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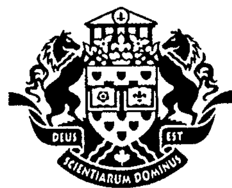

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FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

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Conditionally Activated Therapeutics for the Treatment of Hepatitis C

Marie-Grace Christou

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master's of Science

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"The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact."

-Thomas H. Huxley (1825-1895)

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ABSTRACT

Hepatitis C virus (HCV) is the etiologic agent responsible for the majority of cases of non-A, non-B hepatitis. It is estimated that 2% of the world's population is infected with hepatitis C with roughly 75% of infections progressing into a chronic state. Current interferon therapy is largely ineffective against chronic Hepatitis C due to the induction of interferon-resistance by HCV. The underlying mechanisms for resistance to interferon therapy have been widely studied and documented and as is the case in interferon resistant malignancies, there is some evidence that HCV infected cells are more susceptible to infection by interferon sensitive viruses.

Most current research into novel HCV therapeutics has focused on the discovery of inhibitors of viral replication. However, the high mutation rate of the HCV genome makes it a virtual certainty that viral variants resistant to protease and/or polymerase inhibitors will arise.

As an alternative approach, we have designed a therapeutic strategy that exploits both the expression of a viral protease in infected cells as well as the defects in the interferon pathway to build selectively active therapeutics that target and kill HCV infected cells. Initial experiments showed some promise using this approach and indicated areas for further development. Conditionally replicating viruses failed to rescue in HCV expressing cell lines, thus requiring further modification. A separate approach involving conditional stabilization of the suicide gene HSV-1 TK was successful with a two-fold increase in the expression levels of TK in cells co-expressing HCV protease.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immuno Deficiency Syndrome
ALT	Alanine AminoTransferase
CBV3	Coxsackievirus B3
CPE	Cytopathic Effect
CPV	Cytopathic Vacuoles
CTL	Cytotoxic T-Lymphocytes
dsRNA	Double Stranded RNA
eIF-2α	Eukaryotic Initiation Factor-2α
eIF-2B	Eukaryotic Initiation Factor-2B
EMCV	Encephalomyocarditis Virus
FCS	Fetal Calf Serum
GCV	Gancyclovir
GDP	Guanine Diphosphate
GFP	Green Fluorescent Protein
GTP	Guanine Triphosphate
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSV-1	Herpes Simplex Virus-1
IFN	Interferon
IFN-R	Interferon Receptor
IL-8	Interleukin 8
IRES	Internal Ribosomal Entry Site
IRF	Interferon Response Factor
ISG	Interferon Stimulated Gene
ISRE	Interferon Stimulated Response Element
JAKs	Janus Kinases
LDL	Low Density Lipoprotein
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PERK	PKR-like ER Kinase
PKR	RNA-dependent eIF-2α Kinase
RdRp	RNA-dependent RNA polymerase
STATs	Signal Transducers and Activators of Transcription
TBS-T	Tris-Buffered Saline with Tween 20
TK	Thymidine Kinase
TNF-α	Tumour Necrosis Factor-α
UTR	Untranslated Regions
VAK	Virus Activated Kinase
VSV	Vesicular Stomatitis Virus

1. INTRODUCTION

1.1 Hepatitis C Infection

The hepatitis C virus (HCV) was identified in 1988 as the causative agent for the majority of cases of non-A, non-B hepatitis (1). Today, it is estimated that some 200 million individuals or approximately 2% of the world's population is infected. In approximately 75% of cases infection is persistent, and roughly a third of chronically infected patients suffer serious complications such as liver cirrhosis or hepatocellular carcinoma (2). Thus, Hepatitis C represents a public health problem that is a cause of significant morbidity. Indeed, liver failure due to chronic HCV infection is currently the major indication for liver transplants in the United States.

The course of HCV infection varies between patients, making it extremely difficult to characterize. Infection with HCV may be associated with a transient rise in levels of the liver enzyme alanine aminotransferase (ALT) usually within 7 or 8 weeks of infection, but is asymptomatic in most cases until the manifestation of serious liver damage in the latter stages of disease (3). This makes it difficult to estimate the time between infection and development of liver disease and poses serious problems for the characterization of the natural history of infection, its treatment and the control of viral spread, as infection may go undiagnosed for long periods of time. The rate at which patients progress through the various stages of liver disease can range anywhere from 20 to 30 years and there are no reliable prognostic indicators to predict outcome as attempts to find a biological marker of infection that correlates with either disease stage or prognosis have failed. For example, immunohistochemical analysis shows that viral proteins are present in the cytoplasm of hepatocytes from infected individuals regardless

of the severity or stage of disease (4), while neither ALT nor HCV RNA serum levels have produced a consistent correlation with disease progression (5,6). Notwithstanding, several factors such as age at infection, sex, alcohol consumption and co-infection with HIV-1 or HBV have been shown to play a role (7-9).

Currently, treatment for hepatitis C is limited to interferon-α (IFN-α) either as monotherapy, or in combination with the nucleoside analogue Ribavirin. Although it is highly effective at mediating viral clearance in acute infections, the treatment is effective in only 30% of persistently infected patients, with devastating side effects that range from flu-like symptoms and fatigue to interferon-associated polyneuropathy and paranoia (3). Due to the high error rate of the HCV polymerase, patients that are infected with only a single species of HCV soon develop a large repertoire of variants (quasispecies), many of which are interferon-resistant. In fact, interferon treatment is hypothesized to drive an increase in the rate of emergence of quasispecies through the interferon-mediated upregulation of RNA editing enzymes such as ADAR1(10). In addition to being a major factor in resistance to therapy, the presence and constant evolution of a large number of mutant genomes even within a single individual has made it extremely difficult to develop effective vaccines against HCV and will likely be a barrier to development of drugs that can produce durable cures by blocking HCV replication.

1.2 Molecular Biology of Hepatitis C Virus

Hepatitis C Virus (HCV) is a member of the Flaviviridae family of viruses, genus hepacivirus. The virion is an enveloped, icosahedral particle containing a 9.6kb positive strand RNA genome encoding a single open reading frame (ORF). The ORF is flanked by two untranslated regions (UTRs). The 5'UTR functions mainly as an internal

ribosomal entry site (IRES), whereas the 3'UTR contains three motifs, two of which are necessary for viral replication: a polyuridine tract of variable length and a highly conserved 'X-tail' sequence (11,12). The viral genome is translated into a polyprotein approximately 3000 amino acids long where the 5' third of the ORF encodes the structural constituents of the virion and the remaining two-thirds encodes the non-structural proteins, which form the viral replicase (Figure 1). Upon entry into a cell, the viral particle is uncoated in acidic vesicles (13) and translation of the genome is initiated by the host cell translation machinery through the IRES element present in the 5' UTR of the genome (11,14). The nascent polyprotein undergoes several co- and post-translational cleavages to yield at least ten functional viral proteins. Mature structural proteins are generated through a series of proteolytic cleavages by host cell proteases and signal peptidases (15,16) whereas the non-structural proteins are cleaved by two virally encoded proteases.

1.2.1 The structural proteins

HCV has three structural proteins of known function, Core, E1 and E2. The highly basic core is the first protein to be translated and is thought to form the nucleocapsid. It has been postulated to interact with host cell factors and influence gene expression in a manner facilitating viral replication (17,18) and may also be involved in signaling the switch from translation to replication during the viral life cycle (19).

The envelope glycoproteins El and E2 are thought to be involved in receptor binding and cell entry. *In vitro*, El and E2 can heterodimerize (20) and both appear to be necessary for cell fusion (21). Two molecules have been proposed as receptors for HCV

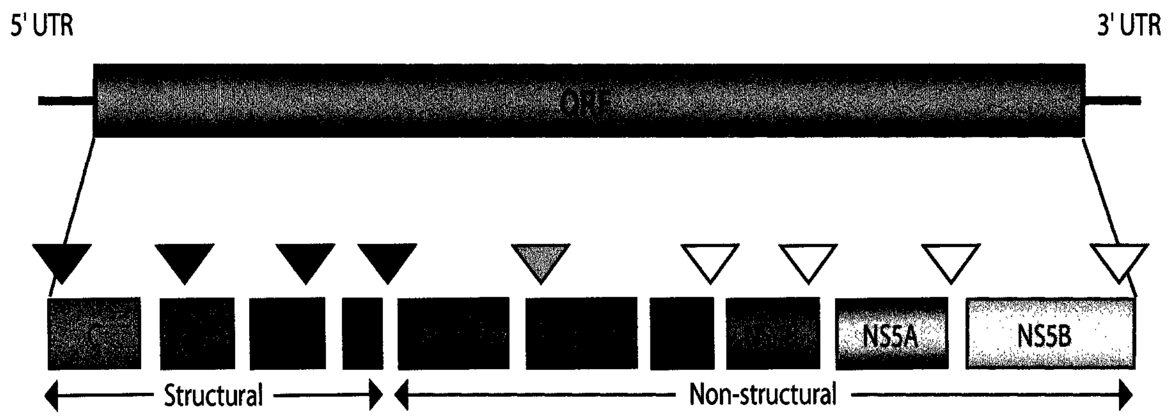

5' UTR
3' UTR
NS5A
NS5B
Structural
Non-structural

Figure 1: Organization of Hepatitis C Genome As a member of the Flaviviridae family of viruses, HCV has a single stranded plus-sense RNA genome. The genome is translated into a polyprotein through the IRES present in the 5' UTR. The polyprotein undergoes a series of cleavages by host cell proteases (▼), viral proteases (▼) and (▽) for NS2/3 and NS3/4A catalyzed cleavages respectively.

thus far: CD81 by binding to E2 and the LDL receptor through interactions with unknown components of the viral envelope (22-24).

1.2.2 The non-structural proteins

As indicated above, the non-structural proteins NS2-NS5B carry out various functions necessary for the replication of the viral genome.

Evidence suggests that most HCV proteins, particularly NS3-NS5B form a tight complex in association with intracellular membranes that serve as the site of viral replication in the cell (25-28). This strategy is commonly used by plus stranded RNA viruses such as polio virus (29) providing the advantage of forming a separate membranous compartment into which viral proteins can be produced and allowing tight coupling of functions that reside in separate viral proteins. The exact mechanism of viral replication has not been fully elucidated although it is known that NS5B is the main RNA polymerase, being sufficient *in vitro* for the synthesis of both positive and negative stranded genomes (30). *In vivo* however it is thought that NS3 and NS5B are also important in viral replication. NS3 helicase function may be important for unwinding RNA secondary structure while NS5A phosphoprotein has been speculated to act as a regulator of replication (11), in a manner analogous to the P protein of Vesicular Stomatitis Virus (VSV) (31) or the NS5 protein of Dengue virus (32).

In addition to its helicase activity, the NS3 protein functions in complex with two other proteins to mediate proteolysis of the non-structural proteins. The first of these, NS2/3 is a cis-acting protease which has been classified as either a metalloproteinase or a thiol protease (14) and is mainly responsible for processing of the NS2-3 junction (33-

35). The second, is a serine-protease NS3/4A that can carry out cis- and trans- cleavages of NS3-NS5B (36-39). Both the crystal structure (40,41) and the consensus sequence of the NS3/4A cleavage site (D/E-D/E-X-X-X-X-C/T-↓-S/A) have been elucidated. The structure of the protease domain was found to be that of a chymotrypsin family serine protease, resembling the Sindbis virus core protein. Owing to the importance of its role in the generation of a functional RNA-dependent RNA polymerase, inhibition of the NS3/4A protease activity is an area of intensive research as will be discussed below.

1.3 HCV Downregulates the Interferon Response in Infected cells

The interferon (IFN) response is a key pathway in the defence against viruses. Interferons are a family of inducible secreted cytokines that possess potent antiviral activity. They are commonly classified into two groups, type I and type II. Type I IFNs, which include IFN-α and IFN-β, are secreted by leukocytes and fibroblasts and are involved in the innate immune response. Type II IFN also referred to as IFN-γ is secreted by immune cells such as natural killer cells and both cytotoxic and helper T-cells (10). In most mammalian cells, viral infection or exposure to double stranded RNA (dsRNA) triggers the activation of a number of molecules which initiate a cascade of downstream signalling events that lead to the rapid secretion of IFN-α/β and impact several vital cellular processes such as translation, proliferation and apoptosis (Figure 2), ultimately leading to the establishment of an anti-viral state in the infected cell and the population as a whole (10). In fibroblasts, viral entry and replication lead to the phosphorylation of the interferon responsive factor 3 (IRF-3) by viral activated kinases (VAKs). Phosphorylated IRF-3 now translocates into the nucleus and along with several transcription factors such as NFκB and c-Jun, leads to the transcription of IFN-β. IFN-β is secreted from these cells

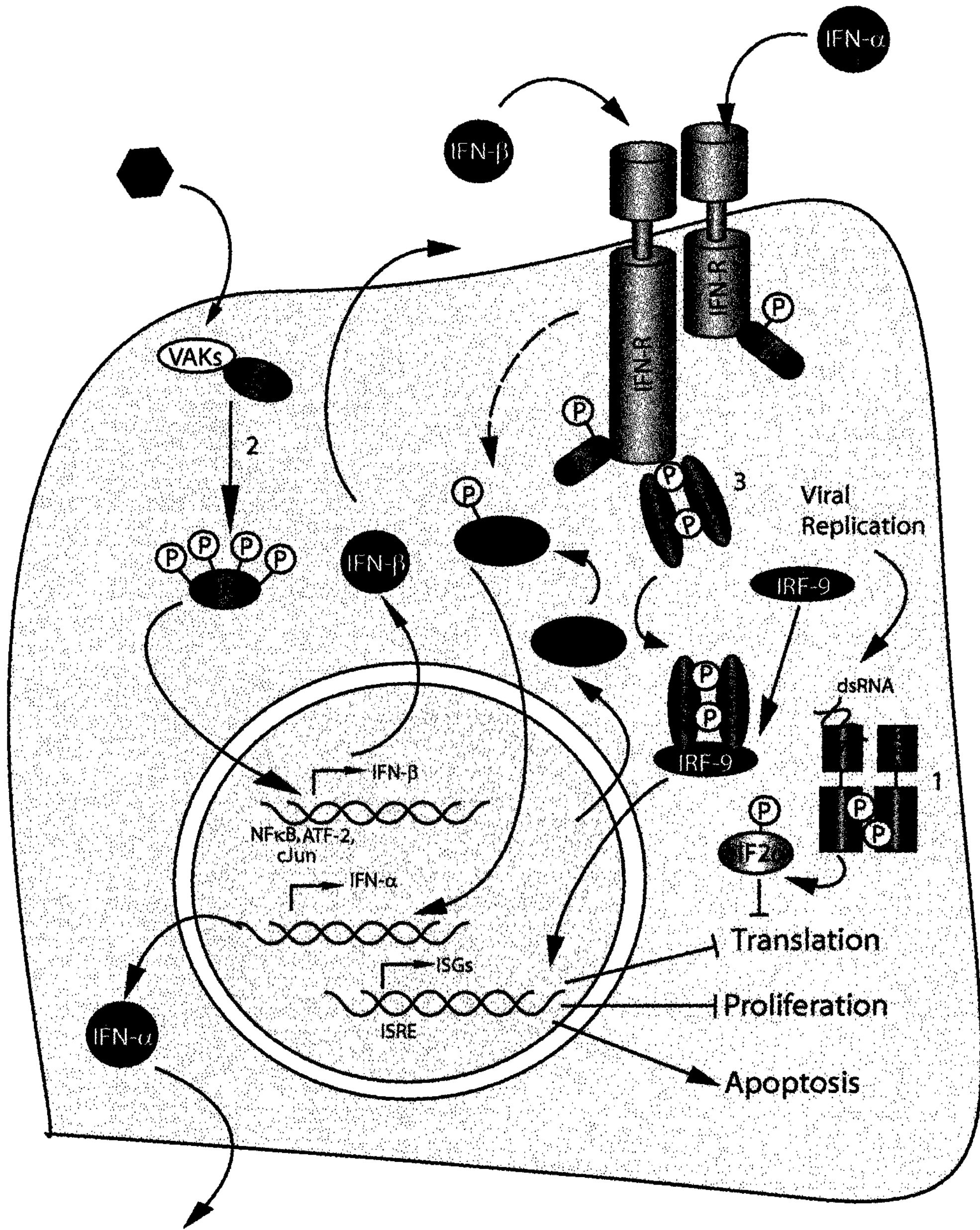

IFN-α
IFN-β
VAKs
2
IFN-β
3
Viral
Replication
IRF-9
dsRNA
IRF-9
1
IFN-β
NFκB, ATF-2,
cJun
IFN-α
ISGs
ISRE
Translation
Proliferation
Apoptosis
IFN-α

Figure 2. HCV Downregulates the Interferon Response Multiple HCV proteins have been implicated in blocking the IFN response: NS5A can disrupt PKR dimerization (1); expression of NS3/4A inactivates IRF-3 (2); expression of the HCV polyprotein impairs JAK-STAT mediated signalling (3).

and binds the type I interferon receptors (IFN-R) to initiate a second wave of signalling events. Binding of IFN-β to its receptor results in the phosphorylation of IFN-R associated kinases such as Janus Kinases (JAKs), which in turn phosphorylate the signal transducers and activators of transcription (STATs) and cause them to form dimers. The activated STATs associate with IRF-9 and the entire complex translocates to the nucleus where it can bind interferon stimulated response elements (ISREs) in the promoters of a host of interferon-stimulated genes (ISGs). One of the most important ISGs whose transcription is induced by the STAT/IRF-9 complex is IRF-7. IRF-7 is a cytoplasmic factor that is activated by phosphorylation and subsequently translocates into the nucleus where it acts to induce IFN-α expression. IRF-7 is only constitutively expressed in resting T-cells where it is immediately activated by viral infection and triggers the rapid production of IFN-α. Like IFN-β, IFN-α is also secreted and binds the IFN-R, initiating a similar sequence of events that results in the induction of a large number of ISGs. Both IFN-α and β induce genes that are involved in inhibition of translation and the induction of apoptosis as well as a number of cytokines that serve to recruit immune cells to the site of infection (42).

Another key event in the anti-viral response is the inhibition of translational initiation through the phosphorylation of the eukaryotic translation initiation factor 2α (eIF-2α). This is mediated by the RNA-dependent eIF-2α protein kinase (PKR), an RNA-sensing serine/threonine kinase that is activated by RNA-dependent autophosphorylation and dimerization. The N-terminus of PKR contains an RNA binding domain with a conserved core sequence referred to as subdomain R. It is this amino acid sequence that is able to bind double stranded RNA (dsRNA) produced during viral

infection and trigger autophosphorylation of the C-terminal catalytic domain. Normally, eIF-2 is part of a ternary complex (eIF2.GTP.methionyl-tRNA) required for the recruitment of the initiator methionyl-tRNA to the 40S ribosomal subunit. eIF-2 is released as eIF-2.GDP and this inactive form is converted into eIF-2.GTP by the guanine exchange factor eIF-2B. PKR phosphorylates eIF-2α on serine 51 and impairs the ability of eIF-2B to function as an exchange factor, thus making eIF-2 unavailable for translation initiation (see (43). Although PKR is constitutively expressed at basal levels, its expression is induced by interferon and thus, it is an integral part of the interferon-response.

The importance of the interferon response in inhibiting viral replication and spread is such that the majority of viruses have evolved strategies to inhibit key players in the pathway (reviewed in (10,44). Although IFN-α is currently used for the management of HCV infection, and is effective as a treatment for acute hepatitis, HCV has long been known to downregulate the interferon response in infected cells, leading to the observed phenotype of interferon non-responsiveness in the majority of persistently infected patients. Previous and ongoing research have uncovered multiple mechanisms for the observed interferon insensitivity involving several HCV proteins. Perhaps the most thoroughly studied of these mechanisms is the inhibition of PKR dimerization by the hepatitis C NS5A protein. Normally, PKR is activated in response to viral replication leading to its dimerization and autophosphorylation. In addition to catalyzing the phosphorylation of eIF-2α, PKR has also been implicated in several cellular functions such as growth and proliferation (45-47) and it is thus not surprising that viruses often

encode proteins that specifically inactivate PKR in order to evade the interferon response (44).

In a Japanese isolate of the interferon insensitive HCV J, NS5A protein was found to contain a stretch of some 40 amino acids termed the Interferon Sensitivity Determining Region (ISDR), so called because mutations in that region were associated with increased IFN sensitivity of variants of the prototypic IFN resistant HCV J genotype 1b (48-50). Further analysis of NS5A from IFN resistant strains revealed that NS5A disrupts PKR function by blocking the critical dimerization step, thus preventing suppression of translational initiation. The introduction of mutations into the ISDR abrogated the ability of NS5A to bind PKR and block its activation (51).

Interestingly, cells expressing NS5A were refractory to induction of apoptosis by double stranded RNA and exhibited a transformed phenotype, forming solid tumours *in vivo* (52). Of particular interest, in the same study, NS5A from IFN-resistant HCV allowed VSV to escape the inhibitory effect of IFN on VSV replication, whereas VSV would otherwise be extremely sensitive to this inhibition. The existence of an ISDR and the extent to which the NS5A plays a role in clinically observed IFN-resistance remains extremely controversial as separate studies using different isolates of HCV have failed to reproduce the phenomenon (53,54). Work using other HCV genomes has shown that genetic differences outside of the ISDR can also influence the ability of NS5A to inactivate PKR (55), indicating that at best, the ISDR is one of possibly several regions that can mediate this effect.

NS5A has also been implicated in blocking other events involved in the establishment of an innate antiviral response such as NFκB mediated Tumor Necrosis

Factor-α (TNF-α) signalling (56). There is evidence that it also induces the secretion of Interleukin-8 (IL-8) (57,58), a pro-inflammatory cytokine that has been shown to inhibit the antiviral effects of IFN-α *in vitro* (59,60) although these mechanisms have not been extensively documented.

Independently of NS5A, several other HCV proteins have also been implicated in resistance to IFN. In one study using the UHCV system (discussed in section 1.4.1), expression of the HCV polyprotein mediated IFN resistance (without affecting PKR activation or eIF2α phosphorylation) by impairing signal transduction through the JAK-STAT pathway (61) leading to a broader inactivation of the IFN response than could be achieved by targeting PKR alone. In a separate study using the same system, NS5A expression failed to affect interferon sensitivity, but expression of HCV structural proteins Core, El and E2 was able to induce IFN resistance and enhance cytopathic effect due to VSV in the presence of type I IFNs (53). Using an HCV replicon system (discussed in section 1.4.2) the serine protease NS3 has recently been reported to inactivate IRF3, a factor critical in the initiation of the interferon signalling cascade in response to viral infection (62). Furthermore, the HCV glycoprotein E2 has been shown to bind and inhibit PKR activation (63).

This brief overview of mechanisms underlying IFN resistance in HCV infected cells demonstrates that although the precise mechanisms by which different strains of HCV achieve IFN resistance in their host cell may be divergent, there is ample evidence that they all result in increased susceptibility of HCV infected cells to infection by IFN sensitive viruses such as VSV or Encephalomyocarditis Virus (EMCV). This represents an excellent opportunity to exploit defects in the IFN pathway by using viral based

therapeutics to clear HCV infected cells, in a manner analogous to that in which viral oncolytic agents can be used to kill cancer cells (64).

1.4 Tissue Culture Systems

Despite the growing burden caused by HCV infection worldwide, interferon alone or in combination with Ribavirin remains the only licensed treatment currently available. The inadequacy of available treatment and the growing numbers of infected individuals have created an urgent need for more effective therapeutics against HCV infection. The search for such agents has been complicated by the unavailability of suitable systems in which to study HCV replication and identify inhibitors. Until the development of a mouse model in 2001(65), the only other animal model that was susceptible to HCV infection was the chimpanzee. Despite many valuable discoveries made using this model (reviewed in (14), ethical and financial considerations make large scale studies of HCV pathogenesis in this primate model impractical.

To further exacerbate the problem, it has been extremely difficult to propagate HCV in cell culture for unknown reasons, despite numerous attempts over the past decade. Methods used ranged from the infection of primary cell cultures or cell lines with HCV to culturing primary cells isolated from persistently infected tissues. Unfortunately, results obtained had poor reproducibility and a low level of HCV replication making them unsuitable as research tools (12). More recently however, a number of systems have been successfully developed to permit the study of HCV replication, the interactions between viral proteins and host cell factors and to serve as tools for screening for inhibitors of replication *in vitro*.

1.4.1 The Inducible UHCV Cell Line

This cell system was the first, and until recently, the only system that allowed the stable expression of the entire HCV polyprotein (66). Essentially, the osteosarcoma cell line U2OS was stably transfected with a construct encoding the HCV genome under the control of a tetracycline-responsive promoter. Although the cell line does not mimic viral replication, it has been extremely useful in studying the cellular effects of expressing the HCV proteins (53,61,67) and because the expressed polyprotein still requires proteolytic processing to yield the mature HCV proteins, the system can also be utilized to screen for inhibitors of either the NS2/3 or NS3/4A viral proteases.

1.4.2 HCV Subgenomic Replicons

This system is based on the stable replication of sub-genomic HCV RNA in the Huh-7 human hepatoma cell line. The replicon sequence is based on that of an infectious clone isolated from a chronically infected patient. The viral proteins C-El-E2-p7-NS2 were deleted and replaced with the selectable marker neomycin phosphotransferase and the IRES element of EMCV. Thus, a bicistronic RNA was constructed, with the first cistron translated via the HCV IRES and the second cistron containing HCV NS3-NS5B translated through the EMCV IRES (68,69). Huh-7 cells were transfected with the RNA and neomycin sulphate (G418) resistant clones were obtained, albeit at a low frequency. The isolated clones contained RNAs that were capable of autonomous replication and have since been extensively characterized (70). Curiously, the isolated RNA species contained mutations that conferred cell culture adaptation (observed as an increase in frequency of formation G418 resistant colonies upon retransfection), but prevented the replication of infectious HCV genomes in the chimpanzee animal model (71,72)

suggesting that the requirements for virus propagation *in vitro* and *in vivo* may be different. It is important to note that attempts to establish HCV replicons in cell lines other than Huh-7 have been unsuccessful. This points to the possibility that there exists a strict requirement for host cell factors that are differentially expressed in Huh-7 cells compared to other liver-derived cell lines such as HepG2 for example.

Sub-genomic and genomic replicons have now been established for both the la and 1b genotypes (73,74) and although no infectious viral particles have been isolated from these systems to date, they represent encouraging developments and a valuable resource for those in the field.

Although the replicon harbouring cells are not useful as a tool for investigating HCV infection and spread, their value as a tool for screening potential inhibitors of multiple targets involved in viral replication is obvious. To date, they have been used to identify at least two such molecules: one is an antibody against the RNA-dependent RNA polymerase (RdRp) (75) and the other is an inhibitor of the NS3/4A protease that is currently undergoing clinical testing (76,77).

1.4.3 Chimeric Viruses

Beyond the development of tissue culture systems for the long-term expression of HCV polyprotein constituents, an innovative approach to screening for HCV protease specific inhibitors in the form of viral chimeras has been reported (78-80). HCV shares many of the properties of other positive stranded RNA viruses such as piconaviruses, pestiviruses or alphaviruses for which chimeras have been generated. In all three classes, genomes are translated into a viral polyprotein that requires proteolytic cleavage by viral and perhaps cellular proteases. The chimeras constructed express the HCV NS3/4A fused

to the genome of the parental virus by means of an NS3/4A recognition sequence. In all cases, propagation of the chimeric viruses is absolutely dependent on proper function of the HCV NS3 protease. Thus, inhibition of cytopathic effect on infected cells can be used as a simple readout for an HCV protease inhibitor screen.

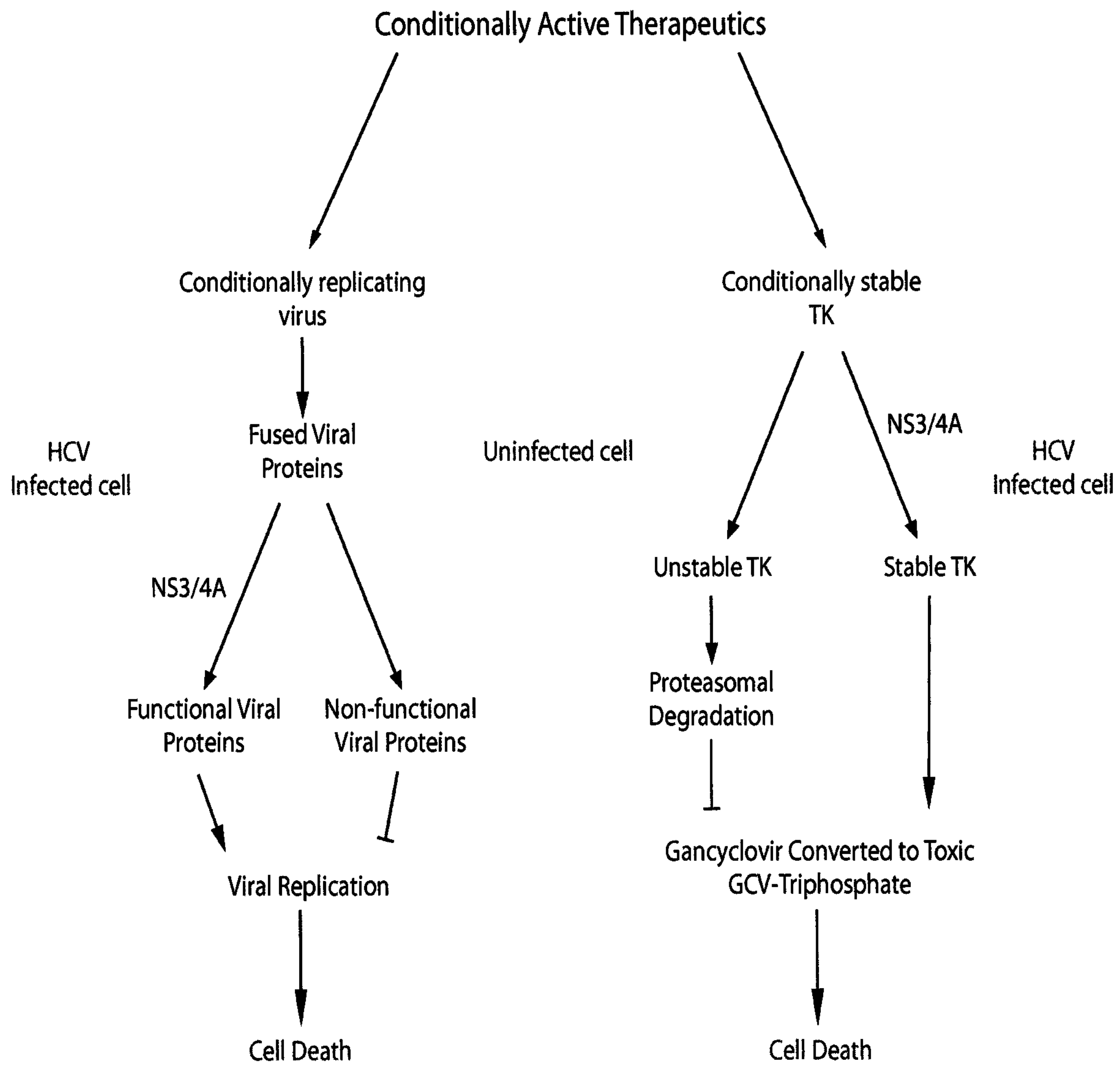
1.5 Hepatitis C Therapeutics

As discussed above, hepatitis C represents a significant and mounting health care problem. The prevalence of HCV infection is four times that of HIV and the available treatment regimen is inadequate. In addition to the fast emergence and spread of interferon resistant variants, interferon associated toxicities (exacerbated with Ribavirin combination therapy) often lead to the cessation of therapy, leaving patients and physicians alike with a lack of treatment options to pursue. To address the growing need for novel therapeutics, valuable effort is being invested in the discovery and development of drugs that block the replication of HCV. Much like enzymatic inhibitors used for the management of HIV, most approaches aim to block enzymes such as the NS3/4A protease or the RdRp. The shallow substrate-binding site of the NS3/4A enzyme poses problems for the design of specific protease inhibitors, due to the lack of folds that are unique to that enzyme, as reviewed (81). One landmark event in the field was the discovery of a small molecule inhibitor of the NS3/4A serine-protease which showed some efficacy in a Phase-I clinical study (77). Other approaches that showed some promise *in vitro* included the use of monoclonal antibodies to block RdRp function (75) and siRNA to block gene expression and RNA synthesis (82).

Due to the high mutation rate in genomes produced by the error-prone HCV RdRp – evidenced by the large number of quasispecies isolated from infected patients—

such approaches will likely also encounter the problem of resistance. One can use the case of another highly mutable virus, HIV, to illustrate the point. Despite having contributed significantly to improving quality of life of HIV positive patients, enzyme inhibitors that are currently used for therapy become ineffective after a certain period of time, making the development of fulminant AIDS a virtual certainty. A typical HIV management regimen includes a cocktail of several inhibitors, including several reverse-transcriptase and protease inhibitors taken concurrently and a similar scenario arising in HCV therapy can easily be envisaged.

The work presented here proposes an alternate approach to the problem by attempting to exploit the function of the HCV NS3/4A protease rather than inhibiting it (Figure 3). Based on work with chimeric NS3/4A dependent viruses discussed earlier and an HIV protease dependent active Caspase-3 (83), we attempted to explore a multi-pronged approach to the design of therapeutics that depend on cleavage by the HCV NS3/4A for their activity, thus only becoming activated in and killing HCV infected cells. Such therapeutics would be less vulnerable to the emergence of resistant strains of HCV because resistance would necessitate a change in both the protease itself and all cleavage sites within the HCV polyprotein. Furthermore, the proposed strategy would function to eliminate reservoirs of viral replication as opposed to simply suppressing viral replication to an undetectable level. In doing so, these therapeutics would mimic the mechanism used by the immune system to eliminate infection. The immune system responds to viral infection so as to first, neutralize the virus and prevent spread of the infection and second, to prevent further production of virus by killing infected cells. The production of antibodies is a function of B-cells, while killing of infected cells is



Conditionally Active Therapeutics
Conditionally replicating virus
Fused Viral Proteins
HCV Infected cell
NS3/4A
Functional Viral Proteins
Non-functional Viral Proteins
Viral Replication
Cell Death
Uninfected cell
Conditionally stable TK
NS3/4A
HCV Infected cell
Unstable TK
Stable TK
Proteasomal Degradation
Gancyclovir Converted to Toxic GCV-Triphosphate
Cell Death

Figure 3. The two-pronged Approach to HCV-dependent Therapeutics Cleavage of either mutCBV3 or TKCL1 by NS3/4A in HCV infected cells leads to the induction of cell death due to viral replication or metabolism of GCV. Uninfected cells on the other hand, are spared.

mediated by T-lymphocytes, particularly CD8 positive cytotoxic T-lymphocytes (CTLs). Here we describe two different attempts at engineering a conditionally active therapeutic against HCV: one in the form of a conditionally replicating virus (Coxsackievirus B3) and the second as a conditionally stable toxic enzyme (thymidine-kinase).

1.5.1 Coxsackievirus B3

Coxsackievirus is an enterovirus belonging to the *Picornaviridae* family, of which poliovirus and hepatitis A are also members. Picornaviruses are non-enveloped particles containing single stranded positive sense RNA genomes. Much like the genomic organization of HCV, the 7.3kb coxsackie genome encodes a single ORF translated through an IRES in the 5'UTR. The ORF is translated into a polyprotein which undergoes a series of co- and post- translational cleavages catalyzed by virally encoded enzymes to yield mature proteins. As in the case of HCV, the virus itself encodes two such proteases, P2A which acts in cis- to release itself from the polyprotein while P3C acts in cis- and in trans- to process the remaining junctions. Due to similarities in replicative strategy used by both coxsackievirus and HCV, we opted to engineer a vaccine variant of the coxsackievirus B3 strain (CBV3) to demonstrate in principle the potential utility of such an approach. In addition to their dependence on proteolytic processing, both viruses replicate in a membranous compartment on the cytoplasmic face of the ER and as such the HCV protease may be more easily accessible to protease dependent CBV3 than other viruses that replicate elsewhere in the cell. Furthermore, picornavirus genomes lend themselves quite readily to being genetically engineered and the recombinant genomes can easily be rescued, unless the mutations inhibit viral replication. Taken together, these features of CBV3 biology made it an ideal candidate

for preliminary work. Unlike the chimeric viruses described in earlier work (78-80), our viruses do not encode the NS3/4A protease within their genomes. Instead, we converted junctions normally cleaved by CBV3 proteases into those that can be cleaved by NS3/4A therefore making a virus that can only replicate in cells expressing a functional HCV NS3/4A.

1.5.2 Thymidine Kinase

Thymidine kinase encoded by Herpes Simplex Virus-1 (HSV-1) has long been used in gene therapy studies as a suicide gene (84,85). Although expression of TK alone does not induce cell death, upon treatment of cells with the guanosine analogue Gancyclovir (GCV), HSV-1 TK catalyzes the first step in the conversion of the prodrug into GCV-triphosphate, a toxic product that results in premature DNA chain termination and ensuing apoptosis. GCV is already approved for use in treatment of Cytomegalovirus infections and the TK-GCV is the most widely used suicide-inducing system. The system induces a strong bystander toxicity (86-89), which is advantageous in that it eliminates the requirement for TK to be expressed in every infected cell. Instead, the induction of apoptosis in one cell can affect cell death in neighbouring cells and mediate a more effective clearance of the infected cell population. Furthermore, the ability of TK to induce cell death in liver-derived cell lines, animal models of hepatocellular carcinoma and primary human hepatocellular tumours has already been well documented (90-94).

In the studies discussed here, we describe the construction of a TK gene that is linked to a degradation signal through an HCV NS3/4A cleavage sequence. Several degron peptides have been described in the literature, one of which is the CL1 degron. CL1 was first identified in *Saccharomyces cerevisiae* through a proteome-wide screen for

degradation signals (95). CL1 is thought to function through the recruitment of components of the ubiquitination machinery. In doing so, it marks attached proteins for ubiquitination and proteaseome-mediated degradation (96). Although of yeast origin, the CL1 peptide has been used in mammalian systems to destabilize green fluorescent protein (97) and ubiquitin (Tsirigotis M, personal communication). It is expected that in the absence of proteolytic cleavage of the degron signal, TK would be efficiently targeted for destruction. On the other hand, removal of the CL1 peptide by NS3/4A would selectively enhance protein stability and increase levels of TK in cell expressing the protease, making them more susceptible to killing by GCV.

The main objective of the work presented here is to engineer the described viruses and suicide gene and to evaluate whether they may be of therapeutic value for the treatment of chronic HCV. The data suggest that the approach under investigation represents an opportunity that warrants further exploration and development.

2. MATERIALS AND METHODS

2.1 Construction of Plasmids

The test substrate NS5A/5B:GFP plasmid was generated by PCR amplification of nucleotides 6256-8541of the HCV genome from the parental HCV genome plasmid p90/HCVconFLlongpU (a gift from Dr. Charles Rice) using the primers NS5-A (CACGAATTCACCATGGGCTCCGGCTCCTGGCTAAGGGACATCTG) and NS5-B (CTGGGTACCATGGTGCAGTCCTGGAGCCCTGCGGCTCGACG). The product was ligated in-frame into pEGFP-Nl (Clontech) using the KpnI and EcoRI restriction sites. The NS3/4A expression construct was generated by PCR amplification of nucleotides 3419-5475 of the HCV genome using primers NS3-5' (GCACG AATTCACCATGGCG

CCCATCACGGCGTACGCCCA) and NS3-3' (GCTGTCTAGATTAGCACTCTTCCA TCTCATCGAACTCCTGGTAG). The full-length NS3/4A PCR fragment was ligated into pcDNA3 expression vector (Invitrogen) using the EcoRI and XbaI restriction sites (See Appendix A for maps of both NS5A/B:GFP and NS3/4A constructs).

The parental wild-type CBV3-0 plasmid (a gift from Dr. Matthias Gromeier) is a non-pathogenic clone of coxsackievirus B3 strain (GenBank Accession number M88483). Mutants were generated using primers that modified the proteolytically cleaved VP3/VP1 and P2A/2B junctions by replacing the naturally occurring P7-P7' amino acids with the amino acid sequence GAVTEDVVCCSMSY occurring at the NS5A/5B junction of HCV (98). The HCV junctions were generated in two steps. First, complementary ends of the junction were generated separately. Next, the two products were mixed and the complementary ends extended for 5 cycles in the absence of primers to generate the fused product. PCR amplification of the full-length product was intiated by the addition of the two flanking primers CBV3BglII (CAACCAGGGTACTCGAG TGTT) and CBV3PauI (TAGTGGGCAAAGTATTGGACA). The Bgl II- Pau I fragment was ligated back into the CBV3-0 backbone to generate the mutant genomes.

The P2A/B:GFP construct was made by PCR amplification as described for the modified CBV3 genomes but the first amino acid of the P2A protein was replaced with a methionine. The flanking primers P2AStartNheI (GGGGCTAGCATGCAATCAGGGGC AGTGTAT) and P2BEnd (GGGCTGCAGTTG GCGTTCAGCCATAGGGAT) were used in place of the CBV3BglII and CBV3PauI primers. The resulting PCR product was cloned into pEGFP-N1 using an NheI and a PstI site, which were supplied by the 5' and the 3' PCR primers respectively.

2.2 Cell Culture and Transfection

All cell lines were maintained in α- Minimal Essential Medium (GibcoBRL) supplemented with 10% fetal calf serum. Growth medium for UHCV-11 and UGFP was supplemented with 0.5mg/ml active G418 (GibcoBRL), 2μg/ml puromycin and 1μg/ml tetracycline (66). To de-repress the tetracycline controlled promoter, cells were washed twice with PBS and growth media not containing tetracycline was added.

The Huh-7 HCV replicon cell line was maintained in media supplemented with 1mg/ml of active G418 (68).

For transfection, cells were plated at $2x10^5$ cells per well in 6-well dishes 24 hours prior to transfection. 293T and Huh-7 cells were transfected using Lipofectamine2000 transfection reagent (Invitrogen) as per company protocol. Cells were transfected with 2-4 μg of DNA and 5-10 μl of transfection reagent in OPTIMEM serum-free media (GibcoBRL). 4 hours post-transfection, transfection media was removed and αMEM media supplemented with 10% fetal calf serum (Clontech) added. U2-OS cells were transfected using Effectene Reagent (Qiagen) as per company protocol. Cells were transfected with 0.5μg of DNA and 10μl of transfection reagent in regular serum-containing media. All cells were incubated for 20 hours prior to treatment with cyclohexamide or collection of proteins.

2.3 Western Blotting

Cells were lysed in a lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X, and 5% glycerol) supplemented with Protease Inhibitor Cocktail (Roche Molecular Biochemicals). Lysis buffer was added directly to cell culture dishes and incubated on ice for 5 minutes. Cells were collected by scraping and SDS sample

buffer containing β-mercaptoethanol was added to lysates. The samples were boiled, and proteins were separated by SDS-PAGE (10% acrylamide). The proteins were transferred to a nitrocellulose membrane (Hybond C+, Amersham) at 400mA for 1 hour. Membranes were incubated with a primary antibody diluted in 5% skim milk and 1% bovine serum albumin in TBS-T (10 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20) for 90 minutes at room temperature. Membranes were washed 3 times with TBS-T for 7 minutes each. The membrane was then incubated with an HRP-conjugated secondary antibody for 30 minutes at room temperature and washed 3 times again. The membrane was incubated with a chemiluminescent substrate (Kirkegaard and Perry Laboratories) for one minute and exposed to X-ray film (Antibody dilutions are summarized in Table 1).

Pixel intensities of scanned X-ray images were determined using Adobe Photoshop Professional version 6.

2.5 Preparation of viral RNA and Electroporations

RNA was transcribed from Cla I-linearized CBV3 DNA with the T7 RiboMax kit (Promega) as per the manufacturer's recommendations. RNA was extracted using a phenol:chloroform:isoamylalcohol (1:1:24) mixture (Sigma), precipitated with ethanol and 3M Sodium Acetate pH 5.3 and quantified by spectrophotometry. The quality of run-off transcripts was verified by running on a 1% agarose gel under denaturing conditions. Huh-7 cells were transfected by electroporation. Cells were harvested by trypsinization and washed twice with PBS. 10^7 cells were resuspended in 1ml of Cytomix solution (120mM KCl; 0.15mM $CaCl_2$; 10mM K_2HPO_4/KH_2PO_4; 25mM Hepes, pH7.6; 2mM EGTA, pH7.6; 5mM $MgCl_2$; pH adjusted with KOH) (99) and 10μg of RNA added. Cells were electroporated using a single pulse (250V, 960μF) from a GenePulser (BioRad) and

recovered on ice for 5 minutes. 10^7 cells were then plated in four 60mm tissue culture dishes and allowed to recover overnight. 20hrs post-transfection, cells were transferred into 150mm tissue culture dishes to allow for monitoring of cytopathic effect.

2.3 Immnuofluorescence

U2OS cells were plated on glass cover slips in 6-well dishes as per usual protocol 24 hours before transfection. 20 hours post-transfection, growth medium was aspirated off, and the cells washed once with 1ml of PBS. Cells were fixed by incubating in 1ml of 4% paraformaldehyde solution for 20 minutes. Fixing solution was aspirated and cells were permeabilized in 1ml of 5% BSA in PBS solution containing 0.2% Triton X-100 for 5 minutes at RT. Non-specific binding to antibodies was minimized by blocking cells with a solution of 5% BSA in PBS for 1hr following the permeabilization step. Primary antibodies were diluted in 100µl of blocking solution and cells were incubated with them for 90 minutes to allow antibody binding. Cells were washed 3 times with 1ml of PBS for 5 minutes each. Cells were incubated with secondary antibodies diluted in blocking solution for 30 minutes in the dark and washed again. All incubations were carried out at room temperature (Antibody dilutions are summarized in Table 2). Coverslips were mounted on microscope slides and visualized by light microscopy.

2.6 Flow Cytometry

Transfected cells were harvested by trypsinization and pelleted by centrifugation. Cells were washed twice with 1ml of PBS each and repelleted. Cells were fixed and permeablized using a Fix and Perm kit (Caltag Laboratories) as per manufacturer's protocol. In brief, cells were incubated in 100 µl of fixing medium A for 20 minutes and then permeabilized in 100µl of medium B. Cells were then stained using the same

protocol described for immunofluorescence. For flow cytometry however, all incubations were carried out on ice. Data was analyzed using Cell Quest software.

ANTIBODY	DILUTION
Mouse α-NS3 1°	1:100
Rabbit α-GFP 1°	1:2000
Mouse α-Actin 1°	1:10000
Rabbit α-His 1°	1:1000
Goat α-Rabbit HRP 2°	1:10000
Goat α-Mouse HRP 2°	1:3000

Table 1: Summary of antibody dilutions used in immunoblots.

ANTIBODY	DILUTION
Mouse α-NS3 1°	1:50
Rabbit α-HSV-1 1°	1:2000
Mouse α-His 1°	1:100
Donkey α-Rabbit Cy2 2°	1:400
Goat α-Mouse Cy3 2°	1:800

Table 2: Summary of antibody dilutions used in immunofluorescence.

3. RESULTS

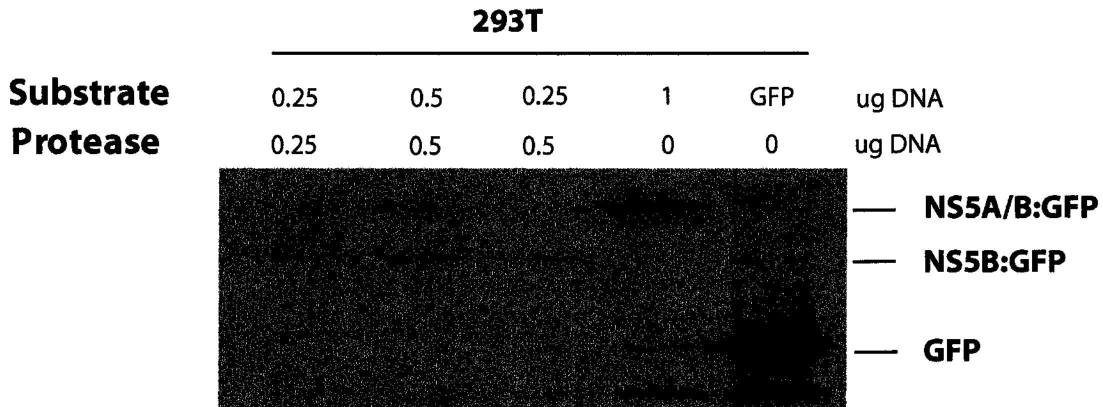
3.1 Trans-cleavage of an HCV Test Substrate

As discussed earlier (section 1.2) HCV encodes two proteases, the cis- acting NS2/3 and NS3/4A capable of both cis- and trans- cleavages. The work being discussed exploits the activity of NS3/4A. Although NS3/4A is known to be able to cleave portions of the viral polyprotein in trans- and in cis-, such studies were for the most part, performed with cleavage substrates that were translated on the same cistron as the

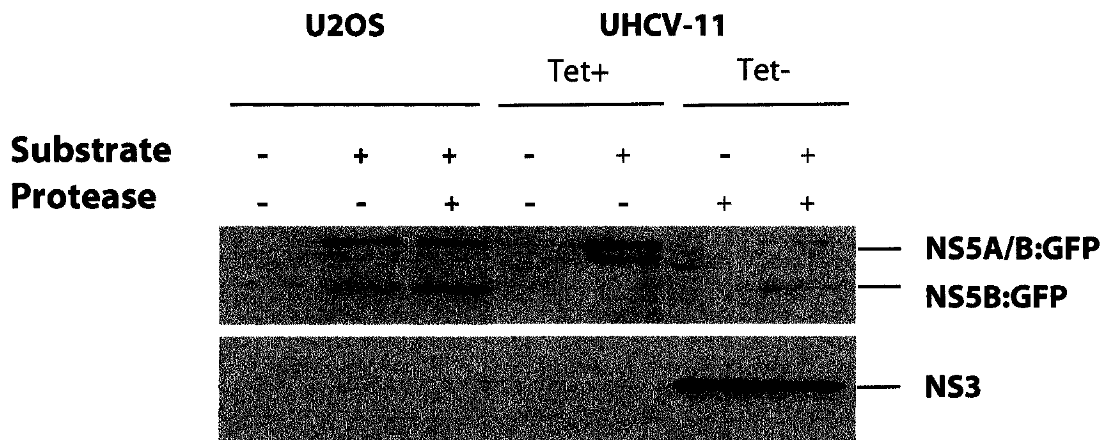
protease (36,98) and there was no solid evidence that NS3/4A could recognize and successfully cleave a substrate that was encoded separately. The function of the proposed therapeutics however, would be dependent on NS3/4A being able to recognize and cleave such a substrate. For that purpose, we constructed a test substrate consisting of the HCV proteins NS5A and part of NS5B fused to a GFP tag to facilitate detection by western blot (See Appendix A). In a screen to select for optimal NS3/4A cleavage sites, the NS5A/B junction was found to be the most efficiently processed (98). In our experiments, 293T cells were either co-transfected with NS3/4A and NS5A/B:GFP or transfected with NS5A/B:GFP alone and protein lysates immunoblotted for GFP. Results presented here strongly suggest that NS3/4A can recognize and cleave a substrate that is not part of the same polyprotein (Figure 4 panel A). First, the absence of a band in untransfected cells confirms that the α-GFP antibody is in fact detecting the NS5A/B:GFP fusion protein. Secondly, the observed shift in molecular weight of the GFP tagged protein in co-transfected cells corresponds to the size of the cleaved NS5A protein and is only observed in the presence of the HCV protease.

Although the substrate was cleaved in the presence of NS3/4A alone, it was important to ascertain whether the observed proteolytic cleavage would still occur when the protease was expressed in the context of the entire HCV genome. Studies using the Huh-7 hepatoma cell line (described in section 1.4.2) harbouring HCV subgenomic replicons suggest that HCV replicates in a separate membranous compartment (28) and the possibility that this compartment may not be permissive to the entry of transfected proteins had to be addressed.

A



B



C

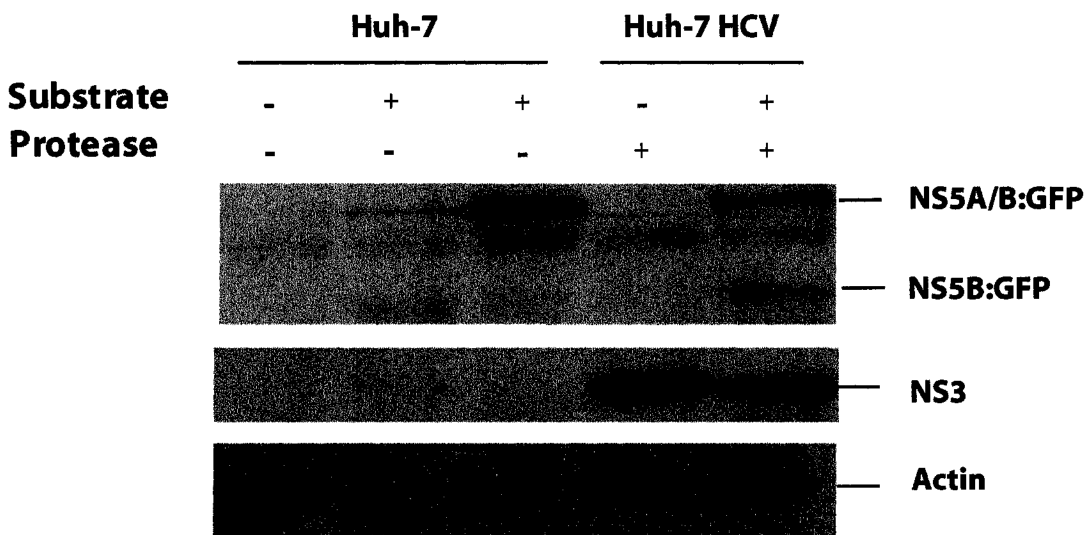


Figure 4: Cleavage of a Test Substrate by NS3/4A The ability of NS3/4A to cleave substrates that are not encoded as part of the same polyprotein was evaluated in three different systems. The protease retained its ability to cleave NS5A/B:GFP whether it was transiently expressed in 293T cells (Panel A) or stably in concert with other HCV proteins as in the UHCV-11 (Panel B) and Huh-7 HCV cell lines (Panel C).

Furthermore, it is formally possible that in the presence of other HCV proteins, NS3/4A would be unavailable to cleave transfected substrates. Thus, ascertaining whether cleavage of exogenously supplied substrate (albeit the preferred substrate) was possible in cells expressing all or part of the HCV polyprotein was central to further development of the therapeutic strategy. To do that, we utilized two systems that express HCV proteins. UHCV-11 cells express the entire HCV polyprotein under the control of a tetracycline-responsive promoter. Huh-7 HCV cells express HCV non-structural proteins NS3-NS5B from autonomously replicating HCV subgenomic replicons and have been used extensively for the study of HCV replication. Both systems have been well characterized as described in sections 1.4.1 and 1.4.2 for UHCV-11 and Huh-7 respectively.

UHCV-11 cells where expression of the HCV polyprotein was either induced or repressed, and Huh-7 cells in the presence or absence of HCV replicons were transfected with NS5A/B:GFP. In both systems, cleavage of transiently transfected NS5A/B:GFP was achieved to levels comparable with those observed in cells transiently transfected with the protease alone (Figure 4 panels B and C). In all cases, roughly 50% of the transfected substrate was cleaved.

3.2 Construction of Mutant Coxsackievirus B3 genomes

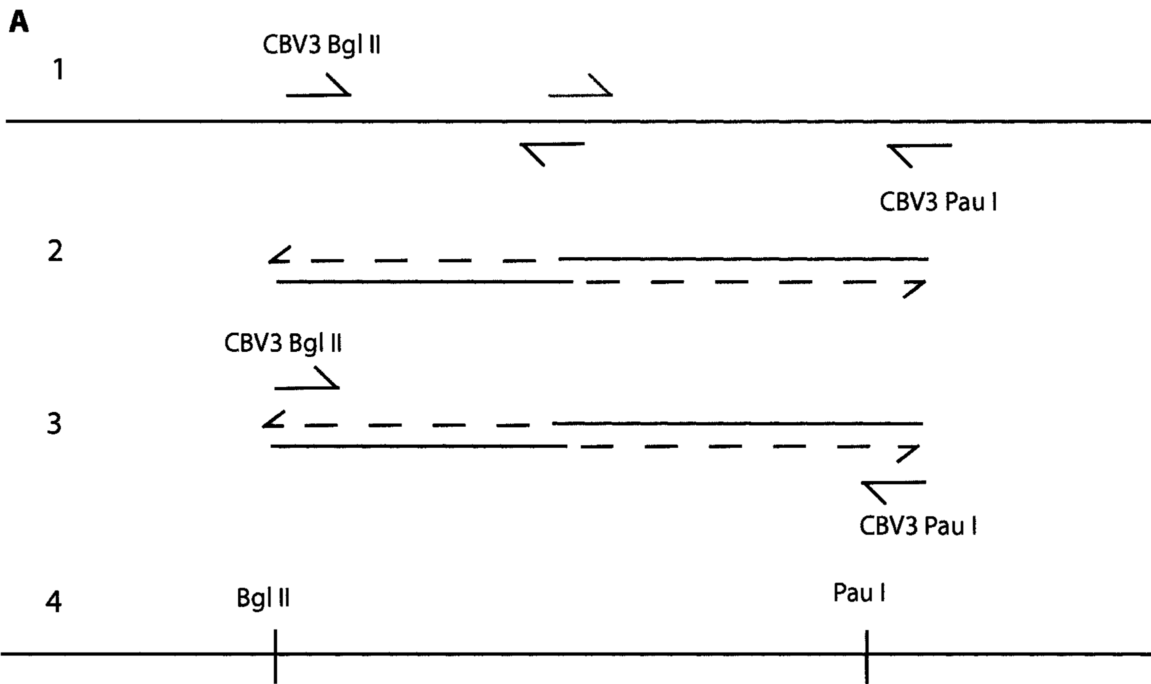
Like that of HCV, the CBV3 genome also encodes a polyprotein that is subject to proteolytic processing to produce functional proteins necessary for virus production. CBV3 encodes its own viral proteases that recognize sites within the polyprotein and cleaves within them. In order to engineer CBV3 viruses that are dependent on NS3/4A

for their replication, at least one of the endogenous cleavage sites needs to be replaced one that can be cleaved by NS3/4A.

To increase the probability that a viable mutant was produced, we chose to engineer two different genomes, one possessing an HCV cleavage site between the structural proteins VP3 and VP1 (mutCBV3 VP3/1) and the other between the non-structural proteins P2A and P2B (mutCBV3 P2A/B). The choice was made to modify structural constituents in one mutant and non-structural components in the other because both of these would potentially have different limitations on their amino acid sequence imposed by their respective functions. In either case, the same 2.3 kilobase segment (Bgl II- Pau I fragment) of the genome would be modified and replaced. The endogenous cleavage site was replaced in three steps. First two fragments with an 18 base-pair overlap in the HCV cleavage site were generated in separate PCR reactions. Next, the products underwent primer extensions to produce the final Bgl II-Pau I fragment containing the complete HCV site. This was then further amplified by PCR using appropriate flanking primers (as described in section 2.1 and Figure 5) and cloned back into the CBV3 genome. Identity of resultant genomes was verified by restriction digest and sequencing of the entire Bgl II- Pau I fragment.

3.3 Rescue of Mutant Coxsackievirus B3 Genomes

Transcription of the CBV3 genomes is driven by a T7 promoter encoded on the plasmid. In order to rescue recombinant CBV3, transcripts must be generated *in vitro* and transfected into a permissive cell line by electroporation.


A
1
CBV3 Bgl II
CBV3 Pau I
2
CBV3 Bgl II
3
CBV3 Pau I
4
Bgl II
Pau I

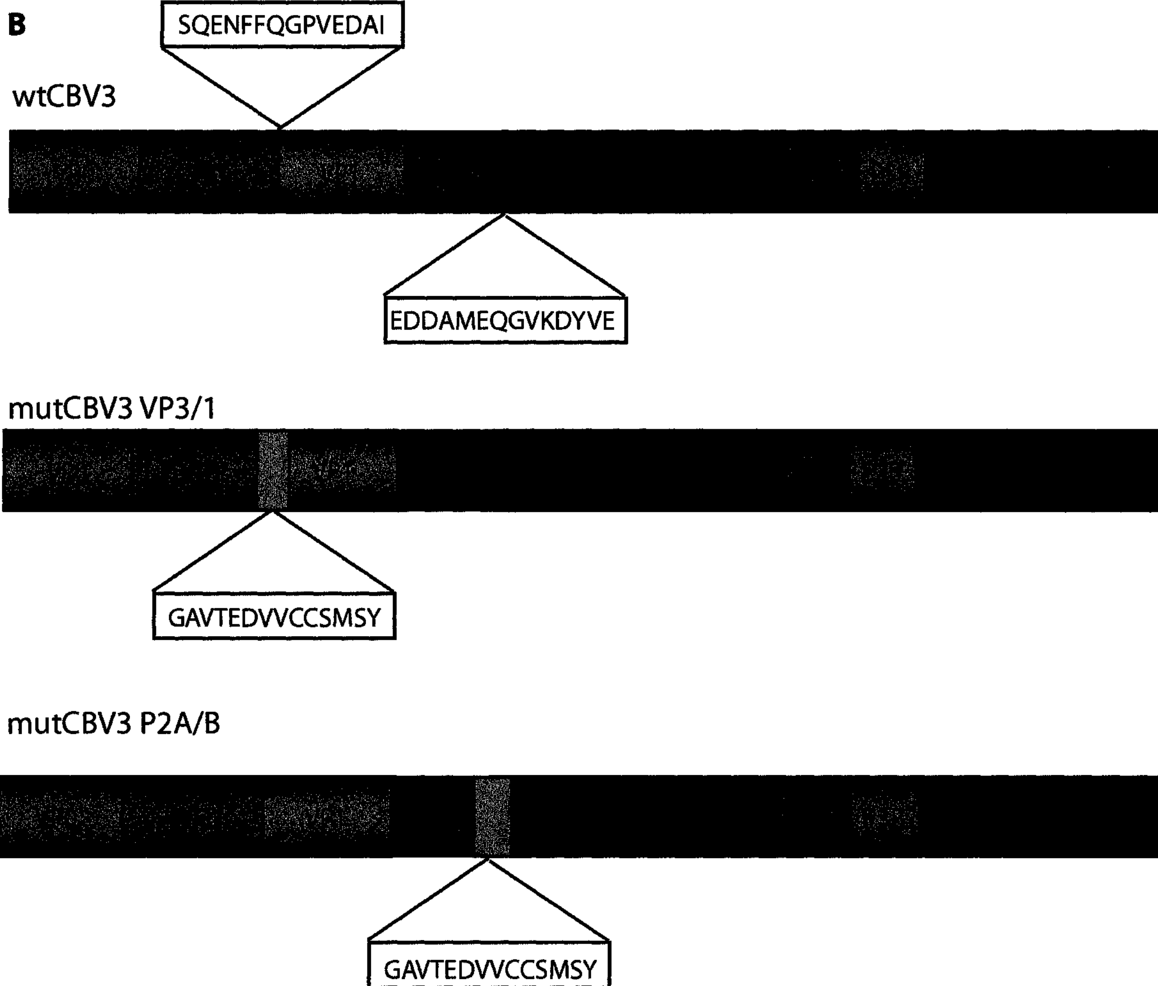

B
SQENFFQGPVEDAI
wtCBV3
EDDAMEQGVKDYVE
mutCBV3 VP3/1
GAVTEDVVCCSMSY
mutCBV3 P2A/B
GAVTEDVVCCSMSY

Figure 5: Construction of HCV Protease Dependent Coxsackievirus B3 Genomes The genomes were engineered using a 4 step process (Panel A) Step 1: Mutations were introduced into the Bgl II-Pau I fragment in two parts that had an 18 bp overlap. Step 2: The full length mutant Bgl II-Pau I fragment was produced by primer extension. Step 3: The fragment was amplified by PCR. Step 4: The modified fragment was cloned back into the parental CBV3 backbone. The resultant genomes encode GAVTEDVVCCSMSY in place of the endogenous amino acid sequence (Panel B).

3.3.1 Identification of an In vitro System for Rescuing Recombinant CBV3

To determine which cell culture system should be used for the rescue of recombinant CBV3 viruses several approaches were evaluated. HeLa, UHCV-11, Huh-7 and Huh-7 HCV cells were all investigated as potential systems for CBV3 rescue.

HeLa cells have been used by others to rescue recombinant picornaviruses (100), and to propagate CBV3 (101), but in experiments conducted here, wild type CBV3 rescued with extremely poor efficiency in HeLa cells. It is possible that requirements for rescue of recombinant CBV3 are different from those for poliovirus.

Next, we analyzed the expression profile of HCV proteins in UHCV-11 cells to determine whether it was adequate for carrying out viral rescues. NS3 protease expression levels were maintained at a high level 48 hours post-induction (Figure 6A). Despite that, UHCV-11 cells were unsuitable for use in rescues because upon induction of the tetracycline controlled promoter, toxicity due to expression of HCV proteins resulted in a significant amount of CPE even without transfection, thus making it difficult to observe CBV3 replication.

Attempts were made to find a concentration of tetracycline at which the level of HCV protein expression was suitable for viral replication, but insufficient to induce toxicity. As a first trial, UHCV-11 and UGFP cells were cultured in 0, 0.1, 0.5 and 1mg/ml of tetracycline and the number of adherent cells counted 24, 48 and 72 hours later. An abrupt difference in cell numbers between UGFP and UHCV-11 cells became obvious when the concentration of tetracycline in the culture medium was reduced to 0.1mg/ml, indicating that induction of the tetracycline-responsive promoter may have occurred at this concentration (Figure 7A). Further reducing the concentration to 0mg/ml

A

Induction Time (hrs) 0 8 16 24 48 +ve ctl

NS3

Tubulin

B

48 hours

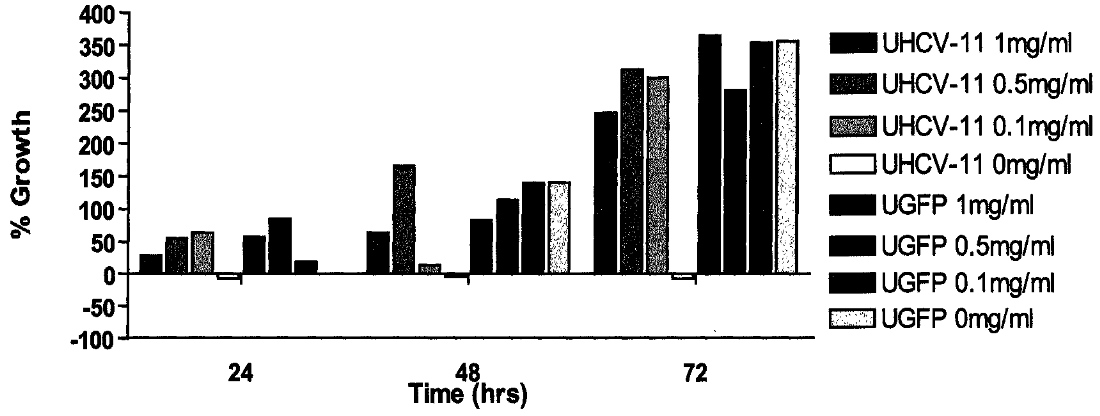
96 hours

Tet Conc. (mg/ml) 0 0.001 0.01 0.1 0 0.001 0.01 0.1

NS3

Figure 6: Patterns of NS3 Expression in UHCV-11 Cells Upon removal of tetracycline, cells reach maximal levels of expression within 16 hours and maintain them for at least 48 hours (Panel A). Titration of tetracycline concentrations did not produce a state where HCV protein expression was induced to intermediate levels conducive to viral rescue, but insufficient for CPE (Panel B).

A



B

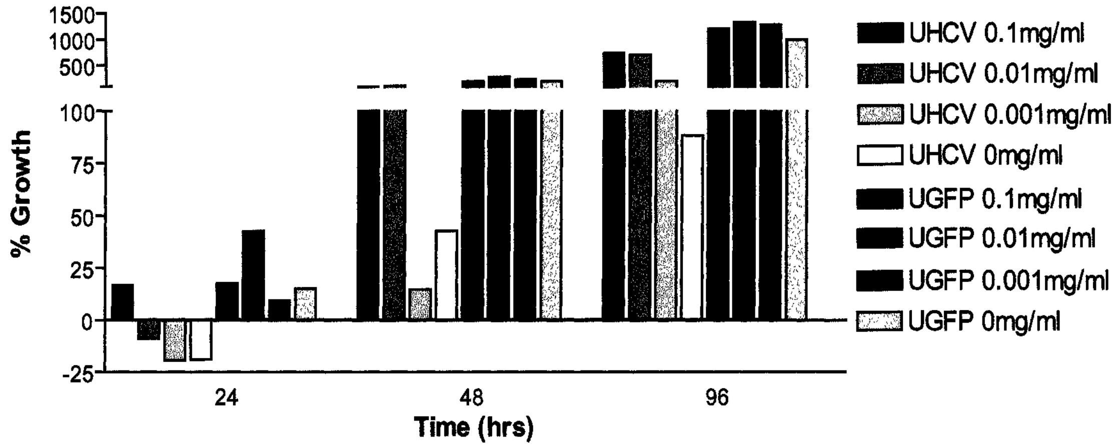


Figure 7: Varying Tetracycline Concentrations Produces Differential UHCV-11 Viability UHCV-11 and UGFP Cells were maintained in 0, 0.1, 0.5 and 1mg/ml of tetracycline. UGFP cells were used as a control since induction of GFP expression is non-toxic. The number of adherent cells was counted 24, 48 and 72 hours later (Panel A). Further analysis using concentrations of 0.1-0mg/ml suggested that at 0.001mg/ml of tetracycline, the tet-sensitive promoter may be only partially induced (Panel B).

however, produced additional toxicity, indicating to us that the induction seen at 0.1mg/ml was not maximal and perhaps it would be possible to see graded expression of HCV proteins at an intermediate concentration. A second trial was carried out with cells cultured in 0.1, 0.01, 0.001 and 0mg/ml of tetracycline. While cell counts suggested that perhaps at 0.01mg/ml of tetracycline cell viability was not compromised (Figure 7B), western blot analysis revealed that the tetracycline-sensitive promoter was still repressed. Experiments were subsequently terminated as the Huh-7 HCV cells became available to us.

Wild type CBV3 genomes were successfully rescued in Huh-7 and Huh-7 HCV cells. Cytopathogenicity was first observed 48 hours post transfection and complete death of the cell monolayer was achieved by 72 hours post-transfection. As published, Vero cells can also be used to propagate CBV3 (102). Therefore, supernatants from cultures where cell death was observed were removed and used to infect fresh monolayers of Vero cells. These cells also exhibited complete CPE within 72 hours of infection. Patterns of CPE were consistent with those of a viral infection as small foci of cell death were first observed in the population 24 hours post infection and subsequently spread throughout the monolayer. A plaque assay was also performed using Vero cells and small viral plaques were observed 72 hours post-infection (Figure 8).

Despite numerous attempts, transfection of Huh-7 HCV or Huh-7 cells with mutant CBV3 genomes however, never resulted in CPE even up to a week post-transfection.

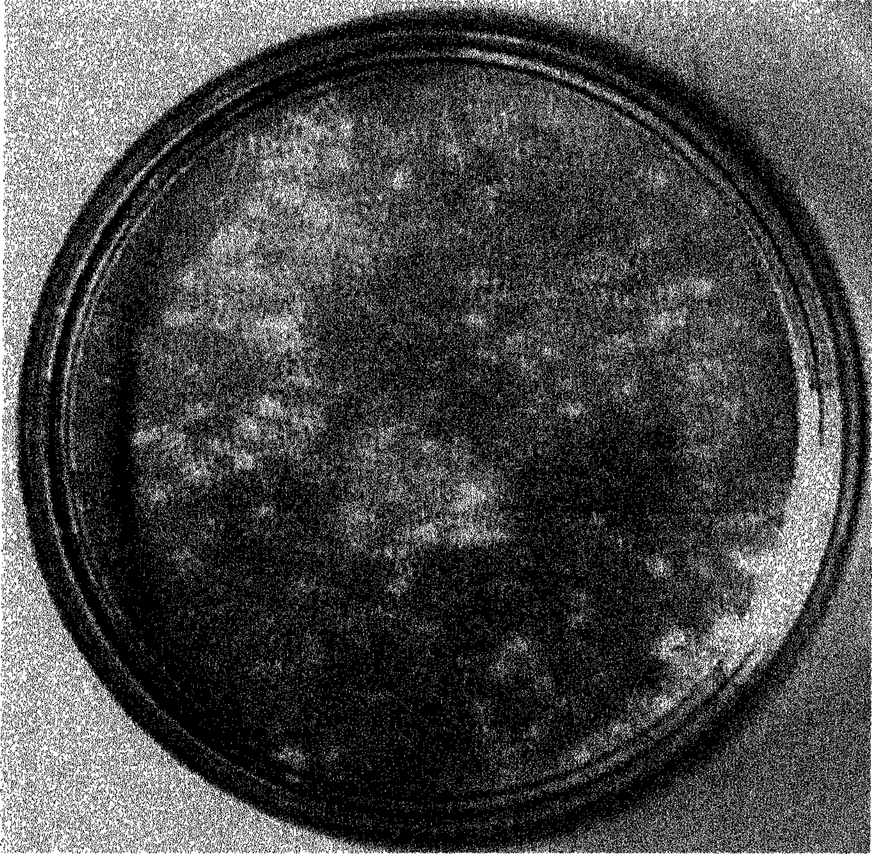
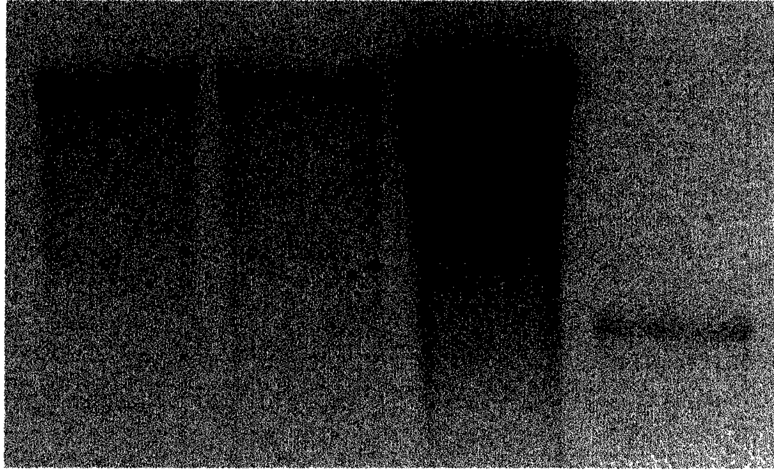


Figure 8: Rescued wild type CBV3 Produces Plaques CBV3 rescued in Huh-7 cells was plaqued on Vero cells to confirm that CPE observed in the hepatoma cell line was due to viral growth and not other factors associated with transfection of CBV3 RNA.

3.3.2 Investigating Factors That Could Inhibit Replication of Mutant CBV3

To eliminate the possibility that *in vitro* transcripts produced from mutated CBV3 templates were of inferior quality to those produced from wild type genomes, all transcripts were generated and simultaneously analyzed by agarose gel electrophoresis or used for transfections. Agarose gel electrophoresis showed the majority of the RNA running at the expected molecular weight indicating that first, most transcripts generated were full genomic-length RNA and second, that no significant RNA degradation had occurred (Figure 9). Moreover, RNA from both wild type and mutant templates was of comparable quantity and quality and as such differential quality of RNA could not explain why mutant CBV3 genomes failed to produce virus.

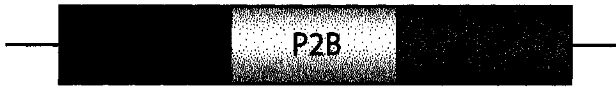
The failure to rescue mutant coxsackievirus genomes could also be due to the inability of the HCV NS3/4A protease to recognize the HCV cleavage signal engineered into coxsackievirus proteins. Studies using chimeric viruses encoding the HCV NS3 suggest that HCV NS3 could recognize and cleave non-HCV viral proteins into which its recognition site had been engineered (78,79). Nevertheless, this did not exclude that CBV3 proteins engineered in a similar manner could not be cleaved due to differences in protein folding which might mask the cleavage sequence or make it inaccessible. An important distinction between chimeric viruses and the strategy being developed here exists in that chimeric viruses encoded NS3/4A in cis- which might have made it more readily available to cleave its substrates in the viral chimeras. To investigate this, an expression construct encoding CBV3 proteins P2A/B separated by the HCV cleavage sequence was co-transfected into 293T cells either with NS3/4A or with an empty pcDNA3 vector as a control. The P2A/B protein was expressed as an N-terminal GFP



CBV3-0
mutCBV3VP3/1
mutCBV3 P2A/B
Luciferase Ctl
CBV-3 genome
Luciferase

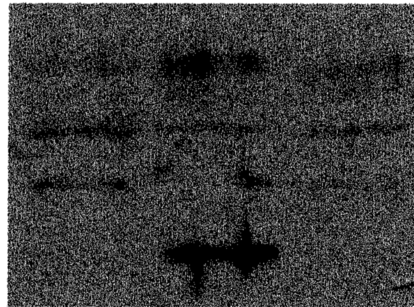
Figure 9: Comparing Quality of RNA Transcripts from Wild Type and Mutant CBV3 Genomes *In Vitro* Transcribed RNA was run on a 1% agarose gel under denaturing conditions to determine whether failure to rescue mutCBV3 could be attributed to inferior transcript quality produced from mutant template.

A

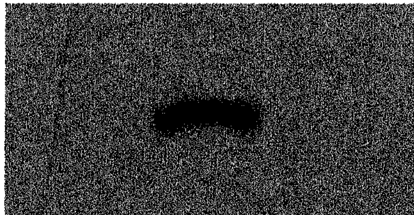


B

Substrate	+	+	-
Protease	-	+	-



— P2B:GFP



— NS3

Figure 10: Cleavage of CBV3 Proteins Containing An NS3/4A Cleavage Site A band corresponding to the size of the cleaved P2B:GFP product could be detected in protein lysates from cells co-transfected with NS3/4A and P2A/B:GFP, but not in cells transfected with P2A/B:GFP and an empty pcDNA3 control vector. Full length P2A/B:GFP could never be detected.

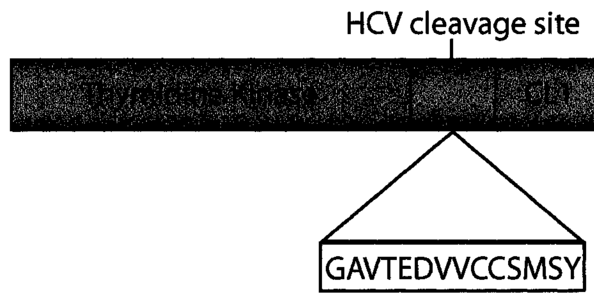
fusion so that it can be detected on Western blot. Excitingly, the HCV protease was able to recognize and cleave the CBV3 substrates as evidenced by the detection of a 35kDa protein corresponding to P2B:GFP in lanes containing both P2A/B:GFP and NS3/4A (Figure 10). Although this does not directly address whether the HCV cleavage sequence is accessible to NS3/4A when expressed as part of the viral polyprotein, it remains an encouraging observation. It is important to note that despite several attempts, the full-length P2A/B:GFP product could not be detected in any of the lysates, nor was GFP visible under fluorescent light in any of the transfected cell cultures, perhaps indicating instability of the full-length GFP fusion protein.

We were unable to explain why mutant CBV3 genomes could not be rescued; yet a final possibility remained. Although regions within 2 kilobases of the altered junctions had been sequenced prior to starting the rescues, it was entirely possible that during the cloning process, we had introduced mutations outside of these areas that may be responsible. Therefore, we undertook the task of fully sequencing the entire length of the 7.3kb genome of the mutCBV3VP3/1 and mutCBV3P2A/B mutants as well as that of wtCBV3 for comparison. As shown in the alignment report presented (Appendix B), upon re-sequencing, the mutCBV3 P2A/B genome contained a single F1018L amino acid substitution. Obviously this may explain why the mutCBV3 P2/A genome did not rescue and further work needs to be done to repair the mutation. In the mutCBV3 VP3/1 genome however, no mutations were identified outside of the targeted junctions.

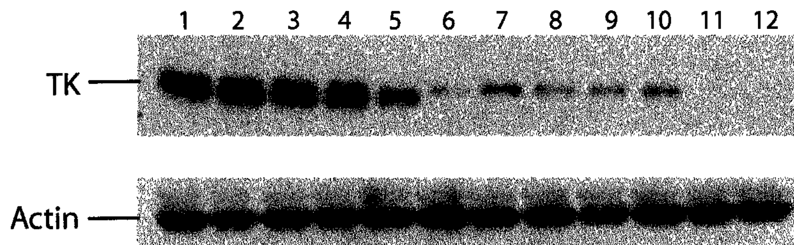
3.4 Conditional Stabilization of Thymidine Kinase in HCV NS3/4A Expressing Cells

Another approach for specifically targeting HCV-infected cells that we wanted to explore was the use of conditionally stabilized toxins. To that end, we engineered a His-

A



B



C

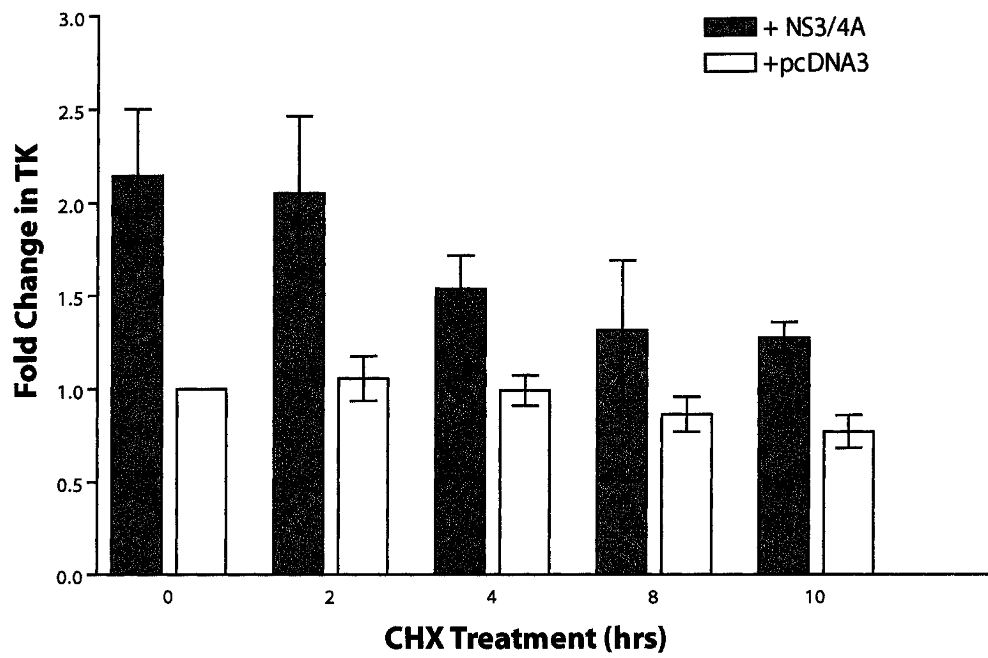


Figure 11: Differential Expression of TKCL1 in the Absence or Presence of NS3/4A
A His-tagged TKCL1 (Panel A) was transfected into U2OS cells either with NS3/4A (lanes 1-5) or a pcDNA3 empty vector (lanes 6-10). 20 hours post-transfection, cells were treated with 0.1mg/ml of CHX for 0, 2, 4, 8 and 10h before harvesting proteins. Western blots show an increase in TK levels upon co-expression of the protease (Panel B). Lanes 11 and 12 show untransfected cells treated with CHX for 0 or 10h respectively. Using densitometry, upregulation of TK is estimated at approximately 2 fold (Panel C).

tagged version of the HSV-1 enzyme TK that is attached to the CL1 degron peptide by means of an NS3/4A cleavage site (Figure 11A). The ability of a degradation signal to reduce protein stability depends on the half-life of the protein to which the degron is being attached, how accessible such a signal is to the ubiquitin conjugating machinery in the nascent or folded protein and on the strength of the degron itself (96). To determine whether the CL1 degron could efficiently destabilize thymidine kinase and whether this effect could be reversed by proteolytic removal of the degron by HCV NS3/4A, U2OS cells were co-transfected with HisTKCL1 and either NS3/4A or a pcDNA3 vector control. Cells were treated with cycloheximide (CHX) for 0, 2, 4, 8 or 10 hours before harvesting proteins in order to determine whether the rate of protein decay as well as the steady-state levels of TK are altered by the presence of the protease. Western blot analysis strongly suggested that levels of thymidine kinase were significantly higher in cells expressing NS3/4A compared to cells receiving the control pcDNA3 vector (Figure 11B). Additionally, on some blots, in lanes where TK levels were sufficiently low due to prolonged treatment with CHX, both the cleaved and the uncleaved forms could clearly be seen in the form of a doublet (Appendix C). It must be stressed that the doublet was only observed in samples from cells co-expressing NS3/4A, further demonstrating that the effects seen are probably due to the proteolytic cleavage of the degron peptide by HCV NS3/4A. Densitometry was used to analyze the signal obtained by western blot on three independent experiments. Results show an average increase of two fold in TK levels when NS3/4A was co-expressed (Figure 11C).

Confusingly, CHX treatments showed a similar half-life of TK regardless of the expression of NS3/4A. This observation was in direct conflict with the increase in levels

of TK in cells co-expressing the HCV protease, as it was hypothesized that the difference in protein levels was a direct result of a difference in the rate of protein degradation. CHX is a translational inhibitor often used to look at protein stability, but there are reports in the literature that it may also differentially affect the rate of degradation of certain proteins (103). It is perhaps not surprising that the processes of translation and protein degradation would be linked, and that changes in one process would affect the rate of the other. Therefore, for a true determination of protein half-life, pulse-chase analysis where rates of protein synthesis and degradation would remain unaltered should be undertaken.

To confirm that cells expressing NS3/4A had higher levels of HisTK than non-protease expressing counterparts, immunofluorescence was carried out on U2OS cells transfected in the same manner used for western blot analysis. In cells not expressing protease, HisTK appeared to be localized in speckles throughout the cytoplasm (Figure 12 and 13) whereas co-expression of the protease resulted in stronger TK staining being observed throughout the cytoplasm and nucleus. Staining patterns obtained using the polyclonal α-HSV-1 antibody were confirmed using the monoclonal α-His antibody used for western blot analysis (Figure 14). However, because both the α-HCV NS3 and the α-His antibodies were raised in mouse, they could not be used for the co-localization studies and the polyclonal rabbit α-HSV-1 antibody was used for that purpose instead.

Furthermore, in agreement with published reports (67), NS3/4A was clearly localized to web-like structures resembling the ER in transiently transfected U2OS cells. This is important because, although for technical reasons the presented

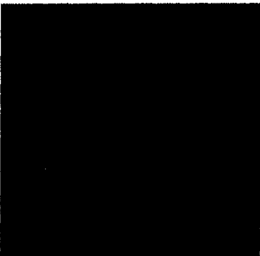
Hoescht α–HSV-1 α–NS3 Overlay

A

B

C

D



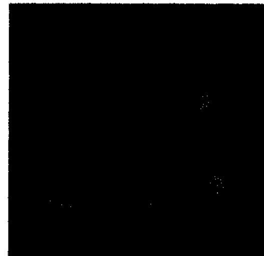
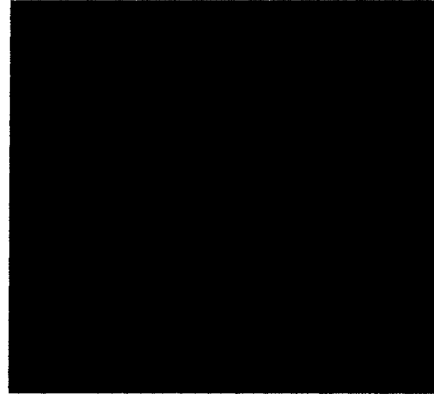


Figure 12: TK Expression is Increased in Cells Co-expressing HCV NS3/4A U2OS cells were transiently transfected with HisTKCL1 and either an empty vector control (Panel C) or NS3/4A (Panels B and D) or left untransfected (Panel A). Cells were stained for both NS3/4A and TK expression (Panels A, C and D). All pictures were taken at equivalent exposures and show an increase in levels of TK expression in cells co-expressing NS3/4A (compare Panels C and D). Background due to non-specific binding with the secondary antibodies alone is shown in Panel B.

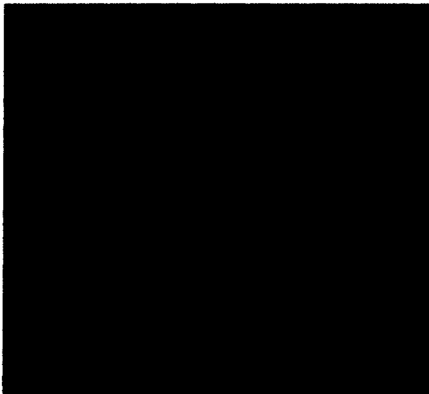
A

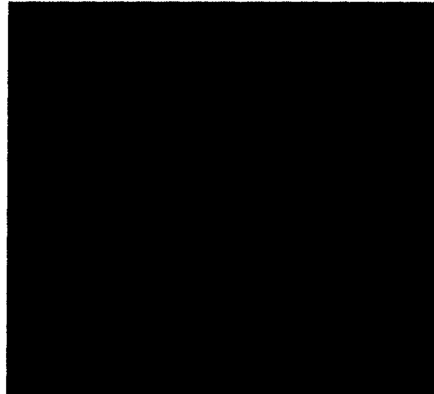
B

α–HSV-1



α–NS3





Overlay

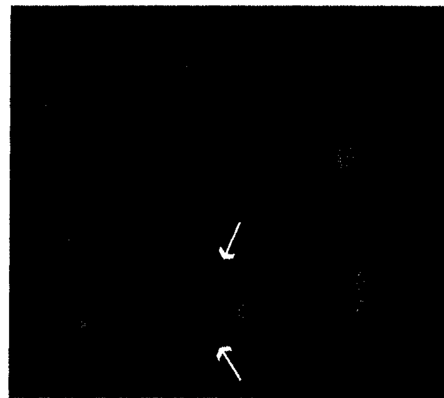
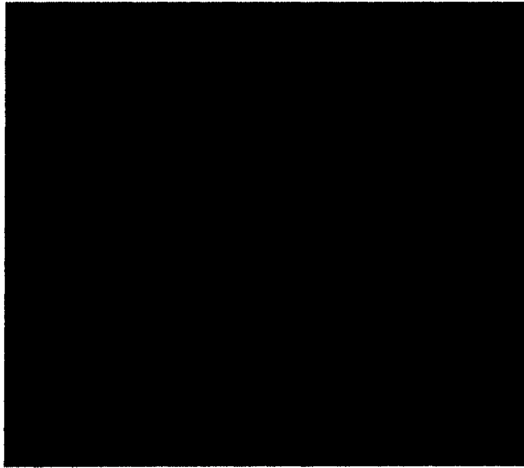


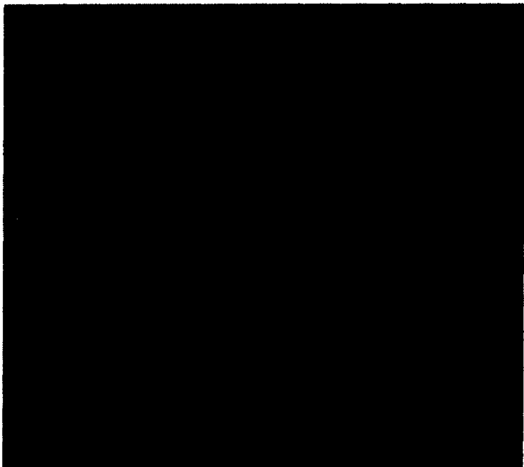
Figure 13: Localization of HisTK Is Different in The Presence of NS3/4A Cells from Panels C and D of Figure 12 were photographed at lower exposures. Staining appears to be more granular and cytoplasmic in cells transfected with HisTKCL1 and pcDNA3 (Panel A) whereas in cells transfected with both HisTKCL1 and NS3/4A, both cytoplasm and nuclei stained more strongly for the presence of TK.

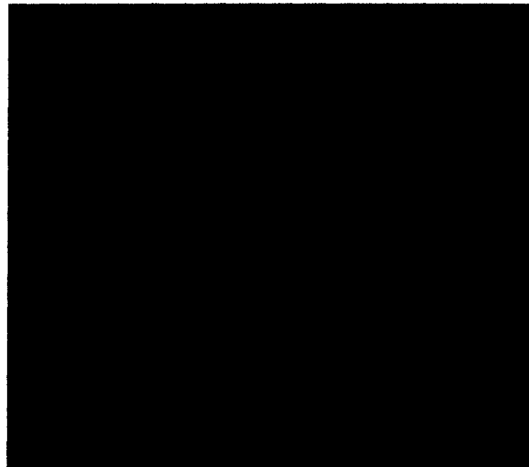
α–His α–HSV-1

A



B





C

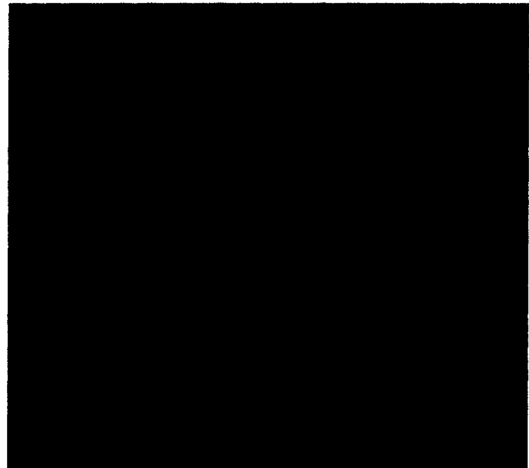


Figure 14: Confirming Patterns of Staining Obtained With Anti-HSV-1 Antibody
U2OS cells transfected with TKCL1 and pcDNA3 were stained with either α-HSV-1 and a Cy2 secondary or the α-His and a Cy3 antibody. Cells were visualized for Cy2 (Panel A) or Cy3 staining (Panel B) and show similar patterns in both cases. Overlay pictures are presented in Panel C.

studies were carried out in cells transfected with NS3/4A, and not other HCV proteins, the distribution of protease appears to be normal in these cells. This lends validity to our results and makes it more likely that our observations would hold true in UHCV-11 or Huh-7

HCV cells, than if the transiently transfected cells had exhibited an atypical pattern of NS3/4A expression – although it does not prove the fact. To get a better estimation of the fold difference in TK levels than that obtained through densitometric analysis of western blots, we sought to quantify the difference in TK levels using a second approach. Efforts were made to use flow cytometry to measure the difference in TK expression levels. Cells were transfected in a manner analogous to that described for both western blot and immunofluorescence analysis and TK was labelled using the same antibodies used for immunofluorescence. Unfortunately, the available antibodies were not suitable for use in flow cytometry and did not yield useful results (Figure 15). The fluorescent signal obtained was extremely low, although the GFP transfection control showed that roughly 30% of cells were transfected. Although inconclusive, the flow data did show a 32% increase in mean Cy2 fluorescence when cells were cotransfected with NS3/4A and TKCL1 compared to cells transfected with TKCL1 and control vector. Taken together, the results obtained from flow cytometry, immunofluorescence and immunoblot clearly indicate that first, the degron sequence targeted TK for degradation and second, that NS3/4A was able to cleave the degron peptide efficiently enough so as to induce a significant increase in expression level and altered sub cellular localization.

Deg Stability 2.131

Counts; M2; M1; DIO

Deg Stability 2.131

Counts; R3; PI

Deg Stability 2.133

Counts; M2; M1; DIO

Deg Stability 2.133

Counts; R3; PI

Deg Stability 2.134

Counts; M2; M1; DIO

Deg Stability 2.134

Counts; R3; PI

Deg Stability 2.132

Counts; M2; M1; DIO

Deg Stability 2.132

Counts; R3; PI

Deg Stability 2.135

Counts; M2; M1

Deg Stability 2.135

Counts; R3

Figure 15: Quantification of Fold Difference in TK Expression by Flow Cytometry
Intensity of the fluorescence signal was low, although the GFP transfection control indicated that roughly 30% of cells were transfected. The percentage of cells that were in the highly expressing group (gate M1) remained relatively unchanged between treatments, but mean fluorescence in the low expressing group (gate M2) increased with the co-expression of NS3/4A. Increase in fluorescence compared to the untransfected group is indicated*. Cells that were stained with either of the two secondary antibodies alone are included for comparison.

4. DISCUSSION

4.1 HCV NS3/4A Cleaves Substrates in Trans-

Both intra- and inter- molecular cleavage activities had previously been reported for NS3/4A, but with the exception of a study by Bartenschlager et al (36) which used recombinant vaccinia viruses to express parts of the HCV polyprotein, most work was done with constructs encoding both protease and substrate (79,98). Although central to our understanding of the dynamics of HCV polyprotein processing, the results obtained with the vaccinia needed to be repeated in a separate system. A key issue was that proteins expressed during the course of vaccinia replication would be co-localized to the cellular compartment where viral replication was occurring and that in the absence of this structure, enzyme and substrate would simply not encounter one another. The assay described here was a logical first step in the design of our strategy and in addition to confirming that the engineered protease was functional, it demonstrated that NS3/4A could cleave a substrate that is expressed separately and that contains non-HCV proteins fused to it. Furthermore, the use of two separate systems where HCV proteins were stably expressed confirmed that this phenomenon is reproducible. The results obtained in the Huh-7 HCV cell line are particularly significant as these cells contain replicating HCV complexes and serve as the closest approximation of a natural HCV infection available. The fact that levels of substrate cleavage were comparable in both the transient transfections and the Huh-7 HCV system (Figure 4) indicated that in cells actively replicating HCV genomes, the protease is not completely sequestered in the replication complex and is still available to cleave an exogenously supplied substrate.

4.2 Replication of mutant CBV3: Conditional or Non-existent?

Several studies have reported the construction of chimeric viruses dependent on cleavage by a co-encoded HCV protease for replication (78-80). In this study, we describe attempts to construct an HCV NS3/4A dependent CBV3 virus that does not encode HCV NS3/4A in its genome. The mutCBV3 viruses would be dependent on the trans-expression of the HCV protease in the cells where they can propagate. We were unable to rescue the genomes of either of the two variants although initial sequencing indicated an absence of any mutations outside of the targeted sequence. Upon re-sequencing however, we were able to identify one amino acid substitution in one of the genomes, and the existence of more mutations cannot be completely ruled out. Although the F1018L substitution may explain why we were unable to rescue the mutCBV3P2A/B genome, it cannot explain our failure to observe any replication of the mutCBV3 VP3/1 genome. To investigate whether the F1018L mutation affects our ability to rescue virus, we will swap this region between the mutant and wild type CBV3 genomes to generate a wtCBV3 with F1018L and a mutCBV3P2A/B wt at position 1018. Regardless of the presence of any mutations, there exist several other potential factors that can prevent the rescue of the conditionally replicating viral genome.

Preliminary studies discussed here show that the junction between CBV3 P2A/B containing an HCV cleavage site can be cleaved by NS3/4A when the target protein is transiently expressed as a GFP fusion, but it cannot be ruled out that NS3/4A is unable to access or cleave the engineered cleavage site in the mutCBV3 polyprotein.

Alternately, the efficiency or the timing of the trans-cleavage may not be conducive to viral replication. There is evidence in the literature that cleavage

intermediates play an important role in the replication of plus-stranded RNA viruses and that their functions are distinct from those of the final protein products. As demonstrated for Semliki Forest Virus, the precursor proteins P123 and P1234 are essential for the formation of cytopathic vacuoles (CPVs), modified lysosomes and endosomes that are the site of alphavirus replication (104). It is conceivable that altering the ratio of precursor to mature proteins can change the rate of viral replication and may even inhibit it in some cases. In a study by Kusov et al (105), increasing cleavage efficiency of the 3A/B junction of Hepatitis A picornavirus (HAV) impaired processing of structural HAV proteins and resulted in the retarded assembly of defective capsid particles. Importantly, *in vitro* transcribed mutant genomes where cleavage of 3A/B was either enhanced or inhibited failed to initiate replication and produce infectious particles indicating that viral replication requires tightly regulated ratios of the 3AB or 3ABC precursor and its cleavage products.

In addition to the possible difference in ratios of precursor to processed CBV3 proteins between the wild type and mutant CBV3 genomes, the extreme C-terminal residues of VP3 and P2A and the N-terminal residues of VP1 and P2B are altered. To engineer the viruses, the seven terminal amino acids of the proteins on either side of the junction were substituted with the fourteen-amino acid sequence that occurs naturally at the NS5A/B site. This change in the amino acid sequence alone may be sufficient to impair function of the CBV3 proteins. In poliovirus P2A, the four C-terminal residues are required for viral RNA replication although they are not essential for the protease function of the protein (106). Interestingly, the replication defect could not be rescued by the substitution of an acidic amino acid cluster (common to all enteroviruses) from

human rhinovirus type2 (HRV2). The reverse chimera where the C-terminus of poliovirus P2A was engineered into a HRV2 background was still viable suggesting that this strict requirement for a specific sequence is not common to all enteroviruses, yet it remains possible that one exists for CBV3. Although non-structural proteins may be able to tolerate amino acid substitutions in non-essential residues, viral capsid assembly poses more severe limitations on amino acid sequence of structural proteins. In order for virion assembly to occur, the various coat proteins must be able to interact in a very specific manner forming repeating structures that then assemble into a viral capsid. Thus, it was not altogether surprising that the VP3/1mutCBV3 was not viable.

A concern for our studies has been that viral replication is not entirely defective, but rather sufficiently attenuated so that viral rescues from *in vitro* transcribed genomes are rare. The frequency of viral rescues might be improved by expression of wild type CBV3 proteins. There is precedent for this approach in the literature as several studies with replication defective viruses have demonstrated that the phenotype can be rescued through trans-complementation with plasmids that encode wildtype viral proteins (104,105,107). We have constructed two such helper plasmids encoding wtVP3/1 and wtP2A/B to be used in future attempts of viral rescues. It is quite possible that the helper plasmids will only be required for the first round of replication and that once high titres of virus are produced, subsequent infections at high multiplicities of infection will result in observable CPE due to viral replication. If after rescuing virus, it cannot be further propagated without helper plasmids however, then it would be reasonable to conclude that mutations introduced into the CBV3 genome have completely abrogated replication of the virus instead of simply rendering it conditionally replicative.

4.3 Challenges and Advantages of Viral Therapeutics for HCV

In the event that an HCV-dependent virus can indeed be produced, several potential hurdles to its efficacy still exist. First, although we were able to demonstrate cleavage of CBV3 proteins in a transient assay, the levels of NS3/4A protease in infected hepatocytes may not be sufficient *in vivo*. One study using hepatocytes from HCV infected patients showed that NS3 and other viral proteins can be detected in about 24% of chronically infected patients that were positive for the C100-3 anti-HCV antibody (4), an antibody whose presence is indicative of infection with HCV. A separate study indicated that roughly 4% of hepatocytes exhibited positive staining using NS3 anti-sera (108). What the data does not indicate is the proportion of infected cells (i.e. those harbouring HCV RNA) that stained positively for viral proteins, including NS3. The limitations that such parameters will pose for our strategy will have to be addressed experimentally using the animal models of HCV and liver biopsy samples from patients with chronic HCV. Another complicating factor will be the level of attenuation of the virus. There will likely exist a trade-off between specificity and efficacy of the virus. A virus that is completely unable to replicate in the absence of the protease may be desirable from the point of view of limiting toxicity, but will likely not be very efficacious. By immunohistochemical staining at least, infected hepatocytes exist as clusters of infected cells in an uninfected background (4), thus elimination of infected cells would require the virus to spread between infected populations by replicating in uninfected hepatocytes. A virus that cannot do this will be limited in its ability to clear infected cells, although this can probably be overcome with multiple dosing.

Even if viral infection and replication are unable to directly mediate clearance of target cells, viral therapy remains an extremely attractive option. A case in point: there is mounting evidence in the field of oncolytic viruses that infecting even a few cells within a tumour can serve to boost the immune response against the tumours (109-111). Work currently underway in our laboratory suggests that in fact, despite excellent efficacy viral infection of tumours by Vesicular Stomatitis Virus (VSV) is very limited and that a healthy immune system may be essential to achieving tumour clearance (Paterson J and Power A, unpublished results). Furthermore, preliminary work points to the possibility that the observed immune stimulation may be a function of CD8+ cytotoxic T-lymphocytes (CTL) and that this requires viral replication (Parato K, personal communication). This raises an intriguing possibility for immune therapy of HCV. It is well known that there exists both a cellular and a humoral immune response to HCV infection, yet neither is effective at mediating viral clearance as evidenced by the high rates of persistent infection with the virus. The general consensus is that first, like many slowly replicating viruses, HCV infection simply does not elicit a strong and well co-ordinated immune response although components of that response are present. Secondly, there is evidence that a robust T-cell response may be essential for resolving HCV infection (112) and that HCV proteins actively suppress T_H cells (113). Thus, infection of HCV-infected liver by a virus that can selectively replicate in infected hepatocytes may provide the stimulus needed to break the relative immune tolerance for HCV and set the stage for immune-mediated clearance of the infection.

4.4 Regulation of Thymidine Kinase levels by NS3/4A

As an alternative approach to conditionally replicating viruses, we have constructed a conditionally stable version of the suicide gene HSV-1 TK. Similar approaches had been investigated for possible treatment of HIV-1. In one case, the cleavage site of procaspase-3 was changed into an HIV-1 protease site and procaspase was successfully cleaved into active caspase-3, effectively inducing apoptosis in HIV-1 infected cells (83). In another case, diphtheria toxin was destabilized by the N-end rule pathway. The degradation signal was engineered upstream of an HIV-1 protease cleavage site. Although this system failed to eradicate HIV infected cells due to low levels of protease in these cells (114), it did at least in principle demonstrate that conditional stabilization of toxins by proteolytic cleavage was a possible route to pursue. In this work, we showed conditional stabilization of TK through cleavage of a C-terminal degradation signal. We estimated that co-expression of the protease increased TK levels by about 2 fold and we hypothesize that this was due to cleavage of the CL1 degron by NS3/4A. A small reduction in size of TK detected in protein extracts of cells transfected with NS3/4A is consistently observed in separate experiments and in some blots, two bands were observed, likely the cleaved and the uncleaved forms of TKCL1. Having said that however, assessment of TK half-life in the presence of the translational inhibitor CHX showed no difference in the presence or absence of HCV protease. Additionally, protein levels remained relatively stable up to 8 hours after treatment and only appeared to diminish at 10 hours post-treatment. It is entirely possible that inhibition of translation affected protein degradation through the ubiquitin pathway. By inhibiting translation, CHX can deplete levels of other proteins needed for degrading TK. It has been

demonstrated for example, that in yeast, treatment with CHX depletes free ubiquitin pools (115).

Although unlikely in our opinion, another possibility is that expression of NS3/4A affects the rate of transcription or translation of TK. Expression of HCV proteins has been shown to induce endoplasmic reticulum stress (ER-stress) and lead to the activation of the PKR-like ER kinase (PERK) (116) . This usually has the effect of transcriptional induction of a specific subset of cells, translational inhibition through phosphorylation of eIF2-α and protein degradation (117), but studies with Huh-7 HCV have shown reduced levels of eIF2-α phosphorylation and 6-fold increase in the levels of cap-dependent translation despite the activation of PERK and other downstream factors in these cells (116). Most reports cite a role for NS5A in this phenomenon and it is unclear whether expression of NS3/4A alone can stimulate the unfolded protein response (UPR). Pulse-chase analysis to determine whether in fact the observed difference in TK levels is due to differential stabilization is currently underway and should help elucidate the exact mechanism of observed TK upregulation.

We did not undertake any assays where cells were transiently transfected with TKCL1 with or without NS3/4A and treated with Gancyclovir because we felt that in the absence of a cell line that stably expresses TKCL1 either alone or with NS3/4A, loss of the plasmid over the course of the experiment would complicate interpretation of results. Future work with this system includes the production of a retrovirus expressing TKCL1 that can be used to stably transduce Huh-7 and Huh-7 HCV cells. This would enable an assessment of both the extent of stabilization and differential killing that can be achieved in that cell line.

It is likely that due to the strong bystander effect of TK discussed earlier, a two-fold difference in TK levels will not translate into a two fold difference in cell death and that a stronger degradation signal is required. On the other hand, results from immunofluorescent staining of transfected cells show a possible difference in the localization of TK under the two sets of conditions that can work to enhance the difference in total levels of the protein. TK activity requires the protein to be present in the nucleus and it is possible that because whatever TK is available in cells not expressing NS3/4A appears to be sequestered in the cytoplasm, the actual difference in TK activity is greater than 2-fold. In any case, these possibilities still need to be addressed experimentally. Due to the promise shown by these preliminary studies, further work is already underway in our laboratory to construct a library to select the optimal degradation signal for this system using a Hygromycin/Thymidine Kinase fusion protein. By selecting for a stronger degradation signal, we might be able to increase the difference in TK levels in cells that are expressing NS3/4A compared to those that do not. There are also plans to extend this system to other toxic proteins or by using other signals that regulate activity by controlling parameters such as sub-cellular localization for example.

Another interesting approach that would combine the potential benefits of a replicating virus with the promise shown by the conditional degradation approach is the construction of a virus with conditionally stable gene products. In the case of a virus such as CBV for example, the entire polyprotein could be destabilized, thus avoiding the complications of altering ratios of various proteins discussed above.

4.5 On The Road to a Cure: Elimination versus Suppression

Traditionally, design of antimicrobial agents has been based on their ability to inhibit various steps of the viral replicative cycle. From general inhibitors of viral replication such as interferon to highly specific enzymatic inhibitors, all have the end result of suppressing viral replication. In acute infections such as influenza, suppression of viral replication improves rates of clearance and reduces symptoms normally associated with viral replication (118). For chronic infections such as HIV, HCV and Hepatitis B Virus (HBV) however, therapy rarely if ever, results in elimination of the virus (119-121). At best, the antiviral drugs are able to slow the progress of infection and delay manifestations of the disease until resistant variants emerge through genetic mutation.

For HCV researchers, the landscape today bears a striking similarity to that of HIV research in the eighties. The aim for the overwhelming majority of drug-discovery efforts has been to discover inhibitors of HCV replication at a multiplicity of steps. The recent development of cell culture systems and animal models as well as the determination of 3D structures of several key HCV proteins has enabled these endeavours and for the first time since cloning of the HCV genome, the development of a plethora of viral inhibitors akin to those available to HIV patients seems possible. There are other lessons to be learnt from HIV however. As even the most superficial survey of HIV literature will reveal, resistance to therapy has emerged as a huge and growing concern. A rapidly mutating virus, such as HIV (and indeed HCV) will quickly give rise to a great number of mutant progeny, and chances are, several of them will be resistant to one compound or another. Thus, even as the inhibitors we develop become more potent,

history suggests that relying on suppression of microbial growth as a means of controlling infection will almost certainly result in a perpetual race to develop novel compounds as pathogens evolve to evade existing ones. As an alternative approach, the work described here represents a different approach to the problem. The therapeutics proposed here do not simply slow the rate of viral replication, but rather work by potentially eliminating the very factories of viral production, either through direct killing or immune stimulation (122). In doing so, they would slow the rate of viral spread and eventually eradicate the virus altogether. Additionally, the design of the proposed therapeutics is such that the development of resistance although not impossible, would be extremely difficult.

Although very preliminary, the work presented here is encouraging and represents a step forward towards understanding both the limitations within which these therapeutics have to function and the endless possibilities for their design. It is our hope that future endeavours will overcome some of the problems described here and that they will put us well on our way towards a cure not only for HCV, but potentially for HIV and other chronic infections.

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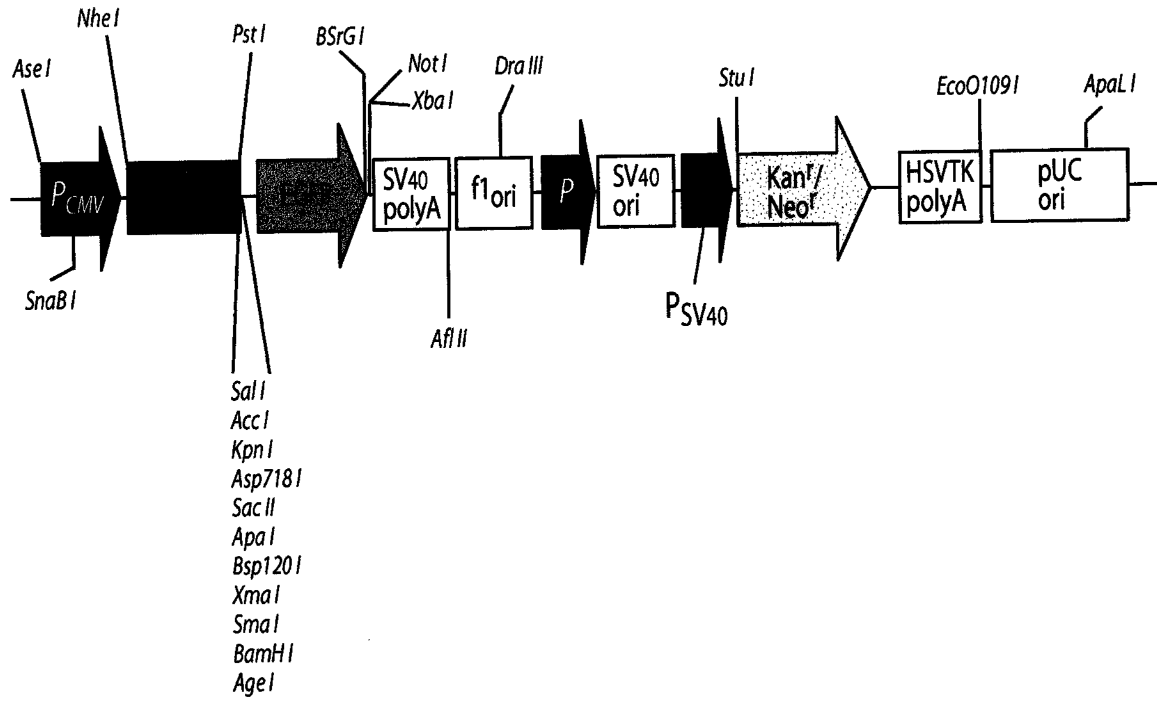
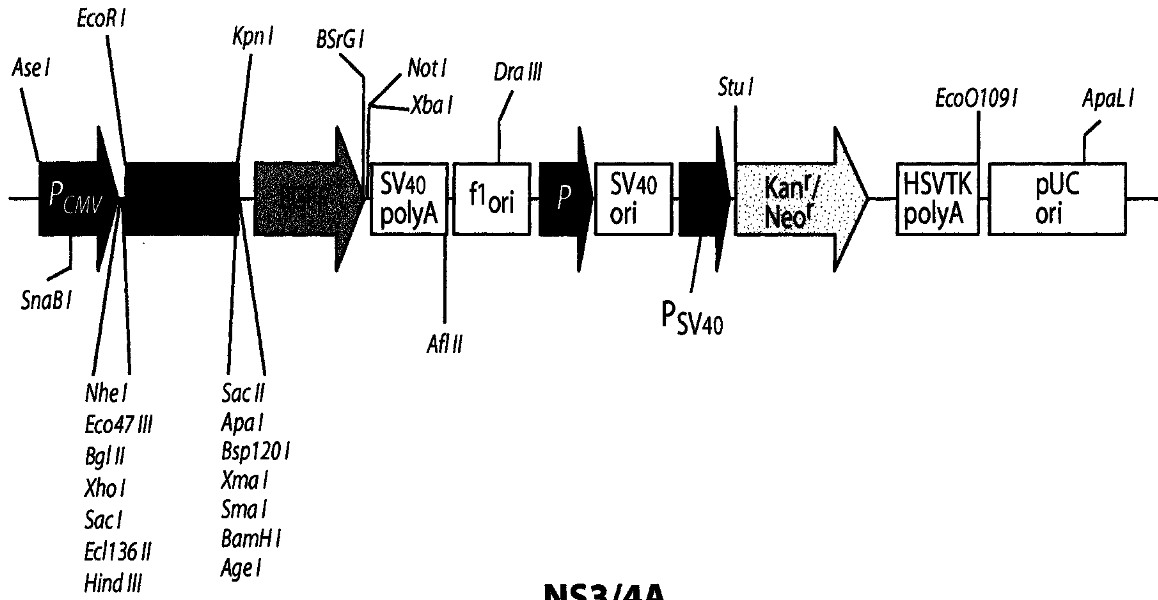
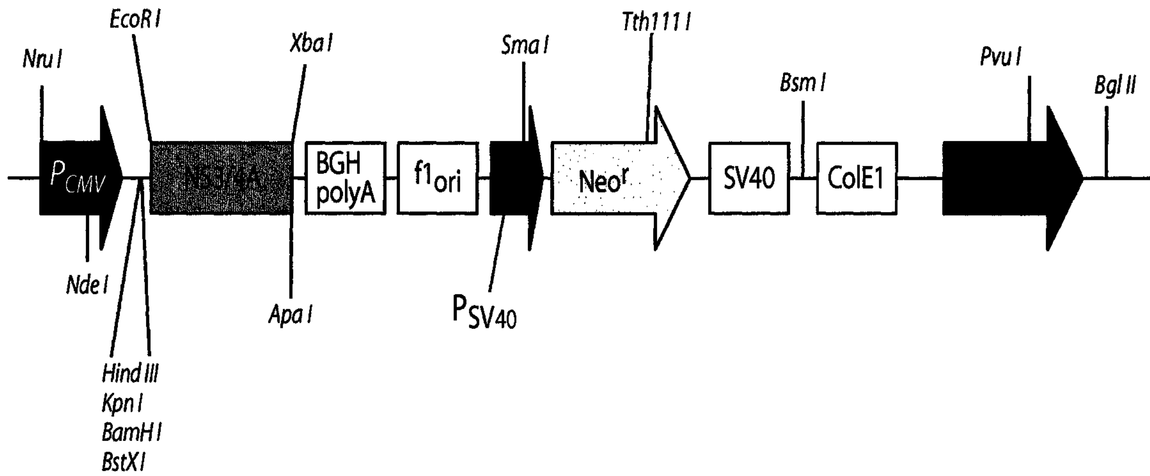
mutP2A/B:GFP



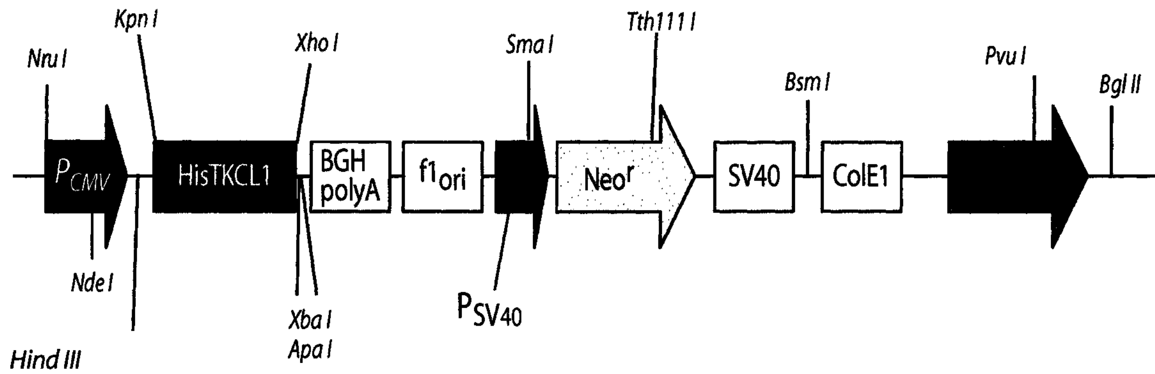
NS5A/B:GFP



NS3/4A



HisTKCL1



APPENDICES

Appendix A Maps of expression constructs NS5A/B:GFP, NS3/4A, TKCL1 and mutP2A/B:GFP.

Start	Sequence	Name
	M G A Q V S T Q K T G A H E T G L N A S	Majority
	10 20	
1	M G A Q V S T Q K T G A H E T G L N A S	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1	M G A Q V S T Q K T G A H E T G L N A S	CBV3 VP3-1 c3-1 Complete ORF.PRO
1	M G A Q V S T Q K T G A H E T G L N A S	CBV3-0 Complete Consensus ORF.PRO
	G N S I I H Y T N V N Y Y K D A A S N S	Majority
	30 40	
21	G N S I I H Y T N V N Y Y K D A A S N S	CBV3 P2A-B c7-2-1 Complete ORF.PRO
21	G N S I I H Y T N V N Y Y K D A A S N S	CBV3 VP3-1 c3-1 Complete ORF.PRO
21	G N S I I H Y T N V N Y Y K D A A S N S	CBV3-0 Complete Consensus ORF.PRO
	A N R Q D F T Q D P G K F T E P V K D I	Majority
	50 60	
41	A N R Q D F T Q D P G K F T E P V K D I	CBV3 P2A-B c7-2-1 Complete ORF.PRO
41	A N R Q D F T Q D P G K F T E P V K D I	CBV3 VP3-1 c3-1 Complete ORF.PRO
41	A N R Q D F T Q D P G K F T E P V K D I	CBV3-0 Complete Consensus ORF.PRO
	M I K S L P A L N S P T V E E C G Y S D	Majority
	70 80	
61	M I K S L P A L N S P T V E E C G Y S D	CBV3 P2A-B c7-2-1 Complete ORF.PRO
61	M I K S L P A L N S P T V E E C G Y S D	CBV3 VP3-1 c3-1 Complete ORF.PRO
61	M I K S L P A L N S P T V E E C G Y S D	CBV3-0 Complete Consensus ORF.PRO
	R A R S I T L G N S T I T T Q E C A N V	Majority
	90 100	
81	R A R S I T L G N S T I T T Q E C A N V	CBV3 P2A-B c7-2-1 Complete ORF.PRO
81	R A R S I T L G N S T I T T Q E C A N V	CBV3 VP3-1 c3-1 Complete ORF.PRO
81	R A R S I T L G N S T I T T Q E C A N V	CBV3-0 Complete Consensus ORF.PRO
	V V G Y G V W P D Y L K D S E A T A E D	Majority
	110 120	
101	V V G Y G V W P D Y L K D S E A T A E D	CBV3 P2A-B c7-2-1 Complete ORF.PRO
101	V V G Y G V W P D Y L K D S E A T A E D	CBV3 VP3-1 c3-1 Complete ORF.PRO
101	V V G Y G V W P D Y L K D S E A T A E D	CBV3-0 Complete Consensus ORF.PRO
	Q P T Q P D V A T C R F Y T L D S V Q W	Majority
	130 140	
121	Q P T Q P D V A T C R F Y T L D S V Q W	CBV3 P2A-B c7-2-1 Complete ORF.PRO
121	Q P T Q P D V A T C R F Y T L D S V Q W	CBV3 VP3-1 c3-1 Complete ORF.PRO
121	Q P T Q P D V A T C R F Y T L D S V Q W	CBV3-0 Complete Consensus ORF.PRO
	Q K T S P G W W W K L P D A L S N L G L	Majority
	150 160	
141	Q K T S P G W W W K L P D A L S N L G L	CBV3 P2A-B c7-2-1 Complete ORF.PRO
141	Q K T S P G W W W K L P D A L S N L G L	CBV3 VP3-1 c3-1 Complete ORF.PRO
141	Q K T S P G W W W K L P D A L S N L G L	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	F G Q N M Q Y H Y L G R T G Y T V H V Q	Majority
	170 180	
161	F G Q N M Q Y H Y L G R T G Y T V H V Q	CBV3 P2A-B c7-2-1 Complete ORF.PRO
161	F G Q N M Q Y H Y L G R T G Y T V H V Q	CBV3 VP3-1 c3-1 Complete ORF.PRO
161	F G Q N M Q Y H Y L G R T G Y T V H V Q	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	C N A S K F H Q G C L L V V C V P E A E	Majority
	190 200	
181	C N A S K F H Q G C L L V V C V P E A E	CBV3 P2A-B c7-2-1 Complete ORF.PRO
181	C N A S K F H Q G C L L V V C V P E A E	CBV3 VP3-1 c3-1 Complete ORF.PRO
181	C N A S K F H Q G C L L V V C V P E A E	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	M G C A T L D N T P S S A E L L G G D S	Majority
	210 220	
201	M G C A T L D N T P S S A E L L G G D S	CBV3 P2A-B c7-2-1 Complete ORF.PRO
201	M G C A T L D N T P S S A E L L G G D S	CBV3 VP3-1 c3-1 Complete ORF.PRO
201	M G C A T L D N T P S S A E L L G G D S	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	A K E F A D K P V A S G S N K L V Q R V	Majority
	230 240	
221	A K E F A D K P V A S G S N K L V Q R V	CBV3 P2A-B c7-2-1 Complete ORF.PRO
221	A K E F A D K P V A S G S N K L V Q R V	CBV3 VP3-1 c3-1 Complete ORF.PRO
221	A K E F A D K P V A S G S N K L V Q R V	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	V Y N A G M G V G V G N L T I F P H Q W	Majority
	250 260	
241	V Y N A G M G V G V G N L T I F P H Q W	CBV3 P2A-B c7-2-1 Complete ORF.PRO
241	V Y N A G M G V G V G N L T I F P H Q W	CBV3 VP3-1 c3-1 Complete ORF.PRO
241	V Y N A G M G V G V G N L T I F P H Q W	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	I N L R T N N S A T I V M P Y T N S V P	Majority
	270 280	
261	I N L R T N N S A T I V M P Y T N S V P	CBV3 P2A-B c7-2-1 Complete ORF.PRO
261	I N L R T N N S A T I V M P Y T N S V P	CBV3 VP3-1 c3-1 Complete ORF.PRO
261	I N L R T N N S A T I V M P Y T N S V P	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	M D N M F R H N N V T L M V I P F V P L	Majority
	290 300	
281	M D N M F R H N N V T L M V I P F V P L	CBV3 P2A-B c7-2-1 Complete ORF.PRO
281	M D N M F R H N N V T L M V I P F V P L	CBV3 VP3-1 c3-1 Complete ORF.PRO
281	M D N M F R H N N V T L M V I P F V P L	CBV3-0 Complete Consensus ORF.PRO

	Sequence	
	D Y C P G S T T Y V P I T V T I A P M C	Majority
	310 320	
301	D Y C P G S T T Y V P I T V T I A P M C	CBV3 P2A-B c7-2-1 Complete ORF.PRO
301	D Y C P G S T T Y V P I T V T I A P M C	CBV3 VP3-1 c3-1 Complete ORF.PRO
301	D Y C P G S T T Y V P I T V T I A P M C	CBV3-0 Complete Consensus ORF.PRO

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    A E Y N G L R L A G H Q G L P T M N T P  Majority
                      330                340
321 A E Y N G L R L A G H Q G L P T M N T P  CBV3 P2A-B c7-2-1 Complete ORF.PRO
321 A E Y N G L R L A G H Q G L P T M N T P  CBV3 VP3-1 c3-1 Complete ORF.PRO
321 A E Y N G L R L A G H Q G L P T M N T P  CBV3-0 Complete Consensus ORF.PRO

    G S C Q F L T S D D F Q S P S A M P Q Y  Majority
                      350                360
341 G S C Q F L T S D D F Q S P S A M P Q Y  CBV3 P2A-B c7-2-1 Complete ORF.PRO
341 G S C Q F L T S D D F Q S P S A M P Q Y  CBV3 VP3-1 c3-1 Complete ORF.PRO
341 G S C Q F L T S D D F Q S P S A M P Q Y  CBV3-0 Complete Consensus ORF.PRO

    D V T P E M R I P G E V K N L M E I A E  Majority
                      370                380
361 D V T P E M R I P G E V K N L M E I A E  CBV3 P2A-B c7-2-1 Complete ORF.PRO
361 D V T P E M R I P G E V K N L M E I A E  CBV3 VP3-1 c3-1 Complete ORF.PRO
361 D V T P E M R I P G E V K N L M E I A E  CBV3-0 Complete Consensus ORF.PRO

    V D S V V P V Q N V G E K V N S M E A Y  Majority
                      390                400
381 V D S V V P V Q N V G E K V N S M E A Y  CBV3 P2A-B c7-2-1 Complete ORF.PRO
381 V D S V V P V Q N V G E K V N S M E A Y  CBV3 VP3-1 c3-1 Complete ORF.PRO
381 V D S V V P V Q N V G E K V N S M E A Y  CBV3-0 Complete Consensus ORF.PRO

    Q I P V R S N E G S G T Q V F G F P L Q  Majority
                      410                420
401 Q I P V R S N E G S G T Q V F G F P L Q  CBV3 P2A-B c7-2-1 Complete ORF.PRO
401 Q I P V R S N E G S G T Q V F G F P L Q  CBV3 VP3-1 c3-1 Complete ORF.PRO
401 Q I P V R S N E G S G T Q V F G F P L Q  CBV3-0 Complete Consensus ORF.PRO

    P G Y S S V F S R T L L G E I L N Y Y T  Majority
                      430                440
421 P G Y S S V F S R T L L G E I L N Y Y T  CBV3 P2A-B c7-2-1 Complete ORF.PRO
421 P G Y S S V F S R T L L G E I L N Y Y T  CBV3 VP3-1 c3-1 Complete ORF.PRO
421 P G Y S S V F S R T L L G E I L N Y Y T  CBV3-0 Complete Consensus ORF.PRO

    H W S G S I K L T F M F C G S A M A T G  Majority
                      450                460
441 H W S G S I K L T F M F C G S A M A T G  CBV3 P2A-B c7-2-1 Complete ORF.PRO
441 H W S G S I K L T F M F C G S A M A T G  CBV3 VP3-1 c3-1 Complete ORF.PRO
441 H W S G S I K L T F M F C G S A M A T G  CBV3-0 Complete Consensus ORF.PRO

    K F L L A Y S P P G A G A P T K R V D A  Majority
                      470                480
461 K F L L A Y S P P G A G A P T K R V D A  CBV3 P2A-B c7-2-1 Complete ORF.PRO
461 K F L L A Y S P P G A G A P T K R V D A  CBV3 VP3-1 c3-1 Complete ORF.PRO
461 K F L L A Y S P P G A G A P T K R V D A  CBV3-0 Complete Consensus ORF.PRO
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	Sequence	
	M L G T H V V W D V G L Q S S C V L C I	Majority
	490 500	
481	M L G T H V V W D V G L Q S S C V L C I	CBV3 P2A-B c7-2-1 Complete ORF.PRO
481	M L G T H V V W D V G L Q S S C V L C I	CBV3 VP3-1 c3-1 Complete ORF.PRO
481	M L G T H V V W D V G L Q S S C V L C I	CBV3-0 Complete Consensus ORF.PRO
	P W I S Q T H Y R Y V A S D E Y T A G G	Majority
	510 520	
501	P W I S Q T H Y R Y V A S D E Y T A G G	CBV3 P2A-B c7-2-1 Complete ORF.PRO
501	P W I S Q T H Y R Y V A S D E Y T A G G	CBV3 VP3-1 c3-1 Complete ORF.PRO
501	P W I S Q T H Y R Y V A S D E Y T A G G	CBV3-0 Complete Consensus ORF.PRO
	F I T C W Y Q T N I V V P A D A Q S S C	Majority
	530 540	
521	F I T C W Y Q T N I V V P A D A Q S S C	CBV3 P2A-B c7-2-1 Complete ORF.PRO
521	F I T C W Y Q T N I V V P A D A Q S S C	CBV3 VP3-1 c3-1 Complete ORF.PRO
521	F I T C W Y Q T N I V V P A D A Q S S C	CBV3-0 Complete Consensus ORF.PRO
	Y I M C F V S A C N D F S V R L L K D T	Majority
	550 560	
541	Y I M C F V S A C N D F S V R L L K D T	CBV3 P2A-B c7-2-1 Complete ORF.PRO
541	Y I M C F V S A C N D F S V R L L K D T	CBV3 VP3-1 c3-1 Complete ORF.PRO
541	Y I M C F V S A C N D F S V R L L K D T	CBV3-0 Complete Consensus ORF.PRO
	P F I S Q E N F F Q G P V - - E D A I T	Majority
	570 580	
561	P F I S Q E N F F Q G P V - - E D A I T	CBV3 P2A-B c7-2-1 Complete ORF.PRO
561	P F I G A V T - - E D V V C C S M S Y T	CBV3 VP3-1 c3-1 Complete ORF.PRO
561	P F I S Q E N F F Q G P V - - E D A I T	CBV3-0 Complete Consensus ORF.PRO
	A A I G R V A D T V G T G P T N S E A I	Majority
	590 600	
579	A A I G R V A D T V G T G P T N S E A I	CBV3 P2A-B c7-2-1 Complete ORF.PRO
579	A A I G R V A D T V G T G P T N S E A I	CBV3 VP3-1 c3-1 Complete ORF.PRO
579	A A I G R V A D T V G T G P T N S E A I	CBV3-0 Complete Consensus ORF.PRO
	P A L T A A E T G H T S Q V V P G D T M	Majority
	610 620	
599	P A L T A A E T G H T S Q V V P G D T M	CBV3 P2A-B c7-2-1 Complete ORF.PRO
599	P A L T A A E T G H T S Q V V P G D T M	CBV3 VP3-1 c3-1 Complete ORF.PRO
599	P A L T A A E T G H T S Q V V P G D T M	CBV3-0 Complete Consensus ORF.PRO
	Q T R H V K N Y H S R S E S T I E N F L	Majority
	630 640	
619	Q T R H V K N Y H S R S E S T I E N F L	CBV3 P2A-B c7-2-1 Complete ORF.PRO
619	Q T R H V K N Y H S R S E S T I E N F L	CBV3 VP3-1 c3-1 Complete ORF.PRO
619	Q T R H V K N Y H S R S E S T I E N F L	CBV3-0 Complete Consensus ORF.PRO

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    C R S A C V Y F T E Y E N S G A K R Y A   Majority
                      650                 660
639 C R S A C V Y F T E Y E N S G A K R Y A   CBV3 P2A-B c7-2-1 Complete ORF.PRO
639 C R S A C V Y F T E Y E N S G A K R Y A   CBV3 VP3-1 c3-1 Complete ORF.PRO
639 C R S A C V Y F T E Y E N S G A K R Y A   CBV3-0 Complete Consensus ORF.PRO

    E W V L T P R Q A A Q L R R K L E F F T   Majority
                      670                 680
659 E W V L T P R Q A A Q L R R K L E F F T   CBV3 P2A-B c7-2-1 Complete ORF.PRO
659 E W V L T P R Q A A Q L R R K L E F F T   CBV3 VP3-1 c3-1 Complete ORF.PRO
659 E W V L T P R Q A A Q L R R K L E F F T   CBV3-0 Complete Consensus ORF.PRO

    Y V R F D L E L T F V I T S T Q Q P S T   Majority
                      690                 700
679 Y V R F D L E L T F V I T S T Q Q P S T   CBV3 P2A-B c7-2-1 Complete ORF.PRO
679 Y V R F D L E L T F V I T S T Q Q P S T   CBV3 VP3-1 c3-1 Complete ORF.PRO
679 Y V R F D L E L T F V I T S T Q Q P S T   CBV3-0 Complete Consensus ORF.PRO

    T Q N Q D A Q I L T H Q I M Y V P P G G   Majority
                      710                 720
699 T Q N Q D A Q I L T H Q I M Y V P P G G   CBV3 P2A-B c7-2-1 Complete ORF.PRO
699 T Q N Q D A Q I L T H Q I M Y V P P G G   CBV3 VP3-1 c3-1 Complete ORF.PRO
699 T Q N Q D A Q I L T H Q I M Y V P P G G   CBV3-0 Complete Consensus ORF.PRO

    P V P D K V D S Y V W Q T S T N P S V F   Majority
                      730                 740
719 P V P D K V D S Y V W Q T S T N P S V F   CBV3 P2A-B c7-2-1 Complete ORF.PRO
719 P V P D K V D S Y V W Q T S T N P S V F   CBV3 VP3-1 c3-1 Complete ORF.PRO
719 P V P D K V D S Y V W Q T S T N P S V F   CBV3-0 Complete Consensus ORF.PRO

    W T E G N A P P R M S I P F L S I G N A   Majority
                      750                 760
739 W T E G N A P P R M S I P F L S I G N A   CBV3 P2A-B c7-2-1 Complete ORF.PRO
739 W T E G N A P P R M S I P F L S I G N A   CBV3 VP3-1 c3-1 Complete ORF.PRO
739 W T E G N A P P R M S I P F L S I G N A   CBV3-0 Complete Consensus ORF.PRO

    Y S N F Y D G W S E F S R N G V Y G I N   Majority
                      770                 780
759 Y S N F Y D G W S E F S R N G V Y G I N   CBV3 P2A-B c7-2-1 Complete ORF.PRO
759 Y S N F Y D G W S E F S R N G V Y G I N   CBV3 VP3-1 c3-1 Complete ORF.PRO
759 Y S N F Y D G W S E F S R N G V Y G I N   CBV3-0 Complete Consensus ORF.PRO

    T L N N M G T L Y A R H V N A G S T G P   Majority
                      790                 800
779 T L N N M G T L Y A R H V N A G S T G P   CBV3 P2A-B c7-2-1 Complete ORF.PRO
779 T L N N M G T L Y A R H V N A G S T G P   CBV3 VP3-1 c3-1 Complete ORF.PRO
779 T L N N M G T L Y A R H V N A G S T G P   CBV3-0 Complete Consensus ORF.PRO
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      I K S T I R I Y F K P K H V K A W I P R   Majority
                        810                 820
799   I K S T I R I Y F K P K H V K A W I P R   CBV3 P2A-B c7-2-1 Complete ORF.PRO
799   I K S T I R I Y F K P K H V K A W I P R   CBV3 VP3-1 c3-1 Complete ORF.PRO
799   I K S T I R I Y F K P K H V K A W I P R   CBV3-0 Complete Consensus ORF.PRO

      P P R L C Q Y E K A K N V N F Q P S G V   Majority
                        830                 840
819   P P R L C Q Y E K A K N V N F Q P S G V   CBV3 P2A-B c7-2-1 Complete ORF.PRO
819   P P R L C Q Y E K A K N V N F Q P S G V   CBV3 VP3-1 c3-1 Complete ORF.PRO
819   P P R L C Q Y E K A K N V N F Q P S G V   CBV3-0 Complete Consensus ORF.PRO

      T T T R Q S I T T M T N T G A F G Q Q S   Majority
                        850                 860
839   T T T R Q S I T T M T N T G A F G Q Q S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
839   T T T R Q S I T T M T N T G A F G Q Q S   CBV3 VP3-1 c3-1 Complete ORF.PRO
839   T T T R Q S I T T M T N T G A F G Q Q S   CBV3-0 Complete Consensus ORF.PRO

      G A A Y V G N Y R V V N R H L A T S A D   Majority
                        870                 880
859   G A A Y V G N Y R V V N R H L A T S A D   CBV3 P2A-B c7-2-1 Complete ORF.PRO
859   G A A Y V G N Y R V V N R H L A T S A D   CBV3 VP3-1 c3-1 Complete ORF.PRO
859   G A A Y V G N Y R V V N R H L A T S A D   CBV3-0 Complete Consensus ORF.PRO

      W Q N C V W E S Y N R D L L V S T T T A   Majority
                        890                 900
879   W Q N C V W E S Y N R D L L V S T T T A   CBV3 P2A-B c7-2-1 Complete ORF.PRO
879   W Q N C V W E S Y N R D L L V S T T T A   CBV3 VP3-1 c3-1 Complete ORF.PRO
879   W Q N C V W E S Y N R D L L V S T T T A   CBV3-0 Complete Consensus ORF.PRO

      H G C D I I A R C Q C T T G V Y F C A S   Majority
                        910                 920
899   H G C D I I A R C Q C T T G V Y F C A S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
899   H G C D I I A R C Q C T T G V Y F C A S   CBV3 VP3-1 c3-1 Complete ORF.PRO
899   H G C D I I A R C Q C T T G V Y F C A S   CBV3-0 Complete Consensus ORF.PRO

      K N K H Y P I S F E G P G L V E V Q E S   Majority
                        930                 940
919   K N K H Y P I S F E G P G L V E V Q E S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
919   K N K H Y P I S F E G P G L V E V Q E S   CBV3 VP3-1 c3-1 Complete ORF.PRO
919   K N K H Y P I S F E G P G L V E V Q E S   CBV3-0 Complete Consensus ORF.PRO

      E Y Y P R R Y Q S H V L L A A G F S E P   Majority
                        950                 960
939   E Y Y P R R Y Q S H V L L A A G F S E P   CBV3 P2A-B c7-2-1 Complete ORF.PRO
939   E Y Y P R R Y Q S H V L L A A G F S E P   CBV3 VP3-1 c3-1 Complete ORF.PRO
939   E Y Y P R R Y Q S H V L L A A G F S E P   CBV3-0 Complete Consensus ORF.PRO
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      G D C G G I L R C E H G V I G I V T M G   Majority
                          970               980
959   G D C G G I L R C E H G V I G I V T M G   CBV3 P2A-B c7-2-1 Complete ORF.PRO
959   G D C G G I L R C E H G V I G I V T M G   CBV3 VP3-1 c3-1 Complete ORF.PRO
959   G D C G G I L R C E H G V I G I V T M G   CBV3-0 Complete Consensus ORF.PRO

      G E G V V G F A D I R D L L W L E D D A   Majority
                          990              1000
979   G E G V V G F A D I R D L L W L G A V T   CBV3 P2A-B c7-2-1 Complete ORF.PRO
979   G E G V V G F A D I R D L L W L E D D A   CBV3 VP3-1 c3-1 Complete ORF.PRO
979   G E G V V G F A D I R D L L W L E D D A   CBV3-0 Complete Consensus ORF.PRO

      M E Q G - - V K D Y V E Q L G N A F G S   Majority
                         1010              1020
999   E D V V C C S M S Y - - Q L G N A L G S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
999   M E Q G - - V K D Y V E Q L G N A F G S   CBV3 VP3-1 c3-1 Complete ORF.PRO
999   M E Q G - - V K D Y V E Q L G N A F G S   CBV3-0 Complete Consensus ORF.PRO

      G F T N R I C E Q V N L L K E S L V G Q   Majority
                         1030              1040
1017  G F T N R I C E Q V N L L K E S L V G Q   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1017  G F T N R I C E Q V N L L K E S L V G Q   CBV3 VP3-1 c3-1 Complete ORF.PRO
1017  G F T N R I C E Q V N L L K E S L V G Q   CBV3-0 Complete Consensus ORF.PRO

      D S I L E K S L K A L V K I I S A L V I   Majority
                         1050              1060
1037  D S I L E K S L K A L V K I I S A L V I   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1037  D S I L E K S L K A L V K I I S A L V I   CBV3 VP3-1 c3-1 Complete ORF.PRO
1037  D S I L E K S L K A L V K I I S A L V I   CBV3-0 Complete Consensus ORF.PRO

      V V R N H D D L I T V T A T L A L I G C   Majority
                         1070              1080
1057  V V R N H D D L I T V T A T L A L I G C   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1057  V V R N H D D L I T V T A T L A L I G C   CBV3 VP3-1 c3-1 Complete ORF.PRO
1057  V V R N H D D L I T V T A T L A L I G C   CBV3-0 Complete Consensus ORF.PRO

      T S S P W R W L K Q K V S Q Y Y G I P L   Majority
                         1090              1100
1077  T S S P W R W L K Q K V S Q Y Y G I P L   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1077  T S S P W R W L K Q K V S Q Y Y G I P L   CBV3 VP3-1 c3-1 Complete ORF.PRO
1077  T S S P W R W L K Q K V S Q Y Y G I P L   CBV3-0 Complete Consensus ORF.PRO

      A E R Q N N S W L K K F T E M T N A C K   Majority
                         1110              1120
1097  A E R Q N N S W L K K F T E M T N A C K   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1097  A E R Q N N S W L K K F T E M T N A C K   CBV3 VP3-1 c3-1 Complete ORF.PRO
1097  A E R Q N N S W L K K F T E M T N A C K   CBV3-0 Complete Consensus ORF.PRO
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       G M E W I A V K I Q K F I E W L K V K I   Majority
                         1130                1140
1117   G M E W I A V K I Q K F I E W L K V K I   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1117   G M E W I A V K I Q K F I E W L K V K I   CBV3 VP3-1 c3-1 Complete ORF.PRO
1117   G M E W I A V K I Q K F I E W L K V K I   CBV3-0 Complete Consensus ORF.PRO

       L P E V R E K H E F L N R L K Q L P L L   Majority
                         1150                1160
1137   L P E V R E K H E F L N R L K Q L P L L   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1137   L P E V R E K H E F L N R L K Q L P L L   CBV3 VP3-1 c3-1 Complete ORF.PRO
1137   L P E V R E K H E F L N R L K Q L P L L   CBV3-0 Complete Consensus ORF.PRO

       E S Q I A T I E Q S A P S Q S D Q E Q L   Majority
                         1170                1180
1157   E S Q I A T I E Q S A P S Q S D Q E Q L   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1157   E S Q I A T I E Q S A P S Q S D Q E Q L   CBV3 VP3-1 c3-1 Complete ORF.PRO
1157   E S Q I A T I E Q S A P S Q S D Q E Q L   CBV3-0 Complete Consensus ORF.PRO

       F S N V Q Y F A H Y C R K Y A P L Y A A   Majority
                         1190                1200
1177   F S N V Q Y F A H Y C R K Y A P L Y A A   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1177   F S N V Q Y F A H Y C R K Y A P L Y A A   CBV3 VP3-1 c3-1 Complete ORF.PRO
1177   F S N V Q Y F A H Y C R K Y A P L Y A A   CBV3-0 Complete Consensus ORF.PRO

       E A K R V F S L E K K M S N Y I Q F K S   Majority
                         1210                1220
1197   E A K R V F S L E K K M S N Y I Q F K S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1197   E A K R V F S L E K K M S N Y I Q F K S   CBV3 VP3-1 c3-1 Complete ORF.PRO
1197   E A K R V F S L E K K M S N Y I Q F K S   CBV3-0 Complete Consensus ORF.PRO

       K C R I E P V C L L L H G S P G A G K S   Majority
                         1230                1240
1217   K C R I E P V C L L L H G S P G A G K S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1217   K C R I E P V C L L L H G S P G A G K S   CBV3 VP3-1 c3-1 Complete ORF.PRO
1217   K C R I E P V C L L L H G S P G A G K S   CBV3-0 Complete Consensus ORF.PRO

       V A T N L I G R S L A E K L N S S V Y S   Majority
                         1250                1260
1237   V A T N L I G R S L A E K L N S S V Y S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1237   V A T N L I G R S L A E K L N S S V Y S   CBV3 VP3-1 c3-1 Complete ORF.PRO
1237   V A T N L I G R S L A E K L N S S V Y S   CBV3-0 Complete Consensus ORF.PRO

       L P P D P D H F D G Y K Q Q A V V I M D   Majority
                         1270                1280
1257   L P P D P D H F D G Y K Q Q A V V I M D   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1257   L P P D P D H F D G Y K Q Q A V V I M D   CBV3 VP3-1 c3-1 Complete ORF.PRO
1257   L P P D P D H F D G Y K Q Q A V V I M D   CBV3-0 Complete Consensus ORF.PRO
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D L C Q N P D G K D V S L F C Q M V S S Majority

1290 1300

1277 D L C Q N P D G K D V S L F C Q M V S S CBV3 P2A-B c7-2-1 Complete ORF.PRO
1277 D L C Q N P D G K D V S L F C Q M V S S CBV3 VP3-1 c3-1 Complete ORF.PRO
1277 D L C Q N P D G K D V S L F C Q M V S S CBV3-0 Complete Consensus ORF.PRO

V D F V P P M A A L E E K G I L F T S P Majority

1310 1320

1297 V D F V P P M A A L E E K G I L F T S P CBV3 P2A-B c7-2-1 Complete ORF.PRO
1297 V D F V P P M A A L E E K G I L F T S P CBV3 VP3-1 c3-1 Complete ORF.PRO
1297 V D F V P P M A A L E E K G I L F T S P CBV3-0 Complete Consensus ORF.PRO

F V L A S T N A G S I N A P T V S D S R Majority

1330 1340

1317 F V L A S T N A G S I N A P T V S D S R CBV3 P2A-B c7-2-1 Complete ORF.PRO
1317 F V L A S T N A G S I N A P T V S D S R CBV3 VP3-1 c3-1 Complete ORF.PRO
1317 F V L A S T N A G S I N A P T V S D S R CBV3-0 Complete Consensus ORF.PRO

A L A R R F H F D M N I E V I S M Y S Q Majority

1350 1360

1337 A L A R R F H F D M N I E V I S M Y S Q CBV3 P2A-B c7-2-1 Complete ORF.PRO
1337 A L A R R F H F D M N I E V I S M Y S Q CBV3 VP3-1 c3-1 Complete ORF.PRO
1337 A L A R R F H F D M N I E V I S M Y S Q CBV3-0 Complete Consensus ORF.PRO

N G K I N M P M S V K T C D D E C C P V Majority

1370 1380

1357 N G K I N M P M S V K T C D D E C C P V CBV3 P2A-B c7-2-1 Complete ORF.PRO
1357 N G K I N M P M S V K T C D D E C C P V CBV3 VP3-1 c3-1 Complete ORF.PRO
1357 N G K I N M P M S V K T C D D E C C P V CBV3-0 Complete Consensus ORF.PRO

N F K K C C P L V C G K A I Q F I D R R Majority

1390 1400

1377 N F K K C C P L V C G K A I Q F I D R R CBV3 P2A-B c7-2-1 Complete ORF.PRO
1377 N F K K C C P L V C G K A I Q F I D R R CBV3 VP3-1 c3-1 Complete ORF.PRO
1377 N F K K C C P L V C G K A I Q F I D R R CBV3-0 Complete Consensus ORF.PRO

T Q V R Y S L D M L V T E M F R E Y N H Majority

1410 1420

1397 T Q V R Y S L D M L V T E M F R E Y N H CBV3 P2A-B c7-2-1 Complete ORF.PRO
1397 T Q V R Y S L D M L V T E M F R E Y N H CBV3 VP3-1 c3-1 Complete ORF.PRO
1397 T Q V R Y S L D M L V T E M F R E Y N H CBV3-0 Complete Consensus ORF.PRO

R H S V G T T L E A L F Q G P P V Y R E Majority

1430 1440

1417 R H S V G T T L E A L F Q G P P V Y R E CBV3 P2A-B c7-2-1 Complete ORF.PRO
1417 R H S V G T T L E A L F Q G P P V Y R E CBV3 VP3-1 c3-1 Complete ORF.PRO
1417 R H S V G T T L E A L F Q G P P V Y R E CBV3-0 Complete Consensus ORF.PRO

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        I K I S V A P E T P P P P A I A D L L K   Majority
                          1450                1460
1437    I K I S V A P E T P P P P A I A D L L K   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1437    I K I S V A P E T P P P P A I A D L L K   CBV3 VP3-1 c3-1 Complete ORF.PRO
1437    I K I S V A P E T P P P P A I A D L L K   CBV3-0 Complete Consensus ORF.PRO

        S V D S E A V R E Y C K E K G W L V P E   Majority
                          1470                1480
1457    S V D S E A V R E Y C K E K G W L V P E   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1457    S V D S E A V R E Y C K E K G W L V P E   CBV3 VP3-1 c3-1 Complete ORF.PRO
1457    S V D S E A V R E Y C K E K G W L V P E   CBV3-0 Complete Consensus ORF.PRO

        I N S T L Q I E K H V S R A F I C L Q A   Majority
                          1490                1500
1477    I N S T L Q I E K H V S R A F I C L Q A   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1477    I N S T L Q I E K H V S R A F I C L Q A   CBV3 VP3-1 c3-1 Complete ORF.PRO
1477    I N S T L Q I E K H V S R A F I C L Q A   CBV3-0 Complete Consensus ORF.PRO

        L T T F V S V A G I I Y I I Y K L F A G   Majority
                          1510                1520
1497    L T T F V S V A G I I Y I I Y K L F A G   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1497    L T T F V S V A G I I Y I I Y K L F A G   CBV3 VP3-1 c3-1 Complete ORF.PRO
1497    L T T F V S V A G I I Y I I Y K L F A G   CBV3-0 Complete Consensus ORF.PRO

        F Q G A Y T G V P N Q K P R V P T L R Q   Majority
                          1530                1540
1517    F Q G A Y T G V P N Q K P R V P T L R Q   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1517    F Q G A Y T G V P N Q K P R V P T L R Q   CBV3 VP3-1 c3-1 Complete ORF.PRO
1517    F Q G A Y T G V P N Q K P R V P T L R Q   CBV3-0 Complete Consensus ORF.PRO

        A K V Q G P A F E F A V A M M K R N S S   Majority
                          1550                1560
1537    A K V Q G P A F E F A V A M M K R N S S   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1537    A K V Q G P A F E F A V A M M K R N S S   CBV3 VP3-1 c3-1 Complete ORF.PRO
1537    A K V Q G P A F E F A V A M M K R N S S   CBV3-0 Complete Consensus ORF.PRO

        T V K T E Y G E F T M L G I Y D R W A V   Majority
                          1570                1580
1557    T V K T E Y G E F T M L G I Y D R W A V   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1557    T V K T E Y G E F T M L G I Y D R W A V   CBV3 VP3-1 c3-1 Complete ORF.PRO
1557    T V K T E Y G E F T M L G I Y D R W A V   CBV3-0 Complete Consensus ORF.PRO

        L P R H A K P G P T I L M N D Q E V G V   Majority
                          1590                1600
1577    L P R H A K P G P T I L M N D Q E V G V   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1577    L P R H A K P G P T I L M N D Q E V G V   CBV3 VP3-1 c3-1 Complete ORF.PRO
1577    L P R H A K P G P T I L M N D Q E V G V   CBV3-0 Complete Consensus ORF.PRO
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Position	Sequence	Name
	L D A K E L V D K D G T N L E L T L L K	Majority
	1610 ... 1620	
1597	L D A K E L V D K D G T N L E L T L L K	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1597	L D A K E L V D K D G T N L E L T L L K	CBV3 VP3-1 c3-1 Complete ORF.PRO
1597	L D A K E L V D K D G T N L E L T L L K	CBV3-0 Complete Consensus ORF.PRO
	L N R N E K F R D I R G F L A K E E V E	Majority
	1630 ... 1640	
1617	L N R N E K F R D I R G F L A K E E V E	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1617	L N R N E K F R D I R G F L A K E E V E	CBV3 VP3-1 c3-1 Complete ORF.PRO
1617	L N R N E K F R D I R G F L A K E E V E	CBV3-0 Complete Consensus ORF.PRO
	V N E A V L A I N T S K F P N M Y I P V	Majority
	1650 ... 1660	
1637	V N E A V L A I N T S K F P N M Y I P V	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1637	V N E A V L A I N T S K F P N M Y I P V	CBV3 VP3-1 c3-1 Complete ORF.PRO
1637	V N E A V L A I N T S K F P N M Y I P V	CBV3-0 Complete Consensus ORF.PRO
	G Q V T E Y G F L N L G G T P T K R M L	Majority
	1670 ... 1680	
1657	G Q V T E Y G F L N L G G T P T K R M L	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1657	G Q V T E Y G F L N L G G T P T K R M L	CBV3 VP3-1 c3-1 Complete ORF.PRO
1657	G Q V T E Y G F L N L G G T P T K R M L	CBV3-0 Complete Consensus ORF.PRO
	M Y N F P T R A G Q C G G V L M S T G K	Majority
	1690 ... 1700	
1677	M Y N F P T R A G Q C G G V L M S T G K	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1677	M Y N F P T R A G Q C G G V L M S T G K	CBV3 VP3-1 c3-1 Complete ORF.PRO
1677	M Y N F P T R A G Q C G G V L M S T G K	CBV3-0 Complete Consensus ORF.PRO
	V L G I H V G G N G H Q G F S A A L L K	Majority
	1710 ... 1720	
1697	V L G I H V G G N G H Q G F S A A L L K	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1697	V L G I H V G G N G H Q G F S A A L L K	CBV3 VP3-1 c3-1 Complete ORF.PRO
1697	V L G I H V G G N G H Q G F S A A L L K	CBV3-0 Complete Consensus ORF.PRO
	H Y F N D E Q G E I E F I E S S K D A G	Majority
	1730 ... 1740	
1717	H Y F N D E Q G E I E F I E S S K D A G	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1717	H Y F N D E Q G E I E F I E S S K D A G	CBV3 VP3-1 c3-1 Complete ORF.PRO
1717	H Y F N D E Q G E I E F I E S S K D A G	CBV3-0 Complete Consensus ORF.PRO
	F P V I N T P S K T K L E P S V F H Q V	Majority
	1750 ... 1760	
1737	F P V I N T P S K T K L E P S V F H Q V	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1737	F P V I N T P S K T K L E P S V F H Q V	CBV3 VP3-1 c3-1 Complete ORF.PRO
1737	F P V I N T P S K T K L E P S V F H Q V	CBV3-0 Complete Consensus ORF.PRO

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	Sequence	
	F E G N K E P A V L R S G D P R L K A N	Majority
	1770 1780	
1757	F E G N K E P A V L R S G D P R L K A N	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1757	F E G N K E P A V L R S G D P R L K A N	CBV3 VP3-1 c3-1 Complete ORF.PRO
1757	F E G N K E P A V L R S G D P R L K A N	CBV3-0 Complete Consensus ORF.PRO
	F E E A I F S K Y I G N V N T H V D E Y	Majority
	1790 1800	
1777	F E E A I F S K Y I G N V N T H V D E Y	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1777	F E E A I F S K Y I G N V N T H V D E Y	CBV3 VP3-1 c3-1 Complete ORF.PRO
1777	F E E A I F S K Y I G N V N T H V D E Y	CBV3-0 Complete Consensus ORF.PRO
	M L E A V D H Y A G Q L A T L D I S T E	Majority
	1810 1820	
1797	M L E A V D H Y A G Q L A T L D I S T E	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1797	M L E A V D H Y A G Q L A T L D I S T E	CBV3 VP3-1 c3-1 Complete ORF.PRO
1797	M L E A V D H Y A G Q L A T L D I S T E	CBV3-0 Complete Consensus ORF.PRO
	P M K L E D A V Y G T E G L E A L D L T	Majority
	1830 1840	
1817	P M K L E D A V Y G T E G L E A L D L T	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1817	P M K L E D A V Y G T E G L E A L D L T	CBV3 VP3-1 c3-1 Complete ORF.PRO
1817	P M K L E D A V Y G T E G L E A L D L T	CBV3-0 Complete Consensus ORF.PRO
	T S A G Y P Y V A L G I K K R D I L S K	Majority
	1850 1860	
1837	T S A G Y P Y V A L G I K K R D I L S K	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1837	T S A G Y P Y V A L G I K K R D I L S K	CBV3 VP3-1 c3-1 Complete ORF.PRO
1837	T S A G Y P Y V A L G I K K R D I L S K	CBV3-0 Complete Consensus ORF.PRO
	K T K D L T K L K E C M D K Y G L N L P	Majority
	1870 1880	
1857	K T K D L T K L K E C M D K Y G L N L P	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1857	K T K D L T K L K E C M D K Y G L N L P	CBV3 VP3-1 c3-1 Complete ORF.PRO
1857	K T K D L T K L K E C M D K Y G L N L P	CBV3-0 Complete Consensus ORF.PRO
	M V T Y V K D E L R S I E K V A K G K S	Majority
	1890 1900	
1877	M V T Y V K D E L R S I E K V A K G K S	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1877	M V T Y V K D E L R S I E K V A K G K S	CBV3 VP3-1 c3-1 Complete ORF.PRO
1877	M V T Y V K D E L R S I E K V A K G K S	CBV3-0 Complete Consensus ORF.PRO
	R L I E A S S L N D S V A M R Q T F G N	Majority
	1910 1920	
1897	R L I E A S S L N D S V A M R Q T F G N	CBV3 P2A-B c7-2-1 Complete ORF.PRO
1897	R L I E A S S L N D S V A M R Q T F G N	CBV3 VP3-1 c3-1 Complete ORF.PRO
1897	R L I E A S S L N D S V A M R Q T F G N	CBV3-0 Complete Consensus ORF.PRO

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      L Y K T F H L N P G V V T G S A V G C D   Majority
                        1930                1940
1917  L Y K T F H L N P G V V T G S A V G C D   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1917  L Y K T F H L N P G V V T G S A V G C D   CBV3 VP3-1 c3-1 Complete ORF.PRO
1917  L Y K T F H L N P G V V T G S A V G C D   CBV3-0 Complete Consensus ORF.PRO

      P D L F W S K I P V M L D G H L I A F D   Majority
                        1950                1960
1937  P D L F W S K I P V M L D G H L I A F D   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1937  P D L F W S K I P V M L D G H L I A F D   CBV3 VP3-1 c3-1 Complete ORF.PRO
1937  P D L F W S K I P V M L D G H L I A F D   CBV3-0 Complete Consensus ORF.PRO

      Y S G Y D A S L S P V W F A C L K M L L   Majority
                        1970                1980
1957  Y S G Y D A S L S P V W F A C L K M L L   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1957  Y S G Y D A S L S P V W F A C L K M L L   CBV3 VP3-1 c3-1 Complete ORF.PRO
1957  Y S G Y D A S L S P V W F A C L K M L L   CBV3-0 Complete Consensus ORF.PRO

      E K L G Y T H K E T N Y I D Y L C N S H   Majority
                        1990                2000
1977  E K L G Y T H K E T N Y I D Y L C N S H   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1977  E K L G Y T H K E T N Y I D Y L C N S H   CBV3 VP3-1 c3-1 Complete ORF.PRO
1977  E K L G Y T H K E T N Y I D Y L C N S H   CBV3-0 Complete Consensus ORF.PRO

      H L Y R D K H Y F V R G G M P S G C S G   Majority
                        2010                2020
1997  H L Y R D K H Y F V R G G M P S G C S G   CBV3 P2A-B c7-2-1 Complete ORF.PRO
1997  H L Y R D K H Y F V R G G M P S G C S G   CBV3 VP3-1 c3-1 Complete ORF.PRO
1997  H L Y R D K H Y F V R G G M P S G C S G   CBV3-0 Complete Consensus ORF.PRO

      T S I F N S M I N N I I I R T L M L K V   Majority
                        2030                2040
2017  T S I F N S M I N N I I I R T L M L K V   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2017  T S I F N S M I N N I I I R T L M L K V   CBV3 VP3-1 c3-1 Complete ORF.PRO
2017  T S I F N S M I N N I I I R T L M L K V   CBV3-0 Complete Consensus ORF.PRO

      Y K G I D L D Q F R M I A Y G D D V I A   Majority
                        2050                2060
2037  Y K G I D L D Q F R M I A Y G D D V I A   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2037  Y K G I D L D Q F R M I A Y G D D V I A   CBV3 VP3-1 c3-1 Complete ORF.PRO
2037  Y K G I D L D Q F R M I A Y G D D V I A   CBV3-0 Complete Consensus ORF.PRO

      S Y P W P I D A S L L A E A G K G Y G L   Majority
                        2070                2080
2057  S Y P W P I D A S L L A E A G K G Y G L   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2057  S Y P W P I D A S L L A E A G K G Y G L   CBV3 VP3-1 c3-1 Complete ORF.PRO
2057  S Y P W P I D A S L L A E A G K G Y G L   CBV3-0 Complete Consensus ORF.PRO
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        I M T P A D K G E C F N E V T W T N V T   Majority
                          2090                2100
2077    I M T P A D K G E C F N E V T W T N V T   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2077    I M T P A D K G E C F N E V T W T N V T   CBV3 VP3-1 c3-1 Complete ORF.PRO
2077    I M T P A D K G E C F N E V T W T N V T   CBV3-0 Complete Consensus ORF.PRO

        F L K R Y F R A D E Q Y P F L V H P V M   Majority
                          2110                2120
2097    F L K R Y F R A D E Q Y P F L V H P V M   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2097    F L K R Y F R A D E Q Y P F L V H P V M   CBV3 VP3-1 c3-1 Complete ORF.PRO
2097    F L K R Y F R A D E Q Y P F L V H P V M   CBV3-0 Complete Consensus ORF.PRO

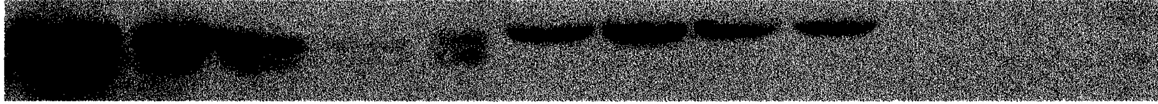
        P M K D I H E S I R W T K D P K N T Q D   Majority
                          2130                2140
2117    P M K D I H E S I R W T K D P K N T Q D   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2117    P M K D I H E S I R W T K D P K N T Q D   CBV3 VP3-1 c3-1 Complete ORF.PRO
2117    P M K D I H E S I R W T K D P K N T Q D   CBV3-0 Complete Consensus ORF.PRO

        H V R S L C L L A W H N G E H E Y E E F   Majority
                          2150                2160
2137    H V R S L C L L A W H N G E H E Y E E F   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2137    H V R S L C L L A W H N G E H E Y E E F   CBV3 VP3-1 c3-1 Complete ORF.PRO
2137    H V R S L C L L A W H N G E H E Y E E F   CBV3-0 Complete Consensus ORF.PRO

        I R K I R S V P V G R C L T L P A F S T   Majority
                          2170                2180
2157    I R K I R S V P V G R C L T L P A F S T   CBV3 P2A-B c7-2-1 Complete ORF.PRO
2157    I R K I R S V P V G R C L T L P A F S T   CBV3 VP3-1 c3-1 Complete ORF.PRO
2157    I R K I R S V P V G R C L T L P A F S T   CBV3-0 Complete Consensus ORF.PRO

        L R R K W L D S F -                       Majority
                          2190
2177    L R R K W L D S F .                       CBV3 P2A-B c7-2-1 Complete ORF.PRO
2177    L R R K W L D S F .                       CBV3 VP3-1 c3-1 Complete ORF.PRO
2177    L R R K W L D S F .                       CBV3-0 Complete Consensus ORF.PRO
```

Appendix B Alignment of Open Reading Frames of CBV3-0, mutCBV3 VP3/1 and mutCBV3 P2A/B/


HisTKCL1+NS3/4A
HisTKCL1+pcDNA3
Mock
0h
2h
4h
8h
10h
0h
2h
4h
8h
10h
0h
10h

Appendix C Western blot showing doublet observable when TKCL1 is co-expressed with NS3/4A. The doublet is only visible in the presence of NS3/4A.

CURRICULUM VITAE

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2001-Present	**University of Ottawa – Human Molecular Genetics (HMG)** **Ottawa Regional Cancer Centre/Cancer Research Group** Ottawa, Ontario MSc. Candidate *Supervisor*: Dr. John C. Bell
2000	**University of Ottawa – Department of Epidemiology and Community Medicine** Special Student – Introductory Epidemiology and Biostatistics courses
1997-2000	**McGill University** Graduated with BSc. Distinction Major Biology

Professional Experience / Educational Programs

2003	**Teaching Assistant** **University of Ottawa – Department of Biochemistry** 3rd year Undergraduate Biochemistry Lab Demonstrator *Course Coordinator:* Dr. G. Guillet
2002-2003	**Teaching Assistant** **University of Ottawa – Department of Biochemistry** 2nd year Undergraduate Biochemistry Lab Demonstrator *Course Coordinator:* Dr. M.A. Rodriguez
2001-2002	**Student Volunteer** **Let's Talk Science Program** *Coordinator*: Dr. B. Vanderhyden

2000-2001 **Research Associate**
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Supervisor: Dr. John C. Bell

2000 **Summer Student**
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Supervisor: Dr. John C. Bell and Dr. Glenwood Goss

2000 **Summer Student**
Ottawa Regional Cancer Center/Clinical Trials
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Supervisor: Simone Dahrouge – Clinical Epidemiologist

1999 **McGill University**
Department of Biology
Undergraduate Independent Studies Thesis
Supervisor: Dr. Richard Roy

Awards

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1. 1st Place Award – Department of Biochemistry, Microbiology and Immunology Graduate Student Poster Competition (April 2002) – Selected to represent the University of Ottawa at Canadian Institutes for Health Research National Poster Competition

Abstracts

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1. **Christou, G.,** Lichty, B.D., and Bell, J.C. Viral Wars: A Virus for the Treatment of Hepatitis C. (Abstract) Canadian Institutes for Health Research National Poster Competition, Winnipeg, Manitoba (June 4-6, 2002)

2. **Christou G.,** Lichty, B.D., and Bell, J.C. Viral Wars: A Virus for the Treatment of Hepatitis C. (Abstract) BioNorth Conference, Ottawa, Ontario (Nov. 17-19, 2003)

CONTRIBUTIONS OF COLLABORATORS

Brian Lichty made the NS5A.B:GFP and the NS3/4A expression constructs.