INVESTIGATION INTO HEPATITIS C NON-STRUCTURAL PROTEIN 3 AND ITS ROLE IN HEPATOCELLULAR CARCINOMA

BY

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A Thesis
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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As an adolescent I aspired to lasting fame, craved factual certainty, and thirsted for a meaningful vision of human life - so I became a scientist. This is like becoming an archbishop so you can meet girls.
- Matt Cartmill

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List of Abbreviations

α	alpha
β	beta
γ	gamma
ATP	adenosine tri-phosphate
bp	base pair
$^{\circ}C$	degrees Celsius
CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagle's Medium
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
E1/E2	envelope 1/envelope 2
E. coli	*Escherichia coli*
HCV	Hepatitis C Virus
INF	interferon
κ	kappa
LB	Luria broth
mRNA	messenger ribonucleic acid
min.	minutes
NK cell	natural killer call
nm	nanometer
nt	nucleotide
PPi	inorganic phosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKR	protein kinase receptor
RNA	ribonucleic acid
STAT3	signal transducer and activator of transcription 3
ssRNA	single-stranded ribonucleic acid
UTR	untranslated region
μl	microlitres
μg	micrograms
wt	wild-type

Abstract

Infection with Hepatitis C Virus (HCV) often becomes chronic and can lead to cellular transformation and ultimately hepatocellular carcinoma in 5% of individuals infected. This phenomenon triggers considerable interest in the analysis of potential interactions between individual HCV proteins and host cell proteins regulating cell growth. Recently, the potential of NS3 protease to elicit a malignant response has been mildly explored. Viral proteases are known to affect cellular metabolism. Without regulatory restrictions, NS3 has the ability to not only disrupt growth pathways by direct protein-protein interaction, but also to degrade regulatory proteins, thereby influencing the initial stages of cellular transformation. It was found that expression of NS3 in Huh 7.0 cells could activate several oncogene - related pathways namely NFkB and *c-fos*. Detection was measured via chemiluminescence and mammalian two hybrid assays. I have currently narrowed down the region of NS3 responsible for this up-regulation to approx. 300 amino acids. Identification of this motif will not only provide powerful insight into the host factors involved in HCV protein interaction but will ultimately serve to provide therapeutic potential in treatment of hepatocellular carcinoma.

1. Introduction

1.1 Hepatitis C Virus

Hepatitis C is a chronic progressive disease of the liver that is caused by infection with hepatitis C virus (HCV). Initially known as "Non-A, Non-B Hepatitis" in the 1970's, this "new" agent turned out to be very difficult to identify and only with the dawn of recombinant DNA technology was it possible to clone the genome of the virus that was termed hepatitis C virus (HCV) in 1989 (Choo *et al.*, 1989). Until recently, HCV has remained frustratingly elusive in the laboratory as a result of its non-culturable nature. Critical successes have since been made, enabling viral clones to replicate efficiently in cultured cells. The initial breakthrough in HCV research came in 1997, with development of the first infectious HCV clone and soon afterward, a subgenomic replicon, enabling replication in cell culture.

Since the year 2000, the hepatitis C virus (HCV) has become the most common cause of liver cirrhosis and is presently the single major reason for liver transplantation in North America (Landford *et al.*, 2004). It has been estimated that 123–170 million people worldwide are living with HCV infection (of whom 250 000 (0.8%) are in Canada) and that about 5000 Canadians are newly infected each year (Public Health Agency of Canada). It was reported that approximately 70% of those with HCV infection suffer from persistent infection, causing active or inactive chronic hepatitis and that about 30% of patients with chronic hepatitis are assumed to develop cirrhosis within their lifetime (Anzola *et al*, 2004).

In the early 1990s, clinical cases of many patients with either community-acquired or transfusion-related hepatitis C documented the sequential development of chronic liver disease and liver cancer. This was the first clue in the identification of HCV as a risk factor for hepatocellular carcinoma (Zemel, 2002). We now know that once HCV infection develops into cirrhosis, HCC develops at an annual rate of 5–7% (Kazuhiko *et al*, 2006). The prognosis of HCC patients is generally very poor. Most studies report a five-year survival rate of less than 5% in symptomatic HCC patients. Furthermore, these tumors have been shown to be quite resistant to radio- or chemotherapy (Colombo, 1999).

1.2 Virus Characteristics

Electron microscopy has revealed the HCV particle to be approximately 50nm in diameter, consisting of an envelope, derived from host membranes, into which viral encoded glycoproteins (envelope proteins 1 and 2) are inserted. The nucleocapsid that is enclosed encircles a linear, single-stranded, positive sense RNA genome of approximately 9,500 nucleotides. The genome contains highly conserved untranslated regions (UTRs) at both the 5' and 3' termini which flank a single open reading frame, encoding a polyprotein of ~3,000 amino acids. Given its high rate of mutation, at least 6 distinct clades of HCV, and more than 100 subtypes, have been identified by nucleotide sequencing (Abid *et al*, 2000). This categorization has become important in predicting both the degree of pathogenicity, as well as responsiveness to treatment in clinical settings.

As with other members of the *Flaviviridae* family, HCV replicates by enzymatically converting its positive-strand RNA genome into a complementary or minus-strand replicative-intermediate RNA and then copying the ladder to produce new progeny plus-strand RNA. Translation of the HCV open reading frame is directed by a 5' UTR, which functions as an internal ribosome entry site (IRES) and permits the direct binding of ribosomes to the start codon of the ORF. A 3' UTR is also present, composed of an ~40 nt long variable region downstream of the HCV coding sequence, a poly (U/UC) tract of heterogeneous length and a highly conserved 98nt long sequence designated the x-tail (Appel *et al*, 2006). The x-tail and poly (U/UC) tract have been shown to be essential for infection (*in vivo*) and replication (*in vitro*) respectively (Lauer and Walker, 2001).

Host and virus intervention mediate translational modification both throughout as well as subsequent to polyprotein production. As a result, at least 10 viral proteins are generated: core, envelope 1 (E1), envelope 2 (E2), p7, nonstructural protein 2 (NS2), NS3, NS4a, NS4b, NS5a and NS5b (Blight *et al*, 2000).

The first major cleavage products (beginning at the N-terminus) reveal nucleocapsid (core) and envelope (E1 and E2) proteins, essential for housing the ssRNA genome and releasing the viral particle respectively. The nascent polyprotein responsible for the generation of these proteins contains a signal sequence that directs it to the membrane of the endoplasmic reticulum (ER). Here, the E1/E2 portions of the protein undergo translocation and extensive N-linked glycosylation within the ER lumen (Figure 1). Cleavage by signal peptidases at the luminal side of the ER separates E1/E2 from the immature form of the core (on the cytosolic side), which then undergoes additional

processing, by the intramembane-cleaving protease (signal peptide peptidases). A mature core protein is developed which is either targeted to other internal organelles within the cell or homo-oligomerized for use in nucleocapsid production. It is this N-terminal portion of the immature polypeptide that includes numerous positively charged amino acids and is mainly involved in RNA binding (Boulant *et al*, 2005).

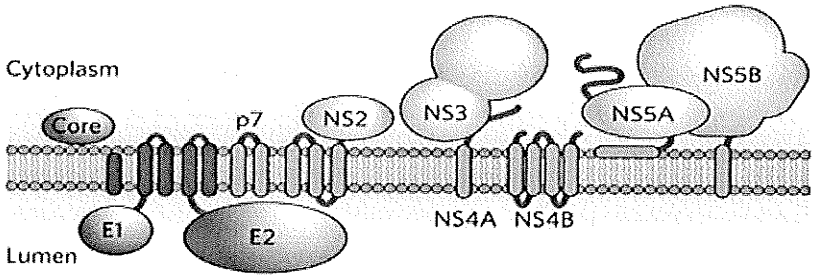

Figure 1: The topology of HCV proteins with respect to a cellular membrane (Lindenbach and Rice, 2005).

E1 and E2 form heterogeneous mixtures of covalently and noncovalently associated heterodimers that are largely retained in the ER via retention sequences located in their transmembrane domains (Drummer *et al*, 2006). However, a small proportion of E1/E2 heterodimers escape the ER and mature through the secretory pathway. The E2 glycoprotein mediates binding to cellular receptors, including the tetraspanin CD81 and the high-density lipoprotein receptor scavenger receptor class B type 1 (Reed and Rice, 2000). In addition to its role in viral entry, the E2-CD81 interaction has been shown to cause inflammatory and immunomodulatory responses *in vitro* (Drummer et al, 2006). For example, E2-CD81 ligation was found to lower the threshold of T-cell activation, stimulating the production of inflammatory cytokines. CD81-E2 ligation also leads to the suppression of NK cell activity, which may decrease the effectiveness of innate immune

responses in clearing virus. These responses are consistent with pathogenic processes observed in infected individuals (Drummer *et al*, 2006).

An additional HCV protein, the F protein, was recently suggested to result from a -2/+1 ribosomal frameshift during translation of the core protein coding sequence. The length of the F protein varies depending on the genotypes. It is found to be localized in the cytoplasm of infected cells (with notable perinuclear localization) and also associated with the endoplasmic reticulum (Xu *et al.*, 2003). The ribosomal frameshift site for the synthesis of the F protein is located in the A-rich sequence at codons 10 to 12 of the core protein sequence (Juan *et al*, 2005). Thus, the F protein and the core protein have the same amino-terminal sequence. Therefore, similarity in subcellular localization between the HCV F protein and HCV core protein raises the hypothesis that the F protein may also participate in HCV morphogenesis or replication (Xu *et al.*, 2003).

The p7 protein of HCV lies at the junction between the structural and non-structural regions of the virus polyprotein. It is small (63 amino acids) and highly hydrophobic in nature, largely comprising two predicted trans-membrane alpha helices, and has been shown to be an integral membrane protein (Carrere-Kremer *et al.*, 2002). Though it is not known whether it is a virion component, the function of p7 has been identified as an oligomeric ion channel, capable of mediating cation flow across artificial membranes. This finding confirms that HCV p7 belongs to an expanding family of viral proteins known as viroporins, which are small hydrophobic proteins encoded by a variety of RNA viruses that oligomerize to form cation channels most often involved in virus assembly or entry/exit, though many have additional functions (Griffin *et al*, 2004). Given that the p7 protein of the related pestivirus, bovine viral diarrhoea virus (BVDV),

has been shown to be essential for the production of infectious virus particles (Harada *et al*, 2000), it is likely that HCV p7 also plays an important role in the virus life-cycle.

Non-structural proteins NS3 and NS4a are involved in polyprotein cleavage and both have unique function and specificity in this process. NS3 has bifunctional properties, operating as both a serine protease (during polyprotein cleavage) and a RNA helicase during viral replication. The enzymatic portion of the protein is located at the N-terminus, and conforms to the typical tertiary structure of serine proteases. Evidence has shown the substrate-binding pocket to be unusually shallow, possibly explaining the method behind its recognition of several cellular substrates (Appel *et al*, 2005). In effect, HCV uses this enzyme to cleave adapter molecules involved in double stranded RNA signaling, thereby counteracting cellular antiviral defense pathways.

The short peptide NS4a serves multiple purposes, but mainly functions to enhance proteolytic activity of NS3 by 4 specific methods: first, it anchors the protease to intracellular membranes via the N-terminal transmembrane segment present in NS4a; second, it contributes one ß strand to the N-terminal protease domain and thereby allows it to complete folding; third, it stabilizes the protease against proteolytic degradation; forth, it activates protease activity by changing the geometry of the catalytic triad (Appel *et al*, 2005).

NS3 also functions as a helicase, essential for translation and replication of the HCV genome. Although its exact function has not been uncovered, it is believed to be involved in initiation of RNA replication by unwinding stable stem loop structures at the termini of positive and negative strand RNA (Appel *et al,* 2005). Approximately 400 amino acids of the c- terminus form the helicase enzyme that is capable of unwinding

RNA/RNA duplexes in an ATP dependent manner. Even though binding of RNA by monomeric NS3 can occur at high affinity, dimeric NS3 is required for RNA unwinding. The suggestion that NS3 protease and helicase function independently is still up for debate, though current evidence supports cross-talk between these domains: the isolated NS3 helicase fragment appears to show a slower kinetic of duplex RNA unwinding as compared with the full-length NS3 protein. Furthermore, evidence has suggested that NS4a acts as an RNA loading factor that greatly enhances productive RNA binding of a full length NS3/4a (Appel *et al*, 2005).

The functions of NS4b and NS5a, although poorly understood, are thought to play a role in viral replication through scaffold production and genome targeting respectively (Brass *et al*, 2006). NS4b is predicted to contain four transmembrane domains and an N-terminal targeting sequence, thought to guide it to intracellular membranes where it integrates and aids in HCV replication. NS5a does not contain transmembrane helices but nevertheless integrates into the membrane of the endoplasmic reticulum by interaction via its hydrophobic N-terminus. It contains a fully conserved polar side that may serve as a platform for the recruitment of proteins involved in RNA replication (Appel *et al*, 2005). NS5a is also found in phosphorylated (p56) and hyperphosphorylated (p58) forms and experimental evidence suggests these forms also play a distinct role in the virus replication cycle. It has been hypothesized that the HCV non-structural proteins form a complex located on the cytoplasmic membrane that catalyze viral genome replication. The replicon system has confirmed this, showing that this complex not only includes native NS5a (co-localized with viral RNA) but also its post translational form p56 (Macdonald and Harris, 2004).

Aside from its role in viral replication, an increasing body of evidence suggests that NS5A may also interfere with host cell functions. For example, NS5a is thought to be involved in the viral resistance to interferon therapy by binding and interacting with IFN-induced double stranded RNA activated PKR (Liang *et al,* 2006). It can also physically associate itself with p53, thereby repressing transcription of the cell cycle repressor gene p21WAF1, while upregulating PCNA expression (Majumder *et al.*, 2001). Recently, it was shown that NS5A was capable of perturbing mitogenic signaling pathways by interacting with Src-family kinases. These proteins regulate a multitude of signaling pathways, including the arrangement of the actin cytoskeleton, the response to growth factor stimulation and, importantly, the response to cytokine stimulation (Macdonald *et al,* 2004). Interestingly, the Src-family kinases are a common target for virus interference with cell signaling, particularly in the case of viruses that establish chronic infections (Collette and Olive, 1997). With respect to HCV, there is no exception; it has been found that NS5A, in the context of the other non-structural proteins, is able to differentially regulate the activity of endogenous Src-family kinases and perturb Src-family regulated signalling pathways. By varying the activity of each individual kinase, the role of NS5A may be to subvert and reroute the response of the cell to best suit HCV replication.

Finally, the NS5b protein of the HCV virus functions as an RNA dependent RNA polymerase and promotes synthesis of both negative stands as well as new RNA genomes. It too contains a hydrophobic region (at the c-terminus), used for integration into the viral replication complex at the cytoplasmic membrane (Lee *et al*, 2006). NS4b (a hydrophobic protein of unknown function) was reported to be a negative regulator of

the NS3-5b replication complex (Lee et al, 2006). NS5b is also required in the localization of NS5a to this "membranous web" (Macdonald and Harris, 2004). Besides its role as an RNA polymerase, NS5a is involved in numerous other cellular mechanisms.

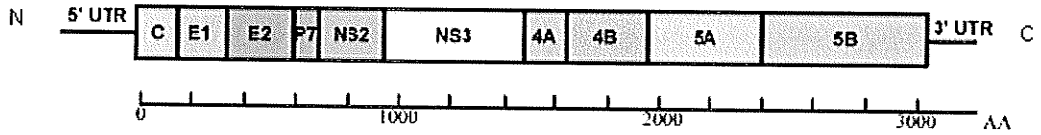

Figure 2: HCV-1 polyprotein organization from N to C-terminus (adapted from *The Springer Index of Viruses*, 2001). C – core; E1/E2 – envelope protein; p7 protein; NS2 – non-structural protein 2; NS3 – non-structural protein 3; 4A – non-structural protein 4a; 4B – non-structural protein 4b; 5A – non-structural protein 5a; 5B – non-structural protein 5b.

1.3 Hepatocellular Carcinoma and HCV

Hepatocellular carcinoma (HCC) is characterized by the imbalance of normal growth-promoting and growth-arresting signal transduction cascade reactions, the net result of which leads to uncontrolled hepatocyte growth (Feng *et al,* 2001). There are two mechanisms by which HCV infection could facilitate liver tumorgenesis. Primarily, an induction of chronic liver inflammation following cirrhosis promotes compensatory proliferation of quiescent hepatocytes by an upregulation of mitogenic pathways. Disruption of this regenesis by external factors such as alcohol or carcinogens targeting the liver could cause irreversible structural alterations in genes and chromosomes. As a result, cell survival and proliferation could occur even in the absence of liver damage causing heptocellular transformation.

Other hepatocellular carcinoma studies have focused on the potential interactions involving individual hepatitis C viral proteins and host cell proteins responsible for

regulating cell growth. Patients with chronic HCV infections have the entire HCV polyprotein expressed within infected hepatocytes (Siavoshian *et al.*, 2004). This therefore triggered considerable interest in the analysis of potential interactions between individual HCV proteins and host cell proteins regulating cell growth. Research to date suggests HCV proteins to be involved in a wide range of activities including signaling, transcriptional modulation, transformation, apoptosis, membrane rearrangements, vesicular trafficking and translational regulation (Levrero, 2006). Many studies involving core and E2 present solid evidence supporting these phenomena as well as their consequence to the host cell.

1.4 Hepatocellular Carcinoma and Core Protein

Expression of HCV core protein has been shown to induce cell proliferation DNA synthesis, and cell-cycle progression either alone or in the context of HCV replication, which is mediated by transcriptional upregulation of growth-related genes (Levrero, 2006). In numerous studies, core protein has been found to decrease the expression of the retinoblastoma protein (pRb). The functional inactivation of the pRb pathway appears to be a major event for multi-step cancer carcinogenesis, consequently resulting in rapid cell proliferation (Kato *et al*, 2000). Core has also been found to bind and therefore possibly inhibit p53. The original function of this tumor suppressor is prevention of the propagation of permanently damaged cells by the induction of apoptosis or growth arrest. Finally, p73 has been found to directly interact with HCV core protein resulting in the prevention of p73 dependent cell growth (Alisi *et al.*, 2003).

1.5 Hepatocellular Carcinoma and E2

The impact of other structural proteins on malignant hepatocyte transformation is more indirect. The E2 glycoprotein has been shown to suppress immunological eradication of virus infected cells on a number of levels: interference with interferon actions *in vitro* have been attributed to E2's inhibition of protein kinase R (PKR), an important intermediate of interferon effects (Levrero, 2006). In addition, E2 has also been found to interact specifically with the CD81 and LDLR surface markers on hepatocytes. Binding to CD81 averts destruction of infected cells by T and natural killer immune response. Binding to LDLR activates the MAPK/protein kinase (ERK) pathway, including the downstream transcription factor ATF-2, promoting cell proliferation and cell survival (Zhao *et al.*, 2005).

1.6 Hepatocellular Carcinoma and NS5a

Hepatitis C virus NS5a protein overexpression has been found to induce chromosome instability via mitotic cell cycle dysregulation. Therefore, many interactions between this viral encoded protein and host proteins are thought to block the apoptotic cellular response to persistent HCV infection, suggesting a potential function of NS5a in inducing chronic liver diseases and HCC associated with HCV infection. Interactions with members of the cell signaling apparatus, transcription activation machinery and cell cycle-regulatory kinases have all been described in literature involving such proteins as: growth factor receptor-bound protein 2, p53, p21WAF1 and various cyclins. The potential mechanism(s) by which NS5A is able to influence key cellular processes are largely unknown. However, one can speculate on the consequence of cell signaling interference by NS5a. One example involves the p53 tumor suppressor protein: NS5a

forms a heteromeric complex with TATA box binding protein (TBP) and p53 (Qadri *et al,* 2002) The p53 tumor suppressor gene serves as a checkpoint in maintaining genomic stability, as its function is impaired in the majority of human cancers. The induction of p21/waf1 is regulated through p53-dependent mechanisms, as p53 acts as a transcriptional activator to upregulate this gene, leading to p53-dependent G1 arrest. The presence of NS5a therefore is speculated to inhibit the binding of both p53 and TBP to each other, as well as to their DNA consensus binding sequence on the promoter of p21WAF1. As a result, suppression of p53-induced apoptosis causes the cell to undergo uncontrolled proliferation.

NS5A-mediated activation of Src-family members also exists (as described above), and its effect has been thought to promote immortality: the activation of Src-family member Fyn, for example, has been found to result in the activation of the transcription factor STAT3 which, when consistently expressed, can promote oncogenesis (Macdonald *et al*, 2004). NS5a has also been found to bind directly to the Src homology 3 domain of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). Stimulation of PI3K by NS5A causes increased phosphorylation and activity of Akt/protein kinase B, providing protection against apoptotic induction (Street *et al*, 2004). Correspondingly then, both Src related NS5a activations are thought to contribute to HCC in HCV infected individuals.

1.7 Hepatocellular Carcinoma and NS3

Recently, the potential of NS3 helicase/protease to elicit a malignant response has been minimally explored. Viral proteases are known to affect cellular metabolism. Without regulatory restrictions, NS3 has the ability to not only disrupt growth pathways

by direct protein-protein interaction, but also to degrade regulatory proteins, thereby influencing the initial stages of cellular transformation. Initial studies indicate that the HCV NS3 protease may elicit a malignant response in host cells however, detailed mechanisms are yet to be characterized. Investigation on the role of HCV NS3 in tumor development is critical to our understanding of HCV oncogenesis and promises a strong foundation for the prevention and therapy of HCV-induced hepatocellular carcinoma.

1.8 The Mammalian *c-fos* Oncogene

As previously elucidated, transformation of hepatocytes can result from a number of modifications within the cellular environment. Overexpression of oncogenes such as *c-fos*, an immediate early response gene involved in the control of growth and differentiation, has been implicated in the process of HCC (Idilman *et al.*, 1998). Cascades of intermolecular reactions mediate the pathway leading to its expression. At the genetic level however, transcription factors belonging to the Ets family dominate its fate.

E-26 transformation specific (Ets) factors are end effector molecules of multiple signal transduction pathways, which act as positive or negative regulators for the expression of genes involved in various biological processes. Among the *c-fos* gene, a multitude of cellular mechanisms are directed by Ets regulation including: control of cellular proliferation, differentiation, hematopoiesis, apoptosis, tissue remodeling, angiogenesis and transformation (Sementchenko and Watson, 2000; Watson and Seth, 2000; Watson *et al.*, 2001; Dittmer, 2003; Oikawa and Yamada, 2003).

Unregulated expression of the Ets gene contributes to malignant transformation and tumor progression. Aberrant expression of Ets factors has been associated with

varying grades of malignancy in several types of tumors including breast, lung and colorectal cancer (Oikawa, 2004). In addition, Ets genes were found to be located at translocation breakpoints, were chromosomally deleted, or were found to have altered expression patterns in leukemia and solid tumors (Hsu *et al.*, 2004). Therefore, the role of Ets in transformed liver cells is not only of relevance, but its relation to the *c-fos* gene expression coupled with hepatitis protein NS3 is of intense interest.

Ets factors are characterized by their highly conserved Ets domain (DNA binding domain), which conveys universal ability to bind the core DNA target 5'GGAA/T 3' *in vitro* (Hsu *et al.*, 2004). They are further subdivided into several subfamilies based on homology within the Ets domain. Additional sorting of Ets factors depends on protein structure and tissue distribution. Ternary complex factors (TCFs) are examples of one type of Ets subfamily and include such proteins as Elk-1 (Ets like protein 1), Sap-1 (serum response factor accessory protein-1) and Net (new Ets factor). As Ets family members, TCF proteins are targets of the Ras-MAPK pathway (Figure 3) and as such, have been adapted to regulate specific genes such as *c-fos*; this is achieved through interactions with other transcription factors at complex sites on DNA termed Ras-responsive elements (RRE). The end result of MAP kinase activation is the generation of a nucleoprotein complex on the promoter of the gene (Figure 4).

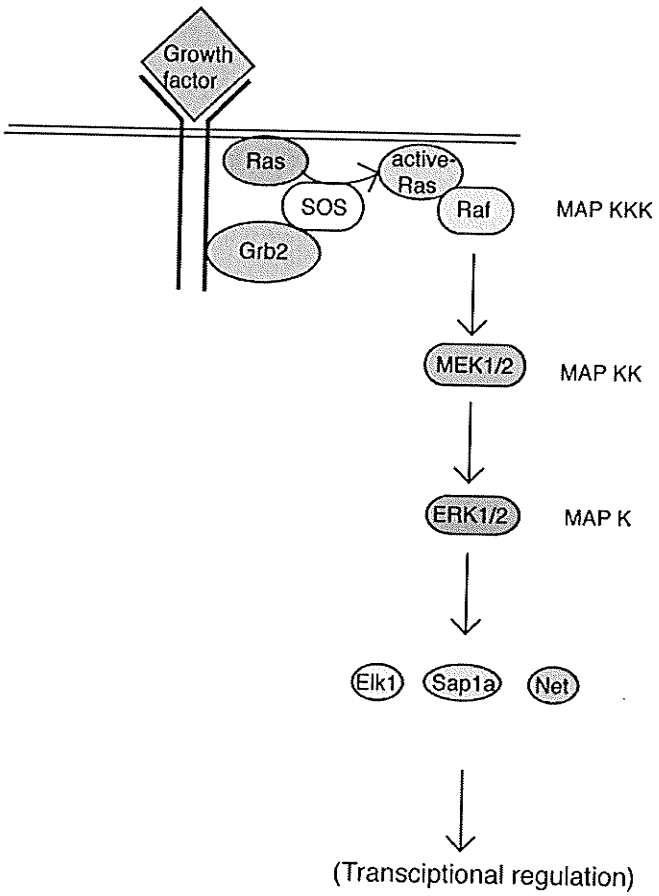

Figure 3: The growth factor ligand binds to its trans-membrane receptor and activates tyrosine kinases inherent in the receptor molecule (RTPK). A series of protein/protein complexes form involving Shc, GRB-2, and SOS. Subsequent to this, GDP-Ras is converted to GTP-Ras which activates Raf, MEK and then MAP kinase. Map kinases phosphorylate a number of transcription factors including TCFs (Elk, Sap1a and Net) (www.brc.riken.jp/lab/dna).

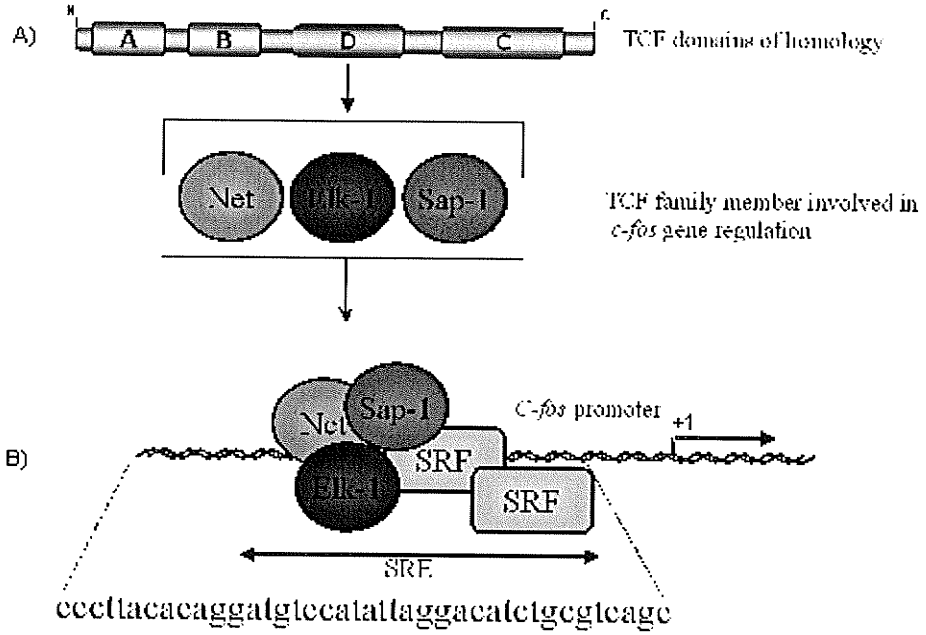

Figure 4a: Homology between TCF family members (such as Net, Elk-1 and Sap-1) is defined by four similar domains A-D: The N-terminal A domain corresponds to the universal Ets binding domain. The B domain interacts with a MADS family serum response factor (SRF). Phosphorylation by MAP kinase takes place at the D domain. The C domain, represents the activation site of the protein. 2b: how TCF factors are situated near the promoter of the c-fos gene and their interaction with SRF.

Homology between family members defines four similar domains A-D (Figure 4). The N-terminal A domain corresponds to the universal Ets binding domain, which enables TCFs to bind to a region near the promoter. The region responsible for TCF binding, near the *c-fos* promoter in particular, is known as the serum response element (SRE). The B domain interacts with a MADS family serum response factor (SRF), which exists as a dimer and allows for ternary complex formation. Phosphorylation by MAP kinase takes place at the D domain of the protein (MAP kinase docking site), which consequently stimulates the c-terminal C domain, representing the activation site of the protein (Buchwalter *et al.*, 2003).

Though many mechanisms are found to govern its transcription, TCF phosphorylation state is the factor that essentially controls the final outcome of *c-fos* expression. While the Ras-MAP kinase pathway is inactivated, *c-fos* expression only occurs at a basal level, due to binding of the ternary complex of Elk-1 and SRF. Complete repression of *c-fos* expression is achieved by the repressive activation of Net and the sumoylation of Elk-1, both leading to the recruitment of histone deacetylases (HDACs) on the promoter (Tootle and Rebay, 2005). When the cell is stimulated however, the Ras-MAP kinase pathway is activated and the Net factor, along with Elk-1 and incoming Sap-1 proteins, undergo phosphorylation. This molecular event causes a conformational change of these proteins and as a result, converts Net's activity from repressor to activator. In addition, intramolecular interactions between the Ets domain and the TAD (transcriptional activation domain) are altered in both Elk-1 and Sap-1, contributing to enhanced transcriptional activation. Thus, with the recruitment of histone acetylases (HATs) to the promoter, coupled with the cooperation of the SRF dimer, gene expression is activated.

Although the TCF family of Ets proteins predominately governs *c-fos* expression, Ets associated proteins (EAPs) have recently been identified. EAPs modulate Ets activities through various mechanisms such as blocking DNA binding, subnuclear sequestering, and inhibition of synergistic interaction with cofactors (Li *et al.*, 2000).

Various methods are possible for NS3 to disrupt the dynamics of *c-fos* oncogene regulation, including the proteolytic cleavage of inhibitors or the un-twisting of secondary structures in mRNA. This prompts curiosity as to whether or not this viral

protein can influence hepatocytes by upregulating growth genes and ultimately causing uncontrolled cellular proliferation.

1.9 Mammalian NFκB, SRC and Cancer

NFκB is a ubiquitously expressed, highly inducible transcription factor that plays an important role in the hepatic acute-phase response, innate and adaptive immunity, and cellular survival through the induction of genetic networks (Fan *et al*, 2005).

Classical NFκB is heterodimer composed of two subunits, usually p65 (RelA) and the p105 precursor p50 (NF-κB1). The C-terminal region of p65, contains a transactivation domain. In non-stimulated hepatocytes, NFκB is sequestered in the cytoplasm by a family of specific inhibitory proteins termed IκBs whose members bind and specifically deactivate Rel A by masking its nuclear-localization sequence and preventing nuclear entry. In response to viral infection, DNA damage, pro-inflammatory cytokines, or partial hepatectomy, the Iκκ complex (composed of α,β, and γ subunits), promotes NFκB activation through phosphorylation-induced ubiquitination of IκB-α (a subunit of IκB), thereby targeting this molecule for proteolysis in the 26S proteasome (Hoffmann *et al*, 2002). Additional levels of control for NFκB activity include the phosphorylation status of the various NFκB/Rel and Iκκ subunits and their selective interaction with co-activator proteins (Lipniackia *et al*, 2004).

In contrast to typical NFκB regulation involving p50 and RelA subunits, an alternative pathway exists involving the p100-RelB NFκB complex. This route is activated by phosphorylation of the C-terminal region of p100 by an IKKα homodimer (lacking IKKγ), which leads to ubiquitination followed by degradation of the p100 IkB-like C-terminal sequences to generate p52-RelB (alternate form of NKkB). In either

pathway, the unmasked NF-kB complex can then enter the nucleus to activate target gene expression. In the classical pathway, one of the target genes activated by NFkB is that which encodes IkBα. Newly-synthesized IkBα can enter the nucleus, remove NF-kB from DNA, and export the complex back to the cytoplasm to restore the original latent state. Thus, the activation of the NF-kB pathway is generally a transient process, lasting from 30-60 minutes in most cells

NFkB has been shown to play a role in the transactivation of over 150 genes. These genes are involved in the regulation of fundamental processes such as the immune response, cell adhesion, physical stress (e.g. liver regeneration), oxidative stress, and cell survival. In certain cell types and in response to certain stimuli, NFkB has also been shown to transactivate pro-apoptotic genes.

The mechanism by which NFkB contributes to neoplastic transformation is partly due to its protective role against apoptosis through transcriptional upregulation of anti-apoptotic target genes (Arsura and Cavin, 2005). NFkB pro-survival target genes include: members of the Bcl-2 family of genes (Bcl-xL, Bfl1/A1 and Nr13) as well as the cellular inhibitors of apoptosis (H-IAP1, H-IAP2, and XIAP) (Perkins, 2006). It is important to note that IkB-α has also been implicated in the regulation of NFkB activity during oncogenic transformation of liver cells (Thompson, 1995).

Studies have implicated members of the NFkB/Rel family in both HBV and HCV induced neoplastic development of the liver. As stated above, the mechanism by which HCV infection facilitates liver tumorgenesis is thought to be the result of induction of chronic liver inflammation that is often followed by cirrhosis or cellular transformation induced by viral particles. With respect to HCV mediated activation of NFkB by direct

protein interaction, two distinct mechanisms have been reported. The HCV structural core protein, when transfected in human HCCs, has been found to bind to TNFR1, subsequently activating NFkB by inhibiting both TNF-α and FasL-induced apoptosis (Vlahopoulos *et al*, 1999). The nonstructural protein 5A has also been found to activate NFκB by inducing endoplasmic reticulum stress, triggering ZAP-70-mediated tyrosine phosphorylation of IκB-α. It would be logical than to investigate possible upregulation of this gene by the presence of HCV NS3.

Transcriptional activity of NFκB, specifically p65, is regulated by transcriptional coactivators and corepressors. The coactivators of p65 include P300/CBP, p/CAF, and p160 proteins (SRC-1, SRC-2 and SRC-3) (Gao *et al*, 2005). These coactivators form an enhanceosome (nucleoprotein complex) that assembles on an inducible enhancer sequence in the regulatory region of the p65 promoter. Both NFkB subunits are needed to complete the enhanceosome, defining a portion of NFkB's autoregulatory effects. CBP/p300 and the p160 proteins both possess intrinsic histone acetylase (HAT) activity, which is necessary to open the chromatin structure through an acetylation-induced conformational change in histone protein. However, the function of p160 protein is dependent upon CBP/p300, as p160 exhibits much less activity in the absence of CBP/p300. The presence of the chromatin-remodeling factors induce formation of an activated preinitiation complex that, in turn, controls DNA-dependent RNA polymerase II activity and subsequent gene expression.

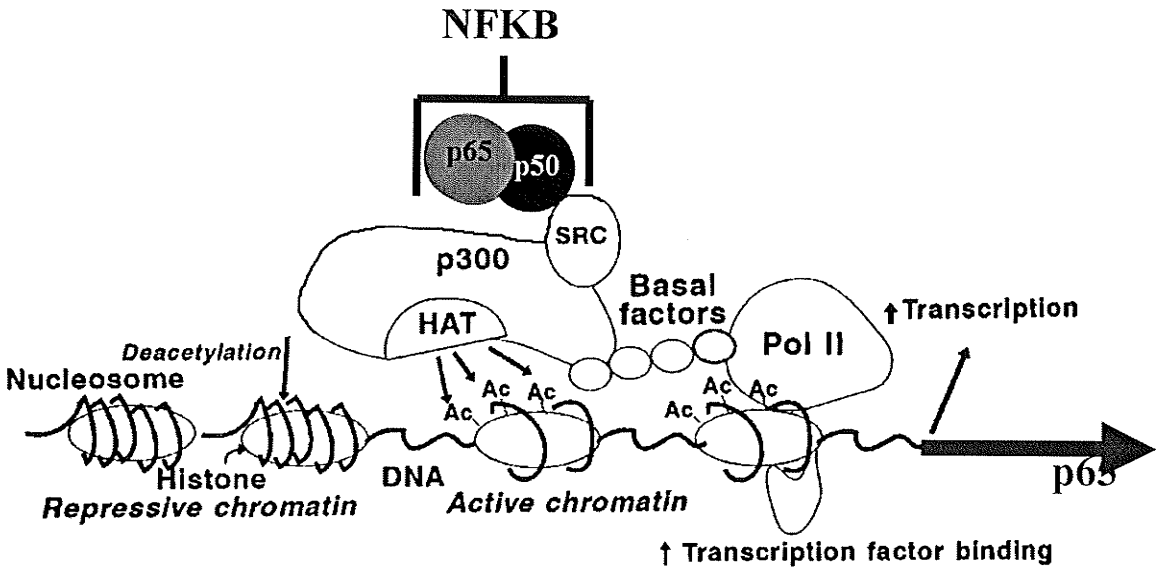

Figure 5 : p65 enhanceosome depicting the autoregulation of NFkB by the control of histone acetyl transferase coactivaors on the p65 promoter (www.clinsci.org/cs/094).

Since autoregulation is recognized as an important controller of network stability, focus on the HCV NS3 protein and its effects on this enhanceosome complex could serve to partly explain the correlation between HCV and liver cancer.

1.10 Hypothesis

HCV-induced hepatocellular carcinoma is caused by the activation of specific tumorgenesis pathways induced by viral interference. In particular, intracellular interactions between viral NS3 and host proteins induce modification within the cellular environment, particularly at the level of host signal transduction. Exploration of these oncogenic interactions will help further our understanding of how HCV influences cell functions, potentially leading to the development of hepatocellular carcinoma.

1.11 Thesis Objectives

1. Through exploration of published findings, identify host signaling pathways that have been found to induce tumorgenesis when altered.
2. Transfect liver cell line with viral NS3 protein and analyze changes in the expression of genes linked to the pathway in question.
3. Map the method by which NS3 interferes with the host-signaling pathway by identifying specific host proteins and/or viral protein domains/motifs responsible for alteration of host gene expression.

2. Material and Methods

2.1 Construction of NS3 Sequential Deletion Mutants

The wild-type HCV NS3 gene (obtained from strain H77-PH21) was cloned into pcDNA 3.1 (-) (Invitrogen Life Technologies, Valentia, CA). PCR amplification was performed using iProof High-Fidelity PCR Kit (Bio-Rad Laboratories, Hercules, CA) on H77-PH21 to retrieve the NS3 amplicon. The primers (Operon, Huntsville, AL) used contained flanking EcoR1/BamH1 restriction sites:

5'GTACGAATTCATGGCGCCCATCACGG 3'

5' GTACGGATCCTCATTACGTGACGACCTCCAGGTC 3'

The resulting amplicon was cleaned using "Freeze N' Squeeze" spin columns (Bio-Rad Laboratories, Hercules, CA), and digested with appropriate restriction enzymes (New England Biolabs, Ipswich, MA) at 37 ˚C for 30min. The digested amplicon was cleaned again and ligated into pcDNA 3.1 (-). Ligation time was overnight at 16˚C. Ligation product was chemically transformed into Top 10 *E. coli* cells (Invitrogen Life Technologies, Valentia, CA). Briefly, 5μl ligation product was added to cells, incubated for 30min. on ice and placed in a 42˚C water bath for 45 sec. The cell solution was then cooled on ice for 2min. 900μl of LB broth was added and the cell solution was aliquoted on 100μg/ml ampicillin selective LB agar plates. Incubation was 37˚C overnight. Colony screenings were done by PCR amplification using Hot StarTaq Master Mix (Qiagen, Valencia. CA). A randomly chosen positive colony was cultured in LB + 100μg/ml ampicillin selective broth and purified with Endo-free Maxi-prep kit (Qiagen, Valencia, CA).

PCR amplification was performed using iProof High-Fidelity PCR Kit on portions of the NS3 insert to generate C-terminal truncated versions and an N-terminal truncated version of wt NS3 (Figure 6).

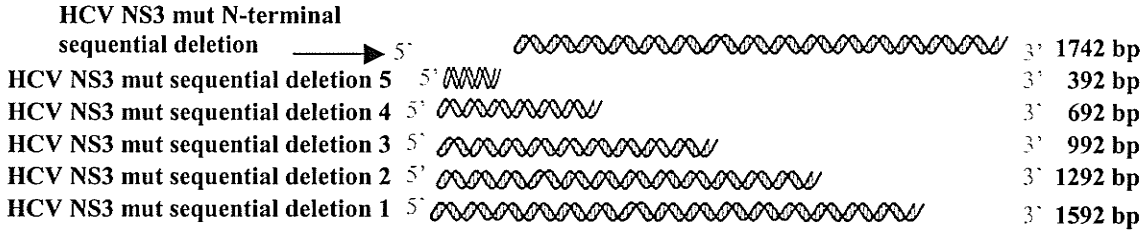

Figure 6: Sequential deletion mutants generated by PCR amplification using internal N/C-terminal primers.

Primers used in this procedure contained flanking restriction enzyme sites EcoR1 and Bam H1. The N-terminal sequential deletion mutant was generated using the primers:

5' GTACGAATTCATGGTATGCTGGACTGTCTAC 3'

5' GTACGGATCCTCATTACGTGACGACCTCCAGGTC 3'

The C-terminal sequential deletion mutants were generated using the following primers:

Table 1: Primer sequences used to construct NS3 c-terminal sequential deletion mutants (reverse primers listed from sequential deletion 1 to 5).

5' GTACGAATTCATGGCGCCCATCACGG 3' (forward)
5' GTACGGATCCTCATTAAACTGTAGTCTCGGCGGGCGTGAG 3' (reverse)
5' GTACGGATCCTCATTGTAGGCCACGGCATTGATG 3' (reverse)
5' GTACGGATCCTCATTGCCGTAGGTGGAGTACGTGATGGGG 3' (reverse)
5' GTACGGATCCTCATTCACGGCGTGTCCCGCGGGGCACAAC 3' (reverse)
5' GTACGGATCCTCATTACCCTCCACTTGGTTTTTGTCC 3' (reverse)

The resulting sequentially deleted NS3 amplicons were cleaned using Freeze N' Squeeze spin columns, digested with appropriate restriction enzymes and cleaned again.

Digested amplicons were ligated into pcDNA 3.1 (-). Ligation time was overnight at 16°C. Ligation products were chemically transformed into Top 10 *E. coli* cells as described above. Colony screenings were done by PCR amplification using Hot StarTaq Master Mix. A randomly chosen positive colony was cultured in LB + 100μg/ml ampicillin selective broth and purified with Endo-free Maxi-prep kit.

2.2 Transfection of Huh 7.0 Liver Cells to Measure *c-fos* Activation by HCV NS3

Effectene Transfection Reagent (Qiagen, Valencia, CA) was used in conjunction with Enhancer and DNA-condensation Buffer. In the first step of Effectene–DNA complex formation, the DNA is condensed by interaction with the Enhancer in a defined buffer system. Effectene Reagent is then added to the condensed DNA to spontaneously produce micelle structures of condensed Effectene–DNA complexes. The Effectene–DNA complexes are mixed with medium and directly added to the cells.

Huh 7.0 cell line (transformed human hepatocytes) was used to study the influence of HCV NS3 on *c-fos* regulation. Cells were seeded in the amount of 125,000/well (12 well plate) and incubated at 37°C overnight prior to transfection (to achieve ~40-60% confluency). Effectene transfection reagent was used to co-transfect Huh 7.0 cells with 0.4μg pc-fos-FA-CMV and 0.2μg pFR-Luc with 0.4μg NS3pcDNA3.1(-). A co-transfection of 0.4μg pc-fos-FA-CMV and 0.2μg pFR-Luc with 0.4μg pcDNA3.1(-) was used as a baseline control.

Briefly, appropriate volumes of each sample were added to 100μl EC buffer and 8μl enhancer in a microcentrifuge tube. Samples were vortexed, spun at maximum speed in a microcentrifuge for 5 sec. and left to incubate at room temperature for 5 min. Following incubation, 13μl of effectene was added to each sample. The samples were

again vortexed, spun at maximum speed in a microcentrifuge for 5 sec. and left to incubate at room temperature for 10 min. Before addition of sample solutions to the 12 well plate, DMEM media was removed from each well and the monolayers were washed with PBS pH 7.4. 1ml of fresh DMEM (incubated at 37°C) was added before sample solutions were added to each well. To ensure even dispersal of samples, the sample volumes were dropped evenly around the area of the well. The plate was then gently shaked vertically and horizontally before incubation for 30 hours at 37°C.

2.3 Measurement of *c-fos* Activation:

PathDetection *In vivo* Signal Transduction Pathway *Trans*-reporting System (Stratagene, La Jolla, CA) was used to measure hepatocyte *c-fos* gene expression in the presence of viral NS3. This transreporting system is designed for **specific** assessment of the *in vivo* activation of transcription activators and upstream signal transduction pathways.

The PathDetect *trans*-reporting system utilizes 3 plasmids. Primarily, the fusion *trans*activator plasmid (pFA-CMV) is used as a mammalian expression vector that expresses a fusion protein (see Figure 7). The fusion *trans*-activator protein consists of the activation domain of *c-fos* fused with the yeast GAL4 DNA binding domain. This plasmid also contains the human cytomegalovirus (CMV) immediate early promoter to drive the constitutive expression of the *trans*-activator protein in the Huh 7.0 cell line. Selection in bacteria is made possible by the kanamycin-resistance gene, which is under control of the prokaryotic β-lactamase promoter.

Secondarily, the plasmid, pFR-Luc acts as the reporter component in the assay. It contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites

that control expression of the *Photinus pyralis* (American firefly) luciferase gene. The DNA binding domain of the fusion *trans*-activator protein (on pFA-CMV) binds to the reporter plasmid at the GAL4 binding sites. Therefore, when the *c-fos* fusion *trans*-activator plasmid, luciferase reporter plasmid, and viral NS3 gene (3^{rd} plasmid) are cotransfected into mammalian cells, expression of the *c-fos* gene (represented by exogenous stimulation of its activation domain) is positively correlated with the amount of chemiluminescent luciferase produced. Luciferase catalyzes the following chemiluminescent oxidation–reduction reaction: luciferase substrate (luciferin) + ATP + O2 → oxyluciferin + light (560 nm) + AMP + PPi + CO2.

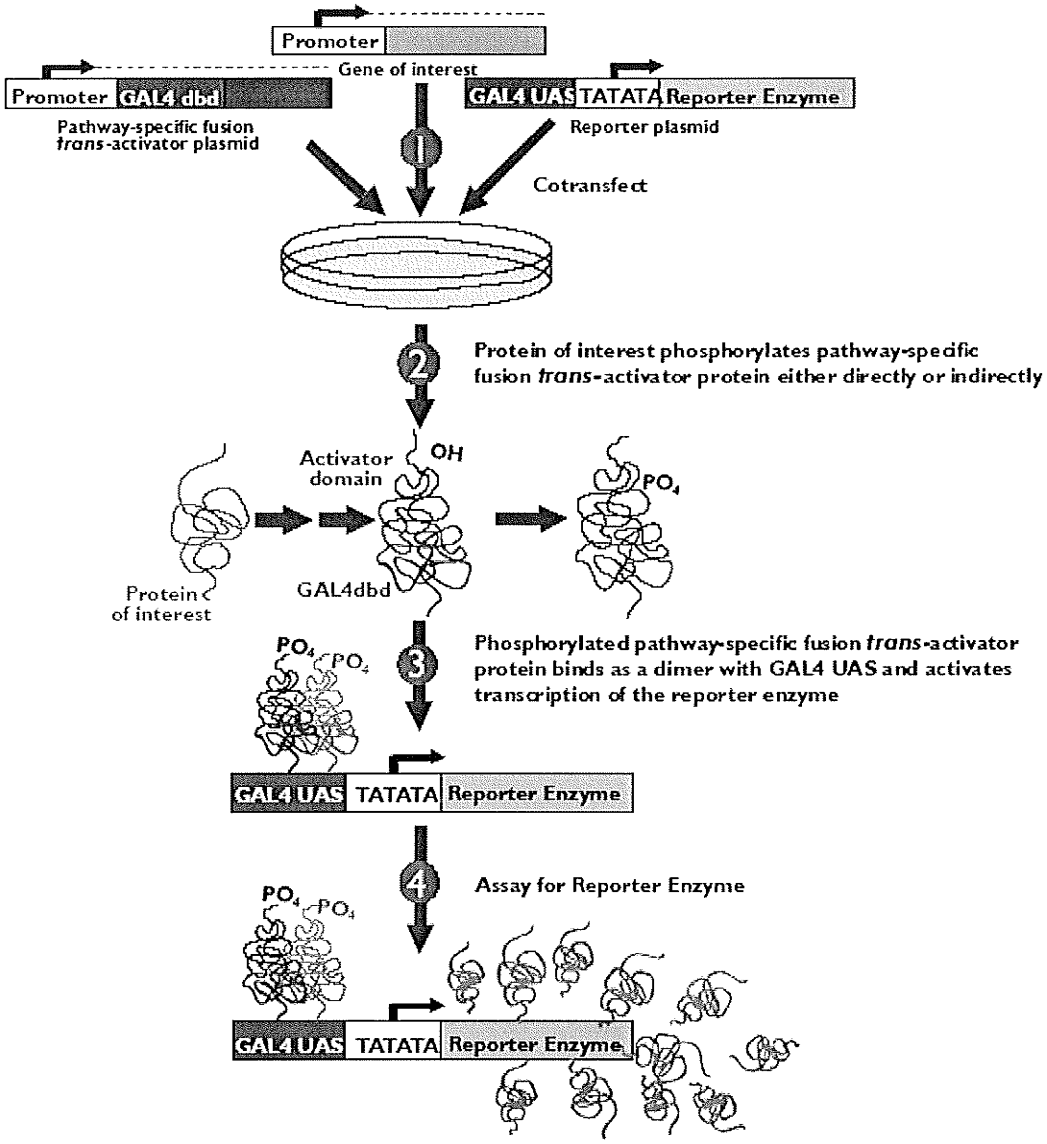

Figure 7: The PathDetect *in vivo* signal transduction pathway *trans*-reporting system (www.stratagene.com/manuals/).

Post 30 hr. cell incubation, DMEM media was removed from the wells and the cells were carefully washed with 1 ml of PBS buffer. After the full removal of PBS, 400 μl of 1× cell lysis buffer was added to the wells. The cells were then scraped from the wells and the solutions from each well were put into separate microcentrifuge tubes. The tubes were rocked at room temperature for 15 min. and spun down at maximum speed using a microcentrifuge. 20 μl of the cell extract was then mixed with 80 μl of room temperature luciferase assay reagent in a Falcon 2054 polypropylene tube. Light emitted from the reaction was immediately measured with a TD-20/20 luminometer (Sunnyvale, CA) at 0 and 30 minutes.

2.4 Transfection of Huh 7.0 Liver Cells to Measure SRE Activation by HCV NS3

Huh 7.0 cell line (transformed hepatocytes) was used to study the influence of HCV NS3 on SRE (serum response element) regulation. Cells were seeded in the amount of 125,000/well (12 well plate) and incubated at 37°C overnight prior to transfection (to achieve ~40-60% confluency). Effectene transfection reagent (Qiagen, Valencia, CA) was used to co-transfect Huh 7.0 cells with 0.9μg NS3pcDNA 3.1(-) and 0.1μg pSRE-SEAP2. A co-transfection of 0.9μg pcDNA 3.1 (-) and 0.1μg pSRE-SEAP2 was used as a baseline control. A transfection with 0.1μg commercial pSEAP2 positive control was also performed in parallel to ensure optimal kit performance. Cells were transfected following manufacture's protocol as described above. The transfected plate was incubated at 37°C for 30h before analysis.

2.5 Transfection of Huh 7.0 Liver Cells to Measure SRE Activation by HCV NS3 C-terminal sequential deletion mutants.

Huh 7.0 cell line (transformed hepatocytes) was used to study the influence of HCV NS3 C-terminal sequential deletion mutants on SRE (serum response element) regulation. Cells were seeded in the amount of 125,000/well (12 well plate) and incubated at 37°C overnight prior to transfection (to achieve ~40-60% confluency). Effectene transfection reagent (Qiagen, Valencia, CA) was used to co-transfect Huh 7.0 cells with 0.9μg NS3pcDNA 3.1(-)/NS3Cdel(1-5)pcDNA 3.1(-) and 0.1μg pSRE-SEAP. A co-transfection of 0.9μg pcDNA 3.1 (-) and 0.1μg pSRE-SEAP was used as a baseline control. A transfection with 0.1μg commercial pSEAP2 positive control was also performed in parallel to ensure optimal kit performance. pSRE-SEAP co-transfected with HCV E pcDNA 3.1(-) was used as a negative control. Cells were transfected following manufacture's protocol as described above. The transfected plate was incubated at 37°C for 30h before analysis.

2.6 Measurement of SRE Activation:

The Great EscAPE SEAP Reporter Assay System (Clontech, Mountain View, CA) was used to measure potential SRE activation by viral NS3 in Huh 7.0 cells. In this *cis*-reporting system, activation of the SEAP reporter gene, rather than a cellular gene, is used to indicate activation status. The pSRE-SEAP plasmid is designed for monitoring the induction of the serum response element and the mitogen-activated protein kinase signal transduction pathway. The unique feature of a *cis* system is that it uses *cis*-acting enhancer elements on its reporter plasmid (pSRE-SEAP) which, unlike a *trans* reporter system, usually binds more than one transcriptional factor and, hence, can interact with a

wide spectrum of signals. Thus, pSRE-SEAP contains the secreted alkaline phosphatase (SEAP) reporter gene along with three tandem copies of the SRE consensus sequence fused to a TATA-like promoter (PTAL) region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter. After transcription factors bind SRE, transcription is induced and the reporter gene is activated.

The SEAP coding sequence is followed by the SV40 late polyadenylation signal to ensure proper, efficient processing of the SEAP transcript in eukaryotic cells. Located upstream of SRE is a synthetic transcription blocker, which is composed of adjacent polyadenylation and transcription pause sites for reducing background transcription. The vector backbone also contains an f1 origin for single-stranded DNA production, a pUC origin of replication, and an ampicillin resistance gene for propagation and selection in *E. coli*.

Post transfection of Huh 7.0 cells with pSRE-SEAP/pSEAP2 (positive control) and NS3, CSPD (a chemiluminescent substrate) was used to monitor expression of the SEAP reporter gene by using the secreted phosphatase activity to produce a quantitative luminescent product. The chemiluminescent signal generally remains constant up to 40 min after the addition of the substrate solution. The detected changes in secreted SEAP activity levels are directly proportional to changes in intracellular concentrations of SEAP protein and thus, SRE activation.

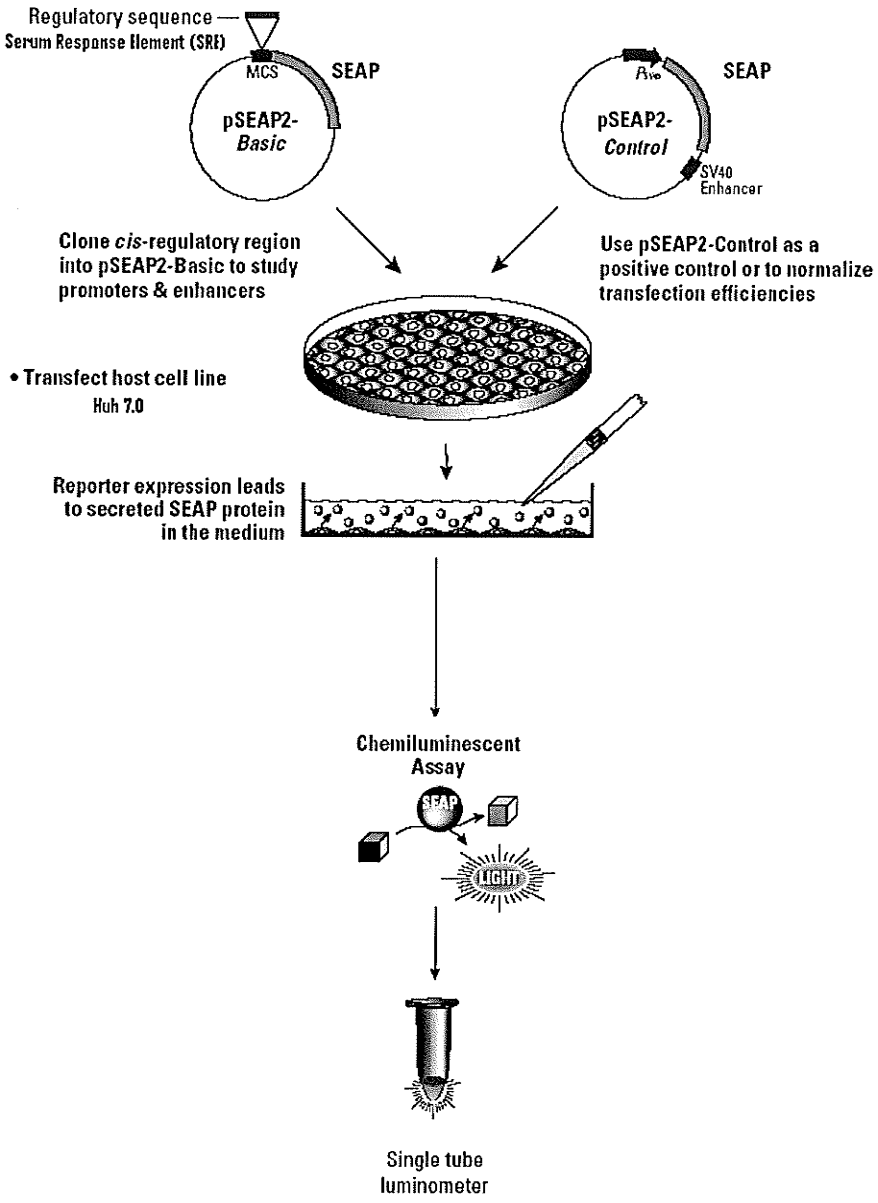

Figure 8: Flowchart of the Great EscAPE SEAP Assay procedure.

30h post transfection of viral NS3 with pSEAP-SRE/pSEAP-control, 110 µl of cell culture media was transferred to a microcentrifuge tube and centrifuged at 12,000 x g for 10 sec (to pellet any detached cells present in the culture medium). The required amount of 1X Dilution Buffer for the entire assay was prepared by diluting a sufficient volume of the provided 5X stock solution 1:5 with ddH20 and allowing it to equilibrate to room temperature. 15 µl of each cell media sample was aliquoted into a 96-well plate. 45 µl of 1X Dilution Buffer was added to each 25 µl sample and mixed gently by pipetting up and down. Diluted samples were then incubated for 30 min at 65°C using a thermocycler. Samples were equilibrated to room temperature by being placed on ice for 2–3 min. 60 µl of Assay Buffer was then added to each sample and incubated for 5 min at room temperature. 1.25 mM CSPD substrate was prepared by diluting 25 mM stock solution 1:20 with Chemiluminescent Enhancer (equilibrated to room temperature). 60 µl of the diluted CSPD Substrate was then added to each sample and incubated for 10 min at room temperature. The solutions were transferred to appropriate luminometer tubes and signals were recorded at 0 and 30min intervals using a TD-20/20 luminometer (Sunnyvale, CA). For multiple readings (sequential deletion mutants), samples were transferred to a 96 well white plate (type brand) and measured with a micro plate reader (Tecan, Switzerland).

2.7 Transfection of Huh 7.0 Liver Cells to Measure NFκB Activation by HCV NS3

Huh 7.0 cell line (transformed hepatocytes) was used to study the influence of HCV NS3 on NFκB regulation. Cells were seeded in the amount of 125,000/well (12 well plate) and incubated at 37°C overnight prior to transfection (to achieve ~40-60% confluency). Effectene transfection reagent (Qiagen, Valencia, CA) was used to co-

transfect Huh 7.0 cells with 0.9μg NS3pcDNA 3.1(-) and 0.1μg pNFκB-SEAP. A co-transfection of 0.9μg pcDNA 3.1 (-) and 0.1μg pNFκB-SEAP was used as a baseline control. A transfection with 0.1μg commercial pSEAP2 positive control was also performed in parallel to ensure optimal kit performance. Cells were transfected following manufacture's protocol as described above. The transfected plate was incubated at 37°C for 30h before analysis.

2.8 Measurement of NFκB Activation:

Great EscAPE SEAP Reporter Assay System was also used to measure potential NFκB activation by viral NS3 in Huh 7.0 cells. pNFκB-SEAP contains the SEAP reporter gene along with four tandem copies of the NFκB consensus sequence fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter. After endogenous NFκB proteins bind to the kappa (κ) enhancer element, transcription is induced and the reporter gene is activated. Like pSRE-SEAP, the pNFκB-SEAP vector also contains: the SV40 late polyadenylation signal; a synthetic transcription blocker (TB); an f1 origin; a pUC origin of replication and an ampicillin resistance gene.

30h post transfection of viral NS3 with NFκB-pSEAP/pSEAP-control a SEAP reporter assay was performed as described in section 2.6.

2.9 Construction of NS3/SRC-1 mammalian two hybrid plasmids:

The wild-type HCV NS3 gene (obtained from NS3-pCDNA 3.1(-)) was sub-cloned into pVP16 (Clontech, Mountain View, CA). NS3-pcDNA 3.1 (-) was digested with EcoR1 and BamH1 restriction enzymes from New England Biolabs (Ipswich, MA), ligated into pVP16 and chemically transformed into Top 10 *E. coli* cells (Invitrogen Life Technologies, Valentia, CA) as described above. Colony screenings were done by PCR

amplification using Hot StarTaq Master Mix (Qiagen, Valencia. CA). A randomly chosen positive colony was cultured in LB + 100ug/ml ampicillin selective broth and purified with Endo-free Maxi-prep kit (Qiagen, Valencia, CA).

The wild-type SRC gene (obtained from colon cancer cDNA) was cloned into pM (Clontech, Mountain View, CA). The primers used contained flanking Mlu1/Xba1 restriction sites:

5'GTACACGCGTATGTAGTGGAAAATATGCTGTGAA 3'

5' GTACTCTAGATCAATACGGGTCGGTC 3'

PCR amplification was performed using iProof High-Fidelity PCR Kit (Bio-Rad Laboratories, Hercules, CA). The resulting amplicon was digested with appropriate restriction enzymes from New England Biolabs (Ipswich, MA), ligated into pM and chemically transformed into Top 10 *E. coli* cells (Invitrogen Life Technologies, Valentia, CA) as described above. Colony screenings were done by PCR amplification using Hot StarTaq Master Mix (Qiagen, Valencia. CA). A randomly chosen positive colony was cultured in LB + 100 μg/ml ampicillin selective broth and purified with Endo-free Maxi-prep kit (Qiagen, Valencia, CA).

2.10 Transfection of Huh 7.0 Liver Cells to Measure SRC-1 interaction with HCV NS3

Huh 7.0 cell line was used to study the interaction of SRC-1 (steroid receptor coactivator-1) with HCV NS3. Cells were seeded in the amount of 125,000/well (12 well plate) and incubated at 37°C overnight prior to transfection (to achieve ~40-60% confluency). Effectene transfection reagent (Qiagen, Valencia, CA) was used to co-transfect Huh 7.0 cells with 0.4μg NS3pVP16, 0.4μg SRCpM and 0.2μg pG5-SEAP. A

co-transfection of 0.4μg pVP16, 0.4μg SRCpM and 0.2μg pG5-SEAP was used as a baseline control. A co-transfection of 0.4μg HCV XpVP16, 0.4μg SRCpM and 0.2μg pG5-SEAP was used as a positive control. A transfection with 0.1μg commercial pSEAP2 positive control was also performed in parallel to ensure optimal kit performance. Cells were transfected following manufacture's protocol as described above. The transfected plate was incubated at 37°C for 30h before analysis.

2.11 Measurement of SRC-1/NS3 Interaction:

The Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech, Mountain View, CA) was used to perform two-hybrid assay of the steroid receptor coactivator-1 and viral NS3 in Huh 7.0 cells. Two-hybrid assays are based on the fact that many eukaryotic transcriptional activators consist of two physically and functionally separable domains: a DNA-binding domain that specifically binds to a promoter and an activation domain that directs RNA polymerase II to transcribe the gene downstream of the DNA-binding site. In this assay, the 2 domains function as two separate proteins found on the pM and pVP16 commercial plasmids. Two proteins of interest (X and Y) are cloned into these plasmids and as a result, are expressed as protein fusions to the activation and DNA-binding peptides, respectively. In this assay, the pM cloning vector was used to generate a fusion of SRC-1 to the GAL4 DNA-binding domain. Similarly, the pVP16 vector was used to construct a fusion of HCV NS3 to an activation domain derived from the VP16 protein of herpes simplex virus. pG5-SEAP acted as the reporter vector, containing the SEAP gene downstream of five consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. Therefore, if the proteins come in close proximity and interact, transcription of the SEAP reporter gene is activated. The potential interaction

between the two proteins of interest is measured using the standard SEAP assay. The commercial positive control compares the interaction between the p53 protein and the SV40 large T-antigen.

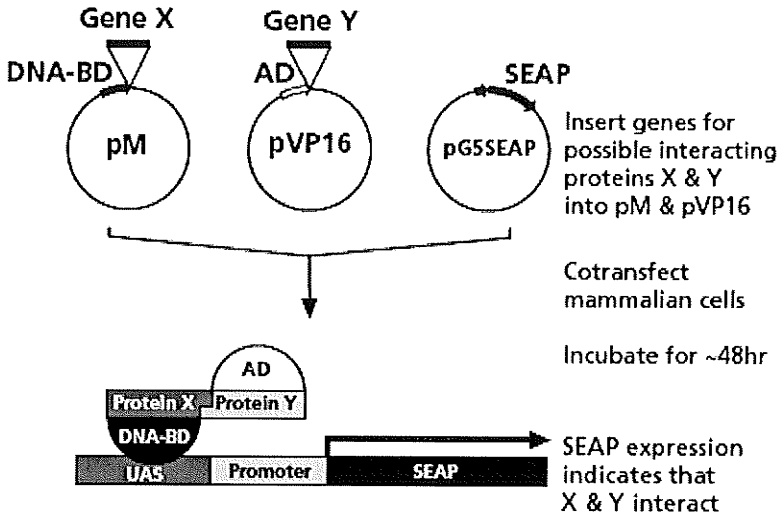

Figure 9: The Matchmaker™ Two-Hybrid Assay Kit 2 procedure. X=SRC, Y=NS3

30h post transfection of HCV NS3pVP16 with SRCpM and pG5-SEAP, 110 µl of cell culture media was transferred to a microcentrifuge tube and centrifuged at 12,000 x g for 10 sec (to pellet any detached cells present in the culture medium). The required amount of 1X Dilution Buffer for the entire assay was prepared by diluting a sufficient volume of the provided 5X stock solution 1:5 with ddH20 and allowing it to equilibrate to room temperature. 15 µl of each cell media sample was aliquoted into a 96-well plate. 45 µl of 1X Dilution Buffer was added to each 25 µl sample and mixed gently by pipetting up and down. Diluted samples were then incubated for 30 min at 65°C using a thermocycler. Samples were equilibrated to room temperature by being placed on ice for 2–3 min. 60 µl of Assay Buffer was then added to each sample and incubated for 5 min at

room temperature. 1.25 mM CSPD Substrate was prepared by diluting 25 mM stock solution 1:20 with Chemiluminescent Enhancer (equilibrated to room temperature). 60 µl of the diluted CSPD Substrate was then added to each sample and incubated for 10 min at room temperature. The solutions were transferred to appropriate luminometer tubes and signals were recorded at 0 and 30min intervals using a TD-20/20 luminometer (Sunnyvale, CA).

2.12 Construction of NS3-HASA mammalian 2 hybrid plasmid:

The NS3 HASA mutant (generated by Mike Carperter's lab) has two amino acid substitutions, unconventionally knocking out the protease function of NS3. Experiments have suggested it to have increased stability compared to the wild type. A mammalian two hybrid plasmid containing HASA was therefore constructed and compared with that of the wild type when exposed to SRC-1 *in vivo*.

The NS3-HASA gene (obtained from NS3-HASA-pCDNA 5) was sub-cloned into pVP16 (Clontech, Mountain View, CA). NS3-HASA-pcDNA 5 was digested with EcoR1 and BamH1 restriction enzymes from New England Biolabs (Ipswich, MA), ligated into pVP16 and chemically transformed into Top 10 *E. coli* cells (Invitrogen Life Technologies, Valentia, CA) as described above. Colony screenings were done by PCR amplification using Hot StarTaq Master Mix (Qiagen, Valencia. CA). A randomly chosen positive colony was cultured in LB + 100µg/ml ampicillin selective broth and purified with Endo-free Maxi-prep kit (Qiagen, Valencia, CA).

2.13 Transfection of Huh 7.0 Liver Cells to Measure NS3/NS3-HASA stability

To analyze HCV NS3 stability in Huh 7.0 cell line, NS3pVP16 and NS3-HASApVP16 were cotransfected in parallel with SRCpM. Huh 7.0 cell line was used to

study the interaction of SRC-1 with HCV NS3/NS3-HASA. Cells were seeded in the amount of 125,000/well (12 well plate) and incubated at 37°C overnight prior to transfection (to achieve ~40-60% confluency). Effectene transfection reagent (Qiagen, Valencia, CA) was used to transfect Huh 7.0 cells with 0.4μg NS3pVP16/NS3-HASApVP16, 0.4μg SRCpM and 0.2ug pG5-SEAP. A transfection of 0.4μg pVP16, 0.4μg SRCpM and 0.2μg pG5-SEAP was used as a baseline control. A transfection with 0.1μg commercial pSEAP2 positive control was also performed in parallel to ensure optimal kit performance. Cells were transfected following manufacture's protocol as described above. The transfected plate was incubated at 37°C for 18h, 24h, 30h and 48h before analysis.

2.14 Measurement of NS3/NS3-HASA stability in Huh 7.0 cells:

The Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech, Mountain View, CA) was used to perform two-hybrid assay of the steroid receptor coactivator-1 and HCV NS3/NS3-HASA to compare NS3/NS3-HASA in Huh 7.0 cells; the HCV genes were cloned into pVP and SRC-1 was cloned into pM. The potential interaction between the two proteins of interest was measured using the standard SEAP assay as discussed above.

2.15 Construction of NS3-pAmCyan C1 Fluorescent construct

The wild-type HCV NS3 gene (obtained from strain H77-PH21) was cloned into pAmCyan C1 (Clontech, Mountain View, CA). PCR amplification was performed using iProof High-Fidelity PCR Kit (Bio-Rad Laboratories, Hercules, CA) on H77-PH21 to retrieve the NS3 amplicon. The primers (Operon, Huntsville, AL) used contained flanking EcoR1/BamH1 restriction sites:

5'GTACGAATTCTATGGCGCCCATCACGG 3'

5' GTACGGATCCTCATTACGTGACGACCTCCAGGTC 3'

The resulting amplicon was cleaned using Freeze N' Squeeze spin columns (Bio-Rad Laboratories, Hercules, CA), and digested with appropriate restriction enzymes (New England Biolabs, Ipswich, MA) at 37°C for 30min. The digested amplicon was cleaned again and ligated into pAmCyan. Ligation time was overnight at 16°C. Ligation product was cleaned and transformed via electroporation of Top 10 *E. coli* electrocompetent cells (Invitrogen Life Technologies, Valentia, CA). A voltage of 2.5 mV was used to transform the cells with 2μl ligation product. 900μl of LB both was immediately added to the cuvette following electroporation and the solution was aliquoted on 15μg/ml kanamycin selective LB agar plates. Colony screenings were done by PCR amplification using Hot StarTaq Master Mix (Qiagen, Valencia. CA). A randomly chosen positive colony was cultured in LB + 15μg/ml kanamycin selective broth and purified with Endo-free Maxi-prep kit (Qiagen, Valencia, CA).

2.16 Construction of SRC pAsRed N1 Fluorescent construct

The SRC gene (obtained SRC-pm) was cloned into pAsRed C1 (Clontech, Mountain View, CA). Due to the large size of SRC, the gene was amplified in 2 segments using iProof High-Fidelity PCR Kit (Bio-Rad Laboratories, Hercules, CA). The SRC-pM construct was used for both PCR amplifications. Segment 1 was amplified using an SRC 5' sense primer with flanking KpnI restriction site and an internal SRC 3' anti-sense primer with flanking BamHI restriction site. Segment 2 was amplified using an internal SRC 5' sense primer with flanking BamHI restriction site and an SRC 3' anti-sense primer with flanking AgeI restriction site (Figure 10).

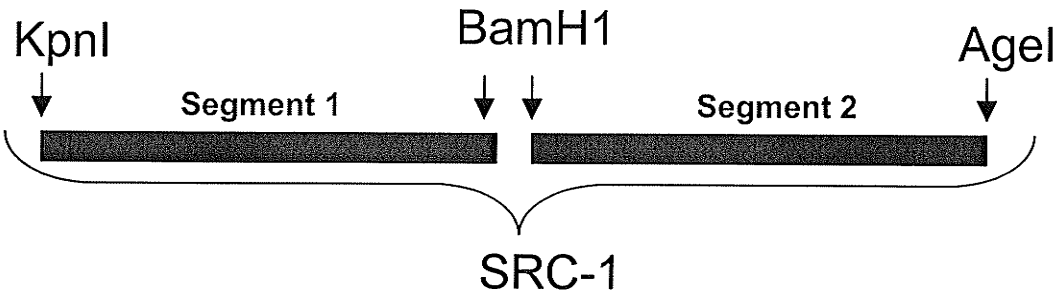

Figure 10: Diagram representing the segmentation of SRC-1 by PCR for the purpose of cloning.

The resulting amplicons were cleaned using MinElute PCR Purification kit (Qiagen, Valencia. CA), which yields higher concentrations than the Freeze and Squeeze kit used above. The amplicons were digested with appropriate restriction enzymes (New England Biolabs, Ipswich, MA) at 37˚C for 30min. The digested amplicons were again cleaned with the MinElute PCR purification kit, combined and ligated into pAmCyan. Ligation time was overnight at 16˚C. Ligation product was cleaned and transformed by electroporation of Top 10 *E. coli* electrocompetent cells (Invitrogen Life Technologies, Valentia, CA) as described above. Colony screenings were done by PCR amplification using Hot StarTaq Master Mix (Qiagen, Valencia. CA). A randomly chosen positive colony was cultured in LB + 15μg/ml kanamycin selective broth and purified with Endo-free Maxi-prep kit (Qiagen, Valencia, CA).

To analyze HCV NS3/SRC co-localization, Huh 7.0 cells were co-transfected with NS3pAmCyan C1 and SRC-pAsRed C1. Cells were seeded in the amount of 125,000/well (12 well plate) and incubated at 37°C overnight prior to transfection (to achieve ~40-60% confluency). Effectene transfection reagent (Qiagen, Valencia, CA) was used to transfect Huh 7.0 cells with 1μg NS3-pAmCyan C1 and 1μg SRC-pAsRed

C1. Cells were transfected following manufacture's protocol as described above. The transfected plate was incubated at 37°C for 48h before analysis.

3. Results

3.1 Construction of HCV NS3 pCDNA 3.1 (-) construct

The wild-type HCV NS3 gene (obtained from strain H77-PH21) was cloned into pcDNA 3.1 (-). Figure 11 shows the HCV NS3 amplicon generated after PCR amplification, using primers flanking the 5' and 3' ends of the NS3 gene. The size of the band is consistent with the size of the HCV NS3 gene at ~1800bp.

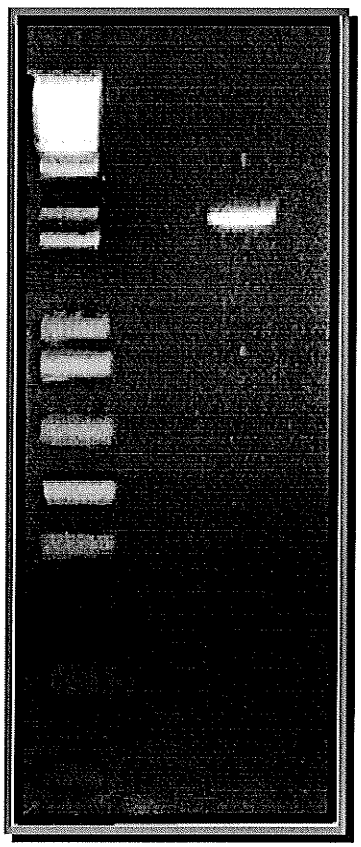

Figure 11: HCV NS3 amplicon generated from the H77-PH21 template using iProof High-Fidelity PCR Kit. 1% Agarose in 1xTBE was used to run the product at 120V for 30min.

Once the product was cleaned, digested and ligated into pcDNA 3.1(-), transformed *E. coli* colonies were selected for screening of the NS3 insert. Figure 12 shows the 8 colonies chosen which were all positive for the NS3 insert. The first colony was used to make a 100ml culture in ampicillin selective Luria broth. The plasmid was later purified and sequenced.

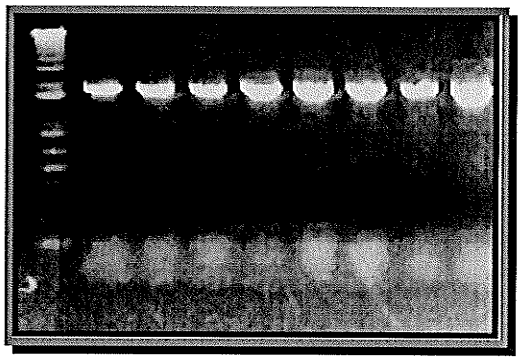

Figure 12: HCV NS3 amplicons generated by PCR amplification from pcDNA 3.1(-) plasmids of transformed *E.coli* cells. 1% Agarose in 1xTBE was used to run the product at 120V for 30min. Inserts are numbered 1-8 from left to right.

3.2 *c-fos* expression in the presence of HCV NS3

Huh 7.0 cells were co-transfected with pc-fos-FA-CMV, pFR-Luc and NS3pcDNA3.1(-). A co-transfection of pc-fos-FA-CMV, pFR-Luc and pcDNA3.1(-) was used as a baseline control. A luciferase assay was performed 30h post transfection to measure the amount of *c-fos* activation when exposed to HCV NS3 mutant. Results indicate close to an 80 fold increase (luminescence = 39.66) in the amount of *c-fos* activation, compared to control (luminescence = 0.025), at 10 minutes post substrate addition and close to a 40 fold increase (luminescence = 76.43) in the amount of *c-fos*

activation, compared to control (luminescence = 0.073), 30 minutes post substrate addition (figure 13).

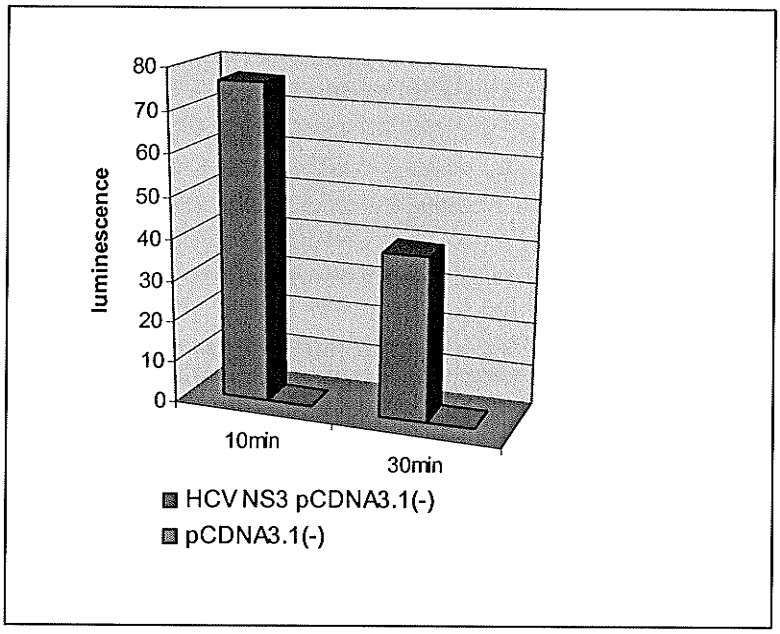

Figure 13: luciferase expression levels in Huh 7.0 cell line transfected with pc-fos-FA-CMV and pFR-Luc in the presence of HCV NS3 pCDNA 3.1 (-) or pcDNA 3.1(-). Luciferase assay was performed 30hr post transfection. Samples were performed in triplicate and measured at 10 and 30 minutes.

3.3 SRE expression in the presence of HCV NS3

Huh 7.0 cells were co-transfected with SRE-SEAP2 and NS3 pcDNA3.1(-). A co-transfection of SRE-SEAP2 and pcDNA3.1(-) was used as a baseline control. A SEAP assay was performed 30h post transfection to measure the amount of SRE activation when exposed to HCV NS3. Results indicate over a 2 fold increase (luminescence = 116.98) in the amount of SRE activation, compared to control (luminescence = 48.04), at both 10 and 30 minutes (luminescence = 98.33/47.11) post substrate addition (figure 14).

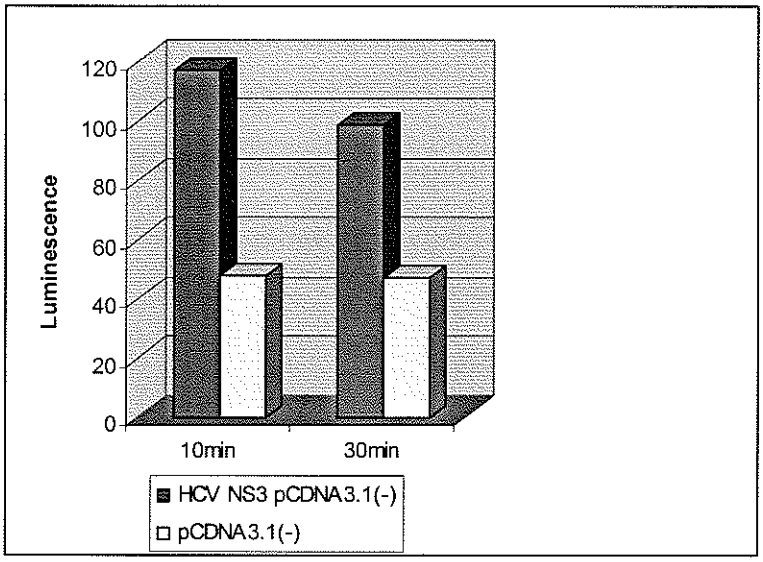

Figure 14: SEAP expression levels in Huh 7.0 cell line transfected with SRE-SEAP2 in the presence of HCV NS3 pCDNA 3.1 (-) or pcDNA 3.1(-). SEAP assay was performed 30hr post transfection. Samples were performed in triplicate and measured at 10 and 30 minutes.

3.4 Construction of NS3 Sequential Deletion Mutants

The wild-type HCV NS3 gene cloned into pcDNA 3.1 (-) was sequentially truncated from the C-terminus. Figure 15 shows the sequentially truncated HCV NS3 amplicons generated after PCR amplification. The sizes of the bands are consistent with the anticipated sizes of the truncated NS3 amplicons (1500bp's, 1200bp's, 900bp's, 600bp's and 300bp's.)

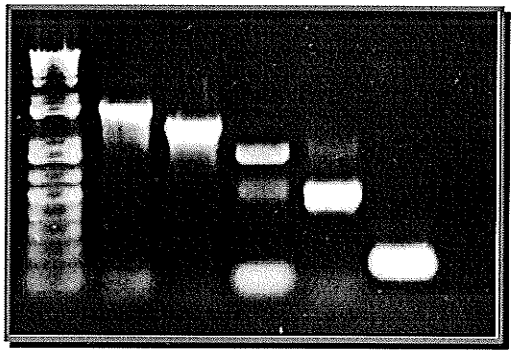

Figure 15: sequentially truncated HCV NS3 amplicons generated from the HCV NS3 pcDNA 3.1(-) template using iProof High-Fidelity PCR Kit. 1% Agarose in 1xTBE was used to run the product at 120V for 30min.

Once the products were cleaned, digested and ligated into pcDNA 3.l(-), transformed *E. coli* colonies were selected for screening of the truncated HCV NS3 inserts. Figure 16a-e shows the 8 colonies chosen for each truncated HCV NS3 colony screen. Selected colonies were used to make a 100ml culture in ampicillin selective Luria broth. The selected plasmids were later purified and sequenced. Colonies were selected as follows:

C-terminal sequential deletion # 1 – colony 1
C-terminal sequential deletion # 2 – colony 5
C-terminal sequential deletion # 3 – colony 7
C-terminal sequential deletion # 4 – colony 1
C-terminal sequential deletion # 5 – colony 1

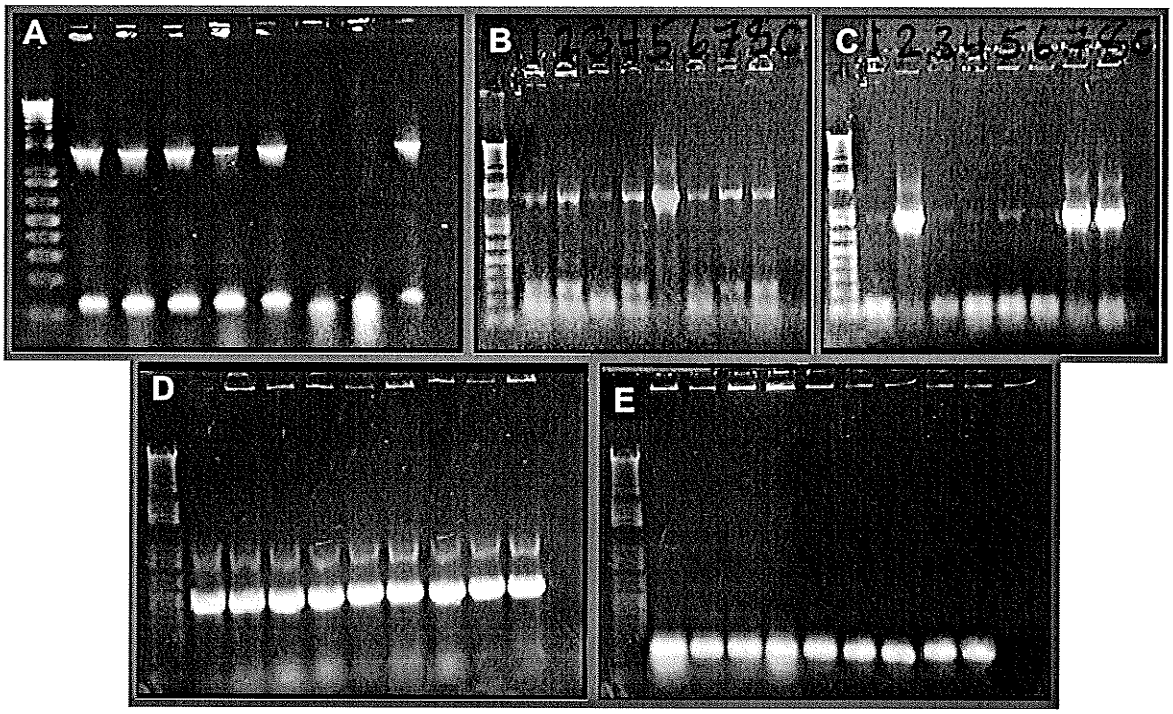

Figure 16: truncated HCV NS3 amplicons generated by PCR amplification from pcDNA 3.1(-) plasmids of transformed *E. coli* cells. 1% Agarose in 1xTBE was used to run the product at 120V for 30min. Inserts are numbered 1-8 from left to right. Lane 9 is a negative control (PCR amplification with no template).

The wild-type HCV NS3 gene cloned into pcDNA 3.1 (-) was truncated from the N-terminus. Figure 17 shows the HCV NS3 amplicon generated after PCR amplification. The size of the band is consistent with the anticipated size of the truncated NS3 gene at ~1500bp.

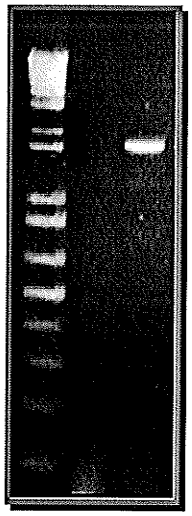

Figure 17: N terminal truncated HCV NS3 amplicon generated from the HCV NS3 pcDNA 3.1(-) template using iProof High-Fidelity PCR Kit. 1% Agarose in 1xTBE was used to run the product at 120V for 30min.

Once the product was cleaned, digested and ligated into pcDNA 3.l(-), transformed *E. coli* colonies were selected for screening of the truncated HCV NS3 insert. Figures 18 shows the 7 colonies chosen for the N terminal truncated HCV NS3 colony screen. Colony 3 was used to make a 100ml culture in ampicillin selective Luria broth. The selected plasmid was later purified and sequenced.

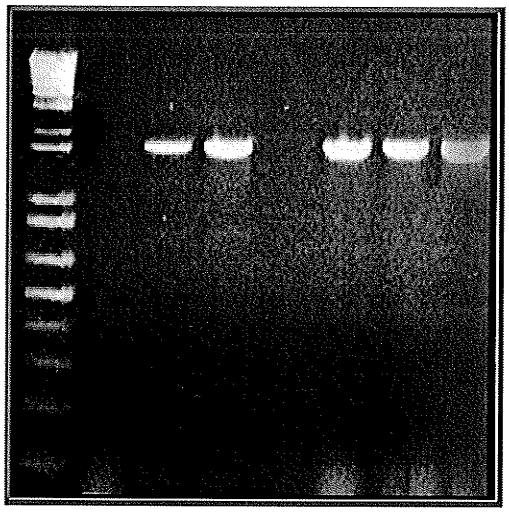

Figure 18: N terminal truncated HCV NS3 amplicons generated by PCR amplification from pcDNA 3.1(-) plasmids of transformed *E. coli* cells. 1% Agarose in 1xTBE was used to run the product at 120V for 30min. Inserts are numbered 1-7 from left to right.

3.5 SRE expression in the presence of HCV NS3 mutants

The created constructs were co-transfected with SRE-SEAP2 into Huh 7.0 cells. A SEAP assay was performed 30h post transfection to measure the amount of SRE activation when exposed to each NS3 mutant. Results indicate strong SRE activation while SRE-SEAP2 was co-transfected with full length HCV NS3 (luminescence = 98.33), first c-terminal truncation product (luminescence = 150.50) and second c-terminal truncation product (luminescence = 161.03). A drop in SRE activation was observed between the second HCV NS3 truncation product and the third HCV NS3 truncation product (luminescence = 49.77). SRE activation remained low while SRE-SEAP2 was co-transfected with the forth (luminescence = 43.90) and fifth (50.39) HCV NS3 truncated products. Empty pCDNA 3.1 (-) vector was used as a baseline control and HCV E gene in pcDNA 3.1 (-) was used as a negative control. Both maintained low

luminescent values of 47.11 and 49.05 respectively. SRE activation was decreased slightly by fusing the NS3 to its co-factor NS4a (luminescence = 72.93).

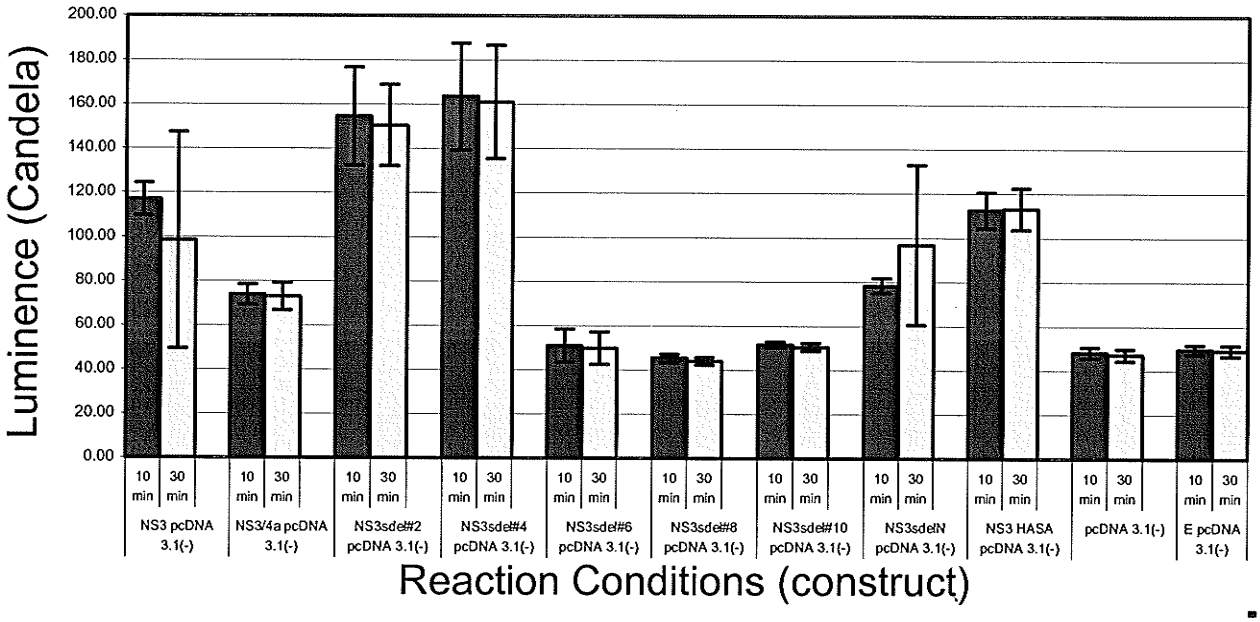

Figure 19: Graphical representation of SEAP reporter assay chemiluminescent results measuring SRE activation in the presence of NS3 and NS3 truncation mutants. pCDNA 3.1 (-) was used as a baseline control. HCV E pCDNA 3.1 (-) was used as a negative control. SEAP assay was performed 30hr post transfection. Samples were performed in quadruplicate and measured at 10 and 30 minutes

3.6 NFκB expression in the presence of HCV NS3

Huh 7.0 cells were co-transfected with NFκB-SEAP2 and NS3 pcDNA3.1(-). A co-transfection of NFκB-SEAP2 and pcDNA3.1(-) was used as a baseline control. A SEAP assay was performed 30h post transfection to measure the amount of NFκB activation when exposed to HCV NS3. Results indicate close to a 6 fold increase (luminescence = 54000) in the amount of NFκB activation, compared to control (luminescence = 6000) (Figure 20).

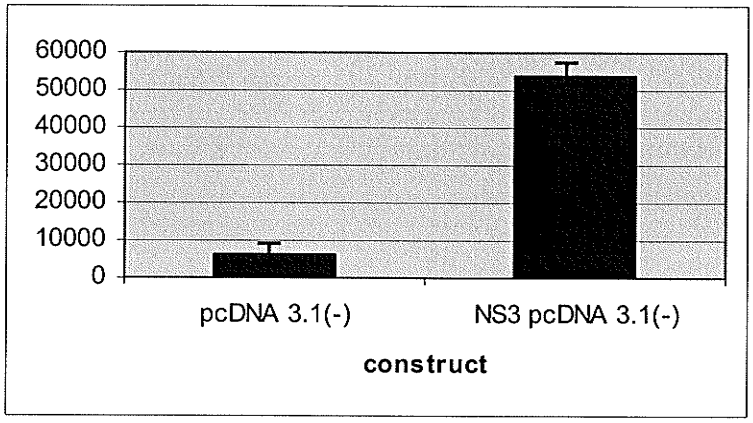

Figure 20: SEAP expression levels in Huh 7.0 cell line transfected with NFκB-SEAP2 in the presence of HCV NS3 pCDNA 3.1 (-) or pcDNA 3.1(-). SEAP assay was performed 30hr post transfection. Samples were performed in triplicate.

3.7 Construction of HCV NS3 pVP16 construct

The NS3 pVP16 construct was created by sub-cloning with use of the NS3 pcDNA 3.1 (-) construct. Figure 21 shows NS3 pCDNA 3.1 (-) digested with EcoR1 and Bam H1 restriction enzymes. Bands are consistent with the anticipated size of the vector (5500 bp) and insert (~1800bp).

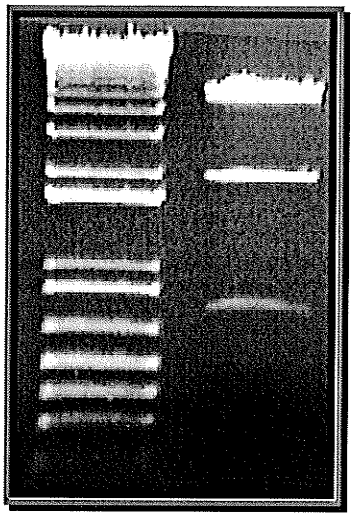

Figure 21: HCV NS3 pCDNA 3.1 (-) digested with EcoR1 and Bam H1 restriction enzymes. 1% agarose in 1xTBE was used to run the product at 120V for 30min.

Once the NS3 insert was cut, cleaned, and ligated into pVp16, transformed *E. coli* colonies were selected for screening of the NS3 insert. Figure 22 shows the 5 colonies chosen for screening. The first colony was used to make a 100ml culture in ampicillin selective Luria broth. The plasmid was later purified and sequenced.

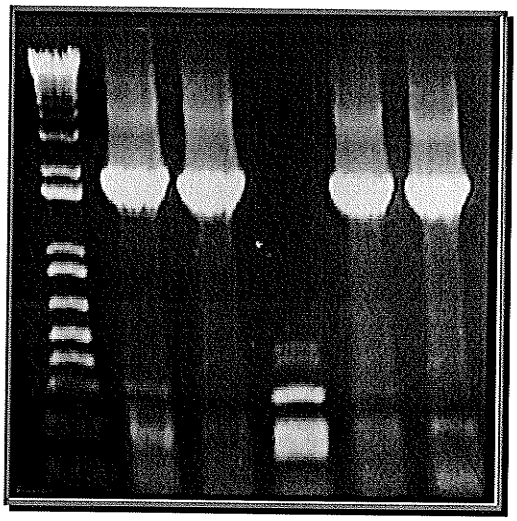

Figure 22: HCV NS3 amplicons generated by PCR amplification from pVP16 plasmids of transformed *E.coli* cells. 1% Agarose in 1xTBE was used to run the product at 120V for 30min. Inserts are numbered 1-5 from left to right.

3.8 Measurement of NS3 SRC interaction

Huh 7.0 cells were co-transfected with SRC-PM and NS3-pVP16. A co-transfection of SRC-PM and pVP16 was used as a baseline control. A SEAP assay was performed 30h post transfection to measure the amount of SRC/NS3 interaction. Results indicate close to a 30 fold increase (luminescence = 28500) in the amount of SRC interaction, compared to control (luminescence = 3000) (Figure 23).

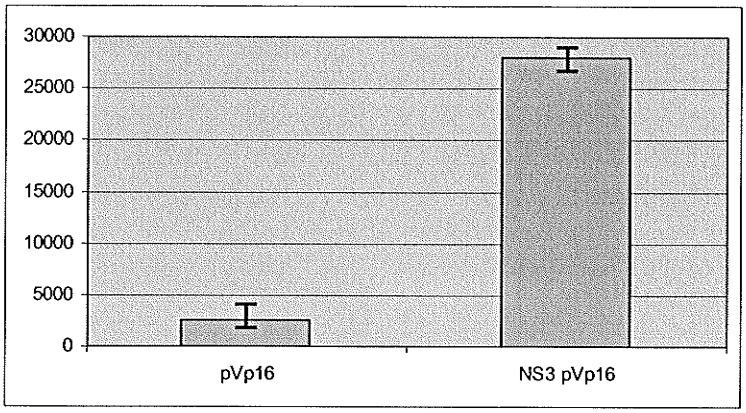

Figure 23: SEAP expression levels in Huh 7.0 cell line transfected with SRC-PM in the presence of HCV NS3 pVP16 or pVP16. SEAP assay was performed 30hr post transfection. Samples were performed in triplicate.

3.9 Construction of NS3-HASA mammalian two hybrid plasmid

The NS3-HASA pVP16 construct was created by sub-cloning with use of the NS3-HASA pcDNA 5 construct. Figure 24 shows NS3-HASA 5 digested with EcoR1 and Bam H1 restriction enzymes. Bands are consistent with the anticipated size of the vector (5137bp) and insert (~1800bp).

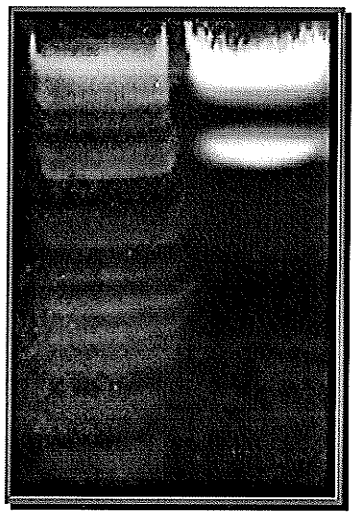

Figure 24: HCV NS3-HASA pCDNA 5 digested with EcoR1 and Bam H1 restriction enzymes. 1% agarose in 1xTBE was used to run the product at 120V for 30min.

Once the NS3-HASA insert was cut, cleaned, and ligated into pVp16, transformed *E. coli* colonies were selected for screening of the NS3-HASA insert. Figure 25 shows the 14 colonies chosen for screening. All yielded a positive screen for the 1800bp amplicon. The first colony screened was used to make a 100ml culture in ampicillin selective Luria broth. The plasmid was later purified and sequenced.

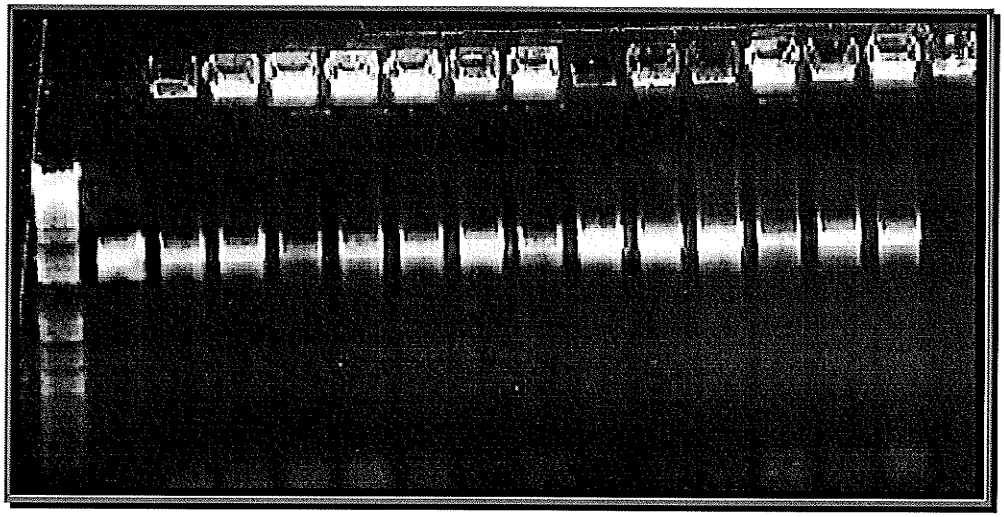

Figure 25: HCV NS3-HASA amplicons generated by PCR amplification from pVP16 plasmids of transformed *E. coli* cells. 1% Agarose in 1xTBE was used to run the product at 120V for 30min. Inserts are numbered 1-14 from left to right. Lane 15 is a negative control (PCR amplification with no template).

3.10 Measurement of NS3 vs. NS3-HASA stability

To analyze HCV NS3 stability in Huh 7.0 cell line, NS3pVP16 and NS3-HASApVP16 were cotransfected in parallel with SRC-1pM. The Huh 7.0 cell line was used to study the interaction of SRC-1 with HCV NS3/NS3-HASA. A SEAP assay was performed 18h, 24h, 30h and 48h post transfection to measure the amount of SRC-1/NS3 and SRC-1/NS3-HASA interaction. Results indicated a continuous increase in SRC-1

expression from 18h to 48h when either NS3/NS3-HASA was present. When compared to NS3 however, a co-transfection of Huh 7.0 cells with NS3-HASA yielded a more prominent increase in SRC-1 expression at 30h and 48h (figure 26).

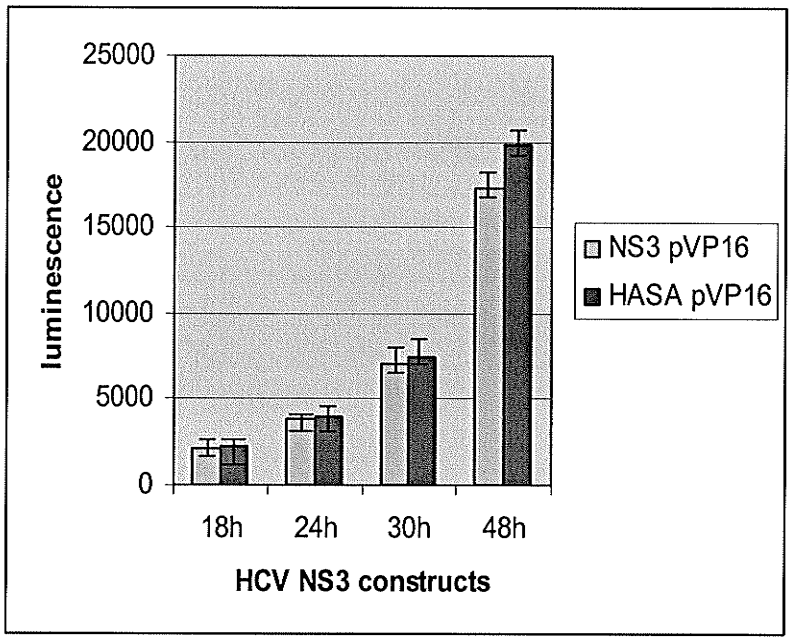

Figure 26: SEAP expression levels in Huh 7.0 cell line transfected with SRC-PM in the presence of HCV NS3 pVP16/NS3-HASA pVP16. SEAP assay was performed 18h, 24h, 30hr and 48h post transfection. Samples were performed in triplicate.

3.11 Construction of HCV NS3 pAmCyan C1 construct

The wild-type HCV NS3 gene (obtained from strain H77-PH21) was cloned into pAmCyan C1. Figure 27 shows the HCV NS3 amplicon generated after PCR amplification, using primers flanking the 5' and 3' ends of the NS3 gene. The size of the band is consistent with the size of the HCV NS3 gene at ~1800bp.

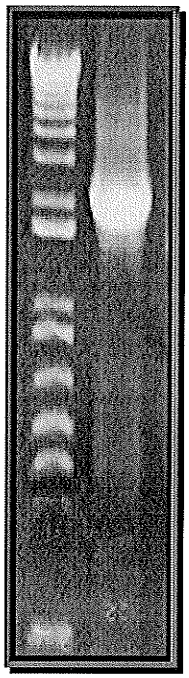

Figure 27: HCV NS3 amplicon generated from the H77-PH21 template using iProof High-Fidelity PCR Kit. 1% Agarose in 1xTBE was used to run the product at 120V for 30min.

Once the product was cleaned, digested and ligated into pAmCyan C1, transformed *E. coli* colonies were selected for screening of the NS3 insert. Figure 28 shows the 12 colonies chosen for screening. The third colony was used to make a 100ml culture in kanamycin selective Luria broth. The plasmid was later purified and sequenced.

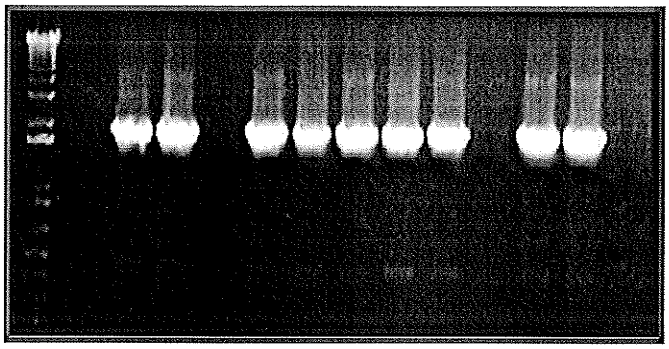

Figure 28: HCV NS3 amplicons generated by PCR amplification from pAmCyan C1 plasmids of transformed *E. coli* cells. 1% Agarose in 1xTBE was used to run the product at 120V for 30min. Inserts are numbered 1-12 from left to right. Lane 13 is a negative control (PCR amplification with no template).

3.12 Construction of HCV SRC-1 pAsRed2 C1 construct

The SRC-1 gene (obtained from SRC-1pM construct) was cloned into pAsRed2 N1. Figure 29 shows segment 1 and segment 2 SRC amplicons generated after PCR amplification. The sizes of the bands are consistent with the anticipated sizes of each of the SRC-1 segments at ~2000bp for both.

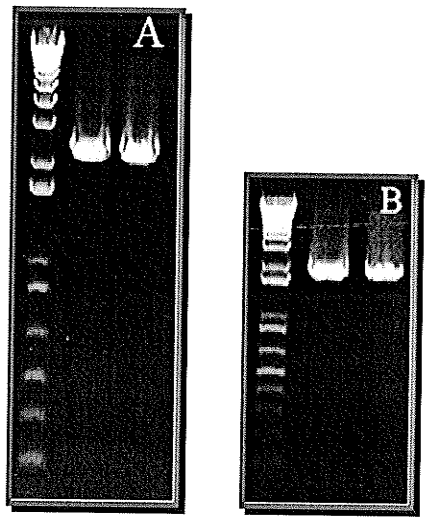

Figure 29a,b: Segmented SRC-1 amplicons generated by PCR amplification. Segments were amplified in duplicate. 1% Agarose in 1xTBE was used to run the product at 120V for 30min.

Once the products were cleaned and digested, they were combined and ligated into pAsRed2 N1. Transformed *E. coli* colonies were selected for screening of the full SRC-1 insert by choosing to amplify a region that contains segment 1, segment 2 and the vector. Figure 30 shows the 48 colonies chosen for screening. Selective colonies of the PCR products circled in red were used to make a 100ml culture in kanamycin selective Luria broth. The plasmids were later purified and sequenced.

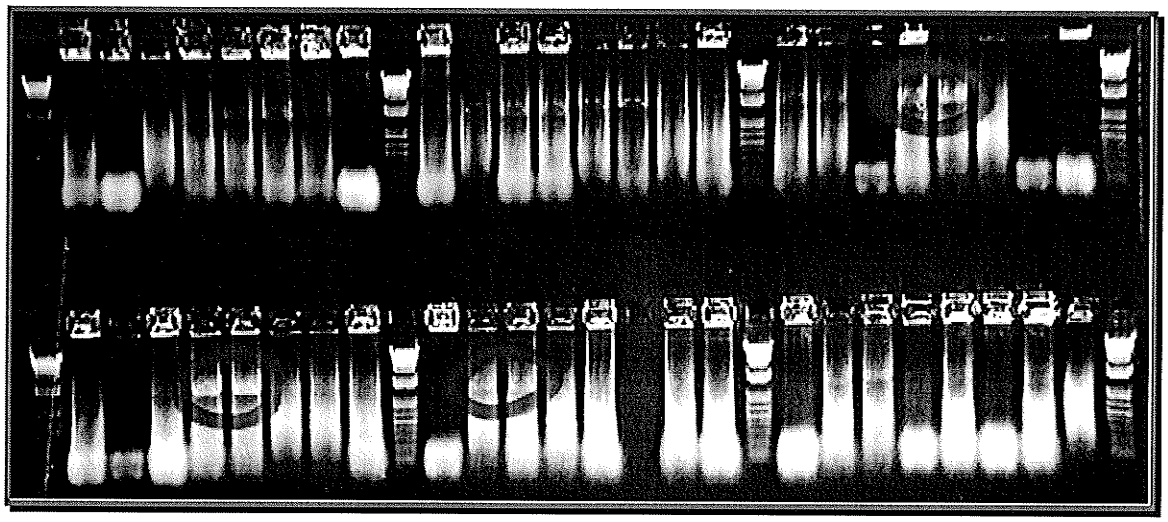

Figure 30: internal SRC-1 amplicons generated by PCR amplification from pAsRed2 C1 plasmids of transformed *E. coli* cells. 1% Agarose in 1xTBE was used to run the product at 120V for 30min. Inserts are numbered 1-48 from left to right. Lane 38 is a negative control (PCR amplification with no template).

3.13 Analysis of SRC-1 and HCV NS3 co-localization in Huh 7.0 cells

SRC-1/HCV NS3 co-localization was analyzed in Huh 7.0 cells using a fluorescent microscope at a magnification of 32x. The image in figure 31 clearly shows interaction between the two proteins: red=SRC-1 blue=NS3 pink=SRC/NS3 interaction.

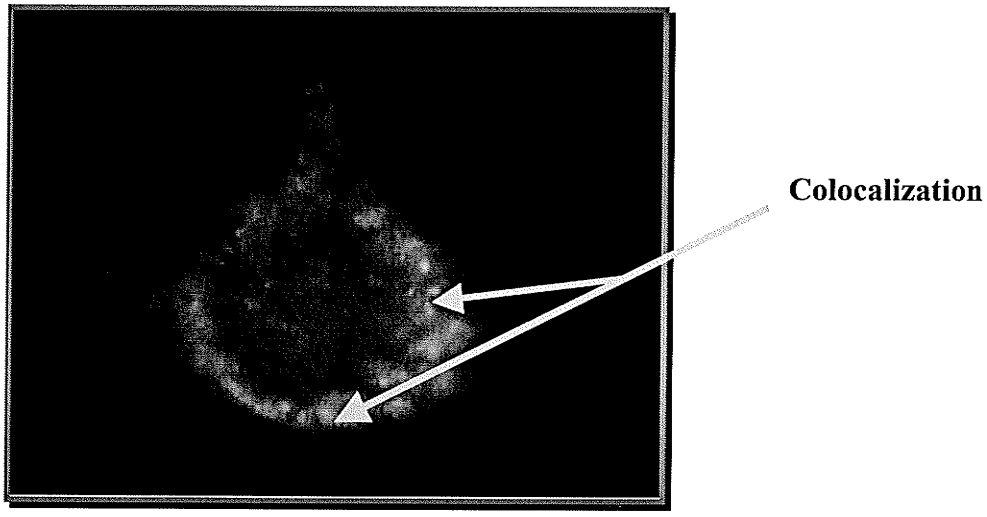

Figure 31: 32x magnification of an Huh 7.0 cell transfected with fluorescent tagged HCV NS3 (blue) and SRC-1 (red).

4.0 Discussion

Chronic hepatitis C, caused by infection with the hepatitis C virus (HCV), leads to a progressive, deteriorating disease of the liver. Since the year 2000, the hepatitis C virus (HCV) has become the most common cause of liver cirrhosis and is presently the single major reason for liver transplantation (Landford *et al.*, 2004). We now know that once HCV infection develops into cirrhosis, hepatocellular carcinoma (HCC) develops at an annual rate of 5–7% (Kazuhiko *et al*, 2006).

There are two mechanisms by which HCV infection could facilitate liver tumorgenesis: irreversible structural alterations in genes and chromosomes from disruptive hepatocyte growth and protein protein interaction between virus and host. Initial studies indicate that the HCV NS3 protease/helicase may elicit a malignant response in host cells however, detailed mechanisms are yet to be characterized. This thesis research has set out to support the fact that intracellular interactions between viral NS3 and host proteins induce modification within the cellular environment, particularly at the level of host signal transduction. Host signaling pathways have been found to induce tumorgenesis when altered by the presence of the HCV NS3.

4.1 *c-fos* expression in the presence of HCV NS3 *c-fos* Oncogene

C-fos, a member of the Fos protein family of AP-1 transcription factors, is able to form a dimer with Jun proteins which bind to the regulatory sequences of target genes. As many genes involved in tumor invasion are AP-1-regulated, it was assumed that Fos family members might be important for invasion of hepatocellular carcinomas. Overexpression of oncogenes such as *c-fos*, have been implicated in the process of HCC

(Idilman *et al.*, 1998). In fact, recently the activation of the AP-1 signaling pathway was demonstrated in Huh 7.0 cells-expressing HCV NS3 protein. Exploration of this regulator proved to support the possibility that the HCV NS3 protein could positively influence *c-fos* expression and therefore activate AP-1. When the HCV NS3 protease/helicase was subjected to Huh 7.0 liver cells, an 80 fold increase in *c-fos* activation was observed compared to cells unexposed to the HCV NS3 protein. The luciferase reporter assay used for this experiment is tailored to measure activated protein - the end product of gene expression. The dynamics therefore reveal NS3's influence to be either transcriptional or translational in nature.

When chemiluminescence was tested 30 minutes later, levels dropped to a 40 fold increase compared to control. This was inevitably due to the very short half-life of the luminescent product. Despite this drawback, luciferase was chosen for its optimality, as it has the capacity to catalyze a reaction so efficient that the quantum yield is the highest of any characterized bioluminescent reaction (www.biocompare.com/).

Similar to my finding, Hepatitis B virus X protein has been found to upregulate AP-1 activation in HepG2 cells by increasing the DNA-binding activity of c-Jun/c-Fos heterodimers (Natoli *et al*, 1994). Further to this, no other hepatitis virus has been found to positively influence *c-fos* expression. Therefore, my investigation reveals a new mechanism by which hepatitis C NS3 protein causes transformation in liver cells, making this finding both novel and credible to the hepatitis research field.

4.2 SRE expression in the presence of HCV NS3

Cascades of intermolecular reactions mediate *c-fos* regulation, ultimately leading to its expression. To determine which region of the *c-fos* pathway was specifically

affected by the HCV NS3 protein, I decided to test the serum response element (SRE) for activation. This 29bp region upstream of the promoter has a significant role in *c-fos* regulation, acting as a docking site for the Ets proteins that amplify *c-fos* transcription. The pSRE-SEAP reporter vector used is designed to measure the binding of transcription factors to SRE, providing a direct measurement of activation for this pathway. Results from this experiment indicate that HCV NS3 induced *c-fos* expression acts through a pathway that utilizes the SRE sequence; over a 2 fold increase in the amount of SRE activation was found, compared to the control. The sensitivity of the SEAP reporter assay is less than that of luciferase, which explains the difference in magnitude when comparing sample and control values. As well, *c-fos* activation depends on more than just transcription factor binding to SRE. The c-*fos* gene includes in its promoter a second major enhancer, the cAMP/Ca^{2+}response element (CRE) (Maturana et al, 2002). Therefore, the two consensus sequences bind different transcription factors and together exponentially antagonize the basal apparatus to transcribe additional *c-fos* mRNA; binding of factors to the SRE is just part of the whole picture which justifies the low levels of SRE activation if compared with total *c-fos* activation.

Various methods are possible for NS3 to disrupt the dynamics of *c-fos* oncogene regulation through the SRE. When the cell is stimulated, the Ras-MAP kinase pathway is activated. Phosphorylation of Elk-1, Sap-1 and Net ternary complex factors occurs, allowing for SRE binding and thus hightened *c-fos* transcription. HCV NS3 protease/helicase can increase *c-fos* expression through the SRE by a number of different methods including a) mimicking a TCF, thereby increasing the demand for all components needed to upregulate the *c-fos* gene through the SRE b) influencing

hightened transcription of a TCF upstream of the SRE. I chose to investigate the latter theory. Fellow biologists in my lab measured mRNA levels of Ets factors in NS3 treated and NS3 untreated Huh 7.0 cells using real-time PCR. Unfortunately, no significant differences between the NS3-transfected cells and the untransfected cells were found, suggesting that the regulation steps may be at a posttranslational level, such as phosphorylation. Due to the limitation of antibodies against these phosphorylated proteins, this theory could not be confirmed and was therefore not measured.

4.3 SRE expression in the presence of HCV NS3 mutants

To further investigate NS3's role in SRE activation, c-terminal truncated NS3 mutants, along with an n-terminal truncated mutant, were exposed to the liver cells in the presence of the SRE reporter construct. A drop in SRE activation was observed between the second HCV NS3 and third HCV NS3 truncation product, and remained low while analyzing the remaining truncated products. To translate this data in terms of protein relevance, amino acid region 334-434 of the 631 amino acid NS3 protein had to be deleted (from the c-terminus) before any significant change in SRE activation was noted (Figure 32).

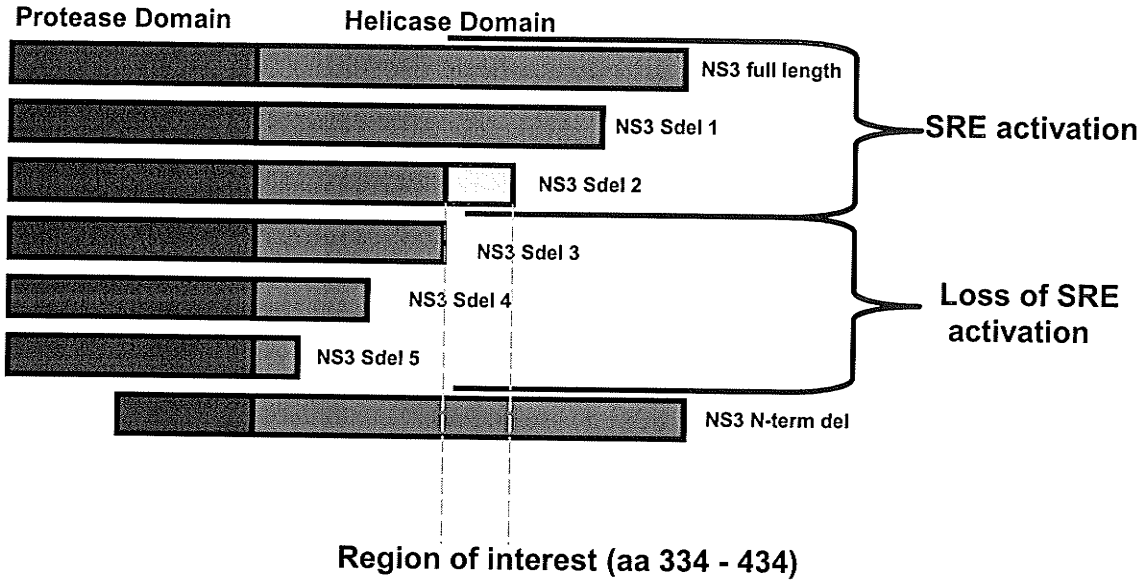

Figure 32: Diagram representing NS3 deletion mutants, their effects on SRE activation and the region that is responsible for SRE deactivation.

Non-structural protein 3 is a bifunctional enzyme exhibiting a protease activity in the N-terminal one-third and an RNA helicase activity in the C-terminal portion. As shown in the diagram, the region responsible for SRE activation falls within the NS3 helicase region.

The HCV NS3 helicase region shares striking homology with another DEAD-box RNA helicase, eIF4A. This important tRNA helicase protein is involved in the initiation of protein translation in eukaryotic cells (Du *et al*, 2002). eIF4A unwinds secondary and tertiary structures in the 5'-untranslated region of mRNA, permitting translation initiation. Reasoning would suggest than that HCV NS3 helicase could act in a similar fashion on eukaryotic mRNA, by assisting in the melting of the 5' proximal secondary structure to facilitate attachment of the 40S ribosomal subunit. However, this could not be the case; conflicting with this theory is the fact that HCV NS3 is unidirectional and can only unwind RNA duplexes in the 3' to 5' direction (Du *et al*, 2002). Therefore, the helicase could not act in assisting translational initiation of TCF's to activate SRE. An alternative

explanation involves the fact that HCV NS3 demonstrates DNA helicase activity as well. Given that HCV has no replicative DNA intermediate suggests that HCV NS3 may have the capacity to affect host DNA. In this case, factors upstream of the *c-fos* pathway may have been affected by the mechanisms of this helicase, ultimately upregulating SRE activation. Furthermore, NS3 RNA helicase activity is promoted by cofactor NS4A. In contrast, NS3 alone is a highly processive helicase on DNA (Pang *et al*, 2002). This could explain why SRE activation was not increased by fusing NS3 to its cofactor, but actually decreased, as depicted in figure 19.

To date, no specific motifs within the HCV NS3 have been identified as crucial to DNA binding. It is possible then that HCV NS3 uses a region between amino acids 334-434 to interact with host DNA. Deleting this region by truncation would then explain a drop in host transcriptional activation and thus SRE activation.

4.4 NFκB expression and SRC interaction in the presence of HCV NS3

NF-κB has been shown to play a role in the transactivation of over 150 genes, including those for cell survival. Studies have implicated members of the NFκB family in both HBV and HCV induced neoplastic development of the liver. When liver cells were subjected to HCV NS3, close to a 6-fold increase in the amount of NFκB activation was observed, compared to cells with no exposure to HCV NS3.

To follow the same methodology as with the *c-fos* investigation, proteins that dock on an NFκB enhancer region were analyzed, in conjunction with the HCV NS3 protein. The regulatory region of interest exists upstream of the NFκB p65 promoter (Rundall *et al,* 2004). Coactivators that dock here regulate transcription by forming a nucleoprotein complex known as the enhanceosome. The majority of proteins that

compose this complex possess intrinsic histone acetylase (HAT) activity, which is necessary to open the chromatin structure and form an activated preinitiation complex, ultimately controlling DNA-dependent RNA polymerase II activity and subsequent gene expression. Focus was shifted to this enhanceosome complex, investigating the potential protein-protein interactions that may occur with HCV NS3.

Interaction between the HCV NS3 protein and SRC-1, a component of the p65 enhanceosome, was measured. SRC-1 is thought to control transcriptional activation by modifying selective sites on histone tails. Results indicate close to a 30 fold difference in SRC/NS3 interaction compared to the control.

Both NFκB subunits are needed to complete the p65 enhanceosome, defining a portion of NFκB's autoregulatory effects. Two different coactivator molecules are recruited by the NFκB components: SRC-1 by p50 and CBP-p300 by p65. SRC-1 is therefore thought to interact with the p50 on the enhanceosome and coactivate the NFκB-mediated transactivations in synergy with the coactivator p300 (Na *et al*, 1998). Since HCV NS3 has been found to both interact with SRC-1 and upregulate NFκB expression, speculation has leaned towards the possibility of NS3 positively influencing the enhanceosome at a cytoplasmic level, creating more p65 and thus more NFκB in the cell. The mechanism by which this occurs is unknown.

4.5 Analysis of SRC-1 and HCV NS3 co-localization in Huh 7.0 cells

SRC-1/HCV NS3 co-localization was analyzed in Huh 7.0 cells using a fluorescent microscope at a magnification of 32x. The image depicted in Figure 31 clearly shows interaction between the two proteins: red=SRC-1, blue=NS3, pink=SRC/NS3 interaction. Both proteins are prominent in the cytoplasm with visible

fluorescent overlap (pink), indicative of protein-protein interaction. This physically supports the results above that HCV NS3 does interact with SRC-1 in Huh 7.0 cells. Fluorescence of both proteins is noticeable in the nucleus and SRC/NS3 interaction is obscure, yet not totally eliminated. The HCV NS3 protein has however been previously detected in the cytoplasm as well as in the nucleus of HCV NS3 transfected cells by immunoblotting (Hassana et al, 2006). Since NS3 lacks a nuclear localization signal, it must interact with another protein to be directed into the nucleus. SRC-1 does contain this signal, and as such is directed into the nucleus, via cytosolic nuclear transport receptors, through the nuclear pore complex. It is possible that HCV NS3 'piggy-backs' to the nucleus while in association with SRC-1 and subsequently translocates to the nucleoplasmic space to participate in the transcriptional activation of cellular genes such as p65. If this is true, the possibility of NS3 mimicking the p50 molecule in the enhanceosome could be theorized. This could in turn increase the demand for all components needed to upregulate the p65 gene through the enhanceosome creating more p65 and thus more NFκB in the cell. More evidence to support nuclear localization is needed.

4.6 Measurement of NS3 vs. NS3-HASA stability

As a supplementary experiment, the positive interaction between HCV NS3 and SRC-1 was analyzed by comparing the luminescent values with that of NS3 HASA and SRC-1. NS3 HASA is a NS3 mutant with specific N-terminal amino acids histidine and serine converted to alanine. This mutant, created by Mike Carpenter's lab, has an unconventional loss in protease activity. Surprisingly, the stability of this protein in eukaryotic cells was found to be higher than that of the wild-type. It is thought that the

native amino acids were targets for host degradation and thus removal of these targets better stabilized the NS3 protein in mammalian cells. My results support this hypothesis, as the HASA mutant delivered continuously higher SRC-1 interaction levels from 18h to 48h compared to the wild type NS3. This phenomenon could explain the low level of SRE expression with wild type NS3 when compared to NS3 truncated mutants.

Conclusion:

Hepatitis C virus has clearly been labeled an inducer of hepatocellular carcinoma by way of a number of different mechanisms. The most prominent explanation involves inflammation of the liver as a by-product of the viral infection, leading to cirrhosis, genetic alterations of hepatocytes and subsequent uncontrolled cellular proliferation. Alternatively, HCV can transform hepatocytes directly via viral/host protein protein interaction.

In this thesis, components generated by Hepatitis C viral infection have been shown to activate *c-fos* and p65 expression *in vitro*. The proteins generated by these genes are responsible for the activation of many molecules including those involved in transformation. In previous studies, HCV core protein was found to activate NFκB, and AP-1 associated signals. NS4B protein was also found to activate the NFκB associated signals. However, the influence of these HCV proteins on the cellular signal transduction pathways has yet to be fully understood. In this thesis, not only was a protein found to activate these pathways, but its functional role was hypothetically mapped.

Hepatitis C non-structural protein 3 has been found to activate the *c-fos* proto-oncogene by way of the serum response element and NFκB component p65 by way of SRC-1 interaction. Both proteins serve to activate genes involved in cellular proliferation.

TNF-α, a cytokine regulated by the activation of these genes, has been reported to be involved in the angiogenesis processes. The proximal region of the TNF-α promoter contains various recognition sequences for several transcription factors including NFκB and AP-1. Research has found that AP-1 and NFκB binding activities were increased in the peritumoral tissue, compared with histologically normal livers in 70% of cases (Macdonald and Harris, 2004). In this regard, the observed neo-angiogenesis in the liver of HCV-infected patients suggests the potential role for TNF-α in the development of viral hepatitis-associated liver tumors, including HCC. What is interesting is the fact that previous research has demonstrated the HCV NS3 protein to induce up-regulation of the TNF-α promoter in liver cells, an effect mediated mainly through the activation of AP-1 and NFκB pathways. Therefore, this data corroborates with my finding that HCV NS3 activates NFκB and *c-fos* (a crucial component of AP-1).

In conclusion HCV NS3 protein might possess a dual function, acting at both the nuclear and the cytoplasmic compartments. The nuclear function of HCV NS3 protein may be mediated by the physical association of HCV NS3 protein with the proteins of the transcriptional machinery, namely SRC-1 where as, the cytoplasmic function of HCV NS3 protein involves the activation of *c-fos*/AP-1 signaling pathways. Nonetheless, NS3 activated NFκB and *c-fos* genes are likely to mediate the induction of cytokines (including TNF-α) leading to either an inflammatory response or transcription of genes involved in hepatocellular carcinoma.

Future Directions:

To further this investigation, there are a number of experiments that could be performed to give a more detailed picture of the actual molecular events occurring in HCV (specifically NS3) infected hepatocytes. Aside from pinpointing the precise motif on HCV NS3, responsible for interfering with mammalian oncogene expression, we should also be interested in investigating the precise region where its influence occurs within the signal transduction pathway of the cell: a) which gene/protein is directly being effected and b) whether NS3's influence is transcriptional or translational in nature with respect to the genes it effects. This is particularly important with regards to the *c-fos* investigation. Which SRE pathway component is being effected and how? Western blot analysis examining the phosphorylation state of different proteins that govern SRE activation could be helpful in our investigation.

Another theory that would be interesting to investigate is whether or not TNF-α is fundamentally involved in HCV NS3 specific transformation. As stated above, the proximal region of the TNF-α promoter contains various recognition sequences for several transcription factors including NFκB and AP-1. Is TNF-α differentially expressed in the presence of NS3 and by what mechanism? Real-time PCR performance or reporter assay analysis, specifically measuring TNF-α gene/protein activation in the presence of NS3 could yield more answers in the investigation of HCV NS3 and its role.

Finally, cellular proliferation assays could be performed to support the assumption that hepatocytes are indeed entering an uncontrolled state of division when exposed to HCV NS3, ultimately leading to hepatocellular carcinoma in HCV infected individuals.

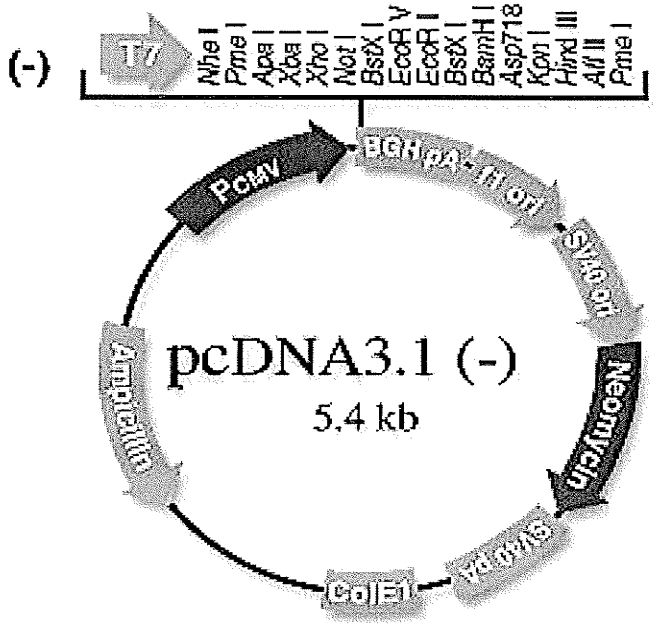

Figure 33: pcDNA 3.1 (-) used to clone NS3 wt and NS3 mutant constructs

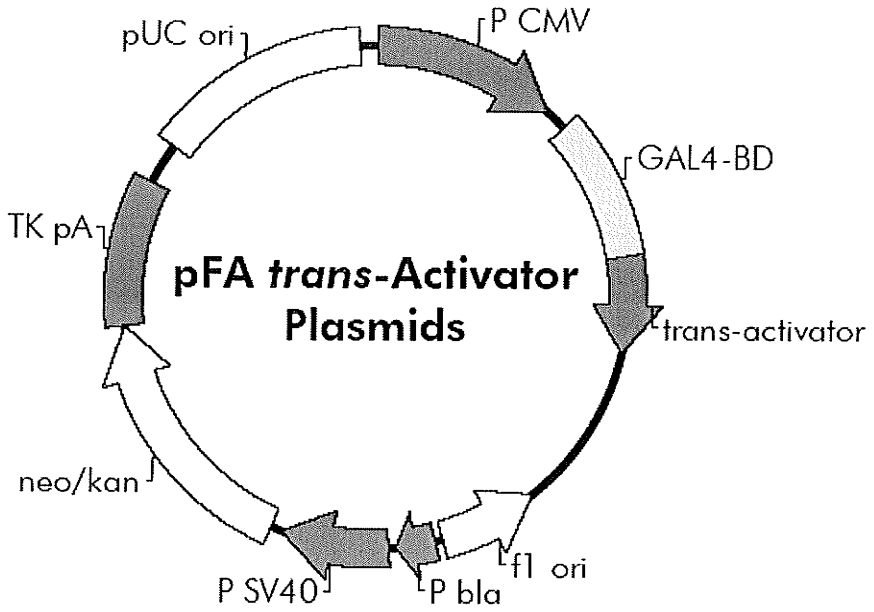

Figure 34: Circular map and configuration of the pFA-C-fos trans-activator plasmid.

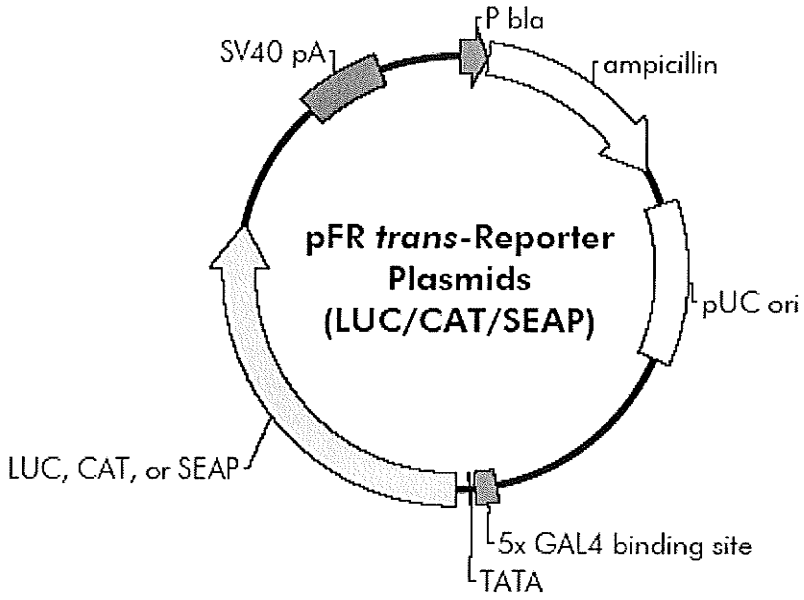

Figure 35: Circular map and configuration of the pFR-SEAP trans-reporter plasmid.

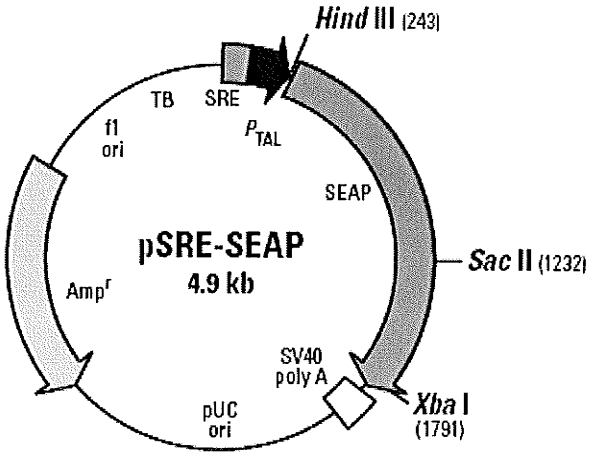

Figure 36: Circular map and configuration of the pSRE-SEAP reporter plasmid.

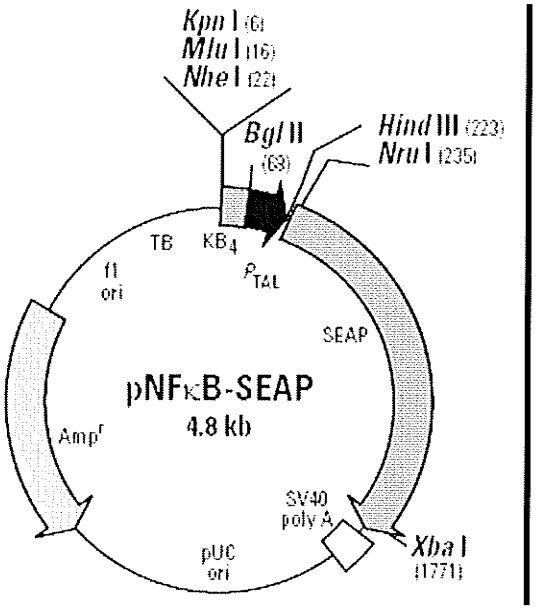

Figure 37: Circular map and configuration of the pNFKB-SEAP reporter plasmid.

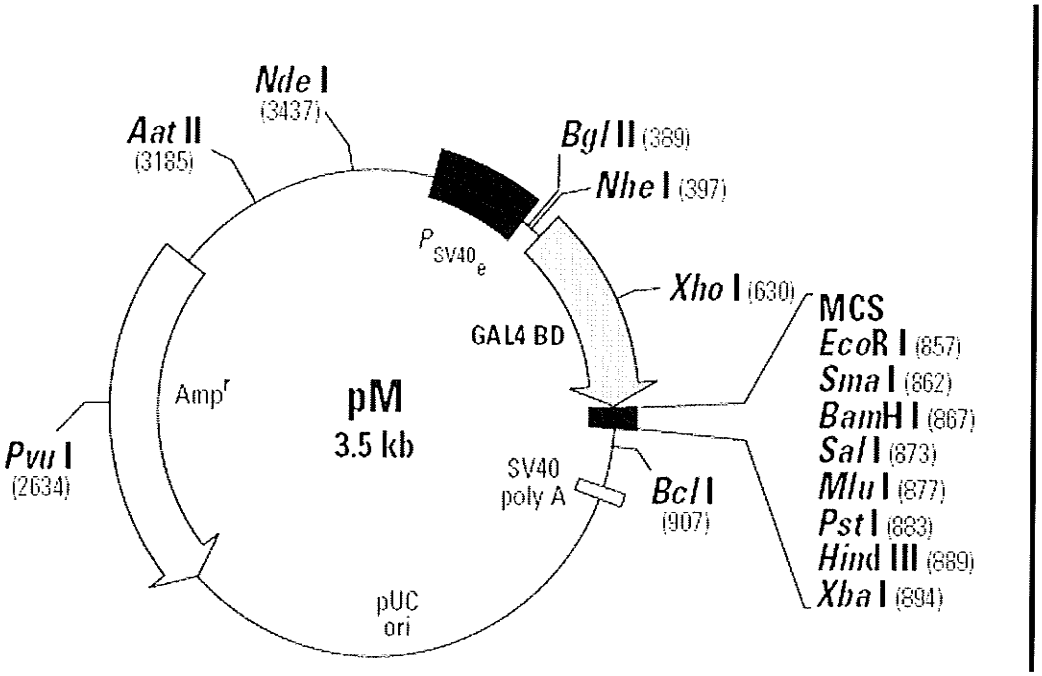

Figure 38: Restriction Map of pM GAL4 DNA-BD Cloning Vector

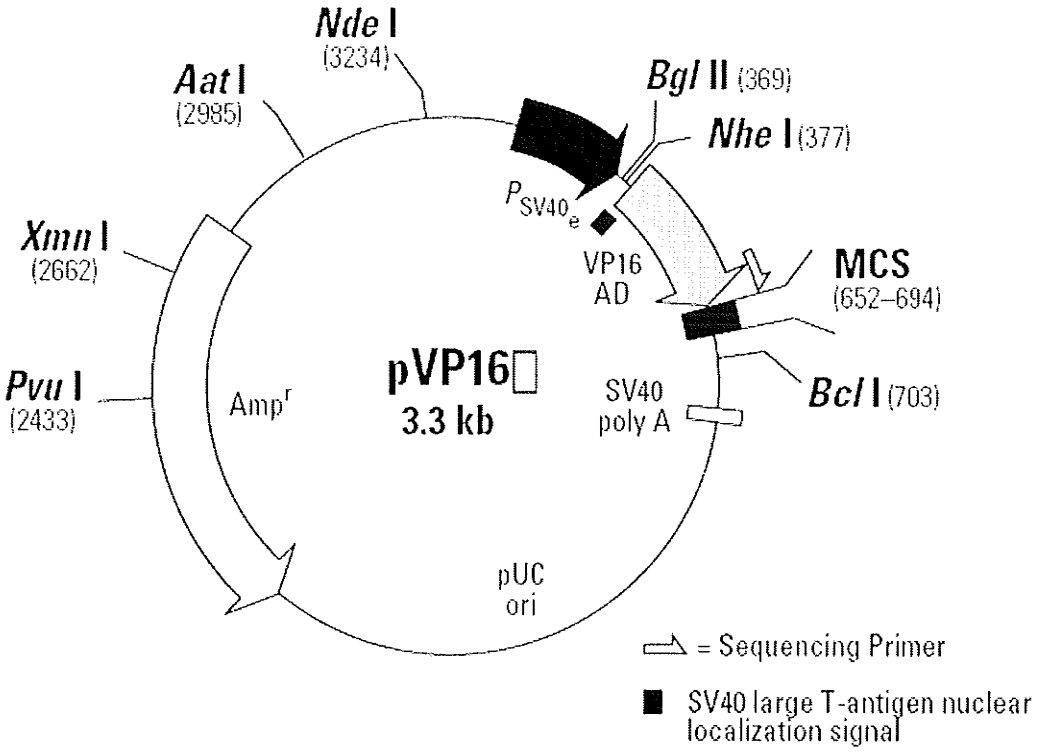

Figure 39: Restriction map of the pVP16 cloning vector

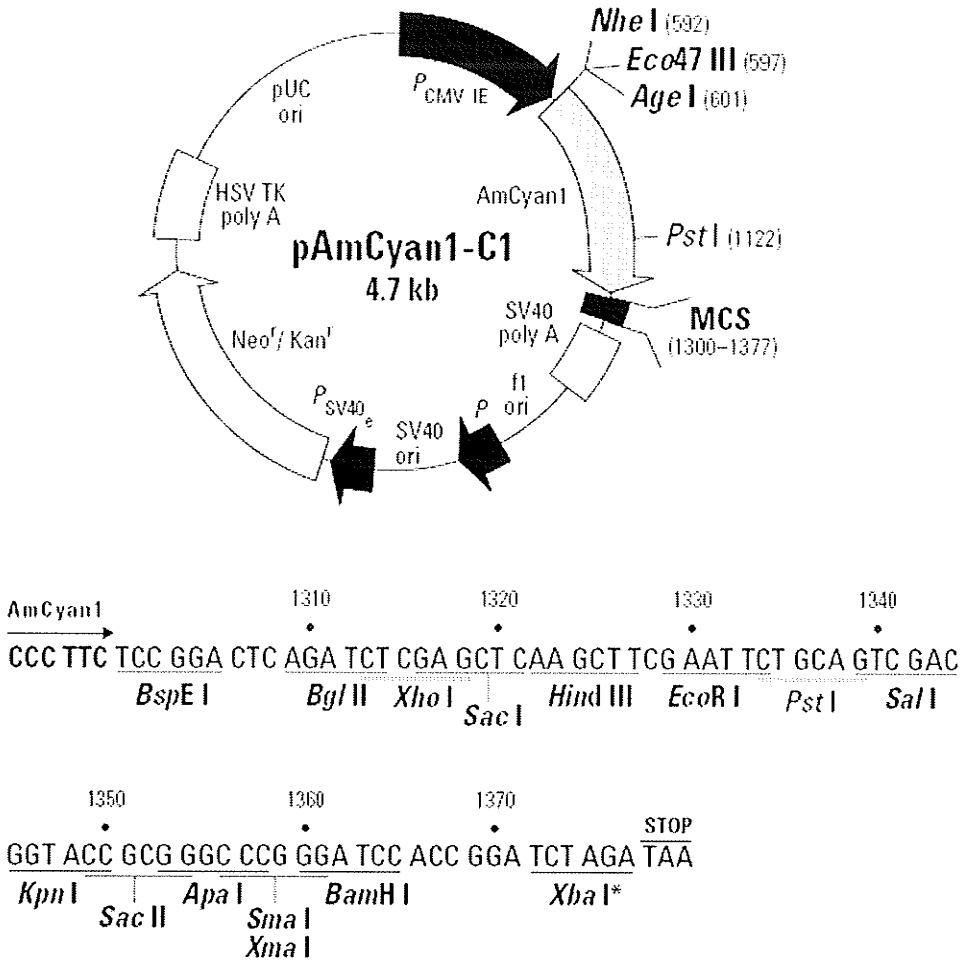

Figure 40: Restriction map and multiple cloning site of the pAmCyan-C1 cloning vector

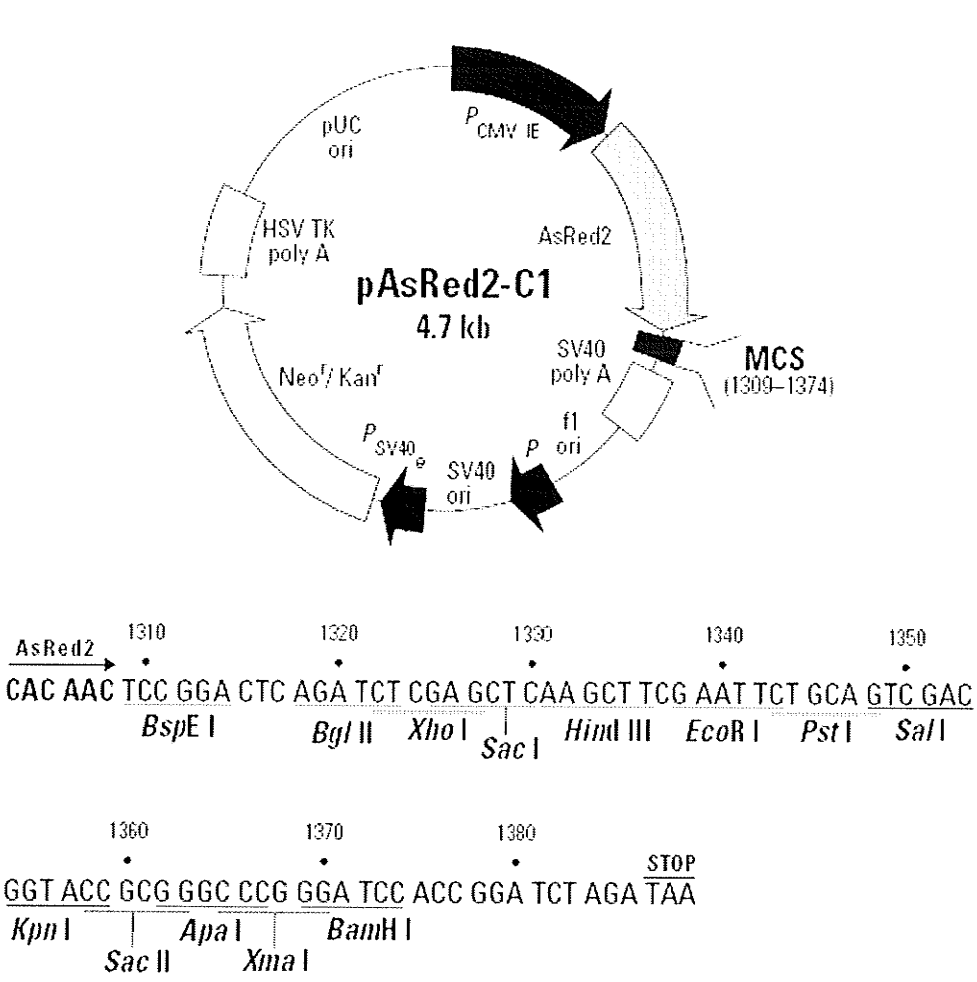

Figure 41: Restriction map and multiple cloning site of the pAsRed2 C1 cloning vector

Figure 42: HCV NS3 amino acid sequence

apitayaqqtrgllgciitsltgrdknqvegevqivstaaqtflatcingvcwtvyhgagtrtiasskgp
viqmytnvdqdlvgwpapqgarsltpctcgssdlylvtrhadvipvrrrgdgrgsllsprpisylkgssg
gpllcpaghavgifraavctrgvakavdfipveglettmrspvfsdnssppavpqsyqvahlhaptgsgk
stkvpaayaaqgykvlvlnpsvattlgfgaymskahgidpnirtgvrtittgspitystygkfladggcs
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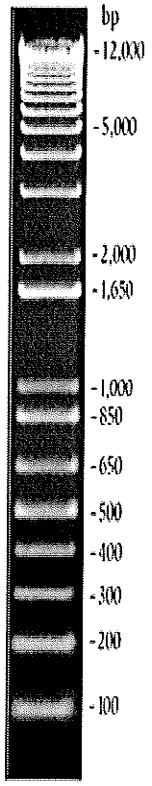
gkaipleaikggrhlifchskkkcdelaaklvalgvnavayyrgldvsvipasgdvvvvatdalmtgftg
dfdsvidcntcvtqtvdfsldptftietttlpqdavsrtqrrgrtgrgkpgiyrfvtpgerpsgmfdssv
lcecydagcawyeltpaettvrlraymntpglpvcqdhlefwegvftglthidahflsqtkqsgenlpyl
vayqatvcaraqapppswdqmwkclirlkptlhgptpllyrlgavqneitlthpitkyimtcmsadlevv
t

Figure 43: 1kb Plus DNA Ladder



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