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HCV-Core Over-expressed Specifically in Liver Cells

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Specifically in Liver Cells

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ABSTRACT

Hepatitis C viruses (HCV) affect 170 million patients, but only a minority of patients develop symptoms and manage to clear the virus, and the pathogenesis remains unknown. Previous studies discovered that some viral proteins may suppress HCV specific T lymphocytes, leading to lower immune responses. Although many mouse models have been tried in laboratories worldwide, none of them mimicked natural Human HCV infection with individual HCV genes *in vivo*.

To study the immunopathogenesis of HCV infections, we constructed some chimeric liver-specific vectors and one was selected to establish promoted mouse models, which express individual HCV genes specifically in the liver. In this research, the over-expression of HCV-Core and the cellular immune responses in mice driven by global and liver-specific promoters were also detected. Although the DNA injection needs to be optimized, our results indicate that liver-specific expression may provide a new way to elucidate the pathogenesis of HCV infections.

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LIST OF ABBREVIATIONS

2apoEs	2 copies of apolipoprotein enhancer in tandem
ADH6	Alcohol dehydrogenase 6 promoter
Amp	Ampicillin
BB	Backbone of plasmid pVAX1 or pGL3
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CMV	Human cytomegalovirus early complete promoter
Core	Hepatitis C Virus Core gene or protein
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic acid
E2	Hepatitis C Virus structural protein 2
Ealb	Human albumin enhancer
EB	Ethidium bromide
EDTA	Ethyl diaminetetraacetic acid
FBS	Fetal Bovine Serum
HAAT	Human a1-antitrypsin
Ham's F12	Ham's F-12 Medium with L-glutamine
HCV	Hepatitis C Virus
HRP	House radish peroxidase
IFA	Indirect immuno-fluorescent assay
IFN-γ	Interferon-γ
IMDM	Iscove's Modified Dulbecco's Medium
IRES	Internal Ribosome Entry Site
Kana	Kanamycin
NK	Natural Killer
NS2	Non-structural protein 2 of Hepatitis C Virus
NS3	Non-structural protein 3 of Hepatitis C Virus
NS5a	Non-structural protein 5a of Hepatitis C Virus
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMA	Phorbol myristate acetate
pLS	Plasmid with liver-specific elements (Ealb+HAAT)
RB	Ribavirin
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SV40	Simian virus 40
TBE	Tris base-boric acid-EDTA buffer
UTR	Un-translational region
UV	Ultra-violet light

1. INTRODUCTION

1.1 General information about HCV

Hepatitis caused by HCV is a worldwide infectious disease, affecting 170 million patients, about 3% of the world population and 5 times more than HIV carriers (1, 2), but the immunopathogenesis is still unknown. This disease was originally termed as non-A, non-B hepatitis with the advent of effective blood screening assays for Hepatitis B Virus at the end of 1970s. The identification of Hepatitis C Virus using molecular methods in 1989 by Houghton et al. was a milestone in modern virology because the virus had never been visualized, grown in cell culture, or detected serologically (3), and it was the first time that a virus was identified by the characterization of the genomic sequence prior to understanding the biochemical properties of the agent (4). Due to the limited amount of virus that can be recovered from patient serum and the lack of efficient cell culture and small animal systems (5), HCV infection has been one of the most difficult challenges the medical scientific community has faced over the past decades.

The prevalence of HCV infection ranged from 0.2~1% in North America and Europe to almost 15% in Egypt (6). In Canada, it is estimated that 1.0~1.2% of the population (300,000~369,000 persons) was affected in 2001 (7). Despite the high prevalence, only a minority of patients (15%) develop symptoms during the acute phase and manage to clear the virus (4) and most of the HCV infections are likely to be unreported (8). Worldwide, a high rate (about 80~85%) of HCV carriers progress

into chronic infection and this is the major cause of liver-related morbidity and mortality (4).

The elucidation of HCV transmission through contaminated blood products had a tremendous impact on the medical system and that directly lead to the creation of a new Canadian blood service, altering the perception of the medical community towards the use of blood products. Routine screening of blood donors since 1990 has detected most of the HCV positive blood donors (9, 10), leading to a remarkable reduction of transmission to transfusion recipients. But HCV transmission is still a major medical problem at present, and this is primarily due to the failure of serologic screening tests to identify infected donors during the early period of infection prior to the development of antibody, and to the hyper-variety of HCV genotypes and subtypes (11). The populations with HIV infection, intravenous drug use or sexual contact were also reported to have a higher risk for HCV infection. However, a study in Thailand discovered the prevalence of anti-HCV antibodies in intravenous drug users was as high as 85%, but the prevalence of anti-HCV antibodies in commercial sex workers and patients with sexually transmitted diseases was as low as 0~2%. Thus, HCV is mainly transmitted by blood contacts, rather than by sexual contacts (12).

Although HCV affects a large population worldwide, there is no effective treatment at present, and neither preventive nor therapeutic vaccines are available. Currently, treatment options for chronic HCV infection are limited to Pegylated Interferon-γ in combination with Ribavirin (RB) (13, 14). However, this treatment is only partially effective, and is often associated with troublesome side effects (15, 16).

Patients with the predominant HCV genotype 1 are the most resistant to IFN-γ and RB treatment (13). Therefore, the development of effective therapies for HCV is quite necessary, but the progress in new drug development is very slow (14). The patients who fail in antiviral therapy remain at risk for disease progression (17), which is the leading cause of blood transmitted chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (18).

1.2 The molecular biology of HCV

HCV is a small, enveloped virus with a positive-sense, single-stranded RNA genome, and is a member of the Hepacivirus Genus of the Flaviviridae Family. The 9.6 kb RNA genome has only one Open Reading Frame (ORF), encoding a large polyprotein of 3010~3033 amino acids (19, 20), which is processed by host and viral proteases to produce 3 structural proteins (Core, E1, E2) at the N-terminal, and 7 nonstructural proteins (p7, NS2, NS3, NS4a, NS4b, NS5a, NS5b) at the C-terminal (Figure 1a and 1b). The Core proteins constitute the viral nucleocapsid, and the E1 and E2 proteins are type I transmembrane glycoproteins (21). Most of the viral NS proteins (NS3–5b) form a membrane-associated replicase complex with cellular proteins to initiate viral RNA replication (4). Of all these non-structural elements, NS5b has the RNA-dependent RNA polymerase activity, and is the key enzyme for the HCV RNA replication. Due to the lack of proofreading activity, NS5b might be responsible for the remarkable sequence variation of HCV genome (5). The ORF is flanked by 5' and 3' untranslated regions (UTR) of 341 and approximately 230 nucleotides respectively, and they play critical roles in the replication, translation, stabilization, and packaging of the viruses (4).

Figure 1: The genomic RNA and protein expression of HCV

HCV has a positive-sense, single-stranded RNA genome of 9.6 kb flanked by 5' and 3' untranslated regions (UTR). The 5'-UTR has an Internal Ribosomal Entry Site (IRES), and the Open Reading Frame (ORF) encodes only one large polyprotein. After translation, this polyprotein was cleaved by endoplasmic reticulum signal peptidase (denoted by diamonds in Figure 1a and scissors in Figure 1b) and viral NS2 and NS3 serine proteases (denoted by arrows in Figure 1a and Figure 1b) into total 10 structural and non-structural proteins.

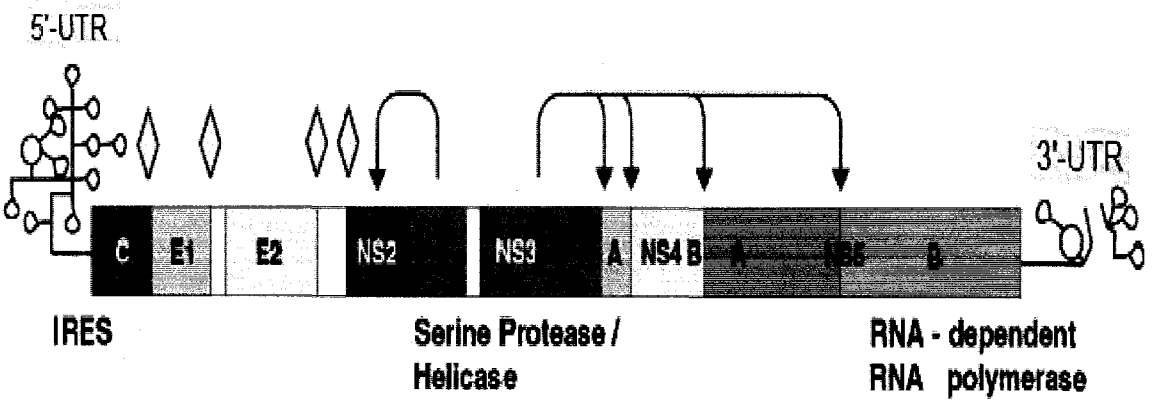


Figure 1a: the genomic RNA structure of HCV (28).

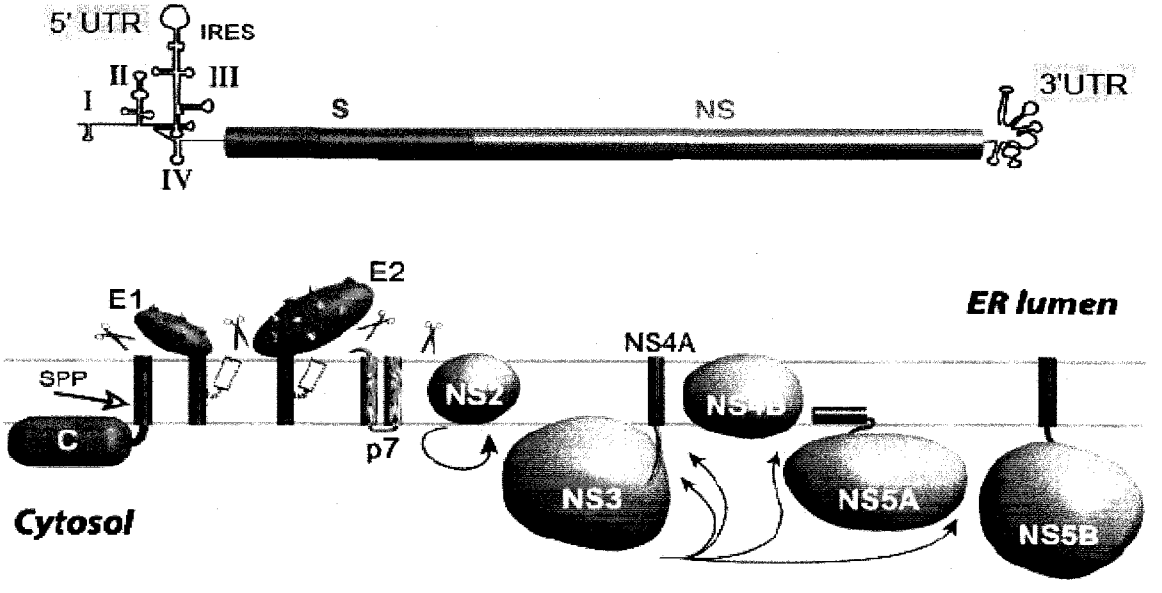


Figure 1b: the protein expression of HCV and the cleavage of structural and non-structural proteins (5).

HCV is a highly mutable hepatotropic RNA virus (22). More than 90 genotypes are distributed around the world and are classified into six main types and 11 subtypes on the basis of nucleic sequence (23, 24, 25). In addition to genotypes, HCV exists within its hosts as a pool of genetically distinct but closely related variants that were termed as "quasi-species" (26, 27, 28). So far, more than 22,300 HCV sequences, including 181 full-length genomes, have been deposited in gene banks (5). The large number of HCV quasi-species might be one of the mechanisms which cause the very challenging problems in HCV therapy, such as escape mutants that evade the humoral and cellular responses and the development of drug resistance (29).

The most conserved region of the HCV genome is the 5'-UTR, and the sequence of this region is widely used for HCV genotyping, qualitative and quantitative diagnostic tests (such as RT-PCR) because it contains type-specific sequence in a conserved region (maximum 10% sequence divergence) (30, 31). Primers or probes designed from these sequences are also used for the universal detection of HCV RNA (4). The 3'-UTR is another highly conserved region in the HCV genome, however, this region is highly structured and it has not been used in the detection of HCV RNA because the well-established tests based on the 5'-UTR sequence are more practical than this region (4). Of the entire HCV encoding region, Core and NS5b genes are also relatively conserved without extensive secondary structures; therefore, they have also been selected for various detection methods (4).

1.3 The problems in HCV infections

Although a number of important breakthroughs have been made in the virology, epidemiology, natural history, and immunopathogenesis of HCV infections,

many scientific questions remain unanswered yet. The key problem in the pathogenesis of HCV is that the immune response caused by HCV infection does not clear the viruses, leading to viral persistence and hepatocyte injury.

Despite the high prevalence of HCV in the worldwide population, only 15% of patients develop symptoms during the acute phase and manage to clear the virus (4). In humans and chimpanzees, a strong and broad HCV-specific Cytoxoxic T Lymphocyte (CTL) response has been associated with viral clearance during acute HCV infections (32, 33). On the other hand, some other studies have pointed out that despite the presence of viral-specific immune responses, the majority of patients still remain chronically infected (29). The existing data suggest that the host-virus interactions during the early phase of HCV infection are critical to the subsequent outcome and that virus-specific T lymphocyte responses play a key role in this process (22). Szabo's study demonstrated that impaired functions of both Plasmacytoid Dendritic Cells (PDC) and Myeloid Dendritic Cells (MDC) in patients with chronic HCV infection might be related to the weak and insufficient immune response for the clearance of the viruses (34). Some other studies also indicated a diminished DC function in HCV infected patients, showing impaired abilities to stimulate allogenic T cells and to produce IFN-γ in HCV infected patients (35). While Sugimoto's research discovered that HCV persistence is associated with a global quantitative and functional suppression of HCV-specific T lymphocytes (36). Individuals with chronic infections are often found to have a relatively weak and narrowly directed CD8+ T-cell response against HCV (37, 38). However, the mechanisms of quantitative and qualitative T cell defect in chronic HCV infections

are not fully understood at present (2), but many studies are ongoing to elucidate why the immune responses to the virus are generally weak.

Existing research results indicate several individual genes might be related to the attenuated immune responses to the virus. You's research indicates that HCV-**Core** protein might induce the apoptosis of HCV-specific cytotoxic CD8+ T lymphocytes via multi-signaling pathways, leading to the viral persistence and lower immune responses (39). By using a position specific scoring system, Hu found that the N-terminal region of **E2** is antigenically and structurally similar to human immunoglobulin variable domains, and that might be correlated with the immune escape, causing the persistence of HCV in humans and experimentally infected chimpanzees (40). Molecular evidence provided by Heo also showed that the neutralizing antibodies from the sera of chronic HCV patients have lower inhibitory activities against the binding of HCV E2 protein to human hepatoma cell lines than to a lymphoma cell line; the incomplete inhibition of one of the receptors leads to only a partial E2 blockade and, possibly, evasion of the host immune response (41). Tseng's study showed that HCV E2 may not only inhibit NK cellular function (proliferation, cytokine production, and cytotoxic granule release) but also activate T cells through its interaction with CD81 (22, 42, 43). Fredrik's research showed that the nonstructural protein **NS3** may trigger the dysfunction and apoptosis of lymphocytes by affecting the roles of a key enzyme in radical formation, the NADPH oxidase (44). Brady discovered that **NS4** suppresses Th1 responses by stimulating IL-10 production from monocytes (45). **NS5a** has an interferon sensitivity determining region (46); it may modulate the response to interferon alpha therapy

(47, 48) and interfere with the activity of the double stranded RNA activated protein kinase *in vitro*. Therefore, it may also play some important roles in the lower immune response in HCV infections (49). Although the findings on the roles of NS5a in the viral replication and resistance to interferon-α are controversial and need more study, it may have many other potential functions, such as the transcriptional activation, cell growth regulation and cellular signaling pathways (28, 50, 51).

Moreover, some other mechanisms may also be involved in the HCV persistence and lower immune responses, and they are likely to be multi-factorial. Host genetic factors may be highly correlated to HCV persistence, as the prognosis of infections varies among individuals, indicating different responses between the individuals who spontaneously cleared the virus and those who failed to (52). One of the other important mechanisms is that the HCV sequences mutate very rapidly, leading to antigen escape variants, this makes the development of preventive and therapeutic vaccines exceedingly difficult (53). Takaki reported that in some cases the patients develop immunity but are unable to clear the virus (54). Cox's findings reveal two distinct mechanisms of sequence evolution involved in HCV persistence: viral escape from CD8+ T cell responses and optimization of replication capacity (55). Rosen's analysis showed that the HCV-specific CD8+ T lymphocyte responses are phenotypically and functionally diverse and may be associated with either viral eradication or chronic hepatic immuno-pathologic states (29). Moreover, Glenda identified a HCV reactive T cell receptor that does not require CD8 for target cell recognition (17). All these are potential mechanisms for the viral persistence.

At the same time, the mechanisms responsible for hepatocyte injury in acute and chronic HCV infection are not well understood either. Although HCV infection is associated with chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, the viral replication does not seem to be cytopathic. Several studies indicate that hepatocellular damage in chronic HCV infection may result from the activation of Cytotoxic T Lymphocyte (CTL) responses, rather than from a direct viral cytopathic effect (52, 56, 57). CTL responses represent a major defensive mechanism in viral infections, but the virus is able to survive in spite of strong CTL responses in chronic hepatitis C patients (56). Due to the long-lasting inflammatory milieu during the chronic phase of the infection, hepatic damage occurs even though the immune response may partially control the viral replication (52). Moreover, the viral factors may also be involved in the hepatocyte injury: genotype 1 was reported to be associated with more severe liver disease (58), whereas genotype 2 has been isolated from a majority of asymptomatic carriers (59, 60). However, many other reports indicate that genotypes do not account for clinical or histological differences (61). All these controversies need further study.

1.4 The immune response caused by HCV infections

HCV infection is known to induce both humoral and cellular immunity in humans. However, so far the role of humoral immune response in the protection against HCV has not been clearly elucidated yet. The anti-HCV antibodies in chronic HCV patients may not be protective; whereas the adaptive cellular immunity may play very important roles in the clearance of HCV particles in acutely infected people.

The adaptive humoral immune response caused by HCV is highly related to the phase of infection, genotype, and the viral load *in vivo*. Chen's research showed that the antibody responses in HCV infections to viral antigens were of relatively low titer and, with the exception of anti-HCV-Core, delayed in appearance until the chronic phase of infection (62). Mondelli's results showed that the vigor and heterogeneity of cross-reactive antibody responses with multiple antigenic peptides were significantly higher in patients with chronic hepatitis compared to those with acute hepatitis, and in patients infected with HCV type 2 compared with patients infected with other viral genotypes (63). The observations from Carlos, et al, demonstrated that among the individuals infected with HCV-1a, those with low viral loads mounted significantly stronger responses against some epitopes than did individuals with high viral loads (64). They also found that quantitatively different antibody responses against HCV depend on the genotype of infecting virus, and the humoral immunity directly against multiple immunodominant epitopes in HCV-1a infected individuals may causes a lower viral load *in vivo* (64). Hadlock's research also indicates that the antibody response to E2 is affected by the genotype of the virus and the induction of a strong humoral immune response to HCV E2 may contribute to a decreased viral load (65).

The CTL responses represent a major defensive mechanism in HCV infections. Many research results indicate the important roles of adaptive cellular immunity, and broad multi-specific CD4+ and CD8+ T cell responses are required for the clearance of HCV in acutely infected people (52). In contrast, individuals with chronic infection are often found to have a relatively weak T cell response against

HCV (38). Folgori's research found that the massive expansion of peripheral and intra-hepatic HCV-specific CD8+ T lymphocytes that cross-reacted with vaccine and virus epitopes suppressed the acute viremia in vaccinated chimpanzees (66). This finding suggests that only cellular responses are effective at clearing heterologous HCV strains. However, even though more than 50 known antigenic epitopes recognized by HCV specific T lymphocytes have been isolated, most of patients still developed into chronic infections (67).

Many attempts have been tried to strengthen the immunity caused by HCV. Encke reported that HCV peptide or protein pulsed Dendritic Cells induce both humoral and cellular immune responses vigorously in mouse models (68). Qiao, et al. described a Hepatitis C Virus like particles (HCV-LPs), and they found the immunization of HCV-LPs with adjuvant AS01B or CpG10105, or the combination of both, increase antibody titers 10 folds compared to the immunization of HCV-LP alone (69). Both approaches elicit very strong immune responses, and provide very promising vaccination candidates against HCV infection. Despite all these endeavors, the immunological mechanism of HCV infection has not yet been elucidated.

1.5 The roles of Core protein in HCV infections

HCV-Core is a 21 KDa viral nucleocapsid protein, which forms homomultimers, binding and packaging the viral genome RNA. Its gene and amino acid sequences are well conserved in the coding region of most HCV genotypes, indicating its important biological functions (70). It interacts with cellular membranes, lipids and envelope glycoproteins, thus, it is critical for the viral morphogenesis and particle

assembly (71, 72). Full length HCV-Core protein exists in the cytoplasm when it is expressed in mammalian cells, but the deletion of C-terminal hydrophobic region translocates it into the nucleus (73). Yasui's study found the Core protein existed not only in the cytoplasm, but also in the nucleus when it was expressed in a transformed CHO cell line, and the nuclear fractions contained the same full length 21 KDa proteins (74). The expressed proteins in bacteria can be efficiently self-assembled into nucleocapsid-like particles *in vitro* (75). Studies on a truncated Core protein demonstrated that the C-terminal is essential for the folding, oligomerization (76) and secretion (77) of this protein.

HCV-Core has been shown to be multi-functional within host cells. It modulates gene transcription, cell proliferation, cell signaling; interferes with lipid metabolism of host cells; and suppresses the immune responses and causes apoptotic cell death (78). The research by Basu, et al. discovered the importance of HCV-Core protein in the maintenance of immortalized human hepatocytes; their conclusion indicates HCV-Core protein might play key roles in hepatocellular carcinoma (79); Brigitte also discovered a truncated HCV-Core protein in a hepatocellular carcinoma (80). HCV-Core disturbs many other signal pathways such as MAPK/ERK (81), JNK (82), NF-κB (39), p38 MAP Kinase (83, 84), and Fas (85), et al. Moreover, HCV-Core interacts with the putative RNA helicase (86), Human Dead box protein DDX3 (87), and participates in the transcriptional repression of the p53 (88) and p21 promoters (89), which are tumor suppressor genes. Furthermore, HCV-Core promotes the immortalization of human hepatocytes (79, 90), causes mitochondrial dysfunction (91), and inhibits HIV, HBV replication, etc.

Immunologically, Core protein presents several epitopes for both T and B cells (92, 93). Kawamura found that the presence of HCV-Core protein in the liver parenchyma protects infected hepatocytes from the attack by the cell-mediated immune system and promotes their proliferation by inhibiting STAT1 and stimulating STAT3 activation (94). All these data indicates the important roles of Core protein in the pathogenesis of HCV infections.

Many researches discovered that Core may induce the apoptosis of HCV specific cytotoxic CD8+ T lymphocytes through multi-signaling pathways, leading to the lower immune responses. You found that HCV-Core protein activates NF-kB in most cell types, which in turn contributes to the chronically activated, persistent state of HCV-infected cells (39). Chang' results suggest that the Core protein promotes the apoptosis of immune cells during HCV infection via the Fas signaling pathway, thus facilitating HCV persistence (95). Zhu discovered that HCV-Core also enhances the FADD-Mediated apoptosis and suppresses the TRADD signaling pathway of tumor necrosis factor receptor (96); Cho's results showed that HCV-Core protein decreases the expression of pRb, thereby allowing E2F-1 to be constitutively active, which is thought to result in rapid cell proliferation or vulnerability to apoptosis (97). Jonathan found that Jurkat cells (T lymphocytes originated), expressing full-length but non-truncated Core protein, exhibit ligand independent apoptosis and cytoplasmic targeting of truncated Core protein recapitulates its ability to induce apoptosis (85). They also found that the activation of caspases 3 and 8 is necessary and sufficient for full-length Core to induce apoptosis; Jurkat expressing full-length, but non-truncated Core protein, induces Fas receptor aggregation, and the apoptotic

pathways activated by HCV-Core in Jurkat cells require cytoplasmic localization of Core proteins (85). Realdon concluded that the pro-apoptotic effect of HCV-Core protein in transiently transfected cells is enhanced by nuclear localization and is dependent on PKR activation (98). Yan, et al. truncated and constructed 7 HCV-Core sequences at various lengths and sites and the corresponding proteins were expressed; they concluded that of all the 7 truncated peptides, the N-terminal of Core protein has a greater effect in inducing apoptosis and necrosis than the C-terminal (99).

1.6 Immune responses caused by HCV-Core immunization

One important characteristics of HCV-Core is that this gene is comparatively conserved in all the genotypes. Many epitopes on HCV-Core protein for both T and B lymphocytes have already been identified (92, 93, 100~102). After studying a group of patients with chronic hepatitis C infections, John concluded that there is no genetic mutation in any of the Core CTL epitopes despite detectable cellular responses (83). Therefore, HCV-Core may be targeted to develop preventive or therapeutic vaccines, and many studies were focused on the immune responses caused by the individual HCV-Core.

DNA immunization is a powerful method to generate both cellular and humoral immune responses and HCV-Core protein induced immune responses are mostly based on intramuscular DNA injections in animal models. However, some studies discovered that the immunization of DNA with the entire HCV-Core sequence only results in weak humoral immune responses with strong CTL activity (103, 104), while in some other studies it induces both humoral and cellular immune response

(100,105); the crucial distinction might be related to the promoters and vectors used to drive the expressions. Encke has tried to co-immunize HCV-Core proteins with adjuvants, and found that the adjuvant CpG-ODN strengthened humoral immune responses without potential effects on cellular immunity (103). Moreover, truncated HCV-Core gene might be a good way to strengthen both humoral and cellular immune responses. Satiago vaccinated BALB/C mice with a plasmid containing a truncated coding sequence of the first 176 amino acids of HCV-Core proteins, both humoral and cellular specific immune responses were successfully induced (106). Julio also intramuscularly inject 2 different mouse strains of another truncated Core proteins (1~120) 3 times with adjuvants, both elicit potent anti-HCV humoral and cellular immune responses (107). Encke proposed a type of HCV vaccine based on *ex vivo* stimulated and matured Dendritic Cells (DC) loaded with HCV specific antigens, and found that mice immunized with HCV-Core pulsed DC generated both therapeutic and prophylactic immune responses (35). This result indicates that HCV-Core pulsed DC may provide a new immunotherapy in chronically infected HCV patients.

1.7 Cell culture system of HCV

The lack of robust cell culture system and small animal models slowed the progress of HCV research. Since the identification of HCV in 1989, many efforts were taken to develop cell culture and small animal models, but only recently efficient cell culture and small animal systems were successfully developed. Despite a bunch of shortcomings, they were believed to be important breakthroughs for the basic research on HCV (108).

A narrow host range featured Hepatitis C Virus, and its replication was highly restricted to humans or experimentally infected chimpanzees. Naturally, HCV could be replicated only in some highly differentiated cells, including primary hepatocytes, peripheral blood mononuclear cells, some hepatoma cells, and lymphoblastoid cells (109). However, the replication level in these cells is extremely low and, and no viral passages could be successfully established (109). Ito, *et al.* have tried to cultivate infected hepatocytes *in vivo* prepared from liver biopsies, but the HCV replication efficiency in this cell culture system was very low and the infected primary human hepatocytes were poorly available (110). Therefore, both the low replication rate and availability circumscribed the application of primary cell culture systems of HCV.

McCaffrey's research showed that the transfected HCV genomic RNA failed to replicate in mouse liver, suggesting a post-entry block to viral replication (111). This hurdle was firstly overcome by "sub-genomic replicons of HCV" developed by Lohmann *et al.* in 1999. They replaced the region encoding from Core to NS2 with a selectable marker, and the Internal Ribosome Entry Site (IRES) mediates the translation of the HCV replicase complex (NS3-5B); transfection of this 'sub-genomic RNA' in Huh-7 cell line (human hepatoma originated cell lines), followed by neomycin selection results in cell clones in which the full length HCV genomic RNA replicates efficiently (112). This cell culture model is very useful in understanding the host cell conditions for HCV RNA replication and antiviral therapies, but efficient HCV genome replication in Huh-7 cells depends on adaptive mutations throughout the NS region, including NS3, NS4B, NS5A and NS5B (108). The adaptive

mutations, often in NS5A, could increase the RNA replication efficiency up to 10,000 folds (113).

The real robust cell culture systems releasing infectious HCV particles were established in 2005 by Lindenbach, et al. (114), Zhong et al. (115), and Wakita et al. (116) respectively. The breakthrough was based on a unique sub-genomic replicon of HCV genotype 2a from a Japanese Fulminant Hepatitis C Virus (JFH1), which replicates the full genome of HCV RNA efficiently in human hepatoma Huh7 cells without adaptive mutations. Lindenbach cloned a full length of JFH1 genome (115) behind a T7 promoter, and then the linearized plasmid DNA was used as the template to transcribe the full-length HCV RNA genome. This HCV RNA transcribed *in vitro* was transfected to Huh7-derived cells, resulting in the secretion of HCV particles that are infectious to both chimpanzee and naïve hepatocytes.

Lindenbach's research showed that the replication of viral particles by the above-mentioned method was robust, producing nearly 10^5 infectious units per milliliter within 48 hours and the viral particles were filterable and neutralized with the monoclonal antibody against the viral glycoprotein E2, which bound to a putative cellular membrane HCV receptor, CD81, and the viral particles could be inhibited by interferon-a or by several HCV-specific antiviral compounds (114). The viral particles cultured from JFH1 genome by Wakita have a density of 1.15~1.17g/ml, and a spherical morphology with an average diameter of about 55nm; the secreted virus can be neutralized by CD81-specific antibodies and by immunoglobulin from chronically infected individuals (116). This cell culture system provides a powerful

tool for the analysis of host-virus interactions that may facilitate the developments of antiviral drugs and vaccines of HCV.

Despite all these indisputable breakthroughs, the understanding of cell culture on HCV still remains obscure. The application of this new HCV cell culture system is still limited in the dependence of JFH1 replicons, and the infectivity is maintained only within genotype 2a (JFH1). Lindenbach has tried to produce infectious particles via this system from a full-length chimeric genome (FL-H77/JFH), which were constructed with the use of the Core-NS2 gene regions from the infectious genotype 1a virus strain H77 (114). Although the genotype 1a/2a chimera is replicable in the primary transfection, it cannot spread within the transfected cell cultures, suggesting that the interactions between the structural and non-structural proteins may be important for HCV replication and particle formation (114). Moreover, the adaptive mutations demonstrated that the host cell conditions also affect the replication of HCV RNA, but little is known about the host cell factors that are necessary for HCV replication (109).

1.8 The animal models of HCV

Due to the narrow host range, the chimpanzee was the only animal species for HCV infection for a long time, but the expense, ethical concerns and short supply highly restricted the application of this large animal model. Thus, an easily accessible small animal model for HCV infection is still in need. Fortunately, just recently, despite a number of disadvantages, small animal models for HCV have been developed successfully.

As the only HCV animal model available for many years, the chimpanzee has provided valuable insights into various aspects of HCV infections. After exposure to HCV, the clinical characteristics observed in chimpanzees and humans are very similar: a significant proportion of experimental chimpanzees failed to clear the virus and developed persistent infections in spite of detectable humoral and cellular immune responses as the infection in humans; the alteration of liver morphology and inflammatory hepatic lesions also closely resemble the pathologic changes in humans. Therefore, the experiments with chimpanzees continue to be very valuable for the studies on the pathogenesis of HCV infections, and the critical evaluation of vaccine candidates (117). However, the diseases caused by HCV in chimpanzees are usually milder than in humans: the liver damage in chimpanzees is not as severe as that in humans; neither fibrosis nor cirrhosis has been observed in chimpanzees as a result of viral replication (118). Some researchers have tried to inoculate the other primate species with serum derived from HCV-infected chimpanzees; unfortunately, due to the susceptibility and the low replication level, all the endeavors failed (119).

Many efforts were undertaken to established small animal models. Galun, et al. transplanted liver fragments from HCV infected patients to "Trimera Mouse", and HCV production can be maintained, however, the short duration of graft survival and the low viral titers limited the application of this model (120). Transgenic mice with full-length HCV cDNA have also been tried. Although both HCV RNA and Core proteins were detected in the transgenic mouse liver, the expression level in this model was quite low, and the histological change could not be observed; moreover,

it could not be used to assess the viral infection processes such as viral binding and entry (121).

The earliest successful HCV animal model was the Alb-uPA transgenic mouse model developed in SCID mice in 2001. Mercer transplanted human hepatocytes into this mice strain to generate chimeric livers (122). This system maintained HCV viremia in the range observed in human infections, persisting up to 35 weeks, and the infection can be serially passaged through three generations of mice (122). Immunohistochemistry detection of liver sections derived from these animals revealed that HCV antigen could only be detected in human originated hepatocytes, indicating that the viral infection is still restricted only to the transplanted human liver cells. Although these chimeric mice may represent the first small animal model for studying the human hepatitis C virus *in vivo* (123), which probably facilitate the studies in some areas of HCV research, they are still limited by the lack of a functional immune system, preventing the study of the immunopathogenesis and the evaluation of vaccines (117); moreover, the SCID/Alb-uPa mice are very fragile animals suffering from bleeding disorders, causing a significant mortality rate of approximately one third of the newborns; furthermore, the accessibility of human liver cells is quite limited and the successful transplantation is technically challenging, requiring microsurgical equipment, skill and expertise (109).

Wu, et al. reported a novel immunocompetent rat model of HCV infection in 2005. They transplanted rats with $5X10^6$ Huh 7 cells (also human originated) one day after birth, and the rats were inoculated with HCV-genotype 1 one week later. HCV levels in serum were 11,000copies/ml at week 4, and peaked at week 12 of

22,500copies/ml. This model was demonstrated to support HCV gene expression, viral replication, and develop biochemical and histologic evidence of hepatitis (124). In 2006, Zhu, et al. reported another reproducible and easily accessible xeno-graft mouse efficacy model. In this model, gamma-irradiated SCID mice were implanted with a mouse-adapted Huh-7 cell line transfected with luciferase replicon. NS3/4A protease inhibitor (BILN 2061), human IFN-a decreased HCV RNA replication, and treatment withdrawal resulted in a rebound of viral replication, which paralleled the clinical treatment outcomes in humans. Their research showed this mouse model could be used as a powerful tool for rapid evaluating potential anti-HCV replication drugs *in vivo* (14).

Despite all of these breakthroughs, the replication of HCV viruses in the small animal models still relied on the exogenous hepatic tissues or cells originating from humans, and restricted only to some genotypes of viruses; which factors of both virus and host play the critical roles in the replication of viral particles still remains unknown. More research and progress are still needed to realize the viral replication in the liver of small animals for the study on the pathogenesis of HCV, as well as drug and vaccine development.

1.9 Research progress on liver-specific expression

HCV belongs to Hepacivirus Genus of the Flaviviridae Family, and the viral protein expression and replication are specific in the liver. Yan, *et al.* explored the replication state of HCV in extra-hepatic tissues, and they concluded that the extra-hepatic viral expression and replication level of HCV is very low (125). On the other hand, Schirren *et al.* studied both liver and blood-derived T-cell lines from 36

patients (18 with chronic hepatitis C and 18 with HCV-associated cirrhosis), they found that HCV-specific CD41 T cells are multi-specific, compartmentalized to the liver and produce IFN-γ (126). Wong also analyzed the liver filtered lymphocytes from 44 chronically infected patients, and nineteen different CTL epitopes were identified, which were distributed throughout the genome (127). All these studies indicate the viral replications and the immune responses are mainly restricted to the liver.

However, none of the model systems studying the immune responses and functions of individual HCV genes at present are restricted to liver-specific expression. There are 4 popular methods to study the pathogenesis of HCV infections: 1) DNA vaccination via intramuscular injection by which the proteins were mainly expressed in the muscles of injected location, and the high level global expression of HCV proteins in various tissues (128) may severely disturb the normal functions of the cells other than hepatocytes, including lymphocytes (22, 39~49, 85, 95); 2) purified viral protein injection, which mainly cause humoral immune responses, but the humoral immune responses were demonstrated non-protective in HCV infections (62~64); 3) HCV protein pulsed Dendritic Cells (68), by which was demonstrated to generate both therapeutic and prophylactic immune response; whereas in real HCV infections, the viral proteins should be presented via liver-specific expression. 4) Transgenic mouse models in which the viral gene expression was driven by global promoters, which could not be used to assess viral infection processes such as viral binding, entry and immune responses, due to the mechanism of tolerance, and the disturbance caused by viral proteins (121).

Although transgenic mice of individual HCV genes could be used to study host-virus interaction, the defects in these mouse models are evident. The antigen presentations in all these pathways are quite different from that in the natural HCV infections. Therefore, none of these studies mimic the natural HCV infections and have some difficulties to elucidate the immunopathogenesis of this disease. To achieve this goal, and to study the functions of individual HCV genes, there is a need to restrict the viral protein expression to hepatocytes both in wild type and transgenic mouse models.

Although numerous liver-specific promoters and enhancers were successively identified over the past decades, the high level expression of exogenous target genes specific in hepatocytes *in vivo* remains the major challenge till recently. The major problems include the inefficient or unstable transductions and the lack of tissue-specificity (129, 130); parts of these problems were firstly solved by Zhang, et al. (128). They demonstrated that a high level expression of plasmid DNA in hepatocytes can be easily obtained by tail vein injections. On the other hand, although some researchers have already tried to liver-specifically express individual HCV genes by transgenic mouse models, and the expression of target genes in liver were confirmed by immunohistochemistry, the transgenic animals were phenotypically similar to the normal littermates and did not exhibit a major histological change within the liver up to 24 months of age (131), which might be related to the lower promoter activity.

Fortunately, the progresses on chimeric liver-specific transcriptional elements in recent years made the over-expression of individual HCV genes only in mouse

liver possible. Kramer et al. reported their research on a collection of chimeric liver-specific elements, and found that a liver-specific enhancer could increase the promoter activity for more than 250 folds without the loss of specificity; among various chimeric constructs they made, the albumin enhancer (Ealb) linked to human a1-antitrypsin (HAAT) promoter could maintain liver-specific expression *in vitro* about 1.8 times as high as CMV early promoter, which was reported as the strongest global promoter so far (132) and the long-term gene expression of this chimera could also be maintained (133). Gehrke et al. reported their tests on a total of 25 chimeric liver-specific transcriptional units, and they concluded that the alcohol dehydrogenase 6 (ADH6) basal promoter linked to two tandem copies of apoplipoprotein E enhancer (2apoEs) is the choice for the liver-specific expression of transgenes, however, they did not detect the long-term expression of this chimera (129).

From the above studies, the over-expression of individual HCV genes specific in mouse liver simply via tail vein injections is possible, and this may also cause immune responses as global expression via intramuscular injection. However, unlike the global expression, liver-specific expressions have the advantages of exactly mimicking the natural HCV infections. This strategy is also potential to further study the functions of individual HCV proteins *in vivo* and to clearly answer which gene, or genes in combination cause the lower immune response and viral persistence. Moreover, liver-specific expression may be used to restrict the expression of both the viral and host factors, which are critical to the HCV replications, to the liver of small animals. Therefore, the work in this field might also provide a brand-new way

of thinking to establish both cell culture and small animal models for HCV infections. However, whether the chimeric liver-specific transcriptional elements lose the specificity in lymphocytes is still unknown; which chimera has high promoter activity with the least impact on immune system and the factors affecting the promoter activity and specificity should be further studied.

1.10 Thesis hypothesis, rationale, objectives and strategies

It is hypothesized that the restricted over-expression of individual HCV genes in hepatocytes will not interfere with the normal function of immune system, but could result in liver damage or immune responses as observed in natural infections. The rationale of this project is the liver specific regulatory elements restrict the expression of HCV individual genes to hepatocytes, without affecting the normal functions of other organs. The objectives are to select a suitable liver-specific vector/expression system, explore the possibility of liver specific over-expression by IV injection, and provide DNA for improved HCV mouse models.

To achieve these goals, some vectors with chimeric liver-specific transcriptional elements were constructed and their promoter activity, specificity and impacts on lymphocytes *in vitro* were compared. One chimera was selected to study the immune responses caused by individual HCV genes in wild type mice and this chimeric DNA can also be used to make liver-specific individual HCV gene transgenic mouse models. In this project, the liver-specific gene expression of HCV-Core protein both *in vitro and in vivo* was detected; the cytotoxic T lymphocyte responses caused by both liver-specifically and globally expressed HCV-Core proteins were also tentatively detected. The vector DNA constructed in this research

may be useful to advance knowledge on HCV, and could be extended to the individual genes of other types of hepatotropic virus such as HAV, HBV, and so forth, the signaling pathways of HCV infections, establishing cell culture systems and small animal models of HCV infections. Therefore, this research may provide the tools to study the immunopathogenesis of HCV infections.

2. MATERIALS AND METHODS

2.1 DNA amplification

The annealing temperatures of all the primers in this project were 52°C and 62 °C (**Table 1**), and were synthesized by the Biotechnology Research Institute (Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road). Plasmid p90 containing the whole genome of HCV-1a was kindly provided by Yanagi (134) for use as the template to amplify individual HCV genes. Briefly, 100ng of template DNA was incubated with 0.5μM of each primer, 0.2mM of each dNTP (Invitrogen, Cat. No. R725-01), 1.5mM $MgCl_2$ and 0.5 unit of Taq DNA Polymerase (Invitrogen, Catalog No. 10342-053) in a total volume of 50μl using PCR buffer provided by the polymerase supplier as described previously (135). All the DNA amplifications use the same PCR program for the Perkin Elmer Cetus Model 9600: denaturation at 94°C for 3 minutes; 5 cycles at '94°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds'; 25 cycles at '94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds'; extension at 72°C for 10 minutes. The DNA products were purified by MinElute PCR Purification Kit (Qiagen, Catalog No. 28004) and detected on 0.7% agarose gel in 1X TBE buffer.

2.2 Agarose gel electrophoresis

5X TBE buffer (0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3) was diluted to 1X TBE, which was used to make 0.7% agarose gel with Ethidium Bromide (final concentration of 1μg/ml); 5μl of 1kb DNA ladder (Promega, Catalog No. G5711)

Table 1: Primers designed for vector constructions and HCV gene cloning

The annealing temperature of all the primers in this project were 52C° **and** 62°C, and all the DNA amplifications use the same PCR program for the Perkin Elmer Cetus Model 9600: denaturation at 94°C for 3 minutes; 5 cycles at '94°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds'; 25 cycles at '94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds'; extension at 72°C for 10 minutes.

Table 1: Primers designed for vector constructions and individual HCV gene cloning

Primer Name	Primer Sequence	Restriction Endonuclease
Complete CMV early promoter	F: TTG GTA CCG GTA AAT GGC CCG CC	Kpn I
	R*: GGTCTAGAATTCCACCACACTGGACT	HinD III*
pVAX1 Backbone	F: TTGGATCCTTCTACTGGGCGGTTTTAT	Bam HI
	R: CCGGTACCATTTACCGTAAGTTATGTAAC	Kpn I
HAAT poly(A)	F: GGT CTA GAT CAG CCT CGA CTG TG	Xba I
	R: TAGGATCCCCAGC TGGTTC CATA	Bam HI
LS-Empty	F: ATAGATCTGTTCCTAGATTACACTACACAT	Bgl II
	R: GGTCTAGAATTCCACCACACTGGACT	Xba I
HCV-Core	F: GAG AAT TCA CCA TGA GCA CGA ATC C	EcoR I
	R: AC TCT AGA CTA GGC TGA AGC GGG CA	Xba I
HCV-E2	F: TTGAATTCACCATGGAAACCCACGTCACCG	EcoR I
	R: TC TCT AGA CTA CGC CTC CGC TTG GGA	Xba I
HCV-NS3	F: TTGAATTC ACC ATG GCG CCC ATC ACG G	EcoR I
	R: AATCTAGACTA CGT GAC GAC CTC CAG G	Xba I
HCV-NS5a	F: TTGAATTCATTATGGGCTCCGGTTCCTGGC	EcoR I
	R: AATCTAGACTAGCAGCACACGACATC TTC	Xba I
Observations	1) F: Forward primer; R: Reverse primer; 2) *The complete CMV early promoter was amplified by this reverse primer but double-digested by Kpn I and HinD III; the later enzyme digestion site is 40bp upstream of the reverse primer binding site, and therefore the sequence recognized by HinD III is not shown in this primer sequence.	

was used as DNA marker; 20μl double-digested DNA products with 4μl of 6X loading buffer (Promega, Catalog No. G5711) were loaded to each well and the gel was running at 80 volts for 45~90 minutes. DNA bands of the expected molecular weights on gels were cut under UV light, and the target DNA was purified by Gel Extraction Kit (Qiagen, Catalog No. 28704).

2.3 DNA digestion, ligation and cloning

DNA Restriction Endonucleases were ordered from New England Biolabs (EcoRI, R0101S; Xba I, 0145S; HinD III, R0104S; Kpn I, R0142S; Bgl II, R0144S; BamHI, R0136S). Before ligation, all the DNAs, including the purified PCR products and vectors, were double-digested by the corresponding enzymes in NEB Buffer 2 at 37°C for more than 1 hour, as recommended by the enzyme supplier. When BamHI was used, a sequential digestion process was applied, in which the appropriate buffer conditions were followed according to the instructions from the supplier. The double-digested and purified DNAs (the insert and vector molar ratio is 3:1) were ligated in a total volume of 20μl with 0.5 unit of T4 DNA Ligase (Invitrogen, Cat. No.15224-017) for 1 hour at room temperature (22~24°C) as described by the ligase supplier. After ligation, 10μl of the reaction mixture was transformed to *E. coli* DH5a competent cells (Invitrogen, Cat. No. 18263-012) as described by the supplier. After shaking in 37ºC for 45 minutes, the bacteria was spread to LB-agar plates with corresponding antibiotics (Ampicillin or Kanamycin) and incubated at 37°C for 16 hours. Single colonies were picked and screened for positive clones as described previously (135).

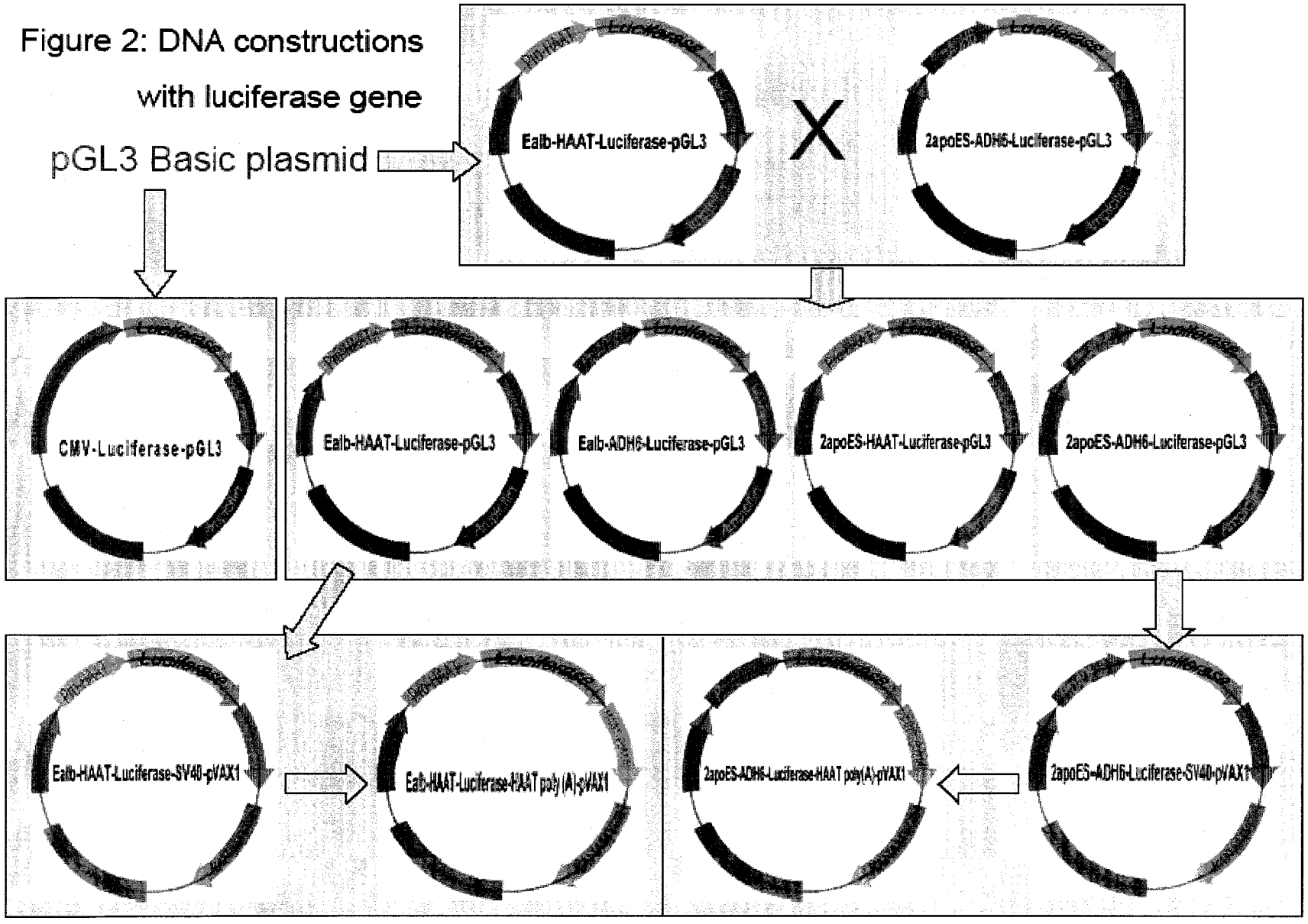
2.4 Reporter DNA constructs with luciferase gene

All the reporter DNA constructs are designed and are shown in **Figure 2** and the primer sequences were listed in **Table 1**. Plasmid Ealb-HAAT-Luciferase-pGL3 was obtained from Dr. M. Gabriela Kramer, University of Navarra, Spain (133), and 2apoEs-ADH6-Luciferase-pGL3 was obtained from Dr. Rolf Müller Philipps University, Germany (129). Plasmid pRSV.hAAT.bpA was provided by Zhi-Ying Chen, University of Stanford, USA (136). Both pGL3 plasmids were double-digested by Kpn I and Bgl II, and the enhancers Ealb and 2apoEs were exchanged to construct two new plasmids Ealb-ADH6-Luciferase-pGL3 and 2apoEs-HAAT-Luciferase-pGL3. The CMV complete Enhancer-Promoter originating from plasmid pVAX1 (Invitrogen, Cat# V260-20) was amplified by PCR and constructed into pGL3-Basic Vector (Promega, Cat. No. E1751). The expression cassettes of Ealb-HAAT-Luciferase-pGL-3 (Lane1) and 2apoEs-ADH6-Luciferase-pGL3 (Lane5) were digested by Kpn I and BamH1 sequentially and cloned into pVAX1 Backbone, which was amplified by PCR with Kpn I and Bam HI on both ends; then Ealb-HAAT-Luciferase-SV40 poly(A)-pVAX1 and 2apoEs-ADH6-Luciferase-HAAT poly(A)-pVAX1 were obtained. The SV40 poly(A) of these two new plasmids was substituted by Human a1-antitrypsin (HAAT) poly(A) (amplified from pRSV.hAAT.bpA by PCR with Xba I and Bam HI on both ends) to obtain the other two plasmids of Ealb-HAAT-Luciferase-HAAT poly(A)-pVAX1 and 2apoEs-ADH6-Luciferase-HAAT poly(A)-pVAX1.

Figure 2: The constructions of plasmid DNA with luciferase gene

To compare the promoter activity, specificity and impact on immune system of different chimeric DNAs, the enhancers Ealb and 2apoEs of Ealb-HAAT-Luciferase-pGL3 and 2apoEs-ADH6-Luciferase-pGL3 were exchanged to obtain two new plasmids Ealb-ADH6-Luciferase-pGL3 and 2apoEs-HAAT-Luciferase-pGL3. The CMV complete Enhancer-Promoter originating from plasmid pVAX1 was amplified by PCR and constructed into pGL3-basic vector as a positive control. To compare the impact of backbone on promoter activity and specificity, the backbone of Ealb-HAAT-Luciferase-pGL-3 and 2apoEs-ADH6-Luciferase-pGL3 were substituted by pVAX1 backbone to obtain the two plasmids Ealb-HAAT-Luciferase-SV40 poly(A)-pVAX1 and 2apoEs-ADH6-Luciferase-SV40 poly(A)-pVAX1. To compare the impact of poly(A) sequence on promoter activity, the SV40 poly(A) of the two new plasmids in pVAX1 backbone was substituted by Human a1 anti-trypsin (HAAT) poly(A).

Figure 2: DNA constructions with luciferase gene



2.5 Individual HCV gene cloning and empty vector construction

The luciferase gene segment in the selected liver-specific plasmid Ealb-HAAT-Luciferase-pGL3 was substituted by individual HCV genes previously cloned into pVAX1 (primers listed in Table 1) to obtain the pLS-Core, pLS-E2, pLS-NS3 and pLS-NS5a, in which the transcriptions of target genes were driven by a liver-specific enhancer and promoter. To construct the empty vector (pLS-Em) as a negative control for liver-specific DNA injection, the Ealb-HAAT-Luciferase segment in Ealb-HAAT-Luciferase-pGL3 was substituted by the Ealb-HAAT segment amplified from plasmid pLS-Core by LS-Empty primers in Table 1.

2.6 Confirmation of plasmid DNA constructs

DNA constructs with luciferase gene were double-digested by Kpn I and Hind III to release the enhancer and promoter segments, and by Hind III and Xba I to release the luciferase gene (~1700bp). The liver-specific empty vector DNA (Ealb-HAAT-pGL3) was double-digested by Kpn I and HinD III to release the Ealb-HAAT segment. This empty vector DNA was also linearized by Xba I digestion to confirm the molecular weight (3700bp). Similarly, the liver-specific plasmid DNA containing individual HCV genes were digested by EcoR I and Xba I to release the HCV gene segments. Following the digestions, the molecular weights of targeted DNA were confirmed by Agarose Gel Electrophoresis. Additionally, the enhancer and promoter segments, individual HCV genes and poly(A) regions of all these constructs were also confirmed by sequencing at the Biotechnology Research Institute of the University of Ottawa.

2.7 Cell culture and transfection rates

Cell lines HepG2 (ATCC Cat. No. HB-8065), Hela (ATCC Cat. No. CCL-2), 293T (ATCC Cat. No. CRL-11268), Jurkat (ATCC Cat. No. TIB-152) and U937 (ATCC Cat. No. CRL-1593.2) were cultured in IMDM (Wisent, Cat. No. 390-000-CL) complete medium, and CHO K1 (ATCC Cat. No. CCL-61) was cultured in Ham's F12 (Wisent, Cat. No. 305-015-CL) complete medium at 37°C with 5%CO_2; both media were supplemented with 10% Fetal Bovine Serum, and 100μg of both Penicillin and Gentamycin. To measure the transfection rates, $1.5X10^5$ cells/well of HepG2, $4X10^4$ cells/well of Hela, $8X10^4$ cells/well of CHO K1 and 293T, $2.0X10^5$ cells/well of Jurkat and U937 were plated into 24-well cell culture plates (BD Primaria, Cat. No. 353847) respectively. The cells were incubated at 37°C with 5%CO_2 for 24 hours, and then each well was transfected with 0.3μg Endotoxin-free pSV-β-galactosidase plasmid DNA and 0.6μl Fugene6 (Roche, Cat. No.1815091) as described by the supplier. Twenty-four hours after the transfection, cells were stained by β-Gal staining reagent (Roche, Cat. No.11828673001). The stained cells were counted and imaged under an inverse microscope (Kruess, MBL-3100). The percentages of cells transfected were calculated by the formula: Transfection Rate = (the number of stained cells/all the cells counted) X 100%.

2.8 Promoter activity assay

Three hundred ng of Endotoxin-free reporter plasmid DNA and two hundred ng of pSV-β-galactosidase plasmid DNA were co-transfected with 1.0μl Fugene6 to all the 6 cell lines pre-cultured in 24-well cell culture plates as described in 2.7.

Transfected HepG2, Hela, 293T and CHO K1 cells were incubated at 37°C with 5%CO_2 for 24 hours; Jurkat and U937 were incubated for 36 hours and one more HepG2 plate was incubated for 48 hours. After incubation, the cells were washed by PBS, lysed in 100µl β-Galactosidase Enzyme Assay lysis buffer (Promega, Cat. No. E2000) on ice for 15 minutes and centrifuged at 12,000g for 1 minute, and 20µl and 50µl of the supernatant were assessed in Luciferase Activity Assay (Promega, Cat. No. E1500) and β-Galactosidase Enzyme Assay respectively.

2.9 Promoter activity normalization and statistical analysis

Means of four independent experiments, standard deviation (SD) and percentage of the promoter activity relative to the complete CMV early promoter was calculated according to the following 6 formulas:

1) Mean value of promoter activity = $(X_1+X_2+X_3+X_4)/4$; Xn is the detection value read directly from the luminometer (Montreal Biotech Inc. Sirius Luminometer) of one independent transfection;

2) Mean value of β-Galactosidase enzyme assay = $(G_1+G_2+G_3+G_4)/4$; Gn is the detection value of β-Galactosidase enzyme activity of one independent transfection read from Microplate Elisa Reader (Bio-Rad, Model 550);

3) Standard deviation (SD) calculation: Standard Deviation (SD)= $\sqrt{\frac{n\sum x^2-(\sum x)^2}{n(n-1)}}$

n=4; X is the detection value from luminometer of one independent transfection;

4) Normalized values of mean or SD = Mean Value or SD/ β-Galactosidase enzyme assay value. Before the division, the mean values of β-Galactosidase enzyme assay were multiplied by 10 or 100 until the final values were between 0.1~2;

5) Percentage of activity relative to CMV = $\frac{\text{Normalized Mean Value}}{\text{Normalized Mean Value of CMV}}$ X 100%;

6) Percentage of SD = Normalized SD/ Normalized mean value of CMV.

2.10 PAGE and Western Blot analysis

For Polyacrylamide Gel Electrophoresis (PAGE) analysis, 1μg plasmid DNA with 2.0μl Fugene6 was transfected to HepG2 cells in 12-well cell culture plates. 24 hours after transfection, samples were lysed in 50μl lysis buffer (125mM Tris, 10mM EDTA, 10mM DTT, 50% glycerol and 5% Triton X-100). Twenty μg of total protein was loaded onto 12% SDS–polyacrylamide gels and separated for 90 minutes at 100 volts. One gel was stained by Coomassie Brilliant Blue G-250. For Western Blots, the proteins on PAGE gel were transferred onto the Immuno-Blot PVDF membrane (Bio-Rad, Cat. No.162-0174) for 45 minutes at 100 volts. Then the membrane was blocked for 1 hour in blocking buffer (PBS, 0.05% Tween, 5% nonfat milk), and incubated with mouse anti-HCV monoclonal antibody (Abcam, Cat. No. ab2740) diluted to 1/2000 in blocking buffer overnight at 4°C. After 3 washes, the PVDF membrane was incubated with goat anti-mouse IgG–peroxidase (Sigma, Cat. No. A9917) conjugated antibody diluted to 1/10,000 in blocking buffer at room temperature for 2 hours. The immunoreactive proteins were developed and imaged by Western Lighting Chemiluminescence Reagent (Santa Cruz, Cat. No. A102).

2.11 Indirect immunofluorescent assay of expression *in vivo*

Three 6~8-week-old Balb/C female mice (Charles River Laboratories, Strain Code: 028) were injected with 100μg Endotoxin-free DNA via tail veins. Forty eight hours after the injection, tissues from treated and control mice were embedded in

liquid Tissue Tek OCT Compound (Somagen Diagnostics, Cat. No. 4583-S) in Cryomolds (Cat. No. 4557). The Cryomolds were then dropped into isopentane pre-cooled on dry ice for more than 10 minutes to freeze completely. Then the tissues were cut in a cryostat machine into 5μm frozen sections, and blocked with 5% normal goat serum and 0.1% Triton X-100 in PBS in a humid chamber at room temperature for 1 hour. The sections were washed in cold PBS 3 times for 5 minutes each, and then were incubated with anti-HCV-Core monoclonal antibody diluted to 1/100 in PBS/1% goat serum for 1 hour at room temperature in a humid chamber. Then the sections were washed 3 times and incubated in goat anti-mouse-FITC conjugated antibody (Sigma, Cat. No. F4108) diluted to 1/200 in PBS/1% goat serum at room temperature for 1 hour. After 3 washes, the sections were dried at 37°C for ~15 minutes, mounted with 50-60% glycerol, 2.5% 1,4 diazobicyclo(2,2,2)-octane in PBS, and imaged under an immuno-fluorescent microscope (Zeiss LSM 510).

2.12 DNA injection via tail vein

Twenty one 6~8-week-old Balb/C female mice (Charles River Laboratories, Strain Code: 028) were randomly distributed into 3 groups, 7 mice each. One hundred μg/mouse of Endotoxin-Free plasmid DNA purified by Giga Kit (Qiagen, Cat. No.12362) in 0.2 ml PBS was immunized into the 3 groups respectively via tail vein injections. The mice were immunized for a total of 3 times every 3 weeks. Seven days after the last injection, they were sacrificed and the liver, kidney, blood, spleen were extracted for protein expression and immune response detections.

2.13 *In vitro* stimulation of splenocytes and Flow Cytometry

Right after the mice were sacrificed, their spleens were homogenized (homogenizer from Thomas Scientific, Cat. No. 3431D7) and filtered by 70μm nylon mesh (BD Falcon Cat. No.: 35 2350). The splenocytes were washed 3 times with PBS and re-suspended at $2X10^6$ cells/ml in RPMI 1640 containing 10% Fetal Bovine Serum and 100u/ml of Penicillin/Gentamycin. A 0.5ml aliquot (10^6 splenocytes) was infected (Multiplicity of Infection = 5) with Vaccinia rVV 6C/Ss (NIH, Cat. No.: 9424; Lot. No.: 11/31/92), which expresses HCV-1a Core and E1 proteins, and incubated in the presence of 10μg/ml Brefeldin A (Sigma B-7651) at 37°C, 5% CO_2 for 16 hours. After the incubation, the splenocytes were washed once with 2ml PBS/0.5%FCS/0.01% azide, centrifuged at 1600rpm for 5 minutes. For extra-cellular staining, the splenocytes were re-suspended in total 9μl of 3 different antibodies, 3 μl each, and incubated at room temperature for 15 minutes in the dark. Antibodies used were rat anti-mouse CD3 molecular complex monoclonal antibody FITC conjugated (BD, Cat. No.555274), rat anti-mouse CD4 monoclonal antibody PE-Cy7 conjugated (BD, Cat. No.: 552775), and rat anti-mouse CD8a monoclonal antibody PerCP conjugated (BD, Cat. No.553036). For intra-cellular staining, the splenocytes were permeabilized by FACSLyse (BD, Cat. No.349202) and FACS Perm 2 (BD, Cat. No.340973) as described by the supplier, and were stained with 2.5 μl rat anti-mouse IFN-γ monoclonal antibody R-PE conjugated (BD, Cat. No.: 554412). Following one wash, the cells were analyzed by BD FACSCanto Flow Cytometry.

3. RESULTS

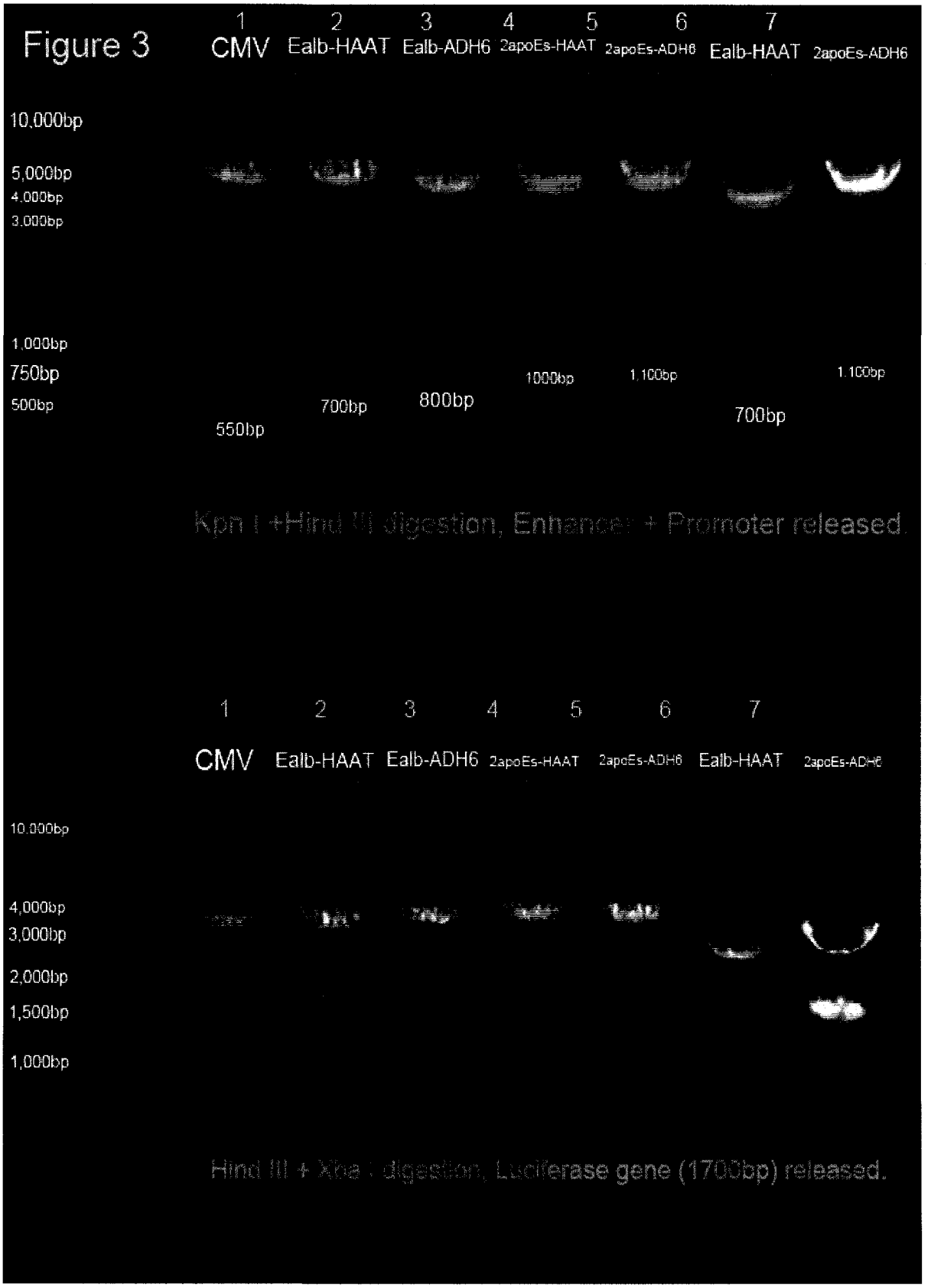
3.1 Reporter DNA constructs with liver-specific elements

Although the chimeras of Ealb-HAAT and 2apoEs-ADH6 were reported by two different labs to be the strongest chimeras of liver-specific transcriptional elements (129, 133), there is no report on their promoter activity in lymphocytes, and it is not yet known which one is the most suitable for liver-specific transcription. To answer these questions, the two plasmids, Ealb-HAAT-Luciferase-pGL3 and 2apoEs-ADH6-Luciferase-pGL3 were obtained; the enhancers Ealb and 2apoEs in these two plasmids were engineered to construct 2 new liver-specific chimeras, and the complete CMV early promoter (132) amplified from plasmid pVAX1 was also constructed into pGL3-Basic vector (Figure 2 and 3) as a control. Moreover, the pVAX1 backbone was reported to have many advantages (137) and the Diaz-Mitoma lab successfully established a transgenic mouse model based on this plasmid (138). In order to see if pVAX1 backbone could increase the promoter activity and specificity, the backbone of Ealb-HAAT-Luciferase-pGL3 and 2apoEs-ADH6-Luciferase-pGL3 was substituted by the pVAX1 backbone. As poly(A) sequence was also reported to affect the protein expression (139, 140), the SV40 poly(A) of these two plasmids was also substituted by liver-specific Human a1-antitrypsin gene poly(A). The restriction endonuclease treatment analysis (**Figure 3**) showed that the plasmid DNA was successfully constructed as designed in Figure 2. All the DNA constructs were also confirmed by sequencing.

Figure 3, DNA constructs confirmed by restriction endonuclease treatment

pGL3 and pVAX1 backbone based plasmids containing liver-specific elements and complete CMV early promoter were successfully constructed with firefly luciferase gene. Top gel: all the constructs are double-digested by Kpn I and Hind III to release the enhancer and promoter segments. Lower gel: all the constructs are double-digested by Hind III and Xba I to release the luciferase gene (~1700bp). For both top and lower gels, Lane1: CMV-Luciferase-SV40 Poly(A)-pGL3; Lane2: Ealb-HAAT-Luciferase-SV40 Poly(A)-pGL3; Lane3: Ealb-ADH6-Luciferase-SV40 Poly(A)-pGL3; Lane4: 2apoEs-HAAT-Luciferase-SV40 Poly(A)-pGL3; Lane5: 2apoEs-ADH6-Luciferase-SV40 Poly(A)-pGL3. Lane 6: Ealb-HAAT-Luciferase-HAAT poly(A)-pVAX1; Lane 7: 2apoEs-ADH6-Luciferase-SV40 poly(A)-pVAX1. The enhancer-promoter segments and poly(A) regions of all the constructs were also confirmed by sequencing.

Figure 3


1 CMV
2 Ealb-HAAT
3 Ealb-ADH6
4 2apoEs-HAAT
5 2apoEs-ADH6
6 Ealb-HAAT
7 2apoEs-ADH6
10,000bp
5,000bp
4,000bp
3,000bp
1,000bp
750bp
500bp
550bp
700bp
800bp
1000bp
1,100bp
700bp
1,100bp
Kpn I +Hind III digestion, Enhancer + Promoter released.
1 CMV
2 Ealb-HAAT
3 Ealb-ADH6
4 2apoEs-HAAT
5 2apoEs-ADH6
6 Ealb-HAAT
7 2apoEs-ADH6
10,000bp
4,000bp
3,000bp
2,000bp
1,500bp
1,000bp
Hind III + Xba I digestion, Luciferase gene (1700bp) released.

3.2 Transfection rate detection by β-gal staining

The lower transfection rates *in vitro* of some cell lines may cause the lower detection values of the promoter activity. However, when the DNA constructs are used to make transgenic animals, all the body cells would have the transfected expression cassettes, and the promoter activity could be high *in vivo* even if the promoter activity detection results *in vitro* are very low. To determine the transfection rates, 0.3μg Endo-toxin free pSV-β-Galactosidase plasmid DNA was transfected to various cell lines and stained by β-Gal staining reagent. The results showed that when 0.3μg DNA and 0.6μl Fugene6 were used, 40 to 50% of HepG2, ~30% of Hela and 293T, 20% CHO K1, ~1% Jurkat and ~2% U937 expressed β-Gal (**Figure 4**). Therefore, if the promoter activity in both Jurkat and U937 cell lines is low, it may not be caused by lower promoter activity, but by the very low transfection rates.

3.3 Promoter activity assay

To determine the promoter activity and specificity from the liver specific elements, 0.3μg of Endotoxin-free plasmid DNA was co-transfected with 0.2μg of pSV-β-galactosidase control vector to all the 6 cell lines. In this research, HepG2 was selected to represent hepatocytes; Hela (141), 293T and CHO K1 were selected to detect the specificity in non-hepatocytes; Jurkat represents T lymphocytes and U937 represents Dendritic Cells/ Monocytes. All the promoter activity results were summarized in **Table 2.** These results showed that the activity of complete CMV early promoter in hepatocytes is much weaker than that in the other cell lines such as Hela, CHO K1 and 293T; the promoter activities in

Figure 4: β-gal staining of various cell lines

To detect the transfection rates of plasmid DNA, 300ng Endo-toxin free pSV-ß-galactosidase plasmid DNA and 0.6μl Fugene6 were incubated in IMDM without serum for 20 minutes and loaded to various cell lines plated in 24 well plates; 24 hours later, the plate was stained with β-Gal staining reagent. The results were observed and counted under an inverse microscope, and the transfection rates were calculated.

Figure 4.1, HepG2 cells,

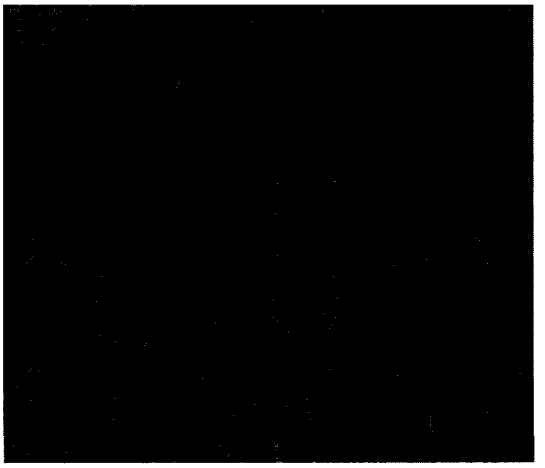


Figure 4.2, Hela Cells;

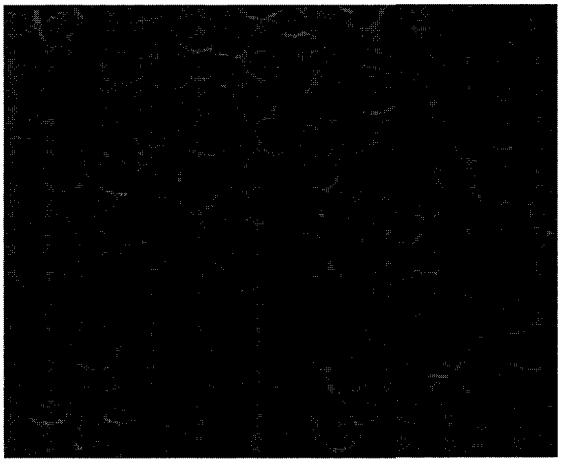


Figure 4.3, 293T cells;

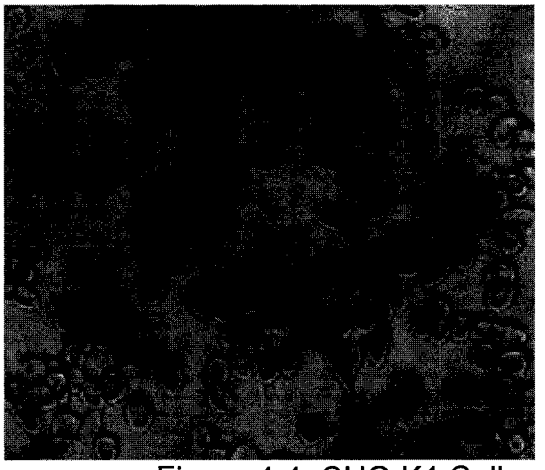


Figure 4.4, CHO K1 Cells;

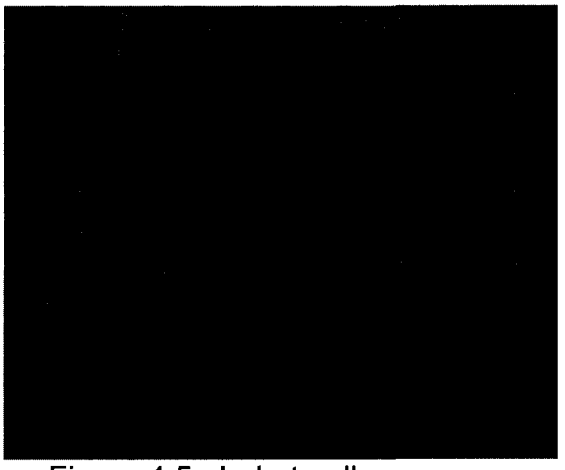


Figure 4.5, Jurkat cells;

Figure 4.6, U937 cells.

Table 2: A summary of promoter activity in various cell lines

To compare the promoter activities of the chimeric elements, the constructed plasmid DNA was co-transfected with pSV-β-galactosidase plasmid into hepatocytes and non-hepatocytes; the mean value ± SD (Standard Deviation) of the promoter activity relative to the complete CMV early promoter was calculated as shown in Appendix 3 and the final results were summarized in Table 2. All these mean values were based on 4 independent experiments, and normalized by β-Galactosidase activity. The digits in parentheses are the percentages relative to the complete CMV early promoter. This table was interpreted into bar charts in Figure 5~8.

Table 2: A summary of promoter activity in various cell lines (normalized by β-gal staining)

Cell line transfe Rate		CMV (100%)	I-pGL3	II-pGL3	III-pGL3	IV-pGL3	I-pVAX1 SV40	I-pVAX1 HAAT	IV-pVAX1 SV40	IV-pVAX1 HAAT
HepG2 (40%) 24Hours	Mean	471,185	765,501(163)	650,932(138)	1,586,855(337)	1,392,771(296)	621,590 (132)	507,259(108)	1,221,391(259)	1,205,561(256)
	SD	23,093(4.9)	49,691(10.44)	49,169(10.34)	79,750(15.9)	52,557(11.15)	57,461(12.1)	18462(3.92)	42,293(8.98)	48999(10.4)
HepG2 (40%) 48Hours	Mean	504,237	2,559,993(508)	2,340,329(464)	10,733,442(2128)	6,794,873(1346)				
	SD	18,051(2.98)	78,628(15.6)	119,117(23.6)	334,615(66.4)	201,363(39.9)				
Hela (30%) 24Hours	Mean	1,920,031	5,628(0.29)	12,211(0.64)	10,297(0.54)	12,934(0.67)	102,037(5.3)	87,388(4.6)	114,346(5.95)	115,071(5.99)
	SD	221,684(6.2)	138 (0.009)	780(0.041)	552(0.028)	1,168(0.055)	5,545(0.28)	6273(0.32)	5216(0.27)	4874(0.25)
293T (30%) 24Hours	Mean	9,221,644	14,491(0.16)	16,955(0.84)	183,799(1.99)	224,962(2.4)				
	SD	157,768(2.3)	1,244(0.0076)	555(0.0036)	11,947(0.076)	13,062(0.084)				
CHO K1 (20%) 24Hours	Mean	6,358,018	60,467(0.95)	82,312(1.3)	91,302(1.44)	42,424(0.66)				
	SD	406,126(5.96)	3,559(0.057)	5,124(0.091)	4,502(0.074)	1,951(0.034)				
Jurkat (1%) 36Hours	Mean	516,442	7,085(1.37)	19,794(3.8)	65,465(12.63)	48,563(9.4)	20,891(4.05)	23,685(4.59)	76,760(14.9)	68,276(13.2)
	SD	8,690(3.4)	431(0.03)	65.7(0.07)	4,167(0.29)	2159(0.112)	1,689(0.33)	1,977(0.38)	3115(0.6)	2298(0.45)
U937 (3%) 36Hours	Mean	27,610(100)	2,665(9.65)	2,897(10.5)	8,492(30.76)	4,716(17.08)	2,395(8.67)	2,815(10.2)	7,132(25.8)	6,529(23.65)
	SD	318(3.1)	185(0.67)	194(0.70)	378(1.36)	189.4(0.69)	183(0.66)	158(0.57)	523(1.89)	391.25(1.42)

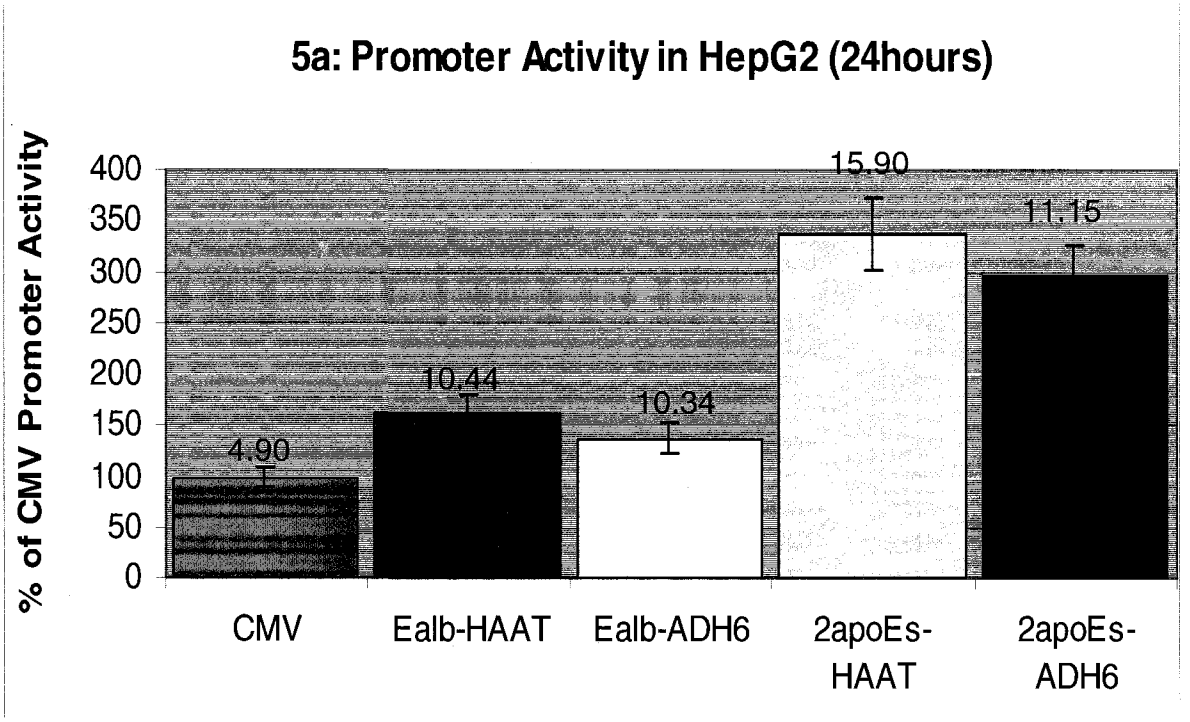
HepG2 of all the 4 chimeric liver-specific elements are stronger than the CMV promoter 24 hours after transfection (**Figure 5a**); significantly, 48 hours later, the promoter activity of liver-specific chimeras could be 5~22 times higher than the complete CMV early promoter, indicating that the liver-specific chimeras have "late promoter activity" in hepatocytes (**Figure 5b**). Although the activity of two copies of the apoplipoprotein E enhancer in tandem (2apoEs) is stronger than Ealb (**Figure 6a**), the specificity of 2apoEs is much worse than that of the albumin enhancer. More importantly, the promoter activity of Ealb-HAAT is more specific than Ealb-ADH6 in Jurkat and U937 (**Figure 6b**), and therefore has the least impact on immune systems. The long-term promoter activity *in vivo* of this combination is also very stable as described by Kramer, et al. (133).

3.4 The impact of vector backbone to promoter activity

The pVAX1 backbone is 800bp shorter than that of pGL3, and has a different PUC origin than pGL3. It is considered to have many advantages, such as containing the fewest unnecessary sequences for gene expression, the minimized possibility of chromosomal integration into the human genome, and the antibiotic (Kanamycin) is less likely to elicit allergic responses in humans (137). Moreover, Naas, et al. have successfully characterized a transgenic mouse model with HCV genes cloned into pVAX1 (138). However, the results summarized in table 2 showed that the pVAX1 backbone did not improve the promoter activity in hepatocytes (**Figure 7a**), but greatly decreased the specificity in nonhepatocytes, and had more impact on lymphocytes (**Figure 7b**). Therefore, the pVAX1 backbone is not ideal for the current research objectives.

Figure 5: The promoter activity in HepG2 cells

To display and compare the promoter activity of the liver-specific chimeras in HepG2 cells, the information in Table 2 is shown in bar charts 5a and 5b. 5a: the promoter activity in HepG2 24 hours after transfection; 5b: the promoter activity in HepG2 48hours after transfection.


5a: Promoter Activity in HepG2 (24hours)
% of CMV Promoter Activity
400
350
300
250
200
150
100
50
0
4.90
10.44
10.34
15.90
11.15
CMV
Ealb-HAAT
Ealb-ADH6
2apoEs-HAAT
2apoEs-ADH6

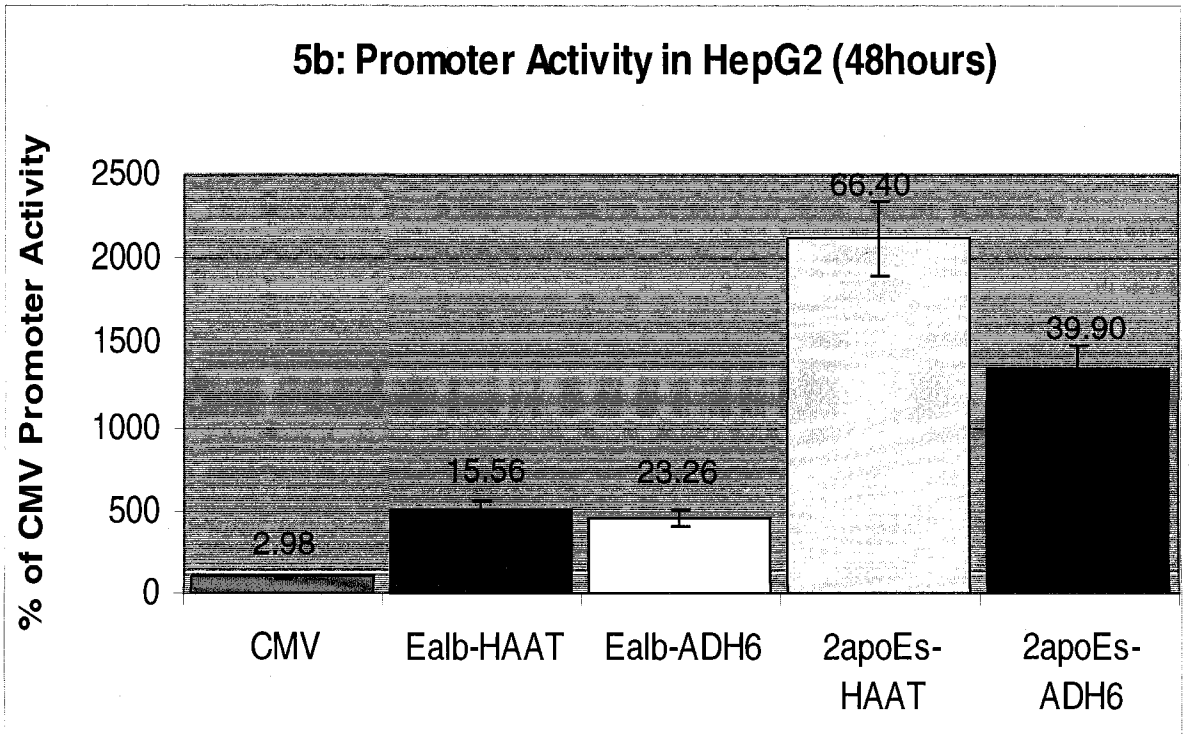
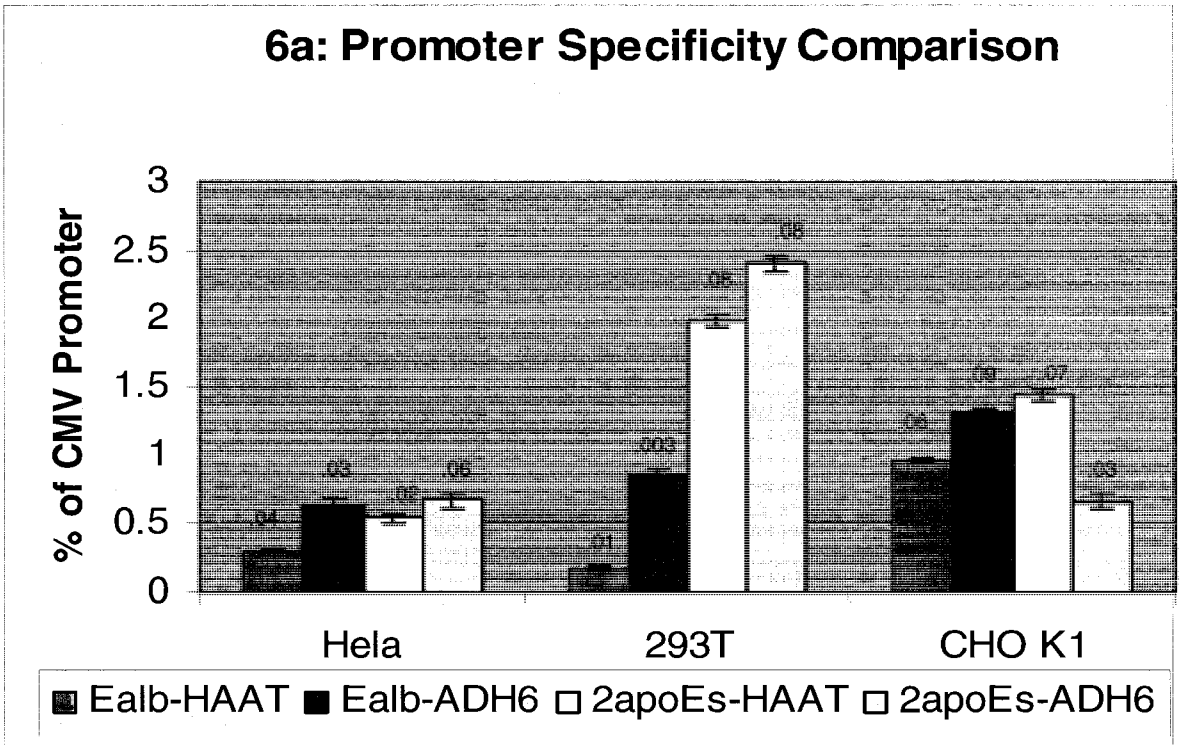

5b: Promoter Activity in HepG2 (48hours)
% of CMV Promoter Activity
2500
2000
1500
1000
500
0
2.98
15.56
23.26
66.40
39.90
CMV
Ealb-HAAT
Ealb-ADH6
2apoEs-HAAT
2apoEs-ADH6

Figure 6: The promoter specificity and impact on lymphocytes

To compare the specificity of the liver-specific chimeras in Hela, 293T and CHO K1 cells and the impacts of the liver-specific chimeras on lymphocytes, the information on these cells in Table 2 is shown in bar charts 6a: Promoter Activity Comparison, and 6b: Impacts to Immune system.


6a: Promoter Specificity Comparison
% of CMV Promoter
3
2.5
2
1.5
1
0.5
0
.04
.03
.02
.06
.01
.003
.05
.08
.06
.09
.07
.03
Hela
293T
CHO K1
Ealb-HAAT
Ealb-ADH6
2apoEs-HAAT
2apoEs-ADH6

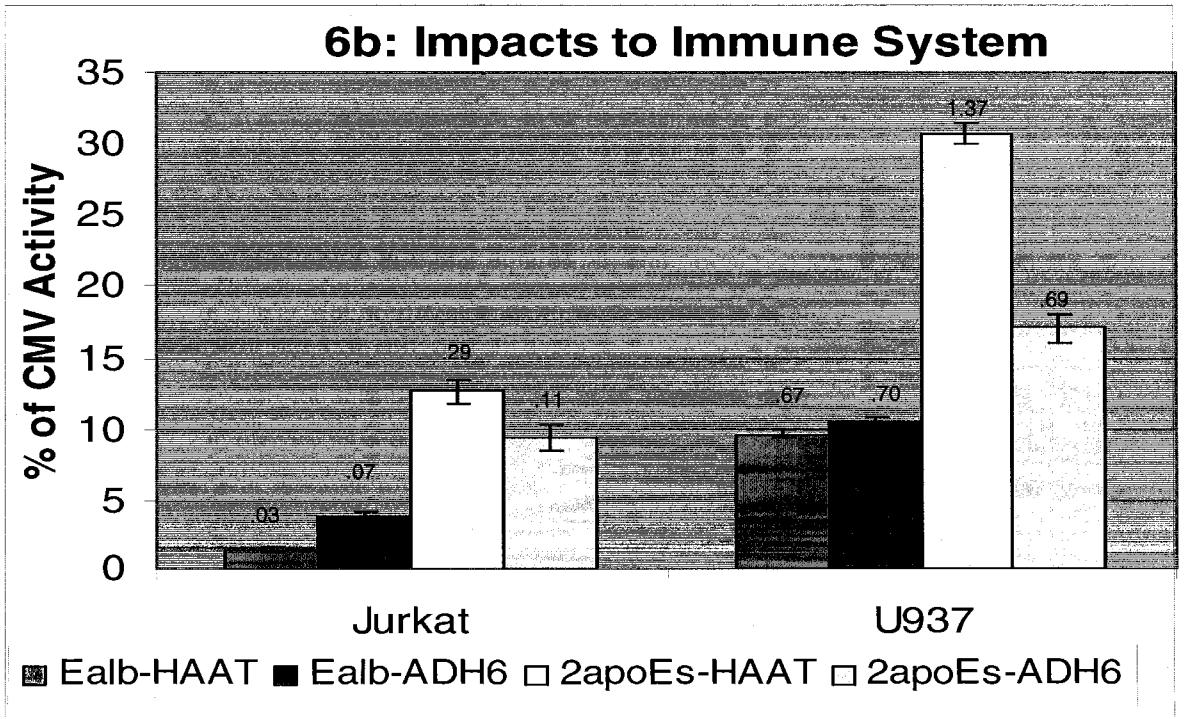
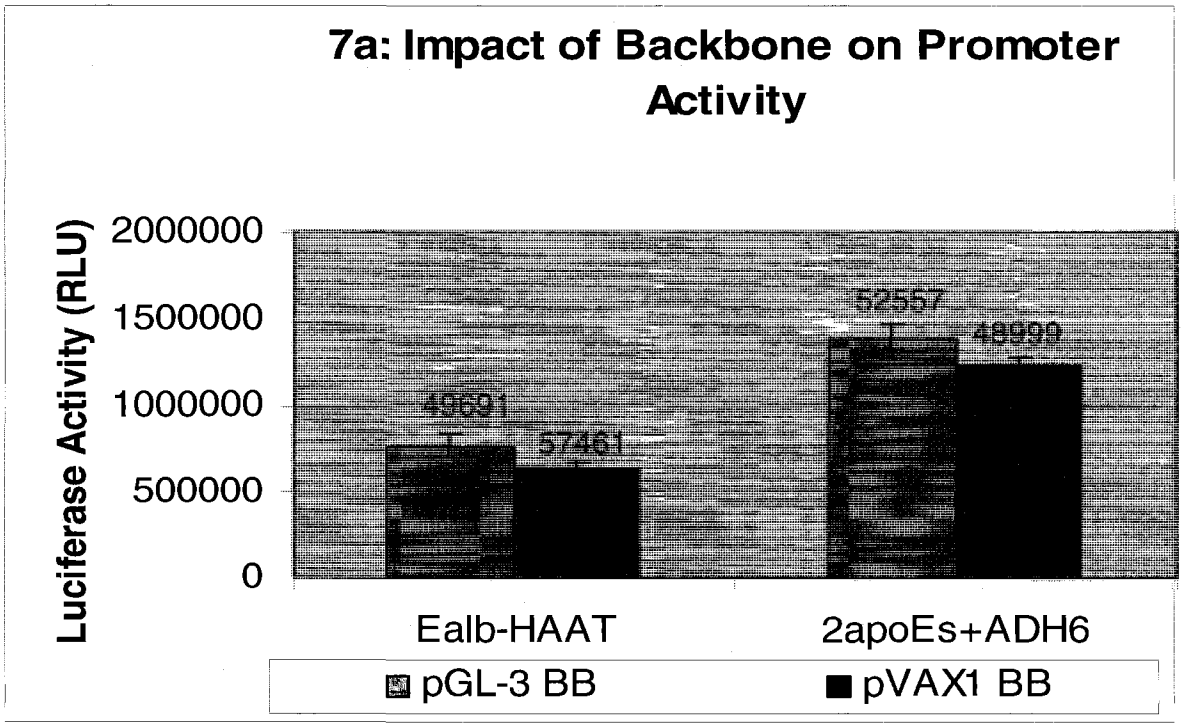
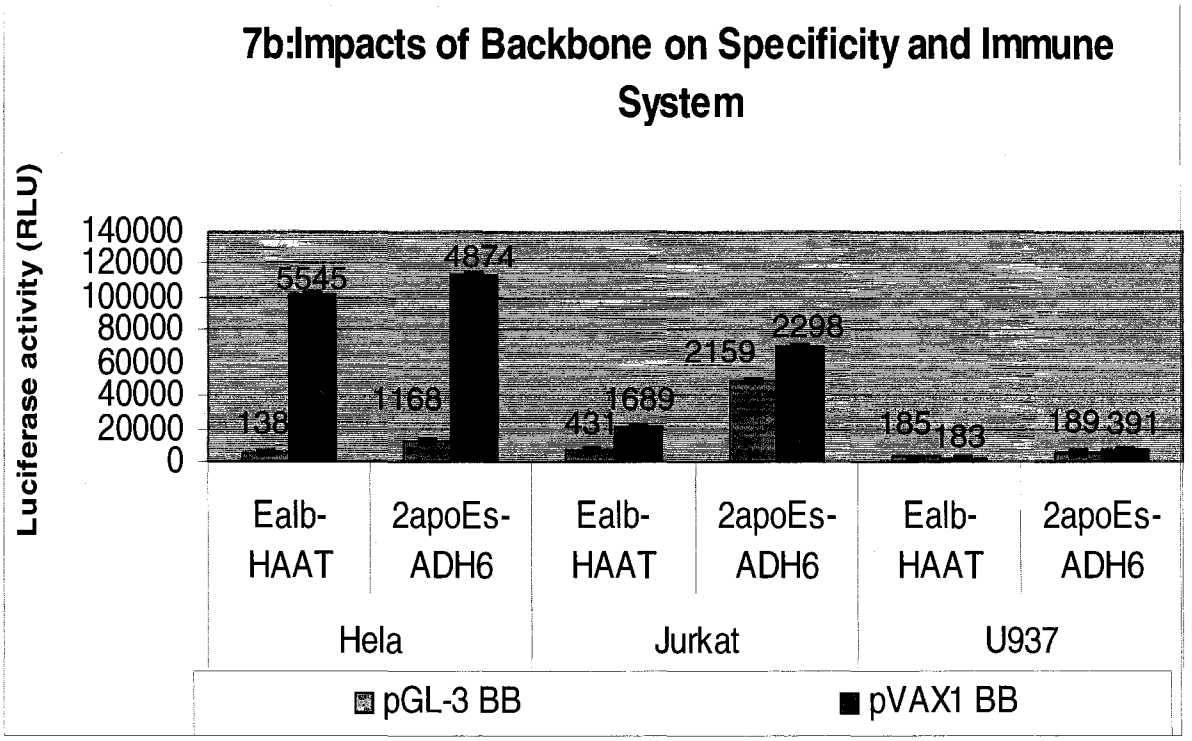

6b: Impacts to Immune System
% of CMV Activity
35
30
25
20
15
10
5
0
.03
.07
.29
.11
.67
.70
1.37
.69
Jurkat
U937
Ealb-HAAT
Ealb-ADH6
2apoEs-HAAT
2apoEs-ADH6

Figure 7: The impact of backbone on promoter activity, specificity and immune system

To know if the pVAX1 backbone could increase the promoter activity and specificity, the information on related backbones and cell lines in Table 2 is shown in bar charts 7a: Impacts of Backbone on Promoter Activity, and 7b: Impacts of Backbone on Specificity and Immune system.


7a: Impact of Backbone on Promoter Activity
Luciferase Activity (RLU)
2000000
1500000
1000000
500000
0
40691
57461
52557
48999
Ealb-HAAT
2apoEs+ADH6
pGL-3 BB
pVAX1 BB


7b:Impacts of Backbone on Specificity and Immune System
Luciferase activity (RLU)
140000
120000
100000
80000
60000
40000
20000
0
138
5545
1168
4874
431
1689
2159
2298
185
183
189
391
Ealb-HAAT
2apoEs-ADH6
Ealb-HAAT
2apoEs-ADH6
Ealb-HAAT
2apoEs-ADH6
Hela
Jurkat
U937
pGL-3 BB
pVAX1 BB

3.5 The effects of poly(A) to liver-specific expression

Poly(A) sequences may also affect the protein expression level by stabilizing mRNA (139, 140), and the Human a1-antitrypsin (HAAT) poly(A) is a natural poly(A) sequence for liver-specific expression. To know if the poly(A) sequence affects the promoter activity and specificity, the late SV40 poly(A) in the above two pVAX1 backbone-based plasmids was substituted by HAAT Poly(A) sequence, and the promoter activity of the plasmids with different poly(A)s was compared. Results showed that there was not much difference between the HAAT poly(A) and late SV40 poly(A) sequences (**Figure 8a and 8b**). Thus, both poly(A) sequences could be selected as the polyadenation signals to stop the liver-specific transcription.

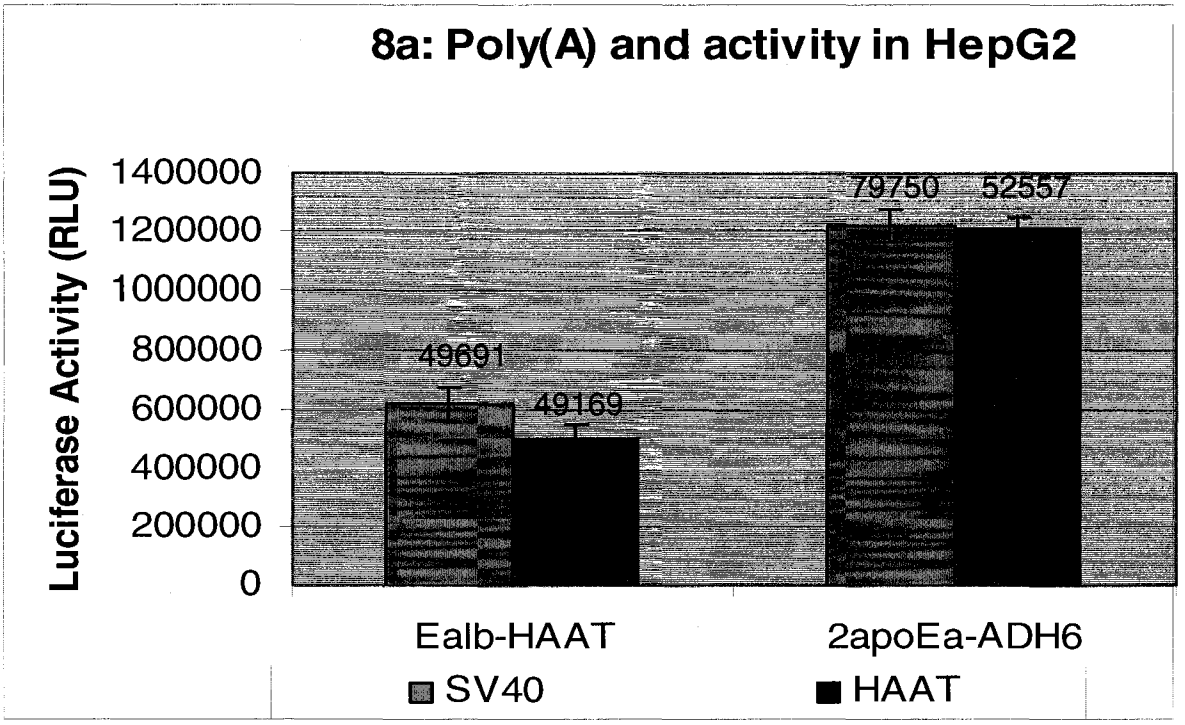
In summary, although the chimera Ealb-HAAT in pGL3 backbone does not have the highest promoter activity in hepatocytes, it is more specific in non-hepatocytes, has less impact on lymphocytes than the others, and can maintain long-term expression *in vivo* (133). Thus, the chimera Ealb-HAAT with SV40 poly(A) in pGL3 backbone could be selected to express the individual HCV genes specifically in mouse liver.

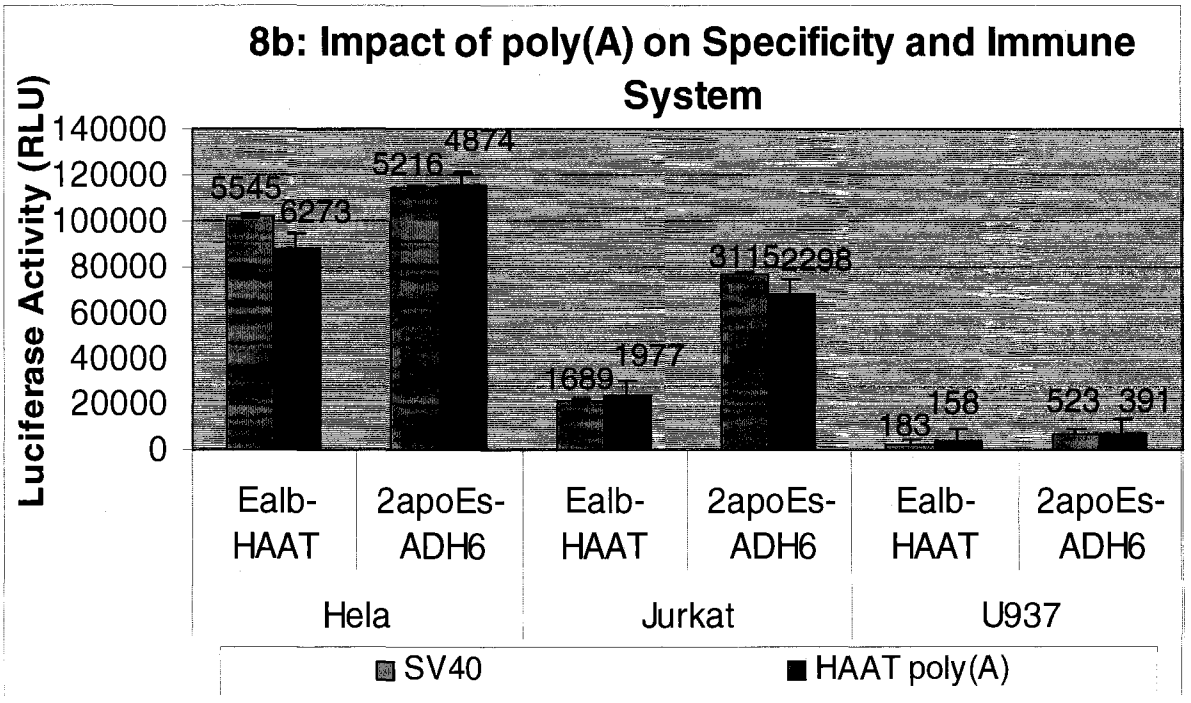
3.6 Individual HCV gene cloning

To express individual HCV genes specifically in mouse liver, the luciferase gene in Ealb-HAAT-pGL3 was substituted by HCV-Core, E2, NS3 and NS5a by sub-cloning the corresponding genes in plasmid pVAX1 previously cloned. An empty vector of Ealb-HAAT-SV40 poly(A)-pGL3 without ORF was also constructed as a negative control. All these constructs were confirmed by EcoR I

Figure 8: The impact of poly(A) on promoter activity, specificity and immune system

To know if poly(A) affects the promoter activity and specificity, the related information in Table 2 is shown in bar charts 8a: Poly(A) and Activity in HepG2, and 8b: the impact of poly(A) on promoter specificity and immune systems.


8a: Poly(A) and activity in HepG2
Luciferase Activity (RLU)
1400000
1200000
1000000
800000
600000
400000
200000
0
49691
49169
79750
52557
Ealb-HAAT
2apoEa-ADH6
SV40
HAAT


8b: Impact of poly(A) on Specificity and Immune System
Luciferase Activity (RLU)
140000
120000
100000
80000
60000
40000
20000
0
5545
6273
5216
4874
1689
1977
31152298
183
158
523
391
Ealb-HAAT
2apoEs-ADH6
Ealb-HAAT
2apoEs-ADH6
Ealb-HAAT
2apoEs-ADH6
Hela
Jurkat
U937
SV40
HAAT poly(A)

and Xba I digestion (**Figure 9**) and sequencing.

3.7 Gene expression of HCV-Core *in vitro*

To confirm the gene expression *in vitro* from the constructs containing HCV-Core, pVAX1-Core, pLS-Core and pLS-Em were transfected to HepG2 and CHO K1 cells. The HepG2 cells were lysed and the proteins were loaded to 12% SDS-PAGE gel, and the CHO K1 cells were trypsinized for IFA detection. Anti-HCV-Core monoclonal antibody (**Appendix 1B**) was used as the primary antibody to stain the PVDF membrane and the microscope slides. Results showed that the HCV-Core gene driven by CMV promoter (pVAX1-Core) was successfully expressed in both HepG2 and CHO K1 cells, but the HCV-Core protein in pLS-Core could be detected only in HepG2 cells (**Figure 10a and 10b**). Compared to the negative control (pLS-Em), the different protein bands between 37.5~54KD (**Figure 10a**) also indicated that the expression of HCV-Core protein disturbed the normal protein expression of hepatocytes; this may affect the normal functions of hepatocytes and may also be a part of the pathogenesis of HCV infections.

3.8 Gene expression of HCV-Core *in vivo*

To confirm the liver-specific expression of HCV-Core *in vivo*, 100μg Endotoxin-free pLS-Em, pVAX1-Core and pLS-Core were injected into the mice by tail veins. 48 hours after the injection, the mice were sacrificed and the frozen sections of the mouse liver were made; anti-HCV monoclonal antibody was used as the primary antibody in Indirect Immuno-fluorescent Assay to detect the expression in mouse liver (**Figure 11**). IFA results showed HCV-Core protein was highly expressed

Figure 9: Liver-specific empty vector construction and individual HCV gene cloning

To confirm that the DNA was correctly constructed, the empty vector (pLS-Em, 3700bp) was double-digested by Kpn I and HinD III to release the enhancer-promoter segment (730 bp, lane 1); it was also linearized by Xba I digestion (3700bp, Lane 2); HCV-Core cloned into pVAX1 was also double-digested by EcorR I and Xba I to release the Core gene (573bp, Lane 3); HCV-Core, E2, NS3 and NS5a in Liver-specific vector were double-digested by EcorR I and Xba I to release the corresponding individual HCV genes (Lanes 4~7).

Figure 9: Liver-specific vector construction and HCV gene cloning

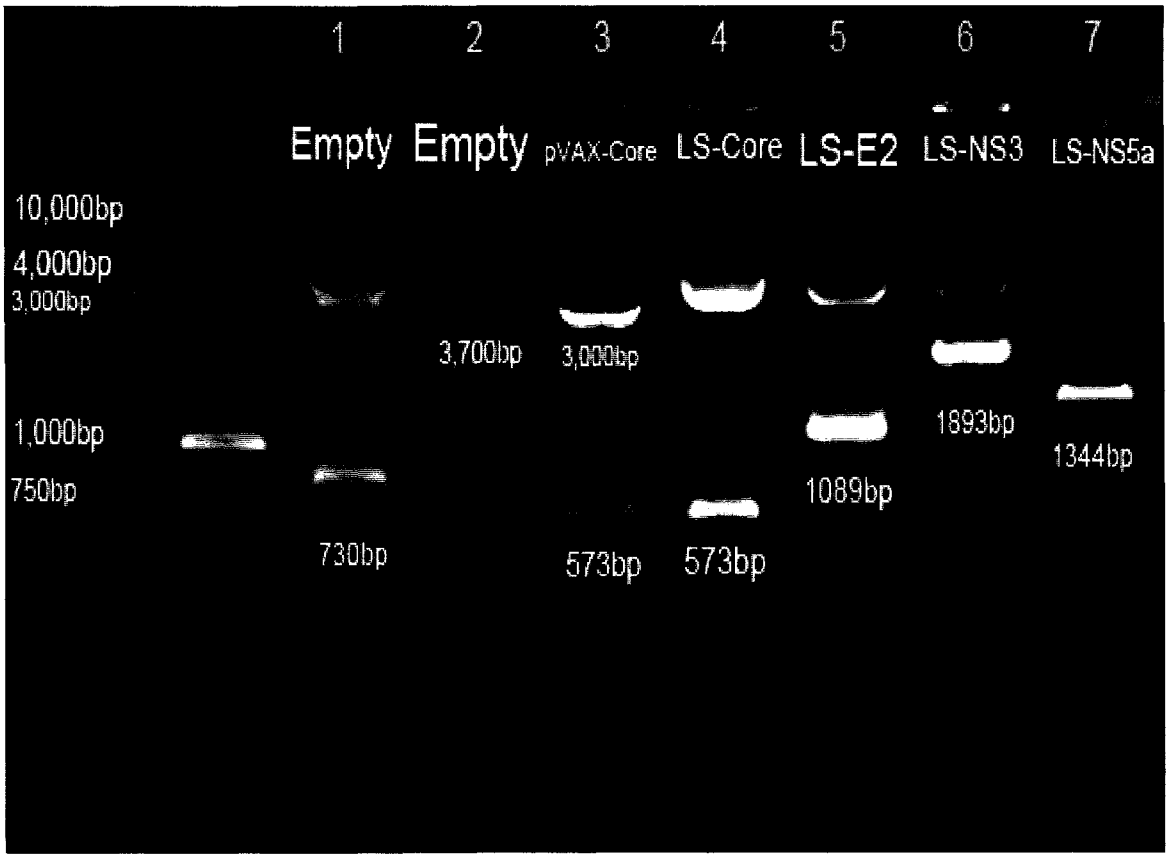
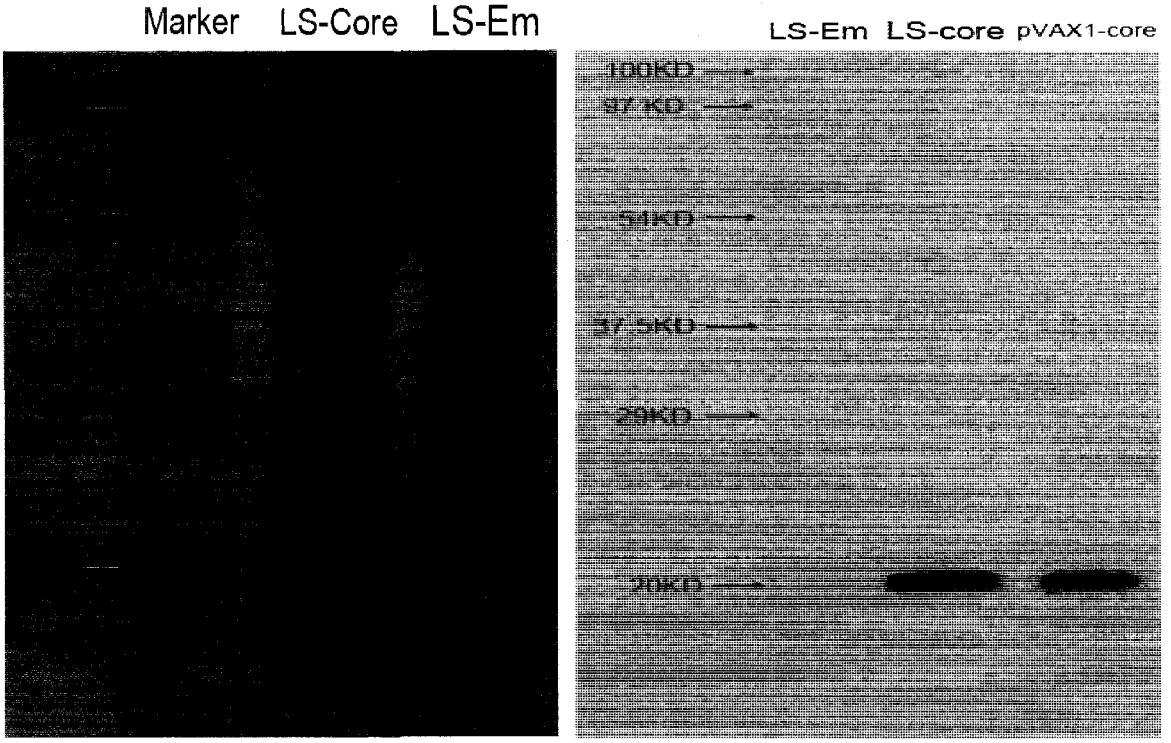


Figure 10: PAGE, Western Blot of HCV-Core expression *in vitro*

To detect the global and liver-specific expressions of HCV-Core *in vitro*, pLS-Empty vector (negative control), pVAX1-Core and pLS-Core were transfected into HepG2 and CHO K1 cells. Coomassie Brilliant Blue G-250 staining and Western Blot were used to show the results. Anti-HCV-Core monoclonal antibody was applied as the primary antibody to stain the PVDF membranes and microscope slides. a) Coomassie Brilliant Blue G-250 staining of liver-specific expression in HepG2 cells; b) Western Blot of both pLS-Core and pVAX1-Core stained by HCV monoclonal antibody.

Figure 10a: PAGE gel, Western Blot and IFA of HCV-Core expression *in vitro*



a) Coomassie Brilliant Blue G-250 Staining; HepG2 cells;

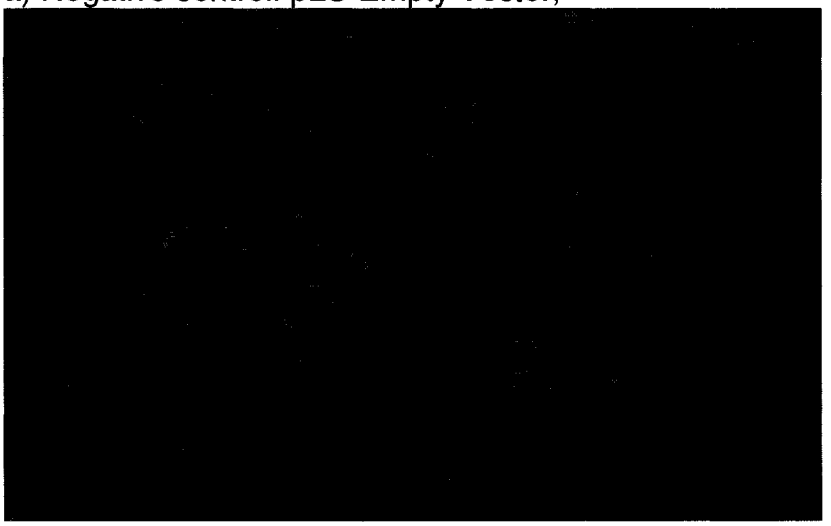
b) Western Blot analysis stained by anti-HCV monoclonal antibody; HepG2 cells;

Figure 11: IFA detection of HCV-Core expression *in vivo*

To confirm the expression of HCV-Core in mouse liver, 100μg Endotoxin- free DNA of pLS-Em, pVAX1-Core, pLS-Core were injected into Balb/C mice by tail veins. Anti-HCV-Core monoclonal antibody was used to stain the frozen sections of mouse livers, and FITC conjugated goat anti-mouse antibody was used as the secondary antibody: a) Negative control; b) pVAX1-HCV-Core; c) pLS-Core.

Figure 11: IFA detection of HCV-Core expression *in vivo*

a) Negative control: pLS-Empty Vector;



b) pVAX1-HCV-Core;

c) pLS-Core.

in mouse liver both by pVAX1-Core and pLS-Core; this also indicated tail vein injection is a good pathway for introducing target DNA into the mouse liver.

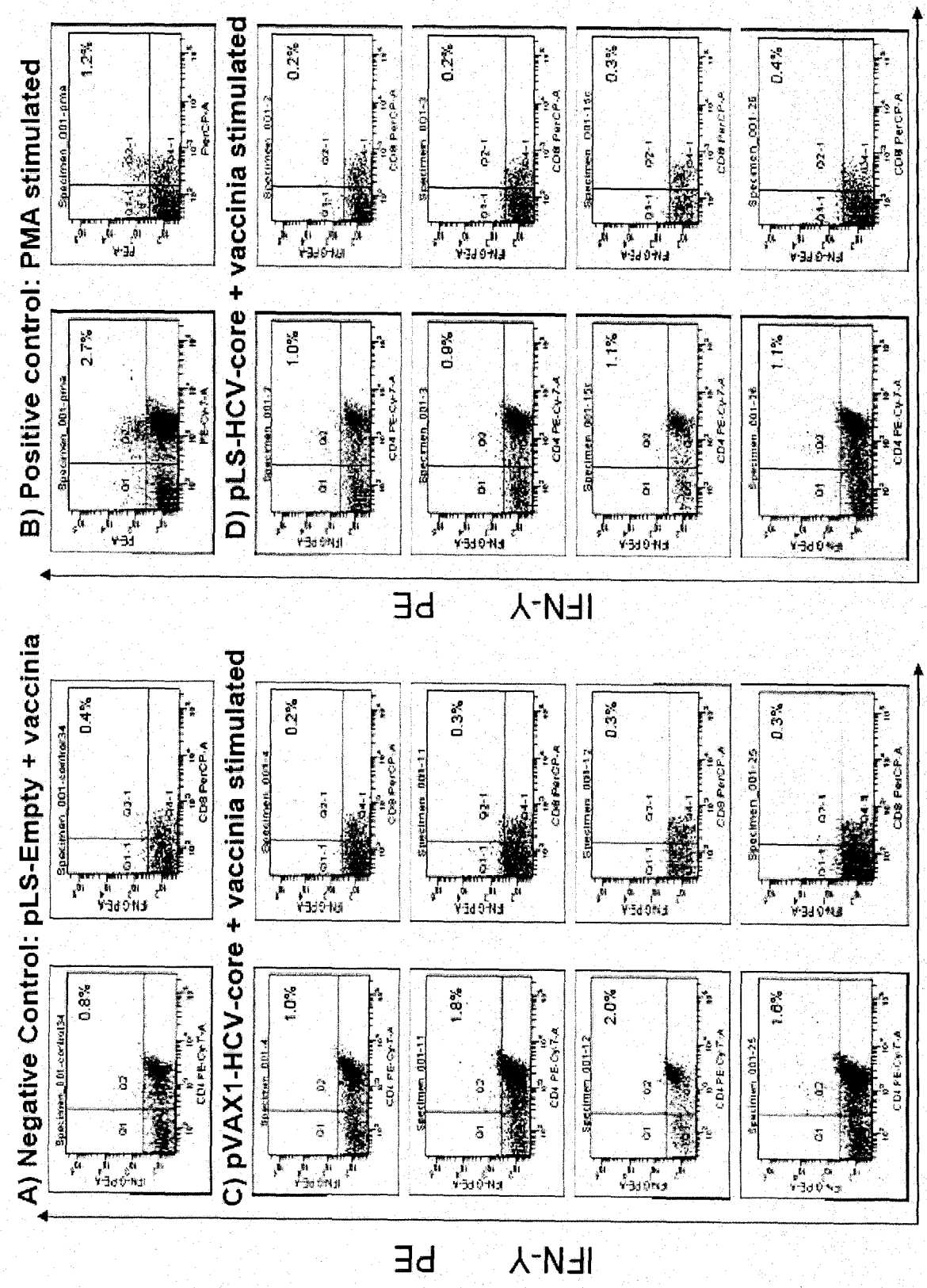
3.9 Immune response detection by Flow Cytometry

To compare the immune responses to HCV-Core after intravenous delivery of the two plasmids, pVAX1-Core and pLS-Core were innoculated into the mice by tail vein. Mice received 3 injections in total, but each one 3 weeks apart; pLS-Empty was also injected as the negative control. Seven days after the third DNA injection, the splenocytes of the immunized mice were extracted and stimulated by vaccinia expressing HCV-Core protein. The CD4+ and CD8+ splenocytes producing IFN-γ were counted by Flow Cytometry. The results showed that the expression of HCV-Core driven by global promoter (CMV) caused an obvious increase of the percentages of CD4+ T lymphocytes, but that of CD8+ T lymphocytes were even lower than the negative control; however, the expression of HCV-Core driven by liver-specific transcriptional elements caused a mild increase of the percentage of CD4+ T lymphocytes, and that of CD8+ T lymphocytes were also lower than the negative control. However, the injection and immune detection should be optimized and repeated to obtain statistical significance in the future.

Figure 12: Flow Cytometry detection of immune responses

To detect the immune responses caused by the expressions of HCV-Core driven by both global and liver-specific transcriptional elements, 6~8-week-old female Balb/C mice were immunized with DNA 3 times every 3 weeks. The splenocytes were extracted 7 days after the last injection, were stimulated by Vaccinia with HCV-Core expression, and were stained by rat anti-mouse CD3, CD4, CD8 and IFY monoclonal antibodies conjugated with different dyes. The stained splenocytes were counted by Flow Cytometry. A) Negative control: pLS-empty DNA injection with vaccinia stimulation; B) pLS-empty DNA injection with PMA stimulation; C) pVAX1-Core injection caused immune responses; D) pLS-Core DNA injection caused immune responses.

Figure 12: Flow Cytometry detection of immune responses



4. DISCUSSION

4.1 The challenges for HCV research

Hepatitis C Virus infection is known to be a leading cause of liver related morbidity and mortality worldwide. Although a number of important breakthroughs have been made in virology, epidemiology, natural history, and immunopathogenesis of HCV infection during the past decade, many scientific questions remain unanswered, there is no effective treatment at present, and neither preventive nor therapeutic vaccines are available.

There are several major challenges for HCV research at present. The first major challenge is the lack of robust cell culture systems and small animal models, which has slowed the progress of HCV research. Despite some indisputable breakthroughs in cell culture and small animal models in recent years, the understanding and application of both systems are quite inaccessible. The cell culture is restricted to only certain genotypes, or some adaptive mutations; on the other hand, in small animal models which are technically challenging (109), the viral replication of HCV viruses still relies on the exogenous hepatocytes or liver tissues originating from humans, and is restricted to only certain genotypes of viruses. The interactions between the viral structural and nonstructural proteins and the host cell factors are believed to be the two critical factors affecting the replication of HCV RNA and particle formation, but to date, only very little is known.

The second challenge for HCV research is the lack of knowledge on the anti-viral mechanisms of the immune response and viral persistence. Although

considerable efforts have been made, the pathogenesis is still quite controversial, at least five individual HCV genes were proposed as being involved in the pathogenesis of HCV. However, the mechanisms of disease causation are likely to be multi-factorial. The HCV viral RNA replication and protein expression in natural infections are restricted to hepatocytes and the extra-hepatic viral replication level of HCV is very low; moreover, the liver is the major organ for HCV induced immune responses. However, in previous research on the immunopathogenesis of HCV, the viral proteins were usually globally expressed in various tissues of transgenic animals, whereas the viral promoter activity in the liver seems to be preferential in human infections. The results of this work suggest that DNA injection resulted in very low levels of viral protein expression in the liver, when the DNA was administered systemically. Other investigators have tried diverse routes of injection with various degrees of immune responses. There is evidence that when mice are immunized with HCV protein pulsed DC, they are able to generate potentially therapeutic and prophylactic immune responses. There is a lack of small animal models of HCV infection and transgenic mice cannot be used in vaccination studies that use the same transgene as these animals are tolerant to these antigens. Therefore, even though various novel injection methods and transgenic mice are utilized in HCV pathogenesis studies, none of them mimic the natural human HCV infection. Thus, there are limitations to elucidate the mechanism for the lack of HCV eradication by the immune response observed in patients with persistent HCV infections. We attempted to address this issue by trying to develop novel DNA

plasmids that have liver expression specificity to develop improved HCV animal models.

4.2 The significance of liver-specific expression

Although some researchers have already tried to make liver-specific transgenic mice that express the individual HCV genes, the transgenic animals were phenotypically similar to the normal littermates and did not exhibit a major histological changes in the liver up to 24 months of age (131). Later research (133) showed that this might be related to the weak promoter activity, and that the lower expression in the liver may not induce the pathological changes as observed in the natural infections. This research and the former two reports (129, 133) on chimeric liver-specific transcriptional control units triggered new hope of developing much stronger liver-specific expression of HCV proteins in mice, which might be very helpful in elucidating the immunopathogenesis of HCV infections.

The results of this research demonstrated that the over-expression of individual HCV genes specific in mouse liver is possible. *In vitro* experiments found that the promoter activity of these chimeric liver-specific elements in hepatocytes could be much stronger than that of CMV promoter. In fact, even the recognized strongest CMV early promoter, which was used for DNA injection, demonstrated weak activity in hepatocytes, whereas its promoter activity in other cells that were not liver derived was very high. The DNA injection based on this global promoter may also severely affect the normal function of other organs, such as kidney, spleen and heart (138), in which there is no viral protein expression during natural HCV infections. One of the consequences of the extra-hepatic expressions of HCV

proteins is the negative impact that such proteins may have on the immune system. More importantly, the high promoter activity of the early CMV promoter in the Jurkat cell line, which is derived from a T lymphocyte, showed that the global promoter may inevitably disturb the normal function of T lymphocytes. Therefore, results on immune responses based on the global expression of HCV proteins using the chimeric plasmids, may not elucidate the immunopathogenesis of HCV in natural infections.

The high promoter activity in hepatocytes and low promoter activity (0.1% of CMV promoter) in the other cell lines of chimeric liver-specific transcriptional control units decrease the impact of extra-hepatic HCV gene expression. The antigen presentation caused by individual HCV protein expression specific in liver cells after the systemic administration of chimeric plasmid DNA may mimic the natural HCV infection, and this may be able to elucidate the role of individual HCV genes. Although the injections with chimeric plasmids needs optimization, the Flow Cytometry results clearly indicate the differences in immune responses caused by protein expressions driven by global and by liver-specific promoters: the pVAX1 based DNA injection caused obvious CD4+ T lymphocyte responses, while the pLS-Core injections induced only mild CD4+ T lymphocyte responses. The Western Bolt result showed the over-expression of HCV-Core specifically in mouse liver. The stronger CD4+ T lymphocyte responses induced by the CMV promoter driven protein expression might be related to the global expressions, in which the amount of expressed HCV protein is much more than the liver-specific expression. On the other hand, the DNA injections of HCV-E2 and NS3 cloned into liver-specific vectors

caused very strong cellular immune responses (data not shown); this further supports that HCV-Core may cause a decrease in the immune response in HCV infections.

Moreover, the successful expression of HCV protein specific in mouse liver could provide an added research tool to increase our knowledge of HCV biology. When the liver-specific transcriptional control units were constructed upstream of the coding area of the HCV whole genome or individual genes, the viral RNA transcription and protein expression would be restricted to hepatocytes. Thus, the application of liver-specific transcriptional elements could partly repeat the natural viral antigen presentation *in vivo*, overcoming the post-entry block to viral replication, which is one of the two major challenges to establishing both cell culture and small animal models of HCV.

Furthermore, although the lower immune responses and viral persistence are widely believed to relate to antigen presentation via the MHC pathway, the exact mechanism is still unknown. The identification of an HCV reactive T cell receptor that does not require CD8 for target cell recognition (17) indicates that mechanisms other than the MHC pathway may also be involved in the pathogenesis of HCV infections, and they are likely to be multi-factorial.

4.3 The optimization of gene expression conditions

Kramer's study showed that *in vitro* expression driven by promoter alone was specific but substantially lower than that of the ubiquitously active CMV promoter and enhancer (133), but enhancers may increase the promoter activity by more than 250-fold without decreasing the specificity of protein expression. Theoretically,

enhancers and promoters are the critical elements affecting transcriptional activity; poly(A) is also involved in regulating the expression and translation levels by stabilizing mRNA or forming the ribosome translational complex. The results in this thesis indicate that substituting a natural liver-specific Human a1-antitrypsin poly(A) with the later SV40 poly(A) does not affect the expression level of the target genes *in vitro*.

The results also suggest that the backbone of the vector affects the promoter activity and specificity. This may be related to the intracellular environment and to other unknown factors. Although random integration into the host genome was believed to be the mechanism of the expression of exogenous DNA in transgenic animals, some studies discovered that the target genes flanked by specific sequences on plasmids were most likely integrated into specific sites on eukaryotic genomic DNA (142, 143), and the enhancers or silencers upstream or downstream of the integration sites on the chromosome may highly affect the promoter activity and specificity. The pVAX1 DNA backbone might be more apt to be integrated into the sites near unknown strong enhancers in some tissues and thus cause lower specificity. Stephan Gehrke's study showed that two copies of the apoE enhancer modules gave rise to a ~3.5-fold higher level of promoter activity compared to the constructs with only single apoE modules, irrespective of the basal promoter used (129). This also indicates that the genetic environment near the integration site of expression cassettes affects the promoter activity introduced into the cellular genome.

Compared with viral genome based vector systems, despite the defects of lower transfection rates and the lack of self-replication, the plasmid DNA vector systems introduce the least exogenous genes into the host genome, and thus the impact of DNA injection on the immune system were reduced. The strategy used was that only individual HCV genes were expressed both *in vivo* and *in vitro*. Thus, the plasmid DNA vector system was used as a form of DNA injection. The successful expression of HCV-Core in mouse liver also demonstrated that, unlike the other injection pathways, the tail vein injection of plasmid DNA might be an optional administration route to introduce exogenous genes into the mouse liver.

4.4 Potential difficulties associated with the animal models

While HCV-Core protein was successfully expressed in mouse liver with the help of liver-specific plasmid vectors, and some immune responses were detected, this was a pilot study to explore the transient expression of HCV genes in an animal model. Further work is required to optimize this model, but the design and the expression of chimeric plasmids should facilitate the development of such animal models.

The route of injection needs to be optimized also as the mouse tail vein plasmid administration may not be ideal. Administration by portal vein injection may cause the plasmid to be directed to the liver via the portal system and to specifically express the exogenous genes in mouse liver; the amount of DNA injected and the choice of mouse strain, etc. need to be optimized as well. In this study, the amounts of DNA and mouse strain were similar to the previous work done in this laboratory. The plasmids were injected intramuscularly into Balb/C mice only (139). Another

important issue is that the mechanisms at play during liver-specific expression and by global expression of viral proteins might be quite different. The amount of viral protein expressed in mice is also distinct. In addition, the liver-specific transcriptional control elements used in this project are of human origin; thus, the mouse strain selected in this research may not have the optimal promoter activity and specificity. Furthermore, the number and the timing of DNA injections through the tail vein may also need to be optimized: unlike the global promoters such as CMV injected intramuscularly, large amounts of DNA may decrease the specificity of the expression vectors. All these factors may have affected the final results.

Moreover, the regulation of liver-specific expression of proteins *in vivo* is very complex. For example, the chimeric plasmids may have non-stable expression of the target genes. In addition, the global expression promoter CMV is known to be inhibited by IFN-Y (144), and cannot maintain long-term expression in vivo. Although many functional sites on the enhancer and promoter regions that we selected (Ealb-HAAT) were well defined (133, 145, 146), the regulation of the liver-specific expression in these vectors may be exposed to other factors that are not known or are not well defined. Furthermore, gene expression level in wild type mouse models may vary among individuals. There may be instability of protein levels and the immune response to these foreign proteins may also have variability among individual animals.

Another difficulty in trying to develop a mouse model for DNA injection and transient expression of viral proteins in the liver is the amount of administered DNA, the number of required injections, and when is the appropriate time to detect the

expression of these proteins or the immune responses. Several studies showed that intramuscular injection of DNA with adjuvants results in a weak humoral immune response but strong CTL activity (103, 104). However, other studies on DNA injections induced both humoral and cellular immune response (100, 105). The antigen presentation and immune responses to HCV proteins expressed in liver through tail vein injections may be quite different from those administered intramuscularly. Adjuvants had not previously been tried under the conditions of liver-specific expression used in this study, and therefore the best choice of adjuvant is unknown. Moreover, compared to the expressions driven by global promoters such as CMV, the protein expressions driven by liver-specific transcriptional elements were restricted only to the liver, maybe more injections and higher doses are necessary to produce good immune responses.

For the optimization of this injection model, a balance between the promoter activity and the specificity may be desirable. The combination of Ealb-HAAT was selected due to the lower impact on the immune system, but the promoter activity *in vitro* is only ~20% of the combination of 2apoEs-HAAT. 2apoEs-HAAT may be able to maintain a much higher level of viral protein expression in the mouse liver and thus result in a better animal model to study the impact of viral protein expression in liver function or in the study of the antiviral immune responses within the liver.

4.5 Future directions

This project explored the possibility of liver-specific expression of individual HCV genes and detected the immune responses after systemic administration of the chimeric plasmids via tail vein injections. The results demonstrated that HCV-Core

was successfully over-expressed in the liver. Although there are some potential difficulties, and more research is needed to optimize this animal model, this research has pioneered a new tool for HCV research. The construction of liver-specific DNA vectors with optimized specificity of expression in the mouse liver can be used to study the roles of individual HCV genes in viral pathogenesis. In addition, the project strategy can be applied to other types of hepatitis such as HAV, HBV, and so forth, and may also provided improved forms of gene therapy. Furthermore, the chimeric liver-specific control units can be used to drive the transcription of the whole HCV genome and therefore the application of these units might be able to overcome the post-entry block of virus replication, thus helping to establish cell culture systems and small animal models for HCV research. Finally, the DNA constructs can be used to make liver-specific transgenic mice with individual HCV genes, or to study point mutations and truncated gene segments.

4.6 Final conclusions

Although HCV infection is a leading cause of liver related morbidity and mortality worldwide, the immunopathogenesis of this disease is still unknown. This project allowed for the cloning of individual HCV gene driven by liver-specific transcriptional control units. Although additional work is required to optimize the described animal model for the transient expression of the chimeric plasmids in the mouse liver as well as immune responses after injection, the results indicate that liver-specific expression may provide a new way to elucidate the pathogenesis of HCV infections, and the project strategy may be extended to the other types of hepatitis, liver-

specific transgenic mice, cell culture systems and small animal models for the research on hepatotropic infections.

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6. APPENDIXES

6.1 HCV Core gene and amino acid sequences in this project

A: HCV-1a Core gene sequence cloned in this project

GAA TTC **ACC ATG AGC** ACG AAT CCT AAA CCT CAA AGA AAA ACC AAA CGT AAC ACC AAC CGT CGC CCA CAG GAC GTC AAG TTC CCG GGT GGC GGT CAG ATC GTT GGT GGA GTT TAC TTG TTG CCG CGC AGG GGC CCT AGA TTG GGT GTG CGC GCG ACG AGG AAG ACT TCC GAG CGG TCG CAA CCT CGA GGT AGA CGT CAG CCT ATC CCC AAG GCA CGT CGG CCC GAG GGC AGG ACC TGG GCT CAG CCC GGG TAC CCT TGG CCC CTC TAT GGC AAT GAG GGT TGC GGG TGG GCG GGA TGG CTC CTG TCT CCC CGT GGC TCT CGG CCT AGC TGG GGC CCC ACA GAC CCC CGG CGT AGG TCG CGC AAT TTG GGT AAG GTC ATC GAT ACC CTT ACG TGC GGC TTC GCC GAC CTC ATG GGG TAC ATA CCG CTC GTC GGC GCC CCT CTT GGA GGC GCT GCC AGG GCC CTG GCG CAT GGC GTC CGG GTT CTG GAA GAC GGC GTG AAC TAT GCA ACA GGG AAC CTT CCT GGT TGC TCT TTC TCT ATC TTC CTT CTG GCC CTG CTC TCT TGC CTG ACT GTG CCC GCT TCA GCC **TAA** TCT AGA

Observations:
1) The underlined are restriction endonuclease sites for EcoR I and Xba I;
2) The bold are Kozac box ACCATGAGC and stop codon TAA; the bold and underlined ATG is the start codon.

B: HCV-1a Core amino acid sequence in this project

MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSE
RSQPRGRRQPIPKARRPEGRTWAQPGYPWPLYGNEGCGWAGWLLSPRGSRPS
WGPTDPRRRSRNLGKVIDTLTCGFADLMGYIPLVGAPLGGAARALAHGVRVLEDG
VNYATGNLPGCSFSIFLLALLSCLTVPASA

Observations: the underlined amino acid sequence is recognized by the monoclonal antibody (Abcam, ab2740) for HCV-Core detections in both IFA and Western Blot analyses.

6.2 Clones, stable cell lines and vectors constructed during two school years

A: Clones and stable cell lines finished in the first year

Gene and Vector	pEF6/Myc His A	pVAX1	pQE-TriSys	**Liver-specific (second year)**	Stable cell lines
Core	√	√		√	√
E1	√	√	√		√
E2	√	√	√	√	√
CoreE1	√	√			√
E1E2	√	√			√
CoreE1E2	√	√			√
E2P7	√	√	√		√
P7(NS1)	√	√	√		√
NS2	√	√			√
NS3	√	√	√	√	√
NS4	√	√	√		√
NS4a	√	√			√
NS4b	√	√			√
NS5a	√	√	√	√	√
NS5b			√		

B: Vectors and clones finished in the second year

	Vector	Luciferase	Gluc	HAAT
CMV in pGL-3	√			
I in pGL-3	**Empty Vector**			√
II in pGL-3	√			√
III in pGL-3	√			√
IV in pGL-3				√
CMV in pVAX1		√	√	√
I in pVAX1-HAAT	√	√	√	√
II in pVAX1-HAAT	√	√	√	√
III in pVAX1-HAAT	√	√	√	√
IV in pVAX1-HAAT	√	√	√	√
I in pVAX1-SV40	√	√		
IV in pVAX1-SV40	√	√		

6.3 The raw data of promoter activity assay and β-gal normalization

A: Percentages of relative activity to the complete CMV early promoter ± SD

Cell line transfe Rate	I-pGL3	II-pGL3	III-pGL3	IV-pGL3	I-pVAX1 SV40	I-pVAX1 HAAT	IV-pVAX1 SV40	IV-pVAX1 HAAT
HepG2 (40%) 24Hours	163±10.44	138 ±10.34	337±15.9	296±11.15	132±12.1	108±3.92	259±8.98	256±10.4
HepG2 (40%) 48Hours	508±15.6	464 ±23.6	2128±66.4	1346±39.9				
Hela (30%) 24Hours	0.29±0.009	0.64±0.041	0.54±0.028	0.67±0.055	5.3 ±0.28	4.6±0.32	5.95±0.27	5.99±0.25
293T (30%) 24Hours	0.16±0.0076	0.84±0.0036	1.99±0.076	2.4±0.084				
CHO K1 (20%) 24Hours	0.95±0.057	1.3 ±0.091	1.44±0.074	0.66±0.034				
Jurkat (1%) 36Hours	1.37±0.03	3.8±0.07	12.63±0.29	9.4±0.112	4.05±0.33	4.59±0.38	14.9±0.6	13.2±0.45
U937 (2%) 36Hours	9.65±0.67	10.5±0.70	30.76±0.13	17.08±0.69	8.67±0.66	10.2±0.57	25.8±1.89	23.65±1.42

B: Promoter activity comparison (in pGL3 backbone)

Date: 20061007;24 hours after transfection;DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: HepG2; Density: 1.5X10^5/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV Promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pGL3	Ealb+ADH6+Luciferase+SV40 poly(A)-pGL3	2apoEs+HAAT+Luciferase+SV40 poly(A)-pGL3	2apoEs+ADH6+Luciferase+SV40 poly(A)-pGL3
1	186	408,234	534,248	480,251	1,565,608	1,268,762
2	179	450,231	487,687	470,392	1,546,495	1,304,560
3	245	431,297	566,612	467,269	1,433,669	1,384,726
4	213	406,506	554,855	404,697	1,420,803	1278,771
Ã	206	424,067	535,850	455,652	1,491,644	1,309,205
β-gal		0.09	0.07	0.07	0.094	0.094
X(%)		471,185(100)	765,501(163)	650,932(138)	1,586,855(337)	1,392,771(296)
SD(%)		23,093(4.9)	49,691(10.44)	49,169(10.34)	79,750(15.9)	52,557(11.15)

Date: 20061008;48 hours after transfection; DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: HepG2 Density: $1.0X10^5$/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV Promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pGL3	Ealb+ADH6+Luciferase+SV40 poly(A)-pGL3	2apoEs+HAAT+Luciferase+SV40 poly(A)-pGL3	2apoEs+ADH6+Luciferase+SV40 poly(A)-pGL3
1	276	609,824	2,994,276	2,648,394	13,753,502	8,472,516
2	279	594,632	3,057,380	2,517,862	12,904,422	8,161,082
3	255	587,577	2,906,824	2,778,396	13,567,908	7,889,768
4	263	628,306	3,124,688	2,820,862	13,012,038	8,092,026
Ã		608,085	3,020,792	2,691,379	13,309,468	8,153,848
β-gal		0.120	0.118	0.115	0.124	0.120
X(%)		504,237(100)	2,559,993(508)	2,340,329(464)	10,733,442(2128)	6,794,873(1346)
SD(%)		18,051(2.98)	78,628(15.6)	119,117(23.6)	334,615(66.4)	201,363(39.9)

Date: 20061007; 24 hours after transfection DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: Hela Density: $4X10^4$/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV Promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pGL3	Ealb+ADH6+Luciferase+SV40 poly(A)-pGL3	2apoEs+HAAT+Luciferase+SV40 poly(A)-pGL3	2apoEs+ADH6+Luciferase+SV40 poly(A)-pGL3
1	130	3,437,471	7,293	5,345	6,592	12,493
2	437	3,404,241	7,134	5,616	7,219	11,566
3	113	3,553,757	6,877	5,775	7,220	10,551
4	258	3,889,582	7,060	6,221	6,566	12,988
Ā		3,571,258	7,091	5,739	6,899	11,810
β-gal		0.186	0.126	0.047	0.067	0.092
X(%)		1,920,031(100)	5,628(0.29)	12,211(0.64)	10,297(0.54)	12,934(0.67)
SD(%)		221,684(6.2)	138 (0.041)	780(0.028)	552(0.019)	1,168(0.055)

Date: 20061007; 24 hours after transfection DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6

Cell line: 293T Density: 8X10^4/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV Promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pGL3	Ealb+ADH6+Luciferase+SV40 poly(A)-pGL3	2apoEs+HAAT+Luciferase+SV40 poly(A)-pGL3	2apoEs+ADH6+Luciferase+SV40 poly(A)-pGL3
1	278	6,404,734	8,675	10,518	99,311	127,762
2	311	6,481,844	7,359	10,031	109,092	142,598
3	318	6,740,497	7,840	9,715	106,205	135,310
4	192	6,673,053	8,847	10,088	116,216	125,690
Ă		6,575,032	8,180	10,088	107,706	132,840
β-gal		0.713	0.5645	0.595	0.586	0.59
X(%)		9,221,644(100)	14,491(0.16)	16,955(0.84)	183,799(1.99)	224,962(2.4)
SD(%)		157,768(2.3)	1,244(0.0076)	555(0.0036)	11,947(0.076)	13,062(0.084)

Date: 20061007; 24 hours after transfection DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6

Cell line: CHO K1 Density: $8X10^4$/well Media:Ham's F12Serum:10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV Promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pGL3	Ealb+ADH6+Luciferase+SV40 poly(A)-pGL3	2apoEs+HAAT+Luciferase+SV40 poly(A)-pGL3	2apoEs+ADH6+Luciferase+SV40 poly(A)-pGL3
1	156	7,244,088	64,887	98,320	96,732	46,425
2	170	6,566,691	56,672	88,894	96,076	45,348
3	146	6,384,680	61,643	86,916	89,762	50,411
4	125	7,067,720	63,746	97,260	101,266	47,704
Ă		6,815,795	61,737	92,848	95,958	47,472
β-gal		1.072	1.021	1.128	1.051	1.119
X(%)		6,358,018(100)	60,467(0.95)	82,312(1.3)	91,302(1.44)	42,424(0.66)
SD(%)		406,126(5.96)	3,559(0.057)	5,124(0.091)	4,502(0.074)	1,951(0.034)

Date: 20061008; 36 hours after transfection DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: Jurkat Density: 2.0X10^5/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV Promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pGL3	Ealb+ADH6+Luciferase+SV40 poly(A)-pGL3	2apoEs+HAAT+Luciferase+SV40 poly(A)-pGL3	2apoEs+ADH6+Luciferase+SV40 poly(A)-pGL3
1	251	241,268	2,393	10,875	24,333	13,887
2	199	261,021	2,761	10,555	21,321	12,861
3	267	252,066	2,556	11,071	24,406	13,181
4	183	257,871	2,493	10,254	24,210	12,520
Ã		253,057	2,551	10,689	23,568	13,112
β-gal		0.049	0.036	0.054	0.036	0.027
X(%)		516,442(100)	7,085(1.37)	19,794(3.8)	65,465(12.63)	48,563(9.4)
SD(%)		8,690(3.4)	431(0.03)	665.7(0.07)	4,167(0.29)	2159(0.112)

Date: 20061008; 36 hours after transfection DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: U937 Density: $2X10^5$/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV Promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pGL3	Ealb+ADH6+Luciferase+SV40 poly(A)-pGL3	2apoEs+HAAT+Luciferase+SV40 poly(A)-pGL3	2apoEs+ADH6+Luciferase+SV40 poly(A)-pGL3
1	206	9,838	647	955	1,901	1,632
2	192	10,594	570	849	2,074	1,554
3	235	10,309	565	945	1,958	1,479
4	154	10,121	563	843	1,880	1,561
Ã		10,216	586	898	1,953	1,557
β-gal		0.037	0.022	0.031	0.023	0.033
X(%)		27,610(100)	2,665(9.65)	2,897(10.5)	8,492(30.76)	4,716(17.08)
SD(%)		318(1.15)	185(0.67)	194(0.70)	378(1.37)	189.4(0.69)

C: Impact of vector backbone and poly(A)

Date: 20061007; 24 hours after transfection DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: HepG2 Density: $1.5X10^5$/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pVAX	Ealb+HAAT+Luciferase+HAAT poly(A)-pVAX	2apoEs+ADH6+Luciferase+SV40 poly(A)-pVAX	2apoEs+ADH6+Luciferase+HAAT poly(A)-pVAX
1	186	408,234	556,736	528,398	1,371,153	1,278,626
2	179	450,231	488,696	490,286	1,324,462	1,172,918
3	245	431,297	588,268	510,269	1,260,778	1,210,001
4	213	406,506	492,138	530,519	1,307,804	1,269,198
Ã	206	424,067	531,460	514,868	1,316049	1,232686
β-gal		0.09	0.0855	0.1015	0.10775	0.10225
X(%)		471,185(100)	621,590 (132)	507,259(108)	1,221391(259)	1,205,561(256)
SD(%)		20,784(4.41)	57,461(12.1)	18,462(3.92)	42,293(8.98)	48,999(10.4)

Date: 20061007; 24 hours after transfection DNA:300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: Hela Density: $4X10^4$/well; Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pVAX	Ealb+HAAT+Luciferase+HAAT poly(A)-pVAX	2apoEs+ADH6+Luciferase+SV40 poly(A)-pVAX	2apoEs+ADH6+Luciferase+HAAT poly(A)-pVAX
1	130	3,437,471	79,361	105,236	136,842	128,896
2	437	3,404,241	72,252	95,225	122,438	134,025
3	113	3,553,757	82,362	91,468	131,256	131,278
4	258	3,889,582	78,259	92,579	129,737	121,317
Ã		3,571,258	78,059	96,127	130,068	128.879
β-gal		0.186	0.0765	0.110	0.11375	0.112
X(%)		1,920,031(100)	102,037(5.3)	87,388(4.6)	114,346(5.95)	115,071(5.99)
SD(%)		221684(6.2)	5,545(0.28)	6273(0.32)	5216(0.27)	4874(0.25)

Date: 20061008; 36 hours after transfectionDNA: 300ng+200ng β-gal/well; 1.0μl Fugene 6

Cell line: Jurkat Density: 2.0X10^5/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pVAX	Ealb+HAAT+Luciferase+HAAT poly(A)-pVAX	2apoEs+ADH6+Luciferase+SV40 poly(A)-pVAX	2apoEs+ADH6+Luciferase+HAAT poly(A)-pVAX
1	251	241,268	7,875	6,769	38,887	31,276
2	199	261,021	8,555	5531	40,861	32,468
3	267	252,066	7,071	6,278	37,181	27,782
4	183	257,871	8,254	6,054	33,520	28,202
Ã		253,057	7,939	6158	33,362	12,182
β-gal		0.049	0.038	0.026	0.049	0.044
X(%)		516,442(100)	20,891(4.05)	23,685(4.59)	76760(14.9)	68,276(13.2)
SD(%)		8,690(3.4)	1,689(0.33)	1,977(0.38)	3115(0.6)	2298(0.45)

Date: 20061008; 36 hours after transfectionDNA: 300ng+200ng β-gal /well; 1.0μl Fugene 6
Cell line: U937 Density: 2X10^5/well Media: IMDM Serum: 10% Recorder: Xin Min Dong

Code	Blank Control (Empty Vector)	CMV promoter in pGL3	Ealb+HAAT+Luciferase+SV40 poly(A)-pVAX	Ealb+HAAT+Luciferase+HAAT poly(A)-pVAX	2apoEs+ADH6+Luciferase+SV40 poly(A)-pVAX	2apoEs+ADH6+Luciferase+HAAT poly(A)-pVAX
1	206	9,838	749	714	1,414	1,653
2	192	10,594	650	682	1,552	1,543
3	235	10,309	774	763	1,679	1,627
4	154	10,121	701	769	1,631	1,445
Ă		10,216	719	732	1,569	1,567
β-gal		0.037	0.030	0.026	0.022	0.024
X(%)		27,610(100)	2,395(8.67)	2,815(10.2)	7,132(25.8)	6,529(23.65)
SD(%)		318(3.1)	183(0.66)	158(0.57)	523(1.89)	391(1.42)