observed with BILN 2061 concentrations as low as 1-2 $\times$ IC_{50} and because only a limited amount of VX-950 was available, the passaging experiment was just carried out with the 3 recombinants J1b1b, J3a3a and J4a4a-19 and PI concentrations of 2-3 $\times$ IC_{50}. An additional obstacle were the relatively high IC_{50} values for VX-950 on Jxxs (650-2,230 nM). Table 6.4 provides the individual schedules for each passaging experiment. As described in section 6.2.1, RNA was extracted from cell pellets at the end of the passaging experiments and clonal analysis of the NS3 protease gene performed (Fig. 6.7 to 6.9). None of the genotypes showed significant substitution rates at any of the described resistance loci (NS3 protease residues 36, 41, 43, 54, 155, 156 and 170) (Table 6.1). This is presumably due to the low PI concentrations in combination with short passaging times (for genotype 1b and 4a). Nevertheless, a few potential resistance mutations were identified (Table 6.5).

Table 6.4: ***Schedule for inducing antiviral (AV) resistance upon VX-950 treatment.*** *PE, post-electroporation.*

Clone	Day PE of AV addition	AV concentration	Days of AV pressure
J1b1b	3	13 days 1,800 nM	13
J3a3a	3	23 days 5,000 nM	23
J4a4a-19	3	13 days 5,000 nM	13

One genotype 1b clone had a Val36Ala substitution, a resistance mutation described in the literature (Table 6.1). In addition, replacement of the wild type codon was observed at position 174 in both replicates. Even though this residue has not been described previously as a resistance locus, the fact that it developed in both replicates but in no other passaging experiment, strongly suggests that it is a resistance mutation towards VX-950. Genotype 3a showed almost complete replacement of the wild type codon at position 77. Again, this position has not been described as a resistance locus towards VX-950, but as it developed in both replicates, involvement in resistance towards VX-950 is suggested. Genotype 4a showed no obvious resistance development. However, one clone each contained a substitution at position 36, 54 and 156, which have been described as resistance loci in the literature (Table 6.1).

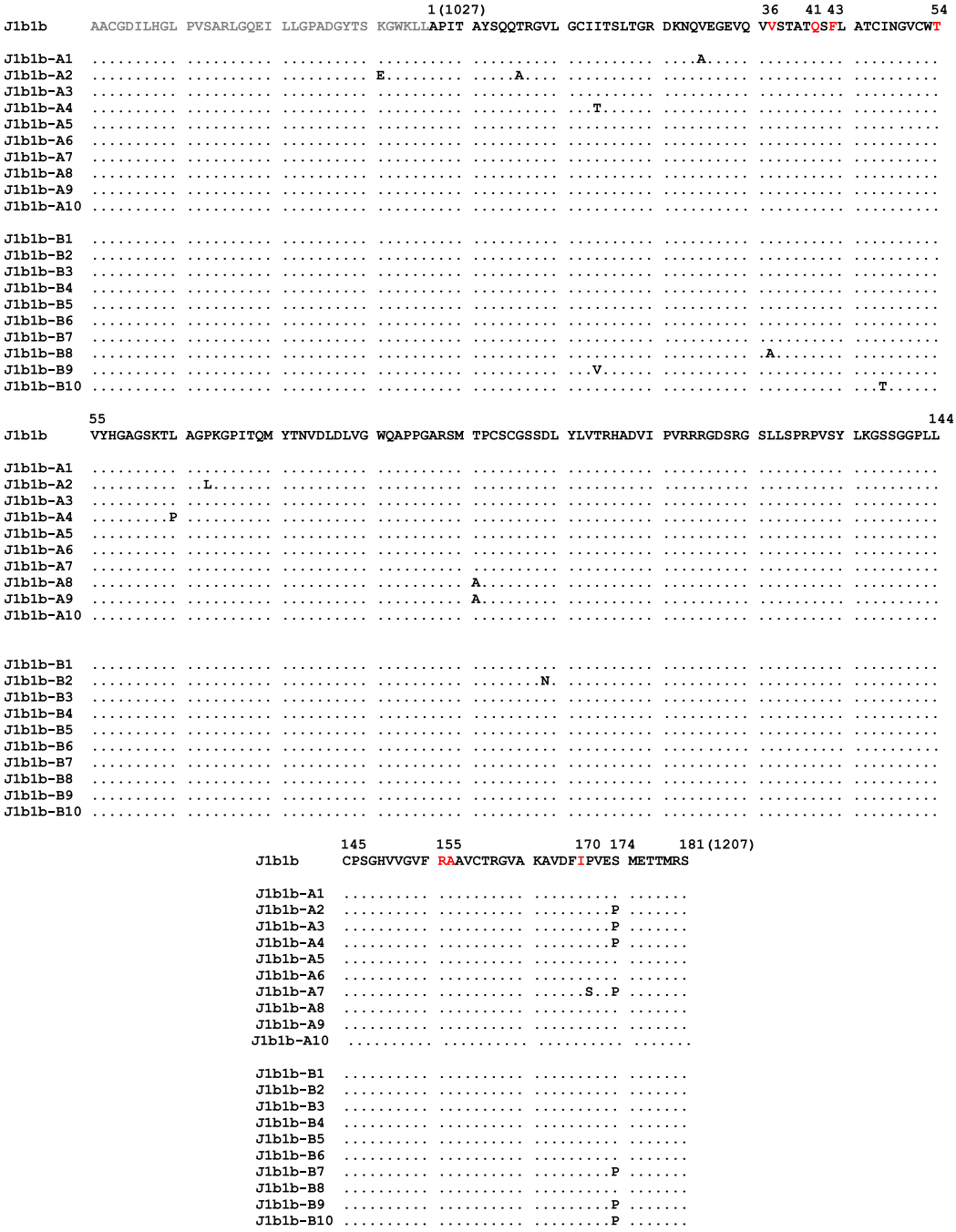

Figure 6.7: ***Acquisition of mutations in J1b1b-NS3 during passaging under VX-950.*** *NS3 residue 1 (1027) to 181 (1207). The J1b1b recombinant was passaged in Huh7.5 cells under VX-950 (1,800 nM) for 13 days. Viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J1b1b-A1 to -A10, J1b1b-B1 to -B10). Previously described residues where resistance mutations can arise under VX-950 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end is indicated in grey.*

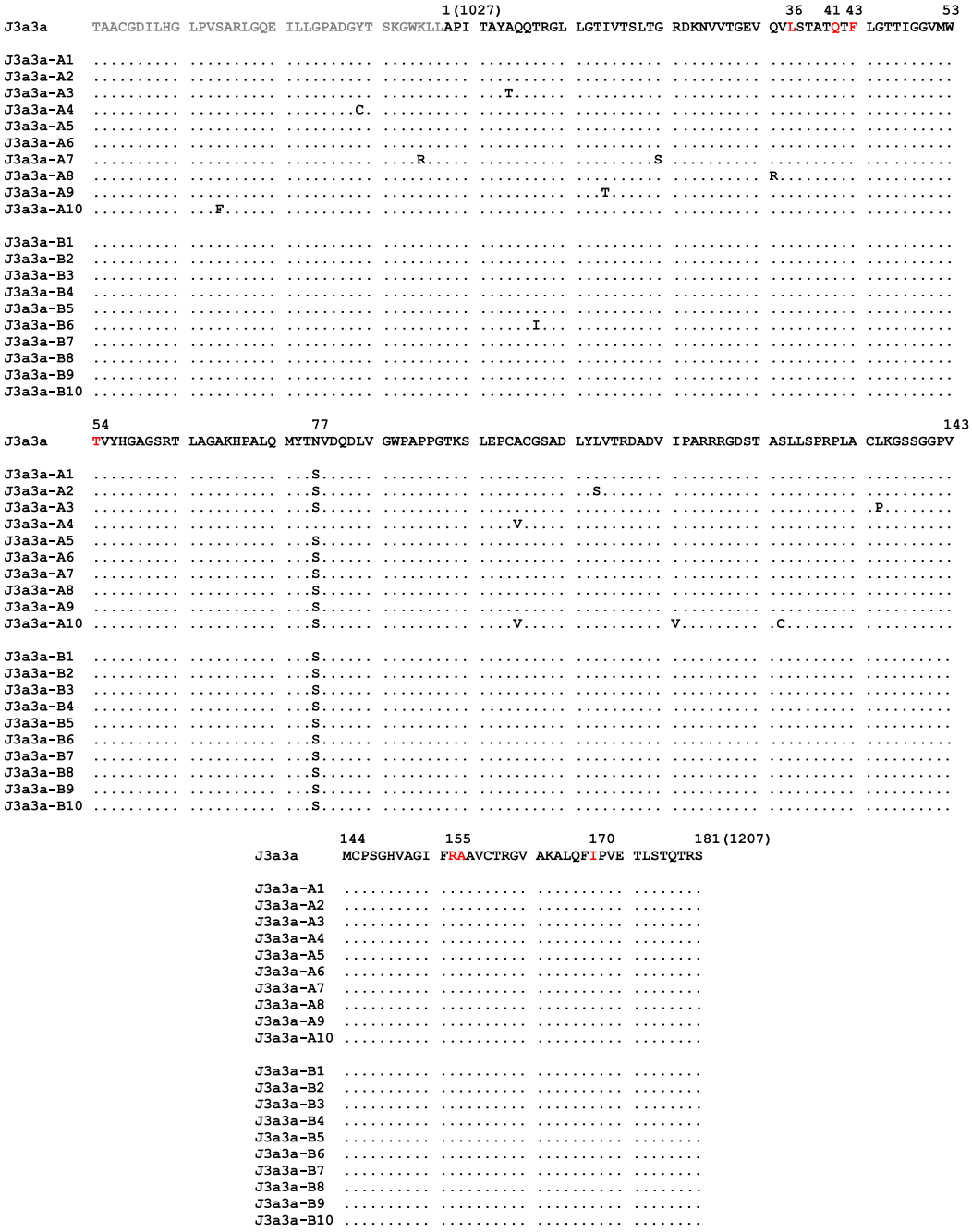

Figure 6.8: ***Acquisition of mutations in J3a3a-NS3 during passaging under VX-950.*** *NS3 residue 1 (1027) to 181 (1207). The J3a3a recombinant was passaged in Huh7.5 cells under VX-950 (5,000 nM) for 23 days. Viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J3a3a-A1 to -A10, J3a3a-B1 to -B10). Previously described residues where resistance mutations can arise under VX-950 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end is indicated in grey.*

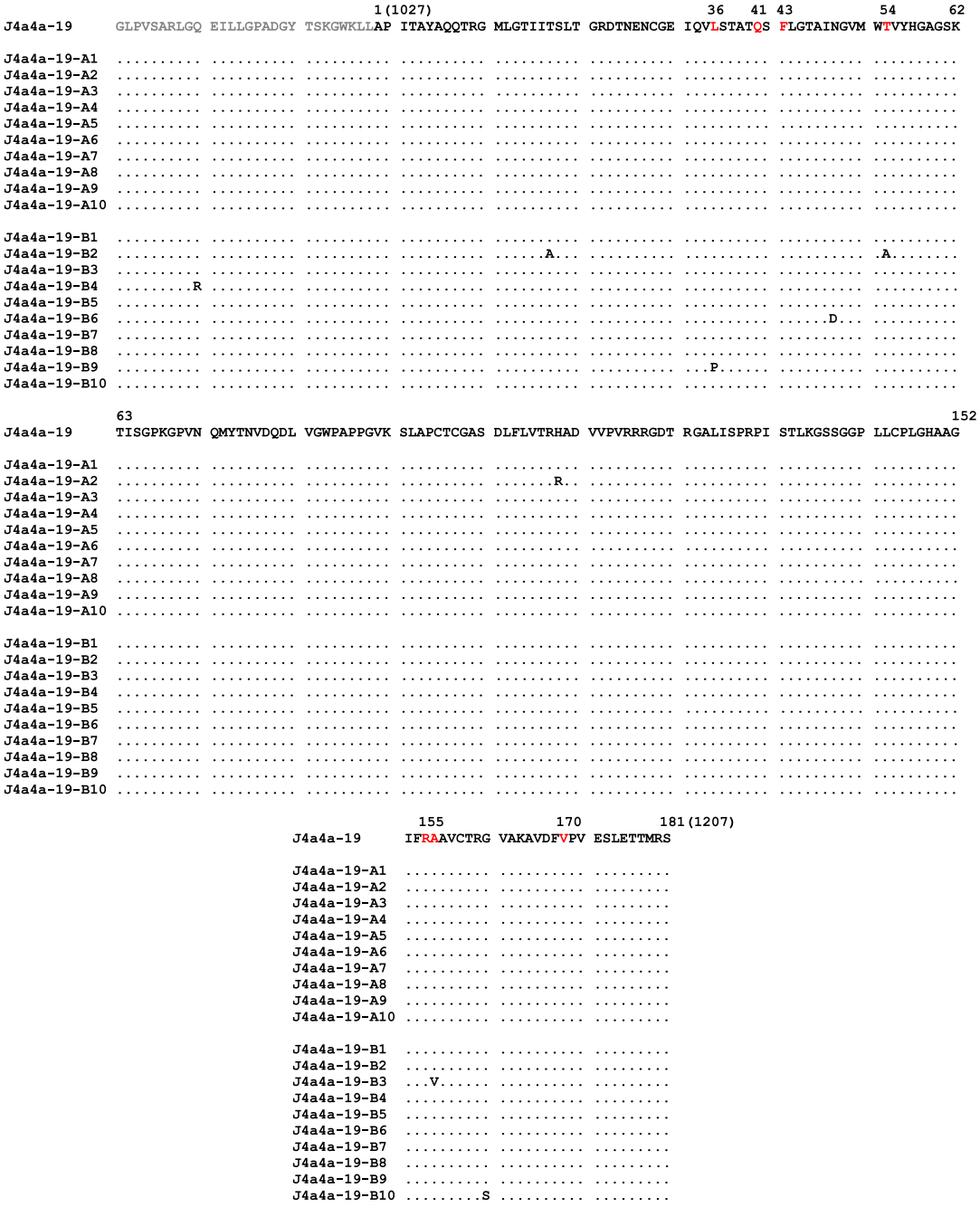

Figure 6.9: ***Acquisition of mutations in J4a4a-19-NS3 during passaging under VX-950.*** *NS3 residue 1 (1027) to 181 (1207). The J4a4a-19 recombinant was passaged in Huh7.5 cells under VX-950 (5,000 nM) for 13 days. Viral RNA was extracted from cells and the NS3 protease gene cloned into the TOPO cloning vector. The passaging experiment was carried out in duplicate and 10 clones analysed in each (J4a4a-19-A1 to -A10, J4a4a-19-B1 to -B10). Previously described residues where resistance mutations can arise under VX-950 are indicated in red; the NS3 protease domain is indicated in black; the NS2 3'end is indicated in grey.*

Table 6.5: ***Summary of mutations acquired within the Jxx NS3 protease gene during passaging under VX-950.***

Viral variant	VX-950	Day	Position	WT	% remaining WT	(mutations[2])	
	(nM)[1]				*Replicate 1*	*Replicate 2*	*Control*
J1b1b	1,800	13	36	V	100	90 (1A)	100
			174	S	60 (4P)	70 (3P)	100
J3a3a	5,000	23	77	N	10 (9S)	0 (10S)	100
J4a4a-19	5,000	13	36	L	100	90 (1P)	100
			54	T	100	90 (1A)	100
			156	A	100	90 (1V)	100

[1] *Final passaging concentration of VX-950.*
[2] *Proportion of clones retaining original amino acid in 2 replicate passaging experiments (10 clones analysed in each) and in a control passaged without VX-950 (10 clones analysed). Mutations were included in this table if they occurred in at least 4 clones, or at a position of previously identified resistance mutations. WT, wild type.*

6.2.3 Influence of BILN 2061-resistance mutations on recombinant viruses replication kinetics

To investigate the influence of the resistance mutations identified in section 6.2.1, individual mutations were reintroduced into the corresponding Jxx recombinants. For genotype 2a, 3a, 5a and 6a, where extracted RNA levels were high, the fragment including the NS3 protease gene plus resistance mutations was directly ligated into the corresponding Jxx recombinant. For genotype 2a, 5a and 6a the cell culture attenuated/adapted recombinants were used for mutant construction. Jxxs including only one resistance mutation and no other mutations were identified through clonal analysis and RNA prepared from the desired clone. For genotype 1b and 4a, where extracted RNA levels were low and alternative primers without restriction sites had to be used, mutations were introduced through site directed mutagenesis. To reduce potential PCR-induced errors, the naturally occurring restriction sites *Not*I and *Spe*I were used to subclone a fragment including the HCV NS3 protease domain into TOPO. After site directed mutagenesis on the TOPO clone and sequence verification, the insert was cloned back into the corresponding Jxx recombinant and RNA prepared from it.

The replication fitness of each mutant recombinant was then assessed in the Huh7.5 cell culture system (Fig. 6.10 to 6.12).

The J1b1b-D168G mutant replicated to similar levels as the wild type J1b1b, whereas replication fitness of the J1b1b-A156V and the J1b1b-A156V-D168G mutants were slightly reduced (Fig. 6.10 top). The D168V substitution completely abrogated the replication ability of J2a2a$_{\text{-T1066S}}$ or reduced it to undetectable levels, whereas the J2a2a$_{\text{-T1066S}}$-D168Y mutant replicated similarly to wild type (Fig. 6.10 bottom). The 2 genotype 3a mutants J3a3a-Q168L and -Q168K replicated similarly to the wild type, whereas the Q168R mutation reduced the replicative fitness of J3a3a after an initial spread (Fig. 6.11 top). For genotype 5a 3 mutants were tested. The E168A mutation did not noticeably affect the replication kinetics of J5a5a$_{\text{-Q1247L}}$. The E168G mutation reduced the fitness of J5a5a$_{\text{-Q1247L}}$ after an initial spread, whereas the E168V mutation slowed the spread of J5a5a$_{\text{-Q1247L}}$ (Fig. 6.11 bottom). The replication kinetics of J6a6a$_{\text{-V1040L}}$ were not noticeably affected by the D168E and D168H mutation, but detectable replication was completely abrogated by the D168V mutation (Fig. 6.12).

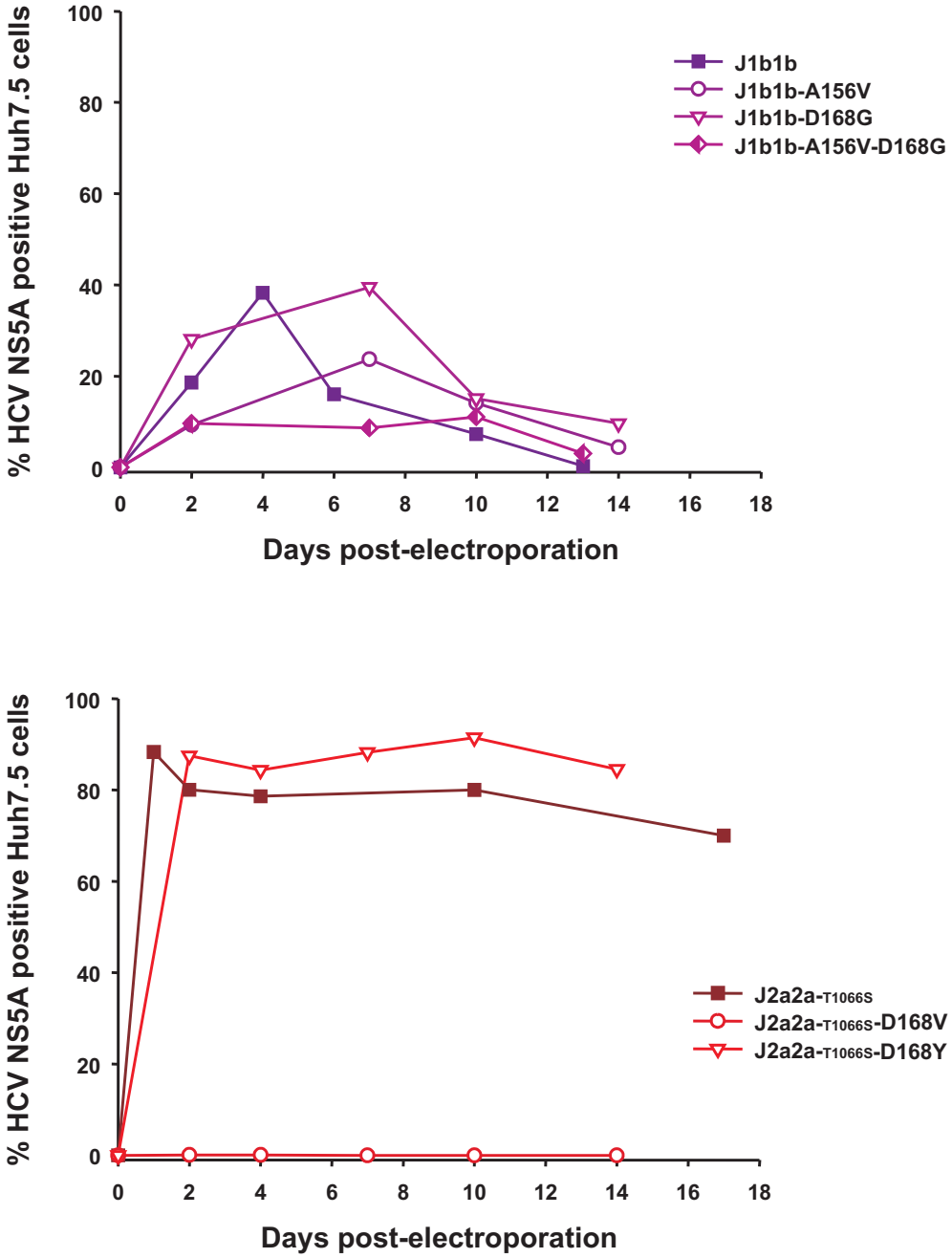

Figure 6.10: ***Influence of BILN 2061-resistance mutations on the recombinants J1b1b and J2a2a$_{\text{-T1066S}}$.*** *Resistance mutations identified during passaging of the J1b1b (top) or J2a2a$_{\text{-T1066S}}$ (bottom) recombinant under BILN 2061 were introduced into the original recombinant and their replication kinetics assessed in the Huh7.5 cell culture system. 10 μg of RNA was electroporated into Huh7.5 cells and cells incubated for 24 hours. The cells were then stained for NS5A or alternatively passaged and assessed for NS5A positive cells every 3 to 4 days. The transcript from the J2a2a$_{\text{-T1066S}}$-D168V clone showed no detectable replication (all values lying on the x-axis line).*

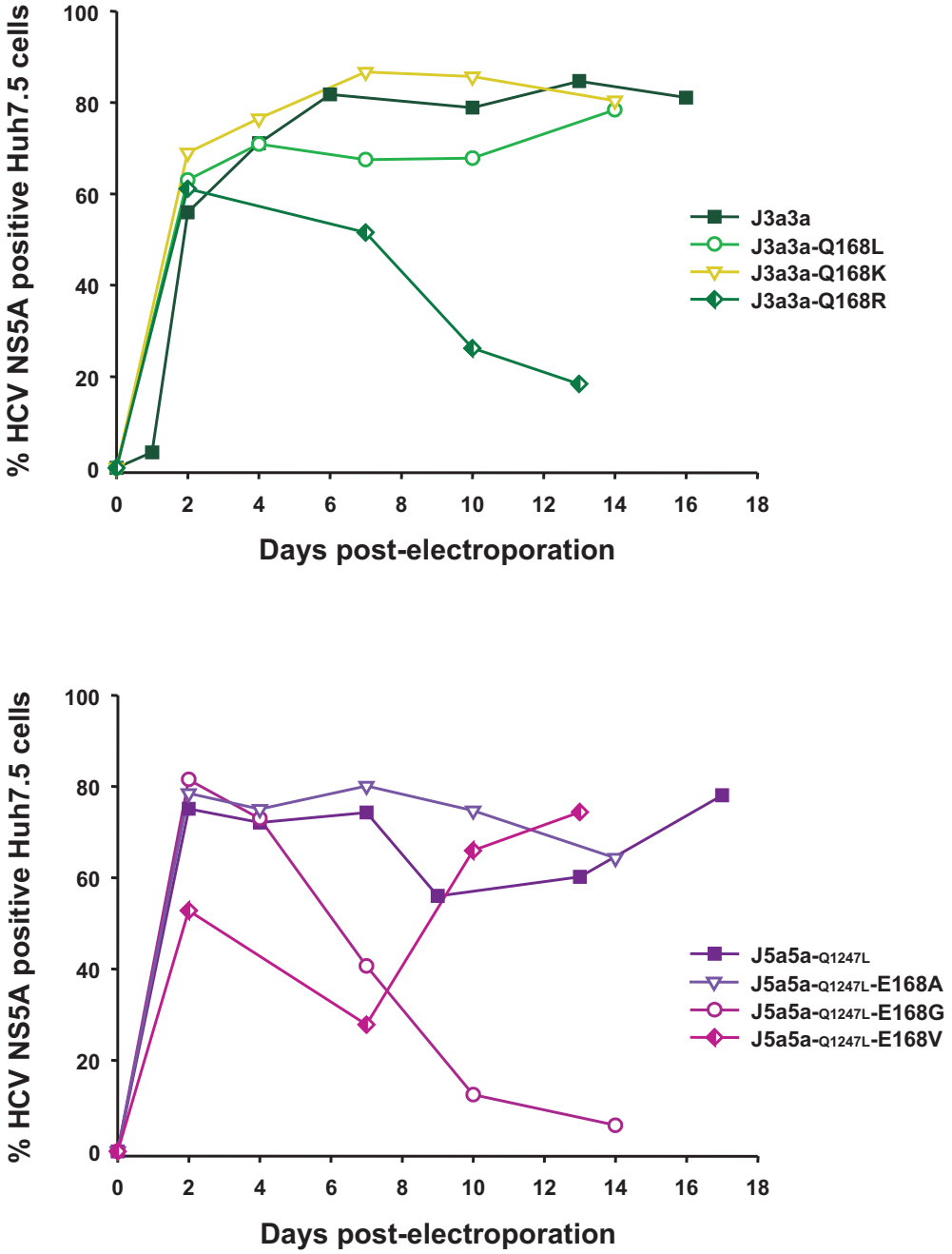

Figure 6.11: ***Influence of BILN 2061-resistance mutations on the recombinants J3a3a and J5a5a$_{-Q1247L}$.*** *Resistance mutations identified during passaging of the J3a3a (top) or J5a5a$_{-Q1247L}$ (bottom) recombinant under BILN 2061 were introduced into the original recombinant and their replication kinetics assessed in the Huh7.5 cell culture system. 10 μg of RNA was electroporated into Huh7.5 cells and cells incubated for 24 hours. The cells were then stained for NS5A or alternatively passaged and assessed for NS5A positive cells every 3 to 4 days.*

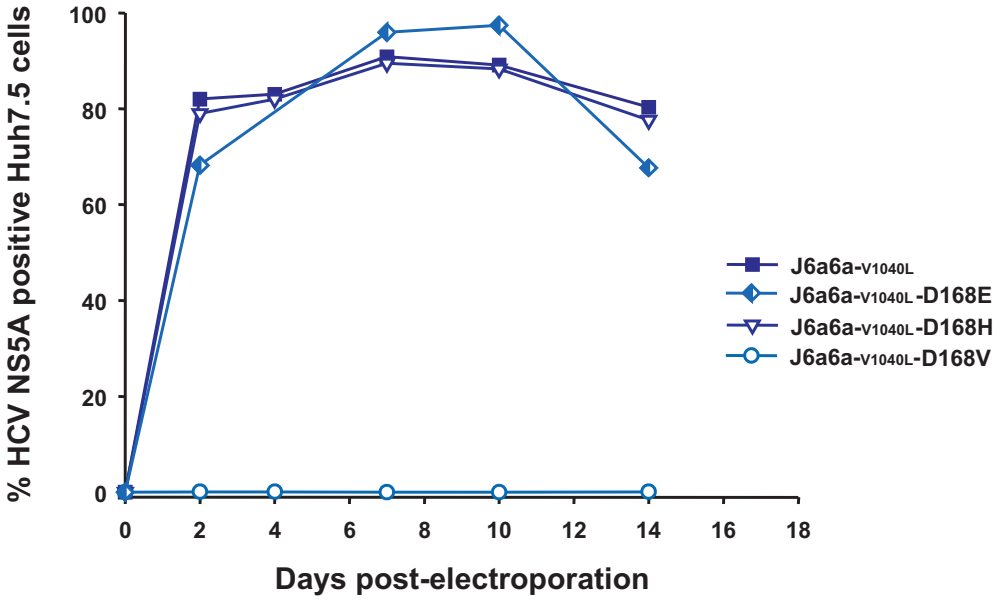

Figure 6.12: ***Influence of BILN 2061-resistance mutations on the J6a6a$_{\text{-V1040L}}$ recombinant.*** *Resistance mutations identified during passaging of the J6a6a recombinant under BILN 2061 were introduced into the original recombinant and their replication kinetics assessed in the Huh7.5 cell culture system. 10 μg of RNA was electroporated into Huh7.5 cells and cells incubated for 24 hours. The cells were then stained for NS5A or alternatively passaged and assessed for NS5A positive cells every 3 to 4 days. The transcript from the J6a6a$_{\text{-V1040L}}$-D168V clone showed no detectable replication (all values lying on the x-axis line).*

6.2.4 BILN 2061 susceptibility of recombinant viruses including BILN 2061-resistance mutations

To assess the impact of resistance mutations identified in section 6.2.1 on BILN 2061 susceptibility, IC_{50} values of Jxx recombinants including resistance mutations described in section 6.2.3, were measured and compared with the wild type (Fig. 6.13-6.17, Table 6.6).

The cell culture adapted/attenuated recombinants J6a6a$_{\text{-V1040L}}$, J2a2a$_{\text{-T1066S}}$ and J5a5a$_{\text{-Q1247L}}$ showed similar IC_{50} values as the wild type recombinants J6a6a, J2a2a and J5a5a (Table 6.6). The A156V mutation provided about 4-fold higher resistance to J1b1b towards BILN 2061 than the D168G mutation (277- vs. 67-fold change in IC_{50} value). Surprisingly, the double mutant J1b1b-A156V-D168G was more susceptible to the PI than J1b1b-A156V but more resistant than J1b1b-D168G. The genotype 2a resistance mutation D168Y only resulted in a very moderate 7-fold increase in the IC_{50} value of J2a2a$_{\text{-T1066S}}$, whereas the genotype 3a resistance mutations Q168K, Q168L and Q168R increased the resistance profile of the J3a3a recombinant 51-, 30- and 43-fold, respectively. Mutations identified for genotype 5a rendered the J5a5a$_{\text{-Q1247L}}$ recombinant only moderately resistant, with E168A increasing the IC_{50} value 5-fold, E168G 1.6-fold and E168V 8-fold. The highest impact on resistance towards BILN 2061 had the D168H mutation on the J6a6a$_{\text{-V1040L}}$ recombinant. The J6a6a$_{\text{-V1040L}}$-D168H mutant recombinant was 527-fold less susceptible to BILN 2061 than the wild type recombinant J6a6a$_{\text{-V1040L}}$. The second mutation identified in genotype 6a, D168E, resulted in a 60-fold increased resistance profile for J6a6a$_{\text{-V1040L}}$.

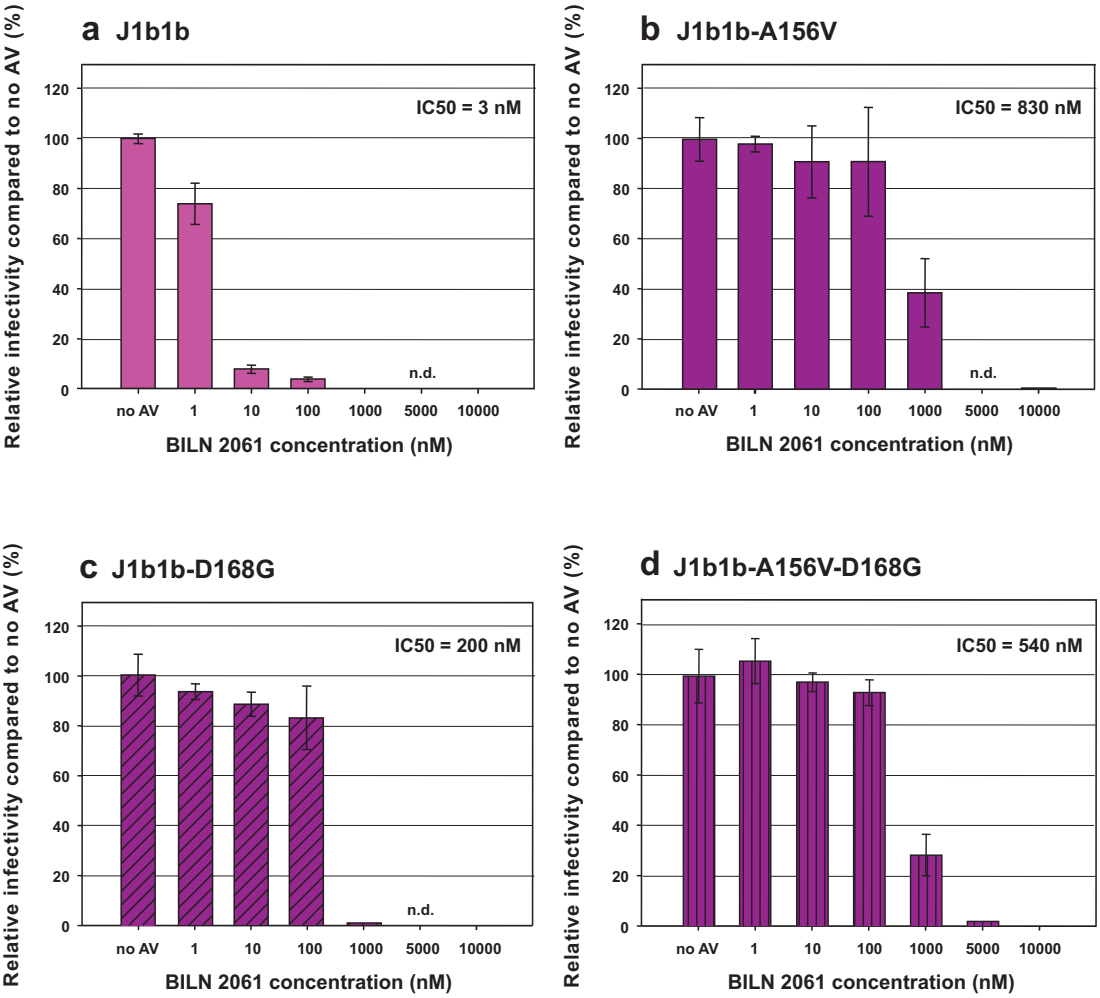

Figure 6.13: ***BILN 2061 susceptibility of J1b1b recombinants including resistance mutations (J1b1b-rm).*** *Resistance mutations identified during passaging of the J1b1b recombinant under BILN 2061 were introduced into the original J1b1b recombinant to create J1b1b-A156V, J1b1b-D168G and J1b1b-A156V-D168G. 1-10 μg (a) J1b1b and (b)-(d) J1b1b-rm RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral. n.d., not determined.*

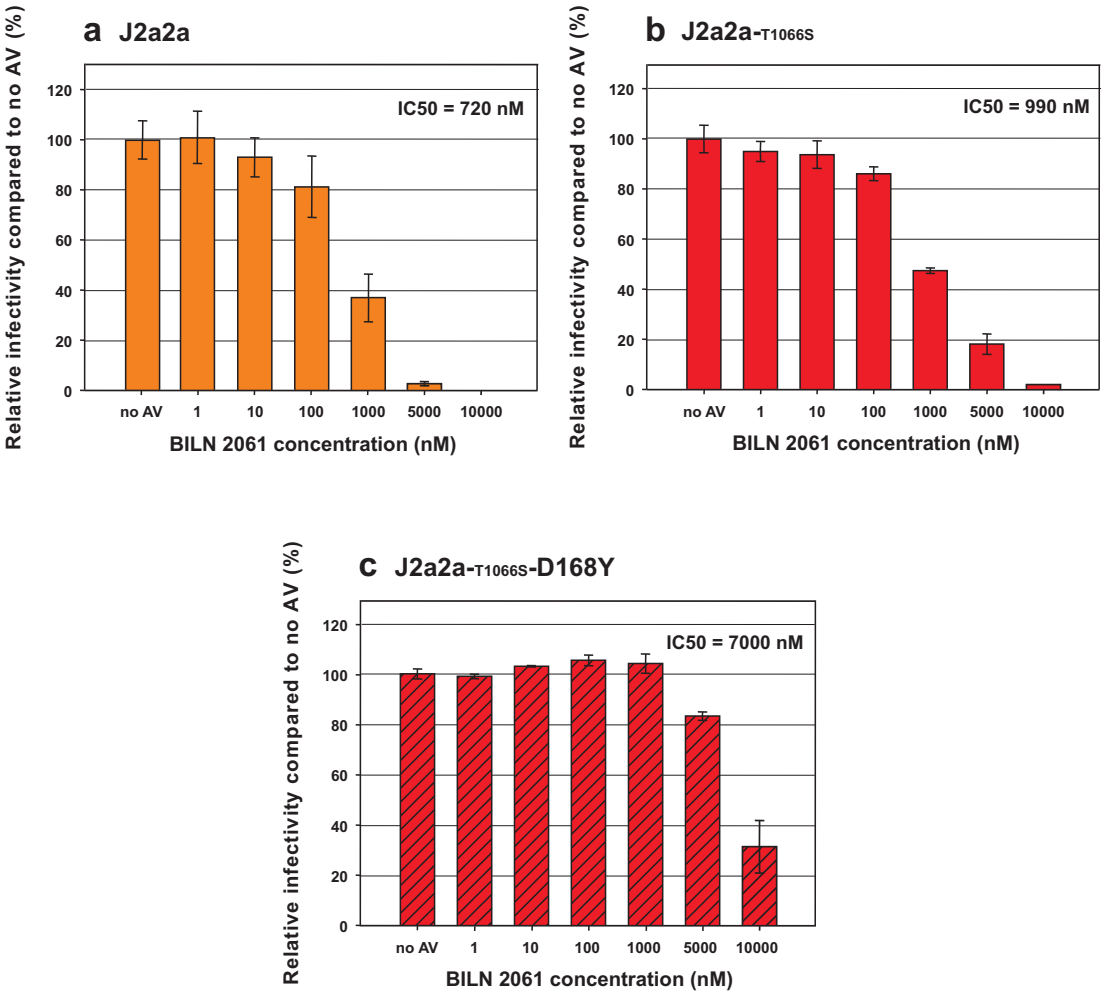

Figure 6.14: ***BILN 2061 susceptibility of J2a2a recombinants including attenuating and resistance mutations (J2a2a-rm).*** *Resistance mutations identified during passaging of the J2a2a$_{\text{-T1066S}}$ recombinant under BILN 2061 were introduced into the original J2a2a$_{\text{-T1066S}}$ recombinant to create J2a2a$_{\text{-T1066S}}$-D168V and J2a2a$_{\text{-T1066S}}$-D168Y. 1-10 μg (a) J2a2a, (b) J2a2a$_{\text{-T1066S}}$ and (c) J2a2a$_{\text{-T1066S}}$-D168Y RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral. J2a2a$_{\text{-T1066S}}$-D168V recombinant replication was below detection limit and was not assessed for BILN 2061 susceptibility.*

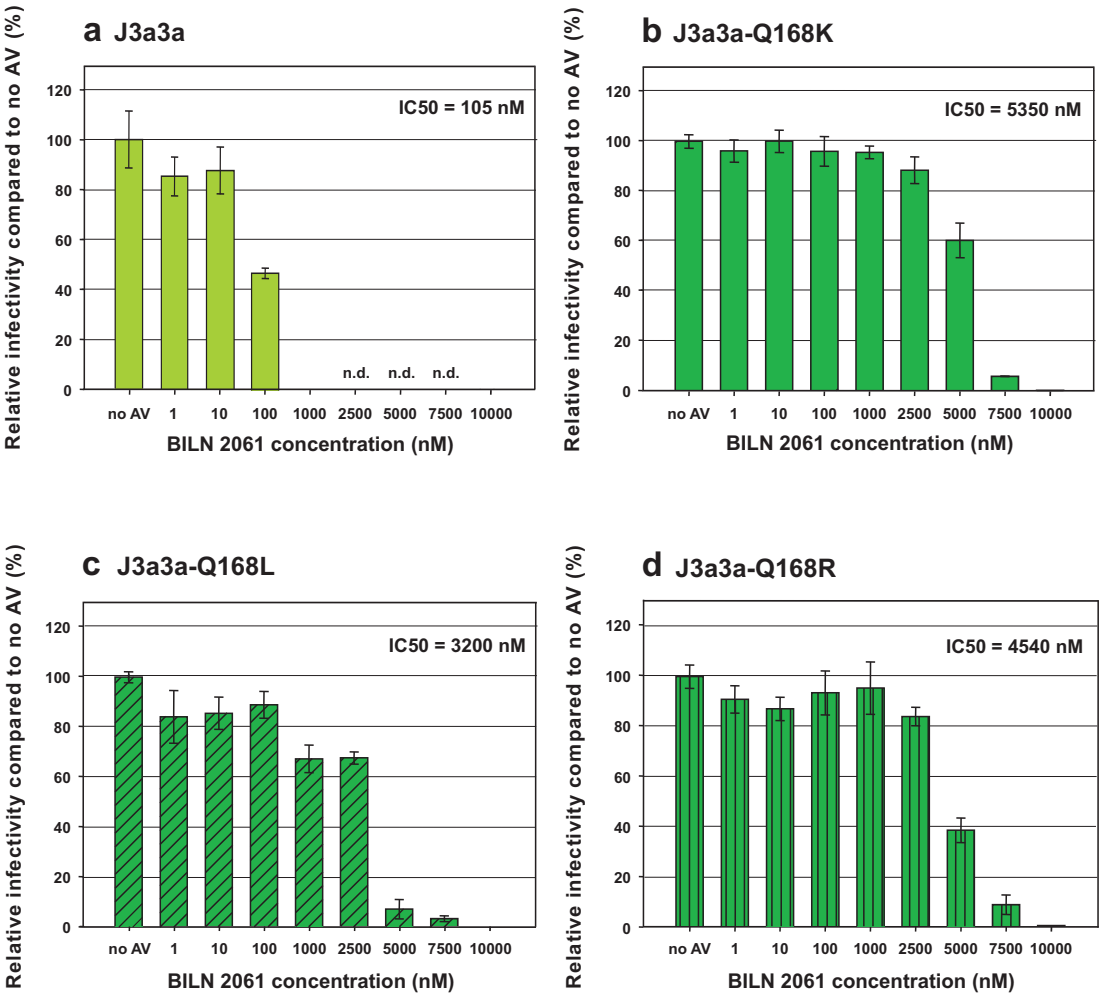

Figure 6.15: ***BILN 2061 susceptibility of J3a3a recombinants including resistance mutations (J3a3a-rm).*** *Resistance mutations identified during passaging of the J3a3a recombinant under BILN 2061 were introduced into the original J3a3a recombinant to create J3a3a-Q168K, J3a3a-Q168L and J3a3a-Q168R. 1-10 μg (a) J3a3a and (b)-(d) J3a3a-rm RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral.*

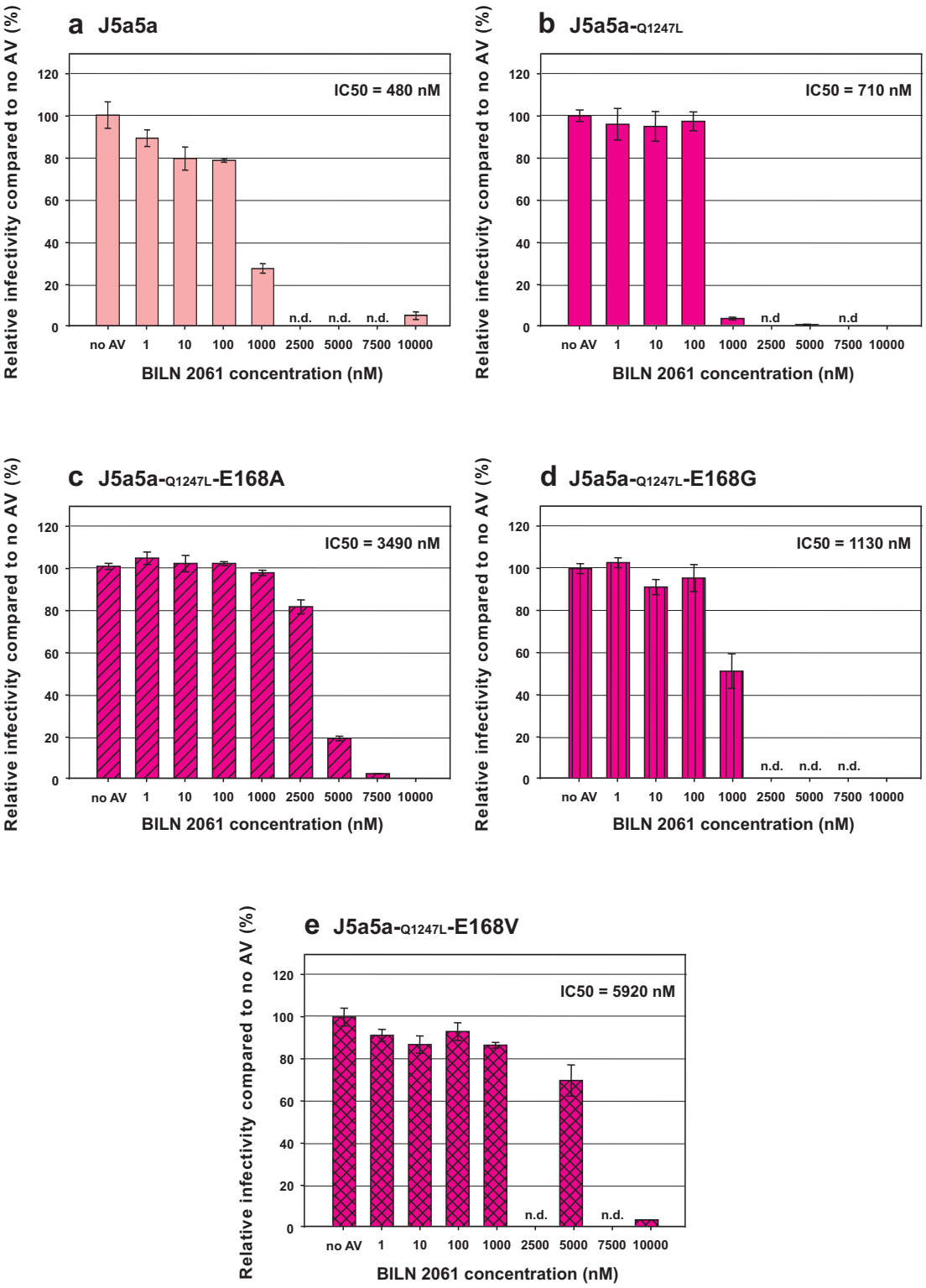

Figure 6.16: ***BILN 2061 susceptibility of J5a5a recombinants including attenuating and resistance mutations (J5a5a$_{-Q1247L}$-rm).***

Figure 6.16: ***BILN 2061 susceptibility of J5a5a recombinants including attenuating and resistance mutations (J5a5a$_{-Q1247L}$-rm).*** *Resistance mutations identified during passaging of the J5a5a$_{-Q1247L}$ recombinant under BILN 2061 were introduced into the original J5a5a$_{-Q1247L}$ recombinant to create J5a5a$_{-Q1247L}$-E168A, J5a5a$_{-Q1247L}$-E168G and J5a5a$_{-Q1247L}$-E168V. 1-10 μg (a) J5a5a, (b) J5a5a$_{-Q1247L}$ and (c)-(e) J5a5a-rm RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral. n.d., not determined.*

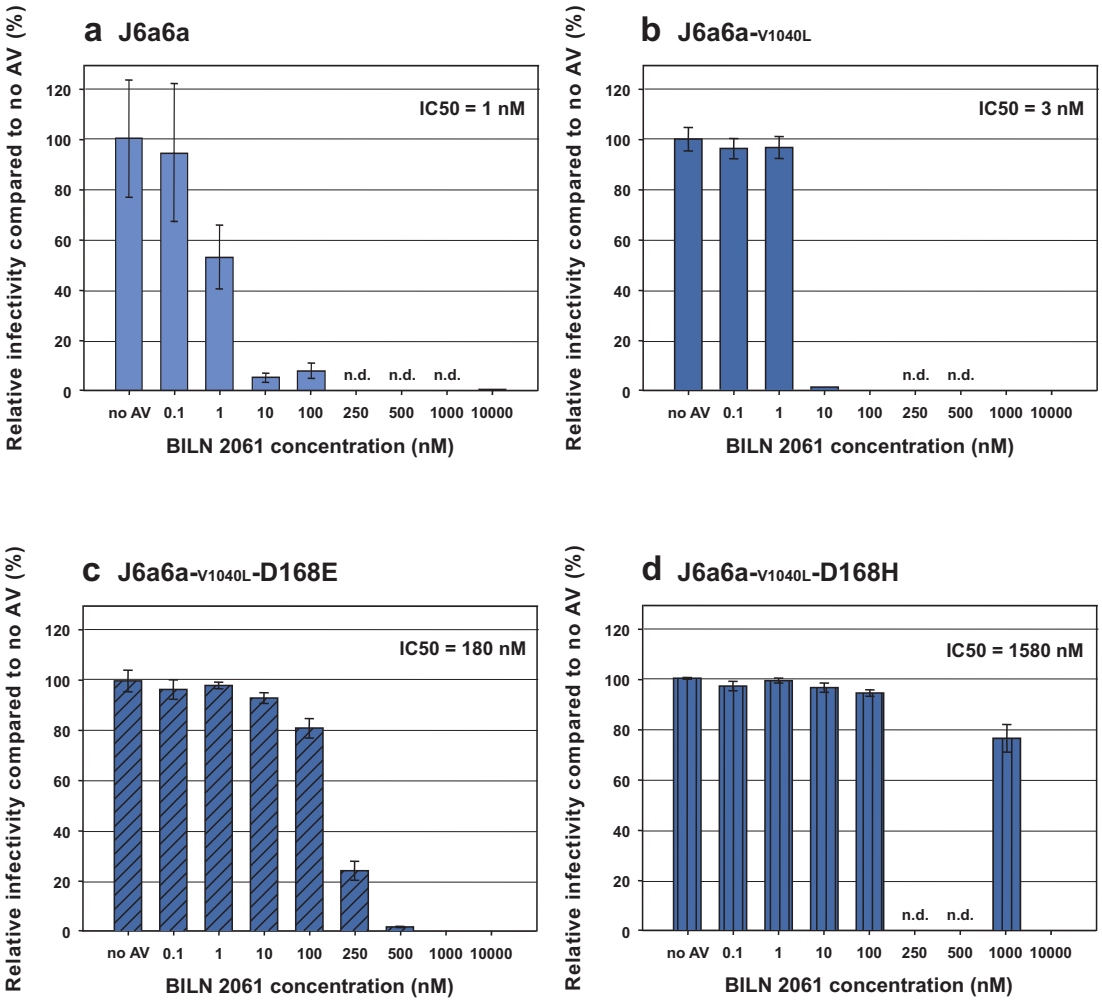

Figure 6.17: ***BILN 2061 susceptibility of J6a6a recombinants including adaptive and resistance mutations (J6a6a$_{-V1040L}$-rm).*** *Resistance mutations identified during passaging of the J6a6a$_{-V1040L}$ recombinant under BILN 2061 were introduced into the original J6a6a$_{-V1040L}$ recombinant to create J6a6a$_{-V1040L}$-D168E, J6a6a$_{-V1040L}$-D168H and J6a6a$_{-V1040L}$-D168V. 1-10 μg (a) J6a6a, (b) J6a6a$_{-V1040L}$ and (c)-(d) J6a6a$_{-V1040L}$-rm RNA was electroporated into Huh7.5 cells and incubated for 24 hours. Cells were then washed and incubated in media containing 0.1 % DMSO, as a carrier control, with or without the indicated doses of BILN 2061 for further 72 hours. The percent inhibition of replication was determined at 96 hours post-electroporation (mean $\pm$SEM; n = 3) and calculated as the ratio of NS5A-positive cells in BILN 2061-treated cells to those of the control without antiviral. J6a6aa$_{-V1040L}$-D168V recombinant replication was below detection limit and was not assessed for BILN 2061 susceptibility.*

Table 6.6: ***Influence of resistance mutations on BILN 2061 susceptibility, fold change in IC$_{50}$.*** *WT, wild type.*

Viral variant	IC_{50} (nM)	Fold change in IC_{50} compared to WT
J1b1b	3	-
J1b1b-A156V	830	277
J1b1b-D168G	200	67
J1b1b-A156V-D168G	540	180
J2a2a	720	-
J2a2a$_{\text{-T1066S}}$	990	1.4
J2a2a$_{\text{-T1066S}}$-D168V	-	-
J2a2a$_{\text{-T1066S}}$-D168Y	7,000	7[1]
J3a3a	105	-
J3a3a-Q168K	5,350	51
J3a3a-Q168L	3,200	30
J3a3a-Q168R	4,540	43
J5a5a	480	-
J5a5a$_{\text{-Q1247L}}$	710	1.5
J5a5a$_{\text{-Q1247L}}$-E168A	3,490	5[1]
J5a5a$_{\text{-Q1247L}}$-E168G	1,130	1.6[1]
J5a5a$_{\text{-Q1247L}}$-E168V	5,920	8[1]
J6a6a	1	
J6a6a$_{\text{-V1040L}}$	3	3
J6a6a$_{\text{-V1040L}}$-D168E	180	60[1]
J6a6a$_{\text{-V1040L}}$-D168H	1,580	527[1]
J6a6a$_{\text{-V1040L}}$-D168V	-	-

[1] *Recombinants including resistance mutations generated from cell culture adapted/attenuated recombinants were compared to the corresponding adapted/attenuated recombinant.*

6.2.5 Summary of results

Intra- and intergenotypic recombinants constructed from HCV genotype 1 to 6 were passaged in initially subinhibitory but increasing concentrations of BILN 2061 to investigate whether antiviral resistance could be induced *in vitro*. Clonal analysis of the HCV NS3 protease gene identified substitutions at position 168 for all genotypes. The identity of the amino acid that replaced the wild type differed between genotypes, where genotype 1b, 2a, 4a and 6a have the same wild type residue but type 3a and 5a an alternative one. Genotype 1b and 4a showed the same substitutions, type 2a, 5a and 6a similar ones and substitutions identified within type 3a did not occur in any other genotype. In genotype 1b and 4a, substitutions were also identified at position 156.

Most substitutions did not affect recombinant replication kinetics substantially, except for Asp168Val which reduced the replicative fitness of J2a2a$_{\text{-T1066S}}$ and J6a6a$_{\text{-V1040L}}$ below detection limit. All mutants created showed an increase in resistance towards BILN 2061 when compared with the wild type recombinant, with the J6a6a$_{\text{-V1040L}}$-D168H mutant followed by the J1b1b-A156V mutant showing the highest fold change in PI susceptibility. Substitutions induced upon VX-950 treatment were identified at previously described resistance loci 36, 54 and 156, but also at the so far undescribed positions 77 and 174.

6.3 Discussion

The rapid selection of viral variants displaying drug-resistant phenotypes is a major concern in HCV treatment. Several resistant phenotypes have been observed in patients experiencing viral rebound during therapy, as well as in replicon experiments (Lin *et al.* (2005a); Lu *et al.* (2004); Cubero *et al.* (2008); Kuntzen *et al.* (2008)). Antiviral drug treatment favours the selection and spread of variants including resistance mutations. Both the relative resistance of a variant and its viral fitness influence the replication phenotype of a resistant variant. Often variants that show high levels of resistance are impaired in their replication kinetics and only circulate in low frequencies. However, upon antiviral drug treatment antiviral susceptible variants will

be cleared and the antiviral resistant variants can spread (section 1.8.4). Resistance mutations arising under BILN 2061 pressure have been described at the 3 main positions Arg155, Ala156 and Asp168 (Lin *et al.* (2005a); Lu *et al.* (2004); Cubero *et al.* (2008)).

In this study, several new *in vitro* resistance mutations in the NS3 protease gene of all 6 major genotypes were identified. The dominant resistance mutations observed against BILN 2061 occurred at position 168, where the Asp residue in genotypes 1b, 2a, 4a and 6a changed to Gly/Glu/Ala/Val (genotype 1b and 4a), Val/Tyr (2a) and to Val/Asn/Glu/His (6a). In the genotype 3a protease gene a substitution of Gln168 to Leu/Arg/Lys was detected, while in genotype 5a Glu168 was substituted with Ala/Gly/-Val. Although conserved in position, a striking feature of this pattern of mutation was the variable nature of the substituted amino acid, suggesting great flexibility in the nature of the structural disruption to the protease induced by these resistance-associated changes. The observation that resistance-associated mutations in one genotype (such as Asp168Glu substitution in genotype 1b, 4a and 6a) may occur as the wild type amino acid in another (genotype 5a), is consistent with significant structural differences in the protease gene implied by the differences between genotypes in their susceptibility to inhibition by BILN 2061. *In vitro* studies with the genotype 1b replicon have associated mutations towards Val/Ala with BILN 2061 resistance at position 168 (He *et al.* (2008)). Interestingly, the Asp168Glu mutation has been described in literature as well, but in association with resistance to ITMN-191, another macrocyclic PI (Seiwert *et al.* (2007a)). Resistance mutations towards a third macrocyclic inhibitor, TMC435, also identified a variety of resistance mutations at position 168 (Asp168Tyr/Glu/His/Ile/Thr/Asn), which support the results described here (Lenz *et al.* (2010)). The Asp168Ile/Thr mutation was not observed, possibly due to the fact that 2 nucleotide changes are necessary for this change and the PI pressure applied here was not high enough to induce this.

These results demonstrate that resistance towards macrocyclic inhibitors can be induced by a variety of viable changes and that the resistance profile towards the different members of one class of PIs is similar. The fact that all genotypes show mutations

at the same position suggests a similar escape route from BILN 2061, something that has been also suggested for TMC435 (Lenz *et al.* (2010)).

All escape mutations resulted from one single nucleotide change. It is unclear why genotype 1b, 4a and 6a show a higher variability in substitutions than genotype 2a. The type 2a resistance mutations Asp168Tyr/Val showed 10- to 100-fold higher resistance to BILN 2061 in the type 1b replicon system than Asp168Ala/His/Glu/ Gly/Asn (Lenz *et al.* (2010)). However, the J2a2a$_{\text{-T1066S}}$-D168Y mutant only showed 7-fold increase in resistance, which is 355-fold lower than that described in the type 1b replicon system. The low level resistance increment of Asp168Tyr in type 2a could possibly explain why only Asp168Tyr/Val arose in type 2a, as the type 2a passaging experiment was shorter than the type 6a and involved relatively lower PI concentrations than the type 1b and 4a experiment. The resulting low PI pressure would not be enough to induce high level resistance mutations. It would be interesting to see whether further substitutions would arise within the genotype 2a recombinant if it was passaged under higher PI concentrations. The discrepancy between the results described here and those reported elsewhere are presumably due to the differences in the genetic backgrounds.

The genotype 5a wild type amino acid at position 168 is Glu, which is very similar to Asp in genotype 1b, 2a, 4a and 6a (both are polar and negatively charged), except that it is one methyl chain longer. As expected, the resistance profile in genotype 5a was very similar with Glu168Ala/Val/Gly substitutions occurring. Substitutions identified within genotype 3a on the other hand were different to those in all other genotypes, reflecting the charge difference in the wild type amino acid at position 168 (Gln is polar neutral).

Although further mutations at positions 155 and 156 have been associated with BILN 2061 resistance (Lin *et al.* (2005a); Lu *et al.* (2004)), substitutions at position 156 were only observed in a minority of genotype 1b and 4a clones. The Ala156Val substitution was found in both genotypes, whereas the Ala156Thr substitution was only found in genotype 4a (Table 6.3). It is unclear why the Ala156Thr substitution only occurred in genotype 4a and not in 1b. Both, Ala156Val and Ala156Thr only need one nucleotide change and should be accessible to all genotypes. However, these geno-

type specific differences are consistent with a report that described the occurrence of the Ala156Val substitution in the genotype 1b, but not in the genotype 1a replicon (Lenz *et al.* (2010)). Residue 195 in genotype 2a has not been described as a resistance locus before and it is unclear what effect the Gln195His substitution has on the recombinant replication and resistance profile.

Interestingly, no substitutions were identified at position 155. The Arg155Gln mutation has been observed during *in vitro* studies in the genotype 1b replicon system, but was associated with lower resistance than the Ala156Thr and Asp168Val mutations (Lu *et al.* (2004)). In that study, Ala156Thr has been shown to confer the highest fold increase in resistance to BILN 2061, followed by Asp168Val and Arg155Gln. These findings could explain the appearance of substitutions at position 156 for only the genotype 1b and 4a passaging experiment. In these, the PI concentrations reached 100 $\times$ IC_{50}, whereas it was about 70 $\times$ IC_{50} for type 3a, 10 $\times$ IC_{50} for type 6a and only 1-2 $\times$ IC_{50} for type 2a and 5a. The observed pattern of substitutions in all 6 genotypes (Table 6.3) suggests that resistance mutations at position 156 only arise when PI concentrations reach 100 $\times$ IC_{50}. Two other studies reporting higher resistance for the Ala156Val/Thr mutation than for the Asp168Val/Ala mutation, support these findings (Lu *et al.* (2004); He *et al.* (2008)). However, contradictory results showing slightly higher levels of resistance for the Asp168Tyr/Val mutation when compared with the Ala156Val mutation, were reported elsewhere (Lenz *et al.* (2010)). These differences are likely a result of differences in the genetic background and type of assay, demonstrating how important it is to characterise resistance mutations in all HCV genotypes. The time of AV pressure does not seem to have a major influence on the resistance profile, as type 1b and 4a were only passaged for 8 and 13 days, respectively.

Using specific site directed mutagenesis, the phenotypic effects of substitutions were determined. Whereas the Asp168Val mutation reduced the replicative fitness of the J2a2a$_{\text{-T1066S}}$ and J6a6a$_{\text{-V1040L}}$ recombinant below the detection limit, it only slowed down the spread of the J5a5a$_{\text{-Q1247L}}$ recombinant (Fig. 6.10 to 6.12). This deleterious effect on genotype 2a and 6a is surprising, as the Asp168Val substitution in the genotype 1b replicon only partly reduced its replicative fitness (He *et al.* (2008)). Pre-

sumably these discrepancies are due to differences in the different genotypic NS3 protease sequences. Viral variants with the Asp168Val mutation potentially have compensatory secondary mutations outside the NS3 protease region. The J3a3a-Q168R and the J5a5a$_{\text{-Q1247L}}$-E168G recombinant initially spread like the wild type recombinant, but were then diluted out as the cell culture was split, suggesting inefficient spread between cells as observed for J1b1b and J4a4a-19 (Fig. 6.11 and 4.11). The remaining tested substitutions did not show any major impact on recombinant fitness. However, as no quantitative real-time PCR was performed it cannot be excluded that there are differences in intra- and extracellular RNA levels between wild type and mutant recombinants.

The different substitutions identified during the selection experiment had different effects on BILN 2061 susceptibility and did not correlate with their effect on replication fitness (Table 6.6). The J6a6a$_{\text{-V1040L}}$-D168H mutant that conferred the highest level of resistance of the mutants analysed, replicated with similar efficiency in the cell culture as the wild type (Table 6.6 and Fig. 6.12). Within genotype 3a and 5a, all mutants analysed showed a similar increase in resistance towards BILN 2061, although the replication of some was impaired (Fig. 6.11). Only within genotype 1b was higher resistance associated with slightly lower replication levels (Fig. 6.10 top). The fold increase in resistance in J1b1b induced by the Asp168Glu/Gly mutation was about the same as described in the literature, whereas the measured increase for Ala156Val was 2- to 10-fold and for Asp168Tyr in genotype 2a 100-fold lower than described before. The observed increase in resistance for the Asp168His mutation in genotype 6a is about twice as high as reported before (He *et al.* (2008); Lenz *et al.* (2010)). However, comparisons should be made with caution as the cited studies were performed using the genotype 1b replicon, whereas results presented in this chapter are derived from 6 different genotypic backgrounds.

The resistance mechanism of HCV to BILN 2061 has been described in great detail (Courcambeck *et al.* (2006)). Two different mechanisms were demonstrated. The direct mechanism is based on the contact between the 2 mutated residues Arg155Gln and Ala156Thr and the inhibitor. Arg155 and Ala156 are part of the S2 binding pocket,

which interacts with the large P2 moiety of BILN 2061. The S2-P2 interaction is the functionally most important interaction for BILN 2061 potency. Important electrostatic interactions between Arg155 and the PI are lost when position 155 is replaced with an uncharged amino acid like Gln, explaining the Arg155Gln mutation. Ala156 is also in close interaction with the PI and a change towards a larger and bulkier amino acid like Thr produces strong steric constraints on the interaction with BILN 2061. This markedly affects the affinity of BILN 2061 for the NS3/4A protease and explains the Ala156Thr substitution. Val is also bulkier than Ala, explaining the Ala156Val substitution.

The second, indirect mechanism involves Asp168, which does not directly interact with BILN 2061. However, Asp168 builds 2 salt bridges with Arg123 and Arg155, 2 residues that directly interact with BILN 2061. Mutations at position 168 prevent the formation of these salt bridges and alter the conformation of Arg123 and Arg155, thereby preventing optimal binding of BILN 2061 (Fig. 6.18). Arg is a positively charged amino acid and will need a negatively charged amino acid to build a salt bridge. All amino acid substitutions towards nonpolar, positively charged or neutral amino acids will therefore disrupt the salt bridges between residue 168 and Arg123 and Arg155, thereby inducing BILN 2061 resistance. All substitutions observed at Asp168 (Ala/Asn/Gly/His/Tyr/Val) are nonpolar, neutral or positively charged, explaining the resistance mechanism of these substitutions (Fig. 6.19). The exception is the Asp168Glu substitution, a change towards another polar and negatively charged amino acid, which would not disrupt the building of the salt bridges. However, the Glu side chain is one carbon atom longer than the Asp side chain, possibly pushing Arg123 and Arg155 further away and rearranging the optimal spatial arrangement.

The highest fold resistance was induced by the Asp168His substitution, which is the only substitution with a positive charge. His168 not only disrupts the salt bridges but also repulses the positively charged residues Arg123 and Arg155, inducing more extensive spatial rearrangements and explaining the increased resistance.

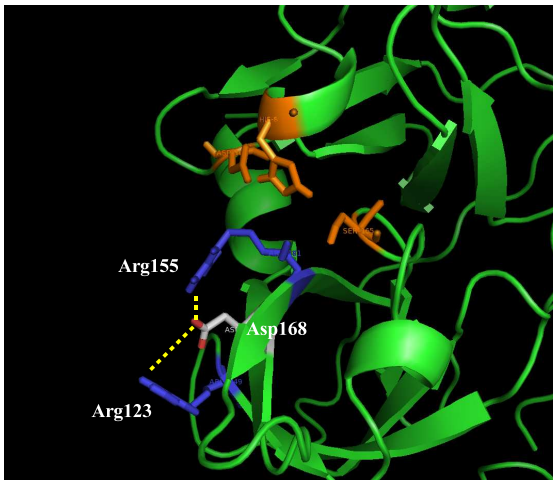

Figure 6.18: ***Structural perspective on Asp168 resistance mechanism.*** *The catalytic triad is depicted in orange and Asp168 in white with nitrogen in red and oxygen in blue. The negatively charged residue Asp168 makes 2 salt bridges with the 2 positively charged residues Arg123 and Arg155 (in blue), which directly bind to BILN 2061. Disruption of these salt bridges changes the optimal PI binding conformation of Arg123 and Arg155.*

The resistance mechanism for genotype 5a works on a similar basis, with changes from the polar and negatively charged amino acid Glu to nonpolar amino acids like Ala/Gly/Val (Fig. 6.20). In genotype 3a, the polar and neutral amino acid Gln is the wild type residue. Furthermore, residue Thr123 is a neutral amino as well, suggesting a slightly different resistance mechanism. Gln can temporarily be positively and negatively charged, as it has an amine and hydroxyl group. It can thereby build a salt bridge with the positively charged Arg155 and the free hydroxyl group of Thr123. All substitutions observed within genotype 3a are towards positively charged residues or nonpolar amino acids that can not undergo both salt bridges and thereby destabilise the interaction between BILN 2061 and the NS3/4A protease (Fig. 6.21).

Analogously to BILN 2061, VX-950 resistance was induced *in vitro* through passaging Jxxs under VX-950 pressure. Due to the limiting amounts of VX-950 available, the PI concentration was kept at 2-3 $\times$ IC_{50}. None of the genotypes showed significant substitution rates at any of the resistance loci described in the literature (NS3 residue 36, 41, 43, 54, 155, 156 and 170) (Table 6.1). As discussed above, this is presumably due to the to low PI concentrations used. Nevertheless, within genotype 1b and 3a

one position (Ser174Pro and Asn77Ser, respectively) showed significant substitution rates in both replicates, suggesting a resistance mutation (Fig. 6.7 and 6.8). Both are at a position which has not been described as a resistance locus and due to the low PI concentrations this suggests a low level resistance mutation. Residue Asn77 lies on a surface β-sheet and it is unclear what the exact phenotypic effect of a Ser substitution would have on fitness and resistance of a viral variant. Ser174 is part of an α-helix and close to the S2 binding pocket. A change towards the bulkier amino acid Pro possibly has a structural impact on PI binding. To get conclusive results on the effect of these mutations, specific site directed mutagenesis and IC_{50} determinations are required.

No significant substitutions were observed within genotype 4a, which can be explained with the high IC_{50} value of J4a4a-19 (2,330 nM). Nevertheless, one clone each was identified carrying a substitution at previously described resistance loci (Leu36Pro, Thr54Ala and Ala156Val, respectively) (Table 6.1 and 6.5). Although it cannot be excluded that these mutations arose due to Taq errors, the fact that they have been described previously, strongly suggests that they represent true resistance mutations. Passaging under higher PI concentrations should allow the identification of more clones containing resistance mutations.

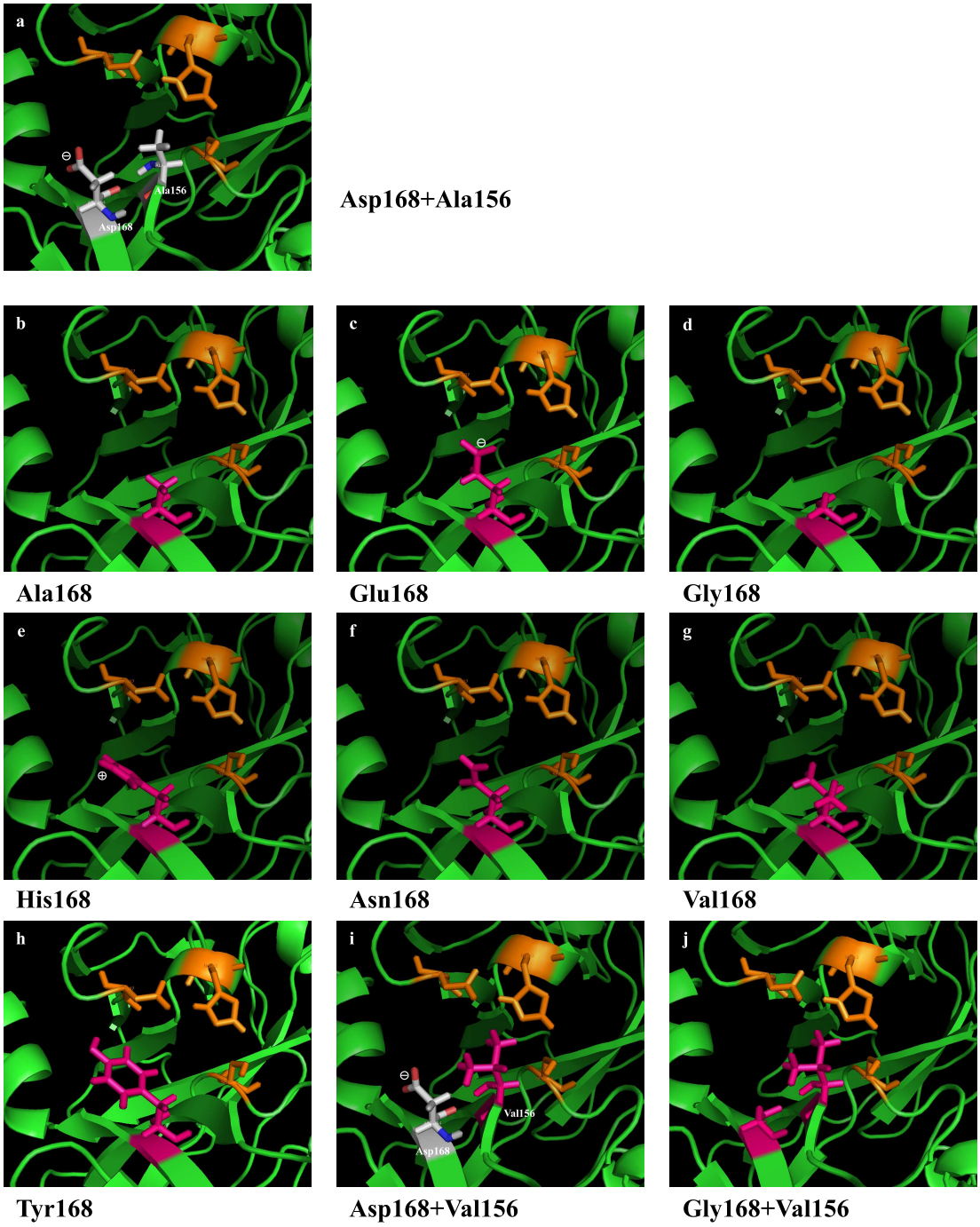

Figure 6.19: ***Structural perspective on Asp168 resistance mutations.*** *The catalytic triad is depicted in orange and Asp168 and Ala156 in white with nitrogen in red and oxygen in blue. Mutations observed during passaging experiments with Jxx under BILN 2061 are depicted in pink.*

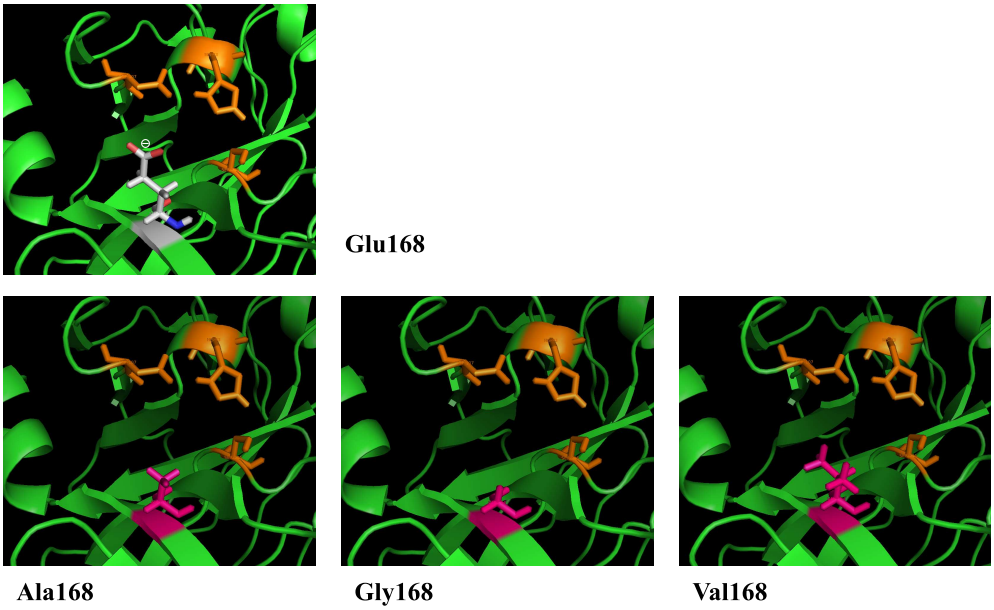

Figure 6.20: ***Structural perspective on Glu168 resistance mutations.*** *The catalytic triad is depicted in orange and Glu168 in white with nitrogen in red and oxygen in blue. Mutations observed during passaging experiments with Jxx under BILN 2061 are depicted in pink.*

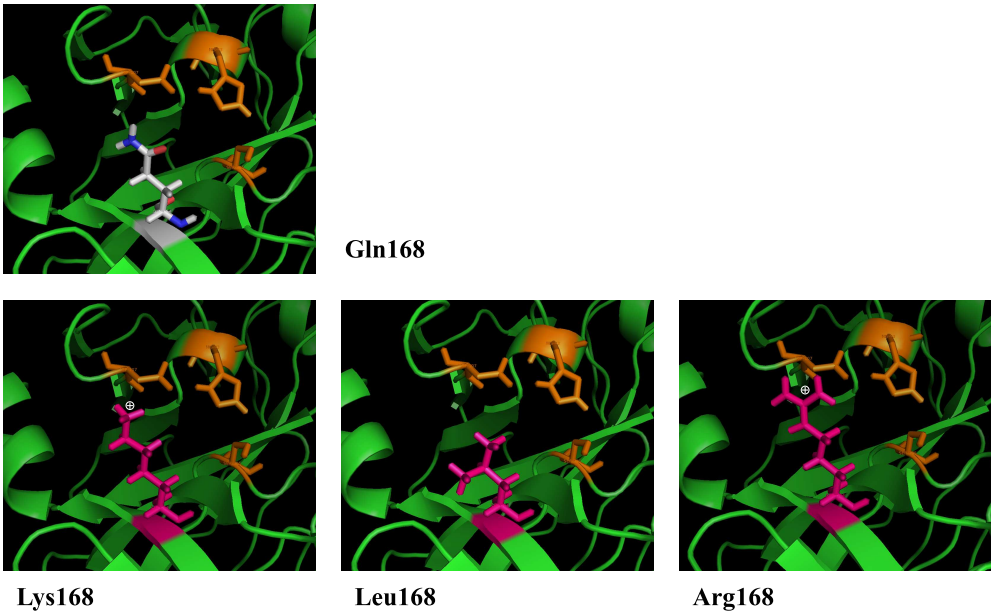

Figure 6.21: ***Structural perspective on Gln168 resistance mutations.*** *The catalytic triad is depicted in orange and Gln168 in white with nitrogen in red and oxygen in blue. Mutations observed during passaging experiments with Jxx under BILN 2061 are depicted in pink.*

6.4 Conclusion

To identify resistance mutations developing during PI treatment, Jxx recombinants of HCV genotype 1 to 6 were passaged under increasing concentrations of BILN 2061. Several new *in vitro* resistance mutations were identified at the previously identified resistance residues 156 and 168. The dominant resistance mutations observed against BILN 2061 occurred at position 168, but within genotype 1b and 4a resistance mutations were also observed at position 156. A high diversity of substitutions was observed at position 168, suggesting great flexibility in the spatial arrangement of the protease to evade PI binding. Using specific site directed mutagenesis allowed investigation of the effects identified resistance mutations have on viral fitness and antiviral resistance. Strikingly, the Asp168Val mutation completely abrogated detectable viral replication, whereas other substitutions only had minor impacts on viral fitness. Amino acid changes from polar and negatively to polar and positively charged residues had the highest impact on viral susceptibility to BILN 2061. Similarly, resistance development upon VX-950 treatment was investigated. Two previously unidentified potentially low level resistance loci were observed together with sporadic mutations at known resistance loci.

Identification of previously described resistance mutations for BILN 2061 and VX-950 underscore the usefulness of the Jxx-recombinant system developed here. Progress towards characterisation of these mutations and combinations will in the long term provide the necessary data for the development of genotype specific drug-resistance databases analogous to those developed for HIV-1.

Chapter 7
Concluding Remarks and Outlook

HCV is globally widespread and a major health concern. It is one of the main causes of severe liver diseases, such as cirrhosis and hepatocellular carcinoma, which can lead to death. The considerable genetic diversity between the 7 genotypes ($>$30 %) leads to substantial differences in persistence, disease associations and treatment response. Furthermore, the pre-existing huge pool of genetically different variants within an individual, the so-called quasispecies, allows a rapid adaptation of HCV to selection pressures, such as immunological recognition and antiviral treatment. The current standard IFN-α/ribavirin combination treatment is associated with severe side effects and patients with genotype 1 and 4 only respond poorly to this treatment. The development of specific antiviral drugs is therefore highly desired. To date, drug development has mainly been based on genotype 1a or 1b-based enzyme structures and the genotype 1b replicon system, leading to genotype 1 specific drugs. Because of substantial sequence divergence between genotypes within the enzymatic gene regions, antivirals developed upon genotype 1 enzymatic structures often show lower efficacies in nongenotype 1 enzymes. The case of the first PI to enter clinical trials, BILN 2061, demonstrated very early on the immense impact structural differences within genotypes can have on drug efficacies. The genotype 1 specific PI showed high efficacy in genotype 1 infected patients, whereas genotype 2 and 3 infected patients were only weakly or non-responsive to BILN 2061 treatment (Lamarre *et al.* (2003); Reiser *et al.* (2005)).

Another problem identified early on in clinical trials with HCV antivirals, was the rapid emergence of antiviral resistance mutations. As shown for HIV-1, large population sizes and the relatively high error rate of the RNA polymerase are the main factors for the huge genetic diversity of HCV. Pre-occurring viral variants with resistance mutations can therefore readily be selected for during antiviral treatment. As shown in the

replicon system, new antiviral specific resistance mutations can also be rapidly induced upon antiviral treatment.

The variable efficacies of PIs on different genotypes and the influence genotypes might have on the effect of naturally occurring resistance mutations demand the development of an *in vitro* system to investigate the effectiveness of antivirals for different genotypes. The aim of the PhD project presented here was to develop an intra- and intergenotypic cell culture system that would allow the *in vitro* assessment of HCV NS3 proteases from genotype 1 to 6.

In a first part, the natural polymorphism within the NS3 protease and NS4A cofactor of genotype 1a and 3a clinical variants was analysed. It was shown that the NS3/4A protease is flexible enough to tolerate substitutions despite the different functional constraints. No mutations were identified within the functional domains of the catalytic triad, the S1 and S2 binding pocket or the zinc binding domain. However, natural polymorphism outside of the functional domains was observed, which might potentially influence the 3D structure of the protease and affect functional sites. The identification of polymorphic sites within the NS3 protease will be valuable in designing new PIs. Inhibitor development could be designed targeting areas which are conserved across genotypes and subtypes, ensuring efficacy in all genotypes and subtypes. In addition, identification of individual polymorphism, known to be associated with treatment failure, will enable patient specific treatment options.

The main part of this PhD project was the development of a full-length HCV cell culture system, which allows the investigation of protease gene function in all 6 major genotypes. This extends the range of available full length replication competent clones that allow analysis of the NS3 protease gene from genotype 2a and 1a to all 6 major genotypes (Lindenbach *et al.* (2005); Wakita *et al.* (2005); Yi *et al.* (2006b); Murayama *et al.* (2007a)). Genetically engineered restriction sites on the NS3 protease boundaries allowed the generation of an intergenotypic replication competent clone for genotype 5a. Extending the cloning strategy to include the homologous NS4A cofactor in the recombinant construction resulted in replication competent intra- and intergenotypic recombinants representing all 6 major genotypes. Different cell

culture adaptive/attenuating mutations were identified that allowed the generation of stably replicating recombinant clones for genotype 2a, 3a, 5a and 6a. The cell culture system presented here provides a powerful tool to study the NS3 protease within the full viral life cycle and adds to the currently available JFH1-based systems for the study of the non-structural genes (Pietschmann *et al.* (2006); Gottwein *et al.* (2007); Yi *et al.* (2007); Jensen *et al.* (2008); Scheel *et al.* (2008); Gottwein *et al.* (2009)). The genetically engineered restriction sites allow the insertion of protease genes amplified from clinical specimens directly into the expression vector. Protease genes from all genotypes and subtypes can thereby easily be assessed for pre-existing antiviral resistance mutations and their antiviral susceptibility.

Using the intra- and intergenotypic recombinants it could be demonstrated that different NS3 proteases react differently to the same PI. The genotype 1b protease was shown to be more susceptible to BILN 2061 than the genotype 2a and 3a derived proteases, confirming previously reported results and underscoring the practicality of the system (Thibeault *et al.* (2004)). Furthermore, inhibitor sensitivity testing within the full length viral lifecycle could be extended to genotype 4a, 5a and 6a. Compared to the replicon system, this cell culture system allows the assessment of antiviral susceptibilities in the context of the entire life cycle of the virus. The effect of antiviral efficacies on replication kinetics as well as supernatant infectivity can be assessed. Analogously, genotype specific differences in susceptibility to VX-950 were also demonstrated.

Passaging the intra- and intergenotypic recombinants under increasing BILN 2061 concentrations allowed the *in vitro* induction of resistance mutations. Several new *in vitro* resistance mutations were identified at previously described resistance loci, emphasising again the practicality of the system. Whereas all genotypes developed resistance mutations at position 168, only genotype 1b and 4a also developed resistance mutations at position 156. The fact that higher PI concentrations were used in type 1b and 4a passaging experiments suggests that mutations at position 156 confer higher levels of resistance than those at position 168. The great diversity of resistance mutations observed between the different genotypes demonstrates the flexibility of the HCV protease and highlights the importance of phenotypic characterisation of resis-

tance mutations for all genotypes. Phenotypic characterisation of the individual resistance mutations identified during passaging experiments identified mutations with no or dramatic effects on viral replication kinetics. All substitutions that were assessed conferred an increase in antiviral resistance, providing proof that they are real resistance mutations and that the cell culture system described here can be used to identify them. Investigation of resistance development upon VX-950 treatment identified previously unidentified substitutions as well as substitutions at already identified resistance loci.

Replication competent intra- and intergenotypic recombinants have successfully been created for genotype 1b, 2a, 3a, 4a, 5a and 6a. The generation of a well replicating recombinant based on the important genotype 1a would complete the set of Jxx recombinants. Possible strategies to create a well replicating J1a1a recombinant include the insertion of further patient-derived protease genes and more extensive laboratory passaging to hopefully induce adaptive mutations. Furthermore, the cloning strategy could be extended to include the NS2 protein in the recombinant construction. NS2 is a zinc-stimulated cysteine protease that cleaves the NS2-NS3 junction, for which it requires the N-terminal part of NS3 as a cofactor (Schregel *et al.* (2009)). Incompatibilities between the heterologous NS2 and NS3 protease proteins in the J1a1a recombinant might contribute to its poor replication kinetic.

Possible next steps in the project also involve the development of a diagnostic phenotypic assay for antiviral resistance. Instead of ligating amplified protease sequences into the expression vector and choosing individual clones representing one viral variant, a more rapid approach involving direct RNA transcription from the ligation product could be pursued. Because the cloning step is omitted, the RNA transcript population will reflect the diversity of HCV sequences within the quasispecies population of the sample. Minor populations of variants including resistance mutations may therefore contribute to resistance development depending on their *in vivo* abundance.

In summary, during this PhD project a full length HCV cell culture system, which allows the investigation of protease gene function in all 6 genotypes within the full viral life cycle, has been developed. Using this system, it could be demonstrated that different NS3 proteins react differently to the same PI. Any PI identified in high-throughput screening can be evaluated for its efficacy on different genotypes and treatments designed according to the outcome. Passaging the recombinants in sub-inhibitory concentrations of antiviral drugs allowed the identification of potential resistance mutations to BILN 2061 and VX-950 and will therefore be of considerable value in preclinical assessment of resistance induction and genotypic variability for newly developed compounds.

The ease with which protease gene sequences directly amplified from clinical specimens can be inserted in the expression vector of the appropriate genotypes allows for the first time direct monitoring of antiviral drug inhibition for specific proteases through assessment of reduction in both supernatant infectivity and replication kinetics. Protease genes from study subjects naïve to treatment can then easily be assessed for sensitivity to a range of antiviral drugs and screened for pre-occurring resistance mutations, as well as providing a phenotypic assay for the rapid assessment of emerging resistance during therapy and the influence of specific mutations on treatment outcome.

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Appendix A
List of Primers

Table A.1: ***Primers used for amplification of the NS3 protease gene from plasma, genotype 1a****. OS, outer sense; OA, outer anti-sense; IS, inner sense; IA, inner anti-sense; OMS, outer middle sense; OMA, outer middle anti-sense; MS, middle sense; MA, middle anti-sense.*

Primer	Position	Sequence (5'-3')
NS3-1a-OS	2903	AGCGCAMYTGCACGTGTGGRTTCC
NS3-1a-OOSb	2823	GCGCTGACTCTGTCACCATATTACAAGC
NS3-1a-OA	4113	CCAAAGCCCAGTGTTGCRGCRAC
NS3-1a-OOA	4164	GCACCAAAGCCCAGYGTTGC
NS3-1a-OOOAb	4182	TGRGCCTTGGACATGTAARC
NS3-1a-IS	2937	GTCCGCGGCGGCCGYGACGCCRTCATCY
NS3-1a-ISb	2940	TATAGCGGCCGYGACGCCRTCATCY
NS3-1a-IS2	3276	ATGGAGACCAAGCTCATCACG
NS3-1a-IA	4103	GAGCACTAGTACTTTATAGCCCTGRGC
NS3-1a-IAb	4083	GGTTGAGCACTAGTACCTTATAGCC
NS3-1a-OMS	3466	GYATAATCACCAGCCTRACYGGC
NS3-1a-MS	3513	GAGGTCCAGATTGTRTCAACWGC
NS3-1a-OMA	3624	GGTATACATCTGGATRACRGGACC
NS3-1a-MA	3569	TGGTAGACAGTCCARCAYACC

Table A.2: ***Primers used for amplification of the NS3 protease gene from plasma, genotype 3a.*** *OS, outer sense; OA, outer anti-sense; IS, inner sense; IA, inner anti-sense; MS, middle sense; MA, middle anti-sense.*

Primer	Position	Sequence (5'-3')
NS3-3a-OS	2899	GCGAGTCYGCCCTYCAWGTGTGGG
NS3-3a-OA	4116	AGAGCCRAAGCCTARTGTGGCCGC
NS3-3a-IS	2937	GCGCGYGGCGGCCGCGACGGTGTCATCY
NS3-3a-ISb	2936	TATAYGGCGGCCGCGACGGTGTCATCY
NS3-3a-IS2	3314	CTTGCGGAGATATTCTTTGCG
NS3-3a-IA	4078	CAGCACTAGTACAKTATATCCTTGTGC
NS3-3a-MS	3478	YTGACTGGYAGGGAYAAGAACG
NS3-3a-MA	3532	TGTACCYARGAAWGTCTGWGTRGC

Table A.3: ***Primers used for amplification of the NS3 protease gene from plasmids.*** *OS, outer sense; OA, outer anti-sense.*

Primer	Position	Sequence (5'-3')
NS3p-1a-OS	3276	ATGGAGACYAAGRTCATYAC
NS3p-1a-OA	4335	TCYGCTTGGTCMAGGAC
NS3p-1b-OS	3323	GGACATCATYTYGGGYYTRC
NS3p-1b-OA	4033	TATAGCTYTTRCCGCTGCCAGTGG
NS3p-3a-OS	3276	ATGGAAATCAAGGTCATCAC
NS3p-3a-OA	4294	AGCGTCTTGGGCATGAC
NS3p-4a-OS	3926	TATAGCGGCCGYGACGCCRTCATCY
NS3p-4a-OA	3326	TGTTGARTTGTCAGTGAACACTGG
NS3p-5a-OS	-	M13Primer
NS3p-5a-OA	4019	TATAGGAGGGTGCATGGAGGTCC
NS3p-6a-OS	3236	CCYATGGAGAAGAARRTYATCACS
NS3p-6a-OA	4286	RTCYGTGGAGTGGCACTCRTCA

Table A.4: ***Primers used for amplification of the NS4A gene, genotype 1a and 3a.*** *OS, outer sense; OA, outer anti-sense; IS, inner sense; IA, inner anti-sense.*

Primer	Position	Sequence (5'-3')
NS4A-1a-OS	4710	TGTGTCACYCAGACAGTCGA
NS4A-1a-OA	5706	GCTGTRAAWGCCATCARTGA
NS4A-1a-IS	4880	TATATCCGTCCTCTGTGAGTGC
NS4A-1a-IA	5622	TTCCACATATGCTTCGCCCA
NS4A-3a-OS	4716	GARCAGTACGTYGACTTCAGC
NS4A-3a-OA	5636	ATCCCRCTCACAAAATTCCAC
NS4A-3a-IS	4714	CCGTCTGGRATGTTTGACTCG
NS4A-3a-IA	4861	YTGAGCTTGCTCGATRTAWGG

Table A.5: ***Primers used for the amplification of the NS3 protease gene from plasmid or plasma, introducing restriction sites.*** *xBstBI-s, primers including a BstBI restriction site; xBglII-as, primers including a BglII restriction site.*

Primer	Position	Sequence (5'-3')
1aBstBI-s	3394	TATATTCGAAGGGGTGGAAGTTGC
1aBglII-as	3941	ATATAGATCTCATGGTTGTCYCTAGG
1bBstBI-s	3394	TATATTCGAAGGGGTGGAAGCTCCTYGCGCC
1bBglII-as	3936	GCACCAAAGCCCAGYGTTGC
2aBstBI-s	3394	TATATTCGAAGGGGTGGAAGCTTCTCG
2aBglII-as	3937	ATATAGATCTCGTGACGATGTCGAGTGTC
3aBstBI-s	3394	TATATTCGAAGGGYTGGAAGCTGTTGG
3aBglII-as	3940	ATATAGATCTAGTCTGTGTRCTRAGGG
4aBstBI-s	3394	TATATTCGAAGGGGTGGAAACTCCTTGCTCCCATTACAGC
4aBglII-as	3935	ATATAGATCTCATRGTRGTCTCAAGRGATTCR
5aBstBI-s	3394	TATATTCGAAGGGYTGGAAACTYCTCGC
5aBglII-as	3935	ATATAGATCTCATCGTGGTYTCCAGGTTTTCR
6aBstBI-s	3394	TATATTCGAAGGGGTGGAAGCTCCTGGCTCCCATTAC
6aBglII-as	3935	TGGTAGACAGTCCARCAYACC

Table A.6: ***Primers used for the amplification of the NS4A gene from plasmids, introducing restriction sites.*** *xSapI-s, primers including a SapI restriction site; xMluI-as, primers including a MluI restriction site.*

Primer	Position	Sequence (5'-3')
1aSapI-s	5282	TATAGCTCTTCCTGACCTTGAGGTCATGACCAGCACCTGG
1aMluI-as	5462	TATACGCGTGGCRCACTCTTCCATC
1bSapI-s	5282	TATAGCTCTTCCTGACCTTGAGGTCATGACCAGCACCTGG
1bMluI-as	5462	TATACGCGTGGCRCACTCYTCCATY
2aSapI-s	5282	TATAGCTCTTCCTGACCTTGAGGTCATGAC
2aMluI-as	5462	TATACGCGTGGCACATTCCTCCATC
3aSapI-s	5282	TATAGCTCTTCCTGACCTTGAGGTCATGACCAGCRCCTGG
3aMluI-as	5462	TATACGCGTGGCRCAYTCYTCCATC
4aSapI-s	5282	TATAGCTCTTCCTGACCTTGAGGTCATGACCAGYACGTGG
4aMluI-as	5462	TATACGCGTGGCRCACTCCTCCATT
5aSapI-s	5282	TATAGCTCTTCCTGACCTTGAGGTCATGACCAGYACGTGG
5aMluI-as	5462	TATACGCGTGGCGCATTCTTCC
6aSapI-s	5282	TATAGCTCTTCCTGACCTTGAGGTCATGACCAGYACATGG
6aMluI-as	5462	TATACGCGTGGCRCACTCCTCCATC

Table A.7: ***Primers used for the amplification of the core gene as a positive control.*** *OS, outer sense; OA, outer anti-sense; IS, inner sense; IA, inner anti-sense.*

Primer	Position	Sequence (5'-3')
Core-OS	287	ACTGCCTGATAGGGTGCTTGCGAG
Core-OAS	750	ATGTACCCCATGAGGTCGGC
Core-IS	320	AGGTCTCGTAGACCGTGCATCATG
Core-IA	723	CAYGTRAGGGTATCGATGAC

Table A.8: ***Primers used for the introduction of point mutations.*** *(a) Introduction of BstBI into pJFH1, (b) introduction of BglII into pJFH1, (c) introduction of BlpI into pJFH1, (d) introduction of MluI into pJFH1, (e) mutation of BglII restriction site within p4a, (f) introduction of cell culture adaptive L1663A amino acid mutation, (g) mutation of SpeI restriction site within the Jc1 backbone.*

Primer	Position	Sequence (5'-3')
(a)		
BstBI-sQC	3384	GCTGATGGCTACACTTCGAAGGGGTGGAAGC
BstBI-asQC	3384	GCT TCCACCCCT TCGAAGTGTAGCCATCAGC
(b)		
BglII-sQC	3944	CGACGTTGTTACAAGATCTCCCACTTTCAGTG ACAACAGC
BglII-asQC	3944	GCTGTTGTCACTGAAAGTGGGAGATCTTGTAA CAACGTCG
(c)		
BlpI-sQC	5281	CATGCATGCAAGCTGAGCTTGAGGTCATGACC
BlpI-asQC	5281	GGTCATGACCTCAAGCTCAGCTTGCATGCATG
(d)		
Mlu-sQC	5468	RGARTGYGCCTCGCGTGCGGCTCTCATCG
Mlu-asQC	5468	CGATGAGAGCCGCACGCGAGGCRCAYTCY
(e)		
p4aBmt-sQC	3685	CCGGAGTCAGATCACTTGCTCCGTGCACC
p4aBmt-asQC	3685	GGTGCACGGAGCAAGTGATCTGACTCCGG
(f)		
NS4AAmt-sQC	5314	GCACCTGGGTGTTGGCTGGAGGGGTCCTCG
NS4AAmt-asQC	5314	CGAGGACCCCTCCAGCCAACACCCAGGTGC
(g)		
MluI-s	Backbone	TATAACGCGTACCAGTCCC
PvuI-as	Backbone	TATACCTCCGATCGTTGTCAG

Table A.9: ***Primers used for whole genome sequencing of JFH1, Jc1 and the intergenotypic recombinants.*** *JFH-B, sense primers; JFH-C, anti-sense primers.*

Primer	Position	Sequence (5'-3')
JFH-B2	316	TATAAGGTCTCGTAGACCGTGCACC
JFH-B4	876	TATACCTGTTGTCCTGCATCACCG
JFH-B6	1516	TATACGTTCCACCAACGTGATTGC
JFH-B8	1549	GATGTCTTCCTACTGAACAGCACC
JFH-B10	2584	CGAAGCAGCATTGGAGAAGTTGG
JFH-B12	3201	TATACCACCTCACACCTATGTCG
JFH-B14	3889	TATACGAGCAGCTGTGTGCTCTCG
JFH-B16	4432	CGATATAGAAGAGGTAGGCCTCG
JFH-B18	5040	TATAGCCTCACACACATAGACGC
JFH-B19	5415	TATACCAGCGAGTCGTCGTTGC
JFH-B20	5714	TCTTCCATGATGGCATTCAGTGC
JFH-B21	5831	TATAGCTTTGTCGTCAGTGGCCTGG
JFH-B22	6284	TATAACGTGTGGGACTGGGTTTGC
JFH-B23	6532	TCCTATCAATTGCTACACGGAGG
JFH-B24	6749	TTTGCACCCACACCAAAGCC
JFH-B25	7002	TACACAGCAACACCTATGACGTGG
JFH-B26	7266	TATACGTTGCTGGTTGTGCTCTCC
JFH-B27	7542	TATAGGACCTGGAGTCTGATCAGG
JFH-B28	7982	TATAAGGTCCGCAGCTTGTC
JFH-B29	8332	ATGCTTCGACTCAACCGTCACTG
JFH-B30	8686	TATAGCCATGACCAGGTACTCTGC
JFH-B32	9191	ATATCCCTCATCTCCCGTGGA
JFH-C18	5545	TATAGCTGCAGCAAGCCTTGGATC
JFH-C19	5793	TATAGATCTGGGACGCTAACCAGC
JFH-C20	5944	GCCAGACATGATCTTGAATGCG
JFH-C21	6414	TATACGTGGTCATGATGCCAGTGC
JFH-C22	6642	CAGTCCTGTTACATAGGAGTACGACC
JFH-C23	6854	TTAGCATGGACCTCAATACGTCTGC
JFH-C24	7120	TATACAAGGTCGCTCTCTTCCTCG
JFH-C25	7383	TACTGGCCAAAGGTCTTGATGG
JFH-C26	7657	GTATGACATGGAGCAGCACACG
JFH-C27	8092	TATAGGTCCACGCAGAACACCTCA
JFH-C28	8430	TCCTACGTAAAGTCTCTCAGTCAGCG
JFH-C29	8811	TAGGTTGGGTCTCTGGTCAGGTAGT
JFH-C30	9298	GGTGAACCAACTGGATAAGTCCAGT

Table A.10: ***Primers used for whole genome sequencing of JFH1, Jc1 and the intergenotypic recombinants.*** *JFH-F, anti-sense primers.*

Primer	Position	Sequence (5'-3')
JFH-F1	34	GTTCCTCACAGGGGAGTGATT
JFH-F2	374	GCGACGGTTGGTGTTTCTTTT
JFH-F3	692	GTCGATGACTTTACCCACGTT
JFH-F4	953	GCTGTCATTGGAGCAGTCATT
JFH-F5	1253	GCCAGGGTAGATGGAGCAATT
JFH-F6	1589	GTTGATGTGCCAACTGCCGTT
JFH-F7	1778	CGGCCTCATATCCTCTGGATT
JFH-F8	2012	CTTGGTGAAACCAGTGGAGTT
JFH-F9	2410	TGAAGGTGGAGAAGACCAGTT
JFH-F10	2633	ATATAGGAGGCCATGGCAGTT
JFH-F11	3058	ACATGTGTCAAAGCGGCCCTT
JFH-F12	3293	TCCCCAGACGATGACCTTCTT
JFH-F13	3611	TAAGCCGGCTAGAGTCTTGTT
JFH-F14	3967	TCACTGAAAGTGGGAGACCTT
JFH-F15	4181	CCTGACTCCAGTCCTAATGTT
JFH-F16	4508	AATCAGGTGTCTCCCTCCCTT
JFH-F17	4768	TGTGGGACAGTCTGTGTGGTT
JFH-F18	5096	GGCTACTAGGTACGCGAAGTT
JFH-F19	5444	AGCCTCATACAGGACCTCCTT
JFH-F20	5780	TAACCAGCCTCCCATGATGTT
JFH-F21	5993	CAGGATCCCAGGCAGTAGATT
JFH-F22	6344	GGGCAGCTTGGGGAACAATTT
JFH-F23	6581	CCAGATGGCGGTCTTGTAGTT
JFH-F24	6806	GGACCCGACAGCATAGGAATT
JFH-F25	7236	AATCTGGCCTCCTCCACGATT
JFH-F26	7377	CAAAGGTCTTGATGGCCAGTT
JFH-F27	7786	TGTGAGGCGCTCTTTGATGTT
JFH-F28	8065	GCCATGATGGTTGTGGGAATT
JFH-F29	8344	ATGTCTCTCTCAGTGACGGTT
JFH-F30	8770	CCCAACGCCACAGACACATTT
JFH-F31	9029	GGCTGGAAGGTCCAAAGGATT
JFH-F32	9269	CTCCGGCAATGGAGTGAGTTT
JFH-F33	9655	TGATCTGCAGAGAGACCAGTT

Table A.11: ***Primers used for sequencing of TOPO vector inserts.***

Primer	Position	Sequence (5'-3')
M13F	-	GTAAAACGACGGCCAG
M13R	-	CAGGAAACAGCTATGAC

Table A.12: ***Primers used for whole genome sequencing of JFH1, Jc1 and the intergenotypic recombinants.***

Primer	Position	Sequence (5'-3')
JFH-3265-s	3265	GGAACCCATCATCTTCAGTCC
JFH-3395-s	3395	TATAGCTGATGGCTACACCTCC
JFH-5230-s	5230	CCTATTACCAATGAGGTCACC
JFH-5536-as	5536	ATCTTGGACTTCAACATCTCG
JFH-5413-s	5413	TATACGTCAACCAGCGAGTCG
JFH-5769-as	5769	CCATGATGTTGAGAAGGATGG
JFH-5834-s	5834	TATAGGCTTTGTCGTCAGTGG
JFH-6918-as	6918	GTAGGCTGGTTATAGTAAGAGAGC
JFH-6691-s	6691	TTGCCAACTACCTTCTCCAGAG
JFH-6954-as	6954	TATATGATAGCTGGCTCACTGAGG
JFH-7093-s	7093	TACGTTCTGGACTTTCTCGAGC
JFH-7416-as	7416	ATATGGACGAGCCTGCATCAC
JFH-7649-s	7649	TATGATACCACCGTGTGCTGC
JFH-6249-s	6249	ATCCCATGCTCCGGATCCTGG
JFH-7549-as	7549	GGTCTTGGTCTACTTGCTCC
JFH-8273-s	8273	CGACTCAACCGTCACTGAG
JFH-8450-as	8450	GGTGCTAACCACTAGCATGG

Table A.13: ***Primers used for amplification of RNA from supernatant.***

Primer	Position	Sequence (5'-3')
JFH1-s	2863	AGTGTCTGTGGTGGTTGTGC
JFH1-as	4158	CCTAATGTTGGGATTGATGCC

Table A.14: ***Primers used for introduction of resistance mutations.***

Primer	Position	Sequence (5'-3')
1bD168E-sQC	3924	TCGCGAAGGCGGTGGAGTTCATACCCGTTGAG
1bD168E-asQC	3924	CTCAACGGGTATGAACTCCACCGCCTTCGCGA
1bD168A/V-sQC	3924	TCGCGAAGGCGGTGGYCTTCATACCCGTTGAG
1bD168A/V-asQC	3924	CTCAACGGGTATGAAGRCCACCGCCTTCGCGA
4aD168E-sQC	3924	TGGCTAAAGCAGTGGAGTTCGTGCCGGTTGAA
4aD168E-asQC	3924	TTCAACCGGCACGAACTCCACTGCTTTAGCCA
4aD168A/V/G-sQC	3924	TGGCTAAAGCAGTGGBTTTCGTGCCGGTTGAA
4aD168A/V/G-asQC	3924	TTCAACCGGCACGAAAVCCACTGCTTTAGCCA
4aA156T-sQC	3924	GCATCTTCCGAACCGCGGTGTGCACC
4aA156T-asQC	3924	GGTGCACACCGCGGTTCGGAAGATGC
4aA156V-sQC	3924	GCATCTTCCGAGTCGCGGTGTGCACC
4aA156V-asQC	3924	GGTGCACACCGCGACTCGGAAGATGC

Appendix B
Common Solutions and Buffers

4% Paraformaldehyde. In a foom hood, 8 mg of paraformaldehyde were added to 100 ml dH_2O, followed by 2 ml 1 M NaOH. The mixture was styred gently and incubated at 60°C until completely dissolved. 20 ml 10X PBS were added and the solution allowed to cool to room temperature. After adjusting the pH to 7.4, using 1 M HCl, dH_2O was added to a final volume of 200 ml. The solution was then filtered through a 0.45 μm filter and aliquoted to 30 ml in 50 ml falcon tubes. Aliquots were wrapped in aluminium foil and stored at -20°C.

Mowiol mounting medium. 2.4 g Mowiol (Calbiochem) were added to 6 g of glycerol and thoroughly stirred. 6 ml dH_2O were added and the mixture incubated at room temperature for 2 hours. Following the addition of 12 ml 0.2 mM Tris (pH 8.5) the solution was incubated at 55°C until Mowiol had dissolved. The solution was clarified by centrifugation at 1,500 $\times$ g for 20 minutes and 1,4-diazobicyclooctane (DABCO, antifade agent, Sigma) added to 2.5 %. Aliquots were stored at -20°C.

Table B.1: ***Reagents used in this thesis.***

Reagent	Company/Source
Cell Lines	
Huh7 and Huh7.5 cell lines	Gifts from Mark Harris, Leeds
DMEM media	Invitrogen
100X non-essential amino acids	Invitrogen
Penicillin-streptomycin	Invitrogen
Hepes	Sigma-Aldrich
FCS	Harlan Sera-Lab
Plastic ware	Nunc/Thermo Fisher Scientific
DMSO Hybri-MAX®	Sigma-Aldrich
RNA Work	
RNA/DNA extraction and purification kits	QIAGEN
T7 RiboMAX™ for RNA production	Promega
PCR	
Primers	Sigma-Aldrich
Access RT-PCR system	Promega
GoTaq® DNA polymerase	Promega
SuperScript™ III RT-PCR system	Invitrogen
KOD Hot Start DNA polymerase	NOVAGEN
VentR® polymerase	New England BioLabs
FastStart Taq DNA polymerase	Roche
SYBR Green I	Invitrogen
Cloning Techniques	
LB-agar and broth	Merck
Glycine	Sigma-Aldrich
2-mercaptoethanol, min 98 %	Sigma-Aldrich
Glycerol	Fisher Scientific Ltd
Phenol:chloroform:isoamyl-alcohol:25:24:1	Fluka/Sigma-Aldrich
Quick-change site directed mutagenesis kit	Stratgene
pCR®-blunt II-TOPO vector	Invitrogen
TOP10 One Shot® chemically competent *E.Coli*	Invitrogen
NEB 5-α	New England BioLabs
NEB 10-β	New England BioLabs

Table B.2: ***Reagents used in this thesis.***

Reagent	Company/Source
Enzymes	
Restriction enzymes	New England BioLabs
Mung Bean nuclease	New England BioLabs
Antarctic Phosphatase	New England BioLabs
T4 DNA Ligase	New England BioLabs
Immunostaining	
Paraformaldehyde powder 95 %	Sigma-Aldrich
Triton-X	Sigma-Aldrich
PBS tablets	OXOID
NaOH tablets	Sigma-Aldrich
Sheep anti-NS5A serum	Gift from Mark Harris, Leeds
Alexa Fluor 488 donkey anti-sheep IgG	Invitrogen
Protease Inhibitors	
BILN 2061	Gift from GlaxoSmithKline
VX-950	Acme Bioscience, Inc. CA.

Table B.3: ***Recipe used to make up 1X PBS.***

Reagent	Quantity
Sodium chloride	8 g
Potassium chloride	0.2 g
Di-sodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
dH_2O	to a final volume of 1 l
pH	7.3

Table B.4: ***Recipe used to make up 50X TAE.***

Reagent	Quantity
Tris-acetate	242 g
Glacial acetic acid	57.1 ml
0.5 mM EDTA (pH 8.0)	100 ml
dH_2O	to a final volume of 1 l

Table B.5: ***NEB restriction enzyme buffers.***

Reagent	Concentration
1	
Bis-Tris-propane-HCl	10 mM
$MgCl_2$	10 mM
Dithiothreitol	1 mM
pH	7
2	
NaCl	50 mM
Tris-HCl	10 mM
$MgCl_2$	10 mM
Dithiothreitol	1 mM
pH	7.9
3	
NaCl	100 mM
Tris-HCl	50 mM
$MgCl_2$	10 mM
Dithiothreitol	1 mM
pH	7.9
4	
Potassium acetate	50 mM
Tris-acetate	20 mM
Magnesium acetate	10 mM
Dithiothreitol	1 mM
pH	7.9

Appendix C
Sequences

Table C.1: ***Amino acid diversity among NS3 sequences derived from HCV-infected plasma of genotype 1b.*** *Sequences were compared to 459 sequences retrieved from the Los Alamos HCV database project (Kuiken* et al. *(2005)) and NCBI GenBank. First letter indicates the amino acid in HC-J4 (D10750), if not dominant in lower case letter. Dominant amino acids are in big capital letters, minor occurring ones at polymorphic sites in lower case. Amino acids not occurring in any other variant are in italic. Amino acid positions are numbered according to their position in the H77 (AF009606) reference strain.*

NS3-1b					
HC-J4	**v/L1039(m,p)**	**I/V1074(t)**	**S1087(t,p,a,c)**	**L/Q1106(k,r)**	**M/L1120**
J1b1b					
J1b1b-2	**L**		**T**	**Q**	**L**
J1b1b-12	**L**	**V**		**Q**	**L**

NS3-1b					
HC-J4	**S/T1124(a)**	**S1151(a)**	**v/A1176(I,l,t)**	**v/I1179(l)**	**I/V1196**
J1b1b					
J1b1b-2	**T**	**A**	**A**	**I**	**V**
J1b1b-12	**T**		**A**	**I**	**V**

Table C.2: ***Amino acid diversity among NS3 and NS4A sequences derived from HCV-infected plasma of genotype 1a.*** *Sequences were compared to 570 NS3 and NS4A sequences retrieved from the Los Alamos HCV database project (Kuiken* et al. *(2005)), NCBI GenBank and those in chapter 3. First letter indicates the amino acid in H77 (AF011751), if not dominant in lower case letter. Dominant amino acids are in big capital letters, minor occurring ones at polymorphic sites in lower case. Amino acids not occurring in any other variant are in italic. Amino acid positions are numbered according to their position in the H77 (AF009606) reference strain.*

NS3-1a

H77	A1033(t/s)	E1056	V1059i	T/A1066(g,s)	V1077(a)	V1097(i,a)
J1a1a-H77*						
J1a1a-1		*G*		A		
J1a1a-2				A		
J1a1a-3	T					
J1a1a-4			I	G	A	
J1a1a-5				A		
J1a1a-6				A		
J1a1a-7						
J1a1a-8						I
J1a1a-9	T					

NS3-1a

H77	I1090(m,l,v)	P1093(s,a)	P1112(s,l, t,a,q)	A1113(s,v,l,t,c)	S/A/T1117(g)	S1127(a)
J1a1a-H77*						
J1a1a-1					A	
J1a1a-2		A	A		A	
J1a1a-3	M				A	
J1a1a-4	L				A	
J1a1a-5		S			A	
J1a1a-6					A	
J1a1a-7					A	
J1a1a-8					A	A
J1a1a-9				V	A	

NS3-1a

H77	H1136(q,n,r)	S1148(g)	I/L1179(v)	N/S1200(d,g,k)	G/E1202	M1205(i,t,v)
J1a1a-H77*						T
J1a1a-1		G	I	S	E	T
J1a1a-2			I	S	E	T
J1a1a-3			I			T
J1a1a-4			I	S		T
J1a1a-5		G	I	S		T
J1a1a-6			I	S		T
J1a1a-7			I		E	T
J1a1a-8	Q		I		E	T
J1a1a-9			I		E	T

NS4A-1a

H77	K1691(r)	I/V1694(l,m)	P1696(y)	Q/R1703(k)	C1711
J1a1a-H77*					
J1a1a-1	R		Y	R	*L*
J1a1a-3		V			
J1a1a-6				R	
J1a1a-7				R	
J1a1a-8		V			

Table C.3: ***Amino acid diversity among NS3 and NS4A sequences derived from HCV-infected plasma of genotype 3a.*** *Sequences were compared to 242 NS3 and 180 NS4A sequences retrieved from the Los Alamos HCV database project (Kuiken* et al. *(2005)), NCBI GenBank and those in chapter 3. First letter indicates the amino acid in HCV3a-Gla, if not dominant in lower case letter. Dominant amino acids are in big capital letters, minor occurring ones at polymorphic sites in lower case. Amino acids not occurring in any other variant are in italic. Amino acid positions are numbered according to their position in the H77 (AF009606) reference strain.*

NS3-3a						
HCV3a-Gla	**A/T1033(p)**	**S1046(g,r)**	**V1054(g,l,m)**	**A1065(s,t)**	**i/V11074(l)**	**A/V1093(I,n,p,s,t)**
J3a3a-3				**T**		
J3a3a-6		***N***				
J3a3a-8		**G**	**L**		**V**	**P**
J3a3a-11	**T**				**V**	

NS3-3a					
HCV3a-Gla	**A1117(t,v)**	**A/T1124(s)**	**D1136(e,n)**	**A1177(m,t,v)**	**T1200(a,s)**
J3a3a-3	**A**	**T**		**T**	
J3a3a-6		**T**			
J3a3a-8	**A**	**T**		**T**	
J3a3a-11	**A**		**E**		**A**

NS4A-3a	
HCV3a-Gla	**L1670(v,f)**
J3a3a-3	
J3a3a-6	
J3a3a-8	
J3a3a-11	***I***

Table C.4: ***Amino acid diversity among NS3 sequences derived from HCV-infected plasma of genotype 4a.*** *Sequences were compared to 39 NS3 sequences retrieved from the Los Alamos HCV database project (Kuiken* et al. *(2005)) and NCBI GenBank. First letter indicates the amino acid in ED43 (Y11604), if not dominant in lower case letter. Dominant amino acids are in big capital letters, minor occurring ones at polymorphic sites in lower case. Amino acids not occurring in any other variant are in italic. Amino acid positions are numbered according to their position in the H77 (AF009606) reference strain.*

NS3-4a						
ED43	**L1039(m)**	**F1040(c,l,v)**	**S1041(g,n)**	**V/I1044**	**T1048**	**C1056**
J4a4a-ED43*					***A***	
J4a4a-7	***T***	**L**	**G**	**I**		***R***
J4a4a-8				**I**		
J4a4a-10	**M**	**L**	**G**	**I**		
J4a4a-19	**M**	**L**	**G**	**I**		

NS3-4a						
ED43	**V1059(l)**	**T1064**	**V/I1074**	**a/S1087(g)**	**R/K1118(t)**	**A/T1121(v,s)**
J4a4a-ED43*		***I***				
J4a4a-7	**I**		**I**	**S**	**K**	
J4a4a-8			**I**	**S**	**K**	**T**
J4a4a-10	**I**		**I**	**S**	**K**	
J4a4a-19	**I**		**I**	**S**	**K**	

NS3-4a						
ED43	**S/A1127**	**a/S1128**	**Y1131(f)**	**i/V1140**	**L1153**	**i/T1160(l)**
J4a4a-ED43*						**T**
J4a4a-7		**S**	**F**	**V**		**T**
J4a4a-8	**A**	**S**		**V**		**T**
J4a4a-10		**S**	**F**	**V**		**T**
J4a4a-19	**A**	**S**	**F**	**V**	***I***	**T**

NS3-4a				
ED43	**L1169**	**r,A1176(v)**	**M1173(l,q)**	**I1179(v,l)**
J4a4a-ED43*		**A**		
J4a4a-7		**V**	**L**	**L**
J4a4a-8		**A**		
J4a4a-10	***P***	**V**	**L**	
J4a4a-19		**A**	**L**	

Table C.5: ***Amino acid diversity among NS3 sequences derived from HCV-infected plasma of genotype 6a.*** *Sequences were compared to 15 NS3 sequences retrieved from the Los Alamos HCV database project (Kuiken* et al. *(2005)) and NCBI GenBank. First letter indicates the amino acid in EUHK2 (Y12083), if not dominant in lower case letter. Dominant amino acids are in big capital letters, minor occurring ones at polymorphic sites in lower case. Amino acids not occurring in any other variant are in italic. Amino acid positions are numbered according to their position in the H77 (AF009606) reference strain.*

NS3-6a						
EUHK2	**T1030(s)**	**V/L1040(m,f,i)**	**d/A1065**	**v/L1070**	**N1075(s)**	**p/A1085**
J6a6a-EUHK2*			**A**	**L**		**A**
J6a6a-4	**S**	**I**	**A**	**L**		**A**
J6a6a-8			**A**	**L**	**S**	**A**

NS3-6a						
EUHK2	**f/S1087(a)**	**K1094(r)**	**l/K1106(q)**	**n/T1124(a)**	**s/N1148**	**A/T1121(v,s)**
J6a6a-EUHK2*	**S**	**R**				
J6a6a-4	**S**		**K**	**T**	**N**	
J6a6a-8	**S**		**K**	**A**	**N**	**T**

Publications

Imhof, I. & Simmonds, P. 2010. Development of an intergenotypic HCV cell culture method to assess antiviral susceptibilities and resistance development of HCV NS3 protease genes from HCV genotypes 1-6. *J Virol*, in Press. Printout page 306.

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