Evolution of the envelope proteins E1 and E2 and of specific humoral immune response to these proteins in a group of patients infected by HCV in a single-source outbreak

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1 Introduction

Hepatitis C virus (HCV) infection causes approximately 40 % of chronic liver diseases worldwide and is considered as a serious public-health problem. According to the estimates of the World Health Organization (2010), over 170 million people are infected with HCV, of whom 60 to 80 % develop chronic hepatitis, leading to cirrhosis in 10 to 20 % and to hepatocellular carcinoma (HCC) in 1 to 5 % of infected individuals. In the western industrial countries, HCV is the major cause of liver transplantations (Brown, 2005). Currently, a combination therapy with interferon-α (IFN-α) supplemented with the nucleoside analog ribavirin is used for treatment of chronic HCV infection but a sustained virological response is achieved only in about 50 to 80 % of patients (Zeuzem, 2004). A response rate depends on virus genotype, so that a sustained response is achieved in 88 % of cases of genotype 2, whereas for genotypes 1, 4, 5 and 6 in only 48 % of cases a sustained response is observed (Poynard *et al.*, 2003). These data emphasize the need for new prophylactic/therapeutic approaches that would prevent spread of HCV and would provide more efficient antiviral therapy of individuals suffering from a chronic hepatitis C. The progress in development of HCV vaccine and new therapeutic approaches will undoubtedly depend on our knowledge of biology of HCV and characteristics of its interaction with the infected host. In the current section of this thesis an attempt is made to summarise a large bulk of information on molecular biology of HCV, including organization of its genome, mechanisms of its replication, characteristics of viral proteins, and peculiarities of antiviral immune responses.

1.1 The HCV Virus

Morphology of the HCV

HCV is a coated ecosaedric virus whose diameter is about 50 to 70 nm (Kaito *et al.*, 1994; Li *et al.*, 1995). HCV has been classified as the only member of the *Hepacivirus* genus and belongs to the *Flaviviridae* family. Due to the similarity with other *Flaviviridae* viruses, HCV contains an envelope consisting of two envelope proteins E1 and E2. These proteins enclose the spheric nucleocapsid, which is composed of the core proteins and in turn contains the viral RNA (Penin, 2003).

The HCV genome

The HCV genome is a single-stranded RNA molecule of positive polarity approximately 9.6 kb in length. It contains a single open reading frame (ORF) of approximately 9 kb that encodes a polyprotein of 3000 amino acids (a.a.). The ORF is flanked at both ends by untranslated regions (UTR), which are the most conserved regions of the genome (Cocquerel *et al.*, 2006). The 5'UTR consists of 342 nucleotides (nt) and contains an internal ribosomal entry side (IRES), which mediates the translation of the polyproteins (Tsukiyama-Kohara *et al.*, 1992). Downstream of the coding region another untranslated region of approximately 200 nucleotides (3'UTR) is located, which can be divided into 3 parts: a variable region (ca. 40 nt), a poly-uracil track of a variable length (20 to 90 nt), and a highly conserved region (98 nt), which is essential for replication (Cheng *et al.*, 1999; Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996; Yamada *et al.*, 1996).The polyprotein consisting of approximately 3000 a.a. is cleaved by viral and host enzymes into 10 polypeptides: core, envelope 1 (E1), envelope 2 (E2), p7, non-structural protein 2 (NS2), non-structural protein 3 (NS3), non-structural protein 4A and 4B (NS4A and NS4B), and non-structural proteins 5A and 5B (NS5A and NS5B) (Fig.1.1.).

HCV can be classified on the basis of the dissimilarity of nucleotide sequences into major genetic groups or genotypes. There are 7 HCV genotypes. Most of them consist of several subgroups or subtypes. Due to a high selective immune pressure, virus circulated in the infected host consist of a large population of closely related but still distinct genetic variants or quasispecies. The causes of a high sequence diversity of HCV are a high rate of nt misincorporation by virus-encoded error-prone RNA polymerase (NS5B protein) and a large size of virus RNA population. The error rates of approximately 1 per 10.000 copied base pairs combined with a high rate of virus production of up to 10^{12} virions per day (Neumann *et al.*, 1998; Simmonds, 2004) lead to appearance of an extremely diverse population of virus genetic variants, which are subjected to an immune selection driven by host.

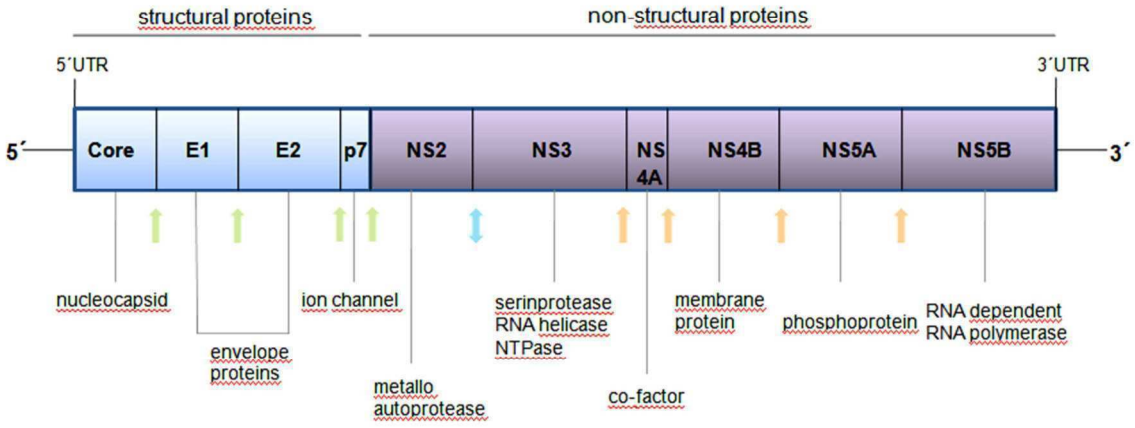


Fig.1.1. Genomic organization of the HCV genome

Cleavage sites of the cellular proteases (green arrows), the NS2 proteases (blue arrow) and the NS3/4A protease-complex (orange arrows) are indicated.

HCV encoding proteins

The ORF of HCV encodes a polyprotein of 3022 a.a.. The polyprotein is co- and post translationally cleaved by viral and host-cell proteases into 10 functional subunits. The structural proteins are located in the N-terminal part, whereas the non-structural proteins are located at the C terminal part of the polyprotein. Core, E1 and E2 are the major constituents of virions.

Core, as the first structural protein is located directly at the N-terminus. It forms the nucleocapsid which encloses and interacts probably the genomic RNA (Baumert *et al.*, 1998). This protein seems to play a role in different cellular signaling transduction cascades and in the oncogenesis (Chang *et al.*, 1998; Matsumoto *et al.*, 1997).

The HCV proteins E1 and E2 are the membrane-associated glycoproteins, forming an integral part of the HCV virion envelope. The proteins are targeted to the endoplasmatic reticulum (ER), where they are cleaved from the viral polyprotein by a host signal peptidase cleavage (Dubuisson *et al.*, 2002). They are type-I transmembrane proteins of about 35 kilo Dalton (kd) and 70 kd (746 aa), respectively, with a large N-terminal ectodomain and a C-terminal transmembrane domain, and they assemble as noncovalent heterodimers (Deleersnyder *et al.*, 1997). Both E1 and E2 transmembrane domains are composed of two short stretches of hydrophobic a.a. separated by short polar segment containing fully conserved charged residues (Cocquerel *et al.*, 2000), whereas the second hydrophobic stretch acts as an internal signal peptide for the downstream protein (Cocquerel *et al.*, 2002).

Before signal-sequence cleavage, the E1 and E2 adopt a hairpin structure and after cleavage by a host signal peptidase the signal-like sequence is re-oriented towards the cytosol, which, in turn, leads to a transmembrane passage (Cocquerel *et al.*, 2001). The deletion of the C-terminal hydrophobic regions of the proteins leads to their secretion (Michalak *et al.*, 1997), suggesting that these regions are involved in membrane anchoring. E1 and E2 can also form heterodimers of both covalent (disulfide-bonded) and non-covalent types (Choukhi *et al.*, 1999).

The E2 glycoprotein contains a hypervariable region I (HVRI), in which, presumably, one of the major neutralization epitopes is located (Farci *et al.*, 1996). High variability of HVRI during infection is considered as a major mechanism that allows the virus to avoid the host humoral immune response (Farci *et al.*, 2000). Experimental deletion of the E2 HVRI results in persistent (albeit low-level) viremia, which suggests that this region is not essential for viral replication, but that its disruption might lead to attenuation of the viral infection (Forns *et al.*, 2000), which might be explained by its involvement in virus entry (Bartosch *et al.*, 2003c; Callens *et al.*, 2005). The E2 glycoprotein contains another hypervariable region, HVR2, which has been described in HCV genotype 1 strains (Kato *et al.*, 2001) and includes a stretch of 7 a.a. (position 91-97) which demonstrate a high level of sequence diversity. It is assumed, that HVRI modulates a binding of E2 to the host receptor CD81 (Roccasecca *et al.*, 2003; Sklan *et al.*, 2009; Zeisel & Baumert, 2009).

HCV envelope glycoproteins are retained in the ER (Deleersnyder *et al.*, 1997; Dubuisson *et al.*, 1994), whereas ER retention signals are present in the transmembrane domains of E1 and E2 (Cocquerel *et al.*, 1999; Cocquerel *et al.*, 1998). The charged residues of the transmembrane domains of E1 (Lys) and E2 (Asp and Arg) play the key role in the ER retention of these glycoproteins (Cocquerel *et al.*, 2000). Furthermore, it was shown, that the transmembrane domains also have an important role in the assembly of E1/E2 heterodimer (Op De Beeck *et al.*, 2000). Although the E1/E2 heterodimer is mainly retained in the ER, HCV glycoproteins can also be detected at the plasma membrane. Importantly, the retention of E1/E2 in the ER has been confirmed in HCV cell culture (HCVcc) - infected cells (Rouille *et al.*, 2006).

The ectodomains of HCV envelope proteins E1 and E2 are highly modified by N-linked glycans, whereas they posses up to 6 and 11 potential glycosylation sites, correspondingly, and most of them are well conserved (Dubuisson, 2007; Goffard &

Dubuisson, 2003). In the early secretory pathway, the glycans play an important role in protein folding, quality control and certain sorting events. Viral envelope proteins usually contain N-linked glycans that can play a major role in their folding, in their entry functions or in modulating the immune response (Ohuchi *et al.*, 1997a; Ohuchi *et al.*, 1997b). Sequence analyses suggest that N-glycosylation sites in the E1 are strongly conserved among HCV genotypes (Goffard & Dubuisson, 2003). For E2 a global sequence analysis indicate that 9 of 11 sites are strongly conserved. The two remaining sites show conservation levels of 75 % and 89 %, respectively (Goffard & Dubuisson, 2003; Lavie *et al.*, 2007). Studies with HCVpp showed that the HCV envelope protein glycosylation is dependent on fewer than 14 a.a. ahead of E1 and 12 a.a. ahead of E2 (Bian *et al.*, 2009). It is worth noting, that some glycans have been shown to play a role in HCV glycoprotein folding or in virus entry (Dubuisson, 2007; Goffard *et al.*, 2005). The most recent data obtained using the HCVcc system confirm that the glycans associated with HCV envelope glycoproteins play an important role at different steps of viral life cycle especially in HCV assembly and/or infectivity. It was also demonstrated that at least five glycans on E2 strongly reduce the sensitivity of HCVcc to antibody neutralization. Four of these sites surround the CD81 binding domain (Helle *et al.*).

HCV envelope glycoproteins have been shown to assemble as a concovalent E1/E2 heterodimer (Deleersnyder *et al.*, 1997). However, in heterologous expression systems, HCV envelope glycoproteins have a tendency to form misfolded aggregates stabilized by disulfide bonds. Therefore, analyses of E1/E2 heterodimer with conformation-sensitive antibodies are necessary to distinguish noncovalent heterodimers from misfolded complexes (Cocquerel *et al.*, 2003b; Deleersnyder *et al.*, 1997). Analysis of the formation of conformation-dependent epitopes demonstrated that the process of folding of HCV envelope glycoproteins is a very slow one (Brazzoli *et al.*, 2005; Dubuisson & Rice, 1996). Analysis of the disulfide bonds formation in attempt to establish a tertiary organization of the E2 molecule revealed that the E2 ectodomain consists of 28 % ß-sheets (Krey *et al.*). The folding of E1 has been shown to be dependent on the co-expression of E2 (Michalak *et al.*, 1997) and vice versa (Brazzoli *et al.*, 2005; Cocquerel *et al.*, 2003a).

C-terminal to the E2 the p7 membrane protein is located. It was assigned to the viroporins group and is essential for formation of ion channels for the last steps of

virus replication (Carrere-Kremer *et al.*, 2002). Moreover, p7 is required for assembly and release of infectious particles (Jones *et al.*, 2007; Sakai *et al.*, 2003).

The non-structural proteins NS2, NS3, NS4A, NS4B, NS5A und NS5B have different functions during the process of viral Ribunucleic acid (RNA) - replication and proteolytic protein processing (Lohmann *et al.*, 1999). NS2 is acting as membrane protein, which is not essential for HCV RNA replication (Blight *et al.*, 2000; Lohmann *et al.*, 1999). The NS2/NS3 protease cleaves the NS2/NS3 junction. Additionally, NS2 domain is further required for the production of infectious virus (Jirasko *et al.*, 2008). The multifunctional NS3 protein consists of a N-terminal serine protease, which catalyzes the processing of all other nonstructural proteins within the C-terminal part of the precursor polyprotein. The C-terminal part of the NS3 protein contains the RNA helicase/nucleoside triphosphate hydrolase (NTPase) domain that is essential for translation and RNA-replication (Bartenschlager *et al.*, 1993). Formation of a heterodimer NS3-NS4A enhances the activity of the protease (Bartenschlager *et al.*, 1994). This complex is essential for processing of all proteins located C-terminal of NS3. Recent studies demonstrated that the NS3/NS4A also targets some host proteins that are involved as signaling intermediates in virus-induced production of Interferon- ß (IFN-ß) (Li *et al.* 2005). The NS4A protein is a membrane protein forming the membrane structure and supporting RNA replication (Egger *et al.*, 2002). The NS4B protein appears as punctuate foci at the ER membrane and consists of four central transmembrane domains with N- and C-termini located in the cytoplasm of the host cell (Lundin *et al.*, 2003). Due to the important function as a component of the replicase complex, NS4B, is thought to create sites for genome synthesis and, as has recently been further suggested, contributes to the processes engaged in virus assembly and release (Jones *et al.*, 2009). The membrane associated NS5A protein is essential for HCV genome replication through its interaction with viral replication complex and several host cell factors (Evans *et al.*, 2004). It was shown, that the interaction of NS5A with NS5B is required for the maintenance of the subgenomic replicon in transfected cells (Shirota *et al.*, 2002). NS5B, as a membrane associated protein acts as a viral RNA-dependent RNA polymerase (Bartenschlager *et al.*, 2004; Behrens *et al.*, 1996). The RNA polymerase activity can presumably be modulated by interaction of NS5B with viral proteins NS3 and NS5A.

Additionally, within the core region, an alternative reading frame is present (Branch et al. 2005). The encoded protein received the name of alternative frame protein (ARFP) or frame shift protein (F protein). It comprises of up to 160 a.a.. Sequencing of the *in vitro* labeled ARFP protein indicated that the frameshift junction is likely to occur at codons 9 to 11 of the core protein sequence (Xu *et al.* 2001). The function of this protein in the lifecycle remains unclear.

1.2 Model systems of HCV infection

1.2.1 *in vivo* models

The only animal species, which supports HCV replication and shows a similar course of disease as human beings, is chimpanzee. This animal model seems to be ideal to get new insights into the progression of the HCV infection and to gain new perceptions of the immune response in acutely infected and chronically infected animals. An obvious advantage of working with chimpanzees is the utilization of the same reagents and methods as used for human beings. Nevertheless, working with this animal model also reveals disadvantages like high maintenance cost, scarcity and ethical considerations. Multiple attempts were made to develop a more suitable small animal model for HCV. Thus, some attention was drawn to the tupaias (*T.belangeri chinensis) (Xie et al., 1998),* a species already shown to be susceptible to infection with hepatitis B virus (Walter *et al.*, 1996; Yan *et al.*, 1996). However, only one quarter of the animals became infected with HCV and develope in most cases only transient viremia with low virus titres. Another approach was the HCV infection of a SCID mouse grafted with human hepatocytes (Mercer *et al.*, 2001). Although the SCID mouse provide an opportunity to study some characteristics of HCV infection, this model also has some disadvantages, including requirement for a special expertise for establishment and handling, and a high mortality rate of animals.

1.2.2 *in vitro* models

To study the complexicity of the HCV life cycle, *in vitro* models had to be invented. At the beginning scientists developed systems that led to the overexpression of HCV proteins in cell cultures (Macdonald & Harris, 2004). Subsequent development of a subgenomic and, few years later, of a full genomic HCV replicons provided us with

new opportunities to study different aspects of HCV replication and HCV replicons were used with a lot of success in many laboratories worldwide (Lohmann *et al.*, 1999) (Blight *et al.*, 2002; Lohmann *et al.*, 2003). (Blight *et al.*, 2003) (Kato, Date *et al.* 2003) (Pietschmann *et al.* 2002). Unfortunately, all generated replicons despite relatively high rates of HCV RNA replication did not produce infectious virions. The breakthrough occurred only after preparation of the replicon on the basis of the virus strain isolated in Japan from a patient with fulminant hepatitis (Kato *et al.*, 2003). The generated JFH-1 replicon was not only able to replicate in Huh7 cells without any adaptive mutations but also produced the mature infectious virus particles. Experiments with this clone in several laboratories led to the development of cell culture systems that allowed for a relatively efficient replication of HCV *in vitro* (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The virus produced by these systems was referred to as a cell culture derived virus or HCVcc. The developed systems were based on transfection of the human hepatoma cell lines Huh7.5 or Huh7.5.1 with genomic HCV RNA of genotype 2a JFH1 strain. In order to allow comparative studies between different HCV strains, chimeric genomes encoding the structural proteins from different genotypes (e.g. from genotype 1a strain H77) and the non-structural proteins from the JFH1 strain have been made (Lavie *et al.*, 2007; Pietschmann *et al.*, 2006). The viruses produced in cells transfected with these chimeric RNAs were infectious for naïve cells, for chimpanzees and for immunodeficient mice transplanted with human hepatocytes (Lindenbach *et al.*, 2006; Wakita *et al.*, 2005). It was also shown that HCVcc could be efficiently neutralized by anti-HCV antibodies derived from human sera (Wakita *et al.*, 2005), as well as by polyclonal anti-envelope antibodies (Pietschmann *et al.*, 2006). Thus, the HCVcc system provides an exellent opportunity to study the complete HCV life cycle.

In parallel to the development of HCV replicon-based systems several other models suitable for studies of some stages of HCV replicative cycle were proposed. The most successful of these systems was based on pseudotyping of particles produced by other viruses, including vesicular stomatitis virus (VSV) or retroviruses, with HCV E1 and E2 glycoprotein (Lagging *et al.*, 1998; Matsuura *et al.*, 2001) (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Lavie *et al.*, 2007). In most of the cases, the murine leukemia virus (MLV) of human immunodeficiency virus (HIV) vectors was used. Retroviruses are known to be able to incorporate into their envelope a variety of cellular and viral glycoproteins (Ott, 1997). Furthermore, it is known that they can

easily package and deliver into the transfected cells different genetic markers (Negre *et al*., 2002). These properties are exploited to produce viral pseudoparticles expressing HCV E1/E2 at their surface and packaging a reporter gene that allows the monitoring of viral infection. The HCV pseudoparticles (HCVpp) are produced by transfection of human embryo kidney 293T cells with two or three expression vectors encoding the HCV E1/E2 proteins, the retroviral gag/pol proteins, and a packaging-competent retrovirus-derived genome containing a marker gene like the green fluorescent gene (GFP) or the firefly luciferase gene (Fig.1.2). The produced HCVpp are infectious for cell lines of hepatocyte origin, principally for Huh7.5 cells and their derivatives as well as for human primary hepatocytes (Bartosch *et al.*, 2003b; Hsu *et al*., 2003). Nowadays, the HCV pseudoparticles model (HCVpp) is widely used for analysis of the mechanisms of HCV entry and studies of HCV-specific humoral immune responses.

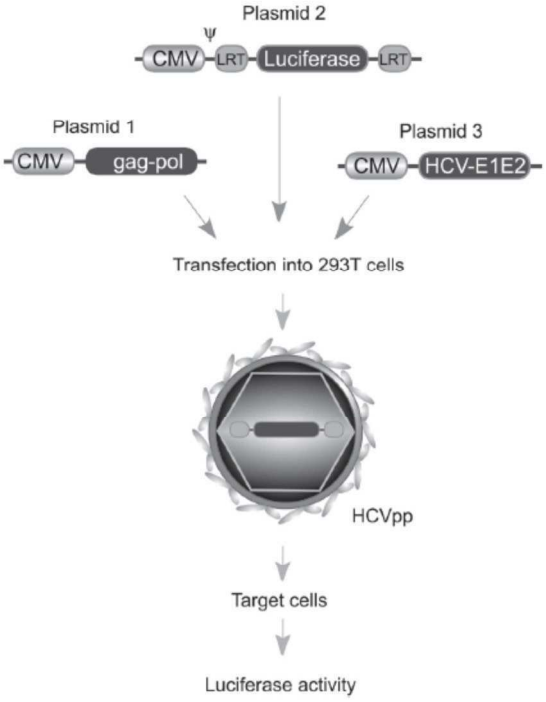


Fig.1.2. Production of HCV pseudopaticles (HCVpp) (Lavie *et al*., 2007)

293T cells are transfected with three expression vectors. The first vector encodes retroviral Gag and Pol protein. Gag proteins are responsible for particle budding at the plasma membrane and RNA encapsidation via recognition of the specific retroviral encapsidation sequence (ψ). The second vector harbors a ψ sequence for encapsidation and encodes a reporter protein (Luciferase). This vector also contains retroviral sequences that are necessary for the reverse transcription of genomic RNA into proviral DNA by retroviral protein Pol encoded by the first vector. The third vector encodes HCV envelope glycoproteins, which are responsible for the cell tropism and fusion of HCVpp with the target cell membrane (Lavie *et al*., 2007).

1.3 The replication cycle

The HCV replication cycle starts with the process of viral entry into the host cell (Fig.1.3.). This complex multistep process is still not clear but it is known that the two envelope proteins E1 and E2 play the major role in virus entry (Bartosch & Cosset, 2006; Cocquerel *et al.*, 2006). HCV enters cells by clathrin-mediated endocytosis (Blanchard *et al.*, 2006). A number of cellular receptors and co-receptors of HCV have been identified. They include glycosaminoglycans, the Low density lipoprotein (LDL) receptor, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), and liver/lymph node-specific intracellular adhesion molecules-3 grabbing non-integrin (L-SIGN), CD81, Scavenger receptor class B member 1 (SR-BI), claudin-1 and occludin-1 (Sabahi, 2009). Recent studies suggest that within the endosome, the low pH environment triggers the fusion process of the virus with the endosomal membrane and the introduction of the HCV genome into the cytoplasm (Lavillette *et al.*, 2006). After the process of entry, RNA is released into the cytoplasm and serves directly as mRNA for the synthesis of viral proteins. Following the polyprotein processing, the proteins retain at the membrane of the ER. The expressed HCV proteins induce intracellular membrane alterations. The generated replication-complex is bound to the altered cell membrane (Moradpour *et al.*, 2003). The RNA with positive polarity serves as matrice for the synthesis of RNA of negative polarity, which is used afterwards for the synthesis of positive strands of RNA. The new RNA with positive polarity is consequently used for synthesis of negative strands of RNA, which is used for protein expression or for virion packaging. The mature virion is thought to possess a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. Virion assembly presumably begins with the interaction of capsid proteins and genomic RNA to form a nucleocapsid, which acquires an envelope and the matured virion is then secreted by following the classical secretory pathway.

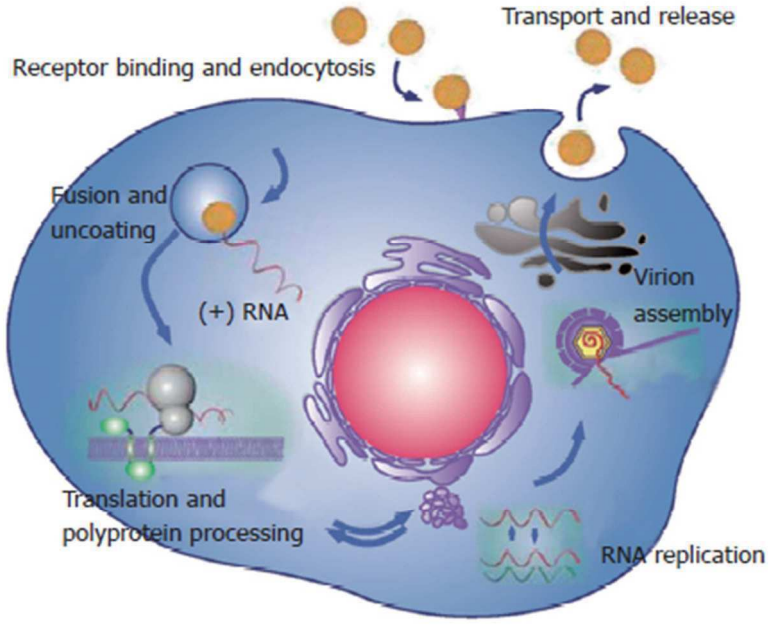


Fig.1.3. Schematic representation of the major steps of HCV replication cycle (Dubuisson *et al.*, 2007)

HCV binds to the receptor complex at the cell surface, which leads to endocytosis of the particle. Fusion of viral envelope and endosomal membrane leads to the release of the HCV genome into the cytosol. Positive stranded RNA is directly translated and all viral proteins are simultaneously produced. Expression of HCV proteins induces intracellular membrane alterations (membranous web), where RNA replication takes place. Accumulation of HCV genomic RNA and the structural proteins leads to the assembly of a nucleocapsid, which acquires its envelope within an intracellular compartment. The viral particle is then secreted by following the secretory pathway.

1.4 The role of envelope proteins during HCV entry

Binding and entry of HCV is believed to be a complex multistep process involving the HCV envelope glycoproteins E1 and E2, as well as several attachment and entry factors like CD81, SR-BI, members of the claudin family and occludin. CD81 was recognized early as an entry receptor for HCV after the experiments, in which a soluble form of HCV glycoprotein E2 was used (Pileri *et al.*, 1998). The tetraspanin CD81 is a widely expressed 25-kd cell surface protein that is involved in pleiotropic activities such as cell adhesion, motility, metastasis, cell activation, and signal transduction (Levy *et al.*, 1998). It is a member of the tetraspanin family type III proteins and contains two extracellular loops and one short intracellular loop. Due to the lack of an *in vitro* infectious system, early studies identified that the long extracellular loop (LEL) is necessary for soluble E2 binding (Petracca *et al.*, 2000). Anti-CD81 antibodies and soluble recombinant forms of the LEL have been shown to inhibit HCVpp and HCVcc entry into Huh7 hepatoma cells and hepatocytes (Bartosch

et al., 2003b; Hsu *et al.*, 2003). Further experiment demonstrating that transduction of HepG2 and HH29 human hepatoma cells lacking CD81, to express CD81 rendered them permissive for HCV infection (Flint *et al.*, 2006). Furthermore, small interfering RNA (siRNA) silencing of CD81 showed a reduction of viral infection (Flint *et al.*, 2006; Lindenbach *et al.*, 2005). Recent studies using the HCVpp and HCVcc model system provided evidence that inhibition of viral entry by anti-CD81 antibodies appears to occur at a post-binding step of HCV (Koutsoudakis *et al.*, 2006). These findings indicate that CD81 plays an important role as an HCV cell entry receptor at a post binding step during the initiation of infection (Barth *et al.*, 2006). Studies analyzing the E2-CD81 interaction and identification of epitopes recognized by antibodies that inhibit these interactions propose that the CD81-binding region contains residues critical for CD81 binding, which were detected via site-directed mutagenesis (Owsianka *et al.*, 2006).

SR-BI was identified as an 82-kd E2 binding protein by demonstration that soluble E2 (sE2) bounds to CD81-negative HepG2 cells by means of SR-BI (Scarselli *et al.*, 2002). SR-BI is highly expressed in liver and steroidogenic tissues (Krieger, 2001), as well as in human monocyte-derived dendritic cells (Yamada *et al.*, 2005). It is involved in trafficking bidirectional cholesterol transport into hepatocytes by uptake of high-density lipoproteins (HDL) and low-density lipoproteins (LDL) into endosomes. HDL enhances HCV entry, a process which depends on the lipid transfer function of SR-BI (Voisset *et al.*, 2005). Different SR-BI ligands have been shown to influence infection, acting to enhance, like HDL (Dreux *et al.*, 2006), or inhibit, like oxidized LDL (Cai *et al.*, 2007; Lavie *et al.*, 2006), viral entry. Kinetics of inhibition with anti-SR-BI antibodies suggest a concomitant acting of SR-BI with CD81 (Zeisel *et al.*, 2007b). It has been shown, that antibodies directed against cell surface-expressed SR-BI inhibits HCV-like particles binding to primary hepatocytes (Barth *et al.*, 2005) as well as HCVpp (Bartosch *et al.*, 2003c) and HCVcc entry (Bartosch *et al.*, 2003c).

Another host cell molecule, a tight junction protein Claudin-1 (CLDN1), was recently identified to be of importance for HCV entry (Evans *et al.*, 2007). Tight junctions are continuous intercellular contacts located at the apical poles of lateral cell membranes (Stevenson & Keon, 1998). The expression of CLDN-1 in several cell lines allowed for HCVpp and HCVcc entry. The siRNA silencing of CLDN-1 expression in permissive hepatoma cells reduced HCV infection. Furthermore, antibody blocking studies, in which the tagged versions of CLDN-1 were used, showed that the first

extracellular loop is an essential co-receptor molecule during late stage of the HCV entry process (Evans *et al.*, 2007). Recently, Claudin-6 and Claudin-9 were identified acting as other HCV co-receptors (Zheng *et al.*, 2007). Anti-CLDN-1 antibodies have shown an inhibition of binding of envelope glycoprotein E2 to HCV permissive cell lines in the absence of detectable CLDN1-E2 interaction. These antibodies also neutralized the HCV infectivity by reducing E2 association with the cell surface and disrupting CD81-CLDN1 interactions (Krieger *et al.*). The most recent data confirmed that the interaction of Claudin with CD81 seems to be essential in HCV infection (Harris *et al.*)

Glycosaminoglycan chains on cell surface proteoglycans serve usually as attachment sites for the binding of number of viruses and other microorganisms. The glycosaminglycan heparan sulfate has been identified as a potential HCV cellular receptor (Barth *et al.*, 2003). Heparin, which is a homologue of highly sulfated heparan sulfate, was able to bind HCV E2 in an ELISA, in a concentration-dependent manner. The pretreatment of HCVpp and HCVcc with heparin and highly sulfated heparin inhibited the infectivity of Huh7 cells.

A recent study identified occludin, a four transmembrane domain protein present in the tight junctions of polarized epithelial cells, as another HCV cellular receptor (Ploss *et al.*, 2009). HCV permissive Huh7 or other cell lines like HepG2 cells were found to express detectable levels of occludin. Silencing of occludin expression lead to inhibition of HCVpp infection in Hep3B cells and, furthermore, to inhibition of infection of HCVpp and HCVcc in Huh7.5 cells.

Additionally, there is evidence that the LDL receptor, a single pass transmembrane glycoprotein, is involved in the HCV infection process. The function of the LDL receptor is a sequestering of cholesterol in the form of LDL and VLDL (very low density lipids) from circulation. It was shown that binding of HCV particles obtained from positive serum of patients to human dermal fibroblasts were inhibited by pretreatment of cells with purified LDL and that the expression of LDLR on COS-7 cells led to HCV binding to cells from 7 out of 12 patient sera (Monazahian *et al.*, 1999). Treatment of hepatocytes with monoclonal antibodies against LDLR or LDL also inhibited HCV infection (Molina *et al.*, 2007).

Thus, the available data indicate that there are at least three cell molecules important for HCV entry: CD81, SR-BI, and claudin-1. Contributions of other factors, such as glycosaminoglycans, LDL receptor, or occludin, have to be analyzed in more detail.

Relatively little is known about domains in the E1/E2 heterodimers involved in reaction with the cell receptors. Several CD81-binding sites were identified in the E2 molecule (Zeisel & Baumert, 2009)- Besides these binding regions, the HVRI, a 27 a.a. segment at the N-terminus of E2 has been suggested to play a role in cell attachment (Penin *et al.*, 2001). This region is known to evolve rapidly in infected individuals due to strong immune pressure driven by the host. HCV clones lacking the HVRI showed attenuated infectivity in chimpanzees, suggesting that the HVRI plays an important role in HCV infectivity (Forns *et al.*, 2000). Additionally, deletion of HVRI significantly reduced HCVpp infectivity (Bartosch *et al.*, 2003c). Most recent data suggest that HVRI is involved in interaction with the SR-BI receptor (Dreux & Cosset, 2007).

1.5 The immune response during a HCV infection

1.5.1 The innate immune response

The innate immune response is induced in the early stages of HCV infection. A group of innate immune receptors, referred to as PRRs, recognize pathogen-associated molecular patterns (PAMPs) and coordinate the innate immune response. During the HCV infection, a viral double strain RNA (dsRNA) is bound by the receptors TLR-3 and RIG-I inducing a signaling cascade, by which the synthesis of Type I interferon is induced. The release of IFN by infected hepatocytes or plasmacytoid dendritic cells induces the transcription of IFN-stimulated genes leading to an increased IFN production via a positive feedback mechanism. Type I IFN enhances furthermore the expression of human leukocyte antigen (HLA) class I on the surface of antigen-presenting cells reinforcing the cellular immune response. Moreover, effector proteins such as PKR, which inhibits translation, or OAS, which induces viral mRNA degradation, are produced (Bode *et al.*, 2007). It was shown, that the signaling cascades are detectable during the first two days of infection (Bigger *et al.*, 2001). Despite the cellular IFN production and IFN therapy of infected patients, in some individuals HCV can persist. HCV developed several mechanisms that allowed

evading the innate immune respnses. Thus, it was shown, that distinct viral proteins like NS3/4A and core are able to inhibit signaling pathways. Besides type I IFN, natural killer cells (NK), natural killer T cells (NKT) and myolide Kupffer cells play an important role in anti-HCV innate immune response (Crispe, 2003; Racanelli & Rehermann, 2006). The Kupffer cells are liver specific macrophages, which eliminate apoptotic cells or microorganisms via phagocytosis. In addition, they induce NK cell- and cytotoxic NK cell differentiation, which, in turn, lead to a production of high amounts of IFNγ (Lauwerys *et al.*, 2000). NK cells are activated by type I IFN, lyse the infected cells and produce IFN-γ. This cytokine stimulates hepatocytes to produce the chemokine CXCL9 for the recruitment of T cells, acting as key factors for the adaptive immune response. NKT cells have the characteristics of NK cells and T cells and exist mainly in the liver where they activate dendritic cells to promote the secretion of high amounts of IFN-γ.

1.5.2 The adaptive cellular immune response

Despite a very early innate immune response during infection, the adaptive cellular immune response seems to be crucial for viral clearance. The acute phase of an HCV infection endures approximately 6 months post infection (p.i.), whereas the viral titer increases exponentially during the first 6 weeks. HCV-specific T cells are typically became detectable 5-9 weeks after infection (Thimme *et al.*, 2001) and are crucial for viral clearance (Lechner *et al.*, 2000). Virus-specific $CD4^+$ and $CD8^+$ T cells are accumulated in the liver by about 8-14 weeks after infection and this event coincides with viral clearance and liver disease. After depletion of $CD4^+$ and $CD8^+$ T cells the chimpanzees were unable to control viral replication (Cooper *et al.*, 1999). Available data allow concluding that spontaneous clearance in the acute phase of infection can be associated with a strong sustained T cell response. Whether IFN-γ is directly involved in HCV clearance or whether it is just a marker for other T cell functions remains unclear.

$CD8^+$ T cells are detectable in the blood of acutely infected patients regardless of virological outcome (Kaplan *et al.*, 2007). They show an impaired proliferation, IFN-γ production, cytotoxicity and increased levels of programmed death-L ((Kasprowicz *et al.*, 2008; Thimme *et al.*, 2001). $CD8^+$ T cells start producing IFN-γ as well as TNF-α (Tumor necrose factor α) after recognition of antigens presented by MHC class I molecules on the surface of infected cells in the liver or in the blood. Afterwards, they

develop a cytotoxic activity, which involves FAS- mediated apoptosis via perforin and granzyme. IFN-γ production leads to a non-cytolytic control of infection involving, for example, the proliferation block, activation of macrophages, and upregulation of MHC molecules. A strong $CD4^+$ T cells response seems to be also essential for viral clearance. These cells are required for the induction and maintenance of virus-specific $CD8^+$ T cells.

Chronic HCV infection is associated with continuous activation yet impaired function and reduced breadth of HCV-specific T cells (Cox *et al.*, 2005). Several studies showed a weak or not detectable $CD4^+$ and $CD8^+$ T cell response during the HCV persistence. Detected cells have only limited functions, namely, decreased cytotoxicity, cytokine secretion or proliferation (Ulsenheimer *et al.*, 2003). Reasons for the impairement of T cell response seem to be dysfunction or an increasing exhaustion during presence of high viremia. Mechanisms causing the T cell failure include an immune escape (Timm *et al.*, 2004), presence of regulatory T cells (Sugimoto *et al.*, 2003), or the increased expression of the co-receptor programmed death-1 on T cells.

1.5.3 The humoral immune response

Viral proteins are recognized as non-self by the immune system of the host and induce the production of antibodies (Abs). During the natural course of infection, Abs targeting epitopes of both structural and non-structural proteins are produced. Only a fraction of Abs that are able to bind the viral particles demonstrate antiviral activity. These Abs are directed against epitopes that play an essential role in the virus entry and reffered to as “neutralizing Abs” (nAbs) (Keck *et al.*, 2008). Virus neutralization correlates with increased antibody binding to virion surface sites independent of the epitope recognized by the antibody. Virus neutralization results depend upon a critical number of occupied binding sites. This process result in prevention of virus entry through steric hindrance (Burton *et al.*, 2001). Higher affinity Abs demonstrate a high virus neutralization, whereas non-neutralizing Abs either do not bind to virion surface or bind with a low affinity. Virus-specific antibodies are usually become detectable approximately 7-8 weeks after infection (Pawlotsky, 1999), whereas HCV RNA is detectable already one week p.i. coinsiding with an early appearance of HCV-specific T lymphocytes. Antibody-mediated neutralization occurs during HCV infection *in vivo* but the exact contribution of humoral immune response to the control

of HCV infection is not completely clear. The occurrence of antibody-mediated neutralization in hepatitis C patients has been proposed after studying the patients undergoing liver transplantation for HCV-related liver cirrhosis - the infusion of anti-HBs hyperimmune globulin containing anti-HCV seemed to reduce HCV infection in the transplanted liver (Feray *et al.*, 1998). Furthermore, it was shown that HCV-infected patients with primary antibody deficiencies have accelerated rates of disease progression (Chapel *et al.*, 2001). Another study demonstrated a passive protection against HCV in a cohort of patients who had been administered immunoglobulin preparations derived from HCV RNA-positive plasma (Yu *et al.*, 2004).

Theoretically, the neutralizing antibodies might be used for therapeutical purposes. At that, one approach to control re-infection of transplanted liver could be the combined use of HCV-specific antibodies together with specific antivirals. However, so far, the role of nAbs in preventing and controlling HCV infection has not been completely defined. Early experimental studies in animals have shown that Immunoglobulin G (IgG) therapy delayed the onset of acute infection in HCV-infected chimpanzees (Krawczynski *et al.*, 1996). In addition, it was shown that a complete protection can be achieved if the HCV inoculums were pre-incubated with HCV–specific Ig (Farci *et al.*, 1996). Studies in patients also demonstrated that serum antibodies from chronically HCV infected patients are directed against the HVRI (Rosa *et al.*, 1996; Shimizu *et al.*, 1994), supporting the supposition that the humoral immune response to this variable region contribute to some extend to protection against HCV infection.

In the acute phase of HCV infection approximately 20 to 25 % of acutely infected individuals clear the viremia. A study of intravenious drug users, in which a comparison of previously HCV–infected individuals (HCV antibody-positive and HCV RNA-negative) with those without evidence of prior infection (HCV antibody-negative and HCV RNA-negative) was performed, demonstrated that individuals with evidence of a previous infection were 12 times less likely than those with first time exposure to develop persistence of HCV. Noteworthy, that the peak viral load was approximately $10^{-1.8}$ lower in previously infected drug users (Mehta *et al.*, 2002), suggesting that the adaptive humoral immunity can prevent a disease progression. Recently, the HCVpp and HCVcc model systems were established. That allowed the initiation of the functional studies directed at analysis of neutralizing antibody responses during acute and chronic HCV infections. Studies using well defined nosocomial or single-source HCV outbreaks with a defined inoculum enabled investigation of a role of

isolate-specific nAbs in a control of HCV infection in humans (Zeisel *et al.*, 2007a). Thus, one recent study, in which serum samples of the well characterized single-source outbreak were used, demonstrated that viral clearance was associated with a rapid induction of neutralizing antibodies in the early phase of infection (Pestka *et al.*, 2007). In contrast, in the same cohort of patients it was shown that chronic HCV infection was characterized by absence or low-titer of nAbs in the early phase of infection (Pestka *et al.*, 2007). Patients, who cleared the virus, exhibited a broader cross-neutralizing activity of antibodies in the early phase of infection. An impaired ability to cross-neutralize viral variants that rapidly emerged during acute infection may thus contribute to viral evasion from neutralizing response in persistent HCV infection (Pestka *et al.*, 2007). These findings suggest that a strong broad neutralizing antibody response may contribute to control HCV in the acute phase of infection.

Patients, who do not clear the virus, usually develop relatively high-titer cross-neutralizing antibodies during the chronic phase of infection (Bartosch *et al.*, 2003a; Pestka *et al.*, 2007; von Hahn *et al.*, 2007). Paradoxically, these antibodies are not able to control the infection. Some data suggest a possible existence of an escape mechanism that allowed the virus to avoid antibody-mediated neutralization (von Hahn, Yoon *et al.* 2007). It is well known that in every infected individual the HCV exists as a pool of quasispecies, closely related but still slightly different variants. These subvariants are changed over time due to an immune pressure driven by the host and this, in turn, might lead to appearance of the virus variants that escape the antibody-mediated neutralization (Bartosch *et al.*, 2003a). The escape mechanism hypothesis, however, needs confirmation. In particular, it would be essential to study if the mechanism of the humoral response escape is operative in all patients, including those with a long history of HCV persistence.

1.6 Envelope proteins and the humoral immune response

It is assumed that the virus-neutralizing antibodies might block the HCV binding to its cell receptors. Development of the HCVpp system open a new avenue of HCV research directed at analysis of interaction of HCV particles and of the E1 and E2 proteins with antiviral antibodies. Available data suggest that the virus-neutralizing antibodies might react with both E1 and E2 proteins, however, most of the antibodies

formed during the course of natural infection are targeting the E2 protein (Zeisel *et al.*, 2007a). Even though most of these nAbs demonstrate a broad virus neutralizing capacity against conformational epitopes within E2, it was reported that there are Abs that are directed against linear epitopes. It was demonstrated that several antibodies targeting different linear regions within E2 can inhibit the E2-CD81 interaction in *in vitro* assays. The first Ab-binding site is located immediately downstream of the second hypervariable region between the positions 480 and 493 (Flint *et al.*, 1999), whereas the second site spands residues 528 to 535, and the third site comprises the residues at positions 544 to 551 (Owsianka *et al.*, 2001). Furthermore, it was shown, that Abs targeting the regions located at positions 412-423 and 432-447 are also able to block CD81 binding. However, the antibodies recognizing the regions 480-493, 528-535 and 544-551 failed to neutralize HCVpp infection of target cells (Hsu *et al.*, 2003). The region corresponding to a.a. positions 432-447 demonstrates a high variability among different HCV isolates, so that antibodies directed against this region have a restricted cross-neutralizing potency (Owsianka *et al.*, 2005). The region spanning aa 412-423 has been shown to contain a potent neutralizing epitope (Triyatni *et al.*, 2002), which is defined by the mouse monoclonal antibody (mAb) AP33 and a rat mAb 3/11. These two mAbs were able to inhibit the interaction between CD81 and a set of different molecules, including a soluble E2, E1/E2 heterodimer and virus-like particles (Owsianka *et al.*, 2001). Moreover, AP33 efficiently neutralized the HCVpp bearing envelope sequences from all six HCV genotypes and major subtypes (Owsianka *et al.*, 2005). The rat mAb 3/11 also neutralized diverse HCVpp, but with a lower potency than AP33. Fine mapping identified four highly conserved residues within the region 412-423 crucial for the binding of AP33. For the mAb 3/11 three critical residues were found, of which two are shared with mAb AP33 (Tarr *et al.*, 2006). A broad sequence analysis of over 5.500 sequences demonstrated that the AP33 epitope is highly conserved across all isolates of HCV. These data led to a suggestion of inclusion of such a conservative fragment of the E2 into the newly generated vaccines. In another study, six murine monoclonal antibodies CET-1 to -6, specific for HCV E2 protein were generated by using recombinant proteins containing truncated E2. The data indicated that three overlapping epitopes recognized by CET-1 to -6 are located in a small domain spanning a.a. residues 528 to 546 (Lee et al. 1999). Early studies have shown that a rabbit hyperimmune serum directed against HVRI of E2 is able to protect chimpanzees against HCV challenge (Farci *et al.*, 1996). In addition, it was

demonstrated that patient antibodies to HVR1 were capable to specifically block the E2 binding to cells (Scarselli *et al.*, 2002), and to inhibit the entry of HCV virus-like particles and serum-derived HCV (Barth *et al.*, 2005; Steinmann *et al.*, 2004), as well as HCVpp into target cells (Bartosch *et al.*, 2003a; Owsianka *et al.*, 2005). Deletion of the HVRI showed an attenuation of virus infectivity in the chimpanzee model as well as in the HCVpp assay (Bartosch *et al.*, 2003c). Recently, it was demonstrated that the deletion of the HVRI led to an enhanced neutralization of HCV particles by E2 specific mAbs (Bankwitz *et al.*) It should be noted, however, that due to a high variability in the region, antibodies directed against the HVRI show relatively low cross-neutralization potency across different isolates. Therefore, the generation of conserved HVRI mimotopes, which might overcome a problem of restricted specificity has been proposed (Wei *et al.*, 2008; Zhang *et al.*, 2003).

Most of the antibodies that are able to interact with diverse strains of HCV are directed to conformational epitopes within E2. One intensive cross-competition study showed that the E2 glycoprotein contains three immunogenic conformational domains A, B and C. These domains are accessible on the surface of HCVpp (Keck *et al.*, 2004). The spatial location of sites recognised by non-neutralizing and neutralizing human mAbs to conformational epitopes of E2 was established in a series of competition studies (Keck *et al.*, 2005). The human mAbs CBH-4G, -4B and -4D are directed against domain A. The second domain B is characterized by reactivity with Abs CBH-2, -5, -8E and -11, and the third domain C is recognized by Abs CBH-7 and -23. Within a domain the human mAbs have significant bidirectional inhibition and minimal competition with each other in other domains. Human mAbs that target the domains B and C also inhibit E2-CD81 interaction. Human mAbs that recognize the domain A are not able to neutralize the HCVpp. Recently, these results were confirmed in experiments with HCVcc (Keck *et al.*, 2007). Antibodies that recognize domains B and C, in contrast to Abs reacting with domain A, were able to neutralize the infectious HCV particles, however, the domain C antibodies showed neutralizing activity only at a much higher concentration. In general, all these experiments support an immunogenic model of HCV E2 containing three functional antigenic domains. It should be noted, however, that the role of these and other antigenic domains of the E1/E2 heterodimer in humoral immune responses *in vivo* is far from being completely clear and needs to be studied in much more details.

1.7 The goals and aims of the study

The major goals of the current project are:

- Analysis of the evolution of the envelope protein genes of HCV in individual chronic hepatitis C patients infected in a single-source HCV outbreak (AD78 cohort).

- Analysis of the dynamic of humoral immune response to these proteins and experimental verification of the hypothesis that chronic HCV infection is associated with a continuous escape of the circulating virus from emerging antibodies.

To reach these goals we aim:

- To characterise the E1 and E2 envelope genes sequences both in contaminated globulin and AD-infected patients chronically infected with the HCV AD78 strain using a phylogenetic analysis.

- To perform a longitudinal analysis of AD78-derived sequences from chronically infected patients, including determination of dS/dN ratio, characterisation of a dynamic, localization and pattern of a.a. substitutions in envelope proteins.

- To generate a series of HCVpp bearing the natural and modified E1/E2 sequences from AD78 isolates obtained from AD-patients at different time points of chronic infection and to test their infectivity.

- To use the generated set of HCVpp to analyse the characteristics of antibodies to envelope proteins present in serial serum samples of patients chronically infected with HCV AD78 strain, in particular, to assess the ability of these sera to neutralize the pseudoparticles bearing the evolving E1/E2 sequences obtained from AD-patients during the follow-up of 28 years post infection.

2 Material and Methods

2.1 Materials

2.1.1 Human sera from chronically infected HCV patients

Sera of patients infected with the AD78 strain were kindly provided by Prof. M. Wiese, St.Georg Hospital, Leipzig, Germany. Between August 1978 and March 1979, 14 HCV1b contaminated batches of anti-D immunoglobulin had been administered to 2867 women throughout East Germany for prophylaxis of rhesus factor incompatibility. After application of the contaminated globulin, 93 % of the recipients developed an acute hepatitis C. After 25 years, 86 % of the 1833 affected women still tested positive for hepatitis C virus antibodies and 46 % for HCV RNA (Wiese *et al.*, 2005). For the current study, nine anti-D patients, who developed a chronic hepatitis C and remain HCV RNA positive, were chosen. All these patients received anti-D globulin, batch #8. Five patients were not treated with IFN-α. For other patients, except patient X, serum samples were obtained before start of the IFN-therapy. Serum sample of patient X was obtained 3 years after unsuccessful IFN-α therapy (Table 2.1.).

Table 2.1. Designation of patients and attitude of therapy

Short form for the patient used in the current study	Year of IFNα-therapy
T	No
N	No
Z	2005
H	No
X	1995
B	No
J	No
M	2003
Y	1998

2.1.2 Chemicals

ß-mercaptoethanol	Sigma-Aldrich
Acrylamide 2 K solution	AppliChem
Agarose	Eurogentec
Ampicillin (50 µg/ml)	Roche Diagnostics
APS	Roth
Bromophenol Blue	SIGMA Aldrich Chemie GmbH
Carbenicillin (50 µg/ml)	Serva
Coomassie Brillant Blue	Serva
D-PBS	Invitrogen
Dimethyl sulfoxide	Roth
DMEM	PAA Laboratories
EDTA Lösung pH8,0	Applichem
ECL Western Blotting Reagents	GE Healthcare
0.5 M EDTA pH8.0	Ambion (UK)
Ethanol	Sigma-Aldrich
Ethidiumbromide	Roth
FBS	Gibco Invitrogen
Geneticin (G 418)	PAA Laboratories
Glycerol	SIGMA
HEPES	PAA Laboratories
Hydrogen Peroxide	Merck
Isopropanol	Roth
Lectin	Sigma

L-Glutamin	PAA Laboratories
Natrium Acetat	Merck
Natrium Acetat 3M pH5,5	Ambion
Natrium Chloride	Merck
OPD	Sigma-Aldrich
Orange G	Sigma-Aldrich
Penicillin/Streptomycin	PAA Laboratories
High Sensitivity Streptavidin-HRP	Pierce
Sodium dodecyl sulfate (SDS)	AppliChem
TBE ultrapure 10x	Invitrogen
TEMED	Roth
Triton X-100	Invitrogen
Trizol	Invitrogen
Trypan Blue	Gibco BRL
Trypsin-EDTA	Gibco BRL
Tween 20	Applichem
X-Gal	Bio Solve LTD

2.1.3 Equipment

Device	**Supplier**
Automatic incubator CO_2	Kendro GmbH
Balance	Vibra
Bioimaging System (Gene genius)	Syngene
Centrifuge 3 LR	Kendro GmbH

Centrifuge 5804	Eppendorf
Centrifuge 5414 R	Eppendorf
Duomax 1030	Heidolph
Electrophoresechamber	Medicin-technique, Uniklinkum Essen
Electrophorese-Power supply	Biometra
Elisa Reader Asys Expert Plus	Asys Hiteck GmbH
Freezer	Liebherr
Fridge	Liebherr
GeneAmp PCRSys 2400	Roche
Gene Pulser II	BioRad Laboratories
Hera Safe Flow	Kendro GmbH
Heraeus CO2 Inkubator	Kendro GmbH
Heraeus Megafuge 1.0 R	Kendro GmbH
Heraeus Multifuge 3 LR	Kendro GmbH
Inverted Microscope	Carl Zeiss
Laminar Flow HSP 12	Kendro GmbH
Luminometer	Siemens
Microplate Scintillation and Luminescences counter	Packard
Microwave	Panasonic
Mini-Protean	Bio-Rad
Mr. Frosty	Nalgene
Pipette- Easypet	Eppendorf
Pipettes	Eppendorf, Gilson

Photometer Gene Quant	Amersham Biosciences Europe GmbH
pH- Meter MP 220	Toledo
Power-Pack	Bio-Rad
Rotary Mixer	Peter Oehmen
Thermocycler	Eppendorf
Thoma counting chamber	Hecht-Assistant
Topcount NXT	Packard
Trans-Blot SD Transfer cell	Bio-Rad
UV transluminator FLX-20M	MWG-Bio Tech
Vacusafe comfort	IBS Integra Biosciences
Vibra AJ-2200CE balance	Shinko Denshi
Vortex Genie 2	Scientific Industries
Waterbath	Julabo
Zentrifuge 5415 D	Eppendorf
Zentrifuge 5804	Eppendorf

2.1.4 Commercial kits

Bio-Rad Protein Assay Kit	Bio-Rad
CalPhos Mammalian Transfection Kit	Clontech
Expand High Fidelity^{PLUS} PCR System	Roche
GloLysis Buffer	Promega
High Speed Midi Kit	Qiagen
Innotest^{TM} HIV Antigen mAb	Innogenetics GmbH
Qiagen Plasmid Maxi Kit	Qiagen

Qiaprep Spin Miniprep Kit	Qiagen
Qiaquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
QuikChange Site-Directed Mutagenesis Kit	Stratagene
TopoTA Cloning Kit	Invitrogen

2.1.5 Plastic products

FACS-Tubes Falcon/Becton	Dickenson
Hybond N+ Membran	Amersham
Dishes	Greiner Bio-One
Nunc-Immuno Plate	Nunc
Pipettes	Greiner Bio-One
Reaction-tubes	Eppendorf
Plastic tube with V-buttom	Falcon/Becton Dickenson
Cell-culture flask	Greiner Bio-One
Cell-culture plates	Greiner Bio-One

2.1.6 Membranes

Hybond-C nitrocellulose	Amersham

2.1.7 Enzymes

Go Taq-Polymerase (1 U/µl)	Promega
T4-DNA-Ligase (20 U/µl)	New England Biolabs GmbH
SalI	Fermentas GmbH
PsiI	Fermentas GmbH

EcoRV	Fermentas GmbH
BsrGI	New England Biolabs GmbH
PvuI New England	Biolabs GmbH
Shrimp Alkaline Phophatase (1 u/µl)	Fermentas GmbH
ThermoScript™ Reverse Transcriptase	Invitrogen

2.1.8 Oligonucleotides

Primer designation	Sequences	Targeted genome region
Sv79s	5'-GAA TGT CGT GAA GGA AGC AG-3'	pHCMV-IRES
Sv655s	5'-TAT AGA TAT CAT GGG GTA CAT TCC GCT CGT C-3'	E1
Sv656as	5'-ATA TGA TAT CTT ACT CAG CCT GAG CTA TCA G-3'	E2
Sv657s	5'-CTA CTT TTC GCT GGC GTC GAC GGC AAC ACC CAC AC-3'	E1
Sv687as	5'-TTC GCC GAC CTC ATG GGG TAC-3'	E1
Sv688as	5'-GCC GCA TTG AGG ACC ACC AGG-3'	E2
Sv690as	5'-GTT TTG AGA GAG TCA TTG CAG-3'	E1
Sv691s	5'-GTG GGG AAC TGG GCT AAG G-3'	E2
Sv692s	5'-GCC TCG CCT ACT ATT CCA TG-3'	E2
Sv709as	5'-TGC CAG CGA GGA GAG AAG GTG-3'	E1
Sv710as	5'-GCC AGC GAG GAG AGA AGG TGA AC-3'	E1
Sv711s	5'-TAT GTA CGT GGG GGA TCT TTG-3'	E1
Sv713as	5'-CGC ACG TCT TGG TAA ACC C-3'	E2
Sv714as	5'-GAA GCA GTC CGT GGG GCA AG-3'	E2
Sv715s	5'-CTC CTT AAC AAC ACG CGG-3'	E2
Sv716s	5'-GGG AGA ATG AGA CGG ACG TG-3'	E2

Sv774s	5’-CGT ATC GCT GGG GGG AGA ATG-3’	E2
Sv826as	5‘-GAA GCA GTC CGT GGG GCA RR-3‘	E2
Sv827as	5‘-CGC ACG TCT TGG TRA ACC C-3‘	E2
Sv828as	5’-AGA ACA GMG CGG CAA KRA ACC-3’	E1
Sv831s	5’-ACT TTT CGC TGG CGT CGA CGG CGC CAC ACA TGC CGT GGG ACA GGT CGG AAG CAG GGG CCT GAA GAG AGG AGA CGC-3’	HVRI
Sv832as	5’-GTT GGT GTT TAT AAG CTG GAT CTT CTG CAC GGA CCC GAT ATC AAA TGC AGC GTC TCC TCT CTT CAG GCC CCT G-3’	HVRI
Sv833s	5’-ACT TTT CGC TGG CGT CGA CGG CGG GAC ACA TGC CCT GGG GGT TAT CGG AGT GAG AGG TGG CGG CGG AGC TGG AAG CGC	HVRI
Sv834as	GTT GGT GTT TAT AAG CGT GAT CTT CGT TCC TGG TCC ACC GCC AAA TGC GGC GCT TCC AGC TCC GCC ACC TCT C-3’	HVRI
Sv864s	5’-GCT CAG AAG ATC CAG CTT ATA AAC ACC AAC GGC AGC-3’	E2
Sv865as	5’-GCT GCC GTT GGT GTT TAT AAG CTG GAT CTT CTG AGC-3’	E2
Sv868s	5’-CT ACT TTT TGC TGG CGT CGA CGG CAT GTC CGG GAC-3’	E1
Sv869as	5’-GTC CCG GAC ATG CCG TCG ACG CCA GCA AAA AGT AG-3’	E1
Sv874s	5’-CAG CGC CGC GTG CAA CTG GAC TCG AGG AGA GCG TTG-3’	E1
Sv875as	5’-CAA CGC TCT CCT CGA GTC CAG TTG CAC GCG GCG CTG-3’	E1
Sv883s	5’-CTA CTT TTT GCT GGC GTC GAC GGC ATG TCC GGG AC-3’	E1
Sv884as	5’-GT CCC GGA CAT GCC GTC GAC GCC AGC AAA AAG TAG-3’	E1
Sv885s	5’-CC CAG AAG ATC CAA CTT ATA AAT ACC AAT GGC AGC-3’	E2
Sv886as	5’-GCT GCC ATT GGT ATT TAT AAG TTG GAT CTT CTG GG-3’	E2
Sv887s	5’-GGA GAC GCT GCA TTT GAC ATC GGG TCC GTG CAG AAG-3’	HVRI

Sv888as	5’-CTT CTG CAC GGA CCC GAT GTC AAA TGC AGC GTC TCC-3’	HVRI
Sv917s	5’-CGA TCC AGC CTC CGG TTG ACC GAT CCT GAG AAC-3’	phCMV-IRES
Sv918as	5’-GTT CTC AGG ATC GGT CAA CCG GAG GCT GGA TCG-3’	phCMV-IRES
Sv929s	5’-CTA CTT TTT GCT GGC GTC GAC GGC ATG TCC GGG AC-3’	E1
Sv930as	5’-GT CCC GGA CAT GCC GTC GAC GCC AGC AAA AAG TAG-3’	E1
Sv931s	5’-CCC AGA AGA TCC AAC TTA TAA ATA CCA ATG GCA GC-3’	E2
Sv932as	5’-CG TGC CAT TGG TAT TTA TAA GTT GGA TCT TCT GGG-3’	E2
Sv933as	5’-GCC GTC GAC GCC AGC AAA AAG TAG C-3’	E1
Sv934s	5’-GGC GTC GAC GCC AGC AAA AAG TAG C-3’	E1
Sv936as	5’-GTA TTT ATA AGT TGG ATC TTC TGG-3’	E2
Sv937s	5’-CAA CTT ATA AAT ACC AAT GGC AGC-3’	E2
Sv939as	5’-GC AGG ACT AGT ATA TGA TAT CTT ACT CAG CCT GAG CCT GAG CTA TCA G-3’	E2
Sv940s	5‘-ATC CG TGT ACA TAT AGA TAT CAT GGG GTA CAT TCC GCT CGT C-3’	E1
Sv941s	5’-CTG TTC TAC ACA CAT AGC TTC AAC TCG TCC GG-3’	E2
Sv942as	5’-CC GGA CGA GTT GAA GCT ATG TGT GTA GAA CAG-3’	E2
Sv945s	5’-CTA GTC CTG CAG GTT TA-3’	pCR4Topo
Sv945as	5’-GCC GCG AAT TCG CCC T-3’	pCR4 Topo
Sv962s	5’-CGA TCC AGC CTC CGG TCT ACC GAT CCT GAG AAC TTC	phCMV-IRES
Sv963as	5’-GAA GTT CTC AGG ATC GGT AGA CCG GAG GCT GGA TCG	phCMV-IRES

Sv969s	5'-TA GAG AAC AAT TGT TAC AAT TAA ATG ATA AGG TAG-3'	phCMV-IRES
Sv970as	5'-CTA CCT TAT CAT TTA ATT GTA ACA ATT GTT CTC TA	phCMV-IRES
Sv971s	5'-CGG CAA AAT CCC TTA CAA ATC AAA AGA ATA GAC C-3'	phCMV-IRES
Sv972as	5'-G GTC TAT TCT TTT GAT TTG TAA GGG ATT TTG CCG-3'	phCMV-IRES
Sv978s	5'-TTA GTG AAC CGT CAG ATC GCC-3'	phCMV-IRES
Sv979as	5'-AAG TGC CAC CTA AAT TGT AAG-3'	phCMV-IRES

2.1.9 Plasmids

Commercial plasmids

The pCR4 Topo plasmid (Invitrogen, Germany) was routinely used for cloning of PCR products.

Plasmid containing the envelope genes of HCV AD78 infected patients

The phCMV-IRES plasmid (5520bp) was used for expression of the E1 and E2 proteins of HCV. This plasmid contains the IRES of EMCV that drives the translation of the HCV Env genes. The inserted envelope protein genes were obtained during the amplification of HCV sequences from serum samples of AD patients collected at different time points. The generated set of plasmids was used for transfection of 293T cells in order to produce the HCV E1/E2 heterodimer for ELISA for the generation of HCVpp.

Plasmid containing HIV-Gag, Pol genes

The lentiviral vector pCMV delta R8.2 (8128) has been obtained from Addgene (USA). This plasmid expresses the HIV Gag and Pol proteins in 293T cells and was used for production of HCVpp.

Plasmid containing the luciferase reporter gene

The HPPT-EF1α- Luc HIV-1-based transfer vector contains the EF1 α internal promoter, which drives the expression of Luc (luciferase reporter gene) gene. The plasmid was used for generation of HCVpp in 293T cells.

2.1.10 Buffers and Solutions

Carbonate Buffer (ELISA)	3.18 g Na_2CO_3
	5.88 g $NaHCO_3$
	0.2 g NaN_3
	5 % FCS (inactivated) (v/v)
	5 µg/ml Lectin
	Add H_20 dest.to 1 liter
	pH 9.6
Blocking Buffer (ELISA)	3.18 g Na_2CO_3
	5.88 g $NaHCO_3$
	0.2 g NaN_3
	5 % FCS (inactivated) (v/v)
	0.1 % Tween 20 (v/v)
Coating Buffer (ELISA)	30 mM Na_2CO_3
	70 mM $NaHCO_3$
	2.5 µg/ml Lectin
	pH 9.6
Coomassie Brillant-Blue-	0.06 % Coomassie brillant-blue R250 (w/v)
Staining solution	50 % Methanol (v/v)
	10 % Acetic acid (v/v)
	2 % negative Celllysat
	2 % Mock
	Add H_20 dest.to 1 liter
	pH 9.6

Destaining Solution	5 % Methanol (v/v) 7.5 % Acetic acid (v/v)
Dilution Buffer (ELISA)	0.1 % Tween 20 (v/v) 3 % FCS (v/v) 1-2 % neg. Celllysat 2 % Mock 2.5 µg/ml lectin 5 % Goat serum Add PBS to 1 liter
DNA-Loading Buffer	5.5 mM Orange G 1.2 M Saccharose
Lysis buffer (SDS-PAGE)	0.5 M Tris, pH 6.8 1 % Glycerol 10 % SDS 5 % 2-Mercaptoethanole 0.05 % Bromphenole-blue
Cell Lysis Buffer	20 mM Tris HCL, pH 8.0 137 mM NaCl 10 % Glycerol 1 % Triton-X 100 2 mM EDTA Add H_20 dest.to 100 %
OPD-Substrate Solution (1 tabl)	10 ml PBS 10 µl H_2O_2
PBS	155 mM NaCl 3 mM Na_2HPO_4 1 mM KH_2PO_4 pH 7.4

Stop Solution	0.5 M H_2SO_4
TBE-Buffer	100 mM Tris 90 mM Boracic acid 1 mM EDTA pH 8.4
Washing Buffer (ELISA)	PBS 0.5 % (v/v) Tween20
4x SDS Loading Buffer	10 % Glycerine 4 % SDS 125 mM Tris, pH 6.8 10 % 2-Mercaptoethanol 0.02 % Bromphenol blue
5x-DNA-loading-buffer	Glycerine 50 % 20 mM Tris, pH 7.5 50 mM EDTA, pH 8.0 0.025 % Bromphenoleblue 0.025 % Xylenecyanole
10x Reaction-buffer	100 mM KCl 100 mM $(NH_4)_2SO_4$ 200 mM Tris-HCl (pH 8.8) 20 mM $MgSO_4$ 1 % Triton X-100 1 mg/ml nuclease-free bovine serum albumin (BSA)
10x TBE-buffer	Tris Base 108g Boric acid 55g 0.5 M EDTA, pH 8.0

2.1.11 Antibodies

Primary Antibodies

- Mouse mAb CET-3 and CET-4 were kindly provided by Prof. Chang-Yuli Kong (South Korea)

- Human mAb CBH-2, CBH-5, CBH-7, CBH-23, HC-1, and HC-12 Anti HCMV RO4 isotype control were kindly provided by Dr.Steven Foung (USA)

Secondary Antibodies

- Peroxidase-conjugated Affini Pure Rabbit anti-mouse IgG + IgM (H+L) – Dianova

- Polyclonal Rabbit Anti-Human IgG/HRP - Dako Cytomation

2.1.12 Medium for culture of E.coli

LB Agar	10 g Trypton
	5 g Yeast extract
	5 g NaCl
	15 g Agar
LB Medium	10 g Trypton
	5 g Yeast extract
	5 g NaCl

Both media were completed to 1000 ml with bidistilled water and sterilized by autoclaving for 10 minutes at 121 °C. Ampicillin was added to each medium at a final concentration of 100 µg/ml.

2.1.13 Bacteria strains

***E.coli* Strains**	**Supplier**
XL-10 Gold Ultracompetent cells	Stratagene GmbH
Top 10 Competent cells	Invitrogen GmbH

Strain	Genotype	Properties
TOP10 (Invitrogen)	F- mcrA Δ(mrr -hsdRMSmcrBC) φ80lacZΔM15ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	• Streptomycin resistant • Contain lacI based on a colony PCR
XL10-Gold (Stratagene)	endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte(mcrA)183 Δ(mcrCBhsdSMR-mrr)173 tet^R F'[proAB $lacI^qZ\Delta M15$ Tn10(Tet^R Amy Cm^R)]	• Tetracycline and Chloramphenicol resistant • Nalidixic acid resistant • Hte phenotype allows high transformation with large plasmid inserts

2.1.14 Eucaryotic cell lines

Human hepatoma cell line Huh7.5

Huh7.5 cells are a subline derived from Huh7 hepatoma cells (Blight *et al.*, 2002). This subline was established by "curing" a cell clone containing a Con1 subgenomic replicon by prolonged treatment with alpha-interferon. The receptors, important for the initiation of virus entry, are expressed on the viral surface and, therefore, these cells can be used for studying virus entry.

Human Embryonic Kidney 293T cells

293T cells are an important variant of the HEK 293 cells. This cell line bears the SV40 large T-antigen that allows episomal replication of transfected plasmids containing the SV40 origin of replication. In the current study 293T cells were used for generation of HCVpp.

2.1.15 Used Software

Codon Code Aligner	Codon Code
Fig Tree	http://www.tree.bio.ed.ac.uk
GraphPad Prism 4.03	GraphPad Software
HCV Sequence Data Base	http://www.hcv.lanl.gov

Microsoft Office	Microsoft
NCBI Homepage	http://www.ncbi.nlm.nih.gov
Nebcutter	http://www.tools.neb.com/NEBcutter2

2.2 Methods

2.2.1 Procaryotic Cells

2.2.1.1 Transformation of E.coli

Transformation of bacterial cells was performed according to the manufacturer's instructions. In brief, bacterial cells (Top10 cells or XL10-Gold cells) frozen at -80 °C were thawed on ice. Two microliters of 2-Mercaptoethanol were added to 45 µl of XL10-Gold cells aliquot and incubated for 10 min. on ice. Top10 cells were used without addition of mercaptoethanol. No more than 10 % by volume of the plasmid DNA was added to the aliquote of bacterial cells. This mixture was incubated on ice for 30 minutes. To improve DNA absorption by bacteria, a heat shock for 30 sec. at 42 °C followed by a subsequent incubation on ice for 2 minutes was performed. After addition of 250 µl LB-medium a mixture was incubated on a shaker for 2 hours at 37 °C. Using a sterile spatula the complete mixture was spread over an LB-agar plate containing a selective antibiotic. The plates were incubated overnight at 37 °C.

2.2.1.2 Plasmid DNA preparation using commercial kits

One bacterial colony was picked up from the LB agar plate, using a sterile pipette tip and transferred into the flask with LB-medium containing the ampicillin (5 ml of medium for minipreps, 100 ml- for midipreps, and 250 ml – for maxipreps). The bacteria culture was then incubated overnight on a shaker at 37 °C. The culture was then centrifuged at 5000 rpm for 10 min and cells were washed once with PBS. The DNA was extracted using the Qiaprep kits (Qiagen), according to the manufacturer's protocol. DNA was quantified by spectrophotometry at 260 nm and the size of the plasmid was checked using the electrophoresis in agarose gel.

2.2.2 Molecular Biological Methods

2.2.2.1 RNA-Extraction from human sera

HCV RNA was extracted from patients' sera using the High Pure Viral RNA Kit (Roche) according to the manufacturer's instructions. In brief, 200 µl serum was mixed with the Binding Buffer supplemented with Poly (A), transferred into a filter minicolumn, and centrifuged for 30 sec. at 8000 x g. Afterwards, 500 µl Inhibitor Removal Buffer was added to the upper reservoir followed by centrifugation for 1 min. at 8000 x g. The column was washed 2 times with 450 µl Wash Buffer. Viral RNA was eluted in 50 µl of Elution Buffer and directly used in the reverse transcription reaction or kept at -80 °C.

2.2.2.2 Reverse-Transcription

HCV RNA isolated from patient's sera was reverse transcribed into cDNA using the Thermoscript kit (Invitrogen) according to the following procedure:

RT reaction Mix I	
Primer (50 pmol/µl)	1 µl
dNTPs (5 mM)	2 µl
RNA	5 µl
RNAse free Water	5 µl
	13 µl

The RNA in Mix I was denatured for 5 min at 65 °C and directly cooled down on ice.

RT reaction Mix II	
5x cDNA Synthesis Buffer	4 µl
0.1M DTT	1 µl
Rnase Out (20U)	1 µl
RNAse free Water	1 µl
	7 µl

Mix II was added to Mix I to a final volume of 20 µl and incubated for 1 h at 50 °C, followed by additional incubation step (5 min. at 85 °C). Five µl of generated cDNA was used for PCR 1.

2.2.2.3 Polymerase Chain Reaction (PCR)

All the cDNA were amplified in a nested touchdown PCR using the Expand High Fidelity PCR System. Two separate master mixes were prepared and mixed at a 50 µl final reaction:

Mastermix PCR1

5 x Reaction-Buffer with $MgCl_2$	10 µl
dNTPs (10 mM)	1 µl
Primer Sens (10 pmol/µl)	5 µl
Primer Anti-sens (10 pmol/µl)	5 µl
cDNA	5 µl
High Fidelity Polymerase (5 U/ml)	0.5 µl
H_2O	23.5 µl
	50 µl

Mastermix PCR2

5 x Reaction-Buffer with $MgCl_2$	10 µl
dNTPs (10 mM)	1 µl
Primer Sens (10 pmol/µl)	5 µl
Primer Anti-sens (10 pmol/µl)	5 µl
DNA	2 µl
High Fidelity Polymerase (5 U/ml)	0.5 µl
H_2O	26.5 µl
	50 µl

Touchdown PCR programme for both reactions:

1.	95 °C	2 min.	
2.	95 °C	1 min.	2x until 57 °C
3.	66-57 °C	1 min.	
4.	72 °C	3 min.	
5.	95 °C	1 min.	15x
6.	57 °C	1 min.	
7.	72 °C	3 min.	
8.	72 °C	20 min	
9.	4 °C	forever	

The size of amplified DNA fragments was assessed using the ethidium bromide agarose gel electrophoresis.

2.2.2.4 Side-directed mutagenesis (SDM)

Quick-Change site-directed mutagenesis kit (Stratagene) was used to introduce single point mutations into the plasmid sequences. In this technique two complementary mutagenic primers, containing the desired nucleotide are used to amplify the entire plasmid. The non-strand-displacing action of the Pfu Ultra HF DNA Polymerase extends and incorporates the mutagenic primers, which results in a formation of nicked circular strands. After touchdown PCR, the methylated parental plasmid DNA is removed by digestion with DpnI.

Standard SDM Mixture:

reaction buffer (10x)	5.0 µl
plasmid (100 ng in 5 µl)	5.0 µl
Sens primer 5'- 3' (10 pmol/µl)	1.5 µl
Anti-sense primer 3'- 5' (10 pmol/µl)	1.5 µl
Stratagene dNTP-mix	1.0 µl
Quick solution	3.0 µl
PfuUltra HF DNA Polymerase (2.5 U/µl)	1.0 µl
H_2O	32.0 µl
	50 µl

Touchdown PCR programme for SDM:

1.	95 °C	3 min.	
2.	95 °C	1 min.	
3.	66°-60 °C	1 min.	3x
4.	68 °C	3 min.	
5.	95 °C	1 min.	
6.	60 °C	1 min.	15x
7.	68 °C	7 min.	
8.	4 °C		

After PCR, 1 µl DpnI was added to the PCR-product and the mixture was incubated 1h at 37 °C. Afterwards, 1 µl of the mixture was used for transformation of XL-10 Gold cells (2.2.1.1.).

2.2.2.5 DNA restriction

Plasmids and PCR-products were restricted either with a single endonuclease or with combination of two enzymes. When the enzymes used for plasmid restrictions had different optimal temperatures or when they did not work in a compatible buffer, sequential restrictions were carried out using the optimal buffers and temperatures.

Restriction of plasmids with one endonuclease:

DNA (3-5 µg)	x µl
10 x Buffer	10 µl
10 x BSA	10 µl
Enzyme (50 U)	y µl
H_2O	z µl
	100 µl

The restriction was carried out overnight at the optimal temperature depending on the endonuclease used.

Restriction of plasmids with two endonucleases:

DNA (3-5 µg)	x µl
10 x Buffer	10 µl
10 x BSA	10 µl
Enzyme 1(50U)	y µl
Enzyme 2(50U)	z µl
H_2O	α µl
	100 µl

The restriction was carried out overnight at the optimal temperature for both endonucleases. Afterwards, the restricted DNA was subjected to a control electrophoresis in agarose gel. The desired DNA fragments were cuted out and subjected to a purification using the QIAprep Gel Extraction Kit (Qiagen).

2.2.2.6 DNA Dephosphorylation

In some cases, e.g. when only one enzyme was used for restriction of the plasmid, in order to prevent a re-ligation of a linearized plasmid, the restricted DNA was incubated with 3 µl Shrimp alkaline phosphatase (1 U/µl) for 1 h at 37 °C.

Afterwards, the enzyme was inactivated by an incubation for 20 min. at 80 °C. Finally, a purification step using the Qiaquick Gel Extraction Kit (Qiagen) was performed.

2.2.2.7 Agarose gel electrophoresis

DNA fragments were separated in a horizontal submerged 1-2 % agarose gel containing the ethidium bromide. The visualization of the separated DNA fragments was performed using a UV- Bioimaging System (Syngene).

2.2.2.8 DNA Extraction from agarose gels

Restricted plasmids or amplified DNA fragments were separated by agarose gel electrophoresis. The fragments of interest were cuted out of the gel and purified with the QIAprep Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

2.2.2.9 Cloning of PCR-products in intermediate plasmids

In most cases the amplified DNA fragments were cloned in pCR4-Topo plasmid (Invitrogen) without additional gel purification, according to the manufacturer's protocol.

pTopo cloning mixture:

PCR product (treated with *Taq*)	2 µl
Salt solution	1 µl
pCR4-Topo - vector	1 µl
H_2O	2 µl
	6 µl

The mixture was incubated for 45 minutes at RT and 2 ul were used for transformation of 25 µl supplied TOP 10 cells according to the standard protocol (2.2.1.1.).

2.2.2.10 Ligation of DNA Fragments

Purified restricted vector DNA and insert DNA were used for the ligation using the T4 DNA ligase. A 1:5 or 1:6 molar ratio of vector to the insert DNA was normally used.

Ligation mixture:	
Vector DNA	100 ng
Insert DNA	x ng
10x ligation buffer	2 µl
T4 DNA ligase (Weiss units)	1 u
Nuclease-Free water	y µl
	20 µl

The ligation mixture was incubated ON at 16 °C and 3 µl were used for the transformation of competent cells (2.2.1.1.). After transformation, single colonies were picked for Mini DNA preparation (2.2.1.2.) and the purified plasmid DNA was subjected to a control restriction (2.2.2.5.), followed by sequencing (2.2.2.11).

2.2.2.11 DNA-Sequencing

Sequencing was performed in both directions and was outsourced to the company AGOWA, Berlin.

Throughout the thesis the following sequence designation system was used:

For the E1 gene, the first nt is located at the position 384, the last nt as a position 576. The a.a. sequence of the E1 protein starts at position 1 and ends at position 192.

For the E2 gene, the first nt is located at the position 577, the last nt at a position 1086. The a.a. sequence of the E2 protein starts at position 1 and includes the four additional a.a. and ends at position 362.

In the result section 3.3 the positions of the additional nt or aa located between E1 and E2 sequences are indicated as positions -1. -2, -3, etc.

2.2.3 Cell culture

2.2.3.1 Culture of Huh7.5 cells

Adherent Huh7.5 cells were grown in monolayers in Dulbecco's modified minimal essential high glucose medium (4,5 g/l) with L-glutamine supplemented with 15 % FBS, 0.5 % gentamycin and 1 % non-essential amino acids. Cells were passaged

two times a week at a dilution of 1:3 or 1:5 depending on the confluence. Briefly, the medium was removed from the cells. Then, the cells were washed with 5 ml PBS and 2 ml of Trypsin-EDTA were added until the cells were covered. After 2 minutes the trypsin-EDTA was removed and after additionally 2-4 minutes, the cells started to detach from the bottom. Immediately, 10 ml of fresh medium was added to the cells. Finally, after resuspension, the cells were transferred to a new flask at a corresponding dilution.

2.2.3.2 Culture of 293T cells

Adherent 293T cells were grown in monolayers in Dulbecco's Modified Eagle Medium 1x (Gibco) containing 4.5 g/l glucose, L-glutamine, and 25 mM Hepes. It was supplemented with 10 % FCS and 1 % Penicillin/Streptamycin. Cells were passaged 2 times a week at a dilution of 1:10 or 1:15 depending on the confluence. Briefly, the used medium was removed from the cells and 10 ml new medium was added. After gentle shaking the cell were detached, resuspended, and distributed to new flasks at proper dilutions.

2.2.3.3 Cryoconservation of cells

The cell suspension was centrifuged in Falcon tubes for 5 min. at 1200 rpm. The cell pellet was resuspended in 900 µl FCS and after that added into a cryotube containing 100 µl DMSO. The cells were frozen slowly overnight at -80 °C using the Mr. Frosty device and were transferred to a liquid nitrogen tank the following day.

2.2.3.4 Thawing of cells

Cryo tubes containing cells were taken out from the liquid nitrogen and thawed under water. Next, the content was added to a flask containing 7 ml medium. After 4 hours culturing at 37 °C, the medium was changed to clear the cells from DMSO.

2.2.3.5 Transfection of 293T cells

293T cells were harvested and seeded in a 10 cm^2 dish at a final concentration of 2.5 x 10^6 cells in 8 ml medium (20-30 % confluence). The next day, the cells were transfected either with the expression plasmid phCMV IRES-E1/E2 (4 µg) for the

production of E1/E2 heterodimers. Alternatively, for the generation of HCVpp, the 293T cells were transfected with the expression plasmid phCMV IRES-E1/E2 in combination with 2 retroviral plasmids encoding for HIV-Gag,Pol or Luc (pCMV delta R8.2; plasmid HPPT-EF1α- Luc) using the Calcium Phosphate Transfection Kit (Clontech). Briefly, 500 µl HBS 2x were put in a facs tube. Next, using an Eppendorf tube 1.5 ml, the mix was prepared as follows:

Mix for the generation of E1/E2 heterodimers:

1. H_2O	x µl
2. phCMV- E1/E2	4 µg
3. $CaCl_2$ solution (added dropwise)	62 µl
Final volume	500 µl

Afterwards, this mix was added dropwise to the tube containing 500 µl of HBS 2x and incubated for 20 to 30 min at Rt until the mixture becomes cloudy. After addition of this cloudy solution dropwise to the cell culture, the dishes were incubated up to 16 h at 37 °C. The medium was changed and the cell culture was additionally incubated for 24 h at 37 °C. Finally, the cells were scrapped from the dishes and lysed.

Mix for the generation of HCVpp:

1. H_2O	x µl
2. phCMV- E1/E2	2.7 µg
3. plasmid encoding for Gag, Pol	8.1 µg
4. plasmid encoding for Luc	8.1 µg
5. $CaCl_2$ solution (added dropwise)	62 µl
Final volume	500 µl

This mix was added dropwise to the tube with 500 µl of g HBS 2x and incubated for 20 to 30 min. at Rt until the mixture becomes cloudy. After addition of this cloudy solution dropwise to the cell culture, the dishes were incubated up to 16 h at 37 °C. The medium was changed and the cell culture was additionally incubated for 48 h at 37 °C. Finally the supernatant containing the generated HCVpp was harvested and centrifuged for 5 min at 1200 rpm. The HCVpp preparation was aliquoted, frozen at -80 °C, and used for infectivity assay or neutralization assay.

2.2.3.6 Infectivity assay

Huh 7.5 cells were seeded in 96 well plates. For each plate, a master mix with 1.4 x 10^6 cells in 20 ml medium was prepared. The next day, the medium was removed and HCVpp in 100 µl of medium were added. After incubation for 4h at 37 °C, 200 µl of fresh pre-warmed medium was added and plates were incubated for additional 72 h at 37 °C. For the infectivity evaluation, the medium was removed and 50 µl of lysis buffer (Promega) was added to each well and incubated for 5 - 10 min at Rt using the shaker. Afterwards, 25 µl of prepared cell lysat were transferred into a luminometer 96 well plate. Twenty five µl of the Bright Glo Luciferase Assay buffer (Promega) were added and the luciferase-activity was immediately measured in the luminescence counter (Berthold Instruments). All cell lysates were measured in triplicates and a mean value was calculated.

2.2.3.7 Neutralization assay

Huh7.5 cells were seeded one day before neutralization as in 2.3.3.6. Dilutions of patients sera or monoclonal antibodies were prepared in DMEM and stored at 4 °C until the next day. The following day, HCV pp were thawed, mixed with the diluted sera or Abs, and incubated for 1 h at 37 °C. Afterwards, the mixture of HCVpp with sera or Abs in the volume of 50 µl was added to Huh-7.5 cells. The plates were incubated for 4 h at 37 °C. Afterwards, 200 µl medium were added to each well. The plates were finally incubated for 72 h at 37 °C. For the infectivity evaluation, the medium was removed and 50 µl lysis buffer (Promega) was added to the cells and incubated for 5 - 10 min. under shaking at Rt. Afterwards, 25 µl of prepared cell lysat was transferred into a luminometer 96 well plate. Twenty five µl of the Bright Glo Luciferase Assay buffer (Promega) was added to each well and the luciferase-activity was immediately measured in the luminescence counter (Berthold Instruments). The luciferase activity of all cell lysates was measured in triplicates and a mean value was calculated. Inhibition of infectivity by more than 50 % was considered as a positive neutralization.

2.2.4 Biochemical and immunological methods

2.2.4.1 Preparation of cell lysates

The medium of the Petri dishes (10 cm^2) containing the transfected 293T cells was removed and dishes were immediately put on ice. In the next step, the cell dish was transferred onto ice and 5 ml of ice cold PBS was added to each dish. The cells were scrapped and transferred into a 15 ml Falcon tubes. A centrifugation step for 5 min. at 1200 rpm at 4 °C was applied. The cell pellets were resuspended in 0.5 ml of non-denaturing lyses buffer and incubated on ice for 30 min. The mixtures were clarified by centrifugation for 20 min at 4 °C at 12.000 rpm. Finally, the supernatant was transferred into another Eppendorf tube and either stored at -20 °C or directly used for SDS-PAGE or ELISA.

2.2.4.2 SDS-PAGE

Proteins were separated using the standard discontinuous SDS-PAGE (sodiumdodecylsulfate-polyacrylamide-gel electrophoresis) in the Mini-Proteam (BioRad).

	Stacking Gel	**Separation Gel 12 %**
30 % Acrylamid solution (29:1)	0.75 ml	4.15 ml
Tris-HCl (1 M, pH 6.8)	0.62 ml	
Tris-HCl (1.5 M, pH 8.8)		2.5 ml
SDS 10 %	0.05 ml	0.1 ml
H_2O	3.55 ml	3.25 ml

For polymerisation of the gels saturated APS solution (1:500) and TEMED (1:1000) were added.

2.2.4.3 Immunoblot analysis (Western Blot)

After the SDS-PAGE, the proteins were transferred from the gel to the nitrocellulose membrane. In this process membrane and gel were soaked in western-blot-transfer-buffer and placed between three layers of Whatman paper without air bubbles. The gel was turned towards the cathode and the membrane was turned towards the anode. Transfer took place for 15 minutes at 7.5 V using the SemiDry-transfer

chamber (Trans-Blot SD, BioRad). After the transfer, binding sites on the membrane were blocked with blocking buffer ON at 4 °C upon a gentle shaking. Anti-HCV- E1 or E2 antibodies were diluted due to their optimal binding capacity in blocking buffer before incubation with the membrane for 1 h or, alternatively, overnight at 4 °C. Following 3 cycles of washing with T-PBS for 10 minutes, the membrane was incubated for 1 h with conjugate (anti-mouse or anti-goat peroxydase labelled IgG) diluted 1:1000 in the blocking buffer. Additional 3 steps of washing with PBS followed. Binding of the conjugate to anti-HCV antibodies on the membrane was detected on a radiographic film (BioMax Film, Kodak) by using ECL Western blotting detection reagents according to the manufacturer's instruction.

2.2.4.4 Enzyme-linked Immunosorbent Assay (ELISA)

The technique of the enzyme-linked immunosorbent assay (ELISA) is used to detect the presence of the AD patient E1/E2 antigen in prepared cell lysates (2.2.4.1.) and to characterize the binding capacity of antibodies included in patients' sera to AD patients' E1/E2 antigen.

The Nunc immunoplates were coated with lectin (5 µg/ml) in a 0.1 M carbonate buffer (pH 9.6) ON at 4 °C. After blocking of the potential unspecific binding sites in the wells with the blocking buffer (incubation ON at 4 °C), the plates were washed 4 times with PBST. Lysates of 293T cells transfected wuth the HCV E1/E2 expressing plasmids were added to each well and plates were incubated ON at Rt. The wells were washed 4 times with PBST and primary antibodies (e.g. monoclonal antibodies to HCV E1 or E2 proteins) were added in a concentration of 1 µg/ml and incubated 3-4 h at 37 °C. Alternatively, human sera, diluted 1:50 in dilution buffer, were added to the wells and incubated 3-4 h at 37 °C. The wells were washed 4 times with PBST. Secondary antibody labeled with HRP (anti-mouse 1:1000, anti-human 1:5000) was added and plates were incubated for 1 h at 37 °C. After additional washing with PBS 100 µl of OPD substrate solution was added to each well, the plates were incubated in the dark for 1-10 minutes, and the reaction was stopped by adding 50 µl of the Stop solution. Finally, the OD_{490nm} was measured using the Elisa Reader Asys Expert Plus (Asys Hiteck).

2.2.4.5 P24 assay

For quantification of HCVpp produced in 293T cells, the commercial Innotest^{TM} HIV Antigen mAb kit (Innogenetics GmbH) for the evaluation of the P24 protein was used according to the manufactor's instruction. Briefly, 100 µl of conjugate working solution 1 were mixed with 100 µl of the sample. The predefined positive control was mixed with the matrice in a ratio 1:1. The mixture was added to the 96 well plate and was incubated for 60 min. at 37 °C. After 5 times washing with a 300 µl washing solution, 200 µl of prepared conjugate working solution 2 was added and incubated for 30 min at 37 °C. After the next round of washing, 200 µl of substrate was added and incubated for 30 min. at Rt. Finally, 50 µl of stop-solution were added and the OD_{450nm} was measured using Elisa Reader Asys Expert Plus (Asys Hiteck).

2.2.4.6 Concentration of HCV pseudoparticles by ultracentrifugation

The supernatant of transfected 293T cells containing the HCVpp was layered on 5 ml of 20 % (w/v) sucrose and centrifuged in rotor SW40 for 2.30 h at 28.000 rpm at 4 °C. The supernatant was discarded, the pellet was dissolved in 30 µl 2x Laemmli buffer and used for SDS-PAGE (2.3.4.2) followed by immonodetection (2.3.4.3).

3 Results

3.1 Evolution of the HCV genome

3.1.1 Generation of HCV AD78-E1/E2 clones in pCR4-Topo

To evaluate the evolution of the HCV genome in patients infected by contaminated AD-globulin, nine patients, who received the batch 8 of contaminated AD-globulin, were chosen (Table 2.1.). Serum samples collected at different time points of infection (Fig.3.7.) were used. Five of these patients have not been treated with IFN-α, in three patients, who received the IFN-ribavirin therapy, serum samples for analysis have been obtained before initiation of the therapy, and in one non-responder patient (patient X) serum sample was obtained 3 years after the cessation of the therapy. For seven patients serum samples collected 20 years after infection were used. For two patients (patients T and H) serial serum samples collected 16, 20, and 28 years p.i. were available for analysis (Fig.3.7.). In parallel, globulin batches 8, 10, and 12 were also used as a source of HCV RNA. The extracted HCV RNA was used for DNA amplification in a nested - pcr reaction using the primers derived from the terminal part of the core gene and terminal part of the E2 gene. The primers used for the second round of PCR contained the EcoRV sites for a subsequent recloning into the expressing vector. Serum samples from some patients have been frozen and thawed during the storage a number of times. Due to degradation of HCV RNA in these samples amplification of the HCV envelope genes were done in two or four overlapping fragments, which were re-assembled into complete envelope sequences upon the results of sequencing of these shorter fragments.

As a result of selective pressure driven by the host HCV circulates in patients as a population of particles with closely related but slightly different genomes (quasispecies). Therefore, to obtain the major quasispecies variants, the amplified E1/E2 fragments were cloned into the vector pCR4-Topo. After transformation into the Top10 cells, at least 5 clones of each fragment were picked up and the DNA was isolated. Insertion of the E1/E2 fragment was confirmed by gel electrophoresis using

the EcoRV restriction (Fig.3.1). As expected, the E1/E2 fragment, amplified in one fragment, showed the correct size of about 1.7 Kb. An example of one E1/E2 fragment inserted in the pCR4-TOPO vector is attached as a vectorcard (Appendix Fig.8.1.). Finally, 1200 ng of DNA was used for sequencing.

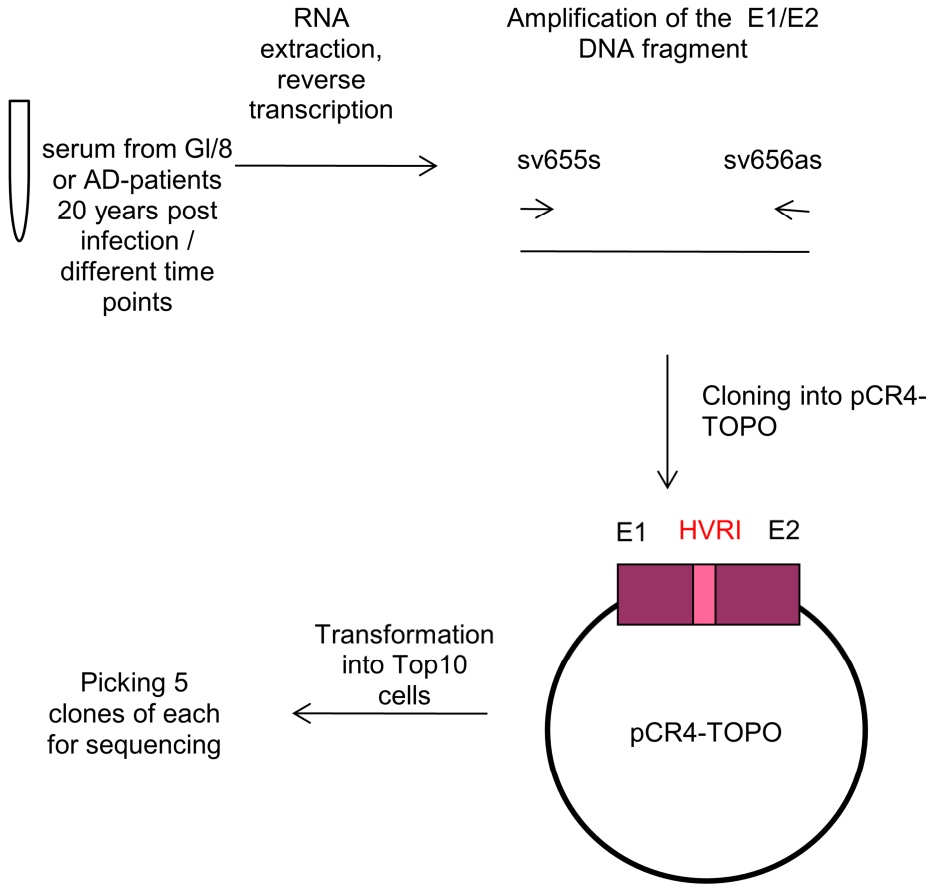


Fig.3.1. Cloning of AD78- E1/E2 DNA fragments into the intermediate plasmid pCR4-TOPO

RNA was extracted from serum samples. E1/E2 DNA fragments were then generated by reverse transcription and amplification using the primers sv655s and sv656as, which contained the EcoRV sites. The DNA fragments were cloned into the pCR4-TOPO vector and transformed into the Top10 cells. At least 5 positive clones, after control restriction with EcoRV, were used for sequencing.

3.1.2 Analysis of the E1/E2 sequences from the contaminated immunoglobulin and infected patients

The first stage of the study assumed the analysis of the HCV envelope genes sequences from the contaminated anti-D globulin (this work has been done in collaboration with Prof. E. Schreier, Berlin). The E1/E2 sequences were amplified from globulin batches GI8, GI10, and GI12. The amplified DNA from batches GI10 and GI12 were used for direct sequencing. The DNA fragments obtained from batch GI8 were further cloned into pCR4-TOPO plasmid and 29 clones were obtained and

sequenced. Both clonal and direct E1/E2 sequences from the anti-D globulin batches were subjected to a phylogenetic analysis using the PHYLIP package to evaluate an evolutionary relationship between these sequences. As expected, the anti-D sequences were clearly separated from E1/E2 sequences of HCV 1b strains randomly chosen from the geneBank (Fig.3.4.). At the same time, the analysis demonstrated clear separation of the anti-D sequences into two distinct clusters (Fig.3.2.) illustrating the position of individual anti-D E1/E2 sequences from batches GI8, GI10, and GI12 in a rooted neighbour-joining consensus tree. As can be seen, the results have demonstrated presence in the contaminated globulin batch GI8 of two distinct sequence variants, reffered to as GI8/A and GI8/B. The separation of sequences into two clades was supported with high bootstrap value of 99. The E1/E2 sequences from the globulin batches GI10 and GI12 obtained after the direct sequencing of the amplified E1/E2 genes clustered together with the sequences of the GI8-B variant.

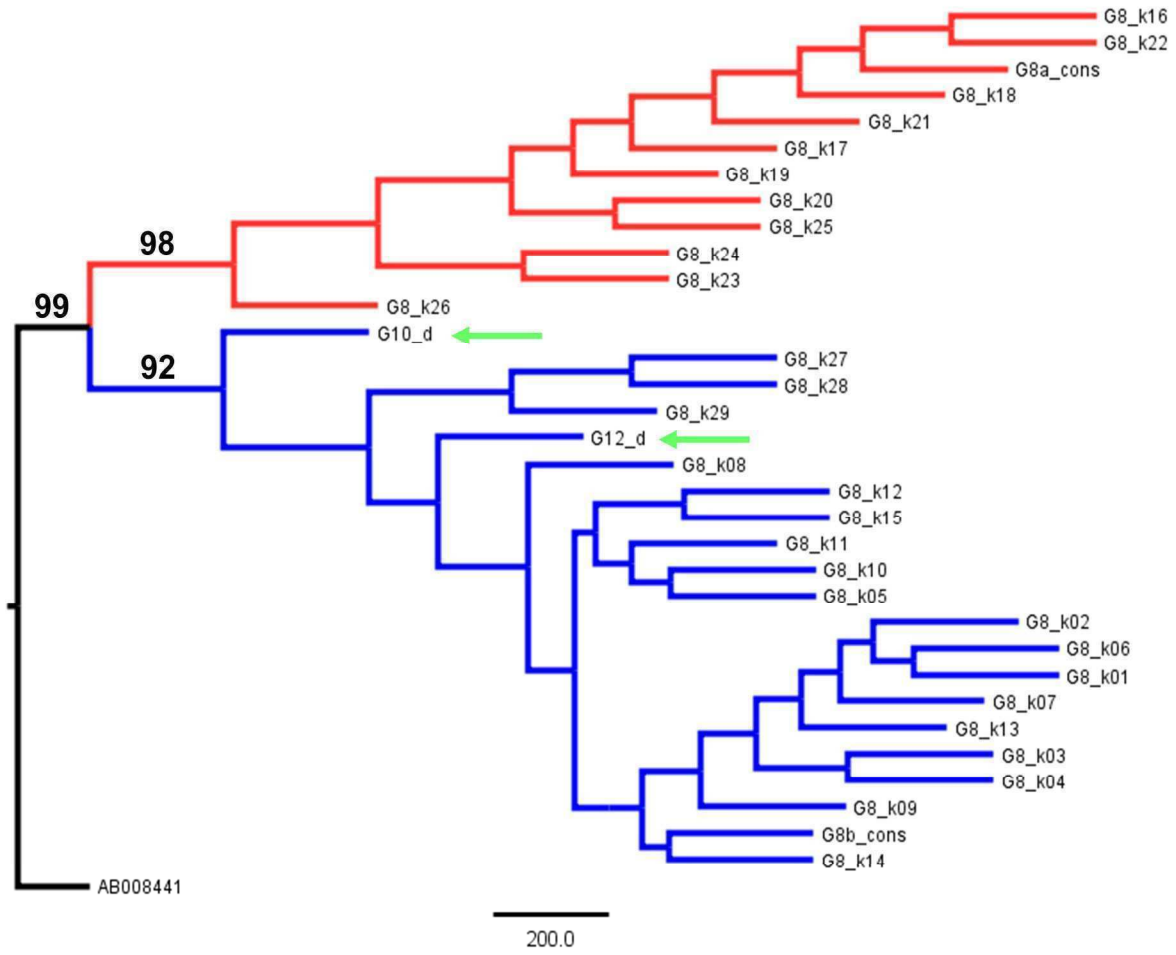


Fig.3.2. Consensus neighbour joining tree of the E1/E2 sequences obtained from HCV AD78 contaminated globulin batches GI8, GI10, and GI12

Phylogenetic analysis of AD78 E1/E2 sequences from globulin batches 8, 10 and 12 was performed using the PHYLIP package. Clonal sequences from batches G8-A and G8-B are indicated in red and blue, correspondingly. The positions of the E1/E2 sequences obtained from batches Gl10 and Gl12 by direct sequencing are marked with green arrows. Bootstrap values for the observed clusters indicated in black. The sequence of a randomly chosen HCV 1b isolate (accession number AB008441) was used to outgroup the tree.

To confirm the heterogeneity of the HCV AD78 strain in the contaminated anti-D globulin we have amplified the NS3 gene sequences obtained from globulin batches Gl8 and Gl12 and from individual AD78-infected patients (this work was done in collaboration with Dr. J.Timm and M. Ruhl). Upon a phylogenetic analysis, these sequences fell into three clusters clearly separated from randomly chosen NS3 sequences from the HCV 1b strains available in the GenBank (Fig.3.3.). Reliability of these clades was highly supported by bootstrap analysis. Thus, the HCV AD78 strain in contaminated anti-D globulin is not homogeneous and is represented by at least three closely related virus variants.

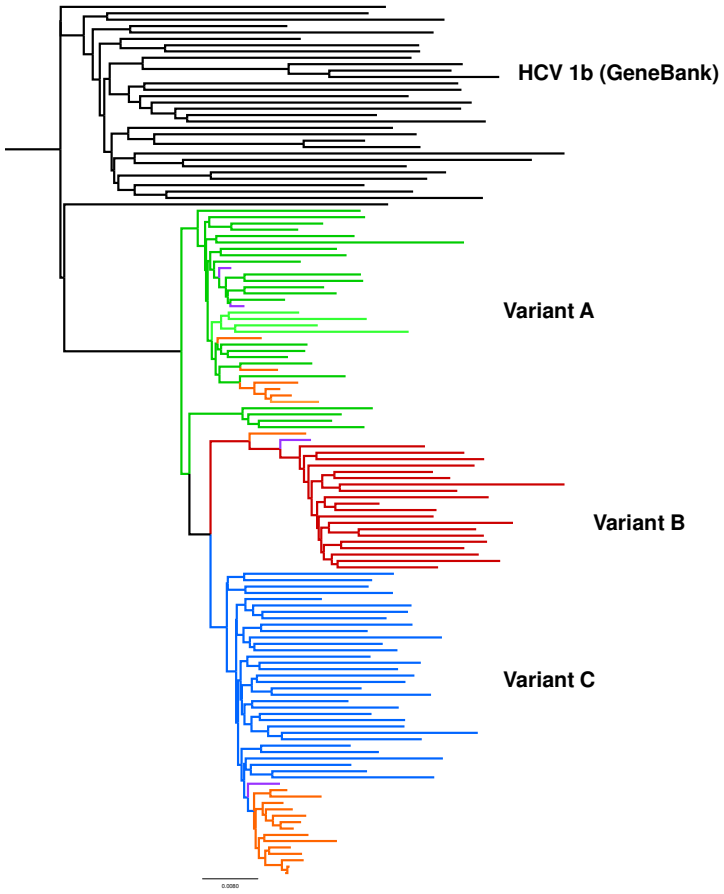


Fig.3.3. Consensus Neighbour-joining tree of NS3 gene sequences obtained from HCV AD78-contaminated globulin 8 and 12, AD-infected patients and HCV 1b strains

There are three NS3 sequence variants present in the NS3 sequences observed by direct sequencing: A (green), B (dark red), C (blue). The clonal NS3 sequences of the globulin 8 are indicated in orange and of globulin 12 in violet, respectively. HCV 1b NS3 sequences from the HCV GeneBank are indicated in black. (Obtained in collaboration with Dr. J.Timm and M. Ruhl)

To analyse the evolutionary relationship between the E1/E2 sequence from the contaminated globulin and infected patients the PHYLIP package was used. The consensus neighbour-joining tree (Fig.3.4.) included 20 clonal E1/E2 sequences from the contaminated globulin, 5 clonal sequences obtained from serum of each infected patient collected at different time points, and, additionally, the E1/E2 genotype 1b sequences randomly chosen from the HCV Los Alamos database. The results demonstrated the clear separation of HCV AD78 sequences from the bulk of HCV 1b sequences indicating the evolutionary relatedness of the viruses present in the globulin and individual patients. These results confirmed that the anti-D outbreak under study was indeed a single source outbreak caused by a single virus strain (HCV AD78). Interestingly, the sequences both from the globulin and individual patients formed two separate clusters, corresponding to two virus variants (A and B) identified in the contaminated globulin batches (Fig.3.2.). These data suggest that two observed clades represent two evolutionary lineages that originated from two closely related but still distinct viruses already present in the contaminated anti-D globulin. This conclusion was further supported by a phylogenetic analysis of separate E1 and E2 sequences (Fig.3.5. and 3.6.). Neighbour-joining trees for these genes have the same topology as the tree for the E1/E2 region thus confirming the fact that the AD78 outbreak was caused by two closely related virus variants already present in the infectious source.

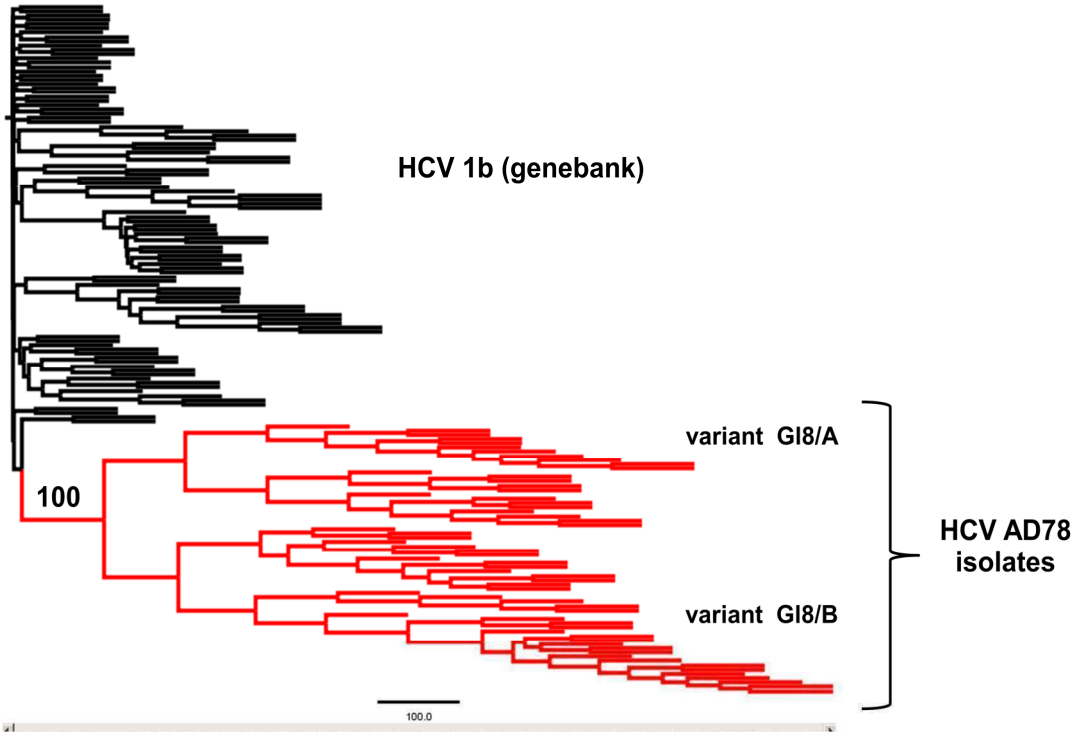


Fig.3.4. Consensus neighbour-joining tree of the envelope gene sequences obtained from HCV AD78-contaminated globulin, AD78-infected patients and HCV 1b strains

Sequences from HCV1b strains are separated from the bulk of AD78 sequences. Sequences from the anti-D globulin and anti-D patients form two distinct clusters. The bootstrap values of the corresponding nodes are indicated.

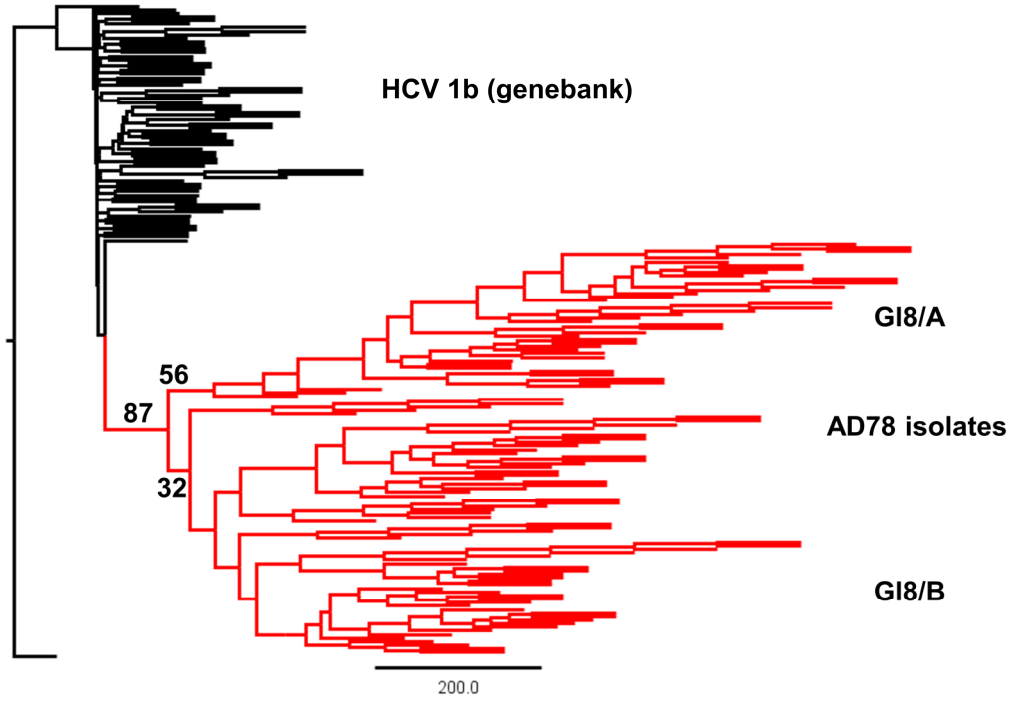


Fig.3.5. Consensus neighbour joining tree of the E1 sequences obtained from HCV AD78-contaminated globulin batch 8, AD78-infected patients and HCV 1b strains

Sequences from HCV1b strains are separated from the bulk of AD78 sequences. Sequences from the anti-D globulin and anti-D patients fell into two distinct clades. The bootstrap values of the corresponding nodes are indicated.

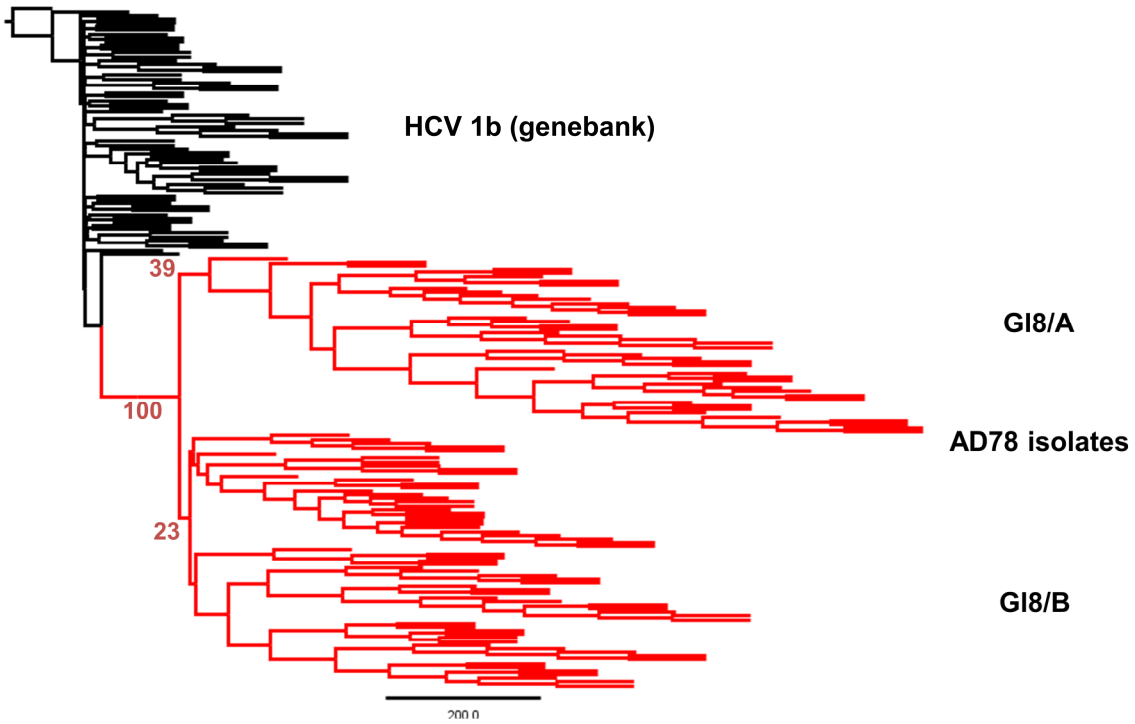


Fig.3.6. Consensus neighbour-joining tree of the E2 sequences obtained from HCV AD78-contaminated globulin batch 8, AD78-infected patients and HCV 1b strains

Sequences from HCV1b strains are separated from the bulk of AD78 sequences. Sequences from the anti-D globulin and anti-D patients fell into two distinct clades. The bootstrap values of the corresponding nodes are indicated.

The planned study of the evolutionary rates of the HCV envelope gene sequences in anti-D cohort was possible only by matching the AD78 virus variants circulating in each patient with the virus variant present in the contaminated source. Phylogenetic analysis of the E1/E2 sequences described above allowed for the identification of individual patients infected either with HCV AD78 variants A or B present in the globulin batch Gl8 (referred to as Gl8/A or Gl8/B virus variants throughout the thesis). Serum samples from 3 patients infected with HCV AD78 variant Gl8/A (patients T, N, and Z) and 6 patients infected with variant Gl8B (patients H, X, B, J, M, and Y) were available for the study (Fig.3.7.).

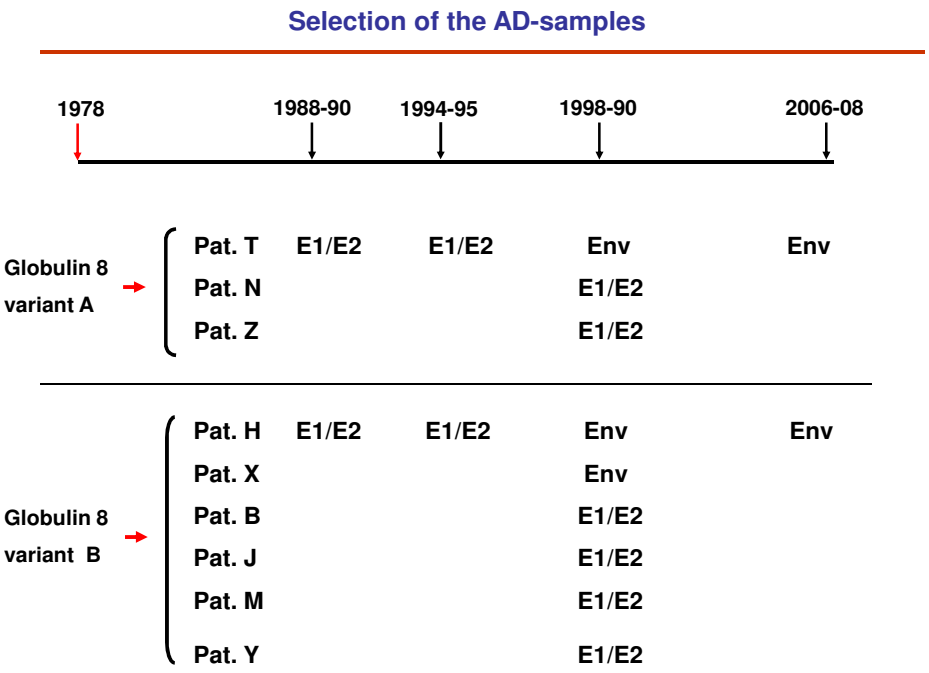


Fig.3.7. Selection of patient's serum samples for amplification of the E1/E2 sequences

Sequences were amplified in two (E1/E2) or one (Env) fragments at indicated time points and cloned into the intermediate vector (pCR4-TOPO). Five clones for each sample were sequenced and used for further experiments.

Serum samples collected in 1998-2000, namely about 20 years after infection, from all 9 patients were used for amplification, cloning in intermediate pCR4-TOPO plasmid, and sequencing of the resulting clones. In addition, amplification of the E1/E2 region was performed from serial serum samples of patients T and H collected approximately 10-12, 16, and 28 years after infection. Alignment of the resulting sequences of the E1 and E2 genome regions presented as supplementary figures 8.2. and 8.3. (see the Appendix).

Generation of the clonal sequences from anti-D globulin and these 9 patients allowed proceeding to the next stages of the project - analysis of the evolutionary rates of the E1/E2 genes of HCV and analysis of the evolution of the humoral immune response to HCV envelope proteins in chronically infected individuals.

3.1.3 Evolution of the HCV E1/E2 sequences in a group of chronically infected anti-D patients

3.1.3.1 HCV AD78 evolutionary rates in different patients

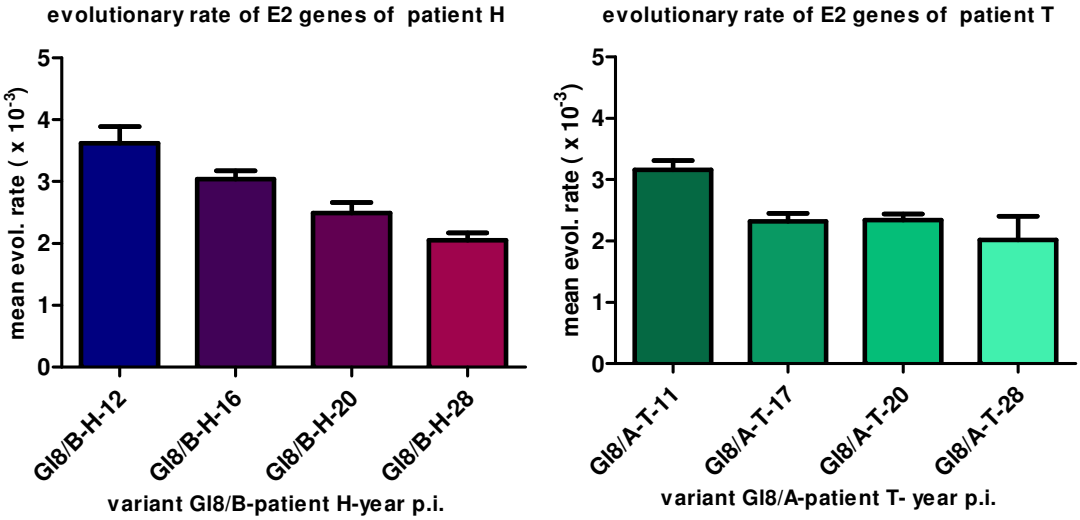
The obtained clonal sequences were used for the evaluation of the evolutionary rates for the E1 and E2 genes in anti-D chronically infected patients (Tab.3.1).The rates for the HCV AD78 isolates tested varied from 0.83 x 10^{-3} to 4.1 x 10^{-3} and from 2.02 x 10^{-3} to 3.62 x 10^{-3} nucleotide substitutions per site per year for E1 and E2 genes, correspondingly. There was no statistically significant difference in evolutionary rates of envelope genes of isolates from patients infected with variants Gl8/A or Gl8/B of HCV AD78. At the same time, both for patient T and patient H a tendency for a decline of substitution rates with duration of HCV persistence was noted for the E1 as well as for the E2 genes (Fig.3.8. A, B).

Tab.3.1. Evolutionary rates of the E1 and E2 genes of HCV AD78 variants in different patients

Variant Gl8 - patient	Duration of HCV persistence	Mean evol.rate (E1 gene) (ntsubstitution/site/year) (x 10^{-3})
Gl8/A-T	11	1,95 ± 0,6
	17	1,06 ± 0,1
	20	0,97 ± 0,1
	28	1,1
Gl8/B-H	11	4,1
	16	1,842 ± 0,1
	20	1,87 ± 0,3
	28	1,08 ±0,1
Gl8/A-N	20	1,24 ± 0,2
Gl8/A-Z	20	1,02
Gl8/B-B	20	1,24
Gl8/B-J	20	0,83 ± 0,1
Gl8/B-M	17	2,86 ± 0,16
Gl8/B-X	20	1,334 ± 0,6
Gl8/B-Y	20	1,12

Variant Gl8 - patient	Duration of HCV persistence	Mean evol.rate (E2 gene) (ntsubstitution/site/year) (x 10-3)
Gl8/A-T	11	3,16 ± 0,1
	17	2,32
	20	2,34± 0,1
	28	2,02 ± 0,18
Gl8/B-H	11	3,62 ± 0,3
	16	3,04 ±0,2
	20	2,48 ± 0,1
	28	2,05 ± 0,1
Gl8/A-N	20	2,76 ± 0,14
Gl8/A-Z	20	2,6 ± 0,2
Gl8/B-B	20	2,47 ±0,06
Gl8/B-J	20	3,12 ± 0,08
Gl8/B-M	17	2,97 ± 0,152
Gl8/B-X	20	2,36 ± 0,06
Gl8/B-Y	20	2,58 ± 0,02

A



B

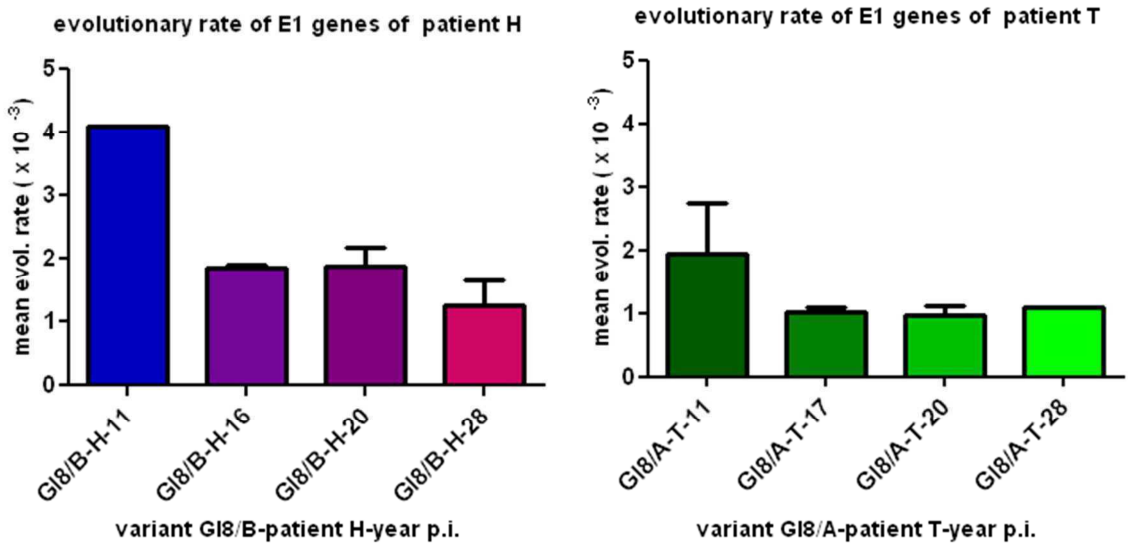


Fig.3.8. Evolutionary rates of the envelope genes of AD78 isolates from two chronically infected patients

(A) The mean evolutionary rates of E2 gene for patient H (left graph) and for patient T (right graph). **(B)** The mean evolutionary rates of E1 gene for patient H (left graph) and for patient T (right graph). The evolutionary rates (nucleotide substitutions per site per year x 10^{-3}) were calculated on the basis of sequences obtained at different time points of infection.

3.1.3.2 Selective pressure on the E1 and E2 genes of HCV AD78 isolates

To evaluate the selective pressure for the E1/E2 regions within different AD78-patients clusters, the ratio of synonymous (dS) to nonsynonymous (dN) nucleotide substitutions was calculated (Fig.3.9.). First, the dS/dN ratio was calculated for E1 and E2 for AD78 isolates obtained from patient T (infected with the virus variant GI8/A) and patient H (infected with the variant GI8/B). The analysis of the changes in the dS/dN ratio in the E1/E2 genes from both patients at different time points p.i. did not show the presence of a strong positive (dS/dN < 1) selection. Quite the opposite, the estimated dS/dN values were higher than 1 suggesting a purifying selection. The comparison of the dS/dN ratio for E1 and E2 separately in different patients 20 years p.i. has shown an evident variation of this parameter even in the absence of a strong positive selection, suggesting that HCV envelope gene evolution is a patient-specific process.

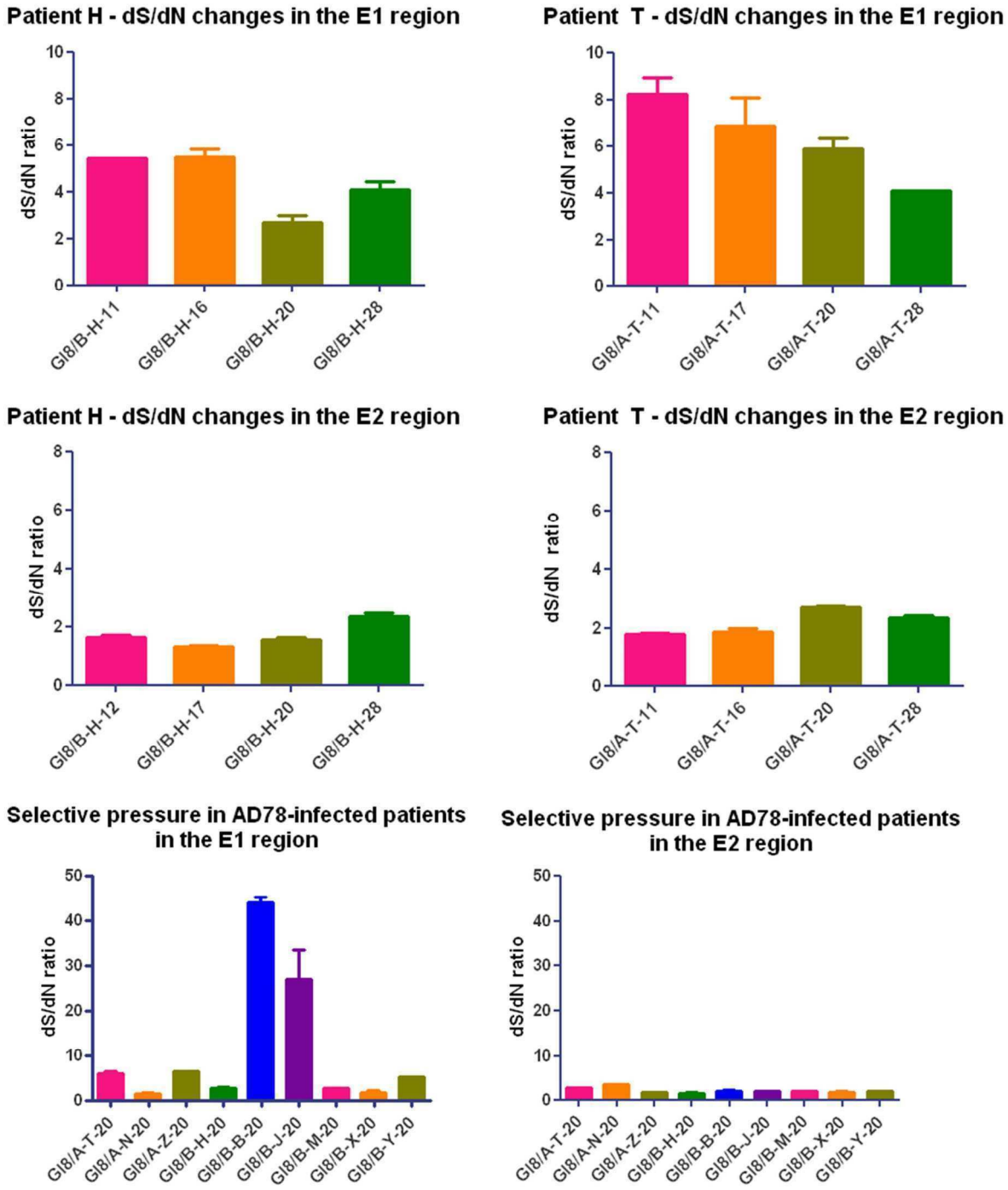


Fig.3.9. Rates of synonymous to nonsynonymous nucleotide substitutions (dS/dN) for E1/E2 genes of HCV AD78 isolates obtained from anti-D patients at different time points post infection

The x –axis indicates the virus variant, patient, and the time interval (delta T in years) between the date of infection and the date of a sample collection.

3.1.3.3 Distribution of amino acid substitutions in E1/E2 sequences of AD78 isolates during persistent infection

Alignment of the clonal HCV E1/E2 sequences from anti-D patients T and H allowed the analysis of a pattern of a.a. substitutions during the chronic phase of infection (Appendix Fig.8.2. and 8.3. and Fig.3.10.). Only evolutionary meaningful substitutions, namely those present in more than 70 % of tested clonal sequences, were included into the analysis. In general, in AD78 isolates obtained from both patients a higher heterogeneity in the E2 region than in the E1 gene was observed. None of the observed mutations occurred in the putative N-linked glycosylation sites. In the E1 sequence of patient T, infected with the GI8/A variant of the virus, two a.a. substitutions D>N and G>S at positions 42 and 60 were fixed during the whole period of observation, whereas two a.a. substitutions R>L at position 40 and V>I at position 96 appear only 16 years post infection (p.i) and remain fixed during the whole period of subsequent observation. In the sequences obtained at the last time point of follow up (28 years p.i.) additional a.a. substitutions appeared at positions 23 (Y>F), 65 (T>A), 79 (A>V), and 138 (A>T). In the E2 sequence of patient T, eight a.a. substitutions at position 65 (V>I), 67 (S>N), 113 (Q>R), 152 (A>E), 195 (L>S), 198 (N>D), 202 (I>V) and 276 (G>D) were fixed during the whole period of observation, whereas only one substitution A>S at position 29 appeared 16 years and a second time 28 years p.i..

In the E1 sequence of patient H, infected with the GI8/B variant of the virus, four a.a. substitutions at positions 42 (D>S), 44 (S>F), 102 (L>F) and 112 (E>D) were fixed during the whole period of observation. One a.a. substitution V>M at position 86 only appears 11 years post infection and furthermore one a.a. substitution at position 122 (V>L) only appears 16 years p.i. After 20 years of infection, four a.a. substitutions at positions 50 (A>E), 136 (S>A), 157 (V>E) and 186 (L>P) appeared. At the last studied time point (28 years p.i.) only one additional a.a. substitution appeared at position 182 (I>V). In the E2 sequences of patient H, three a.a. substitutions L>I, I>T and S>D at positions 58, 202, and 264 were fixed during the whole period of time. An insertion of additional a.a. which also stayed fixed during the whole period of time was at positions 199 (N) and 200 (T). Four a.a. substitutions at positions 63 (F>L), 68 (F>I), 74 (P>S) and 113 (Q>R) only appeared 12 years p.i, whereas two a.a. substitutions at positions 143 (S>F) and 259 (I>V) appeared 12 years p.i and stayed fixed till 20 years post infection. Furthermore, two a.a.

substitutions appeared once 12 years p.i at positions 113 (Q>R) and 327 (F>V) and a second time 28 years p.i. In one case, a.a. substitution appeared at position 65 (A>F) only at the time point 12 years p.i. but 28 years p.i. the a.a. A was again substituted by T (A>T). In another case, a.a. substitution at position 99 (G>S) appeared 12 years p.i. and at the time points 16 years and 20 years p.i. there was a further substitution of the a.a. S by D (S>D). At the last time point of follow up (28 years p.i.) a reversion of the a.a. D back to S (D>S) was observed at the same position. After 16 years of infection, one a.a. substitution N>K at position 55 appeared and whereas another a.a. substitution at position (E>R) appears but 20 years p.i. the amino acid R was substituted by K (R>K). Twenty years after infection one a.a. substitution appeared at position 61 (A>S). Moreover, only one a.a. substitution appeared at postion 198 (N>S) 28 years p.i..

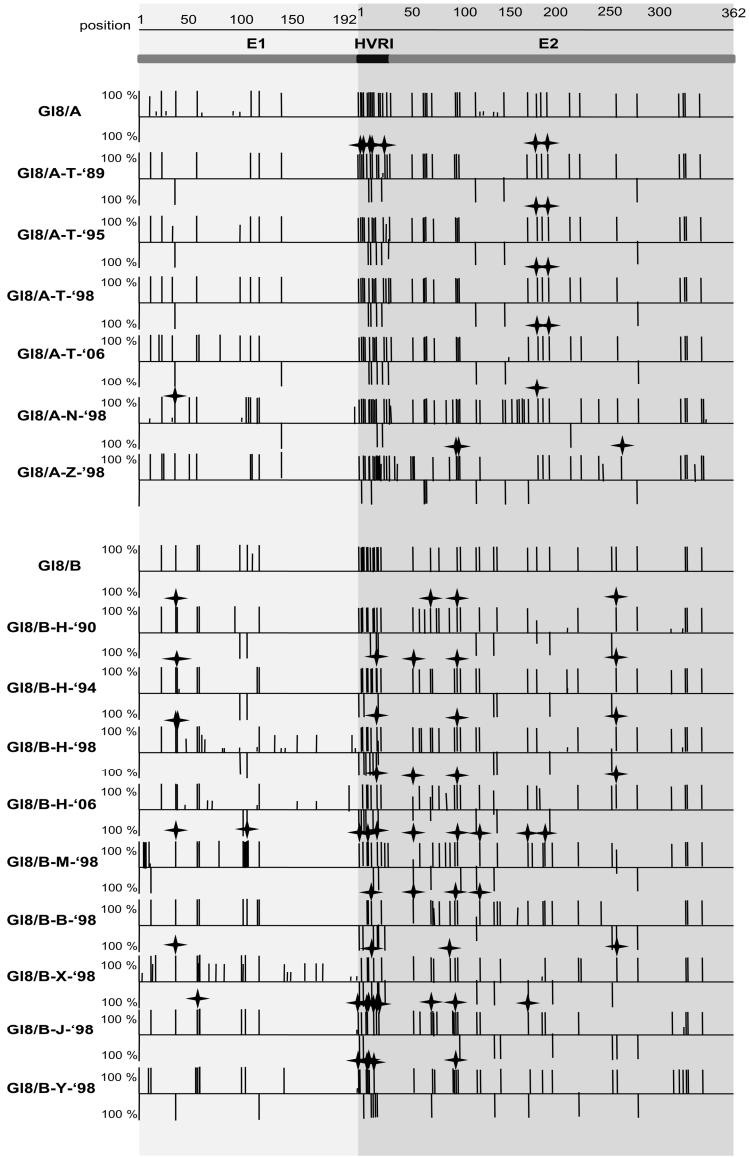
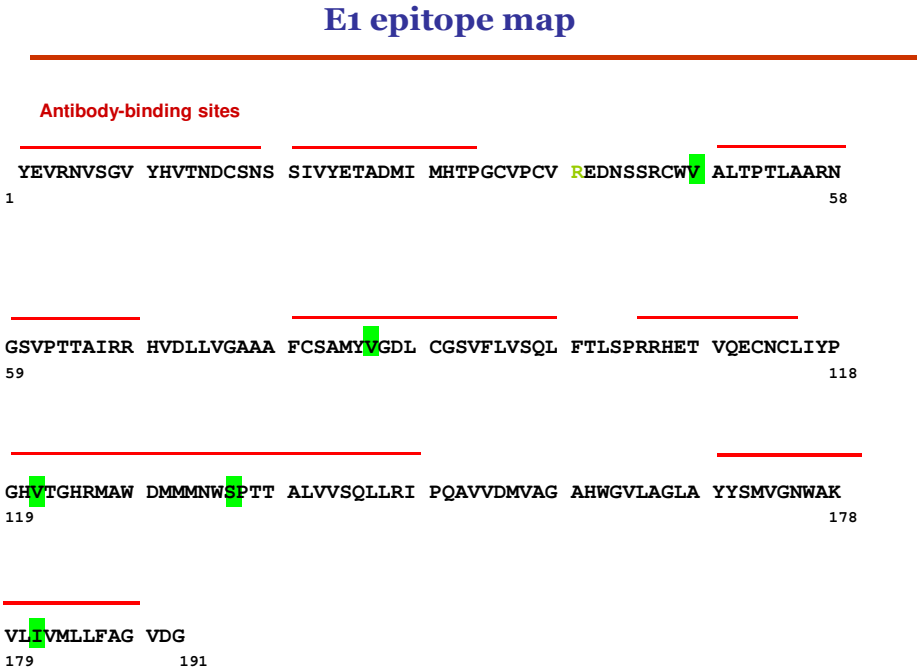


Fig.3.10. Sites of AD78 E1E2 sequence variation

HCV viral genome sequences at sequential time points are illustrated. horizontal lines: Forward mutations are indicated with bars above and reverse mutations are indicated with bars below the horizontal lines that designate the isolate sequences. The length of the bar reflects the occurrence of the mutation observed in all clonal sequences (from 7 to 100 %). Second forward substitutions in already mutated a.a.residues are indicated with an asterics.

During the process of viral evolution in chronically infected host the virus has to evade a host immune pressure. Mutations in the E1/E2 regions, which are crucial for viral entry (e.g. CD81-binding sites) as well as neutralizing Abs-binding sites could provide putative regions for viral escape. Therefore, the occurrence and distribution of mutations separately in the E1 and in the E2 sequences of the AD78 isolates isolated in the current study was analyzed (Tab. 3.2., 3.3. and Fig.3.11.). During the course of chronic infection in patient T, infected with virus variant Gl8/A, and patient H, infected with virus variant Gl8/B, only a few a.a. substitutions occurred in the E1 sequences within a time period of 28 years p.i. Furthermore, only up to 25 % of mutations arose in regions known to be targeted by human mAbs in the E1 sequences from patient H, whereas in the E1 sequences of patient T no mutation occurred in human mAbs-binding sites during the whole period of time. The same tendency was demonstrated for E1 sequences obtained 20 years p.i. from a group of other Gl8 infected patients - most a.a. substitutions were observed in regions outside of those known to be targeted by human mAbs.



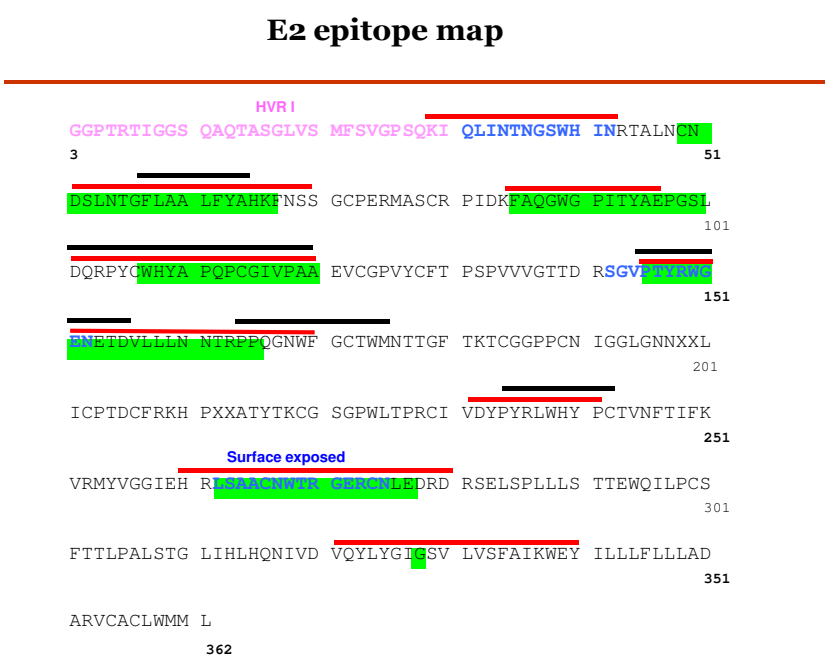


Fig.3.11. Localization of the amino acids substitutions observed in the clonal E1 and E2 sequences of anti-D patients

The E1 and E2 sequences of the Gl8/B variant of the HCV AD78 are shown. Red and black lines indicate positions of known antibody-binding sites and CD81-binding sites. Regions, where amino acid substitutions in the AD78-derived sequences from patients were observed, are shown in green.

In the E2 sequences the number of newly arising mutations varied depending on the phase of infection (Tab.3.3.). As has already been indicated, a tendency for a decline of nucleotide substitution rate with duration of HCV persistence was noted for the E1 as well as for the E2 genes (Fig.3.8. A, B). For the virus persisting in patient T the highest number of a.a. substititions was found in the E2 sequnces obtained at the first sampling date (1989) indicating that most of the mutations arose during the acute/early chronic phase of infection (first 11 years p.i.). About half of these mutations occurred in CD-81- or human mAbs-binding sites. During the next years of HCV persistence the E2 protein of the virus circulating in patient T remained remarkably stable and few newly appeared mutations occurred mainly in the CD-81-binding sites. A different dynamic of a.a. substitutions was observed for the E2 sequences obtained from serial samples of patient H. In this patient, persistence of HCV AD78 was accompanied by a more or less continuous accumulation of mutations throughout the whole folow up period. At that, at each sampling point from 39 to 73 % of newly arising a.a. substitutions in the E2 protein occurred in the CD-81 and human mAb-binding sites. Analysis of the mutations in the E2 proteins of AD78 isolates obtained from seven other AD patients 20 years p.i. (Tab.3.3.) demonstrated

that a significant number of a.a. substitutions (36 o 56 %) present were also localized within described CD-81 binding sites or regions targeted by human mAbs.

Tab.3.2. Occurrence and distribution of mutations in the E1 sequences of AD78 isolates

AD78 isolates	number of new mutations	∑ of mutations	human mAbs – binding sites	Outside of known functional sites
GI8/B-H-11	8	8	0 (0 %)	8 (100 %)
GI8/B-H-16	1	9	1 (11.1 %)	8 (88 %)
GI8/B-H-20	7	16	1 (14.3 %)	6 (85.7 %)
GI8/B-H-26	4	20	1 (25 %)	3 (75 %)
GI8/B-M-20	12	12	4 (33.3 %)	8 (66.6 %)
GI8/B-B-20	2	2	1 (50 %)	1 (50 %)
GI8/B-X-20	12	12	0 (0 %)	12 (100 %)
GI8/B-J-20	2	2	0 (0 %)	2 (100 %)
GI8/B-Y-20	5	5	1 (20 %)	4 (80 %)
GI8/A-T-11	1	1	0 (0 %)	1 (100 %)
GI8/A-T-16	2	3	0 (0 %)	2 (100 %)
GI8/A-T-20	0	3	0 (0 %)	0 (0 %)
GI8/A-T-26	3	6	0 (0 %)	3 (100 %)
GI8/A-N-20	8	8	2 (25 %)	6 (75 %)
GI8/A-Z-20	3	3	1 (33.3 %)	2 (66.6 %)

Abbreviations: AD78 isolate – virus variant-patient-years p.i

Tab.3.3. Occurrence and distribution of mutation in the E2 sequences of AD78 isolates

AD78 isolates	New mutations in the whole E2*	New mutations in the HVR I*	∑ of mutations	new mutations in CD81-binding sites	human mAbs	Outside of known functional sites
GI8/B-H-12	23	9 (39 %)	23	9 (39 %)	0 (0 %)	14 (61 %)
GI8/B-H-16	27	15 (56 %)	50	14 (52 %)	1 (4 %)	12 (44 %)
GI8/B-H-20	15	12 (80 %)	65	11 (73 %)	0	4 (27 %)
GI8/B-H-26	21	11 (52 %)	86	12 (57 %)	0	9 (43 %)
GI8/B-M-20	30	9 (30 %)	30	16 (53 %)	1 (3 %)	13 (44 %)
GI8/B-B-20	28	12 (43 %)	28	14 (50 %)	1 (4 %)	13 (46 %)
GI8/B-X-20	27	11 (41 %)	27	11 (41 %)	0 (0 %)	16 (59 %)
GI8/B-J-20	27	12 (45 %)	27	9 (33.3 %)	1 (3.7 %)	17 (63 %)
GI8/B-Y-20	25		25	9 (36 %)	0 (0 %)	16 (64 %)
GI8/A-T-11	18	10 (55 %)	18	9 (50 %)	1 (5.5 %)	8 (44.5 %)
GI8/A-T-17	7	7 (100 %)	25	6 (85 %)	0 (0 %)	1 (15 %)
GI8/A-T-20	2	2 (100 %)	27	2 (100 %)	0 (0 %)	0 (0 %)
GI8/A-T-26	2	2 (100 %)	29	2 (100 %)	0 (0 %)	0 (0 %)
GI8/A-N-20	26	12 (46 %)	26	8 (32 %)	2 (7.3 %)	16 (61.7%)
GI8/A-Z-20	31	12 (39 %)	31	11 (35.4 %)	1 (3.2 %)	19 (61.4%)

Abbreviations: AD78 isolate – virus variant-patient-years p.i.
* New mutations in the whole E2 in the HVR I include the reverse mutations to the consensus globulin 8 sequence

3.1.3.4 Rate of forward and reverse mutations in the HCV AD78 E1/E2 sequences from patient H and patient T

It is often assumed that evolution of HCV is driven on the one hand by immune pressure, which evidences in appearance of the “forward” or escape mutations, and on the other hand by structural and/or functional constraints, which manifests themselves in sequence preservation or reversion of sequences toward consensus.

In order to assess a possible contribution of structural and functional constrains to HCV evolution in chronically infected individuals we investigated the direction of sequence mutations the E1/E2 sequences from patient T, infected with virus variant Gl8/A, and from patient H, infected with virus variant Gl8/B. The mutations observed in the sequences obtained from patients T and H at different time point p.i. were divided into two groups depending on the time period of infection. Group one (Gl8/A-T-78-89 and Gl8/B-H-78-89) included the mutations observed in the E1/E2 sequences obtained from each patient at the fist sampling time point (covers the first 11 years of acute and chronic infection). The second group (Gl8/A-T-89-06 and Gl8/B-H-89-06) included the mutations, which were detected in the sequences obtained during the subsequent sampling time points and reflected the changes occuring during the late phase of chronic infection (subsequent 17 years of infection) (Fig.3.12.). A number of forward (mutations away from HCV 1b consensus sequence) or reverse (mutations toward the 1b consensus sequence) mutations was calculated. In sequences evolving during the first 11 years p.i. (time period spanning the acute phase of infection and early chronic phase) 13 of 21 a.a. substitutions in the E1E2 sequences from patient H were considered as forward (65 %) and 7 substitutions (35 %) as reverse mutations to the HCV 1b consensus sequence. In the late phase of chronic infection (covering the period between 11 and 28 years p.i.) similar levels of forward (52 %) and reverse mutations (48 %) was noted. For patient T forward mutations constituted 50 of a.a. substitutions occurred during the first 11 years p.i. During the late phase of chronic infection 5 of 6 arosed mutations (83 %) evolved away from the consensus sequence and only 1 mutation (17 %) reverted back to consensus sequence.

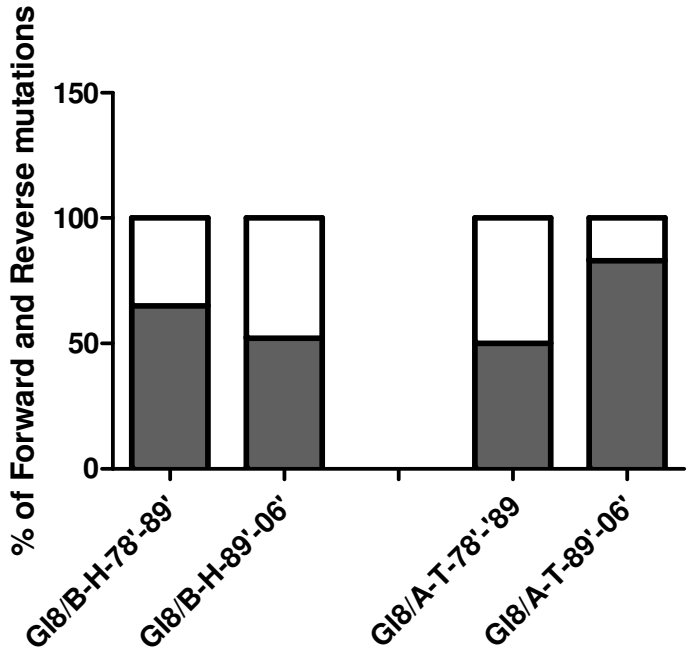


Fig.3.12. Forward and reverse mutations accumulated in E1/E2 sequences of HCV AD78 isolates from patients T and H during two periods of infection – 1978-1989 (first 11 years of infection) and 1989-2006 (subsequent 17 years of infection)

Total number mutations equal to 100 %.

3.2 Analysis of HCV-specific humoral immune responses in AD78-patients

3.2.1 Preparation of the plasmids expressing the envelope proteins of the HCV AD78 isolates

The E1/E2 clonal sequences amplified from the contaminated globulin batch GI8 and from patient's T and H serum samples collected at different time points, as has been indicated above, were cloned into the intermediate pCR4-TOPO plasmids. Clonal sequences for each AD78 isolate were used to create a set of "consensus" sequences for each group of these clones. That allowed the identification of clones with minimal number of a.a. substitutions in comparison to these "consensus" sequences. All these clones were used in the next stage of the project for recloning of the E1/E2 sequences using the EcoRV sites into the expressing plasmid phCMV-IRES according to the protocol described in the "Material and Methods" section.

3.2.2 Generation of artificial HVRI variants and their insertion into the expression plasmid phCMV-IRES

Previous studies have shown that HCV nAbs are directed not only against epitopes within the HVRI but also against epitopes located outside of this region. One of the aims of our study was the analysis of the evolution of the HCV envelope-specific humoral immune response. This task could be facilitated by availability of a tool, which would allow differentiating between antibodies reacting with the epitopes located inside and outside of the hypervariable region I. To generate such a tool, a set of four artificial variants of HVRI was designed (Fig.3.13.).

During the design procedure an attempt was made to preserve the conservative a.a. residues present in all known HVRI sequences, as well as to keep at least some of the basic a.a. residues (R, H, and K) presumably important for HCV infectivity. We assumed that this approach might have led to a generation of a HVRI variant that could preserve the secondary structure of the E2 protein and would not be immunologically active. The peptides corresponding to the designed artificial HVRI variants (AD-M6 to AD-M9) were synthesized and their reactivity in ELISA with sera from anti-D patients were compared with that of the peptides corresponding to the natural HVRI sequences of the AD78 variants Gl8/A, Gl8/B, and HVRI from the AD78 consensus sequence (Fig.3.13.). As expected, the peptides with naturally occurring sequences reacted with the majority (69-80 %) of anti-D sera. In contrast, the peptides with artificial sequences demonstrated much less reactivity. For subsequent experiments the variants M8 and M9, which reacted correspondingly with 4 % and none of the anti-D sera, were chosen. Corresponding oligonucleotides containing the SalI and PsiI restriction sites were synthesized and used for swapping the natural HVRI fragments within the envelope gene sequence of AD78 variants Gl8/A and Gl8/B were cloned into the intermediate pCR4-TOPO vector. The SalI restriction site (pos. 570 bp in the E1 sequence) upstream of the HVRI in the sequence of Gl8/A or PsiI restriction site (pos. 680 bp in the E2 sequence) downstream the HVRI in the sequence of Gl8/B were generated by site-directed mutagenesis (SDM). These sites were used for exchange of the natural HVRI fragments in Gl8/A and Gl8/B sequences with the artificial variants M8 and M9. Afterwards, the whole E1/E2 sequences containing the artificial HVRI, were recloned into the expressing plasmid phCMV-IRES using the EcoRV restriction sites. The scheme of the cloning procedure

is presented on Fig.3.14. and Fig.3.15.. The generated plasmids were used in the series of subsequent experiments for transfection of the 293T cells.

Naturally Occurring variants		Reactivity with AD-sera
	* * * *	
G8/A	PTRTIGGSQAQTASGLVSMFSVGPSQK	41/51 (80%)
G8/B	G.Q....A.SHSVM.VA.I..P..A..	38/51 (75%)
AD78cons	N.H....K...ATG.F.AWLAR....E	35/51 (69%)
Artificial variants		
AD-M6	G.G.L..IG.GG.G.GSGI.GG..G.N	10/51 (20%)
AD-M7	G.HAV..IAGRGLAAGSGA.G...A..	9/51 (18%)
AD-M8	A.HAV.QVGSRGLKRGDAA.DI.SV..	2/51 (4%)
AD-M9	G.HAL.VIGVRGGGAGSAA.GG..G..	0/51 (0%)

Fig.3.13. Reactivity of HVRI synthetic peptides in ELISA with sera from chronically AD78-infected patients

Amino acid residues conserved in all known variants of natural HVRI are indicated in red. Asterics marked the position of basic a.a. residues that are assumed to be important for infectivity.

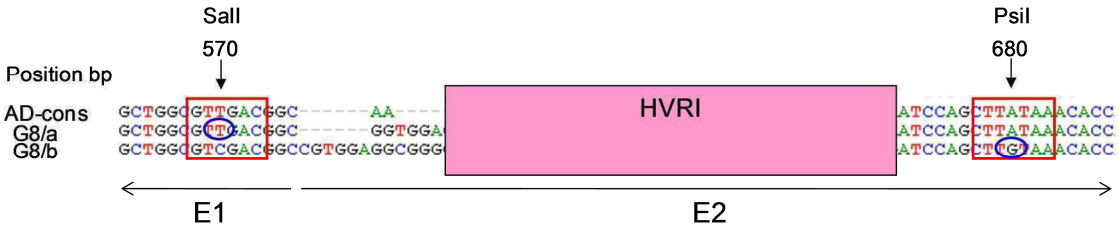


Fig.3.14. Position of the SalI and PsiI restriction sites used for insertion of the artificial HVRI into the E1/E2 sequence

Site-directed mutagenesis was used to generate the SalI restriction site in the GI8/A sequence or the PsiI restriction site in the GI8/B sequence.

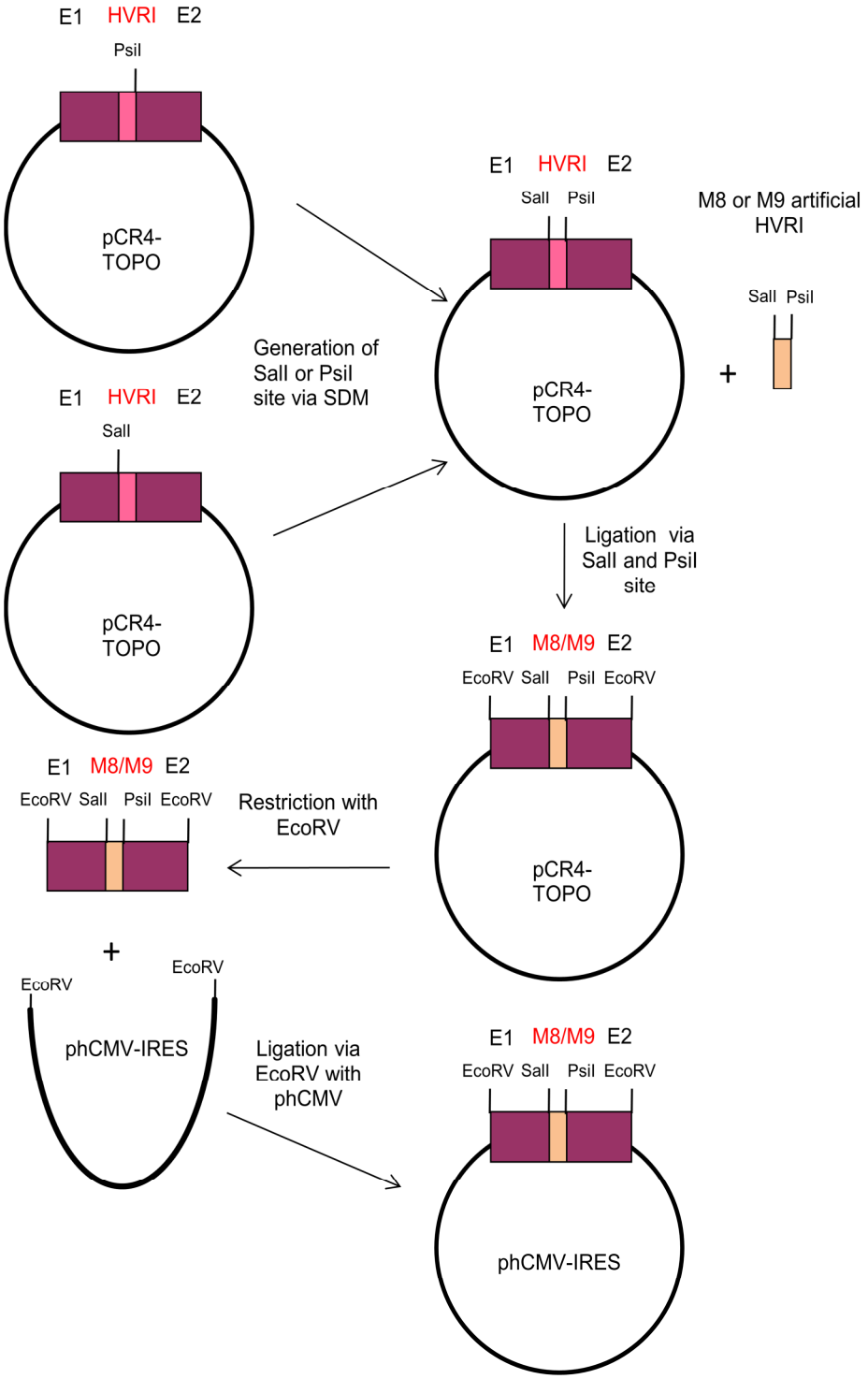


Fig.3.15. Cloning strategy for generation of plasmids expressing the GI8/A and GI8/B E1/E2 sequences containing the artificial variants of the HVRI (M8 and M9)

The first step included a generation of the SalI restriction site in the GI8A sequence or the PsiI restriction site in the GI8B sequence by site-directed mutagenesis (SDM). pCR4-TOPO plasmids containing the GI8/A or GI8/B sequences with generated restriction sites were used for swapping the natural HVRI fragments with the artificial ones. The resulting plasmids were restricted with EcoRV and the E1/E2 sequences were ligated into the EcoRV restricted expression vector phCMV-IRES.

3.2.3 Expression of a full length E1/E2 heterodimer in 293T cells

Studies of the reactivity of the envelope antigen with sera from different patients required expression of the full length E1/E2 heterodimers. Therefore, the 293T cells were transfected with the expression vector phCMV-IRES containing the E1/E2 sequences from AD78 isolates, including the wt, M8 or M9 variant sequences (see 3.2.1.). To see if the transfection led to the expression of the desired proteins, the cell lysates were subjected to SDS-PAGE under denaturating conditions. The proteins were transferred to the nitrocellulose membrane and immunostained with either E1- or E2-specific mouse monoclonal antibodies directed against linear epitopes of E1 (a.a. 1-17) or E2 (a.a. 144-180). As expected, Western blot analysis demonstrated the presence of polypetides with the molecular weights of approximately 30-34 kd and 60-75 kd, which corresponded to E1 and E2 protein monomers, respectively, in each of the tested sample of cell lysate (Fig.3.16.). Due to a low available amount of the first mouse mAb A4 directed against a linear epitope of the E1 protein, the expression of E1 protein could only be confirmed for the virus variants of the HCV AD78 Gl8/A isolate.

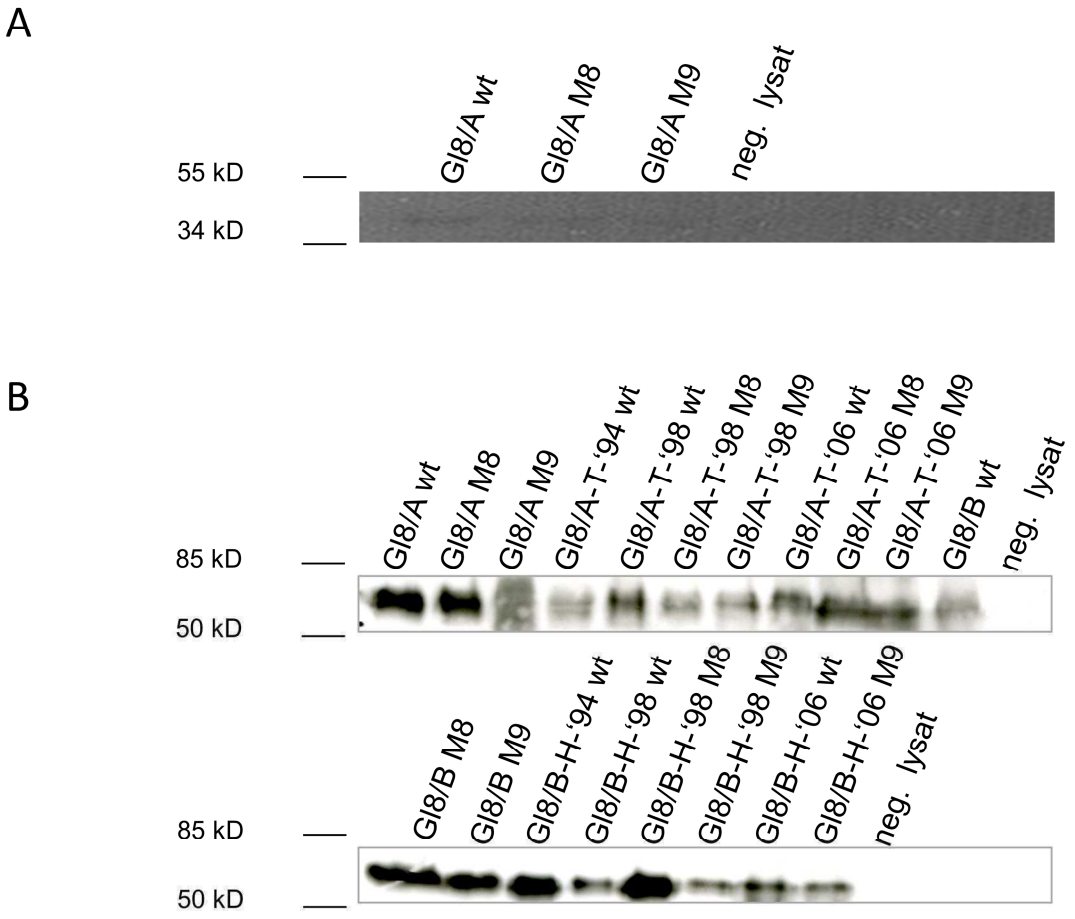


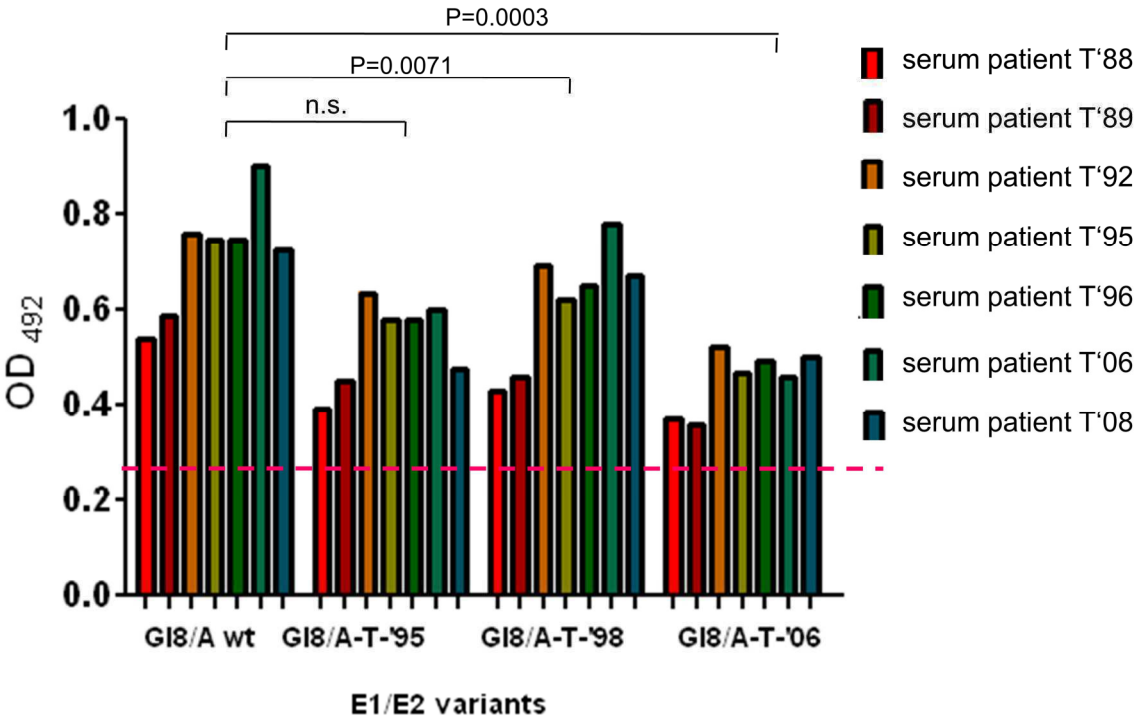
Fig.3.16. Immunodetection of HCV AD78 E1 and E2 proteins expressed in 293T cells

Cell lysates of the 293T cells transfected with the expression vector phCMV-IRES encoding the E1/E2 sequences of different HCV AD78 variants were subjected to SDS-PAGE. The proteins were transferred to the nitrocellulose membrane and immunostained with mouse monoclonal antibodies to E1 (mAb Ab4) (Fig.3.16. A) or to E2 (mAb CET3) (Fig.3.16. B).

3.2.4 Binding experiments of expressed E1/E2 heterodimer with human sera

The prepared and analysed 293T cell lysats containing the expressed E1/E2 heterodimers of HCV AD78 variants, including the original HCV AD78 variants Gl8/A and Gl8/B present in the batch 8 of the contaminated globulin, as well as evolving AD78 variants obtained from patients T and H infected with these virus variants, were used as a source of HCV antigens in binding experiments (immunoenzyme analysis) to assess the HCV-specific immunoreactivity reactivity of homologous and heterologeous sera from AD78-infected patients.

A



B

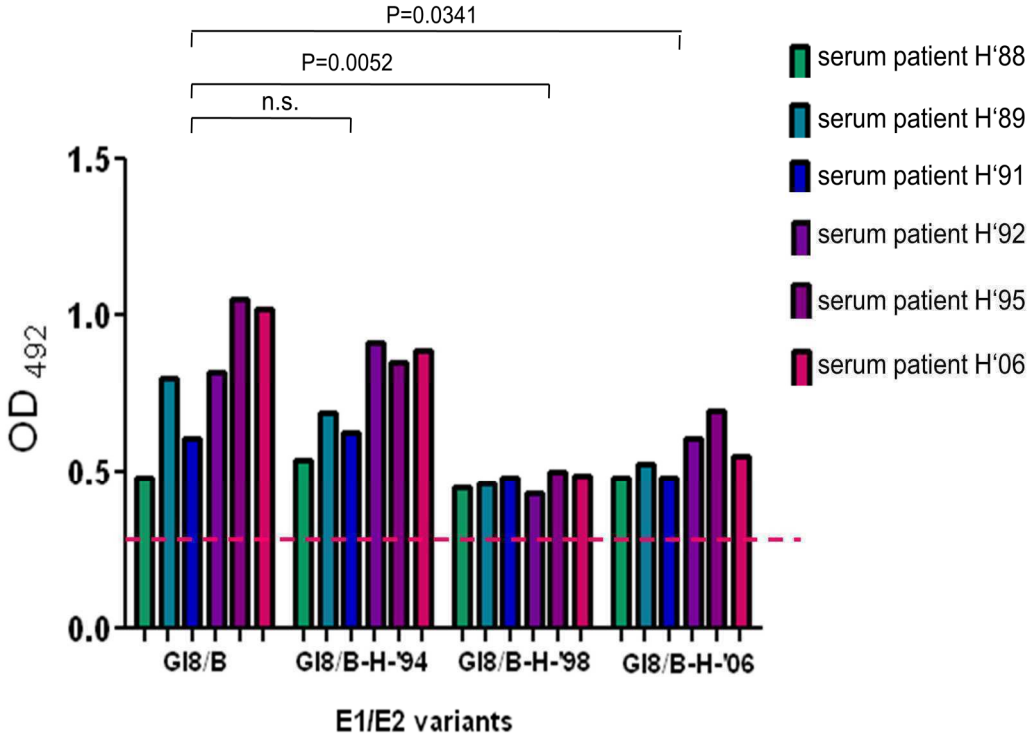


Fig.3.17. Immunoreactivity of the sequential serum samples from HCV AD78-infected patients T and H with four different variants of the homologous AD78 E1/E2 heterodimers

Reactivity of autologous sera with E1/E2 heterodimer was assessed by a solid-phase ELISA**.** For the normalization of antigen (E1/E2) concentration the reactivity with the the mouse mAb CET-3 directed at the conserved sequence present in all tested E1/E2 variants was used. Sera were tested at the dilution 1: 100. The broken line indicates the cut off value.

(A) Reactivity of sera obtained from patient T in 1988-2008 with four variants of the E1/E2 heterodimer corresponding to the original AD78 Gl8/A and variants of the Gl8/A virus present at different time point of infection in patient T. **(B)** Reactivity of sera obtained from patient H in 1988-2006 with four variants of the E1/E2 heterodimer corresponding to the original AD78 Gl8/B and variants of the Gl8/B virus present at different time point of infection in patient H.

Availability of the plasmids expressing different variants of the E1/E2 heterodimers allowed for an analysis of the influence of the evolutionary changes in the sequences of envelope proteins of AD78 isolates on their ability to bind homologous serum samples collected at different time points of infection. In the following experiments the reactivity of the serum samples from patients T, infected with virus variant Gl8/A, and H, infected with virus variant Gl8/B, collected between 1988 and 2008 with Env variants corresponding to isolates from the contaminated globulin and from patients in 1994/95, 1998, and 2006 were measured (Fig.3.17. A, B). The antigen concentration of each antigen was normalized using the mouse mAb CET-3, directed against the linear epitope of E2 (a.a. 144-180) conserved in all AD78 isolates, and allowed the evaluation of reactivity under the same conditions for each antigen. Despite relatively high levels of variability of reactivity of individual sera with different antigen a general tendency was noted, according to which the sera collected a particular time point reacted better with the E1/E2 antigens collected at earlier time points than with those collected at later time points. These data suggested that the accumulation of mutations in the E1/E2 sequences during the evolution of the virus in chronically infected patients might have influence (reduce) their ability to react with the anti-viral antibodies present in infected host at each time point of infection.

In the next step of the binding studies a question of ability of antisera from patients infected with one HCV strain to cross-react with E1/E2 proteins encoded by other HCV strains was addressed. The reactivity of sera from 25 HCV AD78 infected patients or 25 sera from patients infected with genotype 1b with HCV AD78 E1/E2 heterodimers from virus variants Gl8/A or Gl8/B was tested by ELISA (Fig.3.18.). The result has shown that there was no significant difference in the frequency and level of reactivity of sera from patients infected with different subtype 1b HCV strains with two antigens used. On the other hand, additional ELISA experiments performed with the same virus antigens (E1/E2 heterodimers from virus variants Gl8/A or Gl8/B) demonstrated significant difference ($p<0.05$) in the cross-reactivity of sera from patients infected with genotype 1b HCV or from patients infected with other genotypes (2 and 3) (Fig.3.19.).

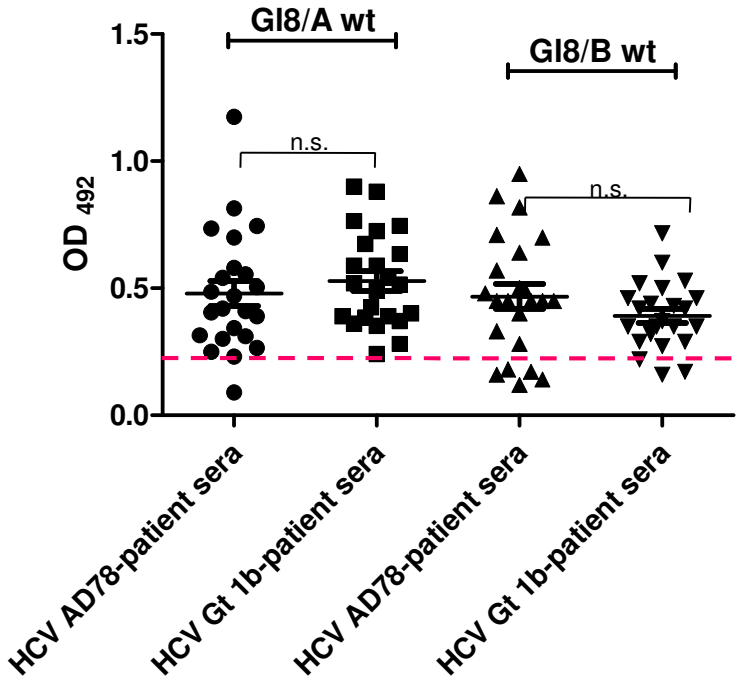


Fig.3.18. Cross-reactivity of sera from HCV AD78 infected patients or from patients infected with HCV subtype 1b strains with two virus antigens (HCV AD78 E1/E2 heterodimers from virus variants Gl8/A or Gl8/B)

Reactivity of patient's sera with E1/E2 heterodimers was assessed by a solid-phase ELISA**.** For the normalization of antigen (E1/E2) concentration the reactivity with the the mouse mAb CET-3 directed at the conserved sequence present in all tested E1/E2 variants was used. Sera were tested at the dilution 1: 100. The broken line indicates the cut off value.

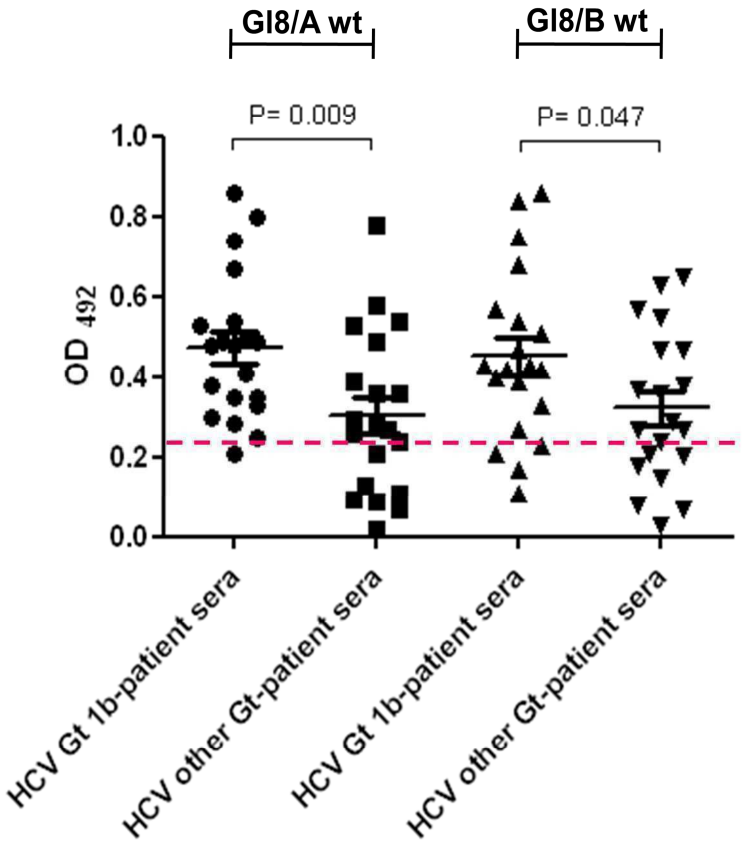


Fig.3.19. Cross-reactivity of sera from patients infected with HCV subtype 1b or other HCV genotype strains with HCV AD78 E1/E2 heterodimers from virus variants Gl8/A or Gl8/B

Reactivity of patient's sera with E1/E2 heterodimers was assessed by a solid-phase ELISA**.** For the normalization of antigen (E1/E2) concentration the reactivity with the the mouse mAb CET-3 directed at the conserved sequence present in all tested E1/E2 variants was used. Sera were tested at the dilution 1: 100. The broken line indicate the cut off value.

Among the variants of the Env sequences inserted into the expressing plasmid were sequences, in which the natural HVRI sequence was substituted with the artificial HVRI variants (M8 or M9). These artificial HVRI sequences have been shown not to react with the human antibodies to natural HVRI but theoretically could preserve the correct conformation of the E1/E2 heterodimers, as already described. Application of such modified version of the E1/E2 heterodimers would allow assessing the anti-Env antibodies directed against the epitopes located outside of the HVRI. Therefore, in the next experiment we studied the reactivity of sera from HCV AD78 patients with Gl8/A variants wt and artificial M8 and M9 variants of HVRI (Fig.3.20.) using the ELISA. The results of these experiments have not demonstrated a significant difference in the reactivity of sera from HCV AD78 patients with Gl8/A variants (wt, M8 and M9). These data suggest that most of the anti-HCV antibodies present in HCV patients' sera are directed against epitopes located outside of the HVRI area.

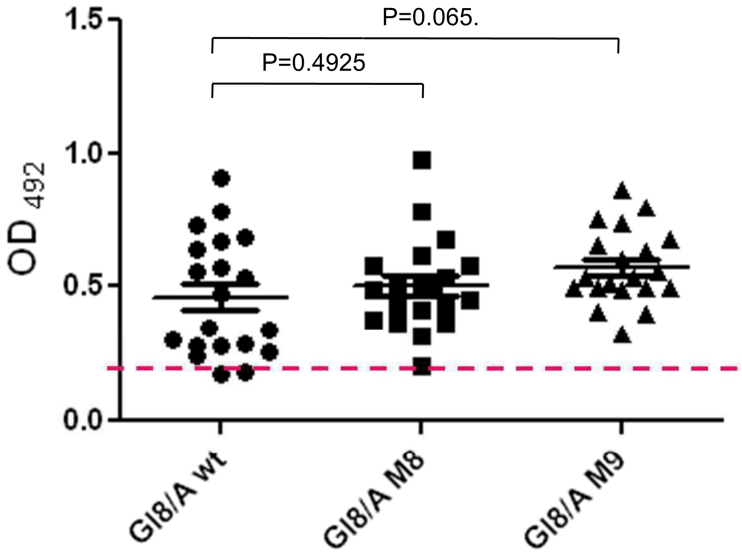


Fig.3.20. Reactivity of sera from HCV AD78 infected patients with HCV AD78 E1/E2 heterodimers from virus variants GI8/A (wt, M8, M9)

Reactivity of 20 patient's sera with E1/E2 heterodimers was assessed by a solid-phase ELISA**.** For the normalization of antigen (E1/E2) concentration the reactivity with the the mouse mAb CET-3 directed at the conserved sequence present in all tested E1/E2 variants was used. Sera were tested at the dilution 1: 100. The broken line indicates the cut off value.

3.2.5 Neutralization of HCV AD78 pseudoparticles by patients sera

Development of the HCV pseudoparticles system (HCVpp) (Bartosch *et al.*, 2003b) made it possible to analyze the HCV-neutralization potentials of human sera. In the current study this system was used to analyze reactivity of sera from AD78-infected patients with the set of HCV AD78-based pseudoparticles. One of the prerequisites for the accurate assessment of the neutralization titre of antibodies is the standardization of the quantity of the antigen – in this case, of HCvpp. In all the subsequent experiments the quantities of different variants of HCVpp was standardized and equalized according to the results of the quantitative determination of the p24 (gag) protein, a constituent of pseudoparticles.

In the first series of experiments ability of sera from different groups of AD patients and individuals infected with other HCV 1b strains to neutralize the HCVpp expressing the E1/E2 proteins encoded by AD78 GI8/A and GI8/B virus variants was tested (Fig. 3.21. A, B). HCVpp bearing envelope proteins of virus variants from AD78 GI8/A wt and GI8/B were neutralized more effectively by sera from patients infected with the same virus variant than by sera of patients infected with another AD78 GI8 virus variant. Even less effective in neutralization of the AD78-based

HCVpp were sera from randomely chosen patients infected with other HCV 1b strains.

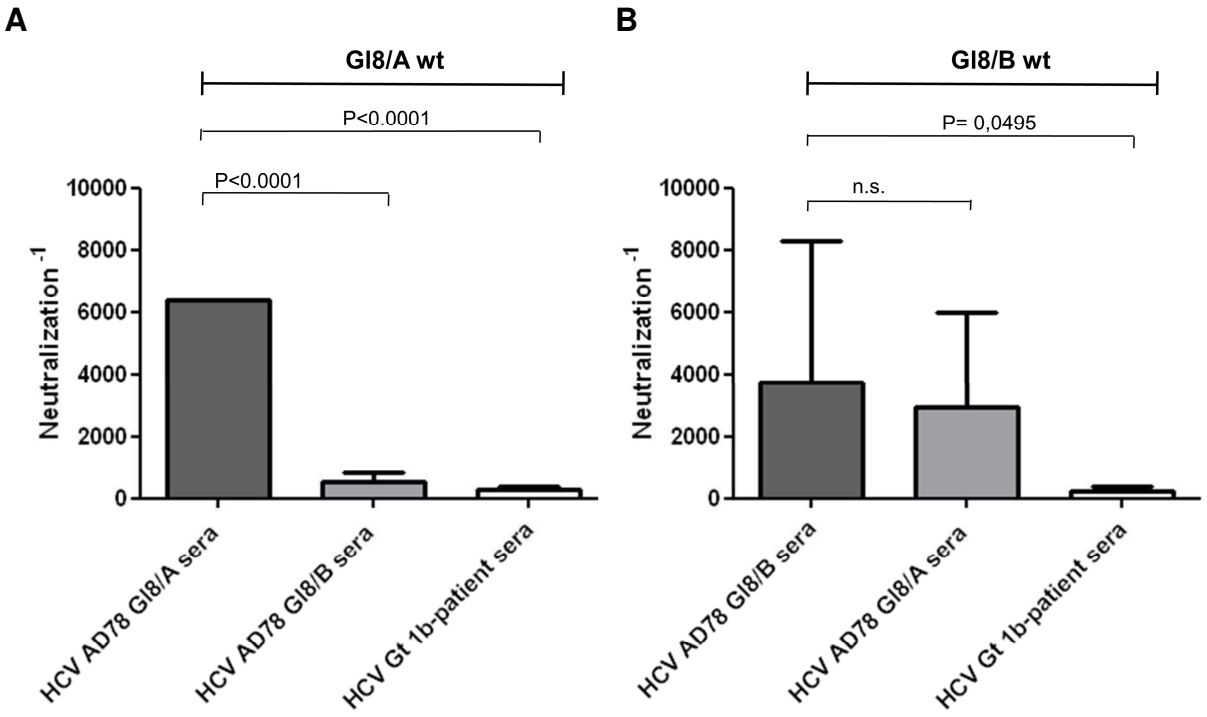


Fig.3.21. Neutralization of HCVpp bearing envelope proteins of virus variants AD78 Gl8/A and Gl8/B by sera of patients infected with Gl8/A, Gl8/B sera or sera from randomely chosen HCV 1b infected patients

HCVpp were normalized by P24 quantification. HCVpp were preincubated for one hour with sera dilutions before the infection of the Huh7.5.1. cells. The means of neutralization titers (IC50) and standard derivations are indicated.

The next series of experiments were directed at the analysis of the ability of sera obtained at different time points of infection from patients T and H to neutralize the series of HCVpp, in which the E1/E2 sequences came from the AD78 Gl8/A or Gl8/B virus variants and from serum samples collected from these two patients after 16 (1994), 20 (1989), and 28 (2006) years p.i. The concentrations of different HCVpp preparations were equalized according to the results of p24 quantification and the infectivity of these HCVpp was tested (Fig.3.22. A, B). As can be seen, the evident reduction of HCVpp infectivity corresponding to the duration of infection was observed for pseudoparticles bearing the E1/E2 sequences from both patients. Thus, the highest infectivity was recorded for HCVpp bearing the envelope proteins sequences derived from the viruses present in contaminated globulin (variants Gl8/A and Gl8/B). In contrast, the pseudoparticles, which included the E1/E2 sequences

from patients collected at different time points p.i., were much less infectious for Huh7.5.1 cells.

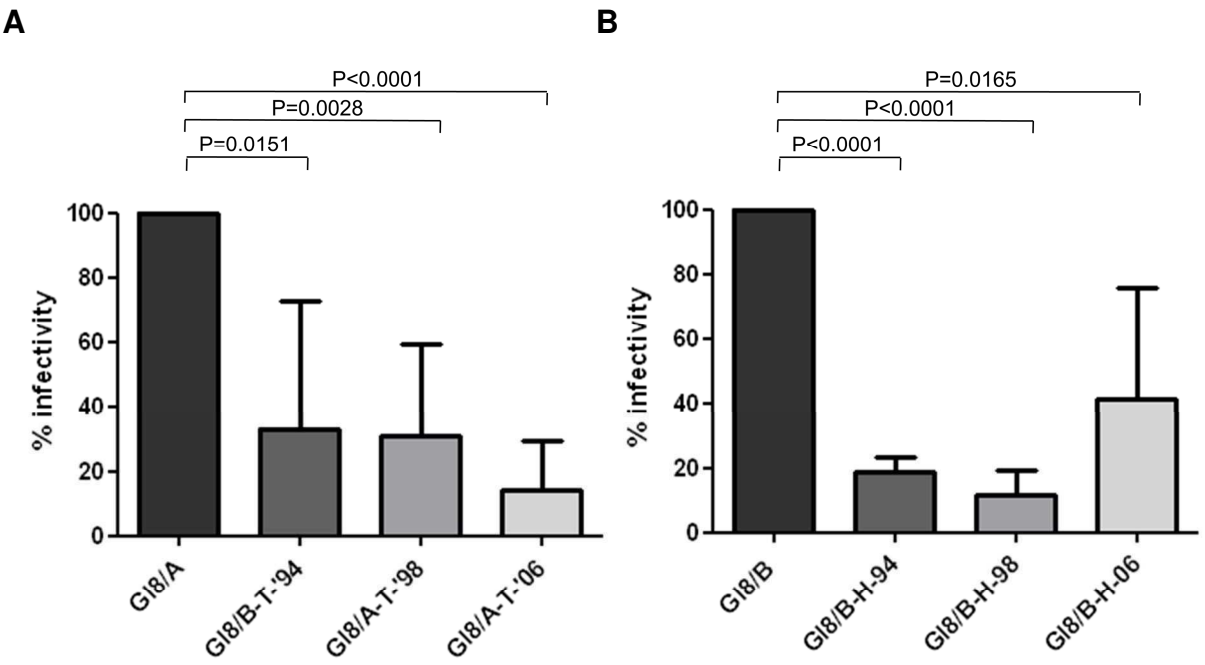


Fig.3.22. infectivity of HCVpp bearing envelope proteins from the AD78 GI8/A or GI8/B virus variants and from serum samples collected at different time points p.i. from patients T and H

The HCVpps were generated on the basis of the E1/E2 sequence of the HCV AD78 virus variant GI8/A, GI8/B and of serum samples from patient T (A), infected with virus variant GI8/A, and from patient H (B), infected with virus variant GI8/B. For the evaluation of the infectivity rate, the amount of HCVpp was normalized on the basis of P24 quantification. The infectivity of HCVpp bearing the envelope proteins of the virus variants GI8/A (A) or GI8/B (B) was considered as 100 %. Data of three experiments are presented.

The set of equalized HCVpp bearing the AD78 E1/E2 sequences derived from the globulin and patients T and H were used in the neutralization assay. The results of these experiments are presented in tables 3.4. and 3.5.. All sera from patient T, infected with the HCV AD78 GI8/A virus variant, irrespective of the sampling date, efficiently neutralized the HCVpp bearing the E1/E2 sequence from the original GI8/A virus. The same sera, however, reacted less effectively with HCVpp bearing the E1/E2 sequences amplified from patient T in 1994 and 1998, or 16 and 20 years p.i, correspondingly indicating that mutations accumulated during this time period reduced the ability of HCVpp to bind the neutralizing antibodies. Unexpectedly, all tested sera turned out to be very efficient in neutralizing the pseudoparticles that included the E1/E2 sequence amplified from the patient sera collected in 2006 or 28 years p.i..

A different pattern of neutralization activities was observed in experiments performed with the materials and sera obtained from patient H. In this case, all sera, irrespective of the time of collection, demonstrated approximately similar levels of neutralization activity against HCVpp bearing the E1/E2 sequences derived either from the virus GI8/B present in the globulin or from the patient H at different sampling time points. In general, the whole series of neutralization experiments demonstrated no evidence of the immune escape.

Tab.3.4. Neutralization of HCVpp from GI8/A and patient T from different time points by serial autologous sera (geometric mean titres are shown)

serum / HCVpp	serum T'89	serum T'91	serum T'95	serum T'98	serum T'08
HCVpp GI8/A-'78	5381	6400	4031	3200	2539
HCVpp GI8/A-T-'95	672	565	672	800	400
HCVpp GI8/A-T-'98	317	400	317	141	400
HCVpp GI8/A-T-'06	6400	6400	3200	6400	3200

Tab.3.5. Neutralization of HCVpp from GI8/B and patient H from different time points by serial autologous sera (geometric mean titres are shown)

serum / HCVpp	serum H'88	serum H'91	serum H'95	serum H'08
HCVpp GI8/B-'78	5381	6400	4031	3200
HCVpp GI8/B-H-'94	672	565	672	800
HCVpp GI8/B-H-'98	317	400	317	141
HCVpp GI8/B-H-'06	6400	6400	3200	6400

As already has been described in section 3.2., the set of plasmids expressing AD78-derived E1/E2 sequences included those, in which the natural HVRI sequences were substituted with the artificial HVRI variants (M8 or M9) that did not react with the antibodies recognizing naturally occuring HVRI sequences. Cells

transfected with such modified plasmids expressed infectious HCVpp. In a series of experiments we tried to assess ability of sera from AD78-infected patients as well as of sera from randomly chosen HCV 1b patients to neutralize the HCVpp generated on the basis of Gl8/A and Gl8/B envelope sequences, in which the wild type (wt) of HVRI was substituted with the M9 variant sequence (Fig.3.23. A, B). The results have shown that both variants of HCVpp bearing the M9 sequence (Gl8/A M9 and Gl8/B M9) were neutralized much more efficiently by sera from HCV infected individuals than HCVpp expressing envelope proteins with naturally occuring HVRI variants (Gl8/A wt and Gl8/B wt). This effect was more evident in case of HCVpp constructed on the basis of the Gl8/A variant of HCV AD78.

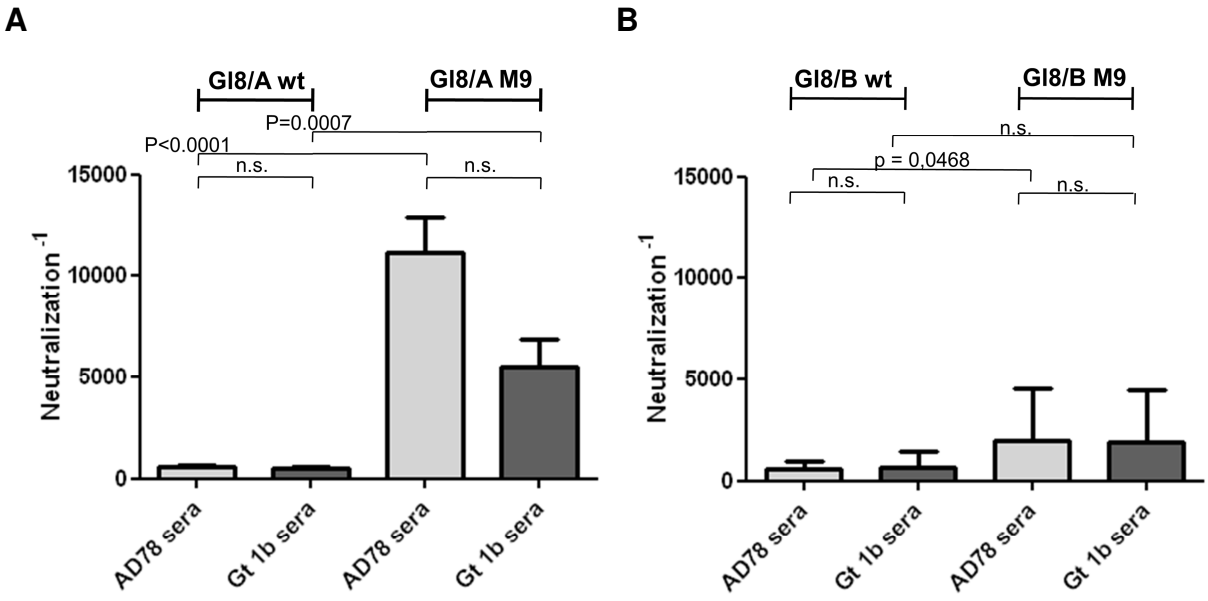


Fig.3.23. Neutralization of HCVpp bearing modified (M9) or unmodified (wt) envelope proteins of AD78 Gl8/A and Gl8/B virus variants by sera of infected patients

3.3 Peculiarities of the AD78 E1/E2 sequences

3.3.1 Presence of additional amino acid stretch at the E1/E2 junction

Analysis of the E1/E2 sequences from the contaminated globulin and HCV AD78 infected patients (Appendix Fig.8.2. and Fig.8.3.) have revealed a presence of additional 1 to 4 codons at the junction between E1 and E2 genes in most of the anti-D sequences. Fig.3.24. and Fig.3.25. demonstrate presence of additional a.a. residues encoded by these codons in the E1/E2 sequence of the HCV AD78 variants Gl8/A (four residues RGGG) and Gl8/B (two residues GG) obtained from the

contaminated globulin batch Gl8. One additional a.a. residue was found also in one of the randomly chosen sequences of the HCV 1b isolates obtained from the HCV database (accession number AB016785).

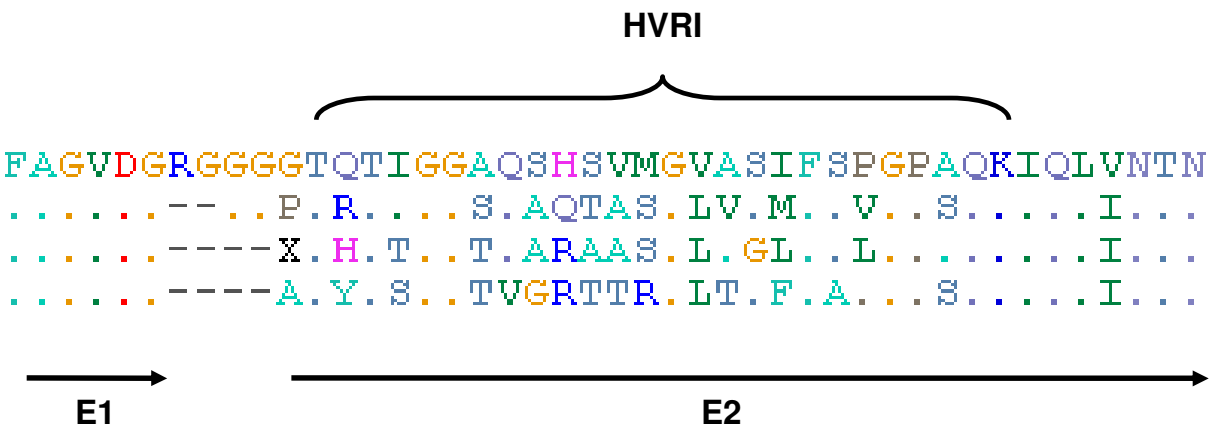


Fig.3.24. Additional amino acid residues at the E1/E2 junction in the sequences of the HCV AD78 variants from the contaminated anti-D globulin

Presence of the additional a.a. residues at the E1/E2 junction raise the question to what protein these residues remain fused after the proteolytic processing of the viral polyprotein. Comparative analysis of the E1 C-terminal sequences of HCV strains from the Los Alamos HCV database, including the signal peptide segment, demonstrated absence of any special amino acid substitutions in this area in strains bearing the additional amino acid residues (Fig.3.26.). Such a conservation of the E1 sequences provides a proper processing signal and strongly suggests the invariable processing pattern. Thus, despite the absence of direct experimental proof, one might reasonably assume that the additional amino acid residues observed at the E1/E2 junction just after the cleavage site upon a processing became a part of the N-terminal sequence of the E2 protein.

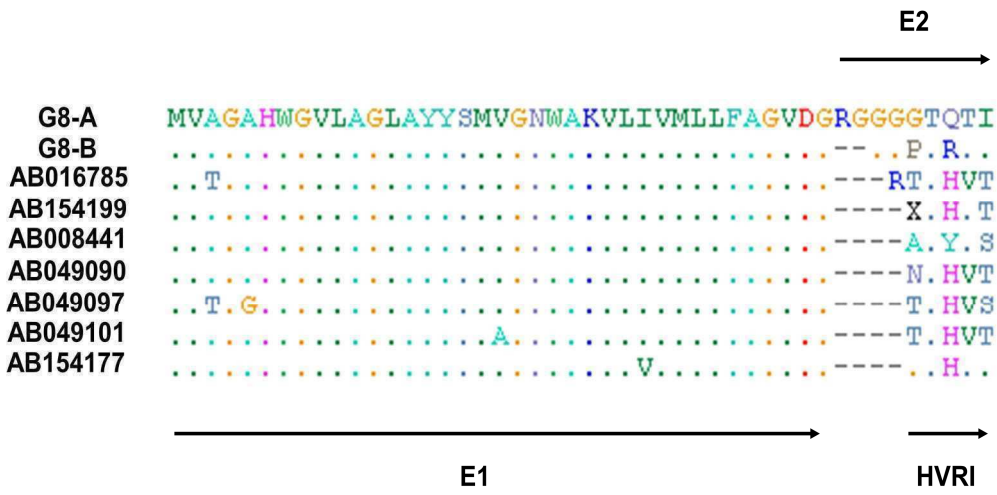


Fig.3.25. Aligment of the HCV AD78 and HCV 1b sequences at the E1/E2 junction

One additional a.a. residue was also found in one of the randomly chosen sequences of the HCV 1b isolates obtained from the EMBL database (accession number AB016785).

3.3.2 Analysis of the distribution of the additional codons at the E1/E2 junction in HCV isolates from the GenBank

Presence of additional codons at the E1/E2 junction in AD78-derived sequences raised a question how often such insertions occurred in HCV strains belonging to different genotypes and subtypes. Table 3.6. demonstrates the results of the analysis of E1/E2 sequences of virus strains included into the Los Alamos HCV database.

None of the 728 strains of HCV 1a or HCV 3a contains additional codons at the E1/E2 junction. In contrast, additional codons were identified in 6 %, 8 %, 0.6 %, 4.5 %, and 1.2 % of type 1b, 2, 4, 5, and 6 sequences, correspondingly. Of note is the fact of variable number of additional codons in sequences of different HCV types. Thus, for example, among HCV1b strains we observed from 1 to 4 additional codons, while among genotype 2 strains this number varied from 1 to 5 codons.

Tab.3.6. Frequency of additional codons at the 5'end of HVR I sequences of HCV isolates from the HCV Los Alamos database

	Gt 1a (n=728)	Gt 1b (n=1788)	Gt 2 (n=809)	Gt 3 (n=230)	Gt 4 (n=157)	Gt 5 (n=88)	Gt 6 (n=81)
1 codon	0	50	14	0	1	2	0
2 codons	0	9	6	0	0	1	0
3 codons	0	12	16	0	0	1	1
4 codons	0	35	0	0	0	0	0
5 codons	0	0	31	0	0	0	0
Any additional codon	0	106 (6%)	67 (8 %)	0	1 (0.6 %)	4 (4.5 %)	1 (1.2 %)

Identification of additional codons at the E1/E2 junction raised some questions. Among them, a question of existence of a mechanism of selection of amino acids encoded by these codons rose. To answer this question, the analysis of a frequency of different classes of a.a. residues at each position of additional a.a. stretches was performed. Table 3.7. demonstrates hydropatic character of residues present in additional a.a. set located at the N-terminus of HVRI of 173 type 1b and 2 strains. As a control, the a.a. composition of proteins from the SWISS-PROT database was used. In contrast to the control proteins, the additional a.a. set under investigation was characterized by a much higher frequency of neutral (43 % vs. 19 %) and lower frequencies of hydrophobic (34 % vs. 47 %) and hydrophilic (23 % vs. 34 %) residues.

Tab.3.7. Amino acid composition of the set encoded by additional codons present at the 5' end of HVRI sequences of 173 type 1b and 2 strains

Hydropatic character of the residues	Source of the amino acid sequences	
	Additional amino acid set at the N-terminus of HVR I	Proteins from SWISS-PROT database
Neutral	43%	19%
Hydrophobic	34%	47%
Hydrophilic	23%	34%

The next approach assumed an analysis of physico-chemical characteristics of residues at different positions of additional a.a. stretches located at the N-terminus of HVRI of some HCV strains. The tables 3.8, 3.9. and 3.10. demonstrate the frequency of different residues at different positions in 106 type 1b, 67 type 2, and 105 AD78-derived sequences.

Tab.3.8. Frequency of amino acid residues encoded by additional codons present at the 5' end of 106 subtype 1b HCV isolates from the HCV Los Alamos database

No. of added codons	Amino acid position with regard to the N-terminus of HVR I			
	-4	-3	-2	-1
One (n=50)				G – 17; R – 10; T – 7; S – 6; A – 5; Q – 3 E – 2
Two (N=9)			S – 3; G – 2; H – 1; N – 1; Q – 1; R – 1	T – 4; A – 2; R – 2; G – 1
Three (n=12)		G – 5; S – 4; I – 1; R – 1; V – 1	G – 5; L – 4; A – 1; E – 1 T - 1	M – 4; P – 3; G – 2; L – 2; Q - 1
Four (n=35)	S – 19; A – 12; H – 2; T – 1; Y – 1	Q – 16; H – 15; S – 2; A – 1; G – 1	G – 11; F – 8; L – 8; P – 3; S – 3; R – 1; Y – 1	L – 26; R – 3: S – 3; M – 2; P – 1

Tab.3.9. Frequency of amino acid residues encoded by additional codons present at the 5' end of 67 subtype 2 HCV isolates from the HCV Los Alamos database

No. of added Codons	Amino acid position with regard to the N-terminus of HVR I				
	-5	-4	-3	-2	-1
One (n=14)					V – 13; I – 1
Two (N=6)				T – 3; A – 2; R – 1	R – 1; H – 1
Three (n=16)			S – 8; R – 5; K – 2; V – 1	G – 7; H – 5; K – 1; L – 1; R – 1; S – 1	A – 6; V – 4; H – 3; T – 3
Five (n=31)	T – 23; A - 8	S - 31	S – 23; A – 8	H – 15; R – 8; S – 5; P – 3	V – 23; M – 6; T – 2

Tab.3.10. Frequency of amino acid residues encoded by additional codons present at the 5' end of 105 HVRI clonal sequences from HCV AD78 contaminated globulin and anti-D patients

No. of added codons	Amino acid position with regard to the N-terminus of HVR I			
	-4	-3	-2	-1
One (n=0)				
Two (N=47)			G – 35; A – 10; S – 2	G – 35; A – 12
Three (n=30)		G – 25; S – 4; R – 1	P – 22; A – 6; S – 1; V – 1	P – 25; S – 4; L – 1
Four (n=28)	R – 17; S – 8; T – 2; A – 1	G – 17; A – 10; R – 1	G – 17; S – 5; L – 4; F – 1; T – 1	G – 18; P – 6; S – 2; T – 2

Results of this analysis have demonstrated a restricted amino acid usage at each of the positions with predominance of neutral and hydrophobic residues. Three residues - Cys, Trp, and Asp were absent among the additional amino acids. Another important characteristic is the predominance of small and flexible residues in these stretches, especially at the very N-terminus, as well as the low frequency of Pro and large hydrophobic residues. Of interest is a very high frequency of Arg at position -4 in anti-D sequences. In contrast, this residue was never present at this position in other HCV 1b sequences. Thus, these results provided an evidence for a selection for particular residues in particular positions in the additional a.a. stretches located at the N-terminus of HVRI sequence.

Another interesting and important question concerns the stability of this additional track of amino acids during the course of HCV infection. Analysis of the E1/E2 sequences obtained at different time points of infection from two chronic anti-D patients (Tab.3.11.) have shown a possibility of two scenarios. In patient T at a particular time during the approximately 10 years interval between infection (1978) and the first time point of serum samples collection (1989) a change of the additional a.a. stretch from RGGG to HGG occurred. The composition of this HGG track remained stable during the rest of the observation period (1989-2006). In patient H, however, quite different chain of events has been observed with regard to evolution of the additional a.a. stretch. Significant changes in the length and composition of this stretch was observed at each time point of observation. Even more so, in contrast to situation with patient T, in patient H a variability of the composition of this track at particular time points was documented. Thus, the additional a.a. stretches at the N-terminus of HVRI are subjected to change and do not always remain stable in the course of infection.

Tab.3.11. Additional a.a. residues at the N-terminus of HVRI at different time points of infection

HCV AD78 Gl8 variants in the inoculum	RGGG	GG
	Patient T	**Patient H**
1989	- HGG	-S
1994	- HGG	TSW
1998	- HGG	MSV MSG MSW MSC ASA TFC
2006	- HGG	-ASA - - AS

3.3.3 Influence of additional a.a. sequences at the N-terminus of E2 protein on HCVpp infectivity

The revealed restriction on physico-chemical characteristics of a.a. residues at each site of additional a.a. stretches located at the E1/E2 junction suggest that these stretches most probably have a particular conformation and/or might influence the conformation of HVRI or the E1/ E2 heterodimer. This might lead in turn to the changes of infectivity of HCV particles. To address this issue, a set of new E1/E2 expressing plasmids was created to analyse the influence of additional a.a. stretches on infectivity of HCVpp (Tab.3.12.). The phCMV-IRES plasmid expressing the envelope gene sequence from HCV AD78 Gl8/A variant was used as a backbone. The four additional codons at the 5' end of HVRI present in this E1/E2 sequence were modified by replacing them with a natural or artificial a.a. stretches (Fig.3.26.).

The procedure included the following steps. A number of sequence-specific primers were used for the amplification of small DNA fragments of the E1/E2 junction. The sense primers encoded the 3'end of the Gl8/A E1 gene that included the SalI restriction site, the sequence that encoded the variant of additional a.a. residues, and the sequence corresponding to the first 20 nucleotides of the Gl8/A HVRI. The anti-sense primer covered the last 20 nucleotides of the Gl8/A HVRI and

15 subsequent nt containing the PsiI restriction site. This protocol allowed for a substitution of the additional codons encoding RGGG, which are present in the Gl8/A E1/E2 junction, with a desired variant of additional codons. The generated DNA fragments were restricted with SalI and PsiI and ligated into the expression plasmid phCMV-IRES-Gl8/A. The prepared constructs were verified by sequencing.

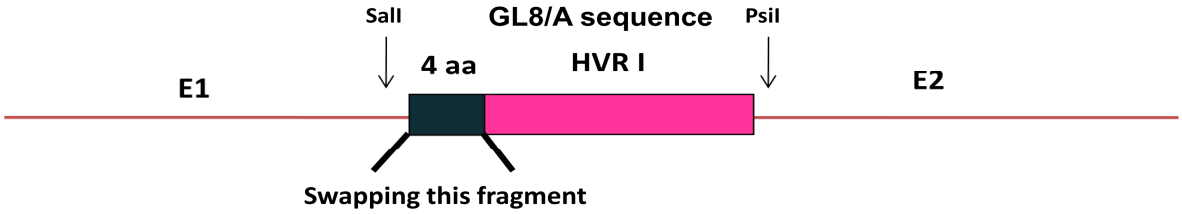


Fig.3.26. Scheme of modification of additional codons located at the E1/E2 junction in the phCMV IRES vector plasmid expressing envelope gene sequence of the HCV AD78 Gl8/A variant

Four additional codons in the original Gl8/A sequence (black rectangle) have been replaced by codons encoding other variants of additional a.a. track (the whole set is indicated in the Table 3.12.) SalI site located before and PsiI site located after the HVRI were used for insertion according to the procedure illustrated in Fig.3.15. The corresponding fragments were obtained by PCR with a specially designed set of primers covering sequence of Gl8/A HVR I and sequences encoding the additional a.a. stretchches.

The prepared set of plasmids, which encode the E1/E2 sequence of the HCV AD78 Gl8/A variant without (wt) or with modification of the additional codons at the E1/E2 junction (Tab.3.12.) was used for transfection of the 293T cells and preparation of the HCVpp. The resulting set of pseudoparticles was used in the infectivity assay in the Huh7.5.1 cell cultures according to the standard protocol. For the exact comparison of HCVpp infectivity, the amount of HCVpp was normalized on the basis of P24 quantification (Gag).

Tab.3.12. Set of expressing plasmids for the analysis of the influence of additional a.a. streches on HCVpp infectivity

Source of sequence	Sequence of additional amino acids
Gl8/A wt -	RGGG
Gl8/A wt – deletion of additional a.a.	----
Pat T‘ 98	-HGG
Pat B‘ 98	-SAR
Consensus of additional a.a. from HCV 1b sequence set	SGQL
Consensus of additional a.a. from HCV 2 sequnce set	TSSHV
Artificial sequence	GGGGG
Residues that are never or exceptionally rare present in the additional a.a. stretches	DNDN

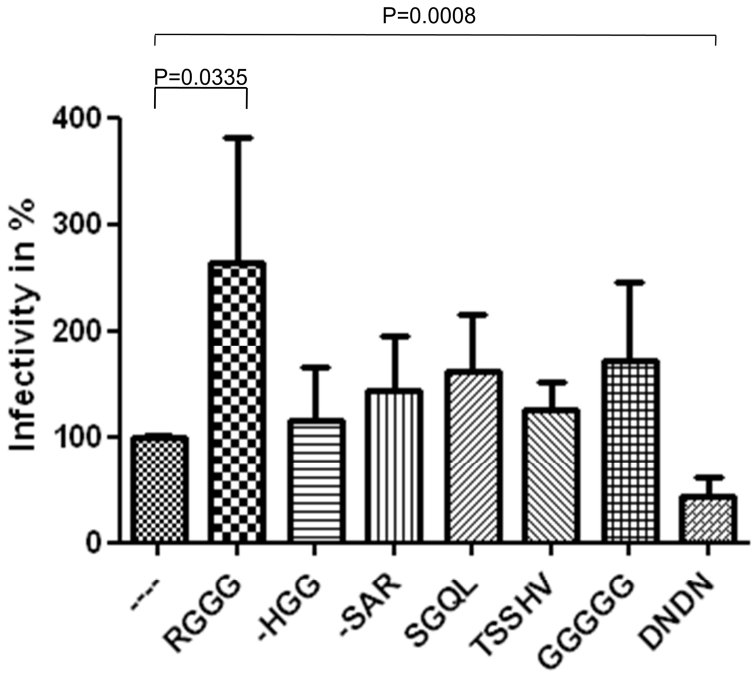


Fig.3.27. Influence of additional amino acid residues at the N-terminus of HVRI on infectivity of HCVpp in Huh 7.5.1. cells

The HCVpps were generated on the basis of the E1/E2 sequence of the HCV AD78 Gl8/A variant (Gl8/A-wt). (A) The set of modified HCVpp was created by deletion or substitution of codons encoding the four additional amino acids RGGG in the Gl8/A-wt/Env sequence either with naturally occuring or artificial codons. (B) For the evaluation of the infectivity rate, the amount of HCVpp was normalized on the basis of P24 quantification. The infectivity of HCVpp with deleted additional a.a. residues was considered as 100 %. Data of four experiments are presented.

As shown in Tab.3.6. 94 % of the HCV 1b sequences available in the Los Alamos database do not contain the insertion of additional a.a. at the N-terminus of the HVRI. Therefore, the infectivity of HCVpp with deleted additional a.a. residues was considered as 100 % (Fig.3.27.). Comparison of the infectivity of generated set of HCVpp bearing different variants of additional a.a. tracks have demonstrated that the presence of additional a.a. track in all cases but one (insertion of DNDN) resulted in increase of infectivity. The most significant increase (P=0.0335) of approximately 2,5 folds, however, was observed with the wild type GI8/A sequence, in which the additional track RGGG was present. Increase of infectivity observed for HCVpp containing the E12/E2 sequences with other variants of additional a.a. residue was not significant, although a statistical trend was noted. Of note is the fact that the presence of additional a.a. residues which are never (D) or exceptionally rarely (N) present in the context of additional a.a. stretches HCV E2 (variant DNDN) leads to a significant decrease in infectivity of HCVpp (P=0.0008).

3.3.4 Influence on HCVpp infectivity by interaction of additional a.a. at the N-terminus of E2 with HDL

Recently, an enhancement of HCVpp infectivity by human high-density lipoprotein (HDL) and involvement of HVRI into this process was described (Bartosch *et al.*, 2005). These results seem logical considering the fact that HDL is a natural ligand of SR-BI, one of the receptors involved in mechanisms of HCV entry. Our results described above (Fig.3.27.) have demonstrated that presence of additional a.a. track at the N-terminus of HVRI might influence the infectivity of HCVpp. One of the mechanisms of such increment might be associated with the changes of HVRI conformation, which, in turn, might influence the interaction of HCVpp with SR-BI through the HDL. To address this issue, the Huh7.5.1. cells were pre-incubated for 1 h at RT with normal DMEM (Fig.3.28. Step1 on top) as negative control or with DMEM containing HDL (10µg/ml) (Fig.3.28. Step1 down). Afterwards, the standard infectivity assay was performed using a set of HCVpp described in Tab.3.12. and Fig.3.28..

Two additional control plasmids, expressing the HCV H77.20 and GI8/A M9 E1/E2 sequences, were also used. As it can be seen from Fig.3.29. these experiments, however, provided the equivocal results. With several preparations of HCVpp containing the variants of additional a.a. tracks RGGG, HGG, TSSHV, and GGGGG,

as well as with the H77-20 HCVpp, an increase of infectivity by HDL was noted. In contrast, with preparations of HCVpp containing the variants of additional a.a. tracks SAR, SGQL and with the variant of the Gl8/A sequence containing the artificial version M9 of HVR1 evident decrease of infectivity was registered. The infection rate of HCVpp bearing the artificial a.a. variant DNDN was reduced to minimal values.

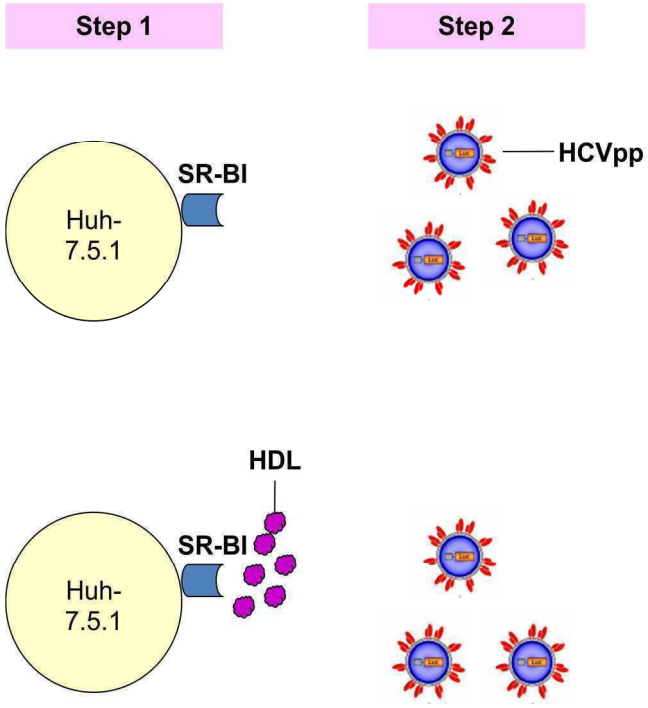


Fig.3.28. Schematic presentation of the experiments on analysis of the influence of HDL on infectivity of HCVpp with modified variants of additional a.a. stretches at the N-terminus of HVRI

Step 1 - Huh-7.5.1. cells were pre-incubated either with normal DMEM or with DMEM containing HDL (10µg/ml). Step 2 - 100 µl of generated HCVpp bearing the natural or artificial a.a. tracks in the Gl8/A wt E1/E2 backbone sequence was added and the infection rate was evaluated after 72 h of incubation at 37 ℃.

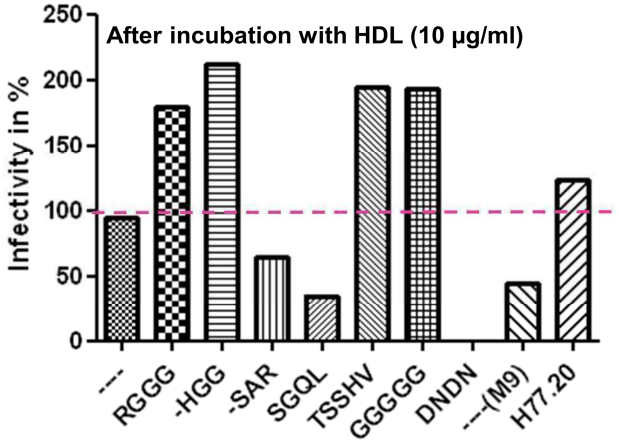


Fig.3.29. Influence of HDL on infectivity of HCVpp with modified variants of additional a.a. stretches at the N-terminus of HVRI

4 Discussion

The data accumulated during the last years suggest that HCV control and clearance during the early phase of infection are associated not only with broad and vigorous HCV specific T cell responses (Lechner *et al.*, 2000) but also with induction of virus-neutralizing antibodies (Pestka *et al.*, 2007). HCV infection in a large number of patients, however, progresses to chronicity and the virus might persist for years despite a presence of relatively high titers of antiviral antibodies, including those that rather efficiently neutralize other HCV isolates (Lavillette *et al.*, 2005; Pestka *et al.*, 2007). The reasons why these cross-neutralizing antibodies are not able to clear the HCV in most of chronically infected individuals remain unclear. In addition, organization of distinct B-cell epitopes located in envelope proteins of HCV as well as the heterogeneity and natural evolution of these epitopes during the different stages of infection, and especially during the chronic phase remains undefined or poorly understood. The aim of the current project was to find answers to some of these important questions and to get an insight into the role of the humoral immune response in control and resolution of HCV infection. Investigations in this field, however, usually are hampered due to the fact that investigators have to deal with patients infected with different HCV and no information on the ancestral virus sequences is available. The essential feature of the current project is the use of a special group of patients – women infected with the same HCV strain (AD78) in a single-source outbreak. This cohort represents a unique model to study the evolution of HCV under the immune selective pressure of the host and to analyze the role of different immune responses, including the impact of virus-neutralizing antibodies on elimination of the virus from infected patients.

Analysis of the E1/E2 sequences obtained from the batches 8, 10 and 12 of the AD78 HCV-contaminated globulin unexpectedly revealed heterogeneity of the virus genome population. The results of the phylogenetic analysis (Fig.3.2.) have shown a separation of the sequences into two major clades that was supported by a high bootstrap value. Heterogeniety of the HCV virus present in contaminated AD-globulin was supported by our data on amplification and analysis of the sequences corresponding to the fragment of the NS3 gene (Fig.3.3.), which demonstrated division of the sequences into 3 clusters. In sum, these results have clearly

demonstrated simultaneous presence of at least two variants of the HCV in different batches of the contaminated AD-globulin. In order to see if this two variants represent different strains of HCV or just variants of the same strain we subjected to a phylogenetic analysis separately E1 and E2 and the whole envelope sequences obtained from the batches of contaminated globulin and from a number of individual patients infected with the virus or viruses present in these batches (Fig.3.4, Fig.3.5. and Fig.3.6.). The results of these experiments have demonstrated a statistically significant separation of HCV AD78 sequences from the bulk of HCV 1b sequences obtained from the Los Alamos HCV database indicating: (a) the evolutionary relatedness of the viruses present in the globulin and individual patients; (b) that the viruses present in the globulin and AD-patients in fact represent different variants of the same HCV strain, which is quite different from all other HCV 1b strains. Of special importance is the fact that the sequences from both globulin and individual patients formed two separate clusters suggesting that these two clades respresent two evolutionary lineages that originated from two closely related but still distinct viruses already present in the contaminated anti-D globulin. In general, these results indicated that the anti-D outbreak under study was indeed a single source outbreak caused by two variants (referred to as Gl8/A and Gl8/B) of a single HCV AD78 strain. At the moment it is not completely clear why upon an analysis of the envelope region a presence of only two variants of HCV AD78 strain was detected in contrast to the results of the experiments with the NS3 region, which demonstrated existence of three variants of this virus. One possible explanation is that the third virus variant was present in the contaminated globulin in a relatively low concentration and only two other more abundant virus variants were preferentially amplified by the primer set used in the current study. Interestingly, we were not able to detect a simultaneous presence of both variants of AD78 strain in any of AD-patients suggesting that despite transmission with contaminated globulin of two (or three) virus variants only one of these variants was able to cause a persistent infection. These data suggest an existence of a selection mechanism, which, probably, is related to a difference in the ability of the incoming virus variants to adapt to a particular host.

The revealed heterogeneiety of the HCV AD78 variants in the contaminated globulin and patients meant that the choice of patients for further analysis of HCV evolution and humoral immune response was critical. We have checked the phylogenetic relatedness of virus isolates in the globulin and individual patients and have identified

a number of matching pairs, including patient T, infected with the Gl8/A variant and patient H infected with Gl8/B variant of HCV AD78 (Fig.3.7.).

Generation of the clonal E1/E2 sequences from the batch 8 of the globulin and selected patients allowed the analysis of the envelope genes evolution during the long term persistent infection. In general, in all AD78 isolates obtained from AD patients a higher heterogeneity in the E2 region than in the E1 gene was observed. The mutation rates for the HCV AD78 isolates tested varied from 0.83 x 10^{-3} to 4.1 x 10^{-3} and from 2.02 x 10^{-3} to 3.62 x 10^{-3} nucleotide substitutions per site per year for E1 and E2 genes, correspondingly. These results are comparable to the estimates reported earlier (Smith *et al.*, 1999; Allain *et al.*, 2000; Brown *et al.*, 2005). Of note is the fact that both, for patient T and patient H, a tendency for a decline of substitution rates in the E1 as well as in the E2 genes with duration of HCV persistence was noted. Most probably, this process reflects the fact of a gradual adaptation of the virus population to a specific host environment.

In order to analyze some characteristics of adaptive evolution within the envelope genes during the chronic infection the rates of synonymous and non-synonymous nucleotide substitution (dS/dN) were estimated. In this study we excluded from the analysis the HVRI because data for this most rapidly diverging fragment of the HCV genome may result in errornous or misleading interpretation of the general mutation rates in the E2 region (McCormack & Clewley, 2002). Analysis of the selective pressure for E1 and E2 regions separatly by calculating the average dS/dN ratio for AD78 isolates obtained for patient T (infected with the virus variant Gl8A) and patient H (infected with the variant Gl8/B) from different time points p.i. did not show a presence of a strong positive selection, suggesting that major driving force in the HCV evolution during the chronic phase of the infection is a purifying or negative selection, which to a significant extent associated with a removal of deleterious mutations. The comparison of the dS/dN ratio for E1 and E2 separately in isolates obtained from nine AD-patients 20 years p.i. shown evident variation of this parameter even in the absence of a strong positive selection, suggesting variability of a selection pressure in individual patients. Of note, is a marked variabilty of the dS/dN ratio in the the E1 region (Fig.3.9.). Our results have demonstrated a significant difference in dS/dN ratios established for patients B and J and those estimated for other patients. At the moment, the reason for such a difference it is not quite clear. Theoretically, one can not exclude a possibility that virus isolates present

in patients B and J are in fact recombinants of two variants of the AD78 strain. In fact, existence of such recombinants in materials of HCV chronic patients has been demonstrated (Brown *et al.*, 2005). Our data, however, did not support such a supposition because a phylogenetic analysis have demonstrated a consistency in topology of the trees constructed separately for the E1 and E2 regions of the AD78 isolates analysed in the current work. Finally, one shoud mention that in the current study an average dN/dS ratio across the E1/E2 region was determined. This approach is quite adequate for a comparison of differences in selective pressures on HCV envelope genes evolution in different patients. At the same time, as has been noted earlier (Sheridan *et al.*, 2004; Brown *et al.*, 2005) this is a highly conservative criterion for detecting positive selection, as only a few codons within the HCV envelope protein may be under diversifying selection. The signal could therefore be diluted in a background of purifying selection, maintained via strong functional or structural constraints. As a result, contribution of the diversifying selection to HCV envelope evolution might be underestimated. To overcome this problem, future studies should involve an estimation of the dN/dS ratio for individual a.a. positions. Our preliminary data (performed not in the frame of the current study), however, suggested an existence of only a restricted number of sites under positive selection (besides those in the HVRI) in the E2 protein of virus isolates obtained from a single-source HCV outbreak (Viazov *et al.*, not published). In general, the analysis of dN/dS ratio for envelope proteins of AD78 isolates as an indicator of a selective pressure on the evolution suggested different dynamic and patterns of HCV evolution in individual patients infected with the same HCV strain.

Other very important and interesting aspects of HCV E1/E2 evolution during chronic infection addressed in the current work are the character, localization, and a potential functional meaning of a.a. substitutions occured during a long-term virus persitence in the infected host. The current investigation included a longitudinal study of a.a. substitutions in HCV AD78 Gl8/A- and Gl8/B evolving variants from pateints T and H as well as comparative analysis of mutations of virus isolates obtained from nine AD-patients at a single time point, namely 20 years p.i. The performed experiments revealed a number of general characteristics of HCV E1/E2 evolution during the chronic infection. A relatively low number of a.a. substitutions occurred in the E1 protein (Tab.3.2.), which might be explained by the fact that in the context of the E1/E2 heterodimers forming the virion envelope this protein presumably is less exposed and accessible to immunocompetent cells. In addition, most of the

mutations observed in this protein were located outside of domains targeted by human mAbs. Relatively larger number of a.a. mutations was detected in the E2 sequences of AD-patients. A significant number of them occurred in known human mAbs targeted sites and, especially often, in CD-81-binding sites or, in other words, in the functionally relevant domains of the protein (Tab.3.3.). These data indicated that in most AD-patients the whole ectodomain of E2 protein and not only the HVRI remains under evident selective pressure during the long term chronic infection.

Theoretically, it is possible that selection pressure on HCV is related to changes in a glycosylation pattern. The HCV envelope proteins are highly glycosylated with 5 to 6 potential N-linked glycosylation sites in E1 and 11 potential sites in E2. Our results, however, provided no evidence that HCV long-term persistence leads to either loss or acquisition of glycosylation sites. Similar results were reported by others (Sheridan *et al.*, 2004).

Of special interest are our results indicating that significant part of mutations within the E2 sequence (around 30 to 80 %) from all patients occurred in HVRI and such a tendency remained in patients T and H throughout the whole follow up period (Tab.3.3.). Especially demonstrative was a situation with the remarkably slowly evolving envelope sequences from patient T, in which all the mutations in the E2 protein in the period between 17 and 26 years p.i. localized in HVRI. It is assumed that HVRI evolution is predominantly driven by the nAb response (von Hahn *et al.*, 2007; Dowd *et al.*, 2009; Liu *et al.*, 2010). In this context, one should mention our data obtained with the E1/E2 variants of env heterodimers in which the natural HVRI sequences were substituted with M8/M9 variants that did not react with anti-HVRI Abs (Fig.3.20.) These modified E1/E2 heterodimers were able to bind antibodies from sera of AD-patients as effectively as the original, unmodified versions of E1/E2 heterodimers, suggesting that significant fraction of Abs present in patients sera are directed against epitopes outside the HVRI. That does not mean, of course, that no Abs to HVRI were induced, as presence of such antibodies has already been described in numerous publications (Kato *et al.*, 1994; Kato *et al.*, 1993). Future studies of sequential sera from patients T and H with a number of specially designed HCVpp and HCVcc bearing different variants of HVRI and a set of synthetic peptides corresponding to HVRI observed in these patients during the follow-up are planned. These experiments should provide an answer to the essential question on contribution of specific antibody responses to HVRI to HCV evolution and escape in

long term chronic infection. In general, results of the analysis of a.a. substitutions in E1/E2 sequences of chronically infected AD-patients complement our data on differences in selection pressure in individual patients and clearly indicate that evolution of envelope sequences during long term HCV persistence is a patient-specific event, which, most probably, is a reflection of differences in genetic and physiological background of the corresponding hosts.

In order to assess a possible contribution of structural and functional constrains to HCV evolution in chronically infected individuals we investigated the direction of mutations the E1/E2 sequences from patients T and H (Fig.3.12.). In sequences evolving during the first 11 years p.i. (acute/early chronic phase) 50 % of a.a. substitutions in the E1E2 sequences from patient T and 35 % of substitutions in sequences from patient H were considered as reverse mutations to the HCV 1b consensus sequence. In the late phase of chronic infection (11 - 28 years p.i.) the proportion of the reverse mutation varied in both patients but remained pretty high (48 %) in patient H, the HCV sequences from which characterized by a relatively high level of evolution, possibly indicating reversion to a more fit viral phenotype in the corresponding hosts. In summary, our results complement the most recent data obtained for a group of acutely infected individuals (Kuntzen *et al.*, 2007; Liu *et al.*, 2010) and emphazied a remarkable impact of reversion on intrahost HCV evolution envelope proteins. It seems that HCV evolution during the long term chronic phase besides being immunologically driven, to a significant extent is limited also by structural and/or functional constraints, which manifests themselves in sequence preservation or reversion of sequences toward consensus.

Analysis of the characteristics and localization of mutations in AD cohort has demonstrated different pace of evolution in HCV AD78-infected patients T and H. In patient H persistent changes in the E1/E2 sequences with approximately similar frequency of a.a. substitutions within and outside of known functional sites and domains was observed. In contrast, in patient T the number of mutations in the E1/E2 sequences remained quite low during the whole follow-up and most of the observed mutations localized in the HVRI and CD-81-binding sites. The reasons for such a dramatic difference are not clear. Patient T demonstrated no evidence of immunosupression and serum samples collected from both patients demonstrated comparable ability to bind to the homologous and heterologous E1/E2 antigens and to neutralize autologous and heterologous HCVpp.

Different trends in the E1/E2 evolution in individual AD-patients observed in the current study and manifested in unequal selection pressure, rates, character, and patterns of a.a. substitutions raise the question how the resulting mutations in the envelope proteins are translated into changes of the properties of the virus and its interaction with the infected host during a long term persistance. In general, our data are in favor of a supposition that accumulation of mutations over time may incur a fitness cost reducing biological properties of the virus in a particular environment. It is clear, that a significant number of observed a.a. substitutions, which have been located in the HVRI, or in CD81- or human mAb-binding sites, would change an ability of the virus to react with cellular receptors and penetrate the intracellular pathways. One should also mention that the mutations located outside of known functional domains and sites may also significantly influence properties of viral particles. Thus, in a recent publication Keck and co-authors (Keck *et al.*, 2009) reported that during the chronic phase of HCV infection and in the presence of broadly neutralizing serum antibodies, mutations occuring outside receptor binding sites resulted in structural changes leading to complete escape from neutralizing antibodies, while simultaneously compromising viral fitness by reducing binding to CD81. Considering location and number of mutations in our set of AD78-derived sequences one might have expected that the HCVpp bearing these sequences would demonstrate different properties in the immunological and infectivity tests. Indeed, results of the immunobinding experiments, in which ability of HCVpp bearing the E1/E2 sequences collected at different time points p.i. from patients T and H to react with autologous patient's sera was asessed, have demonstrated a general tendency. According to this tendency, sera collected at a particular time point reacted better with E1/E2 antigens collected at earlier time points than with those collected at later time points p.i. These data suggested that the accumulation of mutations in the E1/E2 sequences during the evolution of the virus in chronically infected patients might have reduced their ability to bind the anti-viral antibodies present in infected host at each time point of infection. Additional evidence of a dramatic influence of the mutations accumulated over time in the viral envelope on HCV phenotype were obtained in the experiments on HCVpp infectivity (Fig.3.22.). The pseudoparticles generated on the basis of E1/E2 sequences obtained from sequential follow-up samples from both patients demonstrated evident reduction of infectivity during the long term HCV persistence. These data indicate that adaptation of HCV to the

gradual changing environment during the long term persistence may influence the critical stages of the viral replicative cycle, such as virus entry into the cell.

It is generally assumed that virus population is changed over time due to a combination of forces like an immune pressure driven by the host and adaptation to a new host environment. This, consequently, might lead to appearance of virus variants that escape the antibody-mediated neutralization (Bartosch *et al.*, 2003) (Keck *et al.*, 2008). Recently, experimental proofs of this assumption were reported (von Hahn *et al.*, 2007). Using the HCVpp system and sequential virus and serum samples from a patient infected with the Hutchinson strain of HCV the authors demonstrated evidences of a continuous escape of the circulating virus from emerging antibodies and suggested that a humoral immune pressure was a major driving force for the observed diversity in the envelope sequences. In the current study we also tried to assess if the mutations accumulated in the envelope sequences during a long term persistence of HCV AD78 isolates in patients T and H have some influence not only on infectivity but also on ability of the mutated viruses to avoid a specific neutralization.

At first, an ability of antisera from patients infected with one HCV strain to cross-react with E1/E2 proteins encoded by other HCV strains was tested (Fig.3.18.).The result has shown that there was no significant difference in the frequency and level of reactivity of sera from patients infected with different subtype 1b HCV strains. Considering the fact that not all circulating antibodies are the neutralizing ones, a neutralization test was performed, which showed that HCVpp bearing envelope proteins of virus variants from AD78 Gl8/A and Gl8/B were neutralized more effectively by sera from patients infected with the same virus variant than by sera of patients infected with another AD78 Gl8 virus variant or even less effectivly by HCV1b sera. Thus, similar to previous reports (Lavillette *et al.*, 2005), the antibodies present during a chronic phase of infection are able to neutralize the inoculating virus more efficiently than heterologous virus strains.

Interesting and somehow unexpected results were obtained in neutralization experiments with HCVpp bearing the M9 artificial variant of HVRI in the context of E1/E2 sequences derived from Gl8/A or Gl8/B viruses (Fig.3.23. A, B). Both variants of HCVpp bearing the modified envelope sequences (Gl8/A M9 and Gl8/B M9) were neutralized much more efficiently by sera from HCV infected individuals than HCVpp expressing envelope proteins with naturally occuring HVRI variants. These data

suggested that the natural HVRI somehow masks crucial epitopes important for virus neutralization. This observation complement the results of the most recent report, which demonstrated that HCVpp lacking the HVRI are much better neutralized by mAbs than HCVpp bearing envelope proteins with HVR (Bankwitz *et al.*). Both these sets of data are important for our understanding of the organization of the B-cell epitopes of HCV and would have an evident impact on the HCV vaccine development efforts in the future.

A final series of neutralization experiments was performed using the set of HCVpp bearing the E1/E2 sequences both from the ancestral Gl8/A and Gl8/B viruses and from evolving Gl8/A and Gl8/B virus variants obtained from patients T and H 16 (1994-95), 20 (1998), and 28 (2006) years p.i.. Sera from the same patients collected at different time points of infection were used for neutralization (Tab.3.4 and Tab.3.5.). In case of patient T, sera collected at all time points p.i. rather effectively neutralized the HCVpp bearing the envelope sequence of the ancestral Gl8/A virus variant but were much less effective in blocking the infectivity of pseudoparticles with E1/E2 sequences from 16 and 20 p.i. Quite unexpectedly, the HCVpp bearing the envelope sequences corresponding to the virus circulating 28 years p.i. were effectively neutralized by all tested sera from patient T. The reasons for such marked changes in neutralization pattern are not clear. One might suggest, however, that a.a. substitutions accumulated by 2006 (28 years p.i.) played a role of compensatory mutations, which somehow restored the conformation of the E1/E2 heterodimer. In contrast, for patient H no evident differences in ability of sera collected at distinct time points to neutralize HCVpp bearing evolving envelope sequences were noted. In summary, these experiments provided no evidence that humoral immune response lags behind the changes in envelope sequences of the virus circulating in the host at a given time point. In other words, our data suggest that the postulated mechanism of escape from antibody-mediated neutralization is not operative in all chronic patients.

Interpretation of the results of HCVpp neutralization experiments would be incomplete without referring to recently reported data on HCV employing multiple strategies to infect new target cells (Timpe *et al.*, 2008; Witteveldt *et al.*, 2009). It was shown that HCV can be transmitted not only via so-called cell-free route by virus circulating in the bloodstream but also via direct cell-to-cell contacts. At that, as has been indicated by Brimacombe and co-authers (Brimacombe *et al.*, 2010), the extracellular forms of HCV are most likely responsible for spread between hosts and

for liver allograft re-infection following transplantation. However, cell-to-cell transmission may represent the dominant route of virus dissemination within chronically infected individuals. All HCV cell receptors, including the SR-BI, CD-81, claudin-1, and occludin are involved in virus cell-to-cell transmission, however, the SR-BI has a more prominent role in this infection route. One of most interesting features of HCV cell-to-cell transmission is the fact that of all the anti-glycoprotein mAbs tested, the anti-HVRI antibody demonstrated the highest neutralization potentials (Brimacombe *et al.*, 2010), which, in fact, is not surprising considering that HVRI plays a crucial role in HCV entry by interaction with SR-BI. That, in turn, means that *in vivo* HCV can escape the neutralization mediated by anti-HVRI antibodies. In the current study we used the HCVpp assay for measuring neutralization properties of the whole patient sera. Our data (Fig.3.23. A, B) have shown that sera of AD-patients contain relatively high titres of antibodies to AD78 E1/E2 proteins and that most or a significant part of these antibodies are directed against epitopes located outside of the HVRI. In addition, our results clearly demonstrated very intensive rate of mutations of the HVRI sequence in all envelope sequences obtained from AD-patients at different time point p.i. Thus, it is quite possible that anti-HVRI antibodies response in AD-patients is masked by a more vigorous humoral response to other epitopes of envelope proteins. In this case, the experimental approach used in the current study and directed at neutralization of virions transmitted via cell-free route would not detect the HCV escape from anti-HVRI antibodies, which very efficiently neutralize a virus transmitted by a cell-to-cell route. Future studies based on the use of HCVcc bearing different variants of HCV AD78-derived E1, E2, and HVRI sequences should provide an answer to the essential question if the HCV persistence during the chronic infection is associated with the escape of the virus directly transmitted from cell to cell from a specific humoral immune response.

Upon the studies of E1/E2 diversity in HCV AD78-patients an interesting peculiarity of the envelope sequences was noted. The sequences of most of the AD78 isolates contained additional 1 to 4 codon at the junction between E1 and E2 genes (Appendix Fig.8.3.) Conservation of the sequences at the 3' end of the E1 gene, including the signal sequence region, in all of these sequences suggested that the processing of the HCV polyprotein was not disrupted and evidenced for these additional codons being an integral part of the E2 gene. Further studies of HCV sequences retrieved from the Los Alamos database have shown that these additional codons were not a hallmark of the AD78 strain, but were also present in 1.2 to 8 % of

HCV strains belonging to genotypes/subtypes 1b, 2, 4, 5, and 6, but not to type 1a and 3 viruses. Analysis of the physico-chemical characteristics of a.a. residues encoded by the additional codons demonstrated a predominance of neutral and hydrophobic residues in most of the positions suggesting that these additional a.a. stretches might be oriented toward a hydrophobic E2 protein core, thus, providing for additional stability of the HVRI conformation. Predominance of small and flexible residues in these stretches might also facilitate the maintaining of the varied conformation states of HVRI. Absence in the additional a.a. tracks of Trp and Cys, as well as a very low frequency of Pro, namely, the residues that constrain the polyprotein conformation, gives evidence for this hypothesis.

Peculiarities of the physico-chemical characteristics of additional a.a. residues at the N-terminus of the E2 protein as well as their occurrence only in some of HCV subtypes/genotypes suggest that additional a.a. stretches appeared not by chance and that the mechanism of their selection and preservation is operative. That, in turn, means that the additional a.a. track at the N-terminus of E2 might have a particular functional significance. In the current study we used the HCVpp system to assess a possible influence of additional a.a. on virus infectivity. To this end, a set of new E1/E2 plasmids, in which the additional a.a. residues at the N-terminus of HVRI were swapped with a number of natural or artificial a.a. stretches, was generated and tested for infectivity in Huh7.5 cells. These experiments have demonstrated that the presence of additional a.a. track in all cases but one (insertion of DNDN, namely, the sequences that are never or very rarely present in naturally occuring additional tracks) resulted in an increase of infectivity of pseudoparticles (Fig.3.27.) suggesting that the viruses bearing E2 proteins with modified N-terminus might also posess unique biological properties *in vivo*.

The mechanism of formation of the additional a.a. stretches is unknown. Casino and co-workers (Casino *et al.*, 1999) have observed an imperfect direct repeat in the clonal sequence of one HVRI variant and suggested that a C-terminal region of the HVRI was duplicated and replaced the N-terminal region resulting in appearance of one extra amino acid residue. However, our analysis of multiple HCV sequences retrieved from the Los Alamos database as well as from AD78 sequence dataset did not reveal presence of any direct or inverted repeats either in HVRI or in flanking sequences of strains bearing additional codons at the 5' terminus of the E2 gene. We were also not able to identify any disturbances or changes in the secondary structure

of RNA of HCV strains containing additional codons at the E1/E2 junction. Thus, the question of the mechanism of appearance of these extra codons remains unanswered and requires further studies.

5 Conclusion

The current study deals with several important aspects of HCV research, including evolution of the envelope proteins and dynamic of the humoral immune response during the long term virus persistence. The essential feature of the project was the use of a special group of patients - women infected with the same HCV strain (AD78) by a contaminated anti-D globulin in a single-source outbreak.

This unique cohort of patients infected with the same virus strain allowed the minimization of factors, including route of transmission, size of inoculum, duration of infection, age and sex of infected individuals, playing a significant role in evolution of the virus during the chronic infection. Nevertheless, even under these dramatic reduction of factors affecting intrapatient viral evolution, the established parameters, such as a rate of selective pressure, as well as a pattern of mutations in the E1/E2 region, differed markedly between AD-patients. Analysis of the rate and character of nucleotide and amino acid substitutions in the E1/E2 region revealed no evidence for a strong positive selection, while a high frequency of reverse mutations to a consensus HCV 1b sequence was observed, indicating a critical role of functional/structural constrains in evolution of envelope proteins. The obtained data allowed to formulate an unequivocal conclusion that HCV envelope proteins evolution is a patient-specific phenomenon, and that purifying (negative) selection is the major force acting on HCV populations in chronic infection.

Analysis of the character and localization of a.a. substitutions in envelope sequences of AD78 isolates obtained from AD-patients at different time points of chronic infection have shown that a significant part of mutations occurred in the HVRI, suggesting a possibilty of emergence of HCV variants escaping neutralization mediated by anti-HVRI antibodies. A significant number of mutations was also localized in known human mAbs targeted sites and, especially often, in CD-81-binding sites or, in other words, in the functionally relevant domains of the protein. These data indicated that in most AD-patients the whole ectodomain of E2 protein and not only the HVRI remains under evident selective pressure during the long term chronic infection.

The other aim of the current study was the analysis of the dynamic of the HCV-specific humoral immune response in long term chronic patients. Application of the HCVpp system allowed detecting relatively high titres of cross-reacting and cross-neutralizing antibodies in sera of AD-patients; however, no evidence of immune escape during HCV persistence was registered. These experiments provided no evidence that humoral immune response lags behind the changes in envelope sequences of the virus circulating in the host at a given time point. In other words, our data indicated that the postulated mechanism of escape from antibody-mediated neutralization is not operative in all chronic patients. The results of the current study suggested, however, that the absence of escape from neutralization of virions transmitted via cell-free route does not mean that another mechanism of immune escape, namely, the escape from anti-HVRI antibodies, which very efficiently neutralize a virus transmitted by a cell-to-cell route, is not operative and does not contribute to HCV persistence.

Finally, a new interesting structural feature of envelope sequences of some HCV strains belonging to different subtypes and genotypes - presence of additional 1 to 4 a.a. residues at the N-terminus of the E2 protein - was studied using a set of hymeric E1/E2 plasmids, in which the additional a.a. residues were swapped with a number of natural or artificial a.a. stretches. These experiments have demonstrated that the presence of additional a.a. track may result in a significant increase of pseudoparticles infectivity suggesting that the viruses bearing E2 proteins with modified N-terminus might also posess unique biological properties *in vivo*.

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7 Abbreviations

a.a.	Amino Acid
Ab	Antibody
ARFP	Alternative frame protein
APC	Antigen-presenting cell
BCR	B-cell receptor
BSA	Bovine serum albumin
bp	Base-pair
℃	Degree in Celsius
CD	Cluster of differentiation
Con1	Replicon construct
CTL	Cytotoxic T lymphocyte
µg	Micrograms
µl	Microlitre
CLDN1	Claudin-1
CLP(s)	Capsid-like particle(s)
C-terminus	Carboxy-terminus
DC	Dendritic cells
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleotidetriphosphate
dsRNA	Double-stranded RNA

E1	Envelope 1
E2	Envelope 2
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
ER	Endoplasmatic Reticulum
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
Fig	Figure
F-Protein	Frame shift protein
g	Grams
Gt	Genotype
h	Hours
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Hepatitis C virus cell culture
HCVpp	Hepatitis C virus pseudoparticle
HDL	high-density lipoproteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immonudefiency virus
HLA	Human leukocyte antigen
HRP	Horseradish-Peroxidase

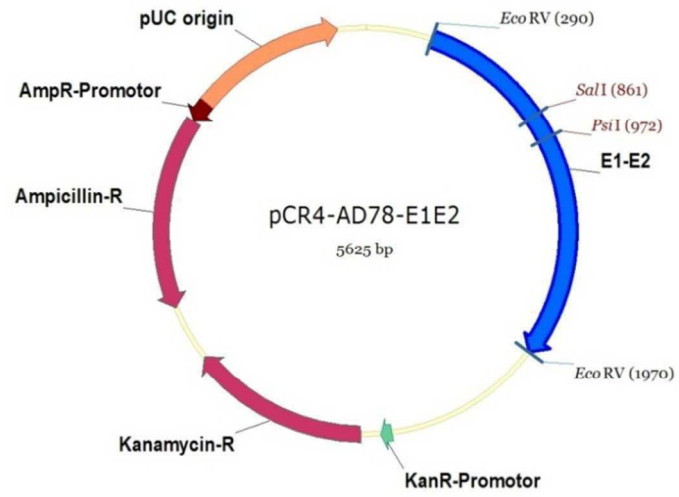
HVRI	Hypervariable region 1
IFN	Interferon
Ig	Immunoglobulin
Ig G	Immunoglobulin G
IL	Interleukin
IRES	Internal Ribosome Entry Site
kD	Kilodalton
LB	Lurian Broth
LEL	long extracellular loop
mAb(s)	monoclonal antibody (ies)
mg	Milligrams
min	Minutes
ml	Millilitre
mM	Millimolar
MLV	murine leukemia virus
mRNA	messenger RNA
Mw	molecular weight
nAb	neutralizing antibody
NK	natural killer cells
NKT	natural killer T cells
nm	Nanometers
NS	non-structural protein
nt	nucleotide
N-terminus	Amino-terminus

NTPase	Nucleoside triphosphate hydrolase
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide-Gelelektrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed Death 1
PD-L	Programmed Death Ligand
p.i.	post infection
RBS	Ribosome Binding Site
RLU	Relative-Light-Units
RNA	Ribonucleic acid
rpm	Revolutions per minute
Rt	Room temperature
RT	Reverse trancriptase
SDS	Sodium dodecylsulfate
sE2	soluble envelope 2
Sec	second
siRNA	small inteferring RNA
SR-BI	Scavenger receptor class B member 1
TTBS	Tris tween buffered saline
U	Units
UTR	Untranslating region

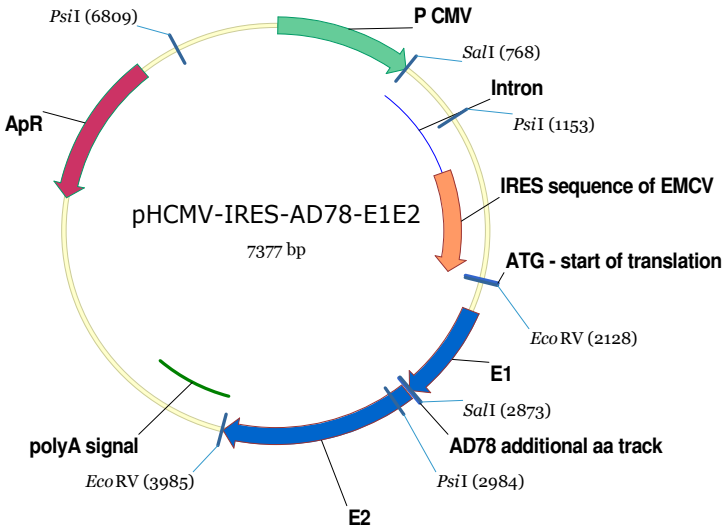
UV-light	Ultra violet light
V	Volt
VLDL	very low density lipids
VSV	Vesicular stomatitis virus
WB	Western blot
WHO	World Health Organization

8 Appendix

8.1 Vectorcards

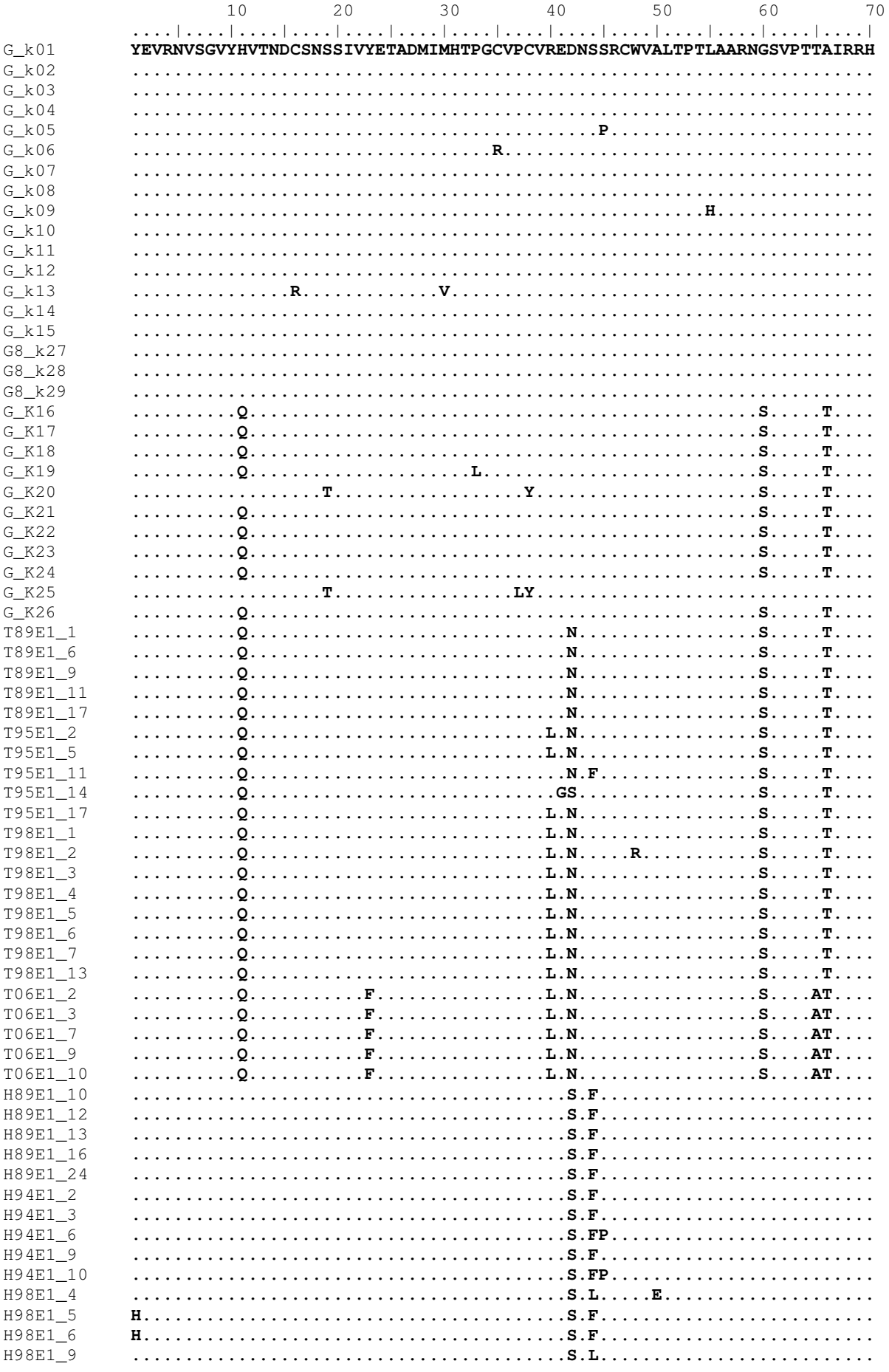


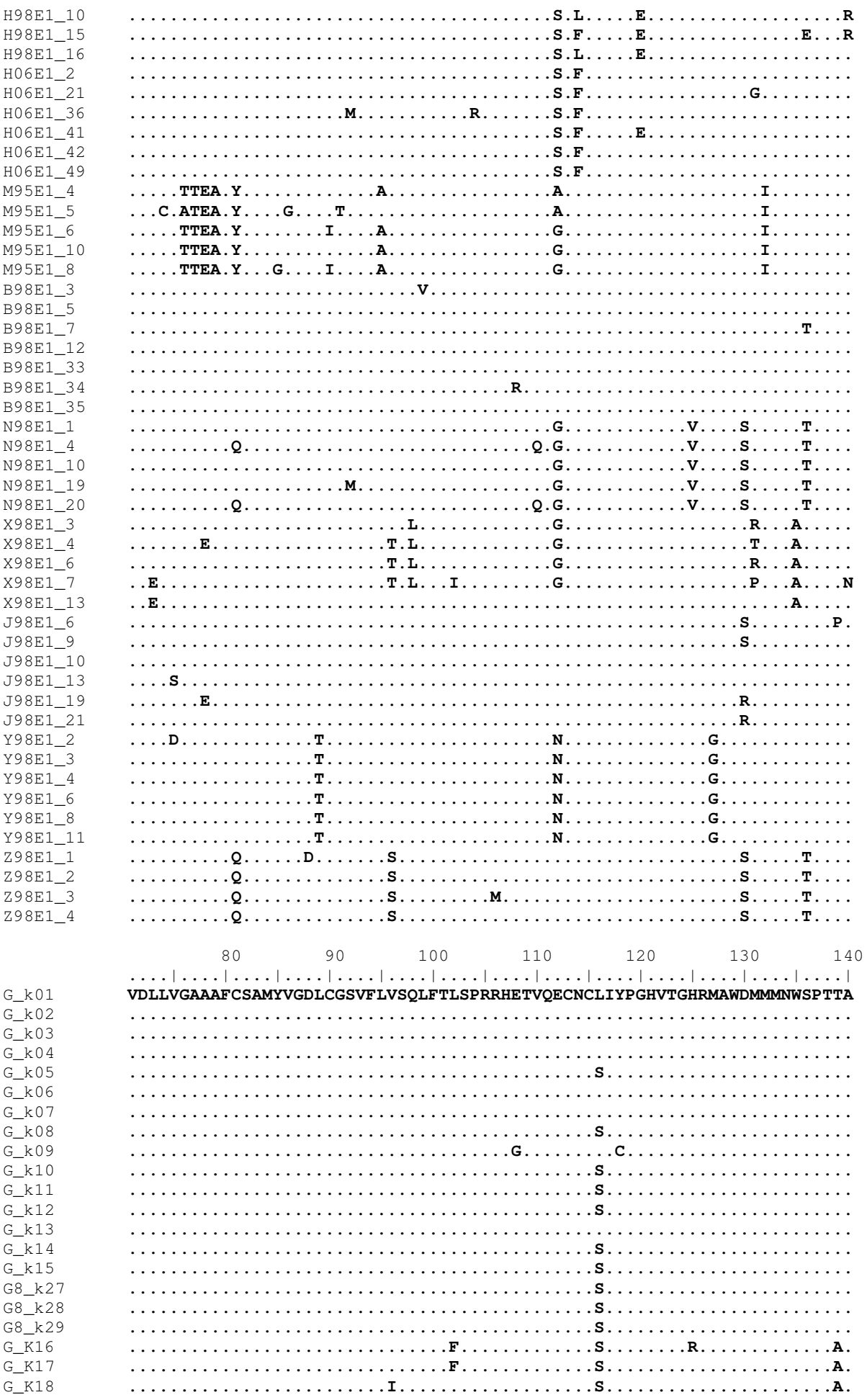
pCR4-AD78-E1E2: The plasmid contains the pUC origin, an Ampicillin resistant Promotor (AmpR-Promotor), the Ampicillin resistant gene (Ampicillin-R), the Kanamycin resistant gene (Kanamycin-R)as well as the Kanamycin resistant- Promotor (KanR-Promotor) into which the AD78 derived E1/E2 sequences were inserted using the EcoRV restriction sites. The hyper variable region as described in (Fig.3.15.) was exchanged by SalI and PsiI restriction.



pHCMV-IRES-AD78-E1E2: The expressing plasmid contains the Ampicillin resistant gene (ApR), the CMV promoter, an Intron, the IRES sequence of EMC. E1 starts at bp 2306-2880 and E2 starts at bp 2881-3979. The restriction sites SalI (768 bp) and PsiI (1153, 6809) were removed by SDM but are mentioned here for better understanding. The polyA signal matures the process of mRNA translation.

8.2 Aligment of E1 a.a. sequences of HCV AD78 Gl8 and infected patients

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H06E1_21 .....S.F.....G.....
H06E1_36 .....M.....R.....S.F.....
H06E1_41 .....S.F.....E.....
H06E1_42 .....S.F.....
H06E1_49 .....S.F.....
M95E1_4 .....TTEA.Y.....A.....A.....I.....
M95E1_5 ...C.ATEA.Y....G....T.....A.....I.....
M95E1_6 .....TTEA.Y.....I....A.....G.....I.....
M95E1_10 .....TTEA.Y.....A.....G.....I.....
M95E1_8 .....TTEA.Y...G....I....A.....G.....I.....
B98E1_3 .....V.....
B98E1_5 .....
B98E1_7 .....T....
B98E1_12 .....
B98E1_33 .....
B98E1_34 .....R.....
B98E1_35 .....
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N98E1_4 .....Q.....Q.G.....V....S.....T....
N98E1_10 .....G.....V....S.....T....
N98E1_19 .....M.....G.....V....S.....T....
N98E1_20 .....Q.....Q.G.....V....S.....T....
X98E1_3 .....L.....G.....R...A.....
X98E1_4 .....E.....T.L.....G.....T...A.....
X98E1_6 .....T.L.....G.....R...A.....
X98E1_7 ..E.....T.L...I.....G.....P...A....N
X98E1_13 ..E.....A.....
J98E1_6 .....S.....P.
J98E1_9 .....S.....
J98E1_10 .....
J98E1_13 ....S.....
J98E1_19 .....E.....R.....
J98E1_21 .....R.....
Y98E1_2 ....D.....T.....N.....G.....
Y98E1_3 .....T.....N.....G.....
Y98E1_4 .....T.....N.....G.....
Y98E1_6 .....T.....N.....G.....
Y98E1_8 .....T.....N.....G.....
Y98E1_11 .....T.....N.....G.....
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Z98E1_2 .....Q.....S.....S.....T....
Z98E1_3 .....Q.....S.....M.....S.....T....
Z98E1_4 .....Q.....S.....S.....T....
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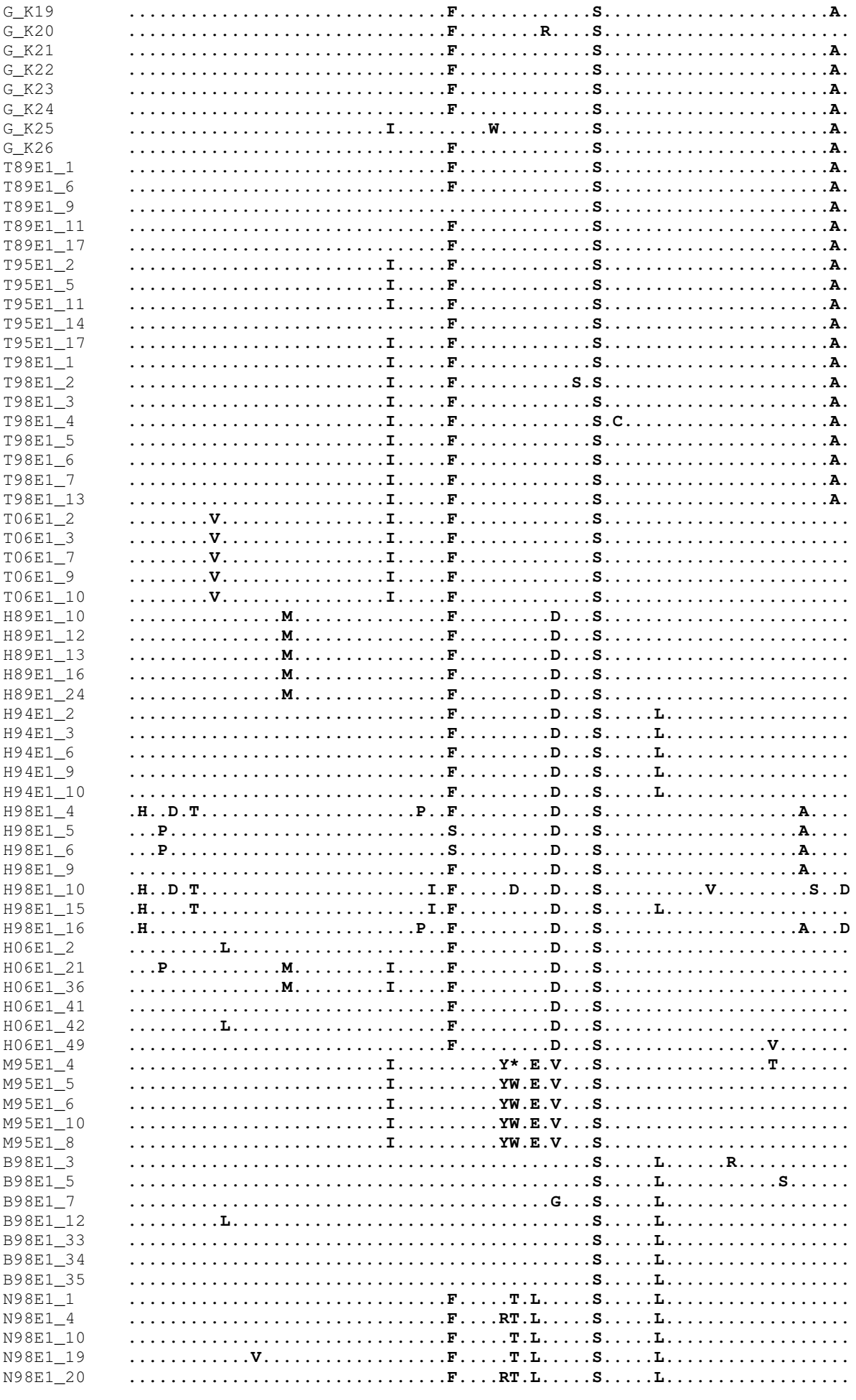
G_k03 .....
G_k04 .....
G_k05 .....S.....
G_k06 .....
G_k07 .....
G_k08 .....S.....
G_k09 .....G.....C.....
G_k10 .....S.....
G_k11 .....S.....
G_k12 .....S.....
G_k13 .....
G_k14 .....S.....
G_k15 .....S.....
G8_k27 .....S.....
G8_k28 .....S.....
G8_k29 .....S.....
G_K16 .....F.....S.....R.....A.
G_K17 .....F.....S.....A.
G_K18 .....I.....S.....A.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T89E1_11
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B98E1_34
B98E1_35
N98E1_1
N98E1_4
N98E1_10
N98E1_19
N98E1_20

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X98E1_3     ..........................................S.....................
X98E1_4     ....D...........Y..........I.......D.......S.....................D
X98E1_6     ..........................................S.....................
X98E1_7     .H..D.T.........WY..........I.......D.PH....S.....................D
X98E1_13    .H..D.T..........Y..........I.......D..H....S.....................D
J98E1_6     ..........................................S.....................
J98E1_9     ..........................................S.....................
J98E1_10    ..........................................S.....................
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J98E1_19    .......................................I.....S.....................
J98E1_21    ..........................................S.....................
Y98E1_2     ..........................................S......S.............S.
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Y98E1_4     ..........................................S......S.............S.
Y98E1_6     ..........................................S......S.............S.
Y98E1_8     ..........................................S......S.............S.
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Z98E1_4     ............V..................F...Q.........S.....................A.

                    150       160       170       180       190
                    ....|....|....|....|....|....|....|....|....|....|..
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G_k03       ..................................................
G_k04       ..................................................
G_k05       ..................................................
G_k06       ..................................................
G_k07       ..................................................
G_k08       ..................................................
G_k09       ..................................................
G_k10       ..................................................
G_k11       ..................................................
G_k12       ..................................................
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G_k14       ..................................................
G_k15       ..................................................
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G8_k28      ..................................................
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G_K25       ..................................................
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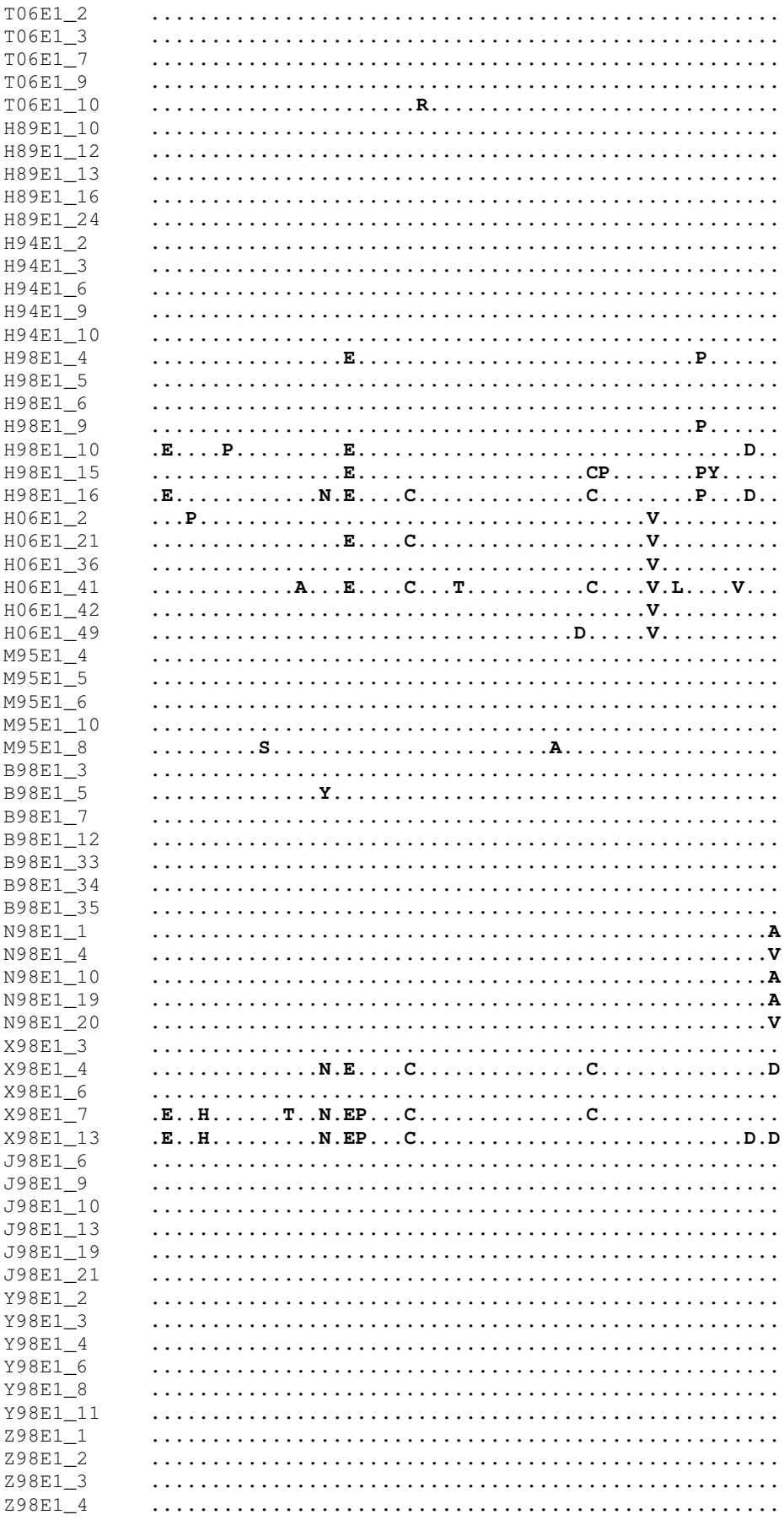
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Z98E1_3
Z98E1_4

8.3 Aligment of E2 a.a. sequences of HCV AD78 Gl8 and infected patients

```
                  10        20        30        40        50        60        70
            ....|....|....|....|....|....|....|....|....|....|....|....|....|
G_k01       --GGPTRTIGGSQAQTASGLVSMFSVGPSQKIQLINTNGSWHINRTALNCNDSLNTGFLAALFYAHKFNS
G_k02       --.............................................................
G_k03       --.............................................................
G_k04       --.............................................................
G_k05       --.............................................................
G_k06       --.............................................................
G_k07       --.............................................................
G_k08       --.............................................................
G_k09       --.............................................................
G_k10       --.............................................................
G_k11       --.............................................................
G_k12       --.............................................................
G_k13       --.............................................................
G_k14       --.............................................................
G_k15       --.............................................................
G8_k27      --.............................................................
G8_k28      --.............................................................
G8_k29      --.........................................................LC......
G_K16       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K17       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K18       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K19       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K20       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K21       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K22       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K23       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K24       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K25       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
G_K26       RG..G.Q....A.SHSVM.VA.I..P..A.....V.............................VRS...
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T98E2_3     -H..G..AV..A.SRYTQRFT.L.TA..A.....V.............................IRN...
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T98E2_6     -H..G..AV..A.SRYTQRFT.L.TA..A.....V.............................IRN...
T98E2_7     -H..G..AV..A.SRYTQRFT.L.TA..A.....V.............................IRN...
T98E2_13    -H..G..AV..A.SRYTQRFT.L.TA..A.....V.............................IRN...
T06E2_2     -H..G..AV..A.SRYTQSFT.L.TA........V.............................IRN...
T06E2_3     -H..G..AV..A.SRYTQSFT.L.TA........V.............................IRN...
T06E2_7     -H..G..AV..A.SRYTQSFT.L.TA........V.............................IRN...
T06E2_9     -H..G..AV..A.SRYTQSFT.L.TA........V.............................IRN...
T06E2_10    -H..G..AV..A.SRYTQSFT.L.TA........V.............................IRN...
H90E2_4     ---SG...V.....RA....T.L.TR....R.............................I...L.V..I..
H90E2_6     ---SG...V.....RA....T.L.TR....R.............................I...L.V..I..
H90E2_8     ---SG...V.....RA....T.L.TR....R.............................I...L.V..I..
H90E2_10    ---SG...V.....RA....T.L.TR....R.............................I...L.V..I..
H90E2_17    ---SG...V.....RA....T.L.TR....R.............................I...L.V..I..
H94E2_2     -TSWT.Y.T..V...VT.S..AWLAP....R.........................K...I.......R...
H94E2_7     -TSWT.Y.T..V...VT.S..AWLAP....R.........................K...I.......R...
H94E2_8     -TSWT.Y.T..V...VT.S..AWLAP....R.........................K...I.......R...
H94E2_18    -TSWT.Y.T..V...VT.S..AWLAP....R.........................K...I.......R...
H94E2_19    -TSWT.Y.T..V...VT.S..AWLAP....R.........................K...I.......R...
H98E2_4     -MS.T.Y.T..A..RYTGAF.GL.AS..A.R.............................I.S.....R...
H98E2_5     -MS.T.Y.T..A.GRLTGR..GL.VS..A.R.............................I.S.....R...
H98E2_6     -MS.T.Y.T..A.GRLTGR..GL.VS..A.R.............................I.S.....R...
H98E2_9     -MS.T.H....A..RLTGS..GWL.P...................................I.S.....R...
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H98E2_10    -TFCT.Y.T..A..RFT.SF.GL.AP....R...........S............I.S.....R...
H98E2_15    -MS.T.H....A..RLTGSI.GL.AP..A.R.......................I.S.....R...
H98E2_16    -MS.T.Y.T..A..RYTGAF.GL.AS..A.R.......................I.S.....R...
H06E2_2     -ASAD.Y.T..AL.R.TH.FT.L..R.A..........................I.....T.S...
H06E2_21    -ASAT.H.T..T.GRA..TF.RL..P.A..N......................K...I.....T.R...
H06E2_36    -ASAT.H.T..THGRA....TGL..P.A..N......................K...I.....R.R...
H06E2_41    -ASAD.Y.T..ML.R.TRRFT.L..P.A..........................I.....T.S...
H06E2_42    -ASAD.Y.T..VL.R.TR.FT.L..P.A..........................I.....T.S...
H06E2_49    -ASAT.H.T..THGRA....TGL..P.A..N...V..................K...I.....R.R...
N98E2_1     -SARG...V..AESYNVR.FT.L.TS..AH.............................VRN...
N98E2_2     -SARG...V..AESYNVR.FT.L.TS..A.....V.........................VRN...
N98E2_11    -SARG...V..AESYNVR.FT.L.TS..AH.............................VRN...
N98E2_12    -SARG...V..AESYNVR.FT.L.TS..A.....V.........................VRN...
N98E2_16    -SARG...V..AESYNVR.FT.L.TS..A.....V.........................VRN...
Z98E2_2     ----Q.H.G..A..RNVKSFAAL.TP..A.T...V..............E.I.........T.....
Z98E2_3     ----Q.H.G..A..RN.KSFAAL.TP..A.T...V..............E.I.........T.....
Z98E2_6     ----Q.H.V..A..RNVKSFAAL.TP..A.T...V..............E.I.........T.....
Z98E2_7     ----Q.H.G..A..RNVKSFAAL.TP..A.T...VS............R.E.I.........T.....
Z98E2_8     ----Q.H.G..A..RNVKSFAAL.TP..A.T...V..............E.I.........T.....
Z98E2_12    ----Q.H.G..A..RNVKSFAAL.TP..A.T...VS............R.E.I.........T.....
Z98E2_13    ----Q.H.V..A..RNVKSFAAL.TP..A.T...VS............R.E.I.........T.....
Z98E2_14    ----Q.H.G..A..RNVKSFAAL.TP..A.T...VS............R.E.I.........T.....
Z98E2_16    ----Q.H.G..A..RNVKSFAAL.TP..A.T...VS............R.E.I.........T.....
X98E2_3     ---SG...V.....RA....T.L.TR....R..................R........I...L.V..ID.
X98E2_4     ---SG...V.....RA....T.L.TR....R..................................I..
X98E2_6     ---SG.H.VE....RA....T.L.TR....R..................I...L.V..I..
X98E2_7     ---SG...V.....RA....T.L.TR....R..................I...L.V..I..
X98E2_13    ---SG...V.....RA....T.L.TR....R...........T......I...L.V..I..
B98E2_2     --S.T..VV..LT...T..FA.L.NY..A.....................S..........NN...
B98E2_5     --S.T...V...A...I..FA.L.RP.........................S..........NR...
B98E2_7     --S.T...V...A...I..FA.L.RP.........................S..........NR...
B98E2_8     --I.T..VV..LT...T..FA.L.RH.........................S..........N....
B98E2_10    --S.T..VV..LT...T..FA.L.NY..A.....................S..........NR...
J98E2_1     ---.G.H.V..AS.RA.G.IATF..P..........S..................I.....VRS...
J98E2_2     ---.G.H.V..AS.RA.R.IATF..P.........................I.....VRS...
J98E2_3     ---.G.H.V..AS.RA.G.IATF..P.........................I.....VRS...
J98E2_6     ---.G.H.V..AS.RA.G.IATF..P.........................I.....VRS...
J98E2_17    ---.G.H.V..AS.RA.G.IATF..P.........................I.....VRS...
M98E2_1     -ALSD..........AVR.FT.RL.T....N.H...S..............Q...I.....T.R...
M98E2_2     -ALSD..........AVR.FT.RL.T....N.H...S..............Q...I.....T.R...
M98E2_4     -ALSD..........AVR.FT.WL.T....N.H...S..............Q...I.....T.R...
M98E2_5     -A.TN...V......A.R.IT.L..A....N.H...S............D...K...I.....T.R...
M98E2_6     -ALSD..........AVR.FT.WL.T....N.H...S..............Q...I.....T.R...
Y98E2_1     --H.S.T....A.GLVTR.FT.L..P.A.......................................T.R...
Y98E2_2     --H.G.T....V.GLVTR.FT.L..P.A.......................................T.R...
Y98E2_3     --H.S.T....A.GLVTR.FT.L..P.A.......................................T.R...
Y98E2_5     --H.G.T....V.GLVTR.FT.L..P.A.......................................T.R...
Y98E2_6     --H.S.T....A.GLVTR.FT.L..P.A.......................................T.R...

                      80        90        100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
G_k01       SGCPERMASCRPIDKFAQGWGPITYAEPGSLDQRPYCWHYAPQPCGIVPAAEVCGPVYCFTPSPVVVGTT
G_k02       ..............................................................................
G_k03       ..........................................................S...................
G_k04       ..........................................................S...................
G_k05       ..........................................................S...................
G_k06       .......V......................................................................
G_k07       ..............................................................................
G_k08       ..........................................................S...................
G_k09       ..............................................................................
G_k10       ..........................................................S...................
G_k11       ..............E...........................................S...................
G_k12       ..............................................................................
G_k13       ..............................................................................
G_k14       ..........................................................S...................
G_k15       ..............................................................................
G8_k27      ..........................................................S...................
G8_k28      ......................T...................................S...................
G8_k29      ..........................................................S...................
G_K16       ......V.....................VG..........................SQ..................
G_K17       ............................VG..........................S...................
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G_K18       ..........................VG..................SQ..................
G_K19       ..........................VG..................SQ..................
G_K20       ..........................VG..................SQ..................
G_K21       ..........................VG..................SQ..................
G_K22       ............N.............VG..................SQ..................
G_K23       ..........................VG..................PQ......R...........
G_K24       ..........................VG..................PQ......R...........
G_K25       ..........................VG..................SQ..................
G_K26       ..........................VG...G..............S...................
T89E2_5     ..........................VG............R.......SQ..................
T89E2_6     ..........................VG............R.......SQ..................
T89E2_8     ..........................VG............R.......SQ..................
T89E2_18    ..........................VG............R.......SQ..................
T89E2_20    ..........................VG............R.......SQ..................
T95E2_4     ..........................VG............R.......SQ..................
T95E2_5     ..........................VG............R.......SQ..................
T95E2_6     ..........................VG............R.......SQ..................
T95E2_7     ..........................VG............R.R.....SQ..................
T95E2_11    ..........................VG............R.......SQ..................
T98E2_1     ..........................VG............R.......SQ..................
T98E2_2     ..........................VG............R.......SQ..................
T98E2_3     ..........................VG............R.......SQ..................
T98E2_4     ..........................VG............R.......SQ..................
T98E2_5     ..........................VG............R.......SQ..................
T98E2_6     ..........................VG............R.......SQ..................
T98E2_7     ..........................VG............R.......SQ..................
T98E2_13    ..........................VG............R.......SQ..........G.......
T06E2_2     ..........................VG............R.......SQ..................
T06E2_3     ..........................VG............R.......SQ............I.....
T06E2_7     ..........................VG............R.......SQ............I.....
T06E2_9     ..........................VG......Y.....R.......SQ..................
T06E2_10    ..........................VG............R.......SQ..................
H90E2_4     ...S....G.....R............S............R.......S...................
H90E2_6     ...S..........R............S............R.......S...................
H90E2_8     ...S..........R............S............R.......S...................
H90E2_10    ...S..........R............S............R.......S...................
H90E2_17    ...S..........R............S............R.......S...................
H94E2_2     ......................T...R.D.....................S...................
H94E2_7     ........................R.D.....................S...................
H94E2_8     ........................R.D.....................S...................
H94E2_18    ........................R.D.....................S...................
H94E2_19    ........................R.D.....................S...................
H98E2_4     ........................K.D.....................S...................
H98E2_5     ........................K.D.....................S...................
H98E2_6     ........................K.D.....................S...................
H98E2_9     ........................K.D.....................S...................
H98E2_10    ........................K.D......C..............S...................
H98E2_15    ........................K.D.........R...........S...................
H98E2_16    ........................K.D.....................S...................
H06E2_2     ..............E.............S............R.......S...................
H06E2_21    ..............E...........K.S............R.......S...................
H06E2_36    ..............E.............S............R.......S...................
H06E2_41    ............................S............R.......S...................
H06E2_42    ...S..........E.V........TK.S............R.......S...................
H06E2_49    ............................S............R.......S...................
N98E2_1     ..............Q..........T..VG.....................LQ..................
N98E2_2     ..............Q..........T..VG.....................LQ..................
N98E2_11    ..............Q..........T..VG.....................LQ..................
N98E2_12    ..............Q..........T..VG.....................LQ..................
N98E2_16    ..............Q..........T..VG.....................LQ..................
Z98E2_2     ......L.......................D............R.......SQ..................
Z98E2_3     ......L.......................D............R.......SQ..................
Z98E2_6     ......L.......................D............R.......SQ..................
Z98E2_7     ......L..............V.......D............R.......SQ..................
Z98E2_8     ......L........V..............D............R.......SQ..................
Z98E2_12    ......L..............V.......D............R.......SQ..................
Z98E2_13    ......L..............V.......D............R.......SQ..................
Z98E2_14    ......L..............V.......D............R.......SQ..................
Z98E2_16    ......L..............V.......D............R.......SQ..................
X98E2_3     ...S..........R............N............R.......S...................
X98E2_4     ...S..........R............S............R.......S...................
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X98E2_6     ...S.........R............S............R.......S..................
X98E2_7     ...S.........R.........H...S............R.......S..................
X98E2_13    ...S.........R.........H...S............R.......S..................
B98E2_2     ..............T........V..D............R.......SG.................
B98E2_5     ..............ST.......D..............R.......SG.................
B98E2_7     ..............ST.......D..............R.......SG.................
B98E2_8     ...............T.......D.....................SG.................
B98E2_10    ...............T.......D.....................SG.................
J98E2_1     ........................TK.IIS.................S..................
J98E2_2     ........................TK.IIS.................S..................
J98E2_3     ........................TK.IIS...........R.....S..................
J98E2_6     ........................TK.IIS.................S..................
J98E2_17    ........................TK.IIS.................S..................
M98E2_1     ..............T..K.......V..N.S...........R.......SK.................
M98E2_2     ..............T..K.......V..N.S...........R.......SK.................
M98E2_4     ..............T..K.R.....V..N.S...........R.......SK.................
M98E2_5     ..............T..K.......V....S...........R.......SK.................
M98E2_6     ..............T..K.R.....V..N.S...........R.......SK.................
Y98E2_1     ........................H.V.DN.................S..................
Y98E2_2     ........................H.V.DN.................S..................
Y98E2_3     ........................H.V.DN.................S..................
Y98E2_5     ........................H.V.DN.................S..................
Y98E2_6     ........................H.V.DN.................S..................

                    150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
G_k01       DRSGVPTYRWGENETDVLLLNNTRPPQGNWFGCTWMNTTGFTKTCGGPPCNIGGLGNNXXLICPTDCFRK
G_k02       ..............................................................................
G_k03       ..............................................................................
G_k04       ..............................................................................
G_k05       ..............................................................................
G_k06       ..............................................................................
G_k07       ..............................................................................
G_k08       ..............................................................................
G_k09       ..............................................................................
G_k10       ..............................................................................
G_k11       ..............................................................................
G_k12       ..............................................................................
G_k13       ..............................................................................
G_k14       ..............................................................................
G_k15       ..............................................................................
G8_k27      ..............................................................................
G8_k28      ..............................................................................
G8_k29      ..............................................................................
G_K16       ..F.....S..A.....................................K.....................
G_K17       ..F.....S..A.....................................K.....................
G_K18       ..F.....S..A.....................................K.....................
G_K19       ..F.....S..A.....................................K.....................
G_K20       ..F.....S..A.....................................K.....................
G_K21       ..F.....S..A.....................................K.....................
G_K22       ..F.....S..A.....................................K.....................
G_K23       ..............................................................................
G_K24       ..............................................................................
G_K25       ..F.....S..A.....................................K.....................
G_K26       ..............................................................................
T89E2_5     ..F.....S.....................................K...S..D...V.........
T89E2_6     ..F.....S.....................................K...SD.D...V.........
T89E2_8     ..F.....S.....................................K...S..D...V.........
T89E2_18    ..F.....S.....................................K...S..D...V.........
T89E2_20    ..F.....S.....................................K...S..D...V.........
T95E2_4     ..F.....S.....................................K...F..D...V.........
T95E2_5     ..F.....S.....................................K...F..D...V.........
T95E2_6     ..F.....S.....................................K...F..D...V.........
T95E2_7     ..F.....S.....................................K...F..D...V.........
T95E2_11    ..F.....S.....................................K...F..D...V.........
T98E2_1     ..F.....S.....................Y...............K...F..D...V.........
T98E2_2     ..F.....S.....................A...............K...F..D...V.........
T98E2_3     ..F.....S.....................T...............K...F..D...V.........
T98E2_4     ..F.....S........P.............R...............K...F..D...V.........
T98E2_5     ..F..S..S.....................................K...F..D...V.........
T98E2_6     ..F.....S.....................................K...F..D...V.........
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T98E2_7     ..F.....S.....................................K...F..D...V.....
T98E2_13    ..F.....S.....................................K...F..D...V.....
T06E2_2     ..F.....S.....................................K...F..D...V.....
T06E2_3     ..F.....S......G..............................K...F..D...V.....
T06E2_7     ..F.....S......G..............................K...F..D...V.....
T06E2_9     ..F.....S.....................................K...F..D...V.....
T06E2_10    ..F.....S.....................................K...F..D...V.....
H90E2_4     ..F.....................................................NT.T.....
H90E2_6     ..F.....................................S.............V..D...T.....
H90E2_8     ..F.....................................................NT.T.....
H90E2_10    ..F.....................................................NT.T.....
H90E2_17    ..F.....................................S.............V..D...T.....
H94E2_2     ..F.....S...............................................NT.T.....
H94E2_7     ..F.....S...............................................NT.T.....
H94E2_8     ..F.....S...............................................NT.T.....
H94E2_18    ..F.....S...............................................NT.T.....
H94E2_19    ..F.....S...............................................NT.T.....
H98E2_4     ..F.....S.........................I.....Q.............NT.T.....
H98E2_5     ..F.....S...............................................NT.T.....
H98E2_6     ..F.....S...............................................NT.T.....
H98E2_9     ..F.....S..G............................................NT.T.....
H98E2_10    ..F.....S...............................................NT.T.....
H98E2_15    ..F.....S.............................................K.NT.T.....
H98E2_16    ..F.....S.........................I.....................NT.T.....
H06E2_2     .........S.............................................NT.T.....
H06E2_21    ..F.....S.S.............................................NT.T.....
H06E2_36    .........S...........................................S.NT.T.....
H06E2_41    .........S...........................................S.NT.T.....
H06E2_42    .H.....S.............................................S.NT.T.....
H06E2_49    .........S...........................................S.NT.T.....
N98E2_1     ..F.....N..A......F..SS...R.G.....................K...R.........
N98E2_2     ..F.....N..A......F..SS...R.S.....................K...R..S......
N98E2_11    ..F.....N..A......F..SS...R.G.....................K...R.........
N98E2_12    ..F.....N..A......F..SS...R.S.....................K...R..S......
N98E2_16    ..F.....N..P......F..SS...R.S.....................K...R..T......
Z98E2_2     ..F.....S.............................S............K.............
Z98E2_3     ..F.....S.........................T.S............K..........V.....
Z98E2_6     ..F.....S.............................S............K......M......
Z98E2_7     ..F.....S.............................S............K.............
Z98E2_8     ..F.....S.............................S............K.............
Z98E2_12    ..F.....S.............................S............K.............
Z98E2_13    ..F.....S.............................S............K.............
Z98E2_14    ..F.....S.....................................................
Z98E2_16    ..F.....S.............................S............K.............
X98E2_3     ..F...S...............................S.......R........F..D...T.....
X98E2_4     ..F.....................................S.............V..D...T.....
X98E2_6     ..F.....................................S.............F..D...T.....
X98E2_7     ..F.....................................S.............V..D...T.....
X98E2_13    ..F.....................................S.............F..D...T.....
B98E2_2     ....A...Q.....................................D.....................
B98E2_5     ....A...Q.................R...................D.....................
B98E2_7     ....A...Q.................R...................D.....................
B98E2_8     ....A...Q.................H...................D.....................
B98E2_10    ....A...Q.................H...................D.....................
J98E2_1     ..F.....S.............................A.....................D...T.....
J98E2_2     ..F.....S.............................A.....................D...T.....
J98E2_3     ..F.....S.............................A.....................D...T.....
J98E2_6     ..F.....S.............................A.....................D...T.....
J98E2_17    ..F.....S.............................A.....................D...T.....
M98E2_1     ..F.....................................A..Y.............AS.............
M98E2_2     ..F.....................................A..Y.............AS.............
M98E2_4     ..F.....................................A..Y.............AS.............
M98E2_5     ..F.....................................A..Y.............DS.............
M98E2_6     ..F.....................................A..Y.............AS.............
Y98E2_1     ..F.....................................SS.............................
Y98E2_2     ..F.....................................SS.............................
Y98E2_3     ..F.....................................SS.............................
Y98E2_5     ..F.....................................SS.............................
Y98E2_6     ..F.....................................SS.............................
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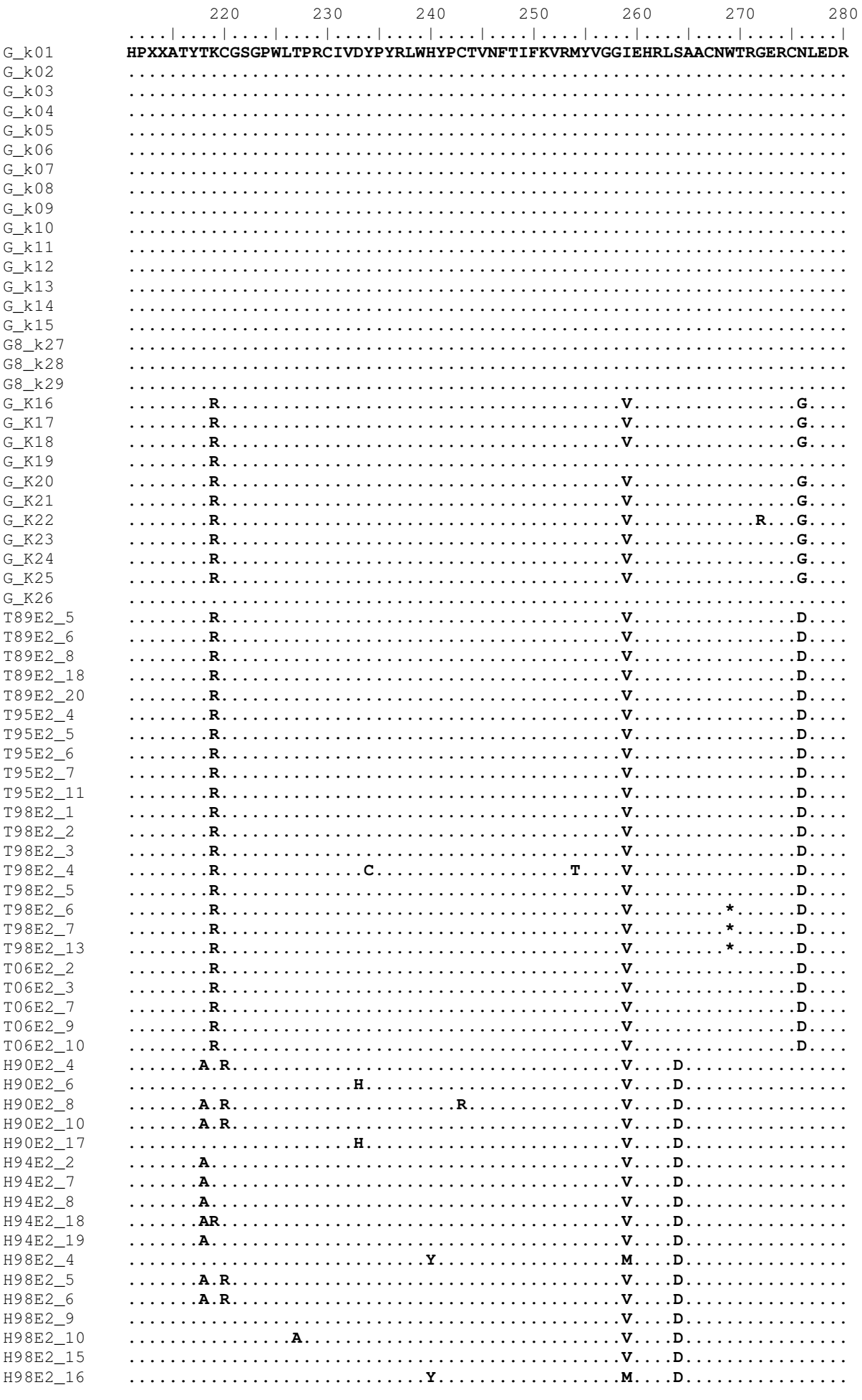
220 230 240 250 260 270 280
G_k01 HPXXATYTKCGSGPWLTPRCIVDYPYRLWHYPCTVNFTIFKVRMYVGGIEHRLSAACNWTRGERCNLEDR
G_k02
G_k03
G_k04
G_k05
G_k06
G_k07
G_k08
G_k09
G_k10
G_k11
G_k12
G_k13
G_k14
G_k15
G8_k27
G8_k28
G8_k29
G_K16
G_K17
G_K18
G_K19
G_K20
G_K21
G_K22
G_K23
G_K24
G_K25
G_K26
T89E2_5
T89E2_6
T89E2_8
T89E2_18
T89E2_20
T95E2_4
T95E2_5
T95E2_6
T95E2_7
T95E2_11
T98E2_1
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T98E2_3
T98E2_4
T98E2_5
T98E2_6
T98E2_7
T98E2_13
T06E2_2
T06E2_3
T06E2_7
T06E2_9
T06E2_10
H90E2_4
H90E2_6
H90E2_8
H90E2_10
H90E2_17
H94E2_2
H94E2_7
H94E2_8
H94E2_18
H94E2_19
H98E2_4
H98E2_5
H98E2_6
H98E2_9
H98E2_10
H98E2_15
H98E2_16

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H06E2_2     ....V.....................................M....D.............
H06E2_21    ..........................................M....D.............
H06E2_36    ..............................................D.............
H06E2_41    ..............................................D.............
H06E2_42    ........................T.....................D.............
H06E2_49    ..............................................D.............
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Z98E2_8     ........R.............A.....................V....D..........D....
Z98E2_12    ........R.............A............L........V....D..........D....
Z98E2_13    ........R.............A............L........V....D..........D....
Z98E2_14    ........R.............A............L........V....D..........D....
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X98E2_4     ......................H.....................V....D.............
X98E2_6     ......................H.....................V....D............*..
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X98E2_13    ......................H.........S...........V....D.............
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Y98E2_6     ....................M.................T.....................D....

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G_k02       ..................................................................
G_k03       ..................................................................
G_k04       ..................................................................
G_k05       ..................................................................
G_k06       ..................................................................
G_k07       ..................................................................
G_k08       ..................................................................
G_k09       ..................................................................
G_k10       ..................................................................
G_k11       ..................................................................
G_k12       ..................................................................
G_k13       ..................................................................
G_k14       ..................................................................
G_k15       ..................................................................
G8_k27      ..................................................................
G8_k28      ..................................................................
G8_k29      ..................................................................
G_K16       ..............................................V.....................
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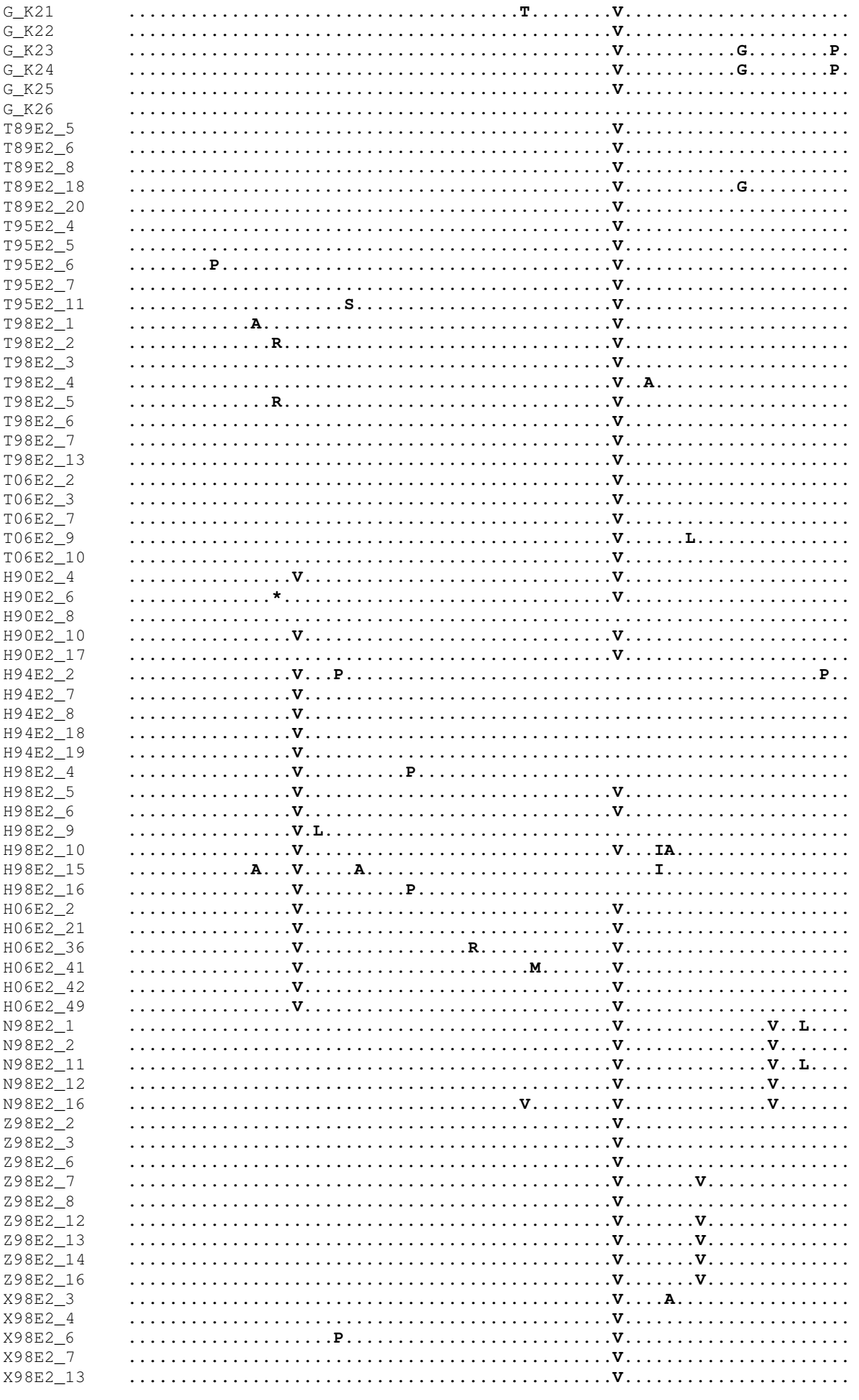
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G_K24
G_K25
G_K26
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T89E2_8
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T89E2_20
T95E2_4
T95E2_5
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T95E2_11
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T98E2_7
T98E2_13
T06E2_2
T06E2_3
T06E2_7
T06E2_9
T06E2_10
H90E2_4
H90E2_6
H90E2_8
H90E2_10
H90E2_17
H94E2_2
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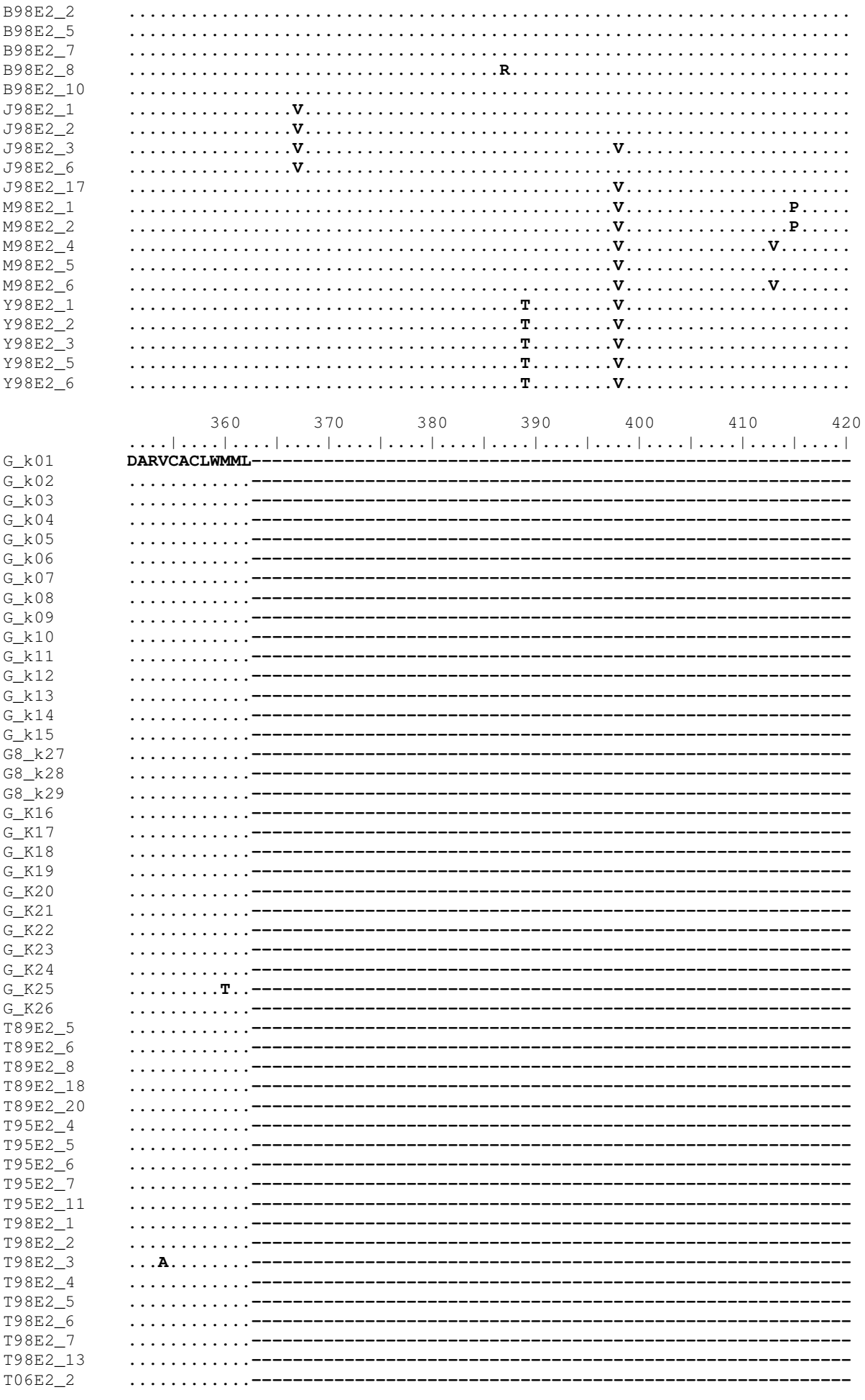
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380
390
400
410
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DARVCACLWMML
G_k02
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G_k07
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G_k14
G_k15
G8_k27
G8_k28
G8_k29
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G_K17
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G_K19
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T06E2_2

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T06E2_3     ............----------------------------------------------------
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T06E2_9     ............----------------------------------------------------
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H90E2_4     ............----------------------------------------------------
H90E2_6     ............----------------------------------------------------
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H94E2_18    ............----------------------------------------------------
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X98E2_3     ............----------------------------------------------------
X98E2_4     ............----------------------------------------------------
X98E2_6     ............----------------------------------------------------
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Y98E2_1     ............----------------------------------------------------
Y98E2_2     ............----------------------------------------------------
Y98E2_3     ............----------------------------------------------------
Y98E2_5     ............----------------------------------------------------
Y98E2_6     ............----------------------------------------------------
```

9 Publications

List of publications and manuscripts in preparation:

1. **Evolution of the HCV envelope genes in association with the humoral immune response in a single-source outbreak.** M.Klein, S.Luppus, E.Schreier,T.Baumert, S.Fakir, M.Wiese, M.Roggendorf, S.Viazov

2. **Characteristics of the additional amino acid track at the N-terminus of the HVRI in the HCV envelope sequences.** M.Klein, M.Lipskoch, S.Luppus, L.Meredith, J. McKeating, M.Roggendorf, S.Viazov

Publication submitted to *Gastroenterology* :

3. **CD8+ T cell selection pressure is a major driving force for evolution of hepatitis C virus nonstructural proteins.** M.Ruhl, T.Knuschke, L.Glavinic, C. Neumann-Haefelin, D.Chang, M.Klein, F. Heinemann, H.Tenckhoff, M.Wiese, P.Horn, S.Viazov, U. Spengler, M.Roggendorf, N.Scherbaum, J.Nattermann, D.Hoffmann, J.Timm and the German Anti-D Study Group

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Finally, I am extremely grateful to Philipp Babranyi for giving me his implicit trust and help. He was extremely patient and supportive all these years.

11 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

12 Statement

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Evolution of the envelope proteins E1 and E2 and of specific humoral immune response to these proteins in a group of patients infected by HCV in a single source outbreak“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Marina Klein befürworte.

Essen, den _____ _____

Michael Roggendorf

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den _____ _____

Marina Klein

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

Essen, den _____ _____

Marina Klein