DEVELOPMENT OF CELL CULTURE REPLICATION SYSTEMS FOR HEPATITIS C VIRUS GENOTYPE 5A

BY

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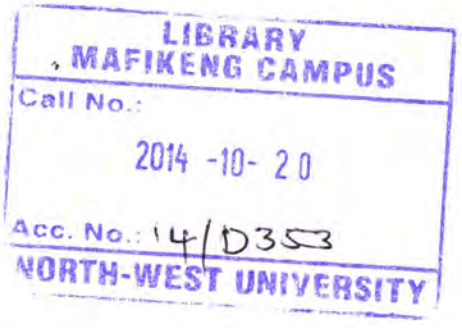
THESIS SUBMITTED FOR THE DEGREE DOCTOR OF PHILOSOPHY IN BIOLOGY (VIROLOGY) AT THE MAFIKENG CAMPUS OF THE NORTH-WEST UNIVERSITY SOUTH AFRICA



DEVELOPMENT OF CELL CULTURE REPLICATION SYSTEMS FOR HEPATITIS C VIRUS GENOTYPE 5A

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DECLARATION

I, the undersigned, declare that this dissertation submitted to the North-West University (Mafikeng Campus) for the degree Doctor of Philosophy in Biology (Virology) and the work contained therein is my own work in design and execution and has not previously, in its entirety or part, been submitted to another university for a degree, and that all the materials contained therein have been duly acknowledged.

PREFACE

This PhD thesis was conducted at The Rockefeller University, New York, USA from April 2011 to November 2013, under the supervision of Dr. N.P. Sithebe, Dr. N. Prabdial-Sing, Dr. M. Saeed, and Professor C. Rice. It is based on the following papers, paper I, which has been published in the PubMed journal Antimicrobial Agents and Chemotherapy in the June 30 2014 issue, and paper II is still under preparation to be submitted for publication in Journal of Virology.

Paper I:

Wose Kinge CN., Espiritu C., Prabdial-Sing N., Sithebe NP., Saeed M., Rice CM. Hepatitis C virus genotype 5a subgenomic replicons for evaluation of direct-acting antiviral agents. Antimicrob. Agents Chemother. 2014 Sep; 58 (9): 5386-94. doi: 10. 1128/AAC.03534-14. Epub 2014 Jun 30.

Paper II:

Constance N. Wose Kinge, Mohsan Saeed, Nomathamsaqa P. Sithebe, Nishi Prabdial-Sing, and Charles M. Rice, 2014. Novel full-length consensus sequences of hepatitis C genotype 5a. J. Virology, 2014; XX (X): XX-XX.

The author at the following scientific meeting will present data included in this thesis:

- 21st International Symposium on Hepatitis C Virus and Related Viruses, Banff, Alberta, Canada, 7-11 September, 2014.

DEDICATION

This work is dedicated to my little cute princess, Priscilla, who has had to endure my absence throughout the years

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ABSTRACT

The hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma with an estimated 170 million people chronically infected with the virus. Among the genotypes of HCV, genotype 5a (GT 5a) was first identified in a cohort of South African patients with HCV-induced hepatocellular carcinoma accounting for more than 30-50% of HCV infections in South Africa. The goal of my research was to establish functional *in-vitro* replication systems for HCV GT 5a as a preclinical tool for better screening and optimization of new and current viral inhibitors. To this end, sub-genomic replicons were generated and RNA transcripts electroporated into Huh-7.5 cells and selected with geneticin (G418 final concentration of 500µg/mL at 48 hours post-electroporation) to test their replication efficiencies. Production of G418-resistant colonies in Huh-7.5 cells was dependent on an NS5A S2205I amino acid substitution, a cell culture adaptive mutation originally reported for genotype 1b replicons. Further, electroporation of naïve Huh-7.5 cells with total cellular RNA isolated from replicon cells transmitted G418 resistance. RNA quantification and NS5A staining of selected colonies revealed high detectable levels of HCV RNA and viral NS5A protein. Sequence analysis revealed potential adaptive mutations, which when introduced back into the original constructs, substantially increased colony formation efficiency. In conclusion, we have established the first functional sub-genomic replicon system for HCV genotype 5a, which together with the recently published system for genotype 6a completes the panel of replicon systems for the clinical significant HCV genotypes 1-6.

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LIST OF ABBREVIATIONS

aa	Amino acid
apoB	Apolipoprotein B
CDC	Centers for Disease Control
cLD	Cytoplasmic lipid droplet
DAAs	Direct acting antivirals
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxy-nucleotide-tri phosphate
E1	Envelop glycoprotein 1
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAG	Glycosaminoglycans
GBV	Hepatitis G virus
GFP	Green fluorescent protein
GSP	Gene specific primer
GT	Genotype
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HCVcc	Hepatitis C virus cell culture
HCVpp	Hepatitis C virus pseudo particles
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HVR	Hypervariable region
IDU	Injecting drug user
IFN	Interferon

IRES	Internal ribosome entry site
ISDR	Interferon-sensitivity determining region
ISG	Interferon stimulated gene
IU	International unit
kb	kilo base
kDa	kilo Dalton
LCS I	Low complexity sequence I
LCS II	Low complexity sequence II
LDs	Lipid droplets
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
luLD	Luminal lipid droplets
LVP	Lipoviroparticle
MHC	Major histocompatibility complex
miR-122	Micro Ribonucleic acid 122
MLV	Murine leukemia virus
MTP	Microsomal triglyceride transfer protein
NANBH	Non-A, non-B hepatitis
NCR	Non-coding region
NEAA	Nonessential amino acids
Neo	Neomycin
NFW	Nuclease free water
NPHV	Non-primate hepacivirus
NPT	Neomycin phospho transferase
NS	Non structural
nt	Nucleotide
NTR	Non-translated region
ORF	Open reading frame
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dentritic cells

PegIFN	Pegulated interferon
PKR	Protein kinase R
pM	pico molar
PRR	Pattern recognition receptors
RACE	Rapid amplification of cDNA ends
RBV	Ribavirin
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
SVR	Sustain virologic response
TdT	Terminal deoxynucleotidyl transferase
μL	micro litre
μM	micro molar
UTR	Untranslated region
VLDL	Very low-density lipoprotein
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 HISTORY OF HEPATITIS C VIRUS (HCV)

After the development of serological tests to screen blood donors for hepatitis A virus (HAV) and hepatitis B virus (HBV) in the 1970s, most cases of hepatits caused by blood transfusion were found to be lacking both of these agents. Therefore, this unknown form of hepatitis was named non-A, non-B hepatitis (NANBH) (Alter, *et al.*, 1978; Feinstone *et al.*, 1975). The causative agent of this hepatitis remained unknown until 1989, when Choo *et al.* (1989) cloned the genome of NANBH agent and named it hepatitis C virus (HCV). This discovery led to a quick development of diagnostic tests for screening of blood donors, which resulted in a drastic decline in the number of HCV infections (Kuo *et al.*, 1989). HCV was later classified as a separate genus *Hepacivirus* in the *Flaviviridae* family, a family that includes the classical flaviviruses such as yellow fever virus, dengue virus and West Nile virus (Robertson *et al.*, 1998).

HCV is a positive-stranded RNA virus that replicates its genome with the help of an RNA-dependent RNA polymerase (RdRp) (Poenisch and Bartenschlager, 2010). Owing to a high error rate of RdRp, the viral genome is highly variable, challenging development of vaccines and therapeutic directly acting antivirals (DAAs). The HCV genome is approximately 9.6 kb long consisting of a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). The ORF is cleaved by host and viral protease (Grakoui *et al.*, 1993; Hijikata *et al.*, 1993) to produce 10 mature functional proteins; the viral structural proteins (Core, E1, and E2), p7, and six nonstructural (NS) proteins, namely NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Bartenschlager and Lohmann, 2000). Due to a large variability in nucleotide sequence, HCV has been classified into seven major genotypes (GT) and more than 70 subtypes. The nucleotide sequence of genotypes differs from each other by more than 30%, while subtypes have a sequence divergence of 10–30% (Smith *et al.*, 2013). Subtypes are designated by lowercase letters following the number of the genotype (for example genotype 1 subtype b is represented as GT1b). HCV genotypes 1 and 2 are geographically widespread in parts of Europe, North America and Japan

(Lauer and Walker, 2001), while genotypes 3 and 4 are found in Africa and the Middle East (Gededzha *et al.*, 2012; Cornberg *et al.*, 2011), 5 in South Africa (Gededzha *et al.*, 2014; Antaki *et al.*, 2013; Prabdial-Sing *et al.*, 2008), and 6 in Southeast Asia and Egypt (Chao *et al.*, 2011; Nguyen *et al.*, 2010). Genotype 7 was isolated from an emigrant from the Congo (Smith *et al.*, 2013).

Infections with HCV are a main cause of acute and chronic liver disease (Jacobson *et al.*, 2010). The worldwide burden of the disease is an estimated 130-170 million chronically infected individuals (Li *et al.*, 2012a). The incidence rate as well as the significance of HCV infection varies considerably from country to country and from region to region, possibly because of cultural factors and social habits that influence HCV transmission (Khaja *et al.*, 2006). With a prevalence of 5.3% and an estimated 32 million people infected with HCV, Sub Saharan Africa has the highest disease burden on a global scale (Karoney and Siika, 2013; WHO, 1999). Other WHO regions with a high prevalence of HCV include Eastern Mediterranean (prevalence 4.6%) and Western Pacific (prevalence 3.9%).

Despite high prevalence estimates and a highly infectious nature of HCV, there is generally less data available to validate the assumptions about the disease burden in Africa. The prevalence of HCV in the general population in Africa ranges between 0.1% and 22%. The highest prevalence of the disease is seen in Egypt (22%), Cameroon (13.8%), and Burundi (11.3%) while countries in the southern parts of Africa like Zambia, Malawi, South Africa and Kenya record the lower prevalence rates (Karoney and Siika, 2013; Antaki *et al.*, 2010). In South Africa, however, the prevalence of HCV infection varies widely in different geographical areas, between urban and rural populations, and among various ethnic groups within regions. Studies have shown prevalence rates of 0.41% to 3.84% in blood donors (Lavanchy, 2011; Ellis *et al.*, 1990).

The risk factors most frequently associated with HCV transmission are blood transfusions from unscreened donors, injection drug use, unsafe therapeutic injections, and other healthcare related procedures. In developed countries the predominant source of new HCV infections is injection drug use. Further, in countries with high seroprevalence in older age groups, unsafe therapeutic injections could

likely have played a significant role in HCV transmission over the past years, and may persist as an important cause of transmission in isolated, hyperendemic areas (Okayama *et al.*, 2002; Guadagnino *et al.*, 1997). Conversely, unsafe therapeutic injections and transfusions are listed as the major modes of transmission in the developing world especially in countries where age-specific seroprevalence rates suggest ongoing increased risks of HCV infection (Sulkowski, 2008; Verbeeck *et al.*, 2006; Wasley and Alter, 2000).

Once in the body, the virus is transported via the blood stream to the liver where it can cause an acute or chronic infection (Figure 1.1). During the acute phase of infection, HCV RNA in blood (or liver) can be detected by polymerase chain reaction (PCR) within several days to eight weeks (Hoofnagle, 1997) and virus titers usually peak at 10^5–10^7 RNA copies/mL between weeks 6 and 10, irrespective of disease outcome. Two to four weeks following onset of viraemia, there is an elevation in serum alanine aminotransferase (ALT) levels, which may reach greater than 10-30 times the upper normal limit (Mauss *et al.*, 2012). HCV antibodies can be found about 8 weeks after exposure although this may take several months. The symptoms of an acute infection include malaise, nausea, and right upper quadrant pain. In patients who experience such symptoms, the illness typically lasts for 2-12 weeks. Due to these nonspecific signs and symptoms, acute infection often remains unrecognized.

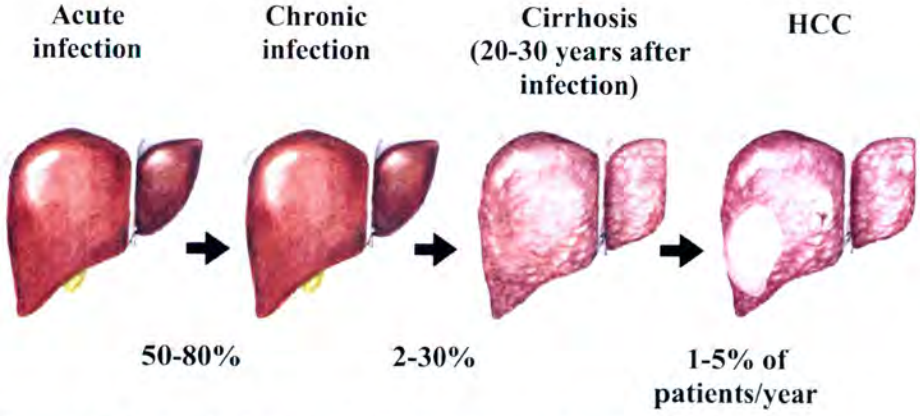

Figure 1.1: Natural history of HCV infection. Adapted from NIH Consensus Statement, 2002.

Approximately 20 to 50% patients are able to clear infection spontaneously during six months following infection. The other 50 to 80%, who cannot eliminate HCV RNA progress to a chronic carrier state. This stage of infection is characterized by relatively mild liver inflammation without significant fluctuations in HCV RNA titers (Mauss *et al.*, 2012). Greater age, obesity, alcohol consumption and HIV coinfection increase the disease progression (Rehermann, 2013). Once chronic infection is established, there is a very low rate of spontaneous clearance. Most patients with chronic infection are asymptomatic or have only mild nonspecific symptoms as long as cirrhosis is not present (Lauer and Walker, 2001, Merican *et al.*, 1993). The most frequent complaint is fatigue. Less common manifestations are nausea, weakness, myalgia, arthralgia, and weight loss (Merican *et al.*, 1993). Aminotransferase levels can vary considerably over the natural history of chronic hepatitis C. About 30% of these chronically infected individuals progress to liver cirrhosis 20 to 30 years after primary infection and hepatocellular carcinoma occurs in up to 2.5% of these patients (Bowen and Walker, 2005). Without treatment, cirrhosis and HCC can be fatal and explain most of the mortality directly attributed to HCV infection, however, for most of the duration of infection, and notably during the period when treatment is possible, the infection is silent and either remain unrecognized or ignored by most persons (Thomas, 2013).

No vaccine for HCV infection is available and the treatment of choice for chronic hepatitis C has been based, for more than 10 years, on the combination of pegylated interferon (PegIFN) and ribavirin (RBV), administered for 24 or 48 weeks. The endpoint for therapy is sustained virological response (SVR), characterized by undetectable HCV RNA (<10–15 international units [IU]/mL) 24 weeks after the end of treatment, which corresponds to viral eradication in more than 99% of cases (Swain *et al.* 2010; Manns *et al.*, 2001). This combination therapy is suboptimal yielding a SVR in approximately 80% and 40–50% of patients infected with HCV genotypes 2/3 and 1/4, respectively (Ghany *et al.*, 2011). Thanks to the development of new model systems to study HCV, insight into multiple steps of the viral life cycle has now been obtained, allowing for the development of new anti-HCV drugs.

The high heterogeneity of HCV and the lack of representative culture systems have hampered HCV vaccine development, preclinical drug testing, assessment of

neutralizing antibodies, and basic HCV research (Li *et al.*, 2012a). The chimpanzee is the only true animal model for HCV and has been extensively used to study various aspects of HCV biology. Although a number of HCV full-length genomes were shown to be infectious in chimpanzees (Yanagi *et al.*, 1997), infection could not be achieved in cell culture until a replicon system was developed in 1999 (Lohmann *et al.*, 1999). The replicon system is an artificial genomic or sub-genomic self-replicating HCV RNA that partially mimics the replication cycle of HCV but without production of infectious particles (Figure 1.2). In the sub-genomic replicon system, the region of HCV genome encoding structural proteins is replaced with a cassette containing a neomycin phosphotransferase II (NPT) gene and an internal ribosome entry site (IRES) from the encephalomyyocarditis virus (EMCV). The latter drives the translation of HCV non-structural HCV proteins.

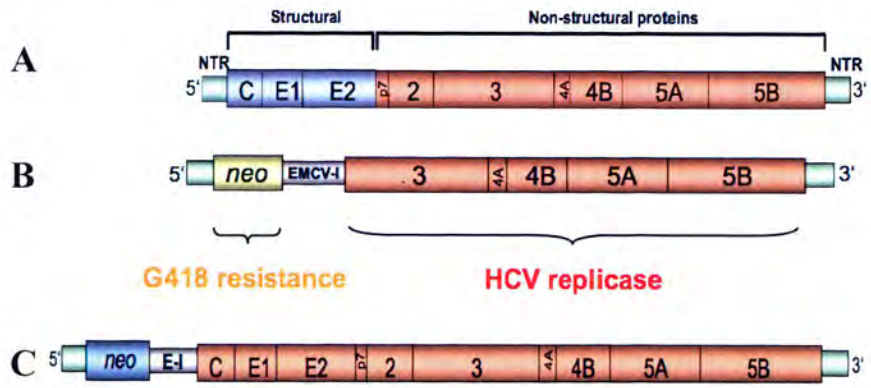

Figure 1.2: Schematic representation of the HCV replicons. (A) HCV full-length genome, (B) a sub-genomic selectable, bicistronic replicon carrying the NS3-NS5B coding region, (C) a selectable full-length bicistronic replicon carrying the C-NS5B coding region. NTR, non-translated region; Neo, neomycin phosphotransferase; EMCV-I, IRES from encephalomyocarditis virus; E-I, EMCV IRES. Adapted from Bartenschlager *et al.*, 2005.

Upon transfection of human hepatoma cell line Huh-7 with *in vitro* transcripts derived from cloned replicon DNA and treatment with geneticin (G418), selection for positive self-replicating RNA clones can be achieved due to neomycin-mediated resistance to G418 (Bartenschlager and Sparacio, 2007). With this approach, cell lines could be

established containing high amounts of self-replicating HCV RNA and proteins (Lohmann *et al.*, 1999). Subsequenst studies showed that the viral sub-genomic RNAs replicating in these cells acquired non-synonymous mutations in the non-structural proteins. These mutations were named cell culture-adaptive mutations and were shown to be essential for the replication of HCV subgenomes in cell culture (Blight *et al.*, 2000). However, when these mutations were introduced in full-length HCV genomes, despite efficient HCV RNA replication, no virus particle production could be detected (Murray and Rice, 2011). One possible explanation for the lack of virus production is that cell culture-adaptive mutations are detrimental for the late steps of HCV life cycle, such as assembly of virus particles (Pietschmann *et al.*, 2009; Bukh *et al.*, 2002). Nevertheless, these replicons provided the first system to study HCV replication in cultured cells and were instrumental for the development of DAAs (Scheel and Rice, 2013). In addition, the replicon system allowed selection of Huh-7-derived subclones, which were highly permissive to HCV RNA replication (Blight *et al.*, 2002).

With the introduction of highly permissive cell clones and cell culture adapted HCV replicons, transient replication systems became available most often based on replicons in which the NPTII gene was replaced with a chimeric gene encoding the firefly luciferase protein fused in-frame with the NPTII gene (Bartenschlager and Sparacio, 2007). Thus far, sub-genomic replicon systems have been reported for genotypes 1, 2, 3, 4, and 6 (Yu *et al.*, 2014; Peng *et al.*, 2013; Saeed *et al.*, 2013; Saeed *et al.*, 2012; Blight *et al.*, 2003; Kato *et al.*, 2003a; Kato *et al.*, 2003b; Blight *et al.*, 2000; Lohmann *et al.*, 1999). The recent establishment of sub-genomic replicon systems for HCV genotypes 3, 4, and 6 by Saeed *et al.*, 2012; Saeed *et al.*, 2013, Peng *et al.*, 2013, and Yu *et al.*, 2014, respectively, provides an important extension to foster HCV research and eventually also therapy development as they open the way to screen DAAs with a pangenotype activity. This study reports the generation of cell culture systems for HCV genotype 5a, the predominant genotype in South Africa.

1.2 PROBLEM STATEMENT

Progress in understanding HCV and developing new and more effective antiviral agents is partly hampered by the lack of *in vitro* replication systems for all major HCV genotypes. Sub-genomic replicon systems for genotypes 1 and 2 were

developed more than a decade ago, and have been extensitvely used to understand different aspects of HCV RNA replication and to screen various antiviral compounds. However, intergenotypic differences in response to antiviral compounds have led to a realization that such systems for other genotypes should also be developed. Recently, replicon systems have been reported for genotypes 3, 4 and 6 (Yu *et al.*, 2014; Peng *et al.*, 2013; Saeed *et al.*, 2013; Saeed *et al.*, 2012), however, no such systems have yet been published for HCV genotypes 5 and 7.

1.3 AIM OF THE STUDY

The aim of the study is to generate functional cell culture replication systems to study HCV genotype 5a, the predominant genotype in South Africa

1.4 OBJECTIVES OF THE STUDY

The objectives of the study are to:

- Determine the full-length consensus sequences of HCV genotype 5a from the plasma of infected individuals from South Africa
- Generate sub-genomic replicons with potential adaptive mutations and test their replication efficiency in Huh-7.5 cells
- Generate chimeric constructs expressing structural proteins of GT 5a and non-structural proteins of JFH1 and examine their ability to replicate and produce infectious virus particles in Huh-7.5 cells

1.5 STATEMENT OF SIGNIFICANCE

With more than 170 million people chronically infected with HCV coupled with the long-term complications associated with HCV infection namely fibrosis, cirrhosis and HCC, HCV contributes substantially to human morbidity and mortality and remains the most common indication of liver transplantation worldwide. Further, the lack of a cell culture replicon system for HCV GT 5a, essential for understanding the viral life cycle and developing antiviral compounds, has hampered research on this particular genotype. The establishment of cell culture replication systems for genotype 5a will therefore be a significant development that will pave the way for testing of the efficacy of new and current viral inhibitors against this genotype, which is a primary cause of HCV infections in the South African population.

CHAPTER 2
LITERATURE REVIEW

2.1 HCV DISEASES, HETEROGENEITY, AND CURRENT TREATMENT

Hepatitis C is a disease with a significant global impact and chronic hepatitis C virus (HCV) infection affects an estimated 150-170 million people worldwide (Lavanchy, 2011; Negro and Alberti, 2011, WHO, 2011). However, acute infections often go unnoticed, while around 70% of infections become chronic. In 10- 20% of patients, chronic hepatitis leads to liver cirrhosis after 10-20 years. Furthermore the yearly incidence rate for development of hepatocellular carcinoma is 1-4% for cirrhotic patients (Pawlotsky and McHutchison, 2004; NIH Consensus Statement, 2002). Chronic HCV infection may also be associated with a range of extrahepatic disease manifestations, which can be mediated by virus-specific immune complex injury and include mixed cryoglobulinaemia vasculitis, a syndrome in which cryoglobulin-containing immune complexes deposit in small- and medium-sized blood vessels causing inflammation in skin, kidney and/or other tissues, membranous glomerulonephritis and arthritis (Rehermann, 2013; Agnello *et al.*, 1992). Genotype 2 is commonly reported in patients with mixed cryoglobulinaemia, B-cell non-Hodgkin lymphoma and autoimmune hepatitis, with a high frequency of 2a seen in monoclonal gammopathies (Zignego *et al.*, 1996), B-cell non-Hodgkin lymphoma (Luppi *et al.*, 1998), and autoimmune hepatitis (Michitaka *et al.*, 1994).

The risk factors for HCV infection include transfusion of unscreened blood and blood products and solid organ transplants from infected donors, injection drug use (IDU), unsafe medical injections, sexual intercourse (especially between men), occupational exposure to blood, and from mother to her infant. Vertical transmission, sex with an infected partner has also been demonstrated by prospective and retrospective studies of persons with acute infection (Alter, 2007; Alter 2002; Terrault, 2002). However, exposure to contaminated blood or blood products, particularly IDU, is the most efficient mode of HCV transmission (Sulkowski and Thomas, 2003). Exposure to blood products and transmission of HCV to haemophiliacs has also been reported (Posthouwer *et al.*, 2006). In most instances the source of the virus is blood (Thomas, 2013). Although HCV RNA has been amplified from semen, saliva, tears and urine,

there is little evidence that these fluids are important sources of transmission of the virus, possibly because there are too few intact viruses in these fluids and/or percutaneous exposure to them is uncommon (Mendel *et al.*, 1997).

Further, transmission of HCV from environmental sources by cross-contamination from reused needles and syringes, multiple-use medication vials, infusion bags, and injecting-drug use equipment has been documented (Alter, 2007; Williams *et al.*, 2004). Coinfection of HCV with HIV is a relatively common clinical occurrence due to the fact that these two viruses have similar modes of transmission (Sulkowski, 2008; Khalili and Behm, 2002). It is estimated that one third of HIV positive persons worldwide are co-infected with HCV (Hoffmann *et al.*, 2012; Jones *et al.*, 2005) and coinfections occur most frequently among injection drug users (Strader, 2005; Verucchi *et al.*, 2004; Borgia *et al.*, 2003). However, based on the relative efficiency of transmission, the prevalence of HCV coinfection varies depending on the route of HIV transmission, which is 10-14% in persons reporting high-risk sexual exposure and 85-90% between those reporting IDU (Sulkowski and Thomas, 2003). Further, coinfection of HCV with HBV accounts for 75% of all cases of liver disease worldwide.

Transmission of HCV by injection drug use is the primary mode of transmission in developed countries like the USA and Australia, where this accounts for 68% and 80% of HCV infections, respectively (Dore *et al.*, 2003; Alter, 2002). Previously in developed countries, an average of 15 years after blood transfusion, approximately 75% of patients became HCV RNA positive and 15-20% of cases developed liver cirrhosis (Prati, 2002; Yamamoto *et al.*, 1994). However, since the screening of HCV blood products in the early 1990's, there were no reported cases of HCV transmission as a result of blood transfusion as far back as 2002 (CDC, 2003; Prati, 2002). In developing countries, the scenario is different, and HCV transmission through blood transfusions remains a major cause of concern (Prati, 2006; Shepard *et al.*, 2005; Alter, 1997). Screening of blood products in these settings is hampered by poverty, lack of infrastructure, frequent electricity breakdowns, unavailability of qualified professionals, inadequate supply of laboratory instruments and reagents, as well as difficulties in the mobilisation of volunteer donors (Aslam and Syed, 2005; Fraser, 2005; Shepard *et al.*, 2005).

Cases of viral hepatitis due to breaks in infection control techniques have been described among dialysed patients since the late 1960s (Kamar *et al.*, 2006). The length of time on dialysis and the number of blood transfusions were the main contributing factors associated with increased prevalence of HCV infection in developed countries. Although the introduction of anti-HCV donor testing and the use of erythropoietin to reduce transfusion requirements did not completely abolish infection, there has been a progressive decrease in the rate of occurrence (Jadoul, 2005; Jadoul *et al.*, 2004). In developing countries, including several of the most populous nations in the world, the risk of acquiring infection through medical procedures is not limited to occasional outbreaks. The use of contaminated injection equipment causes a steady number of unrecognised transmissions on a daily basis and it is the major risk factor for HCV infection (Simonsen *et al.*, 1999).

Data reported by WHO on the worldwide prevalence of HCV shows a high degree of geographic variability in its distribution (Figure 2.1). Although not all nations have adequate means to carry out thorough surveys, epidemiological studies in different regions of the world suggest wide variance in HCV prevalence patterns, with higher incidences of HCV among less developed nations. The highest reported prevalence rates are seen in Africa and Asia whereas industrialised nations in North America, northern and Western Europe, and Australia show a lower prevalence rate. For example in populous nations in the developed world like the USA, France, Canada, and Australia, the HCV prevalence rates are 1.8%, 1.1%, 0.8%, and 1.1%, respectively (WHO, 2007).

In the developing world, there is a wide range of prevalence estimates and generally less available data to validate assumptions about the burden of disease (Shepard *et al.*, 2005). In populous developing countries like India the prevalence is 0.9%. China and Nigeria record an estimated prevalence of HCV infection of 2.1% in the general population, 3.6% in blood donors, and 5.1% in high-risk populations (Madhava *et al.*, 2002). South Africa has a seroprevalence of 1.7% to 3.2% (Lavanchy, 2011) and studies have shown that genotype 5a is the predominant type (Gededzha *et al.*, 2012; Prabdial-Sing *et al.*, 2008; Chamberlain *et al.*, 1997; Sithebe *et al.*, 1996; Smuts and Kannemeyer, 1995). In Egypt, the prevalence is as high as 22% (WHO, 2011). Egypt's extremely high prevalence is presumably as a result of the universal spread of

HCV via blood products but as a result of HCV transmission through widespread anti-schistosomal injection treatment in the 1970s. Although the anti-schistosomal campaigns were stopped in the early 1980s, the prevalence and incidence of HCV in Egypt remains high (Kamal and Nasser, 2008).

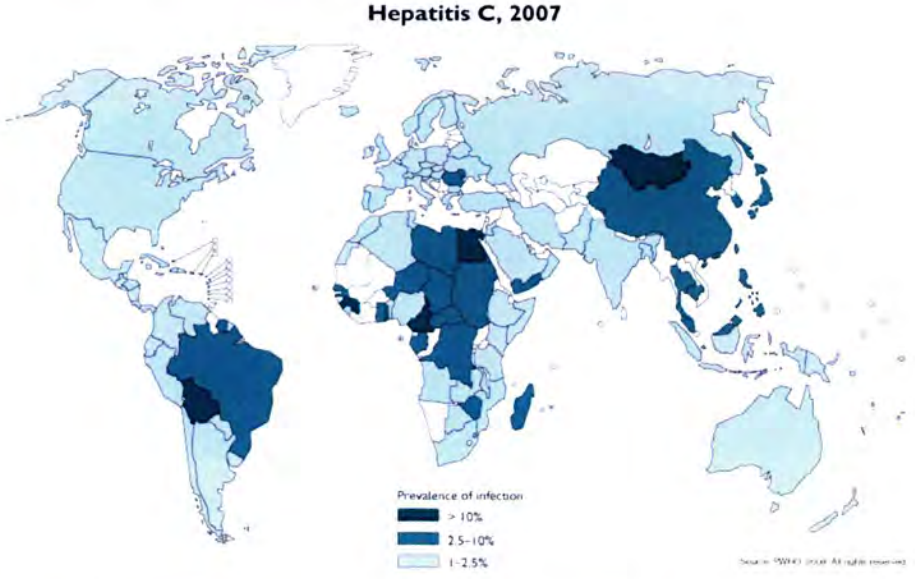

Figure 2.1: Global incidence of HCV. Adapted from WHO, 2007.

HCV is remarkably heterogeneous and consists of seven major genotypes and numerous subtypes (Smith *et al.*, 2013; Bukh *et al.*, 1993). At the nucleotide level genotypes and subtypes differ at around 30% and 20%, respectively; isolates within a subtype differ 2-10% (Simmonds *et al.*, 2005; Bukh *et al.*, 1993). Similar differences are observed at the amino acid level. Most genotypes appear to have very similar pathogenic features; yet HCV genotype 3 has been associated with increased risk of liver steatosis (Negro, 2012). Within each genotype are subtypes and quasispecies (Raghuraman *et al.*, 2003). The HCV genotypes are widely distributed with certain genotypes being predominant in specific areas (Figure 2.2). Genotypes 1-3 are widely distributed with subtypes 1a, 1b, 2a, 2b and 3a commonly seen in Western Europe and USA. Genotype 4 is common in the Middle East and Central Africa, with 4a dominating in the majority of HCV infections in Egypt (Webster *et al.*, 2000),

although new subtypes and variants of genotype 4 have also been reported in South Africa (Gededzha *et al.*, 2012). In Hong Kong, Macau and Vietnam, approximately one-third of HCV infections are attributed to genotype 6a (Webster *et al.*, 2000).

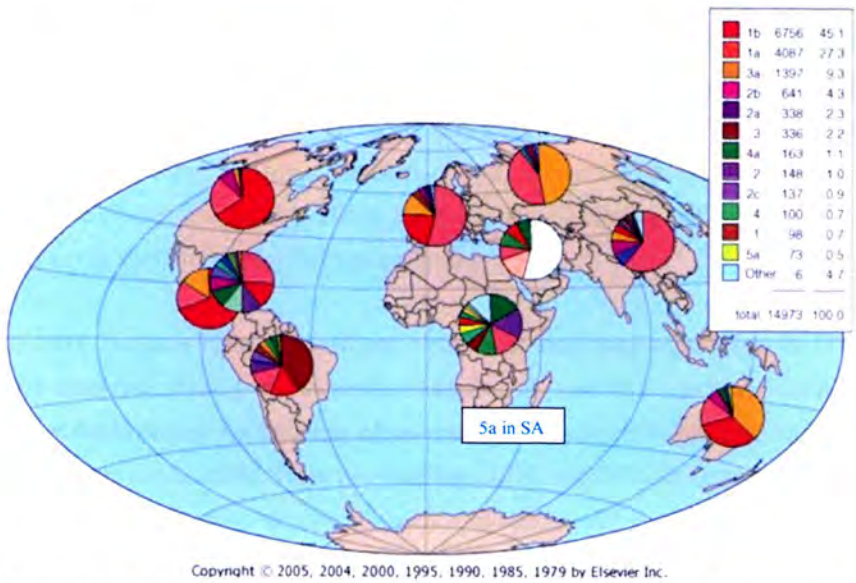

Figure 2.2: Distribution of HCV genotypes and subtypes worldwide. Adapted from Kuiken *et al.*, 2005.

Genotype 5a is thought to be responsible for 30-50% of hepatitis C cases in South Africa; however, the epidemiology of genotype 5a seems to be more diverse as cases have been reported in the Middle East (Antaki *et al.*, 2009). Following a few reports on sporadic HCV genotype 5 infections in the United Kingdom, the Netherlands, Ireland, Australia, Canada, Brazil, and Germany (Levi *et al.*, 2002; Ross *et al.*, 2000), further studies demonstrated that HCV genotype 5 infections can be found worldwide. Three of these studies have reported an unusually elevated and restricted prevalence of HCV genotype 5 in Spain (Jover *et al.*, 2001), in France (Henquell *et al.*, 2004), and in Belgium (Verbeeck *et al.*, 2006). So far, only a few cases of genotype 7 have been reported, all originating from Central Africa (Smith *et al.*, 2013).

Currently, the progression to cirrhosis and end-stage liver disease can be prevented only by viral eradication through drug therapy and the objective of therapy is basically to prevent complications and death from HCV infection. The standard HCV

therapy until 2010 consisting of pegylated interferon-alpha (peg-IFN-α) combined with ribavirin (RBV) results in an overall sustained virological response (SVR) in 45–47% of patients (Manns *et al.*, 2006; Kjaergard *et al.*, 2002; Kjaergard *et al.*, 2001). However, therapy response strongly depends on HCV genotype, with genotypes 1 and 4 being hard to treat compared to 2 and 3. However, the peg-IFN-α + RBV combination therapy is suboptimal since it requires prolonged treatment duration (48 versus 24 weeks for genotype 1 versus 2/3) associated with significant side effects including myalgia, arthralgia, headache, fever, severe depression and haemolytic anaemia (Wong and Terrault, 2005). Further, many patients are not able to adhere to therapy or contraindications prevent treatment.

In 2011, two new FDA approved oral NS3-4A protease inhibitors; telaprevir (TVR) and boceprevir (BOC), in combination with PegIFN and RBV was recommended as the standard of care for treatment of patients with chronic HCV genotype 1 infection (Sulkowski *et al.*, 2014). These two additions, BOC and TVR, represented a new era of therapy, as they were the first clinically available hepatitis C direct acting antiviral (DAA) agents that directly inhibit viral replication (Ferrante *et al.*, 2013; Yee *et al.*, 2012). In clinical trials of HCV genotype 1 infected patients receiving PegIFN and RBV, combined with BOC or TVR, SVR was achieved in 63–75% of treatment-naïve patients, in 69–88% of PegIFN and RBV relapsers, and in up to 33% of PegIFN and RBV nonresponders (Bacon *et al.*, 2011: Jacobson *et al.*, 2011; Poordad *et al.*, 2011; Sherman *et al.*, 2011; Zeuzem *et al.*, 2011). However, the efficacy of these protease inhibitors was limited by the emergence of resistance mutations, and limited efficacy in certain subgroups (including nonresponders to PegIFN/RBV and patients with co-morbidities or progressed liver disease). Furthermore, triple therapy is associated with more adverse side effects, requiring closer patient follow-up than treatment with PegIFN and RBV alone (Pawlotsky, 2011; Sulkowski *et al.*, 2011; Sarrazin and Zeuzem, 2010; Sulkowski, 2008).

Of the two additional HCV NS3/4A protease inhibitors, simeprevir and faldaprevir, which entered phase III clinical trials for the treatment of HCV genotype 1 infection (Gane *et al.*, 2012; Sulkokowski *et al.*, 2012), simeprevir (Olysio-Janssen), an NS4/4A protease inhibitor was approved for use in November 2013 by the United States Food and Drug Administration (FDA) in combination with PegIFN and RBV

for treatment of genotype 1 infections (Zeuzem *et al.*, 2014; Caceres, 2014). Sofusbuvir (Sovaldi-Gilead), a nucleotide analog NS5B polymerase inhibitor, was also approved by the FDA for treatment of genotypes 1, 2, 3, and 4 infections in December 2013. While these drugs show significant improvement over other drugs currently being used, adverse effects like nausea, headache, fatigue etc have been reported (Caceres, 2014). More current research studies, which are focused on the development of protease inhibitors with pan-genotypic activities that also reduce viral escape through commonly detected resistance-associated mutations (Vermehren and Sarrazin, 2012), and ongoing and future trials will determine the best antiviral combinations for treatment of all patients. Table 2.1 below shows a detailed mechanism of action of the above-mentioned antiviral drugs.

Table 2.1: Mechanism of action of antiviral compounds

Compounds	Mechanism of Action
Interferon α-2a	Peginterferon alfa-2a consists of interferon alfa-2a covalently linked to a 40-kd branched polyethylene glycol (PEG). The biologic activity of peginterferon-alfa-2a derives from its interferon alfa-2a moiety, which impacts both adaptive and innate immune responses against hepatitis C virus. This alpha interferon binds to and activates human type 1interferon receptors on hepatocytes, which activates multiple intracellular signal transduction pathways, culminating in the expression of interferon-stimulated genes that produce an array of antiviral effects, such as blocking viral protein synthesis and inducing viral RNA mutagenesis (Ghany *et al.*, 2011).
Ribavirin	Ribavirin is a purine nucleoside analog that has an incompletely understood mechanism of action against hepatitis C virus. Investigators have proposed four main potential sites of ribavirin action against hepatitis C virus: (1) Augmentation of host T-cell immune clearance of HCV, (2) Inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH) that results in depleted pools of guanosine triphosphate, an essential substrate for viral RNA synthesis (3) Direct inhibition of HCV replication, and (4) Induction of RNA virus mutagenesis that drives HCV to an abonormally high error rate (Brok *et al.*, 2010).
Danoprevir	Danoprevir is a NS3/4A protease inhibitor that suppresses HCV replication by inhibiting the NS3 proteolytic activity (Jiang *et al.*, 2013).
Daclatasvir	Daclatasvir inhibits the HCV nonstructural protein NS5A, which plays an important role in replication, assembly, and release of viral particles thereby inhibiting replication (Boulant *et al.*, 2007).
Simeprevir	Simeprevir is a NS3/4A HCV protease inhibitor. Simeprevir is a macrocyclic compound that non-covalently binds to and inhibits the NS3/4A HCV protease, a protein that is responsible for cleaving and processing the HCV-encoded polyprotein, a critical step in HCV viral life cycle (Zeuzem *et al.*, 2014).

Faldeprevir	Faldeprevir is an NS3/NS4A protease inhibitor, which has a chiral cyclopropane, hydroxy propinyl unit (Gane *et al.*, 2014). The HCV NS3/4A protease blocks the phosphorylation and effector action of interferon regulatory factor-3 (IRF-3), a key cellular antiviral signaling molecule (Schoggins *et al.*, 2013).
Boceprevir	Boceprevir is a NS3/4A protease inhibitor. Specifically, boceprevir inhibits the proteolytic cleavage of the HCV encoded polyprotein, an essential step in the viral life cycle for the production of mature forms of the viral proteins NS4A, NS4B, NS5A, and NS5B (Manns *et al.*, 2014).
Telaprevir	Telaprevir (*Incivek*) is a NS3/4A hepatitis C protease inhibitor. Specifically, telaprevir inhibits the proteolytic cleavage of the HCV encoded polyprotein, an essential step in the viral life cycle for the production of mature forms of the viral proteins NS4A, NS4B, NS5A, and NS5B (Kieran *et al.*, 2013).
Ledipasvir	Ledipasvir is a potent inhibitor of HCV NS5A, a viral phosphoprotein that plays an important role in viral replication, assembly, and secretion. Nevertheless, the exact mechanism of action for NS5A inhibitors remains poorly understood (Afdhal *et al.*, 2014; Gane *et al.*, 2014).
Sofosbuvir	Sofosbuvir is a nucleotide analog inhibitor of hepatitis C virus NS5B polymerase—the key enzyme mediating HCV RNA replication. It is metabolized to the active antiviral agent 2'-deoxy-2'-α-fluoro-β-*C*-methyluridine-5'-triphosphate. This triphosphate form mimics the natural cellular uridine nucleotide and is incorporated by the HCV RNA polymerase into the elongating RNA primer strand, resulting in chain termination (Afdhal *et al.*, 2014).

2.2 THE HEPATITIS C VIRUS AND THE VIRAL LIFE CYCLE

2.2.1 HCV viral particle

A hallmark of HCV particles is their tight association with cellular lipoproteins and lipids that thus determine both morphology and biophysical properties of the virion. Because *in vivo* liver cells and cultured human Huh cells can differ in their capability to produce lipoproteins, HCV particles vary in their properties, depending on the host cell in which they are produced (Bartenschlager *et al.*, 2011). The HCV viral particle is a ~50nM enveloped, single-stranded, positive-sense RNA virus (Wakita *et al.*, 2005). In the viral particle, the RNA genome is encapsulated by Core protein monomers. The capsid is surrounded by a host-cell derived double-layer lipid envelope, in which the highly glycosylated envelope proteins E1 and E2 are embedded (Figure 2.3, Panel 7).

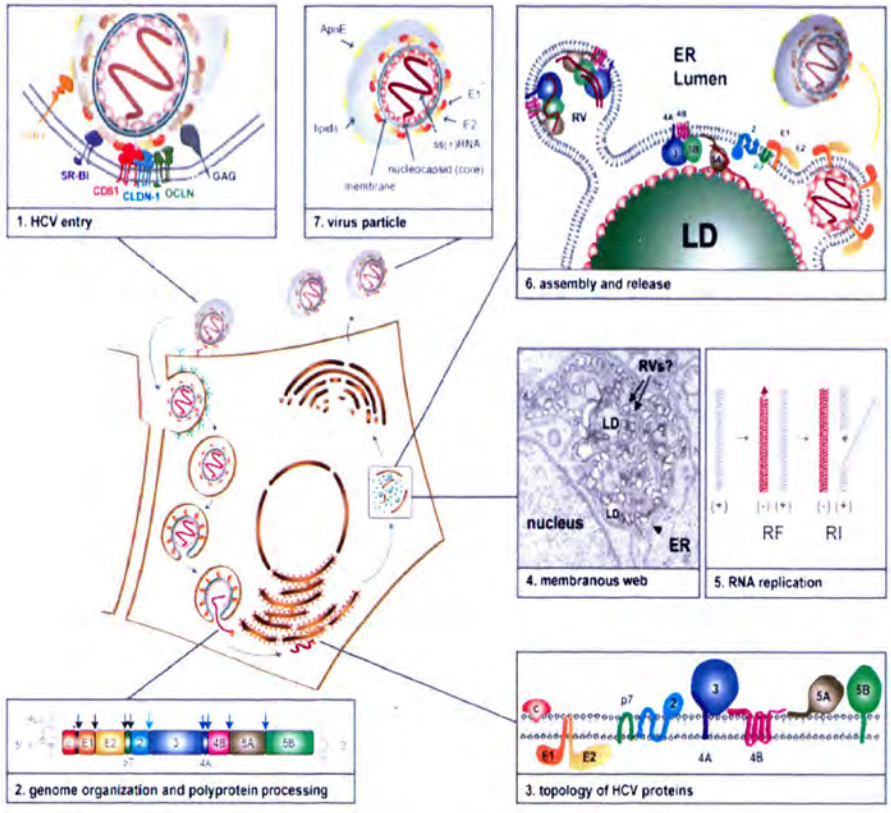

Figure 2.3: Scheme of HCV life cycle. Adapted from Bartenschlager *et al.*, 2010

Viral particles associate with low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) in the infected host (Andre *et al.*, 2005), which is thought to contribute to the heterogenous and low boyant density of HCV (Gastaminza *et al.*, 2006). The E2 protein contains hypervariable regions (HVR) containing major neutralization epitopes thought to function either as an immunological decoy, or to shield conserved neutralization epitopes (Prentoe *et al.*, 2011; Bankwitz *et al.*, 2010). It has been shown that HCV virions produced in cell culture (HCVcc) have a spherical envelope containing tetramers (or dimer of heterodimers) of the HCV E1 and E2 glycoproteins (Yu 2007; Heller *et al.*, 2005; Wakita *et al.*, 2005).

2.2.2 The HCV genome

HCV is an enveloped RNA virus of the *Flaviridae* family, genus *Hepacivirus*. The single-stranded positive sense RNA molecule genome of around 9600 nucleotides contains a single open reading frame (ORF) encoding a polyprotein precursor of 3010-3033 amino acid (aa) residues (Gastaminza *et al.*, 2010a; Suzuki *et al.*, 2007). At the 5' and 3' ends of the RNA are the untranslated regions (UTRs) or non-coding regions (NCRs) that are not translated into proteins but which are important for translation and replication of the viral RNA (The 9.6kb positive- strand RNA genome is schematically depicted in Panel 2 of Figure 2.3). The 5' UTR has a ribosome-binding site (IRES - Internal Ribosomal Entry Site) that initiates translation yielding a polyprotein precursor. The polyprotein precursor is processed by viral and host proteases to produce 10 mature viral proteins: the structural proteins (core, E1, and E2), p7 encoded at the N-terminus, as well as the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Gottwein and Bukh, 2008; Suzuki *et al.*, 2007; Lindenbach and Rice, 2005, Bartenschlager *et al.*, 2004).

2.2.3 The 5' untranslated region (UTR)

The 5'UTR of the HCV genome is around 341 nucleotides (nt). There is more than 90% sequence identity among different HCV genotypes, with some segments nearly identical among different strains (Bukh *et al.*, 1992). The secondary and tertiary structures of this region are also largely conserved (Honda *et al.*, 1999; Honda *et al.*, 1996). A combination of computational, phylogenetic, and mutational analyses of the HCV 5' UTR has identified four major structural domains (domains I-IV), most of which are also conserved among HCV genotypes, GBV-B and nonprimate hepacivirus (NPHV) also from the *Hepacivirus* genus, and the related pestiviruses (Burbelo *et al.*, 2012; Honda *et al.*, 1999; Honda *et al.*, 1996; Smith *et al.*, 1995).

2.2.4 Structural proteins

2.2.4.1 Core protein

Core is a highly basic protein with RNA binding capacity and constitutes the major component of the nucleocapsid. The Core protein is found on endoplasmic reticulum (ER) membranes and it has been shown that Core associates with lipid droplets; this interaction may have a role during viral replication and/or virion morphogenesis (Miyanari *et al.*, 2007; McLauchlan *et al.*, 2002). In addition, it might affect lipid

metabolism, contributing to the development of liver steatosis, which is often seen in hepatitis C (Asselah *et al.*, 2006).

2.2.4.2 Envelope proteins

Both E1 and E2 are heavily glycosylated type 1 transmembrane proteins of 31-35 kDa (E1) and 70-72 kDa (E2), with five and 11 potential N-glycosylation sites, respectively, most of which are well conserved across genotypes and are important for folding and surface expression of the proteins (Op De Beeck *et al.*, 2004). The E1 and E2 glycoproteins are embedded into the lipid envelope surrounding the viral nucleocapsid. They assemble as a non-covalently bound, heterodimeric complex on the surface of the virion, and intra-molecular disulfide bonds stabilize the individual proteins in the folded state (Flint *et al.*, 2004; Bartosch *et al.*, 2003).

2.2.5 The p7 protein

The p7 protein is a small hydrophobic polypeptide membrane protein of 63 amino acid residues with both termini oriented towards the ER lumen (Gentzsch *et al.*, 2013). It contains an N-terminal α-helix followed by two transmembrane (TM) domains connected by a cytosolic loop (aa 33–39). Importantly, p7 monomers can assemble into hexamers or heptamers (Luik *et al.*, 2009; Clarke *et al.*, 2006; Griffin *et al.*, 2003) and thereby form cation-selective ion channels in planar lipid bilayers, thus classifying p7 as a viroporin, and thereby enhancing membrane permeability (Montserret *et al.*, 2010; Premkumar *et al.*, 2004; Griffin *et al.*, 2003; Pavlovic *et al.*, 2003). Viroporins are small, virally-encoded proteins that, once inserted into cellular membranes, homo-oligomerize to form pores increasing permeability to ions and small molecules (Ciampor, 2003; Gonzalez and Carrasco, 2003). In many cases, this channel activity is essential for viral propagation and infectivity. Further, a more recent study has also reported p7-mediated H^+ intracellular conductance (Wozniak *et al.*, 2010).

2.2.6 Non-structural proteins

The HCV non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) play a major role in the viral life cycle. NS2 is a membrane-associated cysteine protease of 21-23kDa responsible for *cis* cleavage at the NS2-NS3 junction within its C-terminal domain (Lorenz *et al.*, 2006). The C-terminal domain of NS2, together with the N-terminal third of NS3, forms the NS2-3 protease, an enzyme that catalyzes a single

cleavage between the two proteins. The crystal structure of the C-terminal domain of NS2 has been determined and reveals a dimeric protease containing two composite active sites (Lorenz *et al.*, 2006). The significance of sequences within NS2 to the infectivity of intergenotypic chimeras has suggested that NS2 may play a role in infectious virus production (Yi *et al.*, 2007; Pietschmann *et al.*, 2006).

The NS3 protein is a fairly hydrophobic protein of 69kDa with a serine protease located at the N-terminal one-third and an RNA helicase/NTPase located in the C-terminal (Yao *et al.*, 1999). The central portion of NS4A is important for efficient processing of the non-structural proteins by NS3 and serves as a co-factor of the NS3 protease. It is also important in tethering NS3 to cellular membranes (Pang *et al.*, 2002). The 27 kDa NS4B protein is an integral membrane protein that plays a role in the formation of a cellular "membranous web", the specific membrane alteration where HCV RNA replication occurs (Romero-Brey *et al.*, 2012; Gossert *et al.*, 2003; Egger *et al.*, 2002). It is predicted to contain at least four transmembrane domains and an N-terminal amphipathic helix that is responsible for membrane association (Lundin *et al.*, 2003). NS4B is required during replication (Jones *et al.*, 2009), where binding of HCV RNA might be necessary (Einav *et al.*, 2008).

The 56-58 kDa NS5A phosphoprotein plays an important role in the regulation of HCV replication, assembly and release (Boulant *et al.*, 2007). It consists of three domains (Tellinghuisen *et al.*, 2004) that are separated by trypsin-sensitive low complexity sequences (LCS I and LCS II) and is a component of the viral replication complex (Gosert *et al.*, 2003). It is anchored to intracellular membranes through the N-terminal amphipathic alpha-helix (Penin *et al.*, 2004; Tellinghuisen *et al.*, 2004; Brass *et al.*, 2002), and is necessary for efficient HCV RNA replication and virus production (Appel *et al.*, 2008; Tellinghuisen *et al.*, 2008; Tellinghuisen *et al.*, 2004). Domain I, of which the crystal structure has been determined, contains a zinc-binding domain that is involved in RNA binding (Love *et al.*, 2009; Tellinghuisen *et al.*, 2005) and a highly basic channel binding RNA (Huang *et al.*, 2005).

The role of domain II and domain III of NS5A in the HCV replication cycle is unknown. However replication-enhancing mutations were mapped to a region spanning the C-terminal part of domain I and LCS I arguing that these sequences are important for efficient RNA replication (Lohmann *et al.*, 2003; Blight *et al.*, 2000). In

contrast, domain III can be deleted or replaced by green fluorescent protein (GFP) with no dramatic effect on RNA replication (Appel *et al.*, 2005; Moradpour *et al.*, 2004). Phosphorylation of NS5A was suggested to influence regulation of HCV replication and virus production (Appel *et al.*, 2005; Evans *et al.*, 2004; Neddermann *et al.*, 2004).

NS5B, on the other hand, is a membrane-anchored 68kDa protein. NS5B has been shown to possess RNA-dependent RNA polymerase (RdRp) activity that is required for the replication of the positive stranded viral genomes (Butcher *et al.*, 2001). Its C-terminal region forms an alpha helical transmembrane domain, which is dispensable for polymerase activity *in vitro* but is responsible for post-translational targeting to the cytoplasmic side of the ER (Moradpour *et al.*, 2004).

2.2.7 The 3' untranslated region (UTR)

The 3'UTR of HCV is approximately 200 and 235 nucleotides long and it is critical for RNA replication. The 3' UTR consists of three distinct regions: a variable region, which follows immediately the termination codon of the HCV polyprotein, and varies in length and composition among different genotypes. It is also highly conserved among viral strains of the same genotype (Yanagi *et al.*, 1998; Blight and Rice, 1997; Yanagi *et al.*, 1997; Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996). Next is a poly U/UC tract, which consists of a poly (U) stretch and a C (U) n-repeat region (referred to as a transitional region) and varies greatly in length and slightly in sequence among different viral isolates (Tanaka *et al.*, 1996), and a highly conserved 98-nt X region, which forms three stable stem-loop structures (3'SLI, 3'SLII, and 3'SLIII) that are highly conserved across all genotypes (Blight and Rice, 1997; Ito and Lai, 1997; Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995). This region is recognized by viral polymerases as the initiation site for plus-strand synthesis of the HCV genome (Ye *et al.*, 2005). Examination of the 3'-terminal sequences of the HCV genome in sera from infected patients revealed that most HCV RNAs contain identical 3' ends with no extra sequence downstream of the X-tail (Tanaka *et al.*, 1996).

2.3 THE VIRAL LIFE CYCLE

2.3.1 Viral entry

Viruses initiate infection by attaching to host cell molecules (receptors) and blocking particle internalization provides attractive targets for therapeutic intervention. The

liver is the primary target organ for HCV infection, but infection of T and B cells, dendritic cells, monocytes and cells of the digestive tract have also been reported (Dustin and Rice, 2007; Sansonno *et al.*, 2007; Pachiadakis *et al.*, 2005). It has been hypothesized that some form of latent infection in those cells might constitute a viral reservoir, allowing HCV to persist in immune cells for many years after it has been eliminated from the serum by successful antiviral therapy. The HCV life cycle begins with the interaction of its glycoproteins, E1 and E2, with attachment factors to the surface of hepatocytes. Entry of the virus into the cell occurs in a pH and clathrin-dependent manner mediated by a number of cellular factors. The current mode for HCV entry involves the initial binding of the virus to glycosaminoglycans (GAGs) and/or the low-density lipoprotein receptor -LDLR (Agnello *et al.*, 1999), which facilitates attachment to the liver cell (Figure 2.3, Panel 1).

The actual entry process starts by receptor-mediated endocytosis and six cell surface molecules contribute to or are essential for infection (Burlone and Budkowska, 2009): low-density lipoprotein receptor (LDLR), glycosaminoglycans (GAG), scavenger receptor class B type 1 (SR-B1) (Scarcelli *et al.*, 2002), testraspanin CD81 (Pileri *et al.*, 1998) and two proteins that are components of tight junctions, namely claudin-1 and occludin. The viral particle is subsequently relocated to tight junctions through claudin-1 (Evans *et al.*, 2007) and occludin (Ploss *et al.*, 2009) (proteins involved in cell-cell adhesion, providing an anchor to the tight-junction complex) binding (Joyce and Tyrell, 2010; Benedicto *et al.*, 2009; Liu *et al.*, 2009; Ploss *et al.*, 2009). The E1 and E2 proteins interact with the co-receptors CD81, SR-B1, claudin-1 and occludin-1 (Ploss *et al.*, 2009; Evans *et al.*, 2007). Upon claudin-1/occludin interaction, clathrin dependent endocytosis delivers the HCV RNA to the cytoplasm (Brazzoli *et al.*, 2008; Blanchard *et al.*, 2006). Once the viral RNA and capsid is released into the cytoplasm, translation of the viral RNA on the endoplasmic reticulum and establishment of the replication complexes follows. E2 is believed to be responsible for fusion with cellular membranes in low-pH compartments (Krey *et al.*, 2010).

2.3.2 Translation and replication of the viral genome

After entry and fusion/uncoating, the HCV viral genome is translated at the rough endoplasmic reticulum (rER) where the polyprotein is cleaved (The topology of the HCV proteins is depicted in Figure 2.3, Panel 3). Mediated primarily by NS4B, the

membranous web (Figure 2.3, Panel 4) is formed where RNA genomes are amplified. RNA progeny is either used for translation, replication or encapsidated into new virions. These are released from the cell by a noncytolytic pathway in tight association with the very low-density lipoprotein (VLDL) pathway. During translation at the rough ER (rER), nascent apolipoprotein B (apoB) is translocated into the ER lumen and loaded by microsomal triglyceride transfer protein (MTP) with phospholipids and triglycerides (Figure 2.4, left panel).

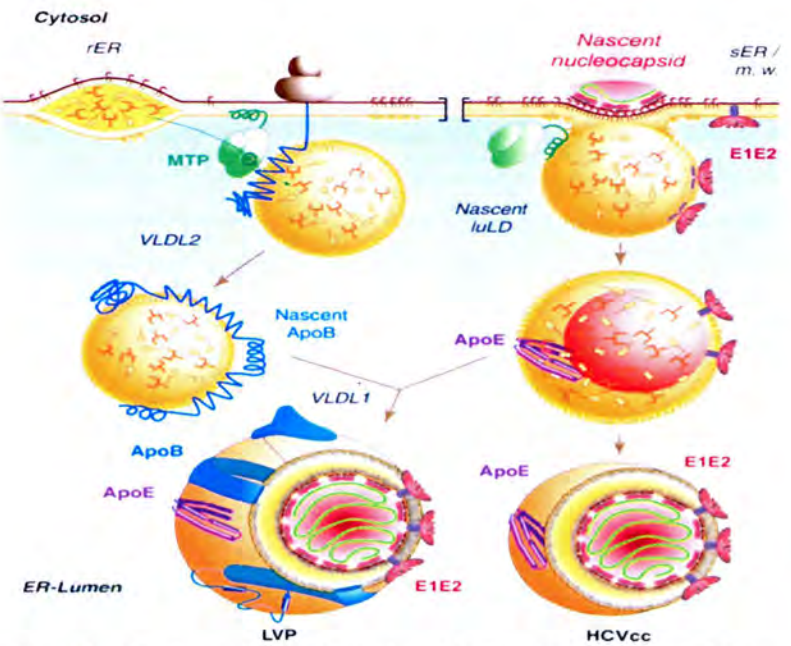

Figure 2.4: Models of structures of infectious HCV particles and their biogenesis. Adapted from Bartenschlager *et al.*, 2011.

This leads to the formation of a neutral lipid core that is converted into a spherical particle (VLDL2) acquiring exchangeable apoE and apoB. In the smooth ER (sER) or membranous web (m.w.), a second precursor (the luminal LD; luLD) is formed from the ER membrane and by MTP-mediated triglyceride enrichment (Figure 2.4, right panel). E1 and E2 retained at the ER membrane might slide onto this luLD prior to pinching-off (Icard *et al.*, 2009). The nucleocapsid would be inserted into the hydrophobic lipid Core of the pinching-off luLD due to the hydrophobic nucleocapsid surface (formed by D2 of the Core protein). In VLDL competent cells such as primary

human hepatocytes, this precursor could fuse with VLDL2 to form the lipoviroparticle (LVP). Alternatively in Huh-7 cells where VLDL1 formation is inefficient (Icard *et al.*, 2009) HCVcc is secreted predominantly as particles lacking apoB.

HCV employs the IRES in the 5' UTR to recruit ribosomes to an internal start site of translation. By the use of this IRES element, the virus avoids the need for any nuclear RNA processing events like capping. This IRES element directs the cap-independent initiation of translation of the viral genome (Firth and Brierley, 2012; Niepmann 2009). In the HCV RNA, the IRES include the stem-loops II to IV, which partially overlap with the replication signals. The IRES extends a few nucleotides into the core-coding region, since the AUG start codon is located in the apical loop of stem-loop IV. In addition to the Core IRES, the sequences and possibly RNA secondary structures in the Core-coding region (stem loops V and VI) also contribute to efficient translation (Vassilaki *et al.*, 2008).

While a variety of cellular RNA binding proteins are recruited by the HCV IRES RNA to modulate its translation efficiency (Niepmann, 2013; Hofmann and Liu, 2011), in 2005, Jopling and coworkers reported that the liver-specific microRNA-122 (miR-122) facilitates HCV RNA accumulation (Jopling *et al.*, 2005), a break-through finding that complemented the previous observation that viruses can encode their own microRNAs to modulate their host cell (Pfeffer *et al.*, 2004). Further, miR-122 was found to stimulate propagation of HCV RNA instead of repressing it and this action of miR-122 was found to be conferred by two target sites near the 5' end of the HCV RNA (Figure 2.5), while the possible roles of two other potential miR-122 binding sites in the 3' UTR and in the NS5B coding region are not yet clear. The interaction of miR-122 with its two target sites in the 5' UTR results in the formation of one or two miR-122/ RNA complexes at the very 5' end of the HCV 5' UTR. This direct interaction of miR-122 with the HCV RNA has been shown to enhance HCV RNA accumulation in Huh 7 hepatoma cells that contain endogenous miR-122 (Jangra *et al.*, 2010; Jopling *et al.*, 2008; Pedersen *et al.*, 2007; Jopling *et al.*, 2005).

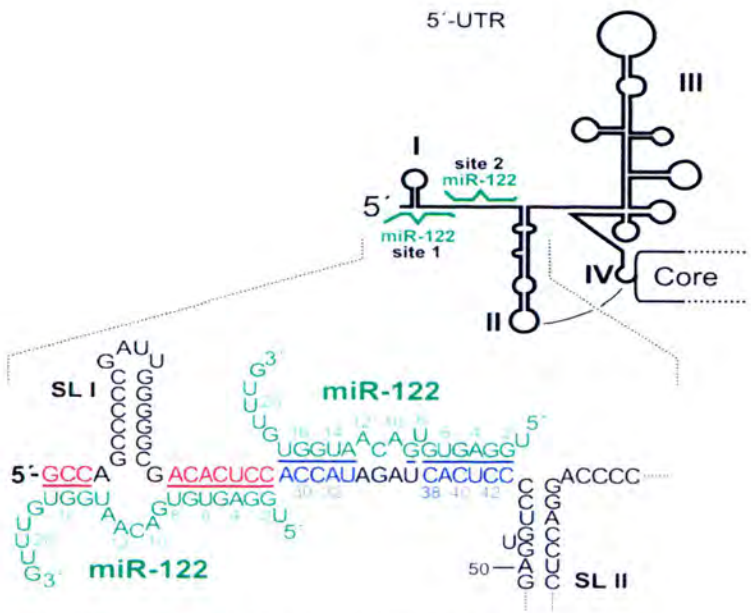

Figure 2.5: Binding of miR-122 to the HCV 5' UTR. The secondary structures of the 5' UTR is shown with the two miR-122 molecules (in green) and their binding sites 1 and 2. In the expanded view, HCV nucleotides (sequence of genotype 1b, Con1 isolate) pairing with miR-122 molecules are shown in red for target site 1 and in blue for target site 2. Adapted from Conrad and Niepmann, 2013.

In addition, ectopic supplementation of miR-122 in hepatoma cell lines that do not express significant amounts of miR-122, like HepG2 cells (Narbus *et al.*, 2011), as well as in non-hepatic cell lines (Chang *et al.*, 2008) enhanced replication of intracellular HCV RNA, showing that miR-122 is a key factor for HCV replication. Moreover, miR-122 is expressed preferentially in the liver, where it constitutes about 60-70% of the microRNAs (Jopling, 2012; Landgraf *et al.*, 2007; Fu *et al.*, 2005; Chang *et al.*, 2004; Sempere *et al.*, 2004). Thus, besides the function of surface receptors in HCV entry (Zeisel *et al.*, 2013; Perrault and Pecheur, 2009) miR-122 can be regarded as an intracellular tissue-specific factor that may contribute to the liver tropism of HCV by promoting its replication in hepatocytes but not in other cells. More recently, other microRNAs have been characterized that also bind directly to the HCV RNA but have inhibitory effects on HCV replication.

The 3'UTR, as mentioned earlier, consists of a genotype-specific variable region, a poly (U/UC) region and a highly conserved terminal 3'X region (Tanaka *et al.*, 1996; Yamada *et al.*, 1996; Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995). The poly (U/UC) region varies greatly in length, even within a single isolate, but a certain length is required for efficient replication (You and Rice, 2008; Yi and Lemon, 2003; Yanagi *et al.*, 1999). The 3' UTR stimulate IRES-dependent translation in human hepatoma (Huh) cell lines (Song *et al.*, 2006) possibly acting as a switch between translation and genome replication. The translated polyprotein is co- and post-translationally cleaved by cellular and viral proteases to release the individual HCV proteins.

According to some authors (Ma *et al.*, 2011; Popescu *et al.*, 2011; Stapleford and Lindebach, 2011; Jirasko *et al.*, 2010), NS2 forms complexes with the envelope proteins, E1 and E2, as well as with p7 and other non-structural proteins in cells supporting the production of infectious virus. They suggest that NS2 may be responsible for mediating interactions between the envelope proteins and non-structural proteins engaged in infectious particle assembly. Although NS2 itself is not required for RNA replication, its cleavage from NS3 is essential for the completion of the viral replication cycle *in vivo* and *in vitro* (Pietschmann *et al.*, 2006; Welbourne *et al.*, 2005; Kolykhalov *et al.*, 2000). The protease activity of NS3 and its NS4A cofactor is responsible for the efficient cleavage of the HCV polyprotein at all sites located downstream of its own sequence: namely, the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junction sites, while the helicase/NTPase is necessary for RNA replication (De Francesco and Migliaccio, 2005; Gossert *et al.*, 2003). Further, NS3 is necessary for assembly and release of viral particles (Ma *et al.*, 2011; Stapleford and Lindenbach, 2011; Jirasko *et al.*, 2010; Ma *et al.*, 2008).

NS4B has emerged as a key player in the viral replication cycle thanks to its several functional domains, which include a nucleotide binding and NTPase activity, and an N-terminal amphipathic helix (Thompson *et al.*, 2009; Elazar *et al.*, 2004). The C-terminal domain of the HCV NS4B protein generates a membrane web, derived primarily from endoplasmic reticulum (ER) membrane, which serves as a platform for the replication complex (Elazar *et al.*, 2004). This web in addition contains ER membranes and lipid droplets (LDs). Here viral RNA is amplified by the NS5B RNA-dependent RNA polymerase (RdRp) in conjunction with most, if not all other NS

proteins and host cell factors such as cyclophilin A (Bartenschlager *et al.*, 2010) and cellular lipid kinase phosphatidylinositol 4-kinase type III alpha (PI4KIIIa) (Reiss *et al.*, 2013). By analogy to flaviviruses (Welsch *et al.*, 2009) RNA replication may occur in invaginations of the ER membrane in a semi-conservative and asymmetric manner (Figure 2.3, Panel 5). The positive strand genome is copied into a negative strand RNA via a replicative form (RF) that is used for the synthesis of excess amounts of positive strand RNAs via a replicative intermediate (RI). Initiation of RNA synthesis requires highly structured RNA elements in the 3' UTR of the corresponding template strand. Newly synthesized RNA genomes are used for translation (production of new viral proteins), RNA replication, or formation of new infectious virions (assembly). NS5A becomes anchored to the intracellular membranes via its N-terminal amphipathic alpha helix and recently, it has been shown to be involved in viral assembly (Appel *et al.*, 2008).

2.3.3 Assembly and release of HCV particles

Assembly of HCV particles occurs through relocation of core and NS5A in close proximity of cytoplasmic lipid droplets (cLDs) where the core protein accumulates (Figure 2.3, Panel 6). These lipid droplets (LDs) are intracellular storage organelles containing a hydrophobic core of neutral lipids and cholesterol esters surrounded by a phospholipid monolayer embedded with LD-specific proteins (Beller *et al.*, 2010). LD- associated proteins are presumably loaded onto LDs at sites of ER contact (Herker *et al.*, 2010). RNA is recruited from replication sites to the core protein on LDs by the viral replicase (Miyanari *et al.*, 2007). Alternatively, NS5A, which has an intrinsic RNA binding capacity, may be released from the replicase complex and transported onto the LD surface. Via a Core–NS5A interaction, viral RNA could be delivered to the Core protein thus triggering nucleocapsid formation. These capsids may bud into the ER lumen in a process that is tightly linked to VLDL synthesis (Huang *et al.*, 2007). Thus, HCV assembly very much depends on VLDL synthesis and requires several enzymes such a microsomal triglyceride transfer protein (MTP) and inhibition of this enzyme might contribute to liver steatosis (Perlemuter *et al.*, 2002), but also apoE.

The HCV structural proteins (C, E1 and E2), which are processed by host cell signal peptidase at the ER membrane (Moradpour *et al.*, 2007), along with copies of the

HCV RNA produced during translation are packaged as infectious virus particles. The p7 channel has been shown to facilitate efficient assembly and release of infectious virions (Gentzsch *et al.*, 2013; Jones *et al.*, 2007; Steinmann *et al.*, 2007) although the precise mechanism of these functions remains unclear. Importantly, p7 is not required for RNA replication *in vitro*, but is essential for the production of infectious virions *in vivo* (Sakai *et al.*, 2003). It has been shown to interact with NS2 (Jirasko *et al.*, 2010; Tedbury *et al.*, 2010), which may be responsible for mediating interactions between the envelope glycoproteins (Phan *et al.*, 2009; Yi *et al.*, 2009) and non-structural proteins (p7, NS3 and NS5A) engaged in infectious particle assembly. According to Ma and coworkers, the absence of functional p7 will effectively abolish the ability of NS2 to interact with these other HCV proteins (Ma *et al.*, 2011). The bulk of the lipid contained in lipid droplets exits hepatocytes as VLDL, and HCV may use the assembly and secretion of VLDL to exit the cell.

2.3.4 Host immune response to HCV infection

Infection with HCV induces a series of intracellular events in the liver of infected individuals. During the viral replication process HCV is sensed as non-self by pattern recognition receptors (PRRs) in the host cell that recognize and bind pathogen-associated molecular patterns (PAMPs) within viral products once infection becomes persistent. This leads to coordinated activation of the innate and adaptive immune responses (Thompson and Iwasaki, 2008). PAMP-receptor engagement activates latent cellular transcription factors that mediate the rapid onset of gene expression, thus marking the immediate-early phase of the host response (Gale and Foy, 2005). A variety of PRRs like the retinoic acid inducible gene-1 (RIG-1)-like receptors, RIG-1, melanoma differentiation antigen 5, and the nontraditional PRR, protein kinase R (PKR) and its double stranded RNA (dsRNA)-binding activity, interacts with mitochondrial antiviral signaling protein (MAVS) to impart PRR signaling of innate immunity (McAllister and Smuel, 2009; Kumar *et al.*, 1997).

PKR binding of HCV dsRNA also activates a kinase-independent signal transduction cascade that drives induction of specific IFN-stimulated genes (ISGs) and IFN-β production by signaling through MAVS, TNF receptor–associated factor 3, interferon regulatory factors (IRFs) and nuclear factors (NF-kB), all before RIG-I activation (Schoggins *et al.*, 2013; Arnaud *et al.*, 2011; Schoggins *et al.*, 2011; Joyce and

Tyrrell, 2010). Both the innate and adaptive arms of immunity, including cross-talk between liver-resident and infiltrating cells (such as hepatocytes, Kupffer cells, plasmacytoid dendritic cells (pDCs), natural killer (NK) cells and other immune cells), contribute to the host's ability to resolve HCV infection (Reherman, 2013; Bowen and Walker, 2005). However, despite these host immune defenses (the effective non-self detection of HCV by RIG-1, TLR3, and PKR to trigger innate immune response), about 70–80% of persons with acute HCV infections do not clear the virus but progress to a chronic state (Seef, 2009). This high frequency of chronic infection reflects the fact that HCV has evolved several mechanisms to evade and suppress innate immunity, resulting in HCV progression to chronicity (Horner and Gale 2013).

2.3.5 HCV evasion of innate antiviral immunity

The NS3-NS4A protease plays a key role in the HCV innate immune evasion strategy (Figure 2.6). This multifunctional NS3-NS4A protease is required for HCV replication, during which it processes the HCV polyprotein at several sites to liberate the viral NS proteins (Morikawa *et al.*, 2011). The NS3-NS4A protease complex is anchored to intracellular membranes through the NS4A transmembrane domain and an amphipathic α-helix at the NS3 N-terminus that facilitates membrane association and cleavage of membrane-anchored substrates (Horner *et al.*, 2012; Brass *et al.*, 2008). NS3-NS4A can block RIG-I signaling, because in addition to proteolytically processing the HCV polyprotein, NS3-NS4A targets and cleaves MAVS from intracellular membranes to prevent signal transduction (Baril *et al.*, 2009; Loo *et al.*, 2006; Li *et al.*, 2005). In addition to its ability to cleave MAVS from intracellular membranes, the NS3-NS4A protease also targets and cleaves mitochondrial-associated membrane (MAM)-localized MAVS (Horner *et al.*, 2011). As MAVS must be anchored to membranes for downstream signaling, this cleavage event prevents activation of the RIG-I pathway during acute infection, abrogates IFN induction and supports the progression to chronic infection (Horner and Gale, 2013).

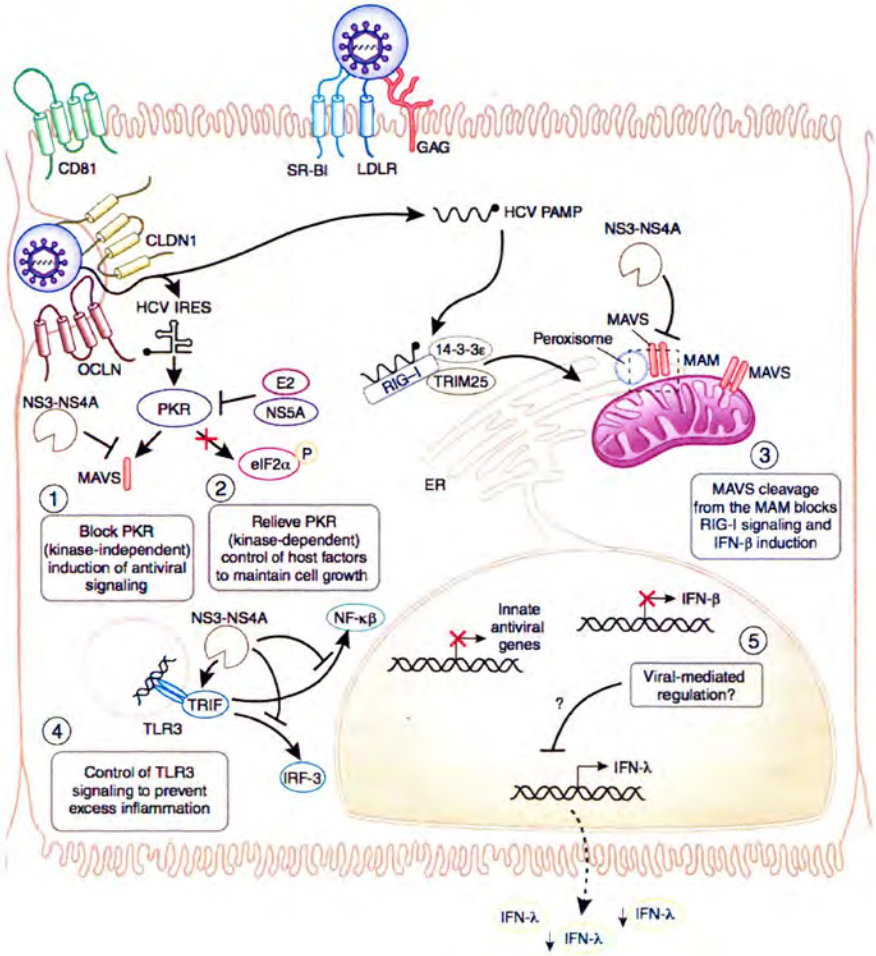

Figure 2.6: HCV control of IFN induction and immune evasion. Immune evasion by HCV in the hepatocyte occurs at several points during viral infection. The proposed regulation is shown here, where the HCV NS3- NS4A protease cleaves the signaling adaptors MAVS (on the MAM; in the region indicated by the dashed box) and TRIF to inactivate PKR (1), RIG-I (3) and TLR3 (4) signaling pathways to prevent induction of immunomodulatory innate antiviral genes and IFN-b, allowing for HCV replication. HCV infection control of IFN-l induction is not yet defined (5); HCV E2 and NS5A proteins inactivate PKR-dependent activation of the host translation factor eIF2a to reactivate protein translation during infection (2). Adapted from Horner and Gale, 2013.

2.4 MODEL SYSTEMS TO STUDY HCV INFECTION

2.4.1 Sub-genomic replicon systems

Since the discovery of HCV in 1989, the lack of a cell culture system for the production of infectious HCV virions represented the major obstacle to study the complete lifecycle of this pathogen and to devise strategies for prophylactic and therapeutic interventions (Scheel and Rice, 2013). The development of molecular clones of strain H77 (genotype 1a) infectious for chimpanzees in 1997 was an important initial advance, but these clones did not produce viral particles in cell culture (Yanagi *et al.*, 1997). A significant improvement in the study of RNA replication was the development of HCV replicon system, which depends on an infectable host cell supporting production of infectious virus progeny, and, a virus that is capable of replicating and assembling infectious particles in these cells (Bartenschlager and Pietschmann, 2005).

2.4.2 Identification of adaptive mutations enhanced HCV replication efficiency

Despite the high levels of sub-genomic RNA replication within a selected cell clone, G418 resistance arose in a very low frequency of transfected cells (Blight *et al.*, 2000; Lohmann *et al.*, 1999). This was due to two restrictions: first, replicon RNAs had to acquire adaptive mutations to efficiently replicate in the Huh-7 cell line; and second, only a low number of cells in the culture support efficient HCV replication. Sequence analysis of Con1-derived HCV RNAs replicating in cell clones after G418 selection identified in most cases at least one mutation in the non-structural coding region, but not in the 5' or 3' UTRs (Lanford *et al.*, 2003; Lohmann *et al.*, 2003; Lohmann *et al.*, 2001; Guo *et al.*, 2001; Krieger *et al.*, 2001; Blight *et al.*, 2000). Testing the impact of individual mutations on HCV RNA replication by introducing mutations into the parental replicon and determining the number of G418-resistant colonies after transfection of a defined amount of *in vitro* transcribed RNA, or by transient replication assays, showed varying degree of RNA replication in Huh-7 cells. Highly adaptive mutations were found in the NS4B, NS5A, and NS5B coding regions, with majority clustering in NS5A. Highly adaptive amino acid substitutions have been identified at nine positions in Con1 NS5A (Lanford *et al.*, 2003; Lohmann *et al.*, 2003; Guo *et al.*, 2001; Krieger *et al.*, 2001; Lohmann *et al.*, 2001; Blight *et al.*, 2000).

The most efficient adapted replicon contains a single serine to isoleucine substitution at position 2204 in NS5A (S2204I) and establishes replication in ~10% of transfected Huh-7 cells (Blight *et al.*, 2000). The >10,000-fold improvement in colony-forming efficiency compared to the parental Con1 replicon was sufficient for the detection of HCV RNA and proteins shortly after RNA transfection. By 96 hours, HCV RNA levels were almost 500-fold higher than a replicon carrying a lethal mutation in the NS5B RdRp (Blight *et al.*, 2000).

Mutations in the integral membrane protein NS4B have a strong impact on Con1 replication with a K1846T substitution enhancing replication to a greater extent than V1897A (Lohmann *et al.*, 2003). In contrast to the highly adaptive mutations in NS4B and NS5A, amino acid substitutions within NS5B are only moderately enhancing. With a single amino acid substitution at position 2884 (R2884G) G418-colony formation was increased by ~500-fold when compared to the parental sequence (Lohmann *et al.*, 2001). Interestingly, the mutations lying within NS3 were always found in conjunction with highly adaptive substitutions. By themselves, these NS3 mutations have minimal or no impact on replicon replication, but can enhance replication synergistically when combined with each other or with highly adaptive mutations in NS4B, NS5A, or NS5B (Lanford *et al.*, 2003; Lohmann *et al.*, 2003; Krieger *et al.*, 2001; Lohmann *et al.*, 2001).

2.4.3 Generation of sub-genomic replicons from other HCV isolates

Of the seven HCV genotypes, viable sub-genomic replicons have been reported for genotypes 1a, 1b, 2a, 3a, 4a, and 6a strains. Six genotype 1b isolates - Con1, HCV-N (Ikeda *et al.*, 2002; Guo *et al.*, 2001), HCV-BK (Grobler *et al.*, 2003), HC-J4 (Maekawa *et al.*, 2004), 1B-2/HCV-O (Kato *et al.*, 2003a), and 1B-1/M1LE (Kishine *et al.*, 2002) - productively replicate in Huh-7 cells. Replication-competent genotype 1a replicons are derived from the Hutchinson strain -H77 or HCV-H (Liang *et al.*, 2005; Yi and Lemon, 2004; Blight *et al.*, 2003; Grobler *et al.*, 2003; Gu *et al.*, 2003) and efficient replication of JFH1, classified as a genotype 2a virus, has also been demonstrated in cell culture (Kato *et al.*, 2003b).

2.4.3.1 Genotype 1b sub-genomic replicons

The only HCV sub-genomic replicons able to autonomously replicate in cultured Huh-7 cells were derived from the Con1 strain. This restriction has now been

overcome by the development of replication-competent sub-genomic RNAs derived from independent genotype 1b isolates. Unlike the Con1 sub-genomic replicons, cell culture adaptation does not appear to be required for efficient replication of sub-genomes derived from the HCV-N isolate, nor for the G418 selection of Huh-7 clones (Ikeda *et al.*, 2002; Guo *et al.*, 2001). For the HCV-BK strain, systematic mutagenesis of the NS3 coding region showed that efficient replicon replication required a mutation in the helicase domain of NS3 (R1496M) in addition to the S2204I substitution in NS5A (Grobler *et al.*, 2003). NS5A adaptive mutations S2197P, S2204I, or S2201 were necessary for productive replication of chimeric HC-J4 replicons containing the 5' NTR and first 75 amino acids of NS3 from the Con1 strain (Maekawa *et al.*, 2004). While the above genotype 1b replicons were derived from cDNA clones, sub-genomic replicons have also been constructed from HCV genomic RNA replicating at low levels in cultured cells infected with human serum containing genotype 1b HCV.

2.4.3.2 Genotype 1a sub-genomic replicons

The identification of efficiently replicating replicons corresponding to genotype 1a strains has proven even more challenging than generating functional genotype 1b sub-genomic RNAs. Attempts to construct a replication-competent replicon from the HCV-1 infectious clone have been unsuccessful, despite the inclusion of adaptive mutations identified in the genotype 1b Con1 replicon (Lanford *et al.*, 2003). Similar negative results were obtained for the H77 strain until highly permissive cell lines were isolated (Blight *et al.*, 2003; Grobler *et al.*, 2003) or H77-Con1 chimeric replicons were constructed (Yi and Lemon, 2004; Gu *et al.*, 2003). Although intrahepatic inoculation of H77 RNA is associated with high viremia during the acute phase of infection in the chimpanzee (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997), replicons derived from this infectious H77 molecular clone require at least two adaptive mutations to productively replicate in cell culture. On the contrary, genotype 1b replicon adaptive mutations can be lethal in chimpanzees (Bukh *et al.*, 2002) and in infectious cell culture systems (Scheel *et al.*, 2012) and are therefore merely boosters of replication in a synthetic system.

Interestingly, the single adaptive mutation S2204I in NS5A that was identified in the Con1 replicon was a crucial prerequisite for obtaining G418-resistant colonies

supporting H77 replication (Yi and Lemon, 2004; Blight *et al.*, 2003; Grobler *et al.*, 2003; Gu *et al.*, 2003), indicating that at least one of the Con1 adaptive mutations is also effective in a genotype 1a sequence. Transfection of highly permissive Huh-7.5 cells with S2204I-containing H77 replicons allowed the establishment of the first G418-resistant colonies supporting H77 replication (Blight *et al.*, 2003; Blight *et al.*, 2002). Analysis of H77 RNAs replicating in these G418-selected cell clones identified a second amino acid substitution in the helicase domain of NS3 (A1226D or P1496L). Both of these NS3 mutations, when combined individually with S2204I in NS5A, increased the replicative capacities of sub-genomic H77 RNA with the greatest enhancement seen with the P1496L substitution. Similarly, Grobler *et al.* (2003) independently found that P1496L (or S1222T) together with S2204I is sufficient for productive replication of H77 RNA in a hyper-permissive Huh-7 subline, MR2.

2.4.3.3 Genotype 2a sub-genomic replicons

The JFH1 sub-genomes replicate with high efficiency in Huh-7 cells in the absence of adaptive mutations; the G418-resistant colony-forming ability of the unmodified bicistronic replicon is 60-fold higher than a Con1 sub-genomic RNA harboring highly adaptive mutations (Kato *et al.*, 2003b). Although adaptive mutations are not a prerequisite for efficient JFH1 replication, amino acid changes in the replicase proteins were identified in the majority of G418-selected replicon containing Huh-7 clones. Of those tested, one mutation, H2476L in NS5B, enhanced the G418 transduction efficiency by only 3-fold, which is well below the level of enhancement seen for single highly adaptive Con1 mutations (Lanford *et al.*, 2003; Lohmann *et al.*, 2003; Guo *et al.*, 2001; Blight *et al.*, 2000).

Nonetheless, the colonies derived from JFH1 sub-genomic replicons containing this NS5B mutation were significantly larger than those obtained after transfection of unmodified JFH1 sub-genomes (Kato *et al.*, 2003b), suggesting that this mutation confers a higher replication phenotype in Huh-7 cells. Furthermore, the high replication efficiency of unmodified JFH1 replicons allowed HCV RNA and proteins to be monitored in transient replication assays. Based on the data presented by Kato and coworkers (Kato *et al.*, 2003b), the JFH1 sub-genomic RNA is the most efficient replicon tested so far. Additionally, it appears that adaptive mutations may not always

be necessary for efficient replication in cell culture. Instead, the requirement for adaptive mutations is dependent on the individual HCV isolate. Identification of the JFH1 determinants that promote this high level of RNA replication could provide insights into the mechanisms of HCV RNA replication (Blight and Norgard, 2006).

2.4.3.4 Genotype 3a sub-genomic replicons

Saeed and coworkers first described sub-genomic replicons for HCV genotype 3a (Saeed *et al.*, 2012, 2013) using the consensus full-length cDNA clones from S310 and S52 (Gottwein *et al.*, 2010). Initial transfection of these sub-genomic replicons in the absence of adaptive mutations into highly permissive Huh-7.5 cells resulted in no observed replication. However, upon transfection of highly permissive Huh-7.5 cells with S2210I (corresponding to S2204I in genotype 1a strain H77) containing S52/SG-neo (I) replicons, the authors obtained stable cell clones (Saeed *et al.*, 2012). Importantly, replicons in these cells had accumulated adaptive, replication enhancing mutations in NS3, NS5A, and NS5B at similar or even identical positions as previously reported for genotype 1b isolates (Lohmann *et al.*, 2003; Lohmann *et al.*, 2001).

2.4.3.5 Genotype 4a sub-genomic replicons

Sub-genomic replicons for HCV genotype 4a were derived (Peng *et al.*, 2013; Saeed *et al.*, 2013) from the consensus full-length cDNA clone of ED43. As in the case with genotype 3a replicons, initial transfection of RNA transcribed from the full-length cDNA clone of ED43 was infectious in a chimpanzee, but failed to replicate in Huh-7.5 cells (Gottwein *et al.*, 2010). However, selectable replicons based on this isolate only gave rise to stable cell clones after a replication enhancing mutation was introduced at a position in NS5A (S232I/S2204I) known to enhance genotype 1 replication (Blight *et al.*, 2000), and, in one of the publications, by using a novel highly permissive Huh-7 cell clone designated 1C (Peng *et al.*, 2013). Further adaptive mutations located in NS3, NS4A, and NS5B were identified in selected replicon clones, increasing replication of this genotype 4a RNA to the level of genotype 1 replicons showing that the mutations observed in these regions were important for the adaptive potential of *de novo* mutations (Peng *et al.*, 2013; Saeed *et al.*, 2012).

2.4.3.6 Genotype 6a sub-genomic replicons

A recent development to the panel of sub-genomic replicons is the generation of genotype 6a sub-genomic replicons (GT6aNeo) by Yu *et al.* (2014), assembled from the consensus sequence of 16 GT 6a genomes that they obtained from the European HCV database. The selection of G418 resistant colonies in Huh-7-derived 1C cells also depended on the NS5A amino acid substitution S2321, corresponding to the S2204I previously decribed. Further analysis of replicon clones revealed two E30V and K272R mutations in NS3 and one K34R mutation in NS4A that acted synergistically with S2321 to efficiently enhance replication of GT 6a replicons (Yu *et al.*, 2014).

2.4.3.7 Replicon reporter assays

With the identification of adaptive mutations that facilitate efficient HCV replication, transient RNA replication assays that allow a more rapid and direct analysis of relative replication efficiencies have been developed. Reporters such as luciferase and ß-lactamase, as well as a transactivator inducing secreted alkaline phosphatase (SEAP), have been used to monitor replication at early times after transfection of Huh-7 cells. Firefly luciferase has successfully replaced the Neo gene in Con1 (Krieger *et al.*, 2001) and HCV-O (Ikeda *et al.*, 2005) bicistronic replicons, thus enabling replication to be monitored at various times following transfection by measuring the luciferase activity relative to a polymerase-defective replicon. The luciferase activity directly correlates with the levels of HCV RNA synthesis, demonstrating that luciferase is a reliable marker of replication (Krieger *et al.*, 2001). To demonstrate that the decrease in luciferase expression correlated with suppression of sub-genomic RNA replication, Saeed and co-workers quantified HCV RNA in IFN-treated cells. Their results showed a dose-dependent decrease in sub-genomic RNA copies indicating that luciferase expression can be used as a measure of RNA replication to evaluate the effects of different inhibitors (Saeed *et al*, 2012).

2.4.4 HCV pseudoparticles (HCVpp)

In the absence of an efficient cell culture system encompassing the entire life cycle of the virus, surrogate models were developed that were useful to study the role of HCV envelope glycoproteins in virus entry (Zeisel *et al.*, 2007). The most successful among the models to investigate early steps of HCV infection was the establishment of

retroviral pseudotypes bearing unmodified HCV glycoproteins (HCVpp) (Bartosch *et al.*, 2003; Hsu *et al.*, 2003). This system is based on the co-transfection of producer cells, typically human 293T cells, with expression vectors encoding HCV E1 and E2, which are necessary for viral tropism and fusion of HCV pseudo type particles with target cell membrane (Afshaq *et al.*, 2011; Bartosch and Cosset, 2009), the gag-pol proteins of either murine leukemia virus (MLV) or human immunodeficiency virus (HIV), and a retroviral genome encoding a reporter gene. Entry of these particles leads to the delivery of the retroviral capsid into the cytoplasm of the target cell following reverse transcription and integration of the viral genome into the host cell genome. The reporter gene is expressed by the integrated provirus to detect productive entry events in a rapid manner (Bartosch *et al.*, 2003; Hsu *et al.*, 2003).

Transfected Human embryo kidney cells (HEK- 293T) secrete assembled viruses into the supernatant, which can be harvested and used to infect naive target cells. Infection of target cells can be monitored by assessment of expression of the viral marker gene (luciferase or GFP) as shown in Figure 2.7, Middle lane. Production of HCVpp is relatively efficient, producing an average of 5×10^5 infectious units/mL supernatant, and can be performed at a convenient safety level. HCVpp allow investigation of all functions mediated by the HCV GPs, comprising cell entry and neutralization with monoclonal antibody against the viral glycoprotein E1, E2 and sera of a HCV infected patient (Cai *et al.*, 2005; Hsu *et al.*, 2003) and are a powerful tool to identify inhibitors, which block HCV entry. HCVpp are also essential to find out the fusion mechanism of the virus. HCVpp have been shown to mimic closely the entry and serological properties of wild-type HCV (Bartosch and Cosset, 2006). The use of HCVpp has contributed to analysis of the roles of the HCV coreceptors CD81 (Pileri *et al.*, 1998), scavenger receptor 1 (Scarselli *et al.*, 2002), low-density lipoprotein receptor (Agnello *et al.*, 1999), and a number of capture receptors, as well as to the identification and analysis of the recently described coreceptor Claudin-1.

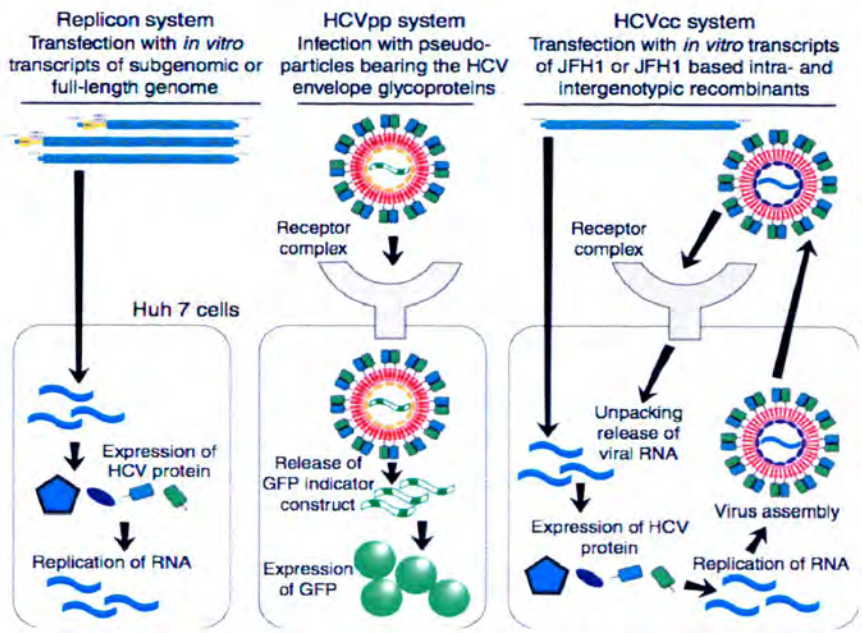

Figure 2.7: *In vitro* systems developed in Huh-7 and derived cell lines for specific isolates of hepatitis C virus (HCV). (Left) HCV replicon systems, (Middle) HCV pseudoparticle systems. Pseudo-particles displaying the HCV envelope glycoproteins E1 and E2 assembled on virus-like structures containing lentiviral or retroviral capsid proteins and a green fluorescent protein (GFP) marker construct can be used to infect Huh-7 and derived cell lines. The resulting intracellular GFP expression can be detected by fluorescent activated cell-sorting (FACS) analysis and is a convenient measure for entry efficiency, (Right) HCV full viral life cycle cell culture systems. In vitro transcripts derived from the JFH1 or intra- and inter-genotypic JFH1-based recombinant cDNA clones are transfected into Huh-7 or derived cell lines. In these systems, expression of HCV protein and HCV RNA replication results in assembly and egress of infectious viral particles. Thus, these systems mimic the complete viral life cycle of HCV. Adapted from Gottwein and Bukh, 2008.

2.4.5 Cell-cultured HCV (HCVcc) system and derivatives

In 2005, Wakita and colleagues developed a cell culture system recapitulating the entire viral life cycle, based on the transfection of the human hepatoma cell line Huh-7 with genomic HCV RNA derived from a cloned viral genome (JFH1)-a genotype 2a strain (Wakita *et al.*, 2005). The success of this isolate was based on the fact that the

JFH1 replicon system did not require adaptive mutations for highly efficient replication. This was a breakthrough in the field. In their study, Zhong *et al.* (2005) showed that transfecting highly permissive Huh-7.5.1 cells could improve virus production. Concurrently, the intra-genotypic recombinant, J6/JFH1, was developed, in which the C-NS2 cassette was replaced by corresponding genes from the J6 isolate, also of genotype 2a (Lindenbach *et al.*, 2005). Surprisingly, J6/JFH1 did not rely on adaptive mutations for efficient virus production in culture (Gottwein *et al.*, 2007), whereas the JFH1 full-length clone only produced efficient viral titers after acquisition of adaptive mutations (Russel *et al.*, 2008; Kaul *et al.*, 2007; Zhong *et al.*, 2006). Because it supports production of infectious HCV particles, it recapitulates the complete viral replication cycle (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005; Kato *et al.*, 2003b). This genome has become the basis of the most widely used HCV cell culture system.

2.4.5.1 Infectious full-length HCV RNAs

After the development of the JFH1 isolate (genotype 2a), which was found to replicate autonomously in Huh-7 cells to produce infectious virus (Wakita *et al.*, 2005), development of other infectious full-length cell culture systems for HCV has been a major challenge, probably because molecular clones of HCV generated from patient sequences do not spontaneously replicate and spread *in vitro* (Ramirez *et al.*, 2014). HCVcc are authentic HCV particles initially produced by hepatoma cell lines transfected with *in vitro* transcribed HCV RNA derived from the JFH1 clinical isolate (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). They are infectious in cell culture as well as in chimpanzee and humanized mice (Lindenbach *et al.*, 2006).

In 2006, an infectious clone of the genotype 1a designated H77S that contained five engineered replication-enhancing mutations (REMs) was generated (Yi *et al.*, 2006). At the time, the H77S (1a) system with H77 replicon mutations was the only genotype 1a isolate reported to be able to infect Huh-7.5 cells and release infectious virus particles. The titers yielded by this virus are however moderate (10-100 times lower than JFH1 virus, with a maximal titer of 10^3 focus forming units (FFU)/mL obtained with a particular Huh-7-derived subclone and an extra titer-enhancing mutation (Yi and Lemon, 2009). Moreover, the specific infectivity is 400-times lower than that of

cell-culture derived JFH1 (Yi *et al.*, 2006). Even after passaging of the full-length H77S genome, viral titers were still very low, probably due to a lower replication efficiency of H77S (Yi *et al.*, 2006). The replication efficiency of H77S was later improved when Shimakami and colleagues incorporated an additional cell culture adaptive mutation in the E2 domain (Shimakami *et al.*, 2011).

With this approach, Li and coworkers developed full-length infectious culture systems of HCV genotype 2a (J6) and 2b (J8) using mutations F1464L/A1672S/D2979G (LSG) identified through studies of J6 recombinants (Li *et al.*, 2012a). Further, the authors developed a robust full-length HCV genotype 1a culture system using genotype 2-derived mutations and an *in vivo* functional HCV genome designated HC-TN (Sakai *et al.*, 2007), which led to the identification of additional mutations A1226G (NS3 helicase) and Q1773H (NS4B). These TN mutations, in combination with genotype 2-adapting mutations, permitted efficient virus production and further adaptation of the TN full-length virus. Thus, according to them, the TNcc system permitted detailed virological studies and applications previously difficult or not possible for genotype 1 viruses (Li *et al.*, 2012b). Recently in their study, Ramirez and colleagues have developed efficient full-length cell-culture systems for genotype 2b strains DH8, J8, and DH10, of which DH8 and J8 yielded high infectivity titers. The high infectivity titers depended on previously described LSG and additional mutations A1951 and V1968 in NS4B, and T2439 or T2440 in NS5A (Ramirez *et al.*, 2014).

2.4.5.2 JFH1 and chimeric genomes

Although the generation of infection models was an important achievement permitting studies of the complete HCV life cycle in cell culture, these systems were constrained to specific isolates and limit comparable studies of all HCV genotypes. But to overcome this, production of viruses from different genotypes and strains mostly relied on chimeric genomes whereby the JFH1 isolate was combined with heterologous strains of all major HCV genotypes. Thus, a comprehensive panel of chimeric genomes was constructed for all major HCV genotypes (Date *et al.*, 2012; Koutsoudakis *et al.*, 2011; Bungyoku *et al.*, 2009; Gottwein *et al.*, 2009; Kang *et al.*, 2009; Russell *et al.*, 2009; Jensen *et al.*, 2008; Russell *et al.*, 2008; Scheel *et al.*, 2008; Delgrange *et al.*, 2007; Gottwein *et al.*, 2007; Kaul *et al.*, 2007; McMullan *et*

al., 2007; Yi *et al.*, 2007; Pietschmann *et al.*, 2006; Yi *et al.*, 2006; Zhong *et al.*, 2006). In most cases, the replicase proteins necessary for generating the membrane-bound replicase complex and nontranslated regions are derived from the highly efficient JFH1 strain. The proteins Core to NS2, which are required for viral morphogenesis are derived from another genotype. With this strategy the yield of HCVcc particles was subsequently enhanced by creating an intra-genotypic chimera using the C-NS2 part of a different genotype 2a isolate, J6 (Lindenbach *et al.*, 2005; Yanagi *et al.*, 1997). Interestingly, some constructs such as the J6/JFH1 designated Jc1 (Pietschmann *et al.*, 2006) or clone 2 (Horwitz *et al.*, 2013) chimeras actually spread more efficiently than the original JFH-1 clone.

To further investigate whether it was also possible to generate inter-genotypic chimeras, an analogous chimeric genome that carried the Core to NS2 part of the GT 1b Con1 isolate was constructed (Pietschmann *et al.*, 2006). As observed this genome produced infectious HCV but with very low virus titers probably due to incompatibilities between the Con1 (genotype 1b) and JFH1 proteins. Using this strategy, a series of inter-genotypic reporter chimeras were generated with different crossover sites varying from the C-terminus of E2 to the NS2-NS3 cleavage site. The results of this mapping analysis identified a cross-over site located right after the first trans-membrane domain of NS2 as the best choice for construction of infectious JFH1-Con1, JFH1-J6 and JFH1-H77 chimeras (Pietschmann *et al.*, 2006).

Similar studies describing the generation of chimeric for all major seven genotypes have been reported (Gottwein *et al.*, 2011a; Scheel *et al.*, 2011a; Gottwein *et al.*, 2009; Jensen *et al.*, 2008; Scheel *et al.*, 2008; Gottwein *et al.*, 2007; McMullan *et al.*, 2007; Yi *et al.*, 2007; Pietschmann *et al.*, 2006). Chimeric genomes have been shown to be highly useful to study entry, neutralization, and virus assembly of all seven known HCV genotypes. They have been further validated to be infectious *in vivo* as human liver-chimeric mice developed high-titer infections after inoculation with HCV of genotypes 1–6 (Bukh *et al.*, 2010). The idea of chimeric genomes was further expanded to also include non-structural proteins. As mentioned above, HCV replicons have so far been described for genotypes 1, 2, 3, 4, and 6, and efficiency or resistance of direct acting antiviral agents (DAAs) targeting non-structural proteins has not been tested for all genotypes. Recently, the construction of viable JFH1-based chimeras in

which sequences encoding NS3/4A or NS5A were replaced with homologous sequences of other genotypes was described (Galli *et al.*, 2013; Gottwein *et al.*, 2011a; Imhof and Simmonds, 2011; Scheel *et al.*, 2011a, b). These technical developments that are based on adaptation approaches allow analyzing effects of antiviral compounds against NS3/4A and NS5A and antiviral resistance for all HCV genotypes in the context of infectious full-length HCV RNAs.

2.4.5.3 Adaptation of cell culture systems to higher titers

The generation of chimeric genomes has been shown to increase viral yields in cell culture. However, genetic incompatibility between JFH1 and the alternative HCV genome segment fused to it often limits production of infectious virus particles. However, through the acquisition of cell culture adaptive mutations as a result of serial passages of the chimeras in cell culture over time, virus yields could be greatly increased (Chan *et al.*, 2012; Gottwein *et al.*, 2011a; Koutsoudakis *et al.*, 2011; Bungyoku *et al.*, 2009; Gottwein *et al.*, 2009; Jensen *et al.*, 2008; Scheel *et al.*, 2008; Abe *et al.*, 2007; Gottwein *et al.*, 2007; McMullan *et al.*, 2007; Yi *et al.*, 2007). In their study, Yi and colleagues demonstrated that mutations in E1, p7, NS2, and NS3 contributed to the ability of a H77/JFH1 chimeric genome to assemble and release high amounts of virus particles (Yi *et al.*, 2007). These mutations acted independently of any detectable effect on viral RNA replication or polyprotein processing indicating the fundamental role of these proteins in virus assembly and relase (Yi *et al.*, 2007).

Cell culture adaptations of JFH1-chimeras were mostly conducted in the highly permissive cell line Huh-7.5 and are based on the passage of JFH1 infected cells or by serial passages of viral supernatants. Over time, viral variants emerge that harbor adaptive mutations leading to increased viral titers up to 100–1,000-fold over the parental genome. Interestingly, several groups found that also the JFH1 wild-type genome can be efficiently adapted in cell culture with an increase in viral titers from 10^3 tissue culture infectious doses (TCID50) /mL to 10^5–10^6 TCID50/mL (Kang *et al.*, 2009; Russell *et al.*, 2008; Delgrange *et al.*, 2007; Kaul *et al.*, 2007; Zhong *et al.*, 2005). This indicates that also JFH1 *per se* is not optimally suited for replication and propagation in Huh-7 cells. However, compared to all other known HCV isolates the degree of replication competence of this particular isolate in these cells is certainly unique. Titer-enhancing mutations were identified throughout the HCV genome

(Core, E2, p7, NS2, NS3, NS5A, NS5B) and interestingly, repetition of an adaptation process showed that none of the mutations identified in the first experiment reappeared in the second selection (Kang *et al.*, 2009; Russell *et al.*, 2008; Delgrange *et al.*, 2007; Kaul *et al.*, 2007; Zhong *et al.*, 2005). Thus, there are likely to be varying independent options to adapt JFH1 to cell culture replication in Huh-7-derived cells.

Further development to the HCVcc was the incorporation of reporter genes that allowed easy, rapid and sensitive detection and quantification of replication or infection (Gottwein *et al.*, 2011b; Reiss *et al.*, 2011; Schaller *et al.*, 2007; Koutsoudakis *et al.*, 2006; Tscherne *et al.*, 2006). So far reporter genes that have been used to generate reporter viruses include; firefly luciferase- FLuc (Koutsoudakis *et al.*, 2006; Wakita *et al.*, 2005), *Renilla* luciferase- RLuc (Liu *et al.*, 2011; Reiss *et al.*, 2011; Schmitt *et al.*, 2011; Wu *et al.*, 2011; Jones *et al.*, 2007; Kim *et al.*, 2007; Tscherne *et al.*, 2006), *Gaussia* luciferase- GLuc (Phan *et al.*, 2009; Marukian *et al.*, 2008), green fluorescent protein- GFP (Masaki *et al.*, 2008; Kim *et al.*, 2007; Schaller *et al.*, 2007), enhanced green fluorescent protein- EGFP (Gottwein *et al.*, 2011b; Wu *et al.*, 2011; Han *et al.*, 2009), enhanced yellow fluorescent protein- EYFP (Yamamoto *et al.*, 2011), red fluorescent protein- RFP (Schaller *et al.*, 2007), yellow fluorescent protein for fluorescence resonance energy transfer (FRET)- Ypet (Jones *et al.*, 2010), and monomeric red fluorescent protein-mCherry (Gottwein *et al.*, 2011b).

Of note is the fact that while luciferase viruses allow easy-to-measure quantification of infectivity (Pietschmann *et al.*, 2009), fluorescent protein-tagged viruses permit infectivity measurement at the single cell level. However, such modified viruses are often not as stable as the original virus in cell culture, which might lead to the loss of the reporter gene expression. They also often do not spread efficiently in cell culture (Koutsoudakis *et al.*, 2006). This makes reporter viruses extremely useful for transient replication assays (Pietschmann *et al.*, 2009) and high-throughput screening of potential HCV antivirals with obvious advantages for antiviral research (Gentzsch *et al.*, 2011; Reiss *et al.*, 2011; Chockalingam *et al.*, 2010; Gastaminza *et al.*, 2010b; Li *et al*, 2009).

CHAPTER 3
MATERIALS AND METHODS

3.1 CELLS, ANTIBODIES, AND CHEMICALS

Huh-7.5 (Blight *et al.*, 2002) and Huh-7.5 Clone 8 (Jones *et al.*, 2010) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1mM nonessential amino acids (NEAA), and penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2). For selection and maintenance of Huh-7.5 cells harboring selectable HCV sub-genomic replicons, G418 (Sigma) was added to the medium to get a final concentration of 500 µg/mL. Alexa Flour® 647 conjugated monoclonal antibody specific for HCV NS5A (9E10) has been previously described (Lindenbach *et al.*, 2005). HRP-conjugated goat-anti-mouse secondary antibody was commercially obtained from Jackson ImmunoResearch, West Grove, PA. HCV compounds IFNα-2a, ledipasvir, and sofosbuvir were purchased from PBL Assay Science (NJ, USA), MedChem Express (NJ, USA) and ACME Bioscience (CA, USA), respectively. Danoprevir (RG7227/ITMN-191) and daclatasvir were purchased from Selleck Chemicals (TX, USA).

3.2 SOURCE OF HCV

HCV RNA-positive plasma samples from eight voluntary blood donors (kindly provided by Dr. N. Prabdial-Sing at the National Institute for Communicable Diseases (NICD) Sandringham, Johannesburg, South Africa, as requested by the North-West University, Mafikeng Campus virology laboratory) were shipped on dry ice to the Laboratory of Virology and Infectious Diseases, The Rockefeller University, New York, USA, where samples were maintained at -80°C until use. These samples were cataloged as SA545, SA546, SA548, SA555, SA556, SA560, SA600, and SA606.

3.3 ETHICAL CLEARANCE

The study is retrospective and has been approved by the ethics committee of the North-West University, South Africa (NWU-00111-10-A2), and has therefore been performed in accordance with the ethical standards of the 1964 Declaration of Helsinki.

3.4 SYNTHESIS OF CONSENSUS FULL-LENGTH HCV GENOME SEQUENCES

3.4.1 RNA isolation and cDNA synthesis

Total RNA was extracted from 140µL of eight plasma samples using the QIAamp viral RNA mini kit protocol (Invitrogen, USA) as specified by the manufacturer. The extracted RNA was subjected to cDNA synthesis by using Superscript™ III reverse transcriptase (Invitrogen, USA) in a two-step reaction. First, a 5µL aliquot of the extracted RNA was mixed with 1µL of 50ng/µL random hexamers, 1µL of 10mM dNTP, and 3µL distilled water. The reaction mixture was incubated at 65°C for five minutes in a thermal cycler (model PTC-220 DYAD™ DNA ENGINE; MJ Research Inc, USA) and placed on ice for one minute. Additional 10µL of master mix (composition: 2µL 10X RT buffer, 4µL of 25mM $MgCl_2$, 1µL of 40U/µL RNase Out, and 1µL of 200U/µL Superscript III), was added to the reaction mix and incubated further at 25°C for 10 minutes, 50°C for 50 minutes, and 80°C for five minutes.

3.4.2 Amplification of viral genome

Partial sequences of three HCV genotype 5a isolate namely SA13 (accession number AF064490), EUH1480 (accession number Y13184), and HCV5_gp1 (accession number NC_009826) were retrieved from the HCV database (http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline. html) and aligned using ClustalW (v1.83) multiple sequence alignment. PCR primers were designed based on the conserved sequences. These primers were used to amplify HCV cDNA of the four novel GT 5a isolates (SA548, SA555, SA556, and SA560) into eight overlapping fragments by nested PCR; fragments A, B, C, D, E, F, G, and H (Figure 3.1).

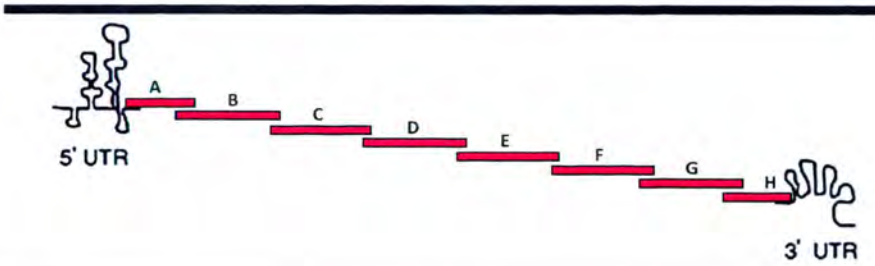

Figure 3.1: Schematic representation of the overlapping fragments of the HCV genome

The primers used were synthesized at Integrated DNA Technologies (IDT), USA, and the sequences are shown in Table 3.1. For each fragment, first round of PCR was carried out with outer primers using Phusion DNA polymerase (NEB, United Kingdom).

Table 3.1: Primers for amplification of HCV GT 5a strains

Fragments	Primer	Orientation	Primer sequence (5'—>3')
5'RACE*	Outer	Antisense	CCACGGGGTTGCGACCGT
	Inner	Antisense	ACTCCACCAACGATCTGA
A	Outer	Sense	GGATAAACCCGCTCAATGCC
		Antisense	GCGGAAGCAATCAGTAGGACAC
	Inner	Sense	GAGCACGAATCCTAAACCTCAAAG
		Antisense	AAGGGCAGTTCTGTTGATGTGC
B	Outer	Sense	GTAATGTCAGTAGGTGCTGGG
		Antisense	CAAATGCCCAAGCAAAGGTAGG
	Inner	Sense	GTAATGTCAGTAGGTGCTGGG
		Antisense	CGCGTTTAGGACAATGAC
C	Outer	Sense	TACTGGCAACTGGTTTGGCTGC
		Antisense	CGACAGATGGATTTAGCACCAGC
	Inner	Sense	CCGCTCTTAGCACTGGTCTCATAC
		Antisense	TCGTGGTTTCCAGGTTTTCAAC
D	Outer	Sense	CGGGACAAAAATGAAGCCGAG
		Antisense	CCTATCTGGGAGACCTGCCATCAT
	Inner	Sense	CGGGACAAAAATGAAGCCGAG
		Antisense	CGGAGCATGCACTTCCACATTGT
E	Outer	Sense	GACTTTGATTCTGTCATAGA
		Antisense	TGAGCAGGGTCTGACAGCATTG
	Inner	Sense	GTTCACAGGGCTTACTAACATCG
		Antisense	CAGGGACACTTCAGATTGTC
F	Outer	Sense	GTCGGCCCGAAGCTGTGCAG
		Antisense	CTTAATGTAGCACGTCATGGT
	Inner	Sense	GTCGGCCCGAAGCTGTGCAG
		Antisense	CAAAGCAGCGGGTATCATAGGAG
G	Outer	Sense	GGATGACTCTGATACCCCATTGC
		Antisense	GTAGGAGTAGGCACAGGAGT
	Inner	Sense	CGACCTTGGAGTCCGCGTCTG
		Antisense	GTAGGAGTAGGCACAGGAGT
H	Outer	Sense	AACTCACTCCATTAGCTGACG
		Antisense	TCATGCGGCTCACGGACCTTTCACAGCTAG
	Inner	Sense	AGCTGGTTCACCGTCGGC
		Antisense	GGACCTTTCACAGCTAGCCGTGACTAGGG
3'RACE	Outer	Sense	CTTTCTTTCTTGGTGGCTCCATC
		Antisense	CCGCTGGAAGTGACTGACAC
	Inner	Sense	TCTTGGTGGCTCCATCTTAGC
		Antisense	CCGCTGGAAGTGACTGACAC

*RACE, Rapid amplification of cDNA ends

The reaction mixture consisted of 4µL of template cDNA, 10µL of 5X Phusion HF buffer, 1µL of 10mM dNTPs, 0.25µL of 100µM forward and reverse primers, 0.5µL of Phusion DNA polymerase, and nuclease free water (NFW). The first-round PCR product (4µL) was further amplified using inner primer pair. Reactions were performed in a thermal cycler and conditions consisted of an initial denaturation step at 98°C for 90 seconds, 35 cycles of PCR (98°C for 30 seconds, 55°C for 30 seconds,

72°C for two minutes), and a final elongation step at 72°C for 10 minutes. The PCR products were resolved by agarose gel electrophoresis (Lonza, USA).

3.4.3 Amplification of the highly conserved 5' UTR

The 5' UTR is the most conserved region of the HCV genome with only 6% sequence divergence between distantly related genotypes compared to 33% over the full genome (Okamoto *et al.*, 1991). Amplification of the 5' UTR was performed using the 5' RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, USA) as per manufacturer's instructions. The 5′ RACE uses an antisense gene specific primer (GSP) for the synthesis of specific cDNA by reverse transcriptase. Prior to PCR, a terminal deoxynucleotidyl transferase (TdT) tailing step attaches an adapter sequence to the unknown 5' sequences of the cDNA. Specific cDNA is then amplified by PCR using a GSP that anneals in a region of known exon sequences and an adapter primer that targets the 5' terminus (Figure 3.2).

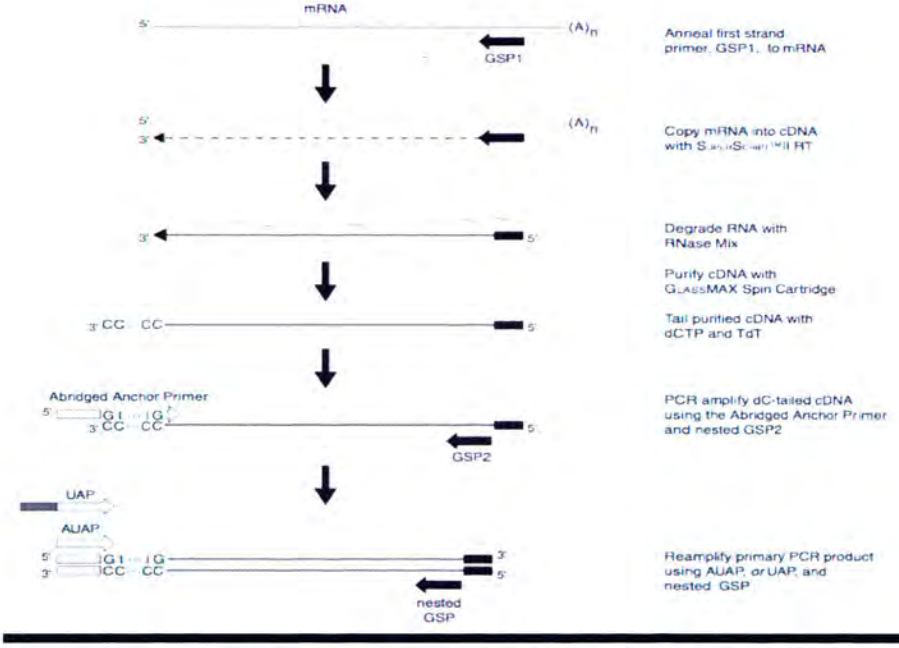

Figure 3.2: Schematic overview of 5' end cDNA amplification using classic RACE

An aliquot of 5µL RNA was used as a template for cDNA synthesis. The cDNA was synthesized using a reverse gene-specific primer (GSP1) 487R 5'-CATAAAGGGGCCAAGGGTA- 3' and SuperScript II in a final reaction volume of 25µL (composition: 5 µL RNA, 2.5µL of 1µM GSP1, 8µL of NFW, 2.5µL 10X PCR buffer, 2.5µL of 25mM $MgCl_2$, 1µL of 10mM dNTP, 2.5µL of 0.1M DTT, and 1µL of SuperScript II). The reaction was carried out in a thermal cycler with an initial incubation at 72°C for 10 minutes, followed by 42°C for 55 minutes and heat inactivation at 70°C for 15 minutes. The tubes were then chilled on ice and 1µL of RNase mix added and the reaction incubated for an additional 30 minutes at 37°C. The synthesized cDNA were purified using the MinElute PCR purification kit protocol (Qiagen, USA).

Tailing of the 5' end of the purified templates was performed using TdT and dCTP in a final reaction volume of 24µL consisting of 10µL of the purified cDNA products, 6.5µL NFW, 5µL of 5X tailing buffer, and 2.5µL of 2mM dCTP. The reaction was subjected to initial 94°C incubation for three minutes in a thermal cycler, chilled on ice for one minute and 1µL of TdT was added into each tube. The reaction was further incubated at 37°C for 10 minutes and deactivated at 65°C for 10 minutes. Tubes were placed on ice and immediately preceded to the next step.

3.4.3.1 First round and nested PCR amplification of TdT-tailed cDNA

A 5µL aliquot of template cDNA was subjected to first round of PCR using Takara LA *Taq* DNA polymerase (TaKaRa BIO INC, Japan) in a final volume of 50µL, consisting of 5µL 10X PCR buffer (without $MgCl_2$), 5µL of 25mM $MgCl_2$, 4µL of 2.5M dNTPs, 2µL each of 10µM sense (5'RACE Abridged Anchored Primer-present in kit) and antisense primer (Table 3.1), 0.5µL LA *Taq* DNA polymerase, and 26.5µL NFW. Cycling conditions were 94°C for three minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for one minute, and a final extension at 72°C for seven minutes. A 5µL aliquot of the primary PCR was first diluted into 495µL TE buffer (10mM Tris-HCl, 1mM EDTA, Qiagen, USA) and 5µL of the diltuted PCR product was used for nested PCR amplification with sense (universal amplification primer) and antisense primers (Table 3.1). Cycling conditions were same as above. The amplified PCR products

were visualized through a 3% LE Agarose gel (Lonza, USA) stained with SyberSafe dye (Invitrogen, USA).

3.4.4 Amplification of the highly conserved X-tail region of the 3'UTR

HCV 3' UTR contains a variable region, a long poly (U/UC) tract of variable length, and a highly conserved 3'X tail. The amplification of the X-tail region of the 3' UTR was performed in a four-step procedure (Figure 3.3). Firstly, total RNA was extracted from 250 µL of plasma samples using the TRIzol LS reagent (Invitrogen, USA) as previously described (Chomczynski, 1993) and tailed at the 3' end using yeast poly A polymerase and GTP. The tailed RNA was then transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, USA) and an antisense gene specific primer (GSP) (Table 3.1) and treated with ribonuclease (RNase) H to degrade residual RNA. The reactions were performed in a gradient as follows: 65°C for five minutes, 50-55°C for one hour, and 70°C for 15 minutes.

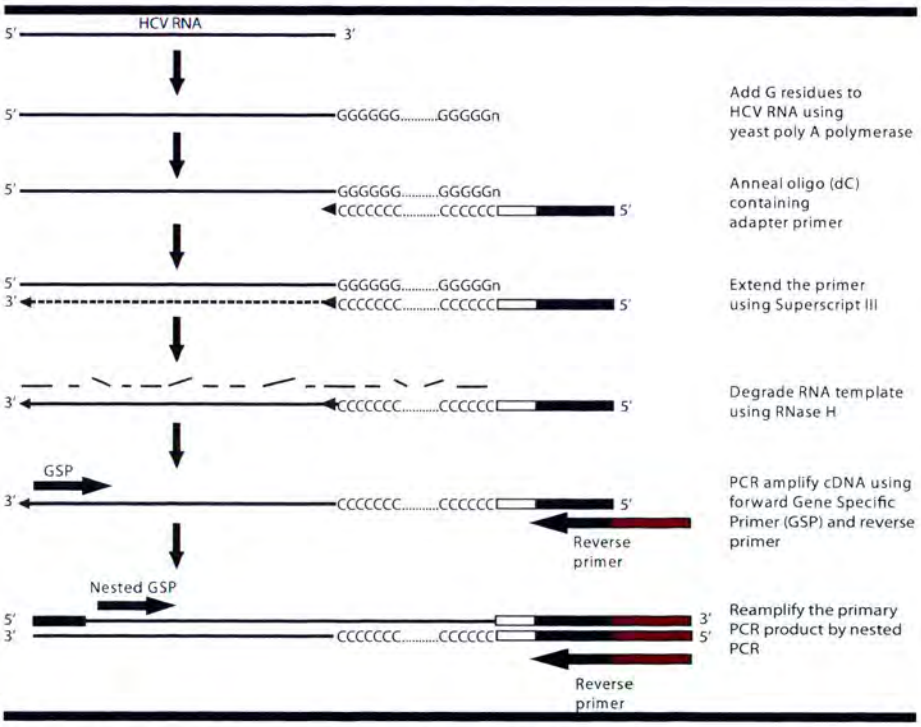

Figure 3.3: Schematic overview of 3' end cDNA amplification using classic RACE

The cDNA was then amplified in a two-step PCR; the first requiring priming with a sense GSP and antisense primers (Table 3.1) using Ex-*Taq* DNA polymerase (Toyobo, Japan). In the second amplification or nested step, a nested sense GSP and antisense primers were used. The reaction was performed in a final volume of 50µL consisting of 5µL of 10X buffer, 4µL of 2.5mM NTP, 5µL each of 10µM sense and antisense primers, 0.25µL of Ex-*Taq* DNA polymerase, and 28.25µL of RNase-free water. The reaction was subjected to an initial denaturation step at 95°C for two minutes, 35 cycles of PCR (95°C for 30 seconds, 58°C for 30 seconds, 72°C for two minutes), and a final elongation step at 72°C for 10 minutes. The amplified PCR products were visualized through a 3% LE Agarose gel (Lonza, USA) stained with SyberSafe dye (Invitrogen, USA). DNA was excised from gel and purified using the MinElute Gel Extraction kit protocol (Qiagen, USA).

3.4.5 Molecular cloning of nested PCR products

Gel-purified PCR products (with the exception of 5' and 3' RACE products) were first monoadenylated at the 3' ends by incubating with *Taq* DNA polymerase (Invitrogen, USA), and purified using the MinElute PCR purification kit protocol (Qiagen, USA). The purified DNA fragments were ligated with pCR 2.1-TOPO TA vector following manufacturer's instructions (Invitrogen, USA). Briefly, a reaction mixture containing 4µL aliquots of the purified monoadenylated PCR product, 1µL each of Salt solution and TOPO vector in a sterile 1.5mL Eppendorf tube was incubated at room temperature for 30 minutes. Thereafter, competent *E. coli* cells (DH5α), prepared using the method of Inoue *et al.* (1990), were transformed with the ligated plasmid DNA and spread on pre-warmed Luria Bertani/ampicillin (100mg/mL) agar plates containing 40µL of X-Gal (20µg/µL). Plates were then incubated at 37°C for 16 hours.

3.4.6 Sequencing and phylogenetic analysis

Five to ten bacterial colonies were picked for each fragment and plasmid DNA, prepared from small-scale bacterial cultures and purified using the QIAprep Spin Miniprep Kit (Qiagen, USA) protocol, was subjected to sequencing to obtain a consensus sequence. Sequence results were analyzed using SeqMan Pro™ DNASTAR Software for Life Scientists version 10.0.0 and alignments performed using MacVector with Assembler version 12.0.6. The consensus sequences obtained

for all overlapping fragments were zipped together to obtain a full-length consensus sequence of each isolate. Phylogenetic analysis was performed using the Neighbour Joining tree building, Tamura-Nei distance, and Best tree methods in MacVector to compare the polyprotein region of genotype 5a isolates and that of other HCV genotypes available in the GenBank database.

3.5 SYNTHESIS OF HCV CONSTRUCTS

3.5.1 Generation of HCV genotype 5a sub-genomic replicons

Based on the full-length consensus sequence of SA548 and SA555, the neomycin-selectable sub-genomic replicons SA548/SG-neo and SA555/SG-neo were generated. Briefly the clones derived from amplicon 3 to 10, covering the NS3-3'UTR region of the viral genome, were pieced together by overlapping PCR or by use of restriction enzymes. Since finding individual clones for each amplicon that were similar to the consensus sequence at all nucleotide positions proved difficult, the clones deviating from the consensus sequence at the nucleotide level, but not at the amino acid level, were sometimes selected to ligate to the neighboring fragment. This resulted in the inclusion of numerous silent mutations in the region of replicon encoding NS3-NS5B proteins. Nucleotides 397 to 1772 were replaced with a cassette containing a neomycin phosphotransferase II (NPTII) gene and an EMCV IRES from S52/SG-neo (GenBank accession number GU814264) as described by Saeed *et al.* (2012). Standard DNA recombination methods were used to assemble different fragments. Wherever PCR was required, a high fidelity KOD DNA polymerase (Toyobo, Japan) was used to avoid PCR-induced mutations. Cycling conditions were as follows: 95°C for two minutes and 25 cycles of 95°C for 30 seconds, annealing (minimum melting temperature, Tm, of primers) for 20 seconds and 70°C for two minutes. A T7 RNA polymerase promoter was cloned immediately upstream of 5' UTR to enable *in vitro* synthesis of RNA. An *Xba*1 runoff site was engineered immediately downstream of 3' UTR. All *Xba*1 enzyme restriction sites within the HCV genome were removed by introducing silent mutations through PCR-mediated mutagenesis.

The SA548/SG-neo plasmid was constructed as follows: The NS5B (nt 6452-7739) gene was amplified using RU-O-8003F (5'-CGACCTTGGAGTCCGCGTCTG-3') and RU-O-18524 reverse (5'- CCGCCGGCGCCGACGGTGAAC-3' with *SgrA*I site)

and the 3' UTR was amplified using RU-O-18453 forward (5'-GGCGGGGGGGACATTTATCAC-3') and RU-O-18451 reverse (5'-CGACTCTAGACATGATCTGCAGAGAG-3') primer set. Both PCR products were purified, mixed, and amplified again with RU-O-8003F and RU-O-18451. The resulting PCR fragment was cloned with JFH1/SG-Feo by *EcoR*I and *SgrA*I digestions to produce the plasmid pNS5B/3'UTR/pUC19. The NS4A-NS5B (nt 1776-6451) region was assembled into pNS4A-NS5B/pUC19 using four plasmids containing overlapping NS genes. The neomycin-resistant gene was amplified from the vector S52/SG-neo using RU-O-18674 forward (5'-ACCGCCGCCCAATGATTGAACAAG-3') and RU-O-17930 reverse (5'-AGAGGGTTTAAACTCAGAAGAAC-3' with *Pme*I site) primer set. The 5' UTR was amplified using RU-O-18673 sense (5'-GTGAATTC**TAATACGACTCACTATAG**ACCCGCCCCTTATTG-3' with *EcoR*1 site and **T7 promoter**) and RU-O-18675 antisense (5'-CTTGTTCAATCATTGGGCGGCGGT-3') primers. Both PCR products were purified, mixed, and amplified again with RU-O-18673 and RU-O-17930. The resulting PCR fragment containing both HCV cDNA (nt 1-396) and the neo gene was cloned with S52/SG-neo by *EcoR*1 and *Pme*I digestions to generate SA548-5'/S52/SG-neo.

The EMCV IRES of S52/SG-neo was amplified using RU-O-Pme1-S (5'-ATTCGCAGCGCATCGCCTTC-3') and RU-O-18676 antisense (5'-GTGATAGGGGCCATGGTATCATC-3') primers. The NS3 protein was amplified using RU-O-9194 forward (5'-CCGCTCTTAGCACTGGTCTCATAC-3') and RU-O-5082R (5'-CGGAGCATGCACTTCCACATTGT-3') primers. The PCR product was purified using MinElute Gel Purification kit (Qiagen, USA) protocol and cloned into TOPO vector as described in section 3.4.5. The purified plasmid was amplified using RU-O-18677 forward (5'-GATGATACCATGGCCCCTATCAC-3') and RU-O-5082R primer set. Both PCR were purified, mixed, and amplified again with RU-O-Pme1-S and RU-O-5082R. The end product, SA548/EMCV-NS3, was cloned with SA548-5'/S52/SG-neo by *EcoR*1 and *Pme*I digestions to generate plasmid SA548-5'/EMCV-NS3. The two plasmids, SA548-5'/EMCV-NS3 and pNS4A-NS5B/pUC19, were cloned by *EcoR*I and *Psi*I digestions to produce the selectable bicistronic SA548/SG-neo construct.

In a similar manner, SA555/SG-Neo was assembled as follows: The 5' UTR was amplified using RU-O-18673 forward (5'-GTGAATTC**TAATACGACTCACTATAG**ACCCGCCCCTTATTG-3' with *EcoR*1 site and **T7 promoter**). The neomycin-resistant gene was amplified from the vector S52/SG-neo using RU-O-18674 forward (5'-ACCGCCGCCCAATGATTGAACAAG-3') and RU-O-17930 reverse (5'-AGAGGGTTTAAACTCAGAAGAAC-3' with *Pme*I site) primer set. Both PCR products were purified, mixed, and amplified again with RU-O-18673 and RU-O-17930. The resulting PCR fragment containing both HCV cDNA (nt 1-396) and the neo gene was cloned with S52/SG-Neo by *EcoR*1 and *Pme*I digestions (SA555-5'/S52/SG-neo). The EMCV IRES was also amplified from vector S52/SG-neo using RU-O-618F (5'-CTATCGTGGCTGGCCACGAC-3') and RU-O-18676 (5'-GTGATAGGGGCCATGGTATCATC-3') forward and reverse primers. The NS3 protein was amplified using RU-O-9194 forward (5'-CCGCTCTTAGCACTGGTCTCATAC-3') and RU-O-18445 reverse (5'-GACGTCCAGACCTCTATAG-3'). The PCR product was purified using MinElute Gel Purification kit (Qiagen, USA) protocol and cloned into TOPO vector as described in section 3.4.5. The purified plasmid was amplified using RU-O-18677 forward (5'-GATGATACCATGGCCCCTATCAC-3') and RU-O-5082R (5'-CGGAGCATGCACTTCCACATTGT-3') primer set. Both PCR were purified, mixed, and amplified again with RU-O-618F and RU-O-18678. The end product, EMCV-NS3, was cloned with SA555-5'/S52/SG-neo by *Pme*I and *BsiW*I digestions to generate plasmid SA555-5'/EMCV-NS3. The NS5B (nt 6452-7739) gene was amplified using RU-O-8003F (5'-CGACCTTGGAGTCCGCGTCTG-3') and RU-O-18454 reverse (5'-GTGATAAATGTCCCCCCCGCC-3') and the 3' UTR was amplified using RU-O-18453 forward (5'-GGCGGGGGGGACATTTATCAC-3') and RU-O-18451 reverse (5'-CGACTCTAGACATGATCTGCAGAGAG-3') primer set. Both PCR products were purified, mixed, and amplified again with RU-O-8003F and RU-O-18451. The resulting PCR fragment was cloned with JFH1/SG-Feo by *EcoR*I and *Xba*I digestions to produce the plasmid pNS5B/3'UTR/pUC19. The NS3-NS5B (nt 1776-6451) region was assembled into pNS3-NS5B/pUC19 using three plasmids containing overlapping NS genes. The two plasmids, SA555-5'/EMCV-NS3 and

pNS3-NS5B/pUC19, were cloned by *EcoR*I and *BsiW*I digestions to produce the selectable bicistronic SA555/SG-neo construct.

3.5.2 Introduction of mutations in HCV constructs

PCR-mediated site-directed mutagenesis was done to introduce amino acid substitutions of possible adaptive value. Three single and two double mutant constructs were generated from SA548/SG-neo, namely SA548/SG-neo S2205I, SA548/SG-neo T1281I, SA548/SG-neo R2888G, SA548/SG-neo T1281I + S2205I, and SA548/SG-neo T1281I + R2888G. Same set of mutants was generated from SA555/SG-neo. To engineer the mutations S2205I (NS5A), R2888G (NS5B), and T1281I (NS3) into plasmid SA548/SG-neo and SA555/SG-neo, PCRs were performed using mutant primers and oligonucleotides, taking advantage of unique restriction enzyme sites within the plasmid genome sequence. To introduce mutation at amino acid position 2205, a 2µl template aliquot was amplified firstly using RU-O-19128 (5'-CTAGCTCCTCGGCCA**T**TCAGCTC-3'; nucleotide change is highlighted in boldface and the resultant codon is underlined) and RU-O-18451 (RU-O-18454 in the case of SA555/SG-neo) forward and reverse primer pairs, respectively and secondly using RU-O-9354 (5'-GCTGAGCAGTTCTGGGCAAAAC-3') and RU-O-19129 (5'-GAGCTGAATGGCCGAGGAGCTAG-3'). The PCR products were purified, combined and amplified using RU-O-9354 and RU-O-18451 (RU-O-18454 in the case of SA555/SG-neo). The resulting products were digested with *EcoR*V-*BstX*I and *EcoR*V-*Spe*I and cloned into similarly digested SA548/SG-neo and SA555/SG-neo fragment to obtain SA548/SG-neo S2205I and SA555/SG-neo S2205I, respectively.

Similarly, the R2888G mutation was introduced by amplifying the *BstX*I-*SgrA*I and *Spe*I-*Xho*I fragment in SA548/SG-neo and SA555/SG-neo template plasmids, respectively in a two-step PCR approach using two primer pairs: RU-O-19130 forward (5'-GCAATCATTCAG**GG**ACTCCATGG-3') and RU-O-18451 reverse primers, RU-O-8003F (5'-GCTGAGCAGTTCTGGGCAAAAC-3') and RU-O-19131 reverse (5'-CCATGGAGTCCCTGAATGATTGC-3') primers. The final PCR product obtained using RU-O-8003F and RU-O-19131 was purified, digested with *BstX*I-*SgrA*I and *Spe*I-*Xho*I, and the digested fragments incorporated into SA548/SG-

neo and SA555/SG-neo by replacing the corresponding digested fragments, generating SA548/SG-neo R2888G and SA555/SG-Neo R2888G.

The mutation T1281I was created by amplifying the *Avr*II-*Psi*I and *Pme*I-*Cla*I sites in template plasmids SA548/SG-neo and SA555/SG-neo, respectively, using RU-O-19126 (5'-GACCCCAACATCAGGATCGGAGTTAG-3') and RU-O-5082R (5'-CGGAGCATGCACTTCCACATTGT-3'). The PCR products were purified, mixed and amplified using RU-O-618F and RU-O-19127 reverse (5'-CTAACTCCGATCCTGATGTTGGGGTC-3') primer sets. The PCR-amplified products were digested with *Avr*II-*Psi*I and *Pme*I-*Cla*I and cloned into these sites in SA548/SG-neo and SA555/SG-neo, respectively. To introduce the double mutations T1281I + S2205I and T1281I + R2888G, plasmids SA548/SG-neo T1281I, SA548/SG-neo S2205I, SA548/SG-neo R2888G were digested with *Avr*II and *Psi*I, and SA555/SG-neo T1281I, SA555/SG-neo S2205I, SA555/SG-neo R2888G with *Pme*I and *Cla*I. The digested products were separated by agarose gel electrophoresis; bands of interest cut and purified and the corresponding purified fragments combined in a ligation reaction mixture. All constructs are depicted in Figures 3.4 and 3.5 below.

3.5.3 *In vitro* transcription and electroporation of cultured cells

Plasmids for *in vitro* transcription were purified from large-scale bacterial cultures by using HiSpeed Plasmid Maxi kit (Qiagen, USA) as previously described (Lindenbach *et al.*, 2005). Briefly, bacterial clones were inoculated in 200mL Luria Bertani broth supplemented with ampicillin (100mg/mL) and grown in a shaker incubator for 16 hours at 37°C. Bacterial pellets obtained after centrifugation were re-suspended with buffer and plasmid DNAs purified. The concentrations were measured using a Nanodrop Spectrophotometer (Nanodrop Products, Wilmington, DE) and DNA diluted with NFW to a final concentration of 1µg/µL. The purified plasmids (20µg each) were linearized with *Xba*I restriction enzyme and the digestion products were purified using the MinElute PCR purification kit (Qiagen Sciences, MD). RNA was transcribed from 1µg of purified templates by using the T7 RiboMAX Express large-scale RNA production system (Promega, Madison, WI) according to the manufacturer's recommendations.

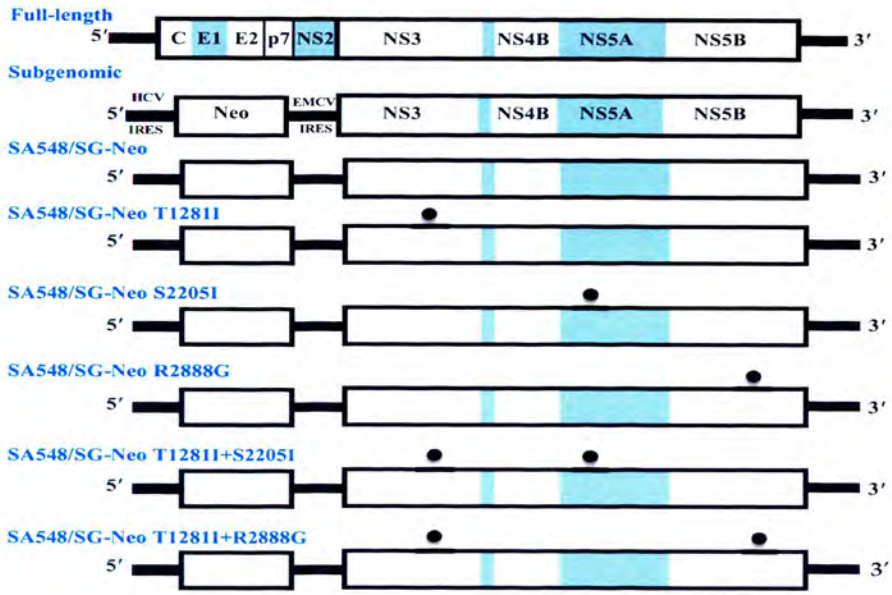

Figure 3.4: Sub-genomic replicons from genotype 5a isolate SA548. Black dots represent the NS3, NS5A, and NS5B point mutations.

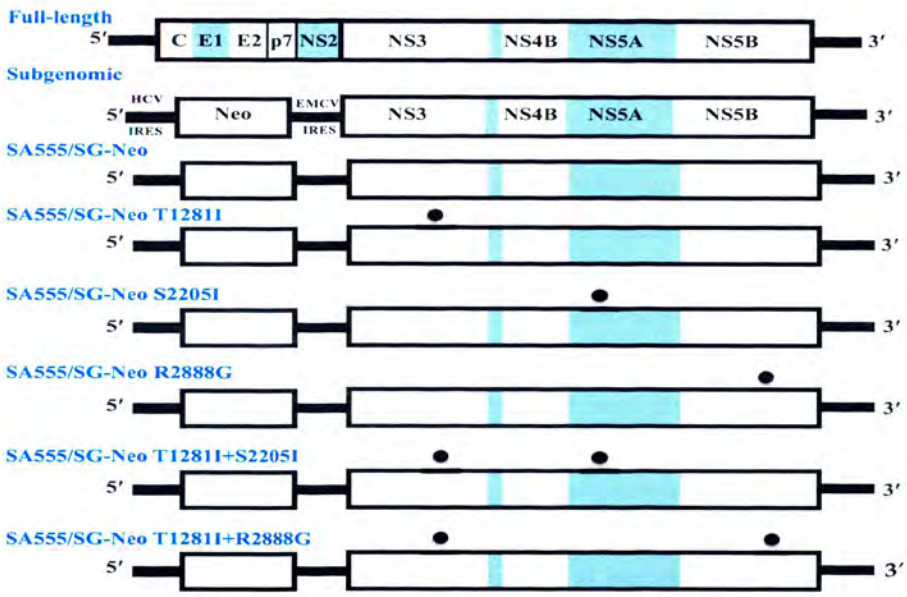

Figure 3.5: Sub-genomic replicons from genotype 5a isolate SA555. Black dots represent the NS3, NS5A, and NS5B point mutations.

RNA transcripts were synthesized at 37°C for 45 minutes in a 20μL reaction mixture containing 10μL of 10X buffer, 2μL of T7 polymerase, and 8μL of 125ng/μL template DNA. Synthesized RNA was treated with RNase-free DNase at 37°C for 15 minutes and then purified using RNeasy Mini kit (Qiagen, USA) according to the manufacturer's protocol. Prior to storage at -80°C, RNA was quantified using a NanoDrop spectrophotometer and the integrity was determined by agarose gel electrophoresis and visualization by SYBR safe dye staining.

3.5.3.1 RNA electroporation

Huh-7.5 cells were electroporated with *in vitro* transcribed RNA as previously described (Lindenbach *et al.*, 2005). Briefly, Huh-7.5 cells were treated with trypsin (0.05% Trypsin-EDTA) and re-suspended in growth media. The cells were pelleted and washed twice with 1X ice-cold Dulbecco's phosphate buffered saline (DPBS) and re-suspended in ice-cold DPBS to a concentration of 12.5 x 10^6 cells/mL. For electroporation, 5μg RNA was mixed with 400μL cell suspension (5.0 x 10^6 cells/electroporation), transferred into 2mm gap electroporation cuvettes (BTX620), and immediately pulsed using a BTX ElectroSquare Porator ECM 830 (BTX, Holliston, MA; 860V, five pulses, 99μs, 1.1s intervals). Electroporated cells were incubated at room temperature for 10 minutes prior to resuspension in 20mL growth media and plated in tissue culture dishes (BD Falcon).

3.5.4 Colony titration assay

Huh-7.5 cells, seeded in 6-well plates at a density of 400,000 cells/well, were grown overnight. Cells were transfected with 2μg of *in vitro* transcribed RNA using TransIT-mRNA transfection kit (Mirus Bio, WI), following manufacturer's recommendations. Six hours later, cells from each well were harvested and plated in new 6-well plates at multiple densities (between 2 X 10^2 and 2 X 10^5 cells/well). The total numbers of cells in each well were maintained at equal level by using cells transfected with RNA transcripts from replication defective HCV genomes. G418 selection was performed as described below. Three weeks post-transfection, colonies were stained with crystal violet and quantified manually. The colony formation efficiency was determined by calculating the percentage of transfected cells survived after selection.

3.5.5 Analysis of G418- resistant cells

For selection of colonies, G418 (final concentration of 500µg/mL) was added to the culture medium at 48 hours post electroporation. Cells were fed every third day with fresh medium containing 500µg/mL G418. Three weeks post electroporation G418-resistant cell colonies were either isolated and expanded for further analysis or fixed with 7% formaldehyde and stained with crystal violet.

3.5.5.1 RNA extraction and RT-PCR amplification

To identify any additional mutations HCV RNA might have acquired during replication, total cellular RNAs were isolated from the replicon cell clones using the RNeasy Mini kit (Qiagen, USA), according to the manufacturer's protocol. 2.5µg of purified RNA was reverse transcribed to cDNA by using a protocol described in section 3.4.1. NS3-NS5B region of the viral genome was amplified by PCR into four overlapping fragments (A, B, C, D) using Ex-*Taq* DNA polymerase. Cycing conditions were the same as mentioned earlier. Fragment A was amplified using PmeI-S (5'-ATTCGCAGCGCATCGCCTTC-3') and RU-O-18842 (5'-CGATGTTAGTGAGCCCTGTGAAC-3'), B using RU-O-18442 (5'-GACTTCAGTCTGGATCCTAC-3') and RU-O-19018 (5'-ACATCAGGTTCTGGCTCACAGG-3'), C using RU-O-18621 (5'-CGACGATATCTGGCCACGTGAAGAACGGAACCATGAGAATCGTCGGCCCAAAGATG-3') and RU-O-18618 (5'- CAGACGCGGACTCCAAGGTCG-3'), and D using RU-O-18619 (5'-GAGCCGCTCAAGGCGGAAG-3') and RU-O-18220 (5'-TCATGCGGCTCACGGACCTTTCACAGCTAG-3'). The PCR products were separated by agarose gel electrophoresis and purified using the MinElute gel extraction kit (Qiagen, USA). The purified PCR products were subjected to direct sequencing.

3.5.5.2 Quantification of HCV RNA by quantitative PCR (qPCR)

HCV RNA was quantified by qPCR as described previously (Saeed *et al.*, 2012). The reaction was carried out in duplicate in a final volume of 25µL consisting 5µL of 5X Genecode Isolution, 0.5µL of 50X Titanium Taq (Clontech, USA), 1µL of 10µM primer mix (forward primer 5'-GGCTCCATCTTAGCCC- 3' and reverse primer 5'-GCTCACGGACCTTTCA-3'), 0.5µL of 250mM MMLV RT, 13µL of NFW, and 5µL of template RNA. *In vitro* transcribed HCV standard (2.0 X 10^7 copies/µL) was

serially diluted 10 fold in NFW. Amplification qPCR was performed in a LightCycler 480 (Roche Applied Sciences) using one cycle of 50°C for five minutes, 95°C for three minutes, 50 cycles (95°C for five seconds, 58°C for 10 seconds, 72°C for 20 seconds), and 70 cycles of 60-95°C. The data was analyzed using MultiCode RTx Analysis software.

3.5.5.3 Flowcytometric analysis

Flow cytometry was used to detect HCV NS5A in cells, by using a protocol described previously (Marukian *et al.*, 2008). Briefly, cells were fixed in 2% paraformaldehyde-DPBS for 20 minutes at 4°C. The cells were pelleted down and resuspended in BD Cytofix/Cytoperm Plus buffer (BD Bioscience, San Diego, CA). After incubation at room temperature for 20 minutes, cells were washed twice in 1X BD Perm/Wash buffer and stained with AlexaFluor 647-conjugated anti-NS5A monoclonal antibody 9E10 (diluted 1:400 in 1X Perm/Wash buffer) for 40 minutes at room temperature. Stained cells were washed once with BD Perm/Wash buffer and suspended in 2% paraformaldehyde-PBS prior to analysis using FACS buffer (PBS/ 3%FBS) prior to analysis using BD FACSCalibur and BD CellQuest™ Pro premier acquisition and analysis software version 5.2.1 (BD Biosciences, San Diego, CA). All data were analyzed using FlowJo software version 8.8.7.

3.5.6 Synthesis of HCV-Feo replicons

To optimize replicons for high-throughput screening and evaluation of antiviral compounds, we generated Feo replicons of SA555/SG-Neo constructs by replacing the NPTII gene with a fusion protein of firefly luciferase and NPTII from JFH/SG-Feo as described by Saeed *et al.* (2012). Firstly, the 5' UTR of SA555/SG-Neo was amplified using sense primer RU-O-18673 (5'-GTGAATTC**TAATACGACTCACTATAG**ACCCGCCCCTTATTG-3' with *EcoR*1 site and **T7 promoter**) and RU-O-17944 antisense (5'-GGCGTCTTCCATTGGGCGACGGTTGGT-3') primer. The Feo-gene was amplified from the vector JFH1/SG-Feo using RU-O-17943 sense (5'-ACCAACCGTCGCCCAATGGAAGACG-3') and RU-O-17930 antisense (5'-AGAGGGTTTAAACTCAGAAGAAC-3' with *Pme*I site) primer set. Both PCR products were purified, mixed, and amplified again with RU-O-18673 and RU-O-17930. The resulting PCR fragment containing both HCV cDNA and the Feo gene

was cloned with JFH1/SG-Feo by *EcoR*1 and *Pme*I digestions to generate SA555/SG-Feo. SA555/SG-neo adaptive mutant replicons together with the SA555/SG-Feo construct were digested with *Pme*1 and *Spe*1. The digested products were separated by agarose gel electrophoresis; bands of interest cut and purified and the corresponding purified fragments combined in a ligation reaction mixture.

Genotype 2a replicon, JFH1/SG-Feo, and genotype 3a replicon, S52/SG-Feo (AII), are previously described (Saeed *et al.*, 2012). Genotype 4a replicon, ED43/SG-Feo (VYG), was made by introducing a newly identified mutation M1205V in NS3 protein of ED43/SG-Feo (Y), described in Saeed *et al.* (2012). It contains three cell culture-adaptive mutations: M1205V (NS3), D1431Y (NS3) and R2882G (NS5B). This replicon replicates to higher levels than ED43/SG-Feo (Y) (Unpublished results). Genotype 1a replicon, H77/SG-Feo (L+8) was generated from H77/SG-neo (L+8) (Tscherne *et al.*, 2007). It contains four adaptive mutations: P1496L (NS3), V1880A (NS4B), A1940V (NS4B), and C1968R (NS4B). Genotype 1b replicon, Con1/SG-neo (I) (Blight *et al.*, 2000) was modified to generate Con1/SG-Feo (I). This replicon contains S2204I substitution in NS5A protein.

3.5.7 HCV Inhibitor assay

Inhibition of HCV replicon replication was determined by measuring the levels of luminescence expressed via the firefly luciferase reporter gene. Replicon cells were seeded in 96-well plates overnight (Packard ViewPlate-96, White cat# 6005181) at a density of 10,000 cells/well in assay medium (DMEM, 10% FBS, 1X NEAA). The following day, HCV compounds were serially diluted five-fold in DMSO (final concentration of 0.125%) added to the cells in duplicate and cells were further grown for 72 hours. After 72 hours of growth, luciferase activity was measured with the luciferase assay system (Promega) according to manufacturer's recommendations using a Lumat LB9507 luminometer (EG and G Berthold, Bad Wild- bad, Germany). The concentrations at which 50% inhibition was achieved (50% effective concentration, EC50s) relative to the DMSO control were determined using the GraphPad Prism software (San Diego, CA). Replicon cell viability in the presence of drug was tested in parallel under the same conditions except that the cells were seeded at 1,000 cells/well in 96-well plates and adenosine triphosphate (ATP) levels were measured using CellTiter-Glo (Promega) according to manufacturer's instructions.

3.5.8 Generation of HCV genotype 5a/JFH1 chimeras

Based on the consensus full-length sequences of SA548 and SA555, chimeric plasmids SA13/JFH1 (SA548EIE2), SA13/JFH1 (SA556EIE2), SA548 (C-NS2)/JFH1, and SA556 (C-NS2)/JFH1 were generated. For E1E2/JFHI chimeras, the *Kpn*I-*Ale*I and *Kpn*I-*Afl*II digested EIE2 fragment of pSA13/JFHI$_{(C3405G-A3696G)}$ (GenBank accession number FJ393024: previously reported by Jensen *et al.*, 2008) was replaced with similarly digested EIE2 PCR fragments of SA548 and SA556. HCV RNAs were extracted from patient plasma and used as a template for cDNA synthesis as previously described above. Template cDNA was first amplified with KOD polymerase as earlier described in section 3.5.1 by PCR using RU-O-9189 forward (5'-GAGCACGAATCCTAAACCTCAAAG-3') and RU-O-9204 reverse (5'-TCGTGGTTTCCAGGTTTTCAAC-3') primer set. A 2µL aliquot of first round PCR products was amplified in a nested PCR using inner forward RU-O-9190 (5'-CTTGAGGACGGGGTAAACTATGC-3') and reverse RU-O-9202 (5'-CAAATGCCCAAGCAAAGGTAGG-3') primers. The PCR products were run on agarose gel, purified and treated with *Taq* DNA polymerase then cloned into TOPO vector as mentioned earlier in section 3.4.5.

To fuse the generated plasmids SA548/EIE2 and SA556/E1E2 with pSA13/JFH1 for *Kpn*I/*Ale*I and *Kpn*I/*Afl*II digestions, respectively, pSA13/JFH1 were amplified using RU-O-20027 forward (5'-GGTCAACCCGGGTACCCTTG-3' with *Kpn*I site) and RU-O-20026 reverse (5'-CATTTCGGTAGGGAACGGCAGAGGTCGGGAC-3') primers. Similarly, plasmid SA548/EIE2 and SA556/EIE2 were amplified using RU-O-20025 forward (5'-GTCCCGACCTCTGCCGTTCCCTACCGAAATG-3') and RU-O-20030 reverse (5'-CAATCAGTAGGACACTTAAGGCTATTG-3' with *Afl*II). The PCR products were run on gel, purified, mixed and amplified using RU-O-20027 and RU-O-20030, to give the intermediate PCR products SA548/EIE2/SA13-JFHI and SA556/EIE2/SA13-JFHI. The purified intermediate PCR products were digested with *Kpn*I and *Afl*II and cloned into these similarly cleaved sites in pSA13/JFHI. The resultant plasmids were named SA13/JFHI (SA548E1E2) and SA13/JFHI (SA556E1E2) as depicted (Figure 3.6).

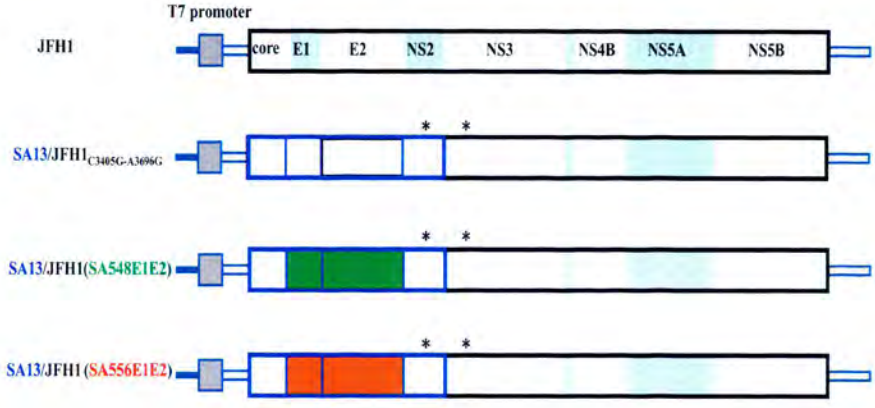

Figure 3.6: E1E2/JFH1 chimeric constructs of genotype 5a and JFH1. *Represent the NS2 (C3405G) and NS3 (A3696G) point mutations.

The C-NS2/JFHI chimeras were assembled via a multistep PCR with high fidelity KOD polymerase and cloning procedure using three plasmids containing overlapping structural and NS2 genes. The first PCR (PCR1) to combine the 5' UTR of pSA13/JFHI with core protein of SA548 and SA556 was done using RU-O-18528 forward (5'-AGCACGAATCCTAAACCTCAA-3') and RU-O18530 (5'-CCAAGGGTACCCGGGTTGAC-3') reverse primers. pSA13/JFHI was amplified (PCR2) using RU-O-15724 forward (5'-TTCGCCAGTTAATAGTTTGC-3') and RU-O-18529 (5'-TTGAGGTTTAGGATTCGTGCT-3') reverse primer set. To fuse core with E1, PCR3 was done using RU-O-20027 and RU-O-20068 reverse (5'-CATTTCGGTAGGGAACTGCAGAGGCCGGGAC-3') primers. In PCR4 plasmids SA13/JFHI(SA548E1E2) and SA13/JFHI(SA556E1E2) were amplified using RU-O-20069 forward (5'-GTCCCGGCCTCTGCAGTTCCCTACCGAAATG-3') and RU-O-20030 primers.

To combine E2 with P7, the same plasmid templates were amplified in PCR5 using RU-O-20028 (5'-CCCCACGCCCGTGCGGAATAG-3' with *Ale*I site) and RU-O-20032 (5'-GACGTTCTCCAGGGCCGCTTGGCCTGAC-3'). The p7-NS2 region was amplified in PCR6 using RU-O-20031 forward (5'-GTCAGGCCGAAGCGGCCCTGGAGAACGTC-3') and RU-O-20071 reverse (5'-

CATAAGCAGTGATGGGAGCGAGAAGTCGCCAGCC-3') primers. The template for PCR7 to fuse NS2 with NS3 proteins was pSA13/JFHI, which was amplified using RU-O-20070 forward (5'-GGCTGGCGACTTCTCGCTCCCATCACTGCTTATG-3') and RU-O-20033 reverse (5'-GCTACCGAGGGGTTAAGCACTAGTAC-3' with *Spe*I site) primer sets. All PCR products were separated by agarose gel electrophoresis and purified. The purified PCR products (PCR1and PCR2) were combined and amplified using RU-O-15724 and RU-O-18530. PCR products were separated on gel, purified, digested with *Kpn*I and *Xmn*I and ligated with similarly digested SA13/JFHI (SA548E1E2) plasmid to generate the intermediate plasmids SA548-Core/SA13/JFHI and SA556-Core/SA13/JFHI (Figure 3.7).

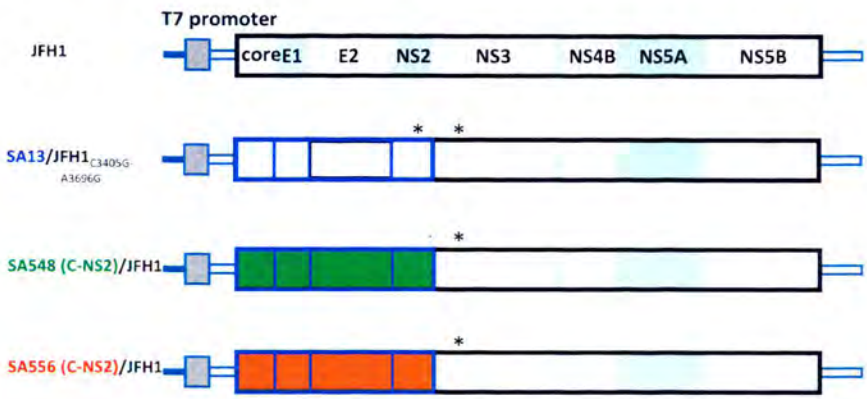

Figure 3.7: C-NS2/JFH1 chimeric constructs of genotype 5a and JFH1. *Represent the NS3 (A3696G) point mutation

In a similar manner, PCR3 and PCR4 products were mixed and amplified using RU-O-20027 and RU-O-20030. The combined product of PCR5/6 amplified using RU-O-20028 and RU-O-20071, was mixed with PCR7 and amplified using RU-O-20028 and RU-O-20033. The purified PCR3/4 product was digested with *Kpn*I and *Afl*II and fused with similarly cleaved SA548-Core/SA13/JFHI and SA556-Core/SA13/JFHI. The resultant plasmids together with PCR products from PCR5/6/7 combined were digested with *Afl*II and *Spe*I and cloned to generate SA548 (C-NS2)/JFHI and SA556 (C-NS2)/JFH1 as illustrated in Figure 3.7. All plasmids were linearized, *in vitro* transcribed and electroporated into Huh-7.5 cells as previously mentioned. Re-

suspended cells were plated into 6-well tissue culture plates in duplicates and in 10cm dishes. The supernatants and cells in the 6-well plates were harvested at 24, 48, and 72 hours post-electroporation for FACS analysis and measurement of infectious virus titers. Some of the supernatants were used to infect naïve Clone 8 cells to monitor for nuclear translocation.

3.5.8.1 Determination of $TCID_{50}$

The 50% tissue culture infectious dose ($TCID_{50}$) of culture supernatants was determined as previously described (Lindenbach *et al.*, 2005). Briefly, Huh-7.5 cells were seeded (6 X 10^3 cells per well) in poly-L-lysine (100µg/mL: Sigma, St Louis, MO) coated 96-well tissue culture plates (BD Biosciences). After approximately 24 hours, cells were incubated with 10-fold serial dilutions of cell culture supernatants in replicates of six. Seventy-two hours later, cells were washed with DPBS and fixed in 100% methanol at -20°C for 30 minutes. Blocking was done at room temperature for 30 minutes in 1X DPBS-T/1% BSA/0.2% Skim milk. Cells were washed twice with 1x DPBS and once with 1x PBS-T and incubated with primary anti-NS5A antibody 9E10 (diluted 1:2000 in DPBS/0.1% Tween-20) for one hour at room temperature. After a second wash, cells were incubated with secondary antibody (Goat-anti-mouse-HRP, Jackson Immuno Research; diluted 1:200 in 1X DPBS/0.1% Tween-20) for 30 minutes at room temperature, followed by incubation with DAB substrate (DAKO, K3468; diluted 1 drop/mL as per manufacturers instructions) for five minutes at room temperature. Washed cells were covered with 1X DPBS and visualized under a light microscope to count and record NS5A-positive wells. Wells were scored positive if at least 1 positive cell was detected and the $TCID_{50}$ values infectious were measured by Reed and Muench method as previously described (Lindenbach *et al.*, 2005).

CHAPTER 4
RESULTS

4.1 ISOLATION OF HCV FROM PATIENT SAMPLES

Blood samples were obtained from eight voluntary blood donors at the National Institute for Communicable Diseases (NICD), Sandringham, Johannesburg, South Africa. The samples were confirmed to be HCV RNA-positive using the qualitative COBAS Amplicor HCV monitor test, version 2.0 (Roche Molecular Diagnostics). Plasma samples (patient ID numbers MD00545, MD00546, MD00548, MD00555, MD00556, MD00560, MD00600and MD00606) were shipped to the Laboratory of Virology and Infectious Diseases, The Rockefeller University, New York, where the samples were renamed as SA545, SA546, SA548, SA555, SA556, SA560, SA600 and SA606. To determine and compare the viral load in these patient samples, total RNAs were extracted and quantified by qPCR using the Eragen protocol. The mean HCV RNA copies were calculated and the results compared to those obtained at the NICD (Figure 4.1). Because of higher RNA titers ($\geq 1.0 \times 10^6$ copies of HCV RNA copies/mL of plasma), samples SA548, SA555, SA556, and SA560 were selected for further studies.

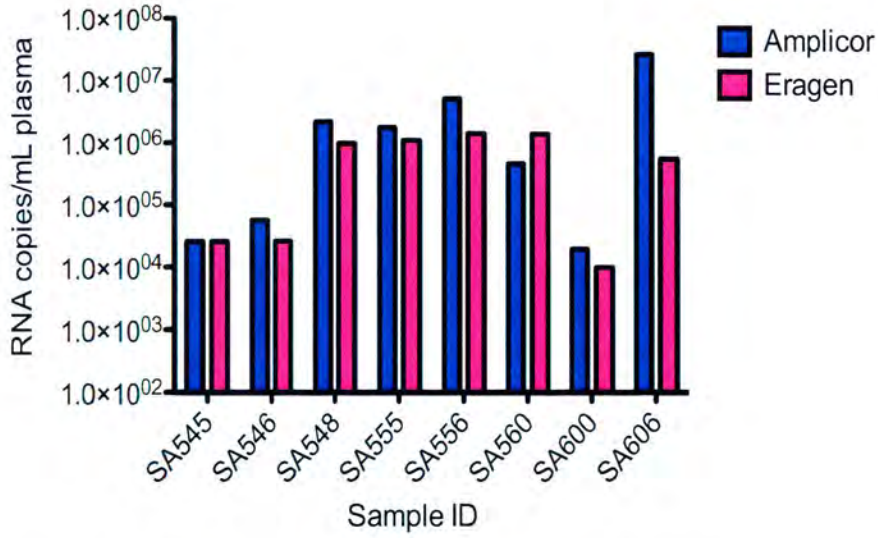

Figure 4.1: HCV RNA quantification in eight patient plasma samples.

4.2 GENETIC ANALYSIS OF THE NOVEL GENOTYPE 5a ISOLATES

4.2.1 Variability in the sequence of the 5' UTR

The heterogeneity of the 5' UTR was determined by cloning and sequence analysis of viruses recovered from plasma pools of infected South African blood donors. The complete 5' UTR of SA548, SA555, SA556, and SA560 was successfully amplified by RACE and amplicons were visualized on 1% agarose gel (Figure 4.2). The purified products were then cloned into the TOPO vector.

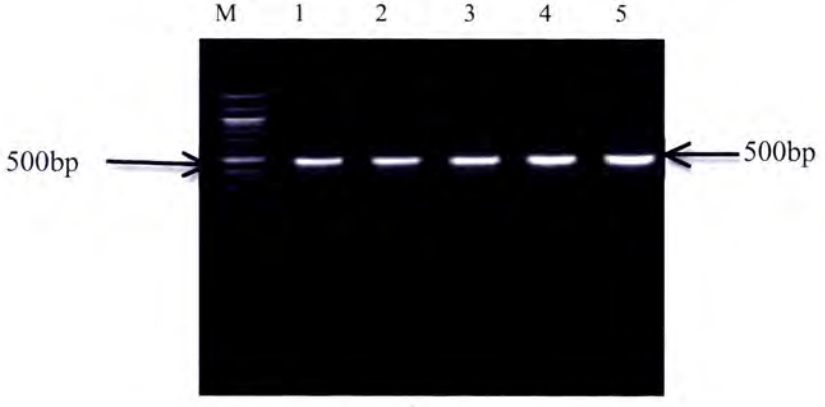

Figure 4.2: Amplification of the HCV 5' UTR by RACE. Lanes 1, 2, 3, and 4 represent isolates SA548, SA555, SA556 and SA560, respectively. Lane 5, positive control (genotype 2a, strain JFH1) Lane M, 100bp DNA ladder (New England Biolabs, NEB).

A consensus sequence of five clones was determined for each isolate. Sequence analysis of the 5' UTR revealed that the entire length was 339bp similar to SA13 (Li *et al.*, 2011) and S52 (Gottwein *et al.*, 2010) as shown (Figure 4.3). In contrast to genotype 1a (strain H77), the 5'-terminal adenine (A) residue was highly conserved for all other genotypes. However, both A and guanine (G) exist naturally as the 5'-terminal nucleotide of HCV isolates (Cai *et al.*, 2004).

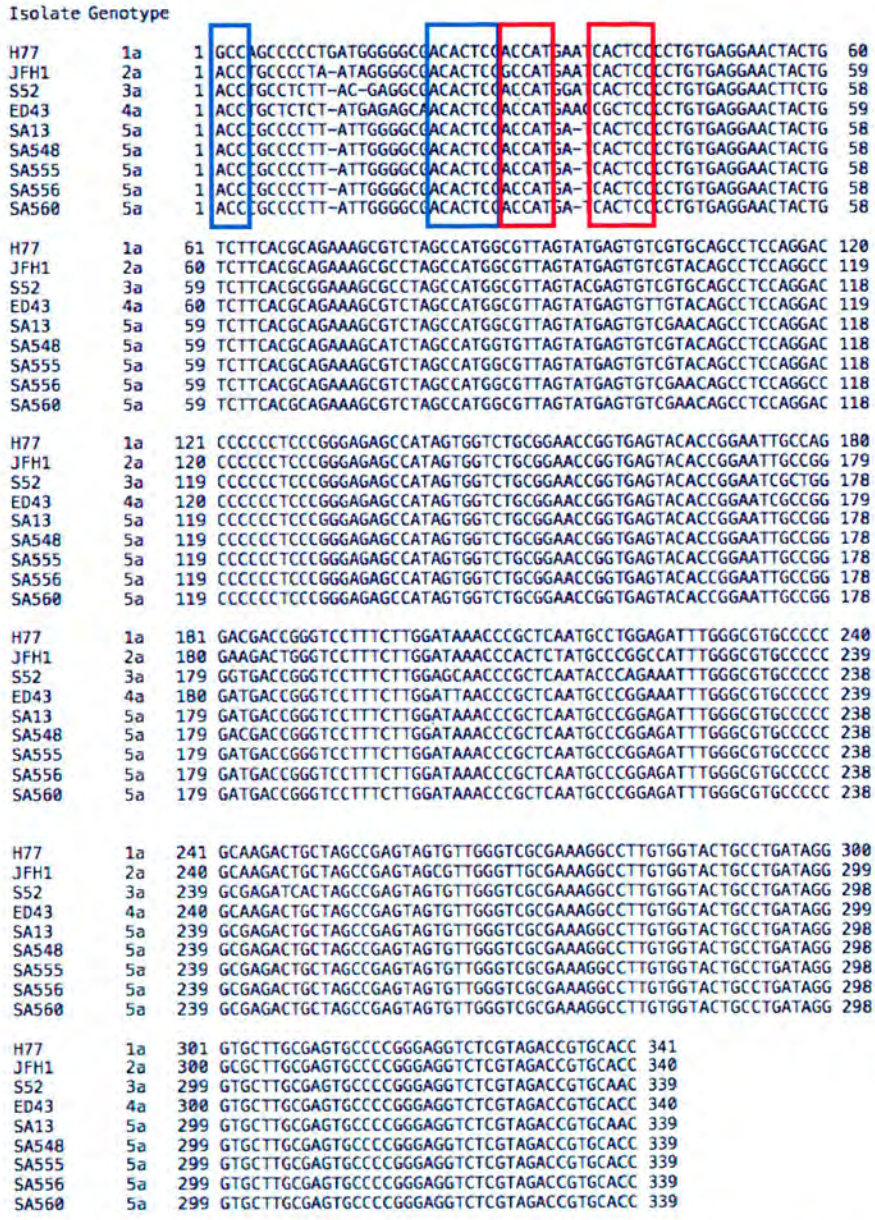

Figure 4.3: Alignment of the complete HCV 5' UTR consensus sequences from GT 5a, H77 (GT 1a), JFH1 (GT 2a), S52 (GT 3a) and ED43 (GT 4a) isolates. miR-122 binding sites 1 and 2 are indicated in blue and red boxes, respectively, dashes (-) indicate deletions.

The degree of deviation of the 5' UTR was determined from the multiple sequence and matrix alignments. The 5' UTR of SA13 was 98.5% identical to SA548 and 99.4% identical to SA555, SA556, and 99.7% identical to SA560. Similarly, SA13, SA548, SA555, SA556, and SA560 had between 96.2 – 96.5% identity with H77, 94.4 – 94.7% with JFH1, 92.1 – 92.9% with S52, and 94.7 – 95.3% with ED43. Overall, the 5' UTR of SA13 was more closely related to SA560 with 99.7% sequence identity, followed by SA555, SA556 with 99.4%, and SA548 with 98.5% identity (Table 4.1).

Table 4.1: Percent nucleotide sequence identity of the 5' UTR among HCV GT 5a and other HCV isolates

	H77	JFH1	S52	ED43	SA13	SA548	SA555	SA556	SA560
H77		**93.8**	**91.5**	**94.7**	**96.2**	**96.5**	**96.8**	**96.2**	**96.5**
JFH1	**93.8**		**90.3**	**92.6**	**94.4**	**94.4**	**95.0**	**95.0**	**94.7**
S52	**91.5**	**90.3**		**91.2**	**92.9**	**92.1**	**92.9**	**92.4**	**92.6**
ED43	**94.7**	**92.6**	**91.2**		**94.7**	**94.4**	**95.3**	**94.7**	**95.0**
SA13	**96.2**	**94.4**	**92.9**	**94.7**		**98.5**	**99.4**	**99.4**	**99.7**
SA548	**96.5**	**94.4**	**92.1**	**94.4**	**98.5**		**99.1**	**98.5**	**98.8**
SA555	**96.8**	**95.0**	**92.9**	**95.3**	**99.4**	**99.1**		**99.4**	**99.7**
SA556	**96.2**	**95.0**	**92.4**	**94.7**	**99.4**	**98.5**	**99.4**		**99.7**
SA560	**96.5**	**94.7**	**92.6**	**95.0**	**99.7**	**98.8**	**99.7**	**99.7**	

4.2.2 Variability in the sequence of the envelope proteins

Multiple sequence alignments of the consensus sequences of E1 and E2 envelope glycoproteins of the novel 5a isolates revealed that the length was 1668 nucleotides (576bp in E1 and 1092bp in E2) and 556 amino acids (192 in E1 and 364 in E2), equivalent to SA13 and HCV-EUH1480 (other GT 5a isolates) but one amino acid residue shorter than genotype 1 isolates, including TN, H77C, J4, and Con-1 (Sakai *et al.*, 2007; Lohmann *et al.*, 1999; Yanagi *et al.*, 1998; Yanagi *et al.*, 1997). Also pairwise alignment of the amino acid sequences of SA13 versus novel genotype 5a isolates showed 89% identity with SA548, SA555 and 560, and 91% identity with SA560. Greatest heterogeneity (percentage of amino acid positions with at least one clone differing from the consensus sequence) was found in the E2 region (Figure 4.4).

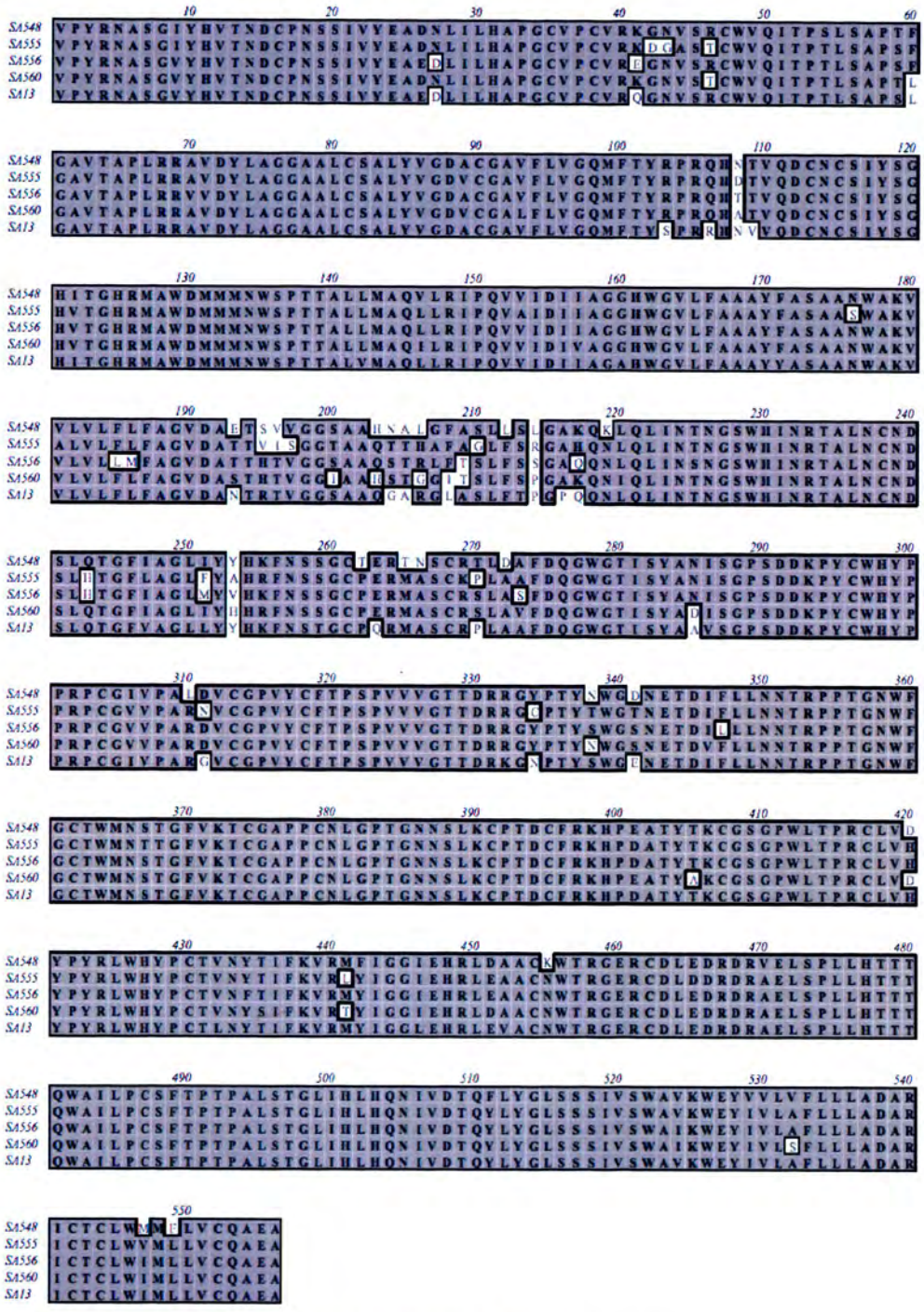

Figure 4.4: Alignment of HCV genotype 5a E1E2 protein sequences

Further more, matrix analysis of the E1E2 consensus sequence of SA548, SA555, SA556, and SA560 showed 94.6 to 96.6% identity to SA13 at the amino acid level (Table 4.2) and 87.3 to 90.5% at the nucleotide level (Table 4.3).

Table 4.2: Percent nucleotide sequence identity of the E1E2 consensus sequence among HCV GT 5a isolates at the amino acid level

	SA548	SA555	SA556	SA560	SA13
SA548		**89.4**	**90.5**	**91.4**	**89.6**
SA555	**94.1**		**91.7**	**90.6**	**89.4**
SA556	**95.1**	**95.7**		**92.6**	**91.9**
SA560	**95.0**	**95.5**	**96.6**		**89.6**
SA13	**94.6**	**95.0**	**96.6**	**95.9**	

Table 4.3: Percent nucleotide sequence identity of the E1E2 consensus sequence among HCV GT 5a isolates at the nucleotide level

	SA548	SA555	SA556	SA560	SA13
SA548		**86.6**	**88.2**	**86.9**	**87.6**
SA555	**86.6**		**89.2**	**87.3**	**87.3**
SA556	**88.2**	**89.2**		**90.0**	**90.5**
SA560	**86.9**	**87.3**	**90.0**		**87.7**
SA13	**87.6**	**87.3**	**90.5**	**87.7**	

4.2.3 Variability in the C-NS2 sequence

The consensus sequences of the C–NS2 of the novel genotype 5a isolates were also aligned. The alignment showed that SA548, SA555, SA556 and SA560 consisted of 3081 nucleotides and 1,027 amino acids. Similar to E1E2, a pairwise alignment of the amino acid sequences of SA13 versus novel genotype 5a isolates was performed. Results showed 91% sequence identity with SA548 and SA560, 92% with SA555, and 93% with SA556. The C-NS2 consensus sequence of SA548, SA555, SA556, and

SA560 was 96.0 to 96.9% identical to SA13 at the amino acid level (Table 4.4) and 89.4 to 91.4% at the nucleotide level (Table 4.5).

Table 4.4: Percent nucleotide sequence identity of the C-NS2 among HCV GT 5a isolates at the amino acid level

	SA548	SA555	SA556	SA560	SA13
SA548		**92.9**	**93.0**	**92.3**	**91.7**
SA555	**96.3**		**93.6**	**92.6**	**92.0**
SA556	**96.2**	**96.7**		**93.5**	**93.6**
SA560	**96.1**	**96.6**	**96.9**		**91.2**
SA13	**96.0**	**96.4**	**96.9**	**96.3**	

Table 4.5: Percent nucleotide sequence identity of the C-NS2 among HCV GT 5a isolates at the nucleotide level

	SA548	SA555	SA556	SA560	SA13
SA548		**89.4**	**89.9**	**88.8**	**89.8**
SA555	**89.4**		**90.2**	**89.2**	**89.4**
SA556	**89.9**	**90.2**		**90.8**	**91.4**
SA560	**88.8**	**89.2**	**90.8**		**89.8**
SA13	**89.8**	**89.4**	**91.4**	**89.8**	

4.2.4 Variability in the sequence of the NS3-NS5B region

In a similar manner to C-NS2, the consensus sequences of the NS3-NS5B of the novel genotype 5a isolates were aligned with SA13. Pairwise alignment of the amino acid sequences of SA13 versus novel genotype 5a isolates showed 97% sequence identity with SA548 and SA556, and 96% with SA555 and SA560. The NS3-NS5B consensus sequence of the novel 5a isolates was 98.6 to 99.2% identical to SA13 at the amino acid level (Table 4.6) and 91.5 to 92.6% at the nucleotide level (Table 4.7).

Table 4.6: Percent amino acid sequence identity of the NS3-NS5B among HCV GT 5a isolates at the amino acid level

	SA548	SA555	SA556	SA560	SA13
SA548		**97.3**	**97.6**	**96.7**	**97.4**
SA555	**98.7**		**96.8**	**96.4**	**96.6**
SA556	**99.1**	**98.5**		**97.4**	**97.2**
SA560	**98.5**	**98.2**	**98.8**		**96.3**
SA13	**99.2**	**98.7**	**99.1**	**98.6**	

Table 4.7: Percent nucleotide sequence identity of the NS3-NS5B among HCV GT 5a isolates at the nucleotide level

	SA548	SA555	SA556	SA560	SA13
SA548		**92.2**	**92.6**	**92.1**	**92.5**
SA555	**92.2**		**92.0**	**91.4**	**91.5**
SA556	**92.6**	**92.0**		**94.1**	**92.6**
SA560	**92.1**	**91.4**	**94.1**		**91.9**
SA13	**92.5**	**91.5**	**92.6**	**91.9**	

4.2.5 Variability in the sequence of the 3' UTR

The heterogeneity of the 3' UTR was also analyzed by cloning and sequencing DNA amplicons obtained in nested RT-PCR. The complete HCV 3' UTR was amplified into two overlapped regions. The first domain from the 3' 131nt of NS5B to the middle part of the 3' X tail was amplified by regular RT-PCR as Fragment H (Figure 4.5A). The second domain containing the 3' X tail was determined by RACE (Figure 4.5B). The two overlapping fragments covering the complete HCV 3' UTR were successfully amplified from all four samples. All fragments were gel-purified and cloned into the TOPO vector.

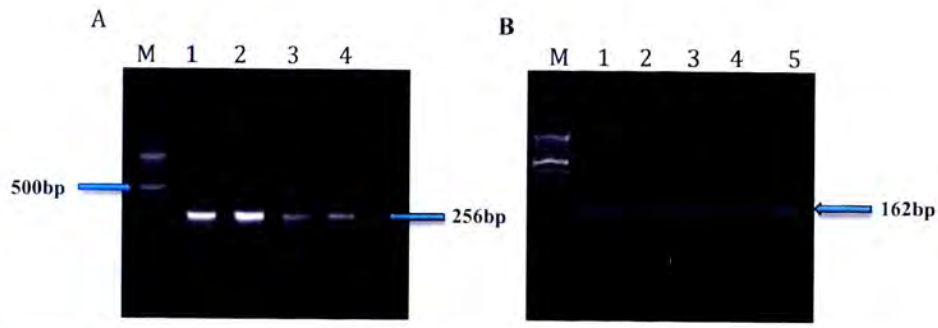

Figure 4.5: Amplification of the HCV 3' UTR. (A) poly (U/UC) tract, (B) 3′ X tail. Lanes 1, 2, 3, and 4 represent isolates SA548, SA555, SA556 and SA560, respectively; Lane 5 in A, negative control, in B, positive control (genotype 2a, strain JFH1). Lane M, 100bp DNA ladder (New England Biolabs, NEB).

The consensus sequence of at least 10 clones, which included sequences of the entire variable domain, the poly U/UC tract, and the first 43 nts of the conserved region, was determined for each isolate. The sequence of the variable domain was highly conserved with only one point mutation in all clones analyzed for each isolate. Sequence analysis showed that the entire length of the 3' UTR was 182 nucleotides long in SA548 and SA556, 178 nucleotides in SA555, and 200 nucleotides in SA560 compared to 269 nucleotides in H77, and 236 nucleotides in JFH1 (Figure 4.6). Although the complete 3' UTR of SA13 hasn't been published, one can expect the 3' X-region to have a similar length as the other genotype 5a isolates, ending with TGT.

The poly U/UC tract varied in length as well as composition amongst the isolates. The clone with the longest poly U/UC stretch was adopted as the clone of choice for generation of full-length constructs. The poly U/UC region observed in SA560 contained two G and one A residues, different from what was observed in the case of SA13, which had one G residue. However, the first 16 nucleotides of the conserved region were identical to SA13, JFH1 and H77. Similar to SA13 (Bukh *et al.*, 1998), the sequence 5'-AAATCTTT-3' was found between the poly UC and the conserved sequence of the 3' UTR. Multiple sequence and matrix alignment of the 3' X-tail revealed 100% sequence homology in all four GT 5a isolates. The percentage nucleotide similarities of GT 5a isolates with H77 and JFH1 were 99% and 96.9%, respectively (Table 4.8).

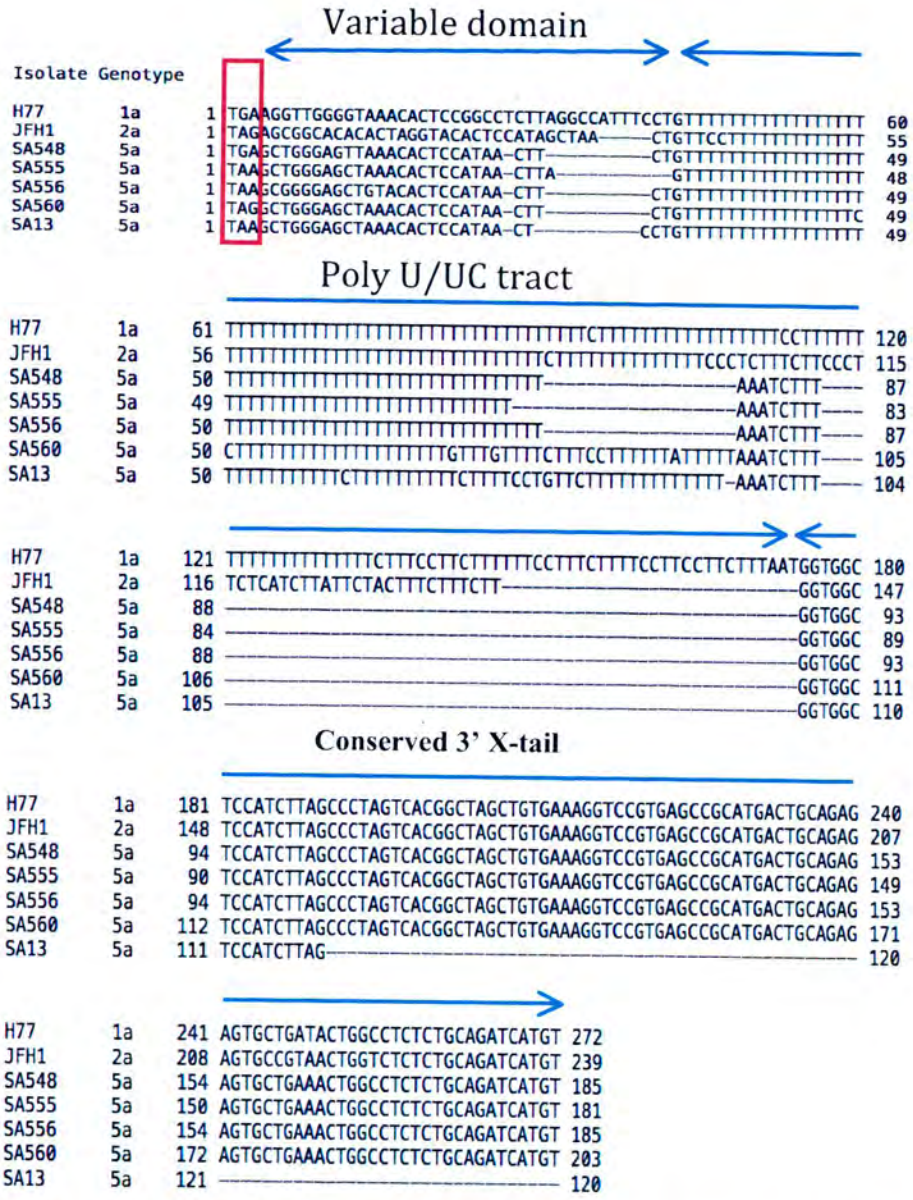

Figure 4.6: Alignment of the complete HCV 3' UTR consensus sequences derived from the novel GT 5a, SA13 (5a- partial sequence), H77 (GT 1a), and JFH1 (GT 2a) isolates. Stop codon indicated in red box, which marks the end of the polyprotein region and dashes (-) indicate deletions.

Table 4.8: Percent nucleotide sequence identity of the 3' X-tail from HCV GT 5a, H77 (1a), and JFH1 (2a) isolates

	H77	JFH1	SA548	SA555	SA556	SA560
H77		**95.9**	**99.0**	**99.0**	**99.0**	**99.0**
JFH1	**95.9**		**96.9**	**96.9**	**96.9**	**96.9**
SA548	**99.0**	**96.9**		**100.0**	**100.0**	**100.0**
SA555	**99.0**	**96.9**	**100.0**		**100.0**	**100.0**
SA556	**99.0**	**96.9**	**100.0**	**100.0**		**100.0**
SA560	**99.0**	**96.9**	**100.0**	**100.0**	**100.0**	

4.2.6 Multiple sequence alignments of the ORF of the novel HCV GT 5a strains and representative isolates of other genotypes

The consensus sequences of the complete genome of HCV from each of the four samples were determined by clonal sequencing of overlapping PCR-amplified genomic regions. Sequence analysis of the full-length genome of genotype 5a 5' UTR – 3'UTR viruses recovered from plasma pools revealed that the length was 9566 nucleotides (in SA548 and SA556), 9562 nucleotides (in SA555), and 9584 nucleotides (in SA560). The difference in lengths of these strains was due to variable lengths of poly (U/UC) region in the 3' UTR. Multiple sequence alignments of the open reading frame (ORF) nucleotide and amino acid sequences were also performed. For all four novel GT 5a isolates the ORF consisted of 9045 nucleotides (nts) encoding a single polyprotein of 3014 aa spanning nts 340-9381 followed by a stop codon. This is the same length at nucleotide and amino acid levels as determined for other GT 5a isolates, SA13 and EUH1480, with no insertions or deletions (Bukh *et al.*, 1998; Chamberlain *et al.*, 1997).

By phylogenetic analysis, genotypes designated 5a from South African specimens, formed a unique, relatively closely related group of isolates (Figure 4.7). The HCV strains analyzed and their corresponding GenBank accession numbers are: SA555 (SA1; KJ925146), SA548 (SA2; KJ925147), SA556 (SA3; KJ925148), SA560 (SA4; KJ925149), K3a/650; D28917, NZL1; NC_009824, S52; GU814263, EUH1480; Y13184, HCV-6a63; DQ480514, Tr Kj; D49374, HC-J8; D10988, BEBE1; D50409, JFH1; AB047639, HCV-02C; DQ418784, H77; AF009606, HCV-JK1; X61596,

SA13; AF064490 and HCV-JT; D11168.

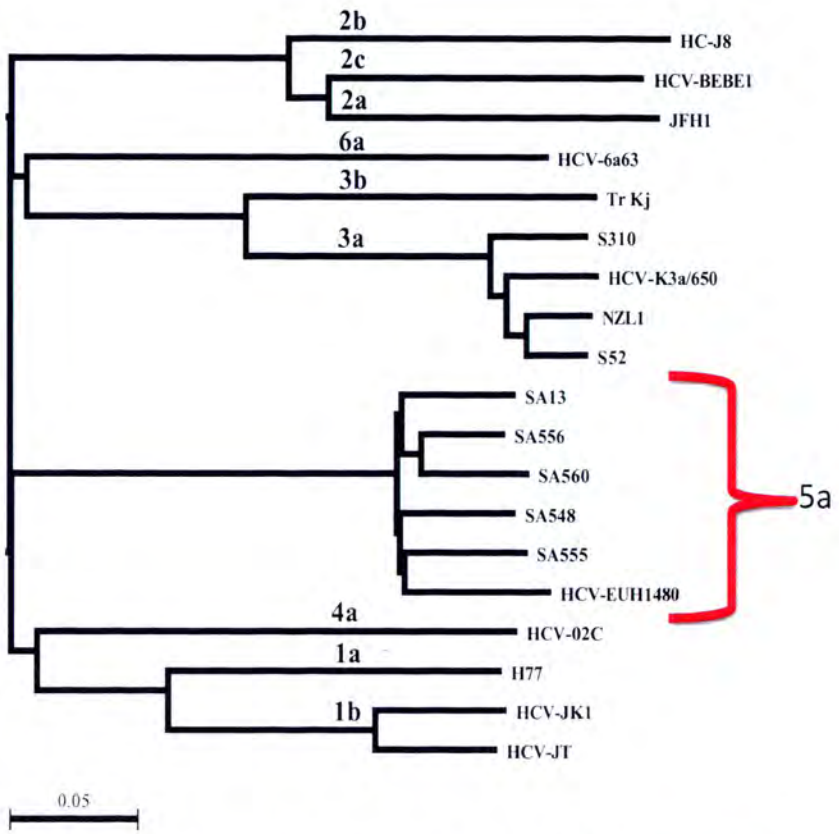

Figure 4.7: Phylogenetic analysis of HCV GT 5a and other HCV isolates. The root of the tree was tentatively taken as the midpoint of the longest path. The length of the horizontal bar indicates the number of nucleotide substitutions per site.

To determine the degree of variation among isolated strains, genetic heterogeneity in each sub-genomic region was calculated (Table 4.9). At the nucleotide level, the mean genetic heterogeneity of the entire ORF was 8.54%. Among the sub-genomic regions, the largest variation was seen in the envelope proteins (E1 11.11%, E2 12.59%) and NS2 (11.44%). The 5' UTR was the most conserved region with a mean diversity of 0.79%. At the amino acid level, genetic heterogeneity of the entire polyprotein was 4.34%. Among the sub-genomic regions, E2, NS2 and E1 displayed greatest diversity (10.53%, 7.68% and 6.60%, respectively).

Table 4.9 Mean genetic heterogeneity between isolated GT 5a strains

	Heterogeneity at the nucleotide level		Heterogeneity at the amino acid level	
Genomic region	Nucleotide position*	% heterogeneity (mean±SD)	Amino acid position*	% heterogeneity (mean±SD)
5' UTR	1-339	0.79 ± 0.48		
Core	340-912	3.93 ± 1.03	1-191	1.83 ± 0.86
E1	913-1488	11.1 ± 0.86	192-383	6.60 ± 1.67
E2	1489-2580	12.6 ± 1.36	384-747	10.5 ± 1.51
P7	2581-2769	9.79 ± 1.60	748-810	1.59 ± 1.74
NS2	2770-3420	11.4 ± 0.63	811-1027	7.68 ± 2.17
NS3	3421-5313	7.16 ± 0.60	1028-1658	1.88 ± 0.53
NS4A	5314-5475	6.28 ± 2.85	1659-1712	0.31 ± 0.76
NS4B	5476-6258	8.17 ± 3.32	1713-1973	1.72 ± 0.79
NS5A	6259-7608	8.98 ± 0.94	1974-2423	6.04 ± 0.99
NS5B	7609-9381	6.91 ± 0.30	2424-3014	2.57 ± 0.39
ORF	340-9381	8.54 ± 0.80	1-3014	4.34 ± 0.41

UTR, Untranslated region, E, envelope, NS, non-structural *The nucleotide and amino acid positions refer to SA13 (GenBank accession no. AF064490)

4.3 SYNTHESIS OF HCV SUB-GENOMIC REPLICONS AND THEIR REPLICATION EFFICIENCY

4.3.1 Replication of SA548 and SA555-derived sub-genomic replicons

Based on the full-length consensus sequences of SA548 and SA555, sub-genomic replicons were established as described in section 3.5. In addition, mutations in the NS3 T1281I (corresponding to T1280I in genotype 1a strain H77; GenBank accession number AF009606), NS5A S2205I (corresponding to S2204I in genotype 1a strain H77; GenBank accession number AF009606), and NS5B R2888G (corresponding to R2882G in genotype 4a strain ED43; GenBank accession number GU814265) previously shown to enhance genotypes 1 and 4 replication, were incorporated. Since combining mutations in NS3 with those in other genes of HCV has been shown to increase adaptation (Blight *et al.*, 2003; Lohmann *et al.*, 2003, Krieger *et al.*, 2001), two additional sets of replicons in which T2181I was combined with S2205I and R2888G mutations to create SA548/SG-neo T1281I+S2205I, SA548/SG-neo T1281I+R2888G, SA555/SG-neo T1281I+S2205I and SA555/SG-neo T1281I+R2888G were generated.

When *in vitro*–transcribed replicon RNAs from all constructs were introduced into Huh-7.5 cells by electroporation and selected with G418 from 48 hours post electroporation and for three weeks, viable colonies were observed for SA548/SG-neo, SA548/SG-neo S2205I and SA555/SG-neo S2205I. All other mutants failed to produce G418-resistant colonies in Huh-7.5 cells. This suggested that the NS5A mutation S2205I was important to the selection of stable replicon colonies, although the SA548 isolate was capable of initiating replication even without mutations (Figure 4.8). From two independent electroporations a total of eight GT 5a replicon colonies were picked and expanded, however, efforts to expand colonies for SA548/SG-neo and SA548/SG-neo S2205I was unsuccessful. No specific reason or conclusion as to why the colonies did not proliferate in culture can be given at this point because no further investigations were made. However, because success was made with the SA555/SG-neo S2205I construct, all efforts were vested on this. Hence only replicons for SA555/SG-neo S2205I are described further.

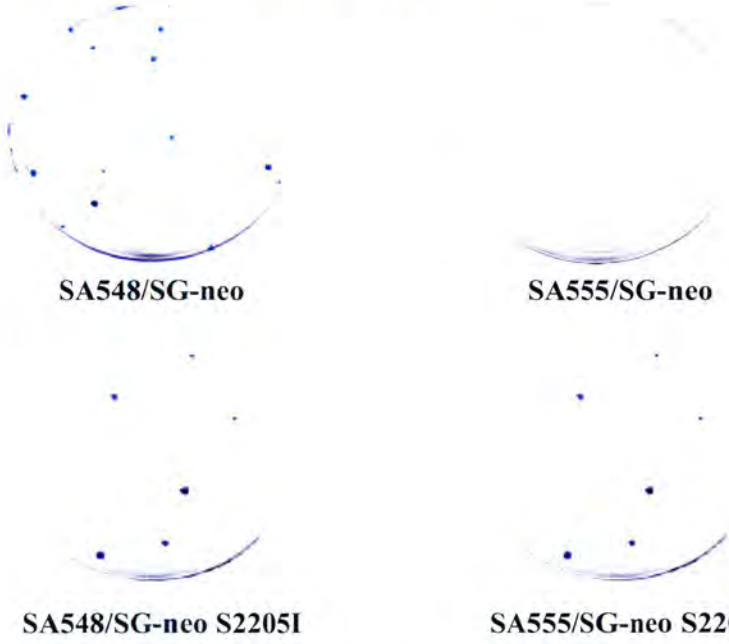

Figure 4.8: Replication efficiency of SA548/SG-neo S2205I and SA555/SG-neo S2205I as well as wild-type replicons in Huh-7.5 cells. Huh-7.5 cells were selected with G418 for 4 weeks following electroporation with RNA transcripts from the indicated replicons. The resulting cell colonies were stained with crystal violet.

HCV replication was confirmed in the eight colonies by real time PCR. High levels of HCV replicon RNA were detected in all selected replicon cells of SA555/SG-neo S2205I ranging from 2.3 x 10^6 to 1.8 x 10^7 copies/μg of cellular RNA, with clone 1 having the highest RNA copy number and clone 5 having the lowest RNA (Figure 4.9A).

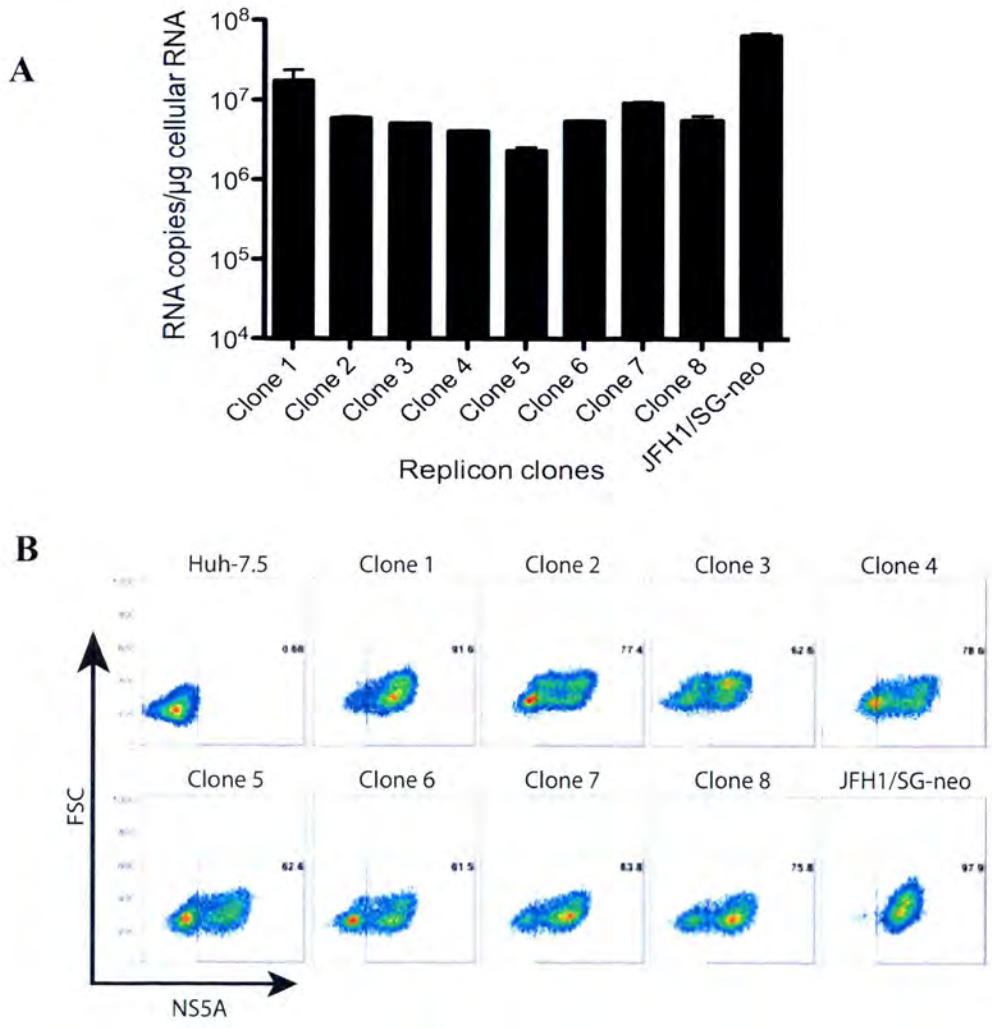

Figure 4.9: Replication of SA555/SG-neo in Huh-7.5 cells. (A) HCV RNA in the isolated cell colonies was measured by qPCR. Results are expressed as mean±SD of two independent measurements. (B) Replicon cells were stained with anti-NS5A antibody and analyzed by flow cytometry.

Expression of viral proteins was also tested by FACS analysis for NS5A staining. The frequency of HCV-antigen positive cells quantified by FACS analysis yielded trends similar to those noted in replicon RNA levels, where 61.5 to 91.6% of cells stained positive for NS5A with colony number 1 having almost as high NS5A signal as JFHI/SG-neo (positive control) as shown (Figure 4.9B). Together these results suggest that self-replicating GT 5a sub-genomic replicon was successfully established in these stable colonies.

4.3.2 Transmission of sub-genomic RNA replication by cellular RNA electroporation

Cellular RNA was extracted from clones 1, 3, 5, 6, 7, and 8 and introduced into naïve Huh-7.5 cells by electroporation to determine whether the G418 resistance of the cells was transmissible by cellular RNA. Cellular RNA was electroporated under the same conditions used for synthetic RNA, and multiple numbers of G418-resistant colonies were visible after 3 weeks of selection with G418 (500μg/mL) (Figure 4.10). Based on previous findings in the literature, we hypothesized that mutations occurring during replication in Huh-7.5 cells had improved the colony formation efficiency. Taken together, these results indicated that the G418-resistant cell colonies carried autonomously replicating viral RNA.

4.3.3 Identification of adaptive mutations

Because replicating genomes have been shown to accumulate cell culture adaptive mutations, which increase their replication potential (Saeed *et al.*, 2012; Blight *et al.*, 2000), the NS3-NS5B coding region in the extracted replicon RNAs was amplified by RT-PCR and sequenced directly to examine whether the increased replication efficiency observed when cellular RNA was electroporated into Huh-7.5 cells was due to additional putative adaptive mutations in the viral genome. While the S2205I mutation was conserved suggesting this NS5A S2205I mutation may be essential for the GT 5a replicon replication, all clones had at least one additional non-synonymous mutation; more than 60% of the mutations were in the NS3 helicase domain (Table 4.10).

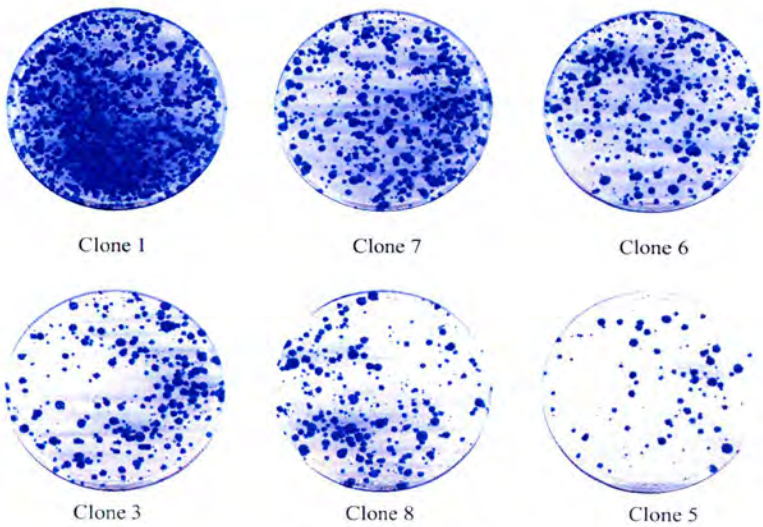

Figure 4.10: Transmission of G418 resistance from replicon cells to naïve Huh-7.5 cells. RNA was extracted from the indicated replicon cell clones and naïve Huh-7.5 cells were electroporated with 5µg of this RNA. These cells were mixed with feeder cells that were electroporated with replication defective sub-genomic replicon. Colonies obtained after selection with G418 were fixed and stained with crystal violet.

Table 4.10: Non-synonymous mutations identified in SA555/SG-neo S2205I replicon clones

Replicon clone	Nucleotide mutation	Amino acid mutation	NS protein
1	3946 (2301) G→A	1203 (176) E→K	NS3
	4556 (2911) A→G	1406 (379) K→S	NS3
	4557 (2912) G→C		
	7435 (5790) C→T	2366 (1339) S→P	NS5A
2	4021 (2376) C→T	1228 (201) H→Y	NS3
3	4165 (2520) G→A	1276 (249) D→N	NS3
	7013 (5368) C→T	2226 (1198) A/V (Mix)	NS5A
4	5453 (3808) A→G	1705 (678) Q→R	NS4A
5	4261 (2616) G→A	1308 (281) G→S	NS3
6	4951 (3306) G→C	1538 (511) V→L	NS3
7	4633 (2988) G→A	1432 (405) D→N	NS3
8	5453 (3808) A→G	1705 (678) Q→R	NS4A

NS, nonstructural; G, guanine, A, adenine; C, cytosine, T, thymine; numbers in brackets indicate the positions within the SA555 sub-genomic replicon and numbers outside the brackets indicates position in the full-length genome

4.3.4 Analysis of adaptive values of mutations identified in the NS3-NS5B coding region

To analyze the possible adaptive potential of identified mutations, these were individually introduced into the SA555/SG-neo S2205I (I) backbone. For C1, all three mutations were introduced independently and in combination to produce SA555/SG-neo (K+I), SA555/SG-neo (S+I), SA555/SG-neo (K+S+I), and SA555/SG-neo (K+S+P+I). For clones C2, C3, C4, C5, C6, and C7, the single mutations were introduced into SA555/SG-neo S2205I to produce SA555/SG-neo (H1228Y+S2205I), SA555/SG-neo (D1276N+S2205I), SA555/SG-neo (Q1705R+S2205I), SA555/SG-neo (G1308S+S2205I), SA555/SG-neo (V1538L+S2205I), and SA555/SG-neo (D1432N+S2205I). The replication efficiencies of these mutants were measured by titrating the colony formation. All the mutants except SA555/SG-neo (S+I) conferred various degrees of cell culture adaptation: a 55 to 1700-fold increase higher than that of SA555/SG-neo S2205I (Figure 4.11). Titration of colony formation was performed as described in section 3.5.4 and the numbers of colonies obtained per 10,000 transfected cells are reported (Figure 4.12A and B). Results were confirmed by two independent experiments.

HCV RNA levels for SA555/SG-neo (S+I) were no greater than those of pol- control suggesting that this construct was replication defective. For C1, which had the highest replication level, it was observed that the colony formation efficiency was increased with the E1203K + K1406S + S2366P + S2205I (K+S+P+I) mutated replicon compared to the other constructs. However, because the frequency of colonies generated after electroporation with HCV RNAs from this construct was lower compared to Con1/SG-neo (I), a highly cell culture-adapted sub-genomic replicon from genotype 1b, we reasoned that additional adaptation might be required for efficient replication. So we isolated three colonies harboring this construct and sequenced the region encoding NS3-NS5B. No additional mutation was found, indicating that this replicon does not need further adaptation.

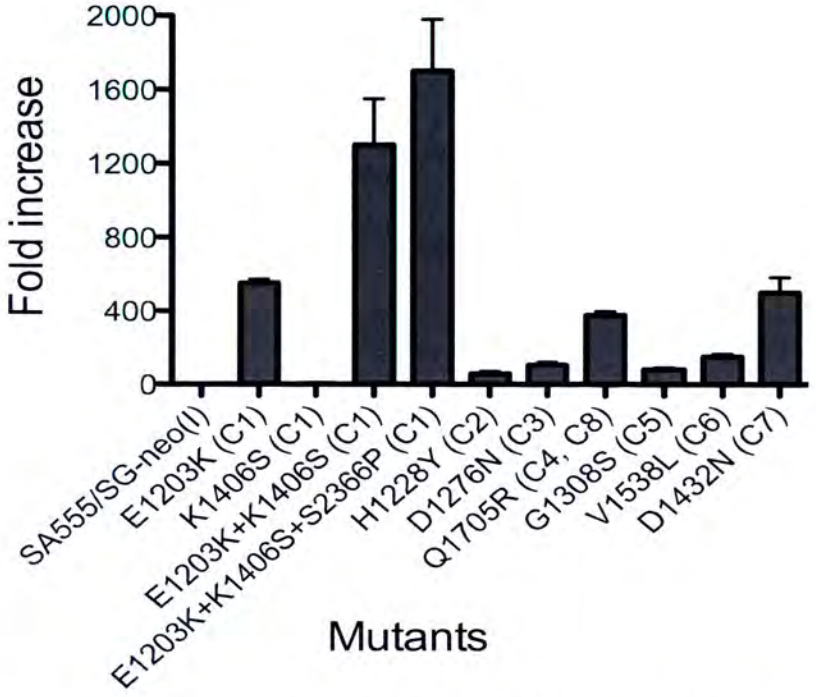

Figure 4.11: Colony titration assay of SA555/SG-neo (I) containing additional mutations identified in the isolated replicon cell clones. Huh-7.5 cells, seeded in 6-well plates at a density of 4 X 10^5 cells/well, were next day transfected with 2μg RNA of the indicated mutants. Six hours after transfection, cells were harvested and seeded in new 6-well plates at densities ranging from 2 X 10^5 to 2 X 10^2 cells/well. The total numbers of cells in each well were brought to 2 X 10^5 by adding feeder cells transfected with RNA from a replication defective HCV replicon (The three residues in the active site of NS5B polymerase were changed from GDD to AAG). The cell colonies resulting from 4 weeks of selection with G418 were counted for at least two cell densities and the percentage of colony formation was calculated (number of colonies in a well/ number of cells initially plated in that well x 100). Shown is the fold-increase in colony formation relative to SA555/SG-neo (I), the value for which was arbitrarily set at 1. Data are mean±SD of two independent experiments.

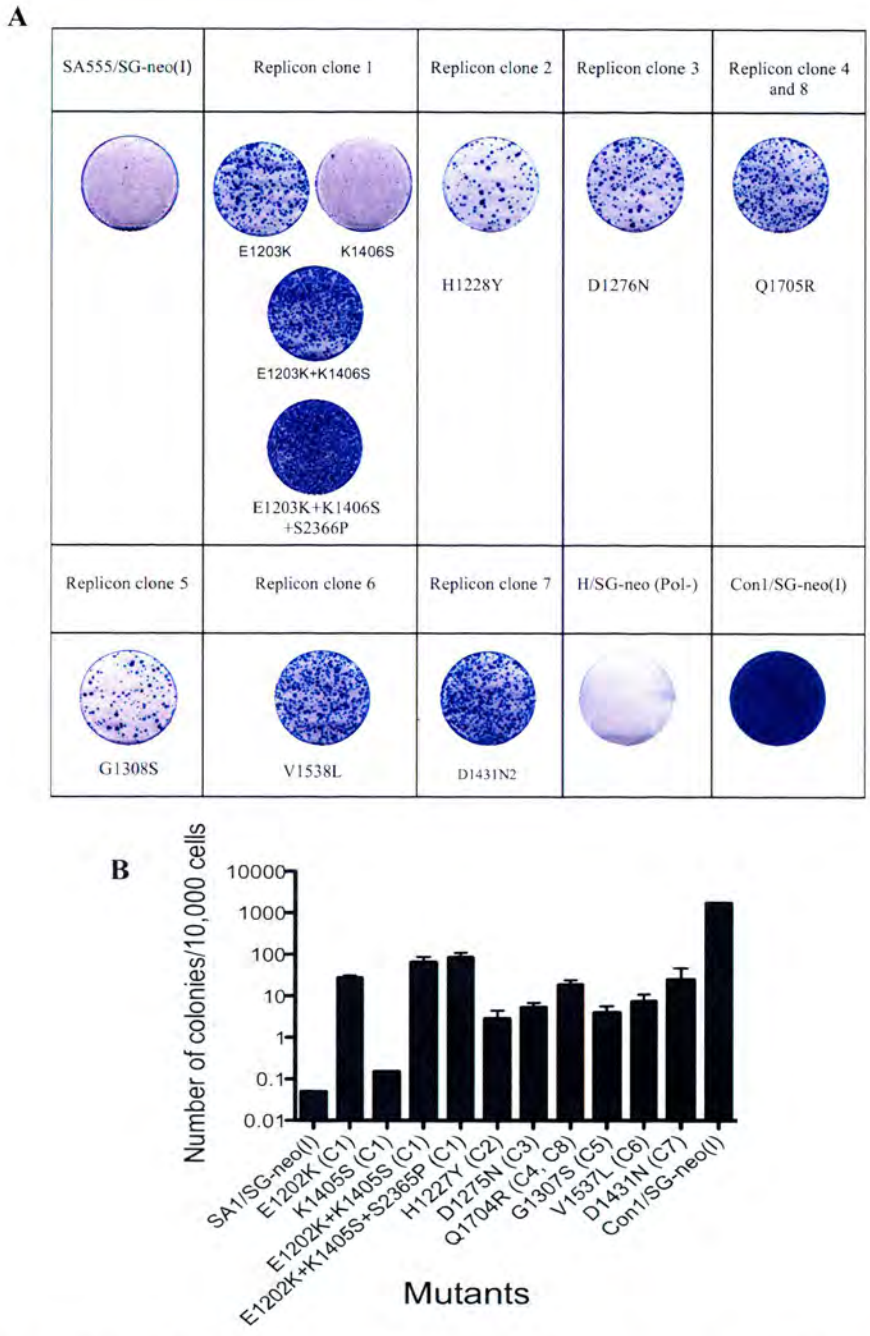

Figure 4.12: Colony titration assay of SA555/SG-neo mutants. (A) Colony formation of SA555/SG-neo (I) mutants. (B) Titration of colony formation.

Next, to address whether these identified mutations could confer a replicative advantage in the absence of the NS5A S2205I mutation, the mutations were individually engineered into the parental SA555/SG-neo construct by PCR. Electroporation of synthesized RNAs from these constructs in to Huh-7.5 and selection with G418 diminished colony formation (data not shown). These results showed that the S2205I mutation was important for the adaptive value of *de novo* mutations. This mutation in NS5A, S2204I (Lohmann *et al.*, 2003; Blight *et al.*, 2000) corresponding with S232I (Peng *et al.*, 2013) or S2210I (Saeed *et al.*, 2013) also enhanced RNA replication of genotypes 1a, 1b, 3a, and 4a replicons.

4.3.5 Synthesis of Feo replicons for genotype 5a

Genotype 5a Feo replicons were synthesized as described in section 3.5.6. Since the mutant SA555/SG-neo E1203K + K1406S + S2366P + S2205I (K+S+P+I) replicated with the highest efficiency, RNA transcribed from this replicon was introduced into Huh-7.5 cells followed by selection with G418 (750 µg/mL). A large number of cell colonies were obtained after 3 weeks, although the number was much lower than that obtained for "neo" version of this replicon. We pooled cell colonies and compared the level of HCV RNA replication with Huh-7.5 cells carrying JFH1/SG-Feo, a previously reported highly efficient replicon derived from genotype 2a isolate JFH1 (Saeed *et al.*, 2012). The luciferase activity of mutant, now referred to as SA555/SG-Feo (SKIP) for convience purpose, was comparable to JFH1/SG-Feo, suggesting high levels of HCV RNA replication (Figure 4.13A). Furthermore, NS5A protein could be detected in 78% of cells carrying SA555/SG-Feo (SKIP) (Figure 4.13B).

4.3.6 Effect of HCV inhibitors on replication of various genotypes

The development of a sub-genomic replicon for genotype 5a provided us an opportunity to compare its response to various antiviral compounds with other genotypes. To this end, we used a panel of Feo replicons from genotypes 1a (H77/SG-Feo (L+8)), 1b (Con1/SG-Feo (I)), 2a (JFH1/SG-Feo), 3a (S52/SG-Feo (AII), 4a (ED43/SG-Feo (VYG)) and 5a (SA555/SG-Feo (SKIP)). JFH1/SG-Feo and S52/SG-Feo (AII) are described previously (Saeed *et al.*, 2012), while H77/SG-Feo (L+8), Con1/SG-Feo (I) and ED43/SG-Feo (VYG) were generated as described in Materials and Methods. For convenience, these replicons were referred to as H77, Con1, JFH1, S52, ED43 and SA555. The G418-resistant Huh-7.5 cells, selected after

electroporation with synthetic RNA from each replicon, were pooled and firefly luciferase assay was done to compare their replication levels. The firefly luciferase values were within 3-fold of each other, indicating similar levels of HCV RNA replication (Figure 4.13A). By flow cytometry, NS5A protein could be detected in 56% - 96% of cells (Figure 4.13B). The lower number of NS5A-positive cells for H77 could be due to a lower sensitivity of used antibody for this genotype.

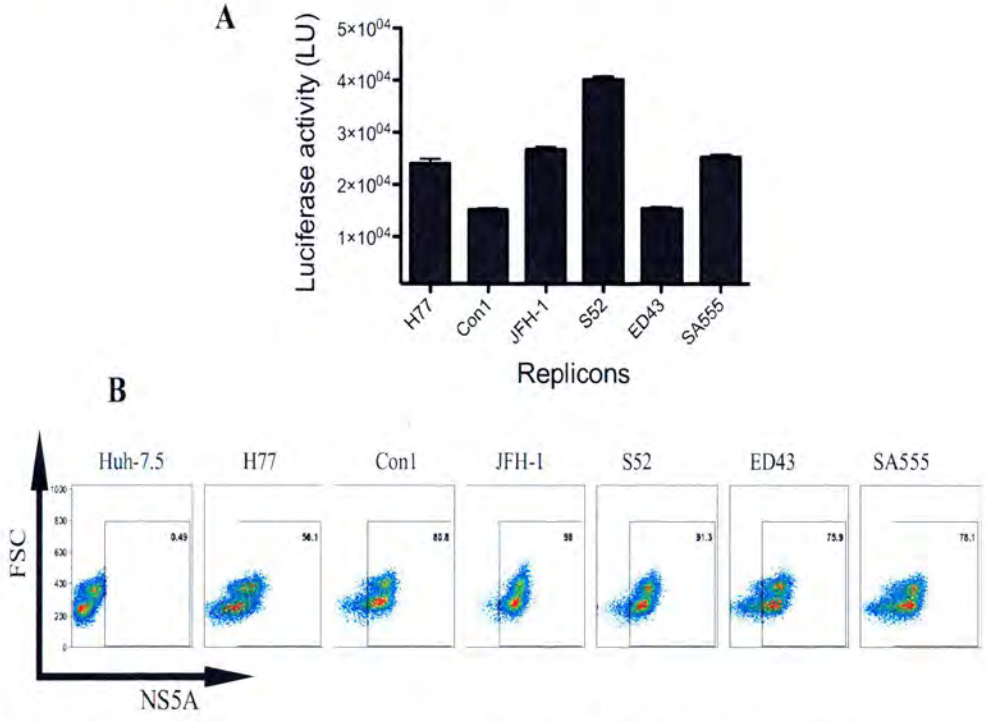

Figure 4.13: Replication levels of Feo replicons from various HCV genotypes. Huh-7.5 cells were electroporated with Feo replicons from genotype 1a (H77), 1b (Con1), 2a (JFH-1), 3a (S52), 4a (ED43) and 5a (SA1) and selected with G418 (750 µg/mL). After 3 weeks, the surviving cells were pooled and passaged twice in G418 containing medium. (A) Replicon cells from each genotype were seeded in 8 wells of a 96-well plate at a density of 2 x 10^4 cells/well. Twenty-four hours later, cells were lysed and firefly luciferase activity was measured. The results are plotted as mean ± SD of eight replicates. (B) HCV NS5A protein in the cells was detected by flow cytometry.

Next, we exposed the replicon cells to various concentrations of interferon α-2a (IFNα2a) and four directly acting antivirals (DAAs): danoprevir (an NS3 protease inhibitor), daclatasvir and ledipasvir (two NS5A inhibitors) and sofosbuvir (an NS5B nucleoside polymerase inhibitor) as shown (Figure 4.14).

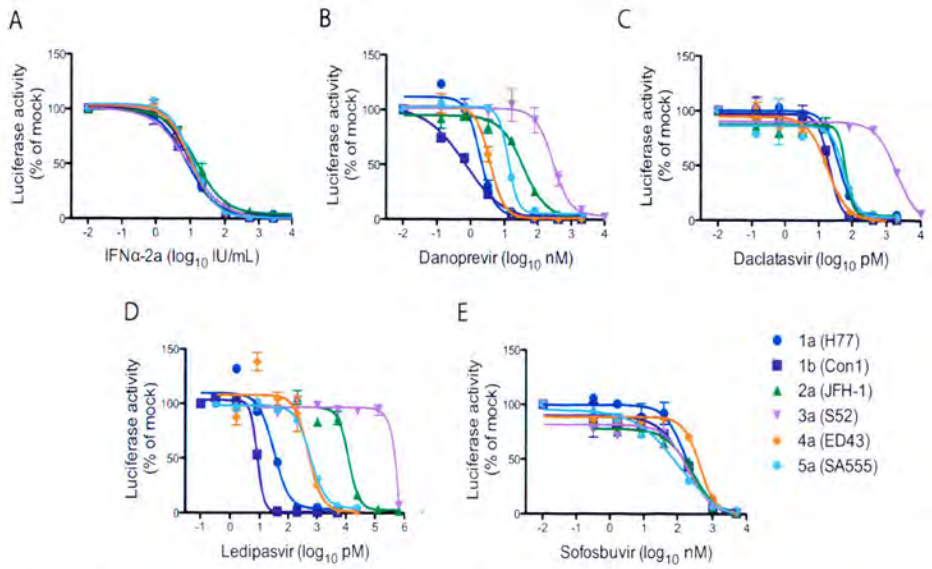

Figure 4.14: Effect of anti-HCV compounds on replication of various genotypes. Cells harboring persistently replicating Feo replicons from genotypes 1a (H77), 1b (Con1), 2a (JFH-1), 3a (S52), 4a (ED43) and 5a (SA555) were seeded in duplicate tissue culture-treated, 96-well, white plates at a density of 1 X 10^4 cells/well using G418-free cell culture medium. The following day, cells were exposed to the indicated concentrations of IFNα-2a (A), Danoprevir (B), Daclatasvir (C), Ledipasvir (D) and Sofosbuvir (E). Seventy-two hours later, cells were lysed in 50μL 1X CCLR (Promega) and firefly luciferase activity was measured. Data is plotted as mean±SD of three independent experiments.

IFNα-2a showed a comparable inhibitory effect across all genotypes (Table 4.11), although a cytotoxic effect was observed at higher concentrations, especially for cells carrying genotype 2a replicons (Figure 4.15A). Among the DAAs, danoprevir was highly potent against genotype 1a, 1b, 4a and 5a, with 50% effective concentrations (EC_{50}) of 1.89nM, 0.67nM, 3.67nM, and 12.79nM, respectively.

Table 4.11: Inhibitory effect of antiviral compounds on HCV replication

Compound	Genotype (isolate)	EC50[a]	95% CI[b]
IFNα-2a (IU/mL)	1a (H77)	7.81	6.75-9.03
	1b (Con1)	10.47	7.06-15.54
	2a (JFH-1)	14.27	11.00-18.51
	3a (S52)	8.95	7.85-10.21
	4a (ED43)	10.67	8.62-13.19
	5a (SA1)	11.52	9.53-13.92
Danoprevir (nM)	1a (H77)	1.89	1.33-2.70
	1b (Con1)	0.67	0.47-0.95
	2a (JFH-1)	36.31	24.81-53.16
	3a (S52)	276.7	189.30-404.50
	4a (ED43)	3.67	3.10-4.34
	5a (SA1)	12.79	11.10-14.73
Daclatasvir (pM)	1a (H77)	38.48	31.29-47.33
	1b (Con1)	20.82	14.84-29.21
	2a (JFH-1)	63.79	11.90-341.80
	3a (S52)	2008	972.10-4147
	4a (ED43)	18.21	13.15-25.23
	5a (SA1)	57.06	31.07-104.80
Ledipasvir (pM)	1a (H77)	29.94	31.29-47.33
	1b (Con1)	8.12	14.84-29.21
	2a (JFH-1)	12062	11.90-341.80
	3a (S52)	4.55×10^5	very wide
	4a (ED43)	498.8	230.2-1081
	5a (SA1)	576.5	463.2-717.5
Sofosbuvir (nM)	1a (H77)	170.1	112.2-258.0
	1b (Con1)	187.1	91.89-380.8
	2a (JFH-1)	263.9	151.9-458.5
	3a (S52)	198.2	92.55-424.5
	4a (ED43)	415.2	293.8-586.8
	5a (SA1)	107.9	47.95-242.2

[a] 50% Inhibitory concentration

[b] 95% confidence intervals

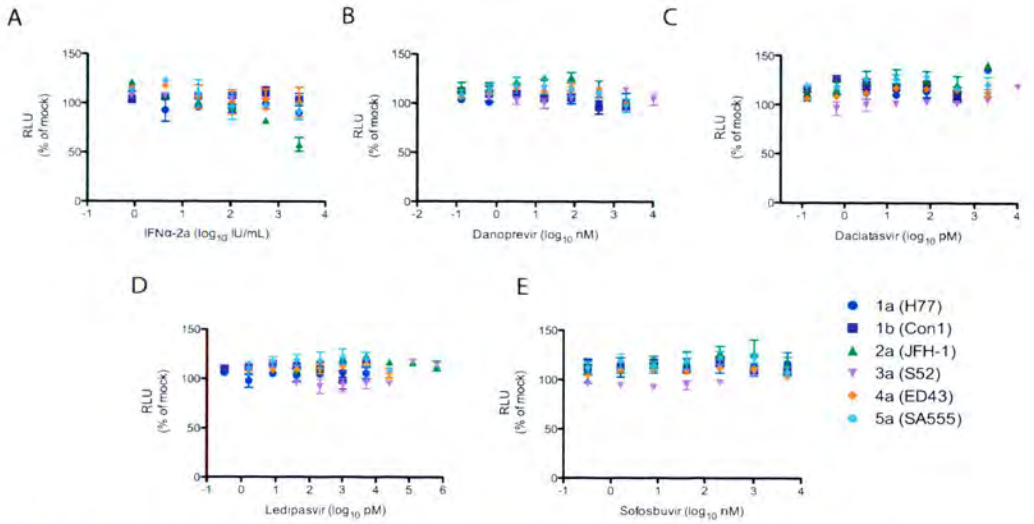

Figure 4.15: Cytotoxic effect of HCV inhibitors. Cells carrying autonomously replicating Feo replicons from genotypes 1a (H77), 1b (Con1), 2a (JFH1), 3a (S52), 4a (ED43) and 5a (SA555) were seeded in duplicate tissue culture-treated, 96-well, white plates at a density of 1 X 10^4 cells/well using G418-free cell culture medium. The following day, cells were exposed to the indicated concentrations of IFNα-2a (A), Danoprevir (NS3 protease inhibitor) (B), Daclatasvir (NS5A inhibitor) (C), Ledipasvir (NS5A inhibitor) (D) and Sofosbuvir (NS5B polymerase inhibitor) (E). Seventy-two hours later, cells were lysed in 50µL 1X CCLR (Promega) and firefly luciferase activity was measured. Data is plotted as mean±SD of three independent experiments.

Intermediary effect was seen against genotype 2a (EC_{50} 36.31nM), while genotype 3a was highly resistant (EC_{50} 276.7nM). Daclatasvir, with EC_{50} values of 38.48pM, 20.82pM, 63.79pM, 18.2pM and 57.06pM was highly effective against genotype 1a, 1b, 2a, 4a and 5a, respectively, whereas the EC_{50} for genotype 3a was ~2nM, indicating a lower inhibitory effect against this genotype. Ledipasvir displayed a broad range of efficacy depending on the genotype: a very strong inhibition of genotype 1a and 1b (EC_{50} values of 29.94pM and 8.12pM, respectively), whereas an extremely weak effect against genotype 3a (EC_{50} 455nM). Sofosbuvir inhibited replication of all genotypes with comparable efficiency. Treatment of cells with the DAAs, at concentrations used in this study, had no effect on cell viability (Figure

4.15), indicating that the decrease in luciferase signal was due to the specific inhibition of HCV RNA replication. Taken together, these results demonstrate differential effects of anti-HCV compounds on various genotypes.

4.4 DEVELOPMENT AND CELL-CULTURE ADAPTATION OF 5a/2a INTER-GENOTYPIC RECOMBINANTS

4.4.1 Development and characterization of SA548/JFH1 and SA556/JFH1 chimeras

HCV genotype 5a/2a-recombinants of NS3-4A (Gottwein *et al.*, 2011a; Imhof *et al.*, 2010), NS5A (Scheel *et al.*, 2011b), and of C-NS2 + NS5A (Galli *et al.*, 2013) have been developed and SA13 is the only 5a isolate for which a C-NS2 recombinant has been developed (Jensen *et al.*, 2008). To develop additional cell culture systems for HCV GT 5 and test the efficiency of replication and infectious virus production in Huh-7.5 cells, the recombinant GT 5a/JFH1 constructs were generated in similar manner to SA13/JFH1. Thus the C-NS2/JFH1 constructs contained the complete 5' and 3' UTR from JFH1, the C-NS2 region from the novel genotype 5a isolates, SA548 and SA556, and NS3-NS5B from JFH1 (nts 3422–9669). Similarly, the E1E2/JFH1 constructs contained the complete 5' and 3' UTR from JFH1, Core from SA13, the E1 and E2 structural genes from the novel 5a isolates (nts 914–2581; nucleotide positions refer to the GT 5a/JFH1 sequence), p7 and the non-structural genes NS3–NS5B, from JFH1 (nts 3422–9669).

4.4.2 Previously identified adaptive mutations in SA13/JFH1 did not confer adaptation to novel inter-genotypic 5a/2a recombinants

From the consensus sequences of SA548 and SA556 isolates, JFH1-based C-NS2 and E1E2 recombinants were constructed. RNAs from these recombinants were electroporated in duplicate to investigate whether adaptation could be conferred by the mutations, C3405G (NS2) and A3696G (NS3), previously identified for SA13/JFH1. From the percentage of NS5A positive cells one day after electroporation of Huh-7.5 cells with RNA transcripts from the novel GT 5a/JFH1 recombinants, as well as with transcripts from SA13/JFH1, it appeared that electroporation efficiency was similar for all the constructs. All recombinants showed 50-60% of HCV NS5A expressing Huh-7.5 cells (Figures 4.16 and 4.17). The percentage of NS5A-positive

cells for SA13/JFH1 (SA548E1E2) remained relatively constant until day 5 (Figure 4.16A). The SA556 (C-NS2)/JFH1 recombinant spread comparably to SA13/JFH1 (Figure 17A), whereas the SA13/JFH1 (SA556E1E2) and SA548 (C-NS2)/JFH1 recombinants had intermediate phenotypes. The HCV infectivity titers were decreased for all constructs compared to SA13/JFH1, peaking around approximately 10^3 $TCID_{50}$/mL on day 5 (Figures 4.16B and 4.17B).

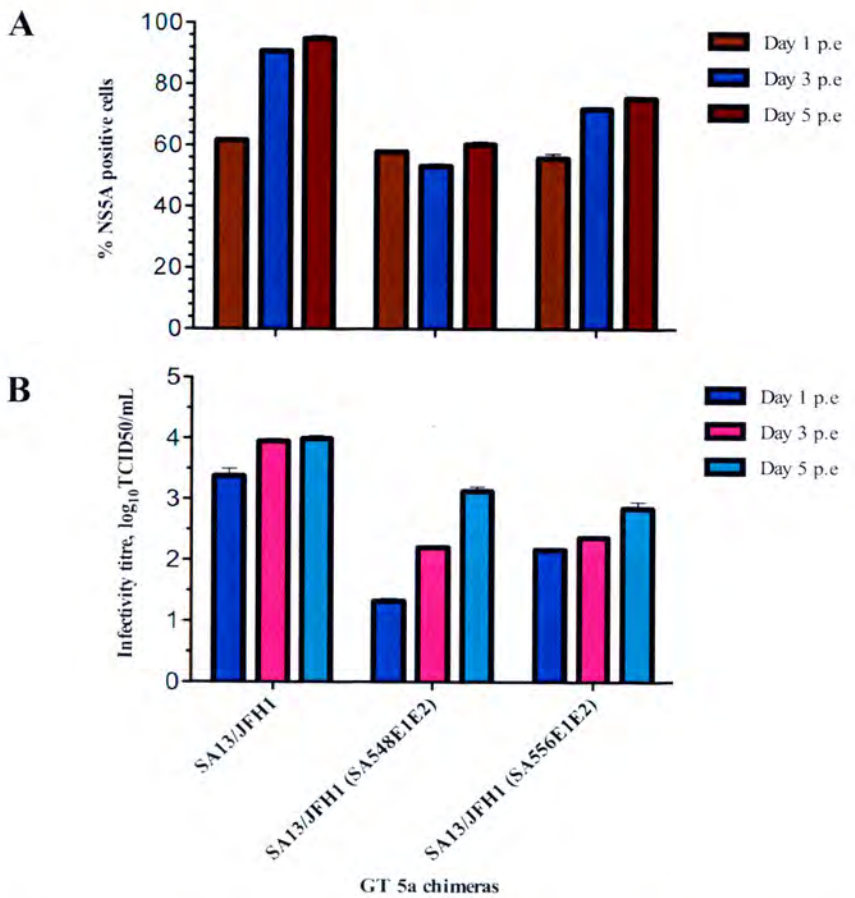

Figure 4.16: Electroporation of Huh-7.5 cells with RNA transcripts of GT 5a E1E2/JFH1 recombinants. (A) %HCV-positive cells, immunostaining for NS5A visualized with Alexa Flour 647. (B) TCID50 of days 1, 3, and 5 post electroporation.

Almost all Huh-7.5 cells in the SA13/JFH1 reference culture became HCV-NS5A positive within 5 days, and titres of around 10^4 TCID50/mL were produced (Figure 4.17B). As observed, there was evidence of viral spread for all the constructs by day 5-post electroporation but not to the level of SA13/JFH1. No NS5A-positive cells in the Con1/SG-Neo-AGG negative control cells were detected throughout the experiment. The delayed viral spread of all GT 5a/JFH1 recombinants compared with SA13/JFH1 indicated the potential of further adaptation of these recombinants by acquisition of additional adaptive mutations.

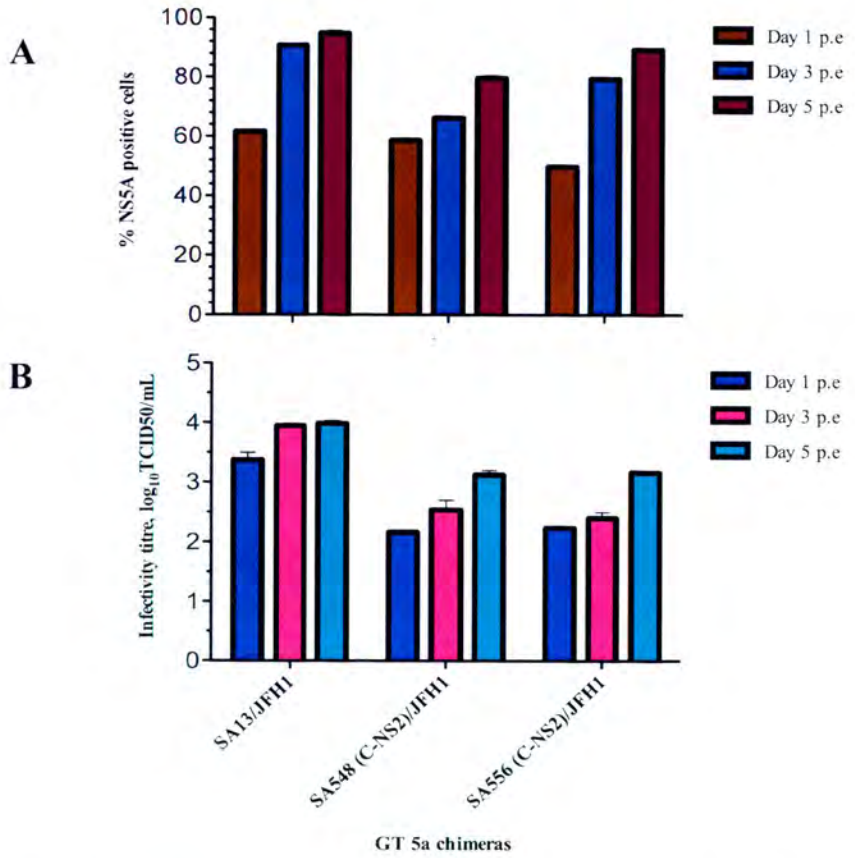

Figure 4.17: Electroporation of Huh-7.5 cells with RNA transcripts of GT 5a C-NS2/JFH1 recombinants. (A) %HCV-positive cells, immunostaining for NS5A visualized with Alexa Flour 647. (B) TCID50 of days 1, 3, and 5 post electroporation supernatant.

To adapt all 5a/JFH1 recombinant viruses, RNA transcripts were transfected into Clone 8 cells (Huh-7.5 cells stably transduced with lentiviruses encoding enhanced green fluorescent protein, EGFP-interferon-β stimulator protein 1-IPS-1) and passaged every third day. On day 3 post-infection (p.i), supernatants from these cells were used to infect naïve Clone 8 cells and all constructs replicated as evidenced by nuclear translocation 24 hours post infection (Figure 4.18).

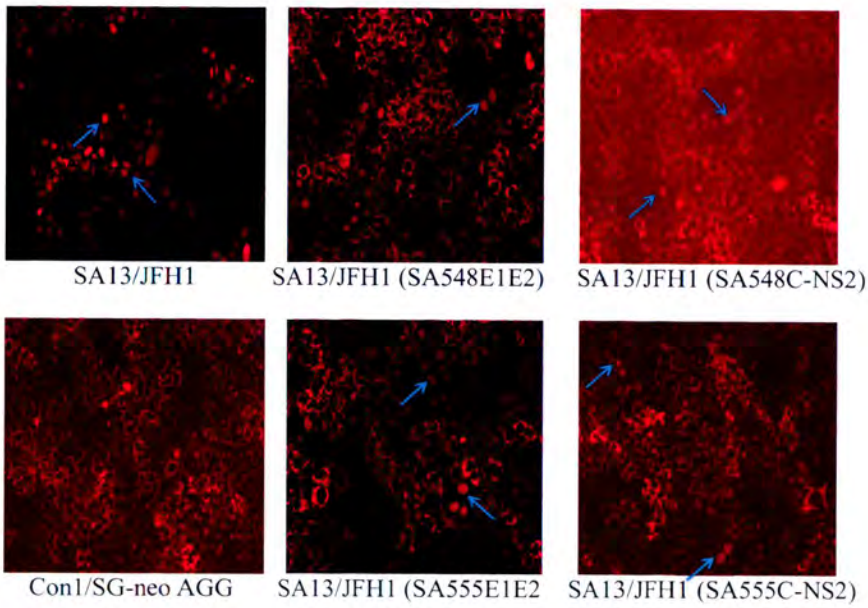

Figure 4.18: Wide field fluorescence images of Clone 8 cells harboring inter-genotypic 5a/JFH1 recombinants at 3 days post-infection. Arrows indicate infected cells.

By day 5 p.i, SA13/JFH1 yielded 100% infectivity of cells as contrasted to what was observed with 5a/JFH1 recombinants (results not shown). However, after several passages of supernatants to naïve Clone 8 cells, all viable 5a/JFH1 recombinants spread rapidly. In line with previous studies (Scheel *et al.*, 2008; Gottwein *et al.*, 2007; Zhong *et al.*, 2006) cell death, followed by proliferation of HCV antigen-negative Clone 8 cells, occurred after infection had spread to most cells.

CHAPTER 5

DISCUSSION

Since the first report of the HCV genome, numerous sequences of HCV isolates have been reported. Comparison of these sequences revealed marked genetic heterogeneity of the HCV genome, and isolates have been classified into seven genotypes (Smith *et al.*, 2013). Among the genotypes of HCV, genotype 5a was first identified in a cohort of South African patients with HCV-induced hepatocellular carcinoma (Bukh *et al.*, 1993); accounting for more than 30-50% of HCV infections in South Africa (Smuts and Kannemeyer, 1995). This clearly highlights the urgent need to develop effective antiviral agents against genotype 5. However, little is known about the virology, clinical features, and treatment susceptibility of this particular genotype. For interferon-based therapy there are indications that GT 5 appear easier-to-treat compared to HCV GT 1 (Antaki *et al.*, 2010), and that like genotypes 2 and 3, it might have a favorable response to treatment (Nguyen and Keeffe, 2005; Legrand-Abravanel *et al.*, 2004). Entering an era of interferon-free regimens, little information is available from clinical trials for genotype 5a; however, broad antivirals effective against genotypes 1-3 are expected also to be effective against genotype 5a (Scheel and Rice, 2013).

The first objective of this study was to determine the full-length consensus sequences of HCV genotype 5a positive samples. To this end, the entire genome of HCV from four plasma samples (SA548, SA555, SA556, and SA560) obtained from South African blood donors confirmed to be HCV RNA and HCV antibody positive, was recovered by overlapping PCRs and cloning methods. The polyprotein region of all four samples was compared with previously identified HCV genotype 5a strains SA13 and EUH1480 by phylogenetic analysis (Bukh *et al.*, 1998; Chamberlain *et al.*, 1997). Based on the data, it was concluded that the novel HCV strains, SA548, SA555, SA556, and SA560, were clustered around genotype 5a (Figure 4.7). Analysis of all HCV genotype 5a isolates in the HCV GenBank database revealed that none had been full-length characterized; hence, this is the first study to identify the complete full-length sequences of the novel genotype 5a isolates and an important step toward a more complete characterization of this particular genotype.

The 5' UTR was highly conserved with a mean genetic heterogeneity of 0.79% (Table 4.9). Comparison with 5' UTRs from H77 (genotype 1a), JFH1 (genotype 2a), S52 (genotype 3a) and ED43 (genotype 4a) revealed differences at 3.54%, 5.24%, 6.93% and 5.16% nucleotide positions, respectively, highlighting inter-genotypic variability. In the initial HCV sequence the 3' end was not identified (Choo *et al.*, 1989) and it took almost a decade to identify the complete 3' end (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995). This was in particular due to the inherent difficulties in amplifying and sequencing through the internal poly (U/UC) tract of around 100 nucleotides. Further, complete HCV 3' UTR sequences occupy a very small percentage in the exponentially growing HCV sequence databases (Combet *et al.*, 2007; Kuiken *et al.*, 2005) and have also not been determined for previously identified genotype 5a isolates. We therefore made significant efforts to amplify this region of the HCV genome in blood samples received.

In the present study, the complete HCV 3' UTR from the four plasma samples was successfully determined in two overlapping PCR fragments using RT and nested PCR to amplify the 5' end of the 3' UTR and the 3' RACE to amplify the extreme 3' end. The 3' X-tail sequence of 98 nucleotides was highly conserved among all four strains; and this was in agreement with previous findings (Tokita *et al.*, 1998; Lemon and Honda, 1997; Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996). The poly U/UC region was found in all isolates of HCV, including the novel HCV genotype 5a isolates. The length of the poly U/UC tract was variable between all four GT 5a isolates (and among individual clones from each isolate) as in the case for other HCV isolates (Yanagi *et al.*, 1998, Yanagi *et al.*, 1997; Kolykhalov *et al.*, 1996). Although this presumably reflects variations introduced by viral polymerase, it might also at least partially be due to PCR artifacts (Yanagi *et al.*, 1997). This was the first time the complete 3' UTR was determined for HCV genotype 5a.

Several published papers have investigated the effects of length on the poly U/UC region, and found some minimal length differences (You and Rice, 2008; Friebe and Bartenschlager, 2002; Yanagi *et al.*, 1999). Kolykhalov *et al.* (1997) reported that HCV RNA transcripts from an infectious cDNA clone with a poly U/UC region of 133 nucleotides seemed to have a replicative advantage in chimpanzees over those with a poly U/UC region of 75 nucleotides, suggesting that the length of the poly

U/UC region might influence viral replication. In contrast, Murayama and coworkers found that a shorter poly U/UC tract in JFH1 contributed to high levels of HCV RNA replication and virus production in cultured cells (Murayama *et al.*, 2010). Using this previous knowledge we selected the longest determined poly U for SA548 and SA555 clones. Within the coding region, a high degree of diversity was seen in the envelope proteins. This was not surprising given that they are the primary targets of neutralizing antibodies. A continuous tug-of-war between host's immune system and virus leads to a high genetic heterogeneity in these proteins.

Since the development of the original genotype 1b (Con1 isolate) sub-genomic replicon in 1999 (Lohmann *et al.*, 1999), and its dependence on adaptive mutations became evident (Lohmann *et al.*, 2001; Blight *et al.*, 2000), the HCV sub-genomic replicon system has since been used as a valuable tool to study the viral and host factors associated with HCV and to screen antiviral inhibitors (Bartenschlager and Sparacio, 2003). However, replicons have been reported so far for only genotypes 1, 2, 3, 4, and 6 (Yu *et al.*, 2014; Peng *et al.*, 2013; Saeed *et al.*, 2013; Saeed *et al.*, 2012; Blight *et al.*, 2003; Kato *et al.*, 2003a; Kato *et al.*, 2003b; Lohmann *et al.*, 2003). The second objective of this study was therefore to generate sub-genomic replicons for HCV GT 5a, and test their replication efficiency in Huh-7.5 cells. To achieve this, selectable sub-genomic replicons for genotypes 5a were constructed from the determined consensus sequence of the HCV isolates of interest. Due to the quasispecies nature of HCV and thereby randomly occurring fatal mutations, this was important to obtain a functional clonal sequence (Gottwein and Bukh, 2008).

In the past, efforts to grow H77, S52, and ED43-derived sub-genomic replicons in Huh-7.5 cells proved unsuccessful until the inclusion of S2204I, a highly adaptive mutation in NS5A of the Con1 replicon (Saeed *et al.*, 2012; Bartenschlager and Lohmann, 2001; Blight *et al.*, 2000). A cluster of single mutations that promoted replication of sub-genomic Con1 in Huh-7 cells were identified in NS5A, in particular, in gene segments upstream of the IFN sensitivity-determining region-ISDR (Lohmann *et al.*, 2001; Blight *et al.*, 2000). Further, Kreiger *et al.* (2001) and Lohmann *et al.* (2001) reported other mutations T1280I (NS3) and R2884G (NS5B), respectively, which displayed increase in RNA levels compared to the parental wild-type Con1 sequence. For these reasons, it was speculated that these mutations in the

backbone of SA548 and SA555 either individually or in combination might also confer replication ability.

Upon electroporation of Huh-7.5 cells with RNA transcripts from the developed GT 5a sub-genomic replicon constructs and selection with G418, a few viable colonies were visible for SA548/SG-neo, SA548/SG-neo S2205I and SA555/SG-neo S2205I (Figures 4.8), however, not for the other mutants. The fact that adaptive mutations in NS5A stimulated HCV replication in cell culture is in agreement with a previous study, which suggested that genomes with single NS5A mutations such as S2204I were found to be more adapted *in vitro* (Bartenschlager and Lohmann, 2001). The association of this protein with other members of the putative replicase was in agreement with the importance of NS5A during RNA replication (Scheel *et al.*, 2012; Appel *et al.*, 2008; Tellinghuisen *et al.*, 2008; Appel *et al.*, 2005; Tellinghuisen *et al.*, 2005; Tellinghuisen *et al.*, 2004).

Despite the observation that mutation T1280I in NS3 acted synergistically with R2882G in NS5B to increase the replication efficiency of genotype 4 (ED43/SG-neo) replicons in Huh-7.5 cells (Saeed *et al.*, 2012), engineering these NS3 and NS5B changes either singly or in combination into SA548/SG-neo and SA555/SG-neo did not enhance replication in these GT 5a sub-genomic replicons. Despite the clear advantage of introducing previously identified replicon enhancing mutations in certain sequence contexts, these results underscore the empirical nature of optimizing adaptive mutations with different HCV genotypes. Also, even though the 1280 mutation (and others) have been enhancing replication in culture, this mutation inhibits replication *in vivo* (in chimpanzees) (Bukh *et al.*, 2002).

Further, electroporation of naïve Huh-7.5 cells with total cellular RNA isolated from SA555/SG-neo S2205I replicon cells transmitted G418 resistance (see Figure 4.11). The fact that only a few colonies were observed in the case of SA555/SG-neo S2205I replicon, suggested that this construct needed additional mutations to achieve efficient replication in Huh-7.5 cells. As suspected, replicons in these cells had acquired adaptive, replication enhancing mutations in NS3, NS4A, or NS5A in the same region or even at identical positions as previously reported for genotype 4a isolates (Peng *et al.*, 2013; Saeed *et al.*, 2012). The majority of mutations were identified in the helicase domain of NS3 protein (Table 4.10). This domain has been proposed to play

a role in HCV replication by unwinding RNA secondary structures and/or double-stranded RNA intermediates (Gu and Rice, 2010).

An active NS3 helicase is essential for replication of sub-genomic replicons (Lam and Frick, 2006). When these acquired adaptive mutations were introduced in the background of SA555/SG-neo (S2205I), colony formation was increased to various degrees (Figures 4.9-4.12), suggesting that there is a high conservation of certain cell culture adaptive mutations among HCV genotypes (Lohmann and Bartenschlager, 2013). Efficient replication of this sub-genomic replicon depended on the unique amino acid insertion in the NS5A ISDR. In their study, Blight and colleagues observed that non-polar amino acid residues such as isoleucine (Ile), valine (Val) or alanine (Ala) at position 2204 greatly enhanced replication of sub-genomic RNA in cell culture, suggesting that replication favors non-polar residues at this locus (Blight *et al.*, 2002). Still to this date, it remains unclear what these adaptive mutations exactly are doing and why they are necessary *in vitro* and not *in vivo*.

The lack of cell culture systems for HCV genotype 5a has hampered studies of responsiveness to anti-HCV inhibitors. Most direct-acting antivirals (DAAs), including the two first approved HCV NS3 protease inhibitors (PI), telaprevir and boceprevir, were developed using the genotype 1b replicon system, and were due to genotype-specific effects initially only approved for treatment of genotype 1 infections (Scheel and Rice, 2013; Kwong *et al.*, 2011). Recently, sofosbuvir and simeprevir inhibitors have been approved for treatment of genotypes 1, 2, 3, and 4 infections (Caceres, 2014). Little is known about the response of genotype 5a to DAAs. Therefore, none of the approved DAAs is currently recommended for the treatment of genotype 5a infections. Having successfully established the first *in vitro* replication system for this genotype, we decided to compare its response to various HCV inhibitors with other genotypes.

The results suggested that genotype 5a was highly sensitive to sofosbuvir; an NS5B polymerase inhibitor currently approved for the treatment of genotypes 1, 2, 3 and 4. In fact, the EC_{50} for genotype 5a was lowest among all genotypes, although the difference was not significant. Recently, a high response of genotype 6a to sofosbuvir is also documented (Yu *et al.*, 2014), suggesting a pan-genotype inhibitory effect of

this nucleoside inhibitor. Unlike sofosbuvir, other DAAs displayed differential activity between genotypes (Figure 4.14 and Table 4.11). For example, danoprevir, a macrocyclic NS3 protease inhibitor, was highly effective against genotypes 1a, 1b and 4a, whereas intermediary effect was seen for genotypes 5a and 2a with EC_{50} values ~12 and 36-fold higher than that for genotype 1b. On the other hand, genotype 3a was highly resistant, with EC_{50} more than 350-fold higher than genotype 1b. This concurs with the previously published reports documenting danoprevir to be less effective against genotype 3a (Saeed *et al.*, 2012; Gottwein *et al.*, 2011a; Imhof and Simmonds, 2011).

Using a cell culture infection system, where NS3 protease domain (NS3P) and NS4A from genotypes 3a, 5a and 6a were expressed in the backbone of genotype 2a J6/JFH1 virus, Gottwein *et al.* (2011a) showed a similar efficacy pattern of genotype susceptibility for danoprevir as is found in this study. In contrast, Imhof and Simmonds (2011) used a similar system as was used by Gottwein *et al.* (2011a) and showed that genotype 5a was highly resistant to danoprevir. This discrepancy might be explained by two different genotype 5a strains used in these studies: while Gottwein *et al.* (2011a) obtained NS3P from SA13, Imhof and Simmonds (2011) used another strain EUH1480. To test if these differences in genetic background could account for discrepant results, we compared NS3P from SA13 and EUH1480 and found amino acid conservation at 96% positions. However, sequence variation was seen at positions 168 and 170 of NS3, two of the several loci associated with resistance of genotype 1 to danoprevir. At both positions, the amino acids present in SA13 (Asp and Ile, respectively) were same as found in susceptible genotype 1 isolates, whereas, EUH1480 had Glu and Val at these positions. Even though Glu168 and Val170 are not reported to be directly associated with resistance, several other residues at these positions mediated high levels of resistance to macrocyclic protease inhibitors in various HCV genotypes (Imhof and Simmonds, 2011; Sarrazin and Zeuzem, 2010). Most notably, similar to SA13, all genotype 5a strains isolated in this study had Asp and Ile at position 168 and 170, which may explain, at least in part, why SA555 and SA13 are highly sensitive to danoprevir. These results clearly highlight the importance of testing multiple strains from various genotypes before drawing general conclusions.

Among the NS5A inhibitors, daclatasvir was highly effective against genotype 5a, whereas ledipasvir showed a moderate effect. Contrary to daclatasvir, which was highly efficient against genotypes 1a, 1b, 2a, 4a and 5a, ledipasvir exhibited a broad range of activity across genotypes. Genotype 3a was resistant to both of these inhibitors. These results agree with the findings of Wang *et al.* (2013) who used hybrid HCV replicons expressing NS5A from either genotype 3a or 4a in a JFH1 backbone to show that EC_{50} of daclatasvir for genotype 3a was 1 to 2 orders of magnitude higher than that for genotype 4a. Thus, we developed the first efficient HCV replicon system for genotype 5a. Together with a recently published system for genotype 6a (Yu *et al.*, 2014), our system completes the panel of replicon systems for the clinical significant HCV genotypes 1-6.

With the advent of *in-vitro* models such as the HCV sub-genomic replicon, HCV pseudoparticles (HCVpp) bearing the envelope glycoproteins and infectious HCV virion and the HCV infectious culture system, aspects of the HCV virus life cycle such as viral entry and neutralizing antibodies (ntAbs) in Huh-7 cells have been unraveled (Ashfaq *et al.*, 2011; Bartosch *et al.*, 2003; Hsu *et al.*, 2003). The replicon system enabled functional studies of intracellular HCV RNA replication (including interaction with host factors) and of inhibitors of NS3-5B. The HCVpp system enabled functional studies of entry (including receptor studies) and studies of ntAbs. The infectious culture system was a huge breakthrough (Wakita *et al.*, 2005). JFH1 (genotype 2a) allowed studies of the complete viral life cycle, including assembly/release (p7, NS2), which could not be studied before. It also reflected real infection better (e.g. certain replicon adaptive mutations that were lethal *in vivo* were also lethal in the HCVcc system (Scheel *et al.*, 2012), and entry could be studied using real HCV particles instead of HCVpp, which are structurally different (e.g. in E1/E2 heterodimers).

Since JFH1 was the only infectious culture system, it allowed development of intra- and inter-genotypic JFH1 recombinants (Galli 2013; Scheel *et al.*, 2011a; Jensen *et al.*, 2008; Scheel *et al.*, 2008; Lindenbach *et al.*, 2005). With such recombinant genomes HCV entry events and related therapeutics (Pawlotsky *et al.*, 2007; Meanwell, 2006), neutralizing antibodies and the functions of the structural proteins, p7 and NS2 can now be studied in a genotype-specific manner. Although a panel of

genotype 1-7/JFH1 recombinants are already available (Gottwein *et al.*, 2009), previous studies have also focused on broadening the panel of available isolates for genotypes 1 (Scheel *et al.*, 2011b), 2 (Pedersen *et al.*, 2013), and 3 (Scheel *et al.*, 2011b) and have used these in genotype- subtype- and isolate-specific studies e.g. of neutralizing antibodies (Pedersen *et al.*, 2013) and functional interaction between E1 and E2 (Carlsen *et al.*, 2013). Therefore we also set out to broaden the panel of genotype 5a/JFH1-based recombinants. Hence, the third objective of this study was to generate chimeric constructs expressing structural proteins of the novel GT 5a isolates and non-structural proteins of JFH1 and also to test their replication efficiencies in Huh-7.5 cells.

To investigate whether previously identified adaptive mutations could be used in the efficient establishment of a broad panel of JFH1-based HCV C-NS2 and E1E2 recombinants, inter-genotypic 5a/JFH1 recombinant systems in which the C-NS2, or the envelope glycoproteins E1E2 of two of the novel genotype 5a strains, SA548 and SA556, were introduced into SA13/JFH1$_{C3405G-A3696G}$ backbone. In reverse genetic studies, HCV RNA transcripts from all the inter-genotypic recombinant constructs were transfected into Huh-7.5 cells and cultures were followed by HCV specific immunostaining and by supernatant infectivity titrations. Upon electroporation of Huh-7.5 cells there was evidence of viral replication and spread of viral particles (Figure 4.18). However, the novel genotype 5a infectious systems produced lower infectivity titers than the previously developed SA13/JFH1 systems (Figures 4.13B and 4.14B), possibly due to suboptimal interactions of proteins of the two different genotypes. For all of the 5a/JFH1 recombinants, previously identified adaptive mutations did not confer efficient adaptation to Huh-7.5 hepatoma cell culture. It is of note though, that the SA13/JFH1 recombinant is among the most efficient HCV culture systems (Jensen *et al.*, 2008).

According to Jensen and coworkers, the original SA13/JFH1 genome showed significant delay in virus spread when compared to the highly adaptive J6/JFH1. But the acquisition of the NS2 (C3405G) and NS3 (A3696G) mutations over a passage of time in culture, led to the highest infectivity titers among JFH1-based inter-genotypic recombinants (Jensen *et al.*, 2008). The lower infectivity titres obtained with the SA548 and SA556 recombinants could probably be due to incompatibilities between

the genotype 5a and JFH1 proteins, but are, however, comparable to genotype 1b (Con1 isolate) C-NS2/JFH1 recombinants (Pietschmann *et al.*, 2006). However, after transfection experiments and passage of supernatants from Clone 8 cells infected with the two novel recombinants to naïve cells over time, the percentage of infected cells was significantly increased. Therefore, future analysis of these 5a/JFH1 recombinants should show whether additional adaptation happened and whether such mutations could enhance virus production to levels comparable to SA13/JFH1 after electroporation. Thus, we developed novel functional and infectious culture systems for the C-NS2 region or E1-E2 of two genotype 5a isolates. Although further culture adaptation should be investigated, these could be important for future genotype- and isolate-specific studies of the C-NS2 region and for inhibitor studies of entry, p7 and NS2. The two additional C-NS2 recombinants from this study add to the previously generated SA13/JFH1 and to the panel of HCV C-NS2 recombinants now available.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATION

With the aim to develop cell culture replication systems for HCV genotype 5a, an entire full-length genome of HCV genotype 5a has been recovered from four plasma samples of four South African blood donors, and these strains were clustered around previously identified genotype 5a isolates. The full-length genome sequences of all four novel isolates will be deposited in the HCV GenBank database. With this significant achievement, a functional replicon system has been generated for HCV GT 5a, a major breakthrough in HCV GT 5a studies. The availability of such a functional genotype 5a replicon system is an important extension and thus will foster HCV research and eventually also therapy development in different respects. It will pave the way for the development of highly effective therapies with pan-genotype activity and possibly development of inactivated HCV vaccine candidates. Also, additional JFH1-based cell culture systems recapitulating the full viral life cycle of HCV genotype 5a inter-genotypic recombinants have been developed. These developed systems will allow genotype 5a-specific functional studies of the structural genes, p7, and NS2 and screening of putative drug candidates and neutralizing antibodies in true cell culture systems.

Overall, the establishment of genotype 5a-specific cell culture systems is an important step toward a broader coverage of the biological diversity of HCV that will clearly foster basic research and help to understand the genotype specificity of DAAs. In parallel, efforts should continue to improve the HCV cell culture systems. One major challenge is to propagate HCV in human hepatoma cells without the need for cell culture-adaptive mutations, as these mutations can modulate virus-host interactions and response to antivirals. In addition, the existing repertoire of HCV *in vitro* replication systems should be expanded to include previously uncharacterized subtypes of major HCV genotypes.

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ANNEXURE A

PAPER I

Antimicrobial Agents and Chemotherapy

Hepatitis C Virus Genotype 5a Subgenomic Replicons for Evaluation of Direct-Acting Antiviral Agents

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Hepatitis C Virus Genotype 5a Subgenomic Replicons for Evaluation of Direct-Acting Antiviral Agents

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Hepatitis C virus (HCV) exists as six major genotypes that differ in geographical distribution, pathogenesis, and response to antiviral therapy. *In vitro* replication systems for all HCV genotypes except genotype 5 have been reported. In this study, we recovered genotype 5a full-length genomes from four infected voluntary blood donors in South Africa and established a G418-selectable subgenomic replicon system using one of these strains. The replicon derived from the wild-type sequence failed to replicate in Huh-7.5 cells. However, the inclusion of the S2205I amino acid substitution, a cell culture-adaptive change originally described for a genotype 1b replicon, resulted in a small number of G418-resistant cell colonies. HCV RNA replication in these cells was confirmed by quantification of viral RNA and detection of the nonstructural protein NS5A. Sequence analysis of the viral RNAs isolated from multiple independent cell clones revealed the presence of several nonsynonymous mutations, which were localized mainly in the NS3 protein. These mutations, when introduced back into the parental backbone, significantly increased colony formation. To facilitate convenient monitoring of HCV RNA replication levels, the mutant with the highest replication level was further modified to express a fusion protein of firefly luciferase and neomycin phosphotransferase. Using such replicons from genotypes 1a, 1b, 2a, 3a, 4a, and 5a, we compared the effects of various HCV inhibitors on their replication. In conclusion, we have established an *in vitro* replication system for HCV genotype 5a, which will be useful for the development of pan-genotype anti-HCV compounds.

Hepatitis C virus (HCV) currently infects approximately 185 million people worldwide, increasing their risk of developing liver cirrhosis and hepatocellular carcinoma (1). No vaccine is available against HCV infection, and the standard of care until 2011, consisting of PEGylated interferon (IFN) and ribavirin, cured only 50% of patients. However, the addition of direct-acting antiviral agents (DAAs) over the past few years has revolutionized HCV treatment, with cure rates now approaching 80 to 90% (2–5). The recently published results of phase 3 clinical trials report even better regimens with >95% cure rates (6, 7). These are exciting developments, yet the issues of drug resistance, side effects, and drug-drug interactions will remain a challenge. Therefore, the quest to find safe drugs with a pan-genotype activity and a high barrier to drug resistance will continue. To aid these efforts, it is important to have cell culture replication systems for all HCV genotypes. Of the six major HCV genotypes, replication systems for HCV genotypes 1 and 2 were developed over a decade ago (8–10) and provided a strong foundation for the development of currently approved antivirals. Recently, replication systems for genotypes 3, 4, and 6 have also been reported (11–14). However, a similar system for genotype 5 is still lacking. This genotype is restricted mainly to South Africa (15) and has been associated with sporadic cases of HCV infection in Canada, Brazil, The Netherlands, Spain, and Belgium (16–18). In 2004, a high prevalence of genotype 5a was reported in central France (19). These findings highlight the importance of developing an *in vitro* replication system for this genotype.

HCV is an enveloped, positive-stranded RNA virus in the family *Flaviviridae*. The viral genome is ~9.6 kb long, with 5′ and 3′ untranslated regions (UTRs) flanking an open reading frame (ORF) of approximately 9,000 nucleotides (nt). This ORF encodes a polyprotein of ~3,000 amino acids that is co- and posttranslationally cleaved by host and viral proteases to yield 3 structural and 7 nonstructural (NS) proteins (20). The observation that the nonstructural proteins were sufficient for replication of a closely related bovine viral diarrhea virus (21) laid the foundation for the development of HCV subgenomic replicons. These replicons comprise three distinct elements: (i) an HCV internal ribosomal entry site (IRES), containing the 5′ UTR and the first 12 to 19 amino acids from the capsid protein, fused with a drug-selectable marker, neomycin phosphotransferase II (NPTII), which upon expression confers resistance to G418, (ii) an IRES from encephalomyocarditis virus (EMCV), which drives expression of the HCV nonstructural proteins, and (iii) the HCV 3′ UTR. When RNA transcripts from these replicons are introduced into Huh-7.5 cells by transfection or electroporation, followed by selection with G418, cell colonies harboring autonomously replicating viral genomes are obtained that can then be used to study various aspects of HCV replication. In this report, we generated the first subgenomic replicon for genotype 5a, using a strain isolated from the

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plasma of an infected blood donor. We identified novel cell culture-adaptive mutations that significantly increased the viral RNA replication levels. Furthermore, we used this newly generated replicon to measure the responsiveness of genotype 5a to various anti-HCV compounds.

MATERIALS AND METHODS

Cells, antibodies, and chemicals. Huh-7.5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 0.1 mM nonessential amino acids (NEAA), at 37°C in a humidified atmosphere containing 5% CO_2. G418 (Sigma, MO) was added to the medium at a concentration of 750 μg/ml to select and maintain cells carrying HCV replicons. Anti-NS5A antibody 9E10 has been described previously (22). Interferon alpha 2a (IFN-α2a), ledipasvir, and sofosbuvir were purchased from PBL Assay Science (NJ, USA), MedChem Express (NJ, USA), and Acme Bioscience (CA, USA), respectively. Danoprevir (RG7227/ITMN-191) and daclatasvir were purchased from Selleck Chemicals (TX, USA).

Human subjects. The samples from volunteer blood donors collected by the South African National Blood Services (SANBS) were anonymized and used for research purposes only after clearance from the ethics committee of the SANBS. Experiments were performed with authorization from the Institutional Review Board at the Rockefeller University.

Amplification of viral genomes. Plasma samples from four HCV genotype 5a-infected blood donors were used to isolate HCV RNA. Briefly, total RNA was extracted from 140 μl of plasma using a QIAamp viral RNA minikit (Life Technologies, CA) by following the manufacturer's protocol. An aliquot of 5 μl was subjected to cDNA synthesis using Superscript III reverse transcriptase (Life Technologies) and random hexamers. The reaction was carried out at 50°C for 50 min, followed by enzyme inactivation at 80°C for 15 min. PCR primers to amplify the cDNA were designed using sequences of two genotype 5a isolates, SA13 and EUH1480 (GenBank accession no. AF064490 and NC_009826), available in the HCV database (http://hcv.lanl.gov). For the sequences of these primers, see Table S1 in the supplemental material. Using these primers, the complete HCV ORF and partial UTRs were amplified into 8 overlapping fragments (see Table S1, fragments 2 to 9) by nested PCR using Phusion High Fidelity DNA polymerase (New England BioLabs, MA). The same conditions were used for both rounds of PCR: initial denaturation at 98°C for 5 min, 35 cycles of PCR (98°C for 30 s, 55°C for 30 s, 72°C for 2 min), and a final elongation step at 72°C for 10 min. The 5′ rapid amplification of cDNA ends (RACE) to amplify the terminal 5′ end of the viral genome was performed exactly as described by Saeed et al. (11). The terminal 3′-end sequence was determined by 3′ RACE. Briefly, RNA extracted from 250 μl of plasma samples, using TRIzol LS reagent (Life Technologies), was tailed at the 3′ end with GTP using yeast poly(A) polymerase (Affymetrix, CA). The tailed RNA was then reverse transcribed into cDNA using SuperScript III reverse transcriptase and a 27-mer reverse primer containing a stretch of eight cytidines at the 3′ end. The resulting cDNA was immediately amplified by nested PCR using *Ex Taq* DNA polymerase (Toyobo, Japan) and the primers described in Table S1. The PCR conditions consisted of an initial denaturation step at 95°C for 2 min, 35 cycles of PCR (95°C for 30 s, 58°C for 30 s, 72°C for 2 min), and a final elongation step at 72°C for 10 min.

Determination of HCV consensus sequences. The PCR amplicons were cloned into the TOPO TA vector (Life Technologies), followed by transformation of DH5α. Plasmid DNA purified from 5 to 10 bacterial colonies for each amplicon was subjected to sequence analysis, and the consensus sequence was determined by adopting the most abundant nucleotide at each position. The strains recovered from four plasma samples were tentatively named SA1, SA2, SA3, and SA4. The subgenomic regions from the isolated strains were aligned using MacVector, and their percent identity was calculated. Furthermore, the polyprotein regions from the newly sequenced genotype 5a isolates and representative isolates from all major HCV genotypes available in the database were subjected to phylogenetic analysis using the neighbor-joining method.

Construction of subgenomic replicons. Based on the consensus sequence of SA1, we assembled SA1/SG-neo. Briefly, clones covering the NS3-3′ UTR region of the viral genome were pieced together by overlapping PCR or by use of restriction enzymes. Since finding individual clones for each amplicon that were similar to the consensus sequence at all nucleotide positions proved difficult, the clones deviating from the consensus sequence at the nucleotide level, but not at the amino acid level, were sometimes selected to ligate to the neighboring fragment. This resulted in the inclusion of a few silent mutations in the region of the replicon encoding NS3-NS5B proteins. A cassette containing the NPTII gene, followed by an EMCV IRES, was cloned upstream of NS3. This construct was then ligated at its 5′ end with the IRES from SA1, consisting of the 5′ UTR and the first 19 codons from the capsid protein. To facilitate *in vitro* transcription, a T7 promoter was cloned upstream of the HCV IRES and an XbaI runoff site was introduced downstream of the 3′ UTR. The NPTII gene of SA1/SG-neo was replaced with a chimeric gene encoding firefly luciferase protein fused in-frame with NPTII to synthesize SA1/SG-Feo, as described elsewhere (12, 23).

RNA synthesis and transfection of cultured cells. Plasmids linearized with XbaI and purified with a MinElute PCR purification kit (Qiagen Sciences, MD) were *in vitro* transcribed using a T7 RiboMAX Express large-scale RNA production system (Promega, WI). To ensure complete removal of template DNA, an additional on-column treatment with RNase-free DNase was performed at room temperature (RT) for 15 min. The quality of *in vitro*-transcribed RNA was assessed by agarose gel electrophoresis. Huh-7.5 cells were electroporated with RNA transcripts using a BTX ElectroSquarePorator as described previously (12). To obtain cell colonies carrying autonomously replicating HCV genomes, electroporated cells were exposed to G418 (final concentration of 750 μg/ml) at 48 h postelectroporation, and medium was replaced every third day with a fresh medium containing 750 μg/ml G418. Three weeks postelectroporation, G418-resistant cell colonies were either isolated and expanded for downstream analysis or fixed with 7% formaldehyde and stained with crystal violet.

Colony titration assay. Huh-7.5 cells, seeded in 6-well plates at a density of 400,000 cells/well, were grown overnight. Cells were transfected with 2 μg of *in vitro*-transcribed RNA using a TransIT-mRNA transfection kit (Mirus Bio, WI), by following the manufacturer's recommendations. Six hours later, cells from each well were harvested and plated in new 6-well plates at multiple densities (between 2×10^2 and 2×10^5 cells/well). The total numbers of cells in each well were maintained at equal levels by using cells transfected with RNA transcripts from replication-defective HCV genomes. G418 selection was performed as described above. Three weeks posttransfection, colonies were stained with crystal violet and quantified manually. The colony formation efficiency was determined by calculating the percentage of transfected cells that survived after selection.

Analysis of HCV RNA replication in replicon cells. The isolation of colonies, RNA quantification, and flow cytometry were performed exactly as described previously (12).

Feo replicons from HCV genotypes 1a, 1b, 2a, 3a, and 4a. The genotype 2a replicon, JFH1/SG-Feo, and the genotype 3a replicon, S52/SG-Feo(AI1), have been previously described (12). The genotype 4a replicon, ED43/SG-Feo(VYG), was made by introducing a newly identified mutation, leading to an M1205V substitution in the NS3 protein of ED43/SG-Feo(Y), as described by Saeed et al. (12). It contains three cell culture-adaptive substitutions: M1205V (NS3), D1431Y (NS3), and R2882G (NS5B). This replicon replicates to higher levels than ED43/SG-Feo(Y) (unpublished results). The genotype 1a replicon, H77/SG-Feo(L+8), was generated from H77/SG-neo(L+8) (24). It contains four adaptive substitutions: P1496L (NS3), V1880A (NS4B), A1940V (NS4B), and C1968R (NS4B). The genotype 1b replicon, Con1/SG-neo(I) (8), was modified to

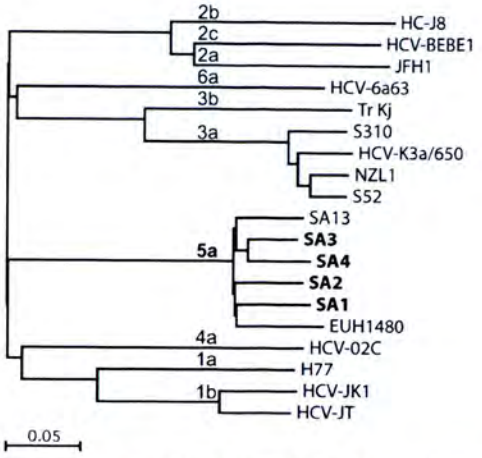

FIG 1 Phylogenetic tree drawn by aligning polyproteins from the HCV strains isolated in this study and representative strains from various genotypes reported in the database. The strains used in the phylogenetic analysis include HC-J8 (GenBank accession no. D10988), HCV-BEBE1 (accession no. D50409), JFH-1 (accession no. AB047639), HCV-6a63 (accession no. DQ480514), TrKj (accession no. D49374), S310 (accession no. AB691595), HCV K3a/650 (accession no. D28917), NZL1 (accession no. NC_009824), S52 (accession no. GU814263), SA13 (accession no. AF064490), EUH1480 (accession no. Y13184), HCV-02C (accession no. DQ418784), H77 (accession no. AF009606), HCV-JK1 (accession no. X61596), and HCV-JT (accession no. D11168). The length of the horizontal bar corresponds to the number of nucleotide substitutions per site.

generate Con1/SG-Feo(I). This replicon contains an S2204I substitution in the NS5A protein.

HCV inhibitor assay. HCV Feo replicons were used to measure the effect of various inhibitors on HCV RNA replication. Huh-7.5 cells carrying these replicons were seeded in 96-well plates with clear bottoms (PerkinElmer, MA) at a density of 1×10^4 cells/well. The following day, the cells were exposed to various concentrations of compounds and incubated for 72 h. Cell lysates were prepared by using 1× cell culture lysis reagent (CCLR; Promega), and firefly luciferase expression was measured with a luciferase assay system (Promega) and a Synergy NEO HTS multimode microplate reader (BioTek). The 50% effective concentrations (EC_{50}) relative to that for the untreated cells were determined using GraphPad Prism software (San Diego, CA). Replicon cell viability in the presence of inhibitors was tested in parallel using CellTiter-Glo assay (Promega), according to the manufacturer's recommendations.

Nucleotide sequence accession numbers. The consensus nucleotide sequences of SA1, SA2, SA3, SA4, and SA1/SG-neo (the subgenomic replicon derived from SA1) have been deposited in the GenBank database under accession numbers KJ925146 to KJ925150.

RESULTS

Isolation of genotype 5a sequences from patients. Plasma from four voluntary blood donors who were found to be infected with HCV genotype 5a served as a source of HCV genomes described in this report. Quantitative PCR (qPCR) showed that these plasma samples contained high levels of HCV RNA, ranging from 9.8×10^5 to 1.4×10^6 copies/ml (see Fig. S1 in the supplemental material). Complete viral genomes were recovered by amplifying 10 overlapping cDNA fragments, and the consensus sequence was determined by sequence analysis of 5 to 10 clones for each fragment. The terminal 5′ and 3′ ends of HCV genomes were amplified by 5′ RACE and 3′ RACE, respectively, as described in Materials and Methods. Strains isolated from the four patients, named SA1, SA2, SA3, and SA4, were composed of 9,562, 9,566, 9,566, and 9,584 nucleotides, respectively, and contained three structural elements: the 5′ UTR (nt 1 to 339), ORF (nt 340 to 9381), and 3′ UTR (nt 9382 to the 3′ end). The difference in lengths of these strains was due to variable lengths of the poly (U/UC) region in the 3′ UTR. In all strains, the ORF was 9,042 nt long, encoding a 3,014-amino-acid-long polyprotein. By phylogenetic analysis, all four strains clustered with previously sequenced genotype 5a isolates (Fig. 1).

To determine the degree of variation among the isolated strains, the genetic heterogeneity in each subgenomic region was calculated (Table 1). At the nucleotide level, the mean genetic heterogeneity of the entire ORF was 8.54%. Among the subgenomic regions, the largest variation was seen in the envelope proteins (E1, 11.11%; E2, 12.59%) and NS2 (11.44%). The 5′ UTR was the most conserved region, with a mean diversity of 0.79%. At the amino acid level, genetic heterogeneity of the entire polyprotein was 4.34%. Among the subgenomic regions, E2, NS2, and E1 displayed the greatest diversity (10.53%, 7.68%, and 6.60%, respectively).

Generation of subgenomic replicons. We generated a G418-selectable subgenomic replicon (Fig. 2A), SA1/SG-neo, from the consensus cDNA clone of SA1, as described in Materials and Methods. Huh-7.5 cells were electroporated with RNA transcripts generated by *in vitro* transcription of SA1/SG-neo, and G418 selection was imposed 48 h later. However, all cells died after 3 weeks of selection, suggesting a failure of SA1/SG-neo to establish replication (Fig. 2B). We have previously shown that some of the cell culture-adaptive changes, originally described for genotypes 1a and 1b replicons, can support replication of other genotypes (11, 12). Based on this observation, we generated three mutants containing single-amino-acid substitutions: (i) SA1/SG-neo(i), containing a T1281I substitution in NS3 (corresponding to T1280I in genotype 1b strain Con1; GenBank accession no. AJ238799) (25), (ii) SA1/SG-neo(I), containing a S2205I substitution in NS5A (corresponding to S2204I in genotype 1a strain H77; GenBank

TABLE 1 Mean genetic heterogeneity between isolated genotype 5a strains[a]

Genomic region	Heterogeneity			
	Nucleotide level		Amino acid level	
	Nucleotides[b]	% heterogeneity (mean ± SD)	Amino acids[b]	% heterogeneity (mean ± SD)
5′ UTR	1–339	0.79 ± 0.48		
Core	340–912	3.93 ± 1.03	1–191	1.83 ± 0.86
E1	913–1488	11.1 ± 0.86	192–383	6.60 ± 1.67
E2	1489–2580	12.6 ± 1.36	384–747	10.5 ± 1.51
P7	2581–2769	9.79 ± 1.60	748–810	1.59 ± 1.74
NS2	2770–3420	11.4 ± 0.63	811–1027	7.68 ± 2.17
NS3	3421–5313	7.16 ± 0.60	1028–1658	1.88 ± 0.53
NS4A	5314–5475	6.28 ± 2.85	1659–1712	0.31 ± 0.76
NS4B	5476–6258	8.17 ± 3.32	1713–1973	1.72 ± 0.79
NS5A	6259–7608	8.98 ± 0.94	1974–2423	6.04 ± 0.99
NS5B	7609–9381	6.91 ± 0.30	2424–3014	2.57 ± 0.39
ORF	340–9381	8.54 ± 0.80	1–3014	4.34 ± 0.41

[a] UTR, untranslated region; E, envelope; NS, nonstructural.
[b] The nucleotide and amino acid positions refer to a previously sequenced genotype 5a strain, SA13 (GenBank accession no. AF064490).

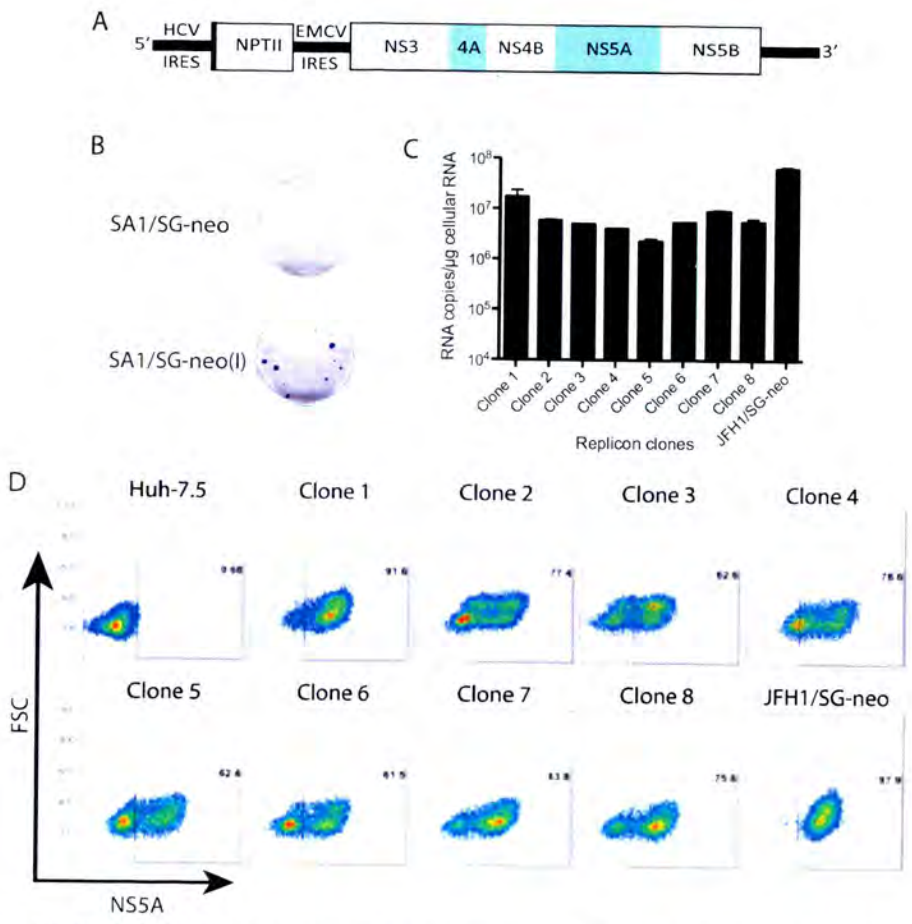

FIG 2 Replication of SA1/SG-neo in Huh-7.5 cells. (A) Schematic diagram of SA1/SG-neo. NPTII, neomycin phosphotransferase II. (B) Huh-7.5 cells were selected with G418 for 4 weeks following electroporation with RNA transcripts from the indicated replicons. The resulting cell colonies were stained with crystal violet. (C) HCV RNA in the isolated cell colonies was measured by qPCR. Results are expressed as means ± standard errors of the means (SEM) of two independent measurements. (D) Replicon cells were stained with anti-NS5A antibody and analyzed by flow cytometry. FSC, forward scatter.

accession no. AF009606) (8), and (iii) SA1/SG-neo(G), containing an R2888G substitution in NS5B (corresponding to R2884G in Con1) (26). Since combining cell culture-adaptive substitutions in NS3 with those in other nonstructural proteins increases the efficiency of G418-resistant colony formation (12, 25, 27), we generated double mutants containing the T1281I substitution together with S2205I or R2888G. All mutants failed to replicate in Huh-7.5 cells, except SA1/SG-neo (I), for which a small number of colonies were obtained (Fig. 2B). Eight cell colonies were isolated, and HCV RNA replication was confirmed by quantification of HCV RNA and detection of NS5A protein. HCV RNA copies ranged from 2.3×10^6 to 1.8×10^7 copies/μg of cellular RNA (Fig. 2C). NS5A protein could be detected by flow cytometry in 61.5 to 91.6% of cells (Fig. 2D). When naive Huh-7.5 cells were electroporated with RNA extracted from replicon cell clones and subjected to selection with G418, a large number of cell colonies were obtained, suggesting transmissibility of G418 resistance by the replicating viral RNAs (see Fig. S2 in the supplemental material). Taken together, these results indicated that the G418-resistant cell colonies carried autonomously replicating viral RNA.

Effect of candidate adaptive mutations on replication. The appearance of a few colonies after electroporation with SA1/SG-neo(I), combined with the fact that significantly more colonies were obtained when cellular RNA from these colonies was introduced into naive Huh-7.5 cells, suggested that additional cell culture-adaptive mutations might have been acquired. To test this possibility, RNA was extracted from the isolated cell clones, and the NS3-NS5B coding region of the viral genome was sequenced. While the S2205I substitution was conserved, at least one additional nonsynonymous mutation was present in each clone (Table 2). We engineered these mutations into SA1/SG-neo(I) and measured their effect on HCV RNA replication by titrating the colony formation in Huh-7.5 cells. Since three nonsynonymous mutations were identified in clone 1, various mutants containing dif-

TABLE 2 Nonsynonymous mutations found in eight SA1/SG-neo(I) replicon clones

Replicon clone	Nucleotide mutation[a]	Amino acid substitution[b]	NS[c] protein
1	3946 (2301) G→A	1203 (176) E→K	NS3
	4556 (2911) A→G	1406 (379) K→S	NS3
	4557 (2912) G→C		
	7435 (5790) C→T	2366 (393) S→P	NS5A
2	4021 (2376) C→T	1228 (201) H→Y	NS3
3	4165 (2520) G→A	1276 (249) D→N	NS3
4	5453 (3808) A→G	1705 (47) Q→R	NS4A
5	4261 (2616) G→A	1308 (281) G→S	NS3
6	4951 (3306) G→C	1538 (511) V→L	NS3
7	4633 (2988) G→A	1432 (405) D→N	NS3
8	5453 (3808) A→G	1705 (47) Q→R	NS4A

[a] Position within full-length SA1 genome and SA1 subgenomic replicon (in parentheses).
[b] Position within full-length SA1 polyprotein and within individual proteins (in parentheses).
[c] NS, nonstructural.

ferent combinations of these substitutions were generated for this clone. A broad range of cell culture adaptation was observed across the mutants; the mutants showed a 55- to 1,700-fold increase in colony formation efficiency compared to that for SA1/SG-neo(I) (Fig. 3) (see Fig. S3A and S3B in the supplemental material). A mutant containing all three substitutions identified in clone 1, henceforth called SA1/SG-neo(SKIP), yielded the highest number of colonies. However, the number of colonies was much lower than that obtained for Con1/SG-neo(I), a highly cell culture-adapted subgenomic replicon from genotype 1b. To examine if SA1/SG-neo(SKIP) acquired an additional mutation(s) which might further increase the colony formation efficiency of this replicon, we isolated three colonies harboring this construct and sequenced the region encoding NS3-NS5B. No additional mutation was found, indicating that this replicon does not need further adaptation. Next, we asked if the identified mutations were able to support colony formation in the absence of S2205I. Elimination of the S2205I substitution from mutants ablated their ability to yield G418-resistant cell colonies (data not shown), suggesting that isoleucine at this position was essential for the adaptive potential of the *de novo* mutations.

Synthesis of the Feo replicon for genotype 5a. To generate a genotype 5a replicon that can be used to facilitate screening of antiviral compounds, we replaced the NPTII gene of SA1/SG-neo(SKIP) with a fusion protein of firefly luciferase and NPTII. When RNA transcribed from this replicon, named SA1/SG-Feo(SKIP), was introduced into Huh-7.5 cells, followed by selection with G418 (750 μg/ml), a large number of cell colonies were obtained; however, the number was ~3-fold lower than that obtained for the "-neo" version of this replicon. We pooled cell colonies and compared the levels of HCV RNA replication with Huh-7.5 cells carrying JFH1/SG-Feo, a previously reported highly efficient replicon derived from the genotype 2a isolate JFH-1 (12). The luciferase activity of SA1/SG-Feo(SKIP) was comparable to that of JFH1/SG-Feo, suggesting high levels of HCV RNA replication (Fig. 4A). Furthermore, NS5A protein could be readily detected in most of the cells carrying SA1/SG-Feo(SKIP) (Fig. 4B).

Effect of HCV inhibitors on replication of various genotypes. The development of a subgenomic replicon for genotype 5a provided us an opportunity to compare its response to various antiviral compounds with those of other genotypes. To this end, we used a panel of Feo replicons from genotypes 1a [H77/SG-Feo(L+8)], 1b [Con1/SG-Feo(I)], 2a (JFH1/SG-Feo), 3a [S52/SG-Feo(AII)], 4a [ED43/SG-Feo(VYG)], and 5a [SA1/SG-Feo(SKIP)]. JFH1/SG-Feo and S52/SG-Feo(AII) have been previously described (12), while H77/SG-Feo(L+8), Con1/SG-Feo(I), and ED43/SG-Feo(VYG) were generated as described in Materials and Methods. For convenience, these replicons are referred to as H77, Con1, JFH-1, S52, ED43 and SA1 in this report. The G418-resistant Huh-7.5 cells, selected after electroporation with synthetic RNA from each replicon, were pooled, and a firefly luciferase assay was done to compare their replication levels. The firefly luciferase values were within 3-fold of each other, indicating similar levels of HCV RNA replication (Fig. 4A). By flow cytometry, NS5A protein could be detected in 56% to 96% of cells (Fig. 4B). The lower number of NS5A-positive cells for H77 may be due to a lower sensitivity of the antibody for this genotype. Next, we exposed the replicon cells to various concentrations of IFN-α2a and four DAAs: danoprevir (an NS3 protease inhibitor), daclatasvir and ledipasvir (NS5A inhibitors), and sofosbuvir (an NS5B nucleotide polymerase inhibitor) (Fig. 5). IFN-α2a showed comparable inhibitory effects across all genotypes (Table 3), although a cytotoxic effect was observed at higher concentrations, especially for cells carrying genotype 2a replicons (see Fig. S4A in the supplemental material). Among the DAAs, danoprevir was highly effective against genotypes 1a, 1b, 4a, and 5a, with 50% effective

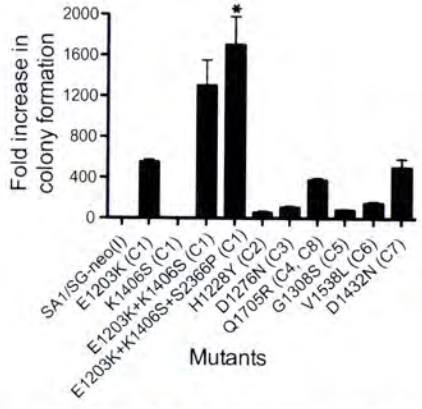

FIG 3 Colony titration assay of SA1/SG-neo(I) containing additional amino acid substitutions described in Table 2. Huh-7.5 cells, seeded in 6-well plates at a density of 4×10^5 cells/well, were transfected the next day with 2 μg RNA from the indicated mutants. Six hours after transfection, cells were harvested and seeded in new 6-well plates at densities ranging from 2×10^5 to 2×10^2 cells/well. The total numbers of cells in each well were brought to 2×10^5 by adding feeder cells transfected with RNA from a replication-defective HCV replicon (the three residues in the active site of NS5B polymerase were changed from GDD to AAG). The cell colonies resulting from 4 weeks of selection with G418 were counted for at least two cell densities, and the percentage of colony formation was calculated (number of colonies in a well/number of cells initially plated in that well × 100). Shown is the fold increase in colony formation relative to the result for SA1/SG-neo(I), the value for which was arbitrarily set at 1. Data are means ± SEM from two independent experiments. *, this replicon had the highest replication levels and was named SA1/SG-neo(SKIP).

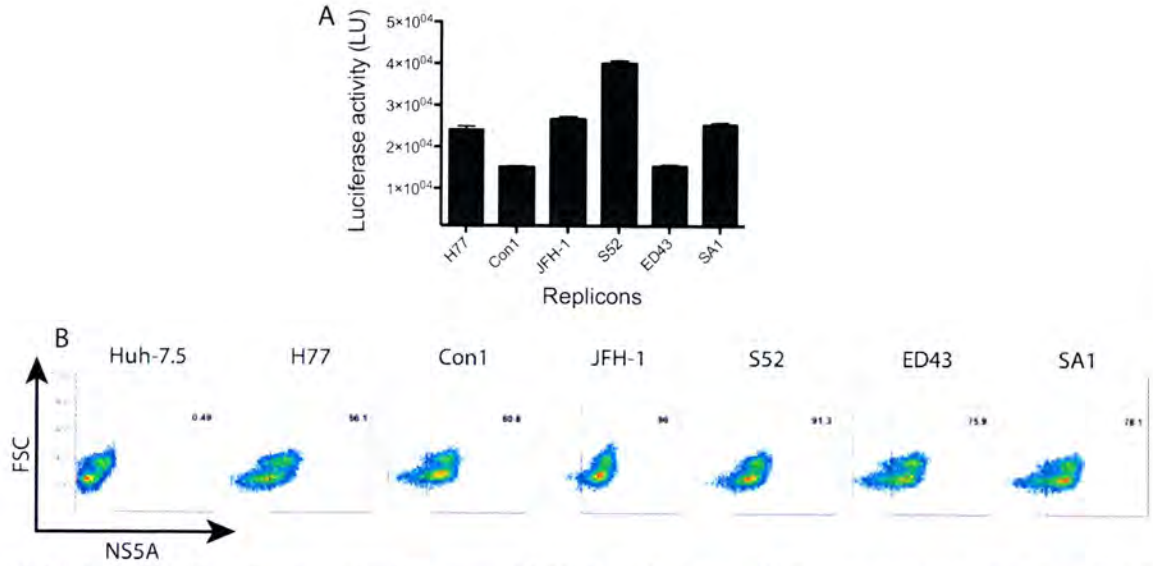

FIG 4 Replication levels of Feo replicons from various HCV genotypes. Huh-7.5 cells were electroporated with Feo replicons from genotype 1a (H77), 1b (Con1), 2a (JFH-1), 3a (S52), 4a (ED43), and 5a (SA1) and selected with G418 (750 μg/ml). After 3 weeks, the surviving cells were pooled and passaged twice in G418-containing medium. LU, luciferase units. (A) Replicon cells from each genotype were seeded in 8 wells of a 96-well plate at a density of 2×10^4 cells/well. Twenty-four hours later, cells were lysed and firefly luciferase activity was measured. The results are plotted as means ± SEM from eight replicates. (B) HCV NS5A protein in the cells was detected by flow cytometry.

concentrations (EC_{50}) of 1.89 nM, 0.67 nM, 3.67 nM, and 12.79 nM, respectively. Intermediate efficacy was seen against genotype 2a (EC_{50}, 36.31 nM), while genotype 3a was least susceptible (EC_{50}, 276.7 nM). Daclatasvir, with EC_{50} of 38.48 pM, 20.82 pM, 63.79 pM, 18.21 pM, and 57.06 pM, was highly effective against genotypes 1a, 1b, 2a, 4a, and 5a, respectively, whereas the EC_{50} for genotype 3a was ~2 nM, indicating a lower inhibitory effect against this genotype. Ledipasvir displayed a broad range of effi-

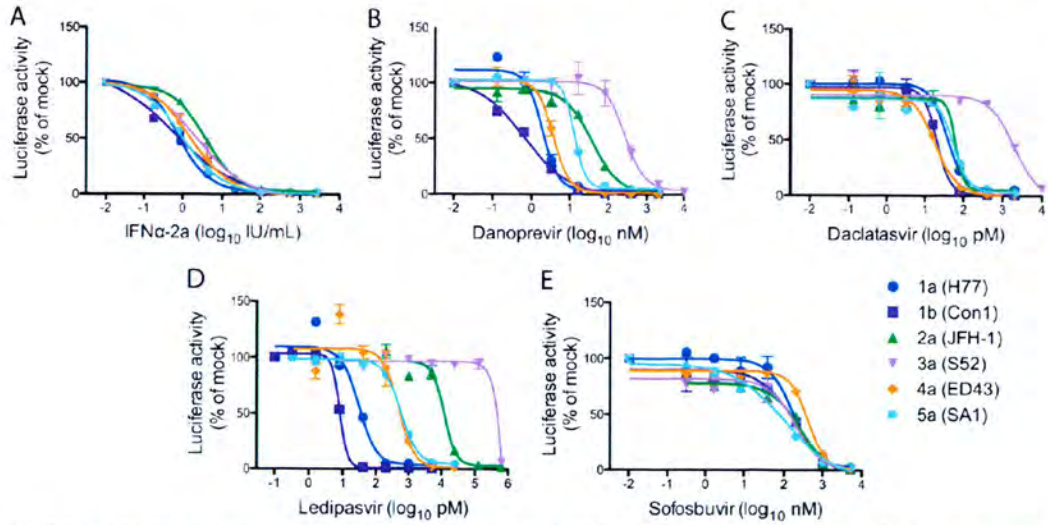

FIG 5 Inhibitory effect of anti-HCV compounds on the replication of various genotypes. Cells harboring persistently replicating Feo replicons from genotypes 1a (H77), 1b (Con1), 2a (JFH-1), 3a (S52), 4a (ED43) and 5a (SA1) were seeded in duplicate in tissue culture-treated, 96-well, white plates at a density of 1×10^4 cells/well using G418-free cell culture medium. The following day, cells were exposed to the indicated concentrations of IFN-α2a (A), danoprevir (B), daclatasvir (C), ledipasvir (D), and sofosbuvir (E). Seventy-two hours later, cells were lysed in 50 μl of 1× CCLR (Promega), and firefly luciferase activity was measured. Data are plotted as means ± SEM from three independent experiments.

TABLE 3 Inhibitory effects of antiviral compounds on HCV replication[a]

Compound	Genotype (isolate)	EC_{50}	95% CI
IFN-α2a (IU/ml)	1a (H77)	0.77	0.66–0.88
	1b (Con1)	0.64	0.36–1.15
	2a (JFH-1)	3.77	2.98–4.76
	3a (S52)	2.46	1.51–4.02
	4a (ED43)	1.63	1.11–2.40
	5a (SA1)	1.01	0.76–1.33
Danoprevir (nM)	1a (H77)	1.89	1.33–2.70
	1b (Con1)	0.67	0.47–0.95
	2a (JFH-1)	36.31	24.81–53.16
	3a (S52)	276.7	189.30–404.50
	4a (ED43)	3.67	3.10–4.34
	5a (SA1)	12.79	11.10–14.73
Daclatasvir (pM)	1a (H77)	38.48	31.29–47.33
	1b (Con1)	20.82	14.84–29.21
	2a (JFH-1)	63.79	11.90–341.80
	3a (S52)	2008	972.10–4147
	4a (ED43)	18.21	13.15–25.23
	5a (SA1)	57.06	31.07–104.80
Ledipasvir (pM)	1a (H77)	29.94	18.46–48.55
	1b (Con1)	8.12	7.77–8.48
	2a (JFH-1)	12,062	8,175–17,796
	3a (S52)	4.55×10^5	Very wide
	4a (ED43)	498.8	230.2–1081
	5a (SA1)	576.5	463.2–717.5
Sofosbuvir (nM)	1a (H77)	170.1	112.2–258.0
	1b (Con1)	187.1	91.89–380.8
	2a (JFH-1)	263.9	151.9–458.5
	3a (S52)	198.2	92.55–424.5
	4a (ED43)	415.2	293.8–586.8
	5a (SA1)	107.9	47.95–242.2

[a] EC_{50}, 50% effective concentration; 95% CI, 95% confidence interval obtained from one experiment. The results were confirmed by two independent experiments, with each experiment performed in triplicate. "Very wide" 95% CI indicates that the standard errors were too high to unambiguously calculate the confidence interval.

cacy depending on the genotype: a very strong inhibition of genotypes 1a and 1b (EC_{50} of 29.94 pM and 8.12 pM, respectively), yet weak activity against genotype 3a (EC_{50}, 455 nM). Sofosbuvir inhibited replication of all genotypes with comparable efficiencies; EC_{50} ranged from 107.9 nM to 415.2 nM. Treatment of cells with the DAAs at the concentrations used in this study had no effect on cell viability (see Fig. S4 in the supplemental material), indicating that the decrease in luciferase signal was due to the specific inhibition of HCV RNA replication. Taken together, these results demonstrate differential effects of anti-HCV compounds on various genotypes.

DISCUSSION

Since its development in 1999, the HCV subgenomic replicon system has been instrumental in studying various aspects of HCV RNA replication in cultured cells. However, for many years, this system was restricted only to genotypes 1 and 2, partly because these are the most predominant genotypes in the United States, Europe, and Japan. This restriction was recently overcome with the development of subgenomic replicons for genotypes 3a, 4a, and 6a (12, 13). In this study, we report the first genotype 5a replicon that successfully replicated in Huh-7.5 cells, producing high levels of HCV RNA and proteins (Fig. 2C and D). These high levels of replication, however, were obtained only after inclusion of cell culture-adaptive changes identified in various regions of the viral genome (Fig. 3) (see Fig. S3 in the supplemental material). The replicon with the highest replication capacity was further modified to express a convenient reporter, facilitating its use for high-throughput screening and evaluation of antiviral compounds. Taking advantage of this newly developed replicon, we compared the responsiveness of genotype 5a to various anti-HCV compounds with that of other genotypes (Fig. 5).

In the present study, we used plasma from HCV-infected blood donors in South Africa to recover the viral genomes. Phylogenetic analysis showed clustering of these genomes with genotype 5a isolates. The genomic sequences from three different strains of genotype 5a, namely, SA13 (GenBank accession no. AF064490), EUH1480 (GenBank accession no. NC_009826), and ZS631 (GenBank accession no. KC844046.1), are available in the HCV database (http://hcv.lanl.gov); however, none of them contains the complete 3′ UTR sequence. The 5′ and 3′ UTR of HCV, like UTRs from many positive-strand RNA viruses, contain important sequences and structural motifs critical for translation and RNA replication (reviewed in reference 28). Although these regions are highly conserved across HCV genotypes, intergenotypic differences exist which may affect the level of RNA replication. We therefore performed 5′ and 3′ RACE to determine the authentic termini of the genotype 5a strains. As expected, a high degree of conservation was seen among the isolated strains on both termini: >99% identity in the 5′ UTR and 100% identity in the X-tail region of the 3′ UTR. Within the coding region, envelope proteins exhibited the highest diversity (Table 1). This was not surprising, given that these are the primary targets of neutralizing antibodies and that a continuous tug-of-war between the host's immune system and the virus leads to a high genetic heterogeneity in these glycoproteins.

As the NS3-NS5B region was highly conserved among the isolated genotype 5a strains (97% identity), we selected one representative strain, SA1, for which to develop a subgenomic replicon. Replication and G418 selection of SA1/SG-neo required introduction of a previously reported, broadly active, cell culture-adaptive substitution, S2205I (Ser-to-Ile substitution at NS5A position 232). This, however, was not sufficient for efficient replication, as all replicons isolated from randomly chosen cell colonies had acquired additional mutations. Most of these mutations resulted in coding changes in the NS3 protein, although a few in NS4A and NS5A were also seen (Table 2). Interestingly, one of the identified changes, D1432N, corresponds to the D1431N substitution described previously for genotype 4a (12). All of the *de novo* mutations, when combined with S2205I, led to various degrees of increased colony formation efficiency (Fig. 3) (see Fig. S3 in the supplemental material). These, however, failed to support HCV RNA replication in the absence of S2205I (data not shown). These results concur with most of the existing data showing that adaptive mutations in different parts of the viral genome enhance each other (12, 25, 27). Exactly how these adaptive changes cooperate and their mechanism of action are still not known.

Genotype 5a is localized mainly to South Africa, where it accounts for approximately 40% of HCV infections (15, 29). Information about disease severity and treatment outcome of this genotype is very limited. Although preliminary studies suggested

that genotype 5 responds well to combination therapy of interferon and ribavirin (30), no large-scale clinical trials were performed to solidify these results. Furthermore, little is known about its response to DAAs, and none of the approved DAAs are currently recommended for the treatment of genotype 5a infection. Chimeric systems, where one or multiple proteins from genotype 5a were substituted in the backbone of JFH-1 genotype 2a replicase, have been used for cell culture-based inhibitor studies (31–33). However, the results obtained with these systems can be affected by suboptimal intergenotypic interactions between replicase components and diminished replicative fitness. Therefore, replicons with the entire replicase from the same genotype offer more desirable systems for testing antivirals. With the first *in vitro* replication system in hand, we examined the response of genotype 5a to various HCV inhibitors. The results suggested a high sensitivity of this genotype to sofosbuvir, an NS5B polymerase inhibitor currently approved for the treatment of genotypes 1, 2, 3, and 4 (Fig. 5, Table 3). In fact, the EC_{50} for genotype 5a was lowest among those for all of the genotypes, although the difference was not significant. Recently, a high response of genotype 6a to sofosbuvir was also documented (13), suggesting a pan-genotype profile of this nucleotide inhibitor. Unlike sofosbuvir, other DAAs displayed differential activity between genotypes. For example, danoprevir, a macrocyclic NS3 protease inhibitor, was highly effective against genotypes 1a, 1b, and 4a, followed by genotypes 5a and 2a, for which EC_{50} were ~12- and 36-fold higher, respectively, than that for genotype 1b. In contrast, genotype 3a, in accordance with previously published reports (12, 32, 33), was highly resistant to danoprevir, with an EC_{50} more than 350-fold higher than that of genotype 1b. Conflicting data exist on the sensitivity of genotype 5a to danoprevir. Using a cell culture infection system, where the NS3 protease domain (NS3P) and NS4A from various genotypes were expressed in the backbone of genotype 2a J6/JFH1 virus, Gottwein et al. showed that the EC_{50} of danoprevir for genotype 5a was approximately 30-fold lower than that for genotype 3a (32). In contrast, Imhof and Simmonds, using a similar system, reported that genotype 5a was less susceptible than genotype 3a (33). This discrepancy might be explained by two different genotype 5a strains used in these studies: while Gottwein et al. obtained NS3P and NS4A from SA13, Imhof and Simmonds used another strain, EUH1480. To test if these differences in genetic background could account for discrepant results, we compared NS3Ps from SA13 and EUH1480. Although 96% of amino acids were conserved, sequence variation was seen at positions 168 and 170, two of the several loci associated with resistance of genotype 1 to danoprevir (34, 35). At both positions, the amino acids present in SA13 (Asp and Ile, respectively) were the same as those found in susceptible genotype 1 isolates, whereas EUH1480 had Glu and Val at these positions. Even though Glu168 and Val170 are not reported to be directly associated with resistance, several other residues at these positions mediated high levels of resistance to macrocyclic protease inhibitors in various HCV genotypes (33, 36). Most notably, among genotype 5a strains isolated in this study, SA1 had Asp at position 168, while the remaining 3 strains had Glu. Similarly, at position 170, SA1 and SA2 had Ile, while SA3 and SA4 had Val. This may explain, at least in part, why SA1 and SA13 are highly sensitive to danoprevir. In addition, these observations clearly highlight the importance of testing multiple strains from various genotypes before drawing general conclusions. Among the NS5A inhibitors, daclatasvir was highly effective against genotype 5a, which concurs with previously reported results using recombinant HCV genomes expressing the genotype 5a NS5A protein in the J6/JFH1 backbone (37). In fact, with picomolar EC_{50} in the cell culture replication systems, daclatasvir is thus far the most potent drug against various HCV genotypes. As these potencies have translated into robust activity in the clinical studies with genotype 1 patients (38), a favorable clinical response for genotype 5a can be expected.

In conclusion, we have developed a subgenomic replicon for genotype 5a that will be a useful research tool to study various aspects of this poorly studied genotype and to evaluate its response to antiviral compounds. With this report, the replication systems for all major HCV genotypes are now established; however, this repertoire should be further expanded to include hitherto uncharacterized subtypes. In addition, efforts should be made to propagate HCV in human hepatoma cells without the need for cell culture-adaptive mutations, as these are typically not found in natural isolates and may affect virus-host interactions and responses to antiviral compounds.

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ANNEXURE B

Paper II

Novel full-length consensus sequences of hepatitis C virus genotype 5a

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ABSTRACT

Persistent infection with hepatitis C virus (HCV) has emerged as one of the leading causes of chronic liver disease with an estimated 170 million people infected by HCV. Based on genetic differences between HCV isolates, the virus species is classified into seven major genotypes (1-7) with several subtypes within each genotype. HCV RNA was detected and quantified in the plasma of four infected South African blood donors by quantitative polymerase chain reaction (qPCR). The entire genome of infected HCV was recovered by PCR amplification into 10 overlapping fragments, cloned, and 5-10 clones for each fragment sequenced from these samples. The 5' and 3' untranslated regions (UTR) of HCV were amplified by 5' and 3' RACE (rapid amplification of cDNA ends), respectively. Multiple sequence alignments of all clones were performed and a consensus file generated. Strains isolated from the four samples, named as SA1, SA2, SA3 and SA4, were composed of 9562, 9566, 9566, and 9588 nucleotides, respectively, and contained three structural elements: 5' UTR (nt 1-339), ORF (nt 340-9381) and 3' UTR (9382-3' end). The open reading frame (ORF) was 9042 nucleotides long in all strains, encoding a 3014-amino acid long polyprotein followed by a stop codon. By phylogenetic analysis, these strains were clustered into genotype 5a. In conclusion, we have established full-length sequences for four HCV genotype 5a isolates, which did not exist previously in the fast growing HCV database.