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Chimeric Orthohepadnavirus Core Particles for Oral Delivery of Vaccines

Part I: Transformation of Tobacco Plants with a Gene Encoding a C-Terminus Truncated Hepatitis B Virus Core Protein

Part II: Construction of a Woodchuck Hepatitis Virus Core Protein-Based Universal Epitope Carrier and Test Expression in *E. coli*

Maximilian W. Callaghan

Thesis presented in partial fulfillment of the requirements

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Department of Biochemistry, Microbiology and Immunology

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I would like to acknowledge in a special way the following people, whose help and guidance made this work possible:

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and my beloved wife Diann, who knew how to say the right things at the right time.

Abstract

Recombinant hepatitis B virus (HBV) core particles have been successfully used as particulate carriers exposing viral and bacterial antigens on their surface. The objective of this research was to explore the use of recombinant core particles from HBV and its close relative the woodchuck hepatitis virus for edible vaccine technology. This was accomplished in two parts. Part 1 was the transformation of a truncated HBV core protein gene into transgenic tobacco plants and characterization of the gene's expression with respect to mRNA levels, protein levels, and particle self-assembly. Part 2 was the construction of a "universal antigen carrier" based on the woodchuck hepatitis virus (WHV) core protein and generation and characterization of chimeric WHV core proteins carrying two different epitopes from the hepatitis C virus (HCV) core protein.

A truncated HBV core gene corresponding to the first 150 amino acids of the core protein was inserted in frame into the CamterIII binary vector for *Agrobacterium*-mediated transformation of *Nicotiana tabacum* cv. Wisconsin 38 plants. The gene was placed under the control of the cauliflower mosaic virus 35S promoter. Twenty-eight transformed plants were confirmed by a PCR using isolated genomic DNA. mRNA levels were assessed by Northern blot and RT-PCR analysis. No HBV core mRNA was detected in the transformed plants. Immunological methods failed to detect any HBV core protein in extracts from the leaves of the transformed plants.

A truncated WHV core gene corresponding to the first 150 amino acids of the core protein was inserted in frame into the prokaryotic expression vector pK233-2(+) that carries an IPTG-inducible promoter. Two unique restriction sites were engineered into the WHV core gene: *Xba*I at nucleotide 234 corresponding to amino acid 78 of the core protein, and

*Xho*I at nucleotide 420 corresponding to amino acid 140 of the core protein. The *Xba*I site would permit the insertion of a foreign epitope into the immunodominant loop of the WHV core protein. The *Xho*I site would permit the insertion of a foreign epitope very close to the C-terminus of the WHV core protein. In this study, two constructs carrying inserts of truncated HCV core protein were generated. One construct carried an insert at the *Xba*I site corresponding to 37 amino acid residues from the N-terminus region of the HCV core protein. Another construct carried an insert at the *Xho*I site corresponding to 39 amino acid residues from the N-terminus region of the HCV core protein.

The pKK233-2/WHVc vectors were transformed into *E. coli* JM109 and high-level expression of the modified WHV core genes was achieved upon induction with IPTG. The recombinant WHV core proteins were purified by sucrose density gradient ultracentrifugation and their antigenicity assessed by ELISA and Western blotting. The recombinant WHV core proteins were assessed for their ability to self-assemble into core particles by electron microscopy. It was found that the truncated and chimeric WHV core proteins retained WHV core antigenicity, as determined by Western blotting. Both chimeric WHV core proteins carrying HCV epitopes possessed HCV antigenicity, as determined by ELISA. Present electron microscopy data suggests that the recombinant WHV core proteins may have lost their ability to self-assemble into core particles.

In conclusion, it appears that a different approach may be required to express core proteins from HBV-like viruses in transgenic tobacco plants. Further attempts may focus on codon optimization for plant expression and/or use of different promoter, enhancer, or signal sequences. HCV core epitopes fused with WHV core proteins are antigenic, suggesting that chimeric WHV core proteins may have a future role in edible vaccine technology.

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List of Abbreviations

BA	6-benzylaminopurine, a plant cytokinin
DHBV	duck hepatitis B virus
BSA	bovine serum albumin
CS	coding sequence
DM	dry milk powder
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
GSHV	ground squirrel hepatitis virus
HBV	human hepatitis B virus
HBcAg	core antigen of human hepatitis B virus = HBV core protein
HCV	human hepatitis C virus
HHBV	heron hepatitis B virus
IgA	class A immunoglobulin
IgG	class G immunoglobulin
IgM	class M immunoglobulin
IPTG	isopropylthio-beta-D-galactoside
LB	Luria-Bertani bacterial growth medium
MCS	multicloning site
MMO	Murashige minimal organics medium for plant tissue culture
NAA	alpha-napthaleneacetic acid, a plant auxin
OD	optical density
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
sIgA	secretory class A immunoglobulin
STE	buffer solution containing sucrose, Tris-HCl, and EDTA
TE	buffer solution containing Tris-HCl and EDTA
WHV	woodchuck hepatitis virus
WHcAg	core antigen of woodchuck hepatitis virus = WHV core protein
YEP	bacterial growth medium containing yeast extract and peptone

Chapter 1: Introduction

Oral Immunization

Viral and bacterial infections cause the largest number of infant deaths worldwide, accounting for the loss of millions of children each year. Antibiotics are often critical for treatment, and are administered when available. Conversely, they are often inappropriately applied, which leads to the development of antibiotic-resistant bacterial strains. New, targeted immunization strategies are consequently required to prevent rather than cure infectious diseases. Many bacteria and viruses, transmitted either through contaminated water or food or by sexual contact, invade epithelial membranes. Often, mucosal immune response is attained more efficiently by oral rather than parenteral antigen delivery. Parenteral inoculation with whole-cell and whole-virus vaccine preparations can lead to defensive serum IgG and delayed type hypersensitivity reactions against organisms that have an important serum phase in their pathogenesis. This strategy is limited by the fact that it does not stimulate mucosal secretory IgA responses, and thus is inadequate against bacteria invading mucosal surfaces (Ruedl and Wolf, 1995). However, oral immunization can successfully elicit specific secretory IgA responses and systemic immune reactions if antigens are presented to T- and B-lymphocytes and accessory cells. New, oral immunization approaches should use antigens that engender specific mucosal immunity, and are inexpensive to produce, store and utilize, in order to meet the requirements of the developing world.

Current systems which can be used to produce recombinant immunogenic proteins include prokaryotic systems such as *Escherichia coli* (Bowden and Georgiou, 1990; Kudo, 1994) and *Bacillus* (Ebisu et al., 1996; Udaka and Yamagata, 1993) as well as eukaryotic

systems such as yeast (Harashima, 1994; Buckholz and Gleeson, 1991), *Aspergillus* (Archer and Peberdy, 1997), mammalian (Hesse and Wagner, 2000) and insect cell cultures (Goosen, 1993; Luckow, 1993; Lanford *et al*, 1989), transgenic animals (Lubon, 1998; Van Cott *et al*. 1997), and plants (Hood and Jilka, 1999; Tackaberry *et al*, 1999, Ganz *et al*, 1996). After identification and cloning, a gene encoding an antigen can be introduced into many cell types and produced in large amounts. *E. coli* continues to be the most important laborer of the biotechnology industry but it is poorly suited for expressing those eukaryotic genes where the expressed protein must be glycosylated and processed. In recent times, transgenic animals and insect and mammalian cell cultures have been used to produce several important pharmaceutical proteins. These eukaryotic systems are effective due to their capacity to glycosylate and process the recombinant protein analogously to the native host. However, the fermentation bioreactors, stringent purification methods and development programs required to yield the necessary amounts of purified recombinant protein with these systems are very expensive. This technology is incapable of producing vaccines below the US$1 mark per dose, which has been estimated to counter the problems of world health care costs (Fox, 1996).

Plant-Based Oral Vaccines

Transgenic plants have the potential to be one of the most cost-effective systems for large-scale production of recombinant proteins for the industrial and pharmaceutical industries. Plants are the most significant renewable resource materials available. Advantages of the plant systems include the low cost of growing plants on large tracts of land, established processes for efficient harvesting, transport, storage and processing, and

the availability of natural protein storage organs for targeted expression of recombinant protein. Plants offer several other benefits which include: (1) the elimination of the need to purify the product when the plant tissue containing the recombinant protein can be provided as a supplement in food or feed; (2) the reduction or elimination of the need to refrigerate the recombinant protein since it may be compartmentalized in a stable fashion in different storage tissues; and (3) freedom from possible contamination by human pathogens. *Nicotiana tabacum* (tobacco) has been widely used as a model expression system due to the relative ease of transformtion and the rapid regeneration of these plants (Giddings *et al*, 2000). Other plants that have been used include Nicotiana bethamina (McCormick *et al*, 1999). Arabidopsis thaliana (Boothe *et al*, 1997), tomato (McGarvey *et al*, 1995), oilseed rape (Goddijn and Pen, 1995), potato (Richter *et al*, 2000) , rice (Ganz *et al*, 1996), wheat (Stoger *et al*, 2000) and maize (Zhong *et al*, 1999).

Since the 1980s, considerable progress has been made in the genetic engineering of plants and in the regulation and stabilization of recombinant protein expression. It is now possible to use genetically modified plants as bioreactors in the production of a wide assortment of valuable pharmaceutical proteins. A decade ago, it became possible to produce antibodies in transgenic plants, consisting of the correctly assembled light and heavy chains (Hiatt *et al*., 1989; During *et al*., 1990). Since that time, several research groups have focused their efforts on producing antibodies targeted for distinct antigens of human pathogens (Ma and Hiatt, 1996; Ma et al., 1995; Richardson and Marasco, 1995). Stably transformed transgenic plants have yielded whole antibody totaling as much as 1% of total soluble protein (Hiatt, 1990). These antibodies can be extracted from plant tissues and used to prevent infectious disease.

A different strategy, the one of concern in this work, is based on oral delivery of antigens to stimulate sIgA production (Bergmann and Waldman, 1988). In 1992, Charles Arntzen's group from Texas A and M University in Houston (now at Boyce Thompson Institute for Plant Research, Ithaca) presented the concept of antigen production in transgenic plants for vaccine applications (Mason et al., 1992). Large amounts of protein can be produced at relatively low cost in an agriculturally based system.

The manufacture of vaccines in the edible tissue of plants would combine production and delivery systems. Immunization may be possible simply through food consumption. Several reports of oral delivery of plant-produced vaccines to animals have suggested that the immune response may range from a modest increase in the level of serum antibodies to protection against pathogen challenge (Sandhu *et al*, 2000; Kapusta *et al*, 1999, Haq *et al*, 1995). To date, transgenic plant-based production of vaccines has included antigens from norwalk virus (Tacket *et al*, 2000), human cytomegalovirus (Tackaberry *et al*, 1999), hepatitis B virus (Kapusta *et al*, 1999, Domansky *et al*, 1995), foot and mouth disease virus (Wigdorovitz *et al*, 1999), transmissible gastroenteritis coronavirus (Gomez *et al*, 1998 and 2000), rabies virus (Yusibov *et al*, 1997; McGarvey *et al*, 1995), human immunodeficiency virus (Yusibov *et al*, 1997), mink enteritis virus (Dalsgaard *et al*, 1997), enterotoxigenic *Escherichia coli* (Haq *et al*, 1995), and rabbit hemorrhagic disease virus (Castanon *et al*, 1999), among others. The first human clinical trials of plant-derived edible vaccines have demonstrated similarly encouraging results (Tacket *et al*, 2000 and 1998).

Reports of experiments in plant-based vaccine production can be classified into several categories including: (1) reports of expression of immunoreactive protein antigens in plants (Domansky *et al*, 1995; Mason *et al*, 1992; McGarvey *et al*, 1995;

Tackaberry *et al*, 1999), (2) reports of induction of a targeted immune response in laboratory animals by parenteral delivery of plant-produced antigens (Yusibov *et al*, 1997), (3) reports of plant-derived parenteral vaccines that conferred protection against the targeted disease in laboratory animals (Castanon *et al*, 1999; Dalsgaard *et al*, 1997; Wigdorovitz *et al*, 1999b), (4) reports of oral delivery of plant-produced antigens that induced a targeted immune response in laboratory animals (Gomez *et al*, 1998; Richter *et al*, 2000; Sandhu *et al*, 2000; Tariq *et al*, 1995), (4) reports of oral delivery of plant-produced vaccines that conferred protection against the targeted disease in laboratory animals (Wigdorovitz *et al*, 1999a), and (5) reports of oral delivery in humans of plant-produced vaccine antigens that induced a targeted immune response (Kapusta *et al*, 1999; Tacket *et al*, 2000). The first report of a plant-derived oral vaccine for humans conferring protection against pathogen challenge has yet to be published.

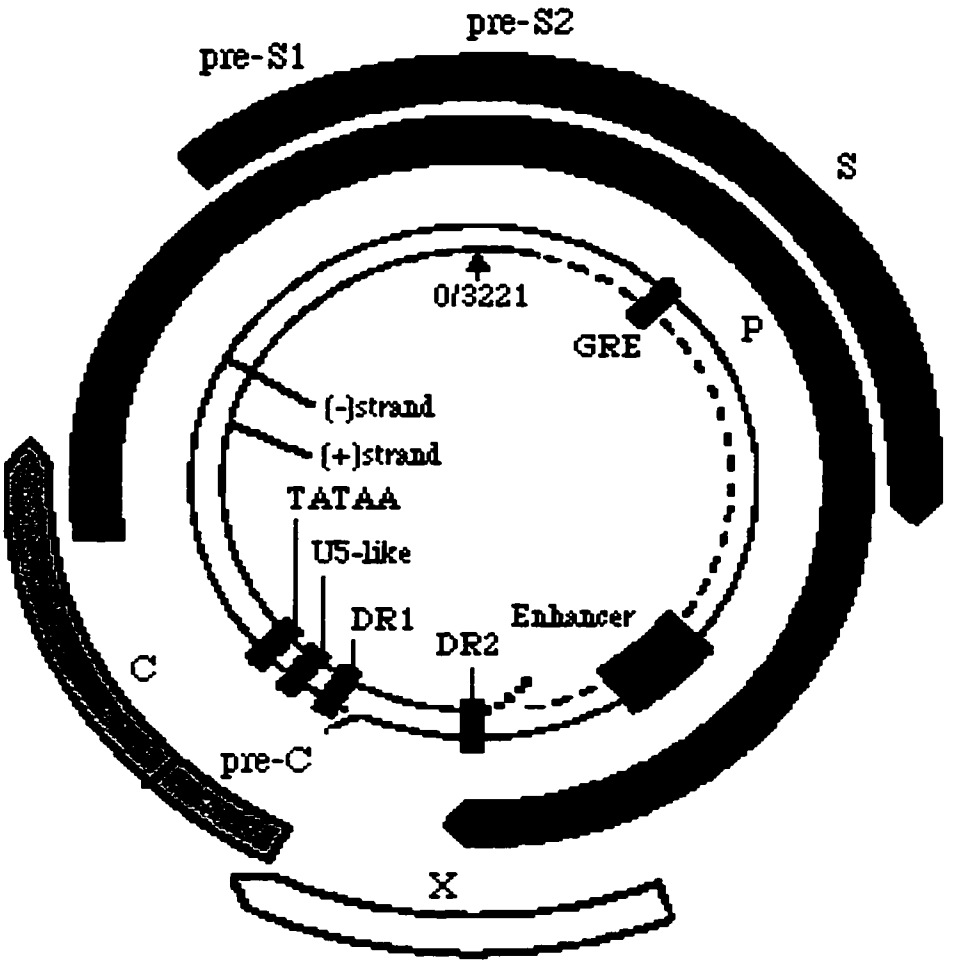
The goal of this research project has been to develop and test plasmid DNA vectors designed for plant-based expression of human hepatitis B virus (HBV) core protein (HBcAg)- and woodchuck hepatitis virus (WHV) core protein (WHcAg)-based particulate carriers for the insertion of foreign peptide sequences (antigens). Modified HBcAg and WHcAg proteins expressed in transgenic plants could then be orally delivered to the T- and B-lymphocytes and accessory cells of the digestive system. These modified proteins could carry a wide assortment of antigens from pathogens to which a mucosal immune response is desired.

Hepadnavirus Properties

Both HBV and WHV are members of the family *Hepadnaviridae* that derives its name from its hepatotropic nature and double-stranded DNA genome. The hepadnavirus family includes viruses in the genus Orthohepadnavirus: HBV, WHV, and Ground Squirrel Hepatitis B Virus (GSHV); and in the genus Avihepadnavirus: Duck Hepatitis B Virus (DHBV), and Heron Hepatitis B Virus (HHBV) (Sixth Report of the International Committee on Taxonomy of Viruses). Each of these viruses contains a very small genome of partially double stranded DNA. In HBV, the complete viral particle, called the Dane particle, is 42-47 nm in diameter. The Dane particle is named in honour of the British pathologist David S. Dane who first discovered the particle in 1970 (Dane *et al*, 1970). The Dane particle is made up of an outer coat containing HBsAg (surface antigen) and an inner 28-32 nm diameter core particle made up of HBcAg. The nucleic acid is packaged within the core particle. There are four known genes in the virus: C, the core protein; P, the polymerase; S, the three polypeptides of the surface antigen – produced from alternative translation start sites; and X, a transactivator of viral transcription (Figure 1). Gene X is conserved among the Orthohepadnaviruses but not among the Avihepadnaviruses (Blum *et al*, 1989).

Figure 1: Schematic representation of the HBV genome and gene products. There are 4 known genes in the virus: C, the core protein; P, the polymerase; S, the three polypeptides of the surface antigen – produced from alternative translation start sites; and X, a transactivator of viral transcription (adapted from Alan Cann, www-micro.msb.le.ac.uk/335/hepatitis1.gif). The inner circles represent the viral genome. The arrow shows nucleotide 0 of the 3221 bp genome. Protein products are represented by the thick arrows surrounding the inner circles.

Figure 1: Schematic representation of the HBV genome and gene products.



Orthohepadnavirus Core Proteins as Vaccine Carriers: HBcAg and WHcAg

The HBV core gene codes for two partly collinear protein products: e antigen (HBeAg). and core antigen (HbcAg). HBeAg is a secreted, non-structural protein of unknown function translated from the pre-C and C coding sequences. HbeAg is composed of ten N-terminal non-HBcAg amino acid residues followed by the HBcAg amino acid sequence from position 1-149. However, HbcAg is a 21-kD protein composed of 183 predominantly hydrophilic and charged amino acid residues. This protein is neither glycosylated nor does it have any lipid attached. However, it appears in eukaryotic cells that this protein may be phosphorylated. HBcAg is synthesized in the cytoplasm and self-assembles into subviral 28-32 nm nucleocapsid particles called core particles that normally contain the viral RNA and polymerase (Schodel *et al*, 1996). The ability for particle assembly lies within the first 144 amino acids. Expression of the HBcAg protein in bacteria has shown it packages RNA in a non-specific fashion. Four arginine clusters within the last 36 to 38 residues appear involved in the packaging of nucleic acids. Phosphorylation occurs on either serine 170 or 172. These amino acids are located between arginine clusters 3 and 4. As such, phosphorylation of the serine residues may hamper nucleic acid binding. Four hydrophobic residues that occur in a small region of the core protein gene appear to be required for assembly of the core particles. These residues, including leucine 101, leucine 108, valine 115, and phenylalanine 122 are spaced every seventh residue and are conserved in all mammalian hepadnaviruses (Yu *et al*, 1996).

The C-terminal border for HBcAg sequences required for self-assembly has been established between amino acid residues 139 and 144 (Pumpens *et al*, 1995). Furthermore, C-terminal truncations beyond this region do not affect the yield, size or morphology of

particles assembled in *E. coli* (Gallina *et al*, 1989). Truncated HbcAg protein particles with 39 amino acid residues removed from the C-terminus showed high-level synthesis in *Escherichia coli* cells and a low affinity to package nucleic acids but were slightly less stable than the corresponding full-length protein particles (Borisova *et al*, 1996). Additionally, the core antigen has been shown to assemble and form particles even when produced in the absence of other HBV gene products in a broad spectrum of bacterial, yeast, insect and mammalian cells (Schodel *et al*, 1996). Two particle species are observed, a rare smaller type composed of 180 subunits and a more abundant type composed of 240 subunits (Crowther *et al*, 1994).

The singular feature of HBcAg that makes it attractive as a carrier molecule for foreign antigens is that at least 20 percent of the molecule is dispensable for self-assembly. Of particular interest is the central part of the molecule, including amino acid residues 72-88 that has been predicted as a nonstructured loop. The origin of this discovery is that duck hepatitis B virus (DHBV) core antigen was observed to contain a nonhomologous insertion of 39 amino acid residues in the proposed tip of this loop (Argos and Fuller, 1988). This region of HbcAg has since been used successfully for insertion of foreign peptide sequences ranging in length from 5 to 238 amino acid residues, without affecting assembly of the core particles (Pumpens *et al*, 1995, Kratz *et al*, 1999). Early work suggested that the insertion capacity of the loop was limited to around 40 amino acids. However, it has recently been conclusively demonstrated that larger inserts are tolerated, provided the individual parts of the corresponding fusion protein are able to fold independently (Kratz *et al*, 1999). In their work, Kratz *et al* used the green fluorescent protein (GFP) as a model insert. The chimeric protein efficiently formed fluorescent particles with GFP domains exposed on the surface.

Therefore, all of its structurally important parts must have been properly folded. This group also demonstrated that the chimeric particles elicited a potent humoral response against native GFP.

In its native form, the loop has been found to contain the primary antigenic sites of HBcAg, and is thus termed the immunodominant loop. The immunodominant loop appears to form the most protruding spikes of the assembled core particle (Pumpens and Grens, 1999). N-terminal and C-terminal regions are also accepted as target sites for the introduction of foreign epitopes. C-terminus deletion mutants have been shown to accept insertions exceeding 100 amino acids in length while the upper limit for N-terminus insertions appears to be in the range of 50 amino acid residues (Pumpens *et al*, 1995). Inserts at the N-terminal, C-terminal, or immunodominant loop regions are exposed on the outer surface of the particles. Exposure is complete in the case of inserts within the immunodominant loop and at least partial in the case of inserts at the N-terminus and C-terminus. Despite these insertions, chimeric core particles still self-assemble from the modified core genes and present the foreign epitopes on their surface in a way that elicits strong B-cell and T-cell responses against them (Kratz *et al*, 1999; Schodel *et al*, 1996; Pumpens *et al*, 1995). Thus, the original immunological activity of the inserted sequences is not only retained, but may even be enhanced due to the spatial organization of the assembled particles (Pumpens *et al*, 1995; Pumpens *et al*, 1999; Ulrich *et al*, 1999). Foreign epitopes which have been successfully expressed on the surface of chimeric HBV core particles include among several others: human rhinovirus (Brown *et al*, 1991), simian immunodeficiency virus (Yon *et al*, 1992), hepatitis C virus (Yoshikawa *et al*, 1993), and hantavirus (Ulrich *et al*, 1999). The chimeric HBV core particles carrying hantavirus

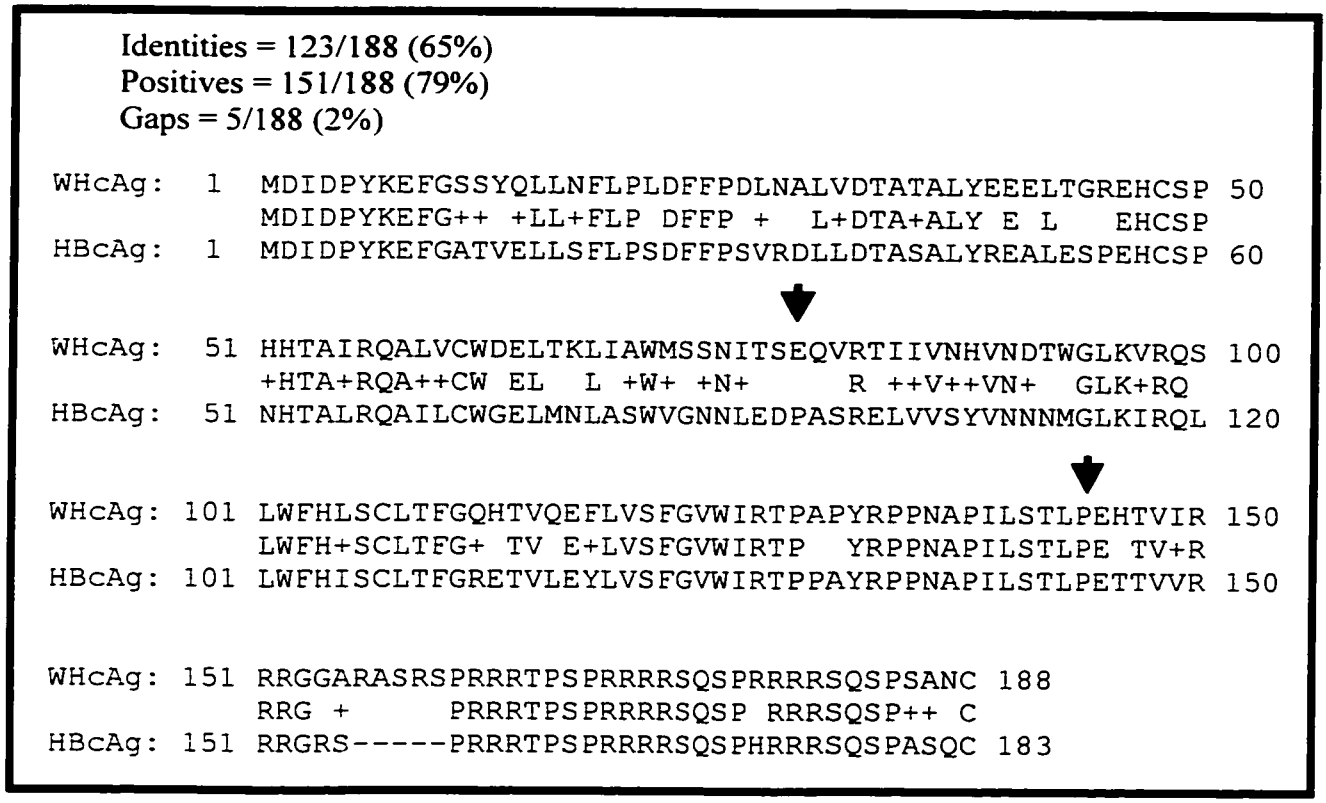
epitopes were immunogenic when administered to bank voles, even in the absence of adjuvant, and conferred partial protection against the disease.

HBcAg is considered as an exceptionally good immunogen in humans and animals (Pumpens et al, 1995). The immunogenicity of HBcAg can probably be accounted for by the multimeric structure of the particles. Epitopes are presented in a polymeric array in both a T cell dependent and T cell independent manner. The repetitiveness of several viral surface antigens has been shown to act as a marker for foreignness that is strongly recognized by B cells as part of the host defense system (Bachman and Zinkernagel, 1996). In chimeric core particles, the three-dimensional conformation and high density of introduced peptides per particle ensure the induction of an immune response against the presented epitopes. To be effective as vaccines, most monomeric proteins and peptides require either application together with adjuvants or chemical coupling to high molecular weight carriers. However, chimeric HBV core particles have been shown to induce B and T cell immune responses against the inserted epitopes, even in the absence of adjuvants (Ulrich *et al*, 1998). Therefore, HBcAg may prove to be a good candidate for oral vaccine delivery.

The HBV core particle is one of the most intensively studied particulate carriers for the insertion of foreign peptide sequences. However, its close relative the woodchuck hepatitis virus has not received the same attention despite the fact that extensive genomic sequence homology between the two viruses, and particularly between the core particle genes, was well characterized nearly two decades ago (Galibert *et al*, 1982). Figure 2 shows a comparison of the complete amino acid sequences of the woodchuck hepatitis virus core protein and the hepatitis B virus core protein.

Figure 2: Comparison of the amino acid sequence of woodchuck hepatitis virus core and hepatitis B virus core proteins. The alignment was generated using the "Position Specific Iterated Basic Local Alignment Search Tool" (Altschul *et al*, 1997) at the NCBI website (www.ncbi.nlm.nih.gov) using the WHV core protein sequence (Genbank accession J02442) as the query. The alignment shown is with the HBV core protein (Genbank accession AAF37215). A comparison of amino acid residues in both sequences is shown between the horizontal lines of text: identical amino acid residues are indicated by their single letter code, similar amino acid residues (positives) are indicated by a "+", and mismatches are indicated by a blank. Gaps are indicated by "-". Arrows indicate the sites in the WHV core protein sequence targeted for insertion of foreign epitopes in this research.

Figure 2: Comparison of the amino acid sequence of woodchuck hepatitis virus core and hepatitis B virus core proteins.



The sequences are 65% identical at the amino acid level. Furthermore, if similar amino acid residues are considered, the sequences are 79% similar. A few years ago, it was shown that sufficient homology exists between the two viruses that HBV and WHV polymerases are each competent to recognize both HBV and WHV packaging signals and can encapsidate each other's pregenomic RNA (Ziermann and Ganem, 1996). This is significant since effective encapsidation of hepadnaviral pregenomic RNA requires the coordinated interaction of the viral capsid and polymerase proteins with each other and with the RNA packaging substrate. A possible advantage of using WHV instead of HBV core particles in edible vaccines is that inoculation with chimeric WHV particles may not interfere with serological diagnosis of HBV infection in humans. Sensitive diagnostic assays could be used which would differentiate between the immune responses to the two core antigens.

A further use for plant-produced native and chimeric orthohepadnavirus core particles would be in the development of serological diagnostic tools. To maximize the availability and minimize the cost of sensitive radioimmunoassays, a large amount of inexpensive, purified antigen is required (Tsuda *et al*, 1998). Mass production in plants of antigenic proteins would be very economical, in terms of equipment and overall cost, if the cost of downstream processing of the harvested plant tissue can be kept to a minimum. Due to their sedimentation and density properties, it is a relatively uncomplicated process to purify virus-like particles from plant tissue. Several groups have already demonstrated the feasibility of isolating virus particles and virus-like particles from transgenic plants. Based on previous work, one research group has already developed effective protocols for extraction of HBV surface antigen from transgenic plants in a manner that preserves the antigenicity of the protein (Dogan *et al*, 2000). Other virus-like particles that have been

purified from plant tissue include chimeric cowpea mosaic virus particles carrying a mink enteritis virus epitope (Dalsgaard *et al*, 1997), and Norwalk virus particles (Mason *et al*, 1996). The size, density and three dimensional structure of orthohepadnavirus core particles make them very good epitope carriers, but it may also make them good candidates for plant-based production of antigens for use in diagnostic kits. However, the goal of this work was the development of novel plant-based oral vaccine technology, not plant-based antigen production for subsequent purification.

The two hypotheses that guided this work were that: (1) orthohepadnavirus core protein gene expression in plants would lead to accumulation of correctly-assembled core particles, and that (2) the WHV core protein would allow heterologous insertions of amino acids in a similar fashion to the HBV core protein, thereby allowing assembly of chimeric WHV core particles that display foreign antigens on their surface. The objectives of the work included the following: (1) to construct a plant transformation vector for Agrobacterium-mediated transformation of dicot plants (like tobacco) with the coding sequence for an orthohepadnavirus core protein, (2) to construct a plant transformation vector for Agrobacterium-mediated transformation of monocot plants (like wheat and rice) with the coding sequence for an orthohepadnavirus core protein, (3) to obtain transgenic tobacco and rice plants for analysis transformed with the orthohepadnavirus core protein coding sequence, (4) to modify the coding sequence of the WHV core gene to include unique restriction sites for insertion of gene fragments encoding heterologous epitopes, (5) to generate chimeric WHV core protein coding sequences with heterologous inserts, and (6) to express the chimeric WHV core protein coding sequences in *E. coli* for analysis of the expressed protein.

Figures 3a and 3b show a simplified summary of the manipulations carried out in Parts I and II of this work, respectively. Part I of this report describes an attempt to express a gene coding for C-terminus truncated HBcAg protein in transgenic tobacco plants. Part II describes the construction of a modified WHcAg protein gene designed to accept insertions of foreign epitopes and its expression in *E. coli*. Figure 4 shows the location of the unique restriction sites, *Xba*I and *Xho*I, that were introduced into the coding sequence for a C-terminus truncated WHV core protein to allow epitope insertion. This diagram is extrapolated from the determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy (Bottcher *et al*, 1997). The polypeptide folds of the HBV core protein were hypothetically extended to represent the fold and shell architecture of the WHV core protein. Figure 5 shows the hypothetical structure of the chimeric protein products generated in Part II by introducing hepatitis C virus (HCV) epitopes into the novel *Xba*I and *Xho*I restriction sites. Epitopes from the C-terminus of the HCV core protein were selected for insertion since a commercial diagnostic kit (HCV EIA 2.0 kit, Abbott) was available that could be used to detect these epitopes. These chimeric WHV core proteins, called WHVc150-2.3 and WHVc150-8.8, carried inserts of 37 and 40 amino acid residues of the HCV core protein, respectively.

Figure 3a: Summary of manipulations in Part I - Expression of a C-terminus truncated HBcAg protein gene in transgenic tobacco plants. Plasmid constructs are indicated in bold type. Arrows indicate the sequence of manipulations. Smaller type briefly describes manipulations and plasmid constructs - see the Materials and Methods chapter for details.

Figure 3a: Summary of manipulations in Part I - Expression of a C-terminus truncated HBcAg protein gene in transgenic tobacco plants.

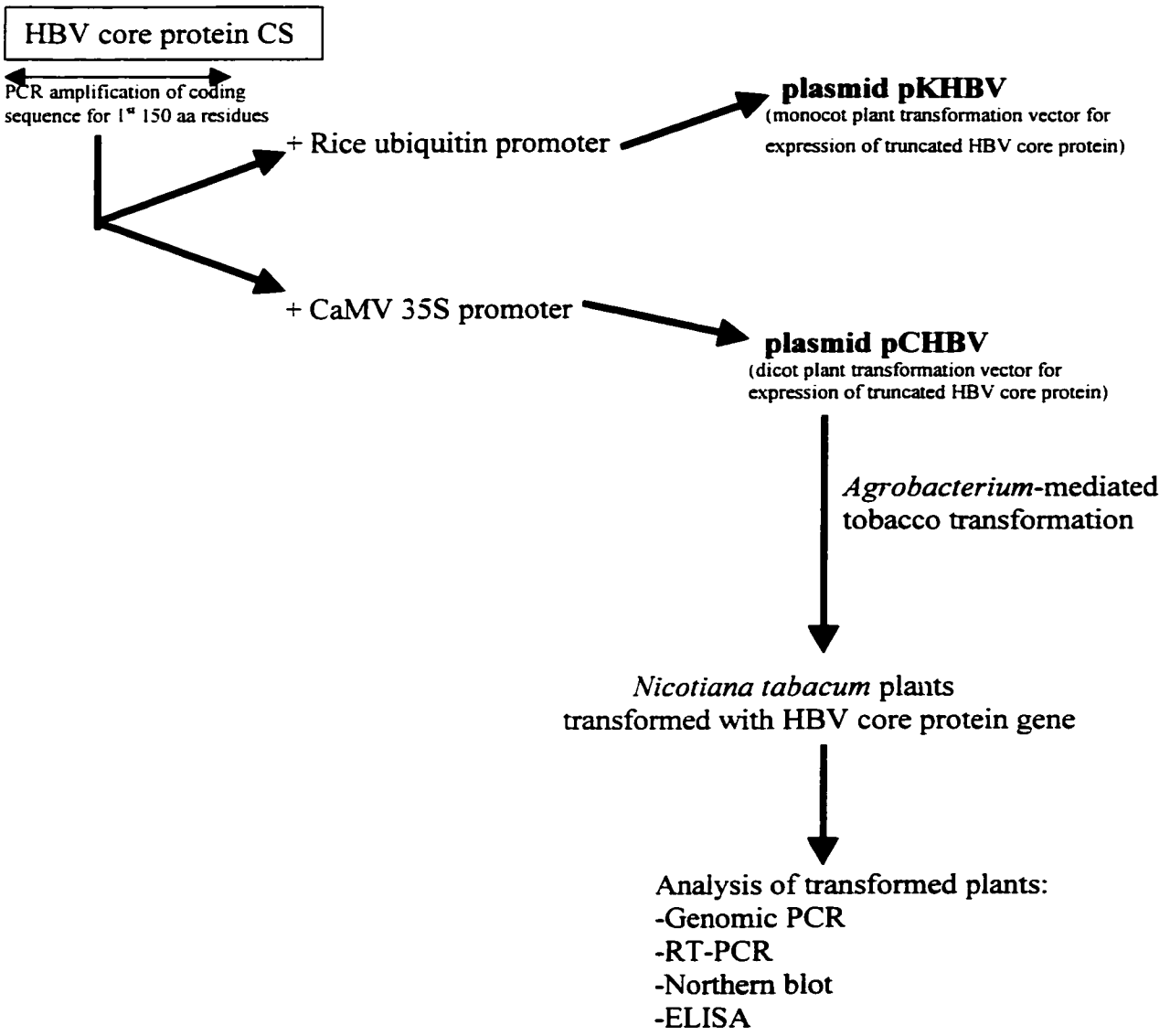


Figure 3b: Summary of manipulations in Part II - Construction of a WHcAg-based epitope carrier protein and test expression in *E. coli*. Plasmid constructs are indicated in bold type. Arrows indicate the sequence of manipulations. Smaller type briefly describes manipulations and plasmid constructs - see the Materials and Methods chapter for details.

Figure 3b: Summary of manipulations in Part II - Construction of a WHcAg-based epitope carrier protein and test expression in *E. coli*.

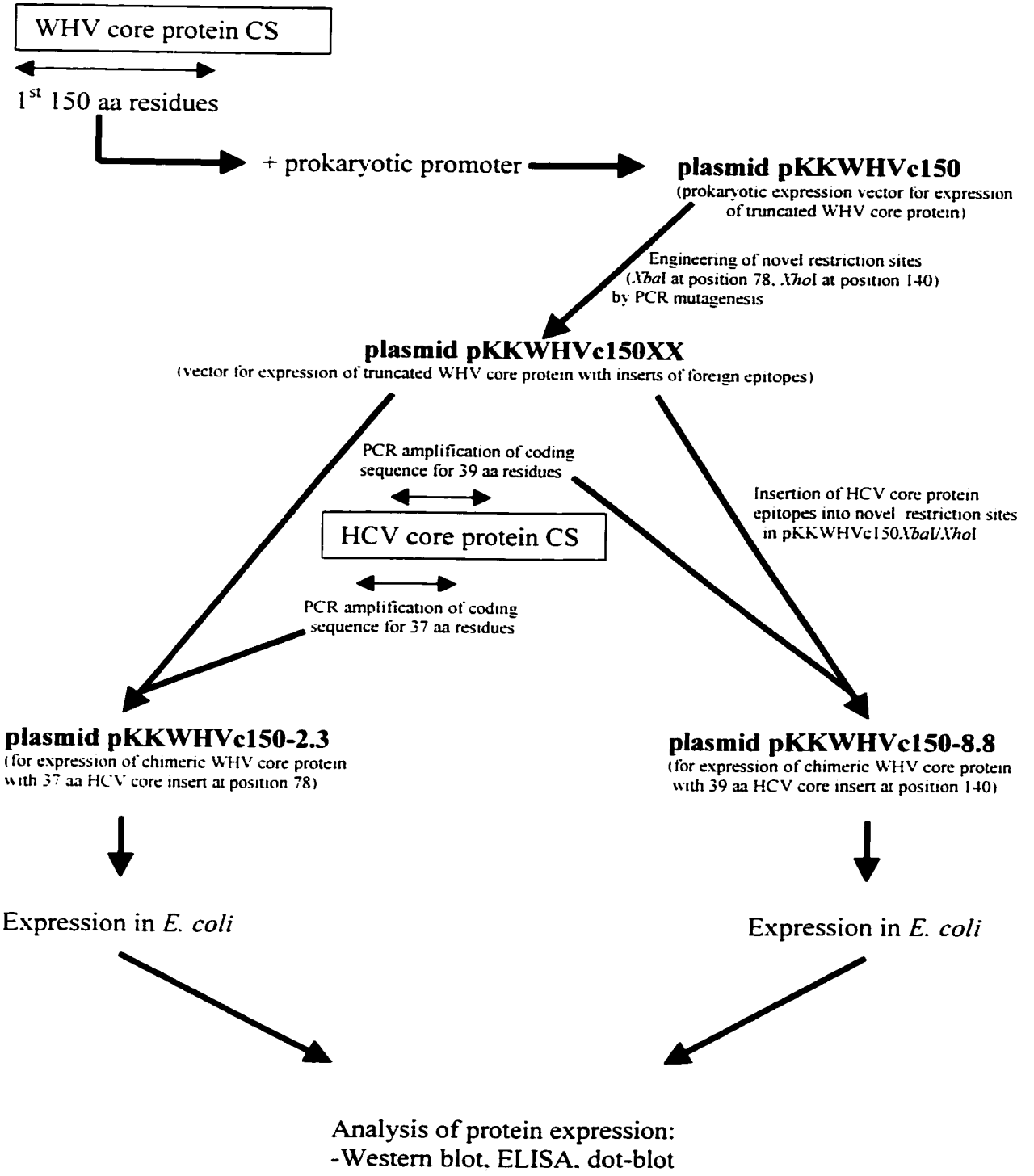


Figure 4: Location of the unique restriction sites *Xba*I and *Xho*I introduced into the truncated pKKWHVc150XX construct. The diagram of the polypeptide folds of the HBV core protein is hypothetically extended to represent the fold and shell architecture of the WHV core protein. The α-helical regions of the polypeptide are indicated as cylinders and the N and C termini are marked. An approximate numbering system for the amino acid residues is given. This model was adapted from Bottcher *et al*. 1997.

Figure 4: Location of the unique restriction sites *Xba*I and *Xho*I introduced into the truncated pKKWHVc150XX construct.

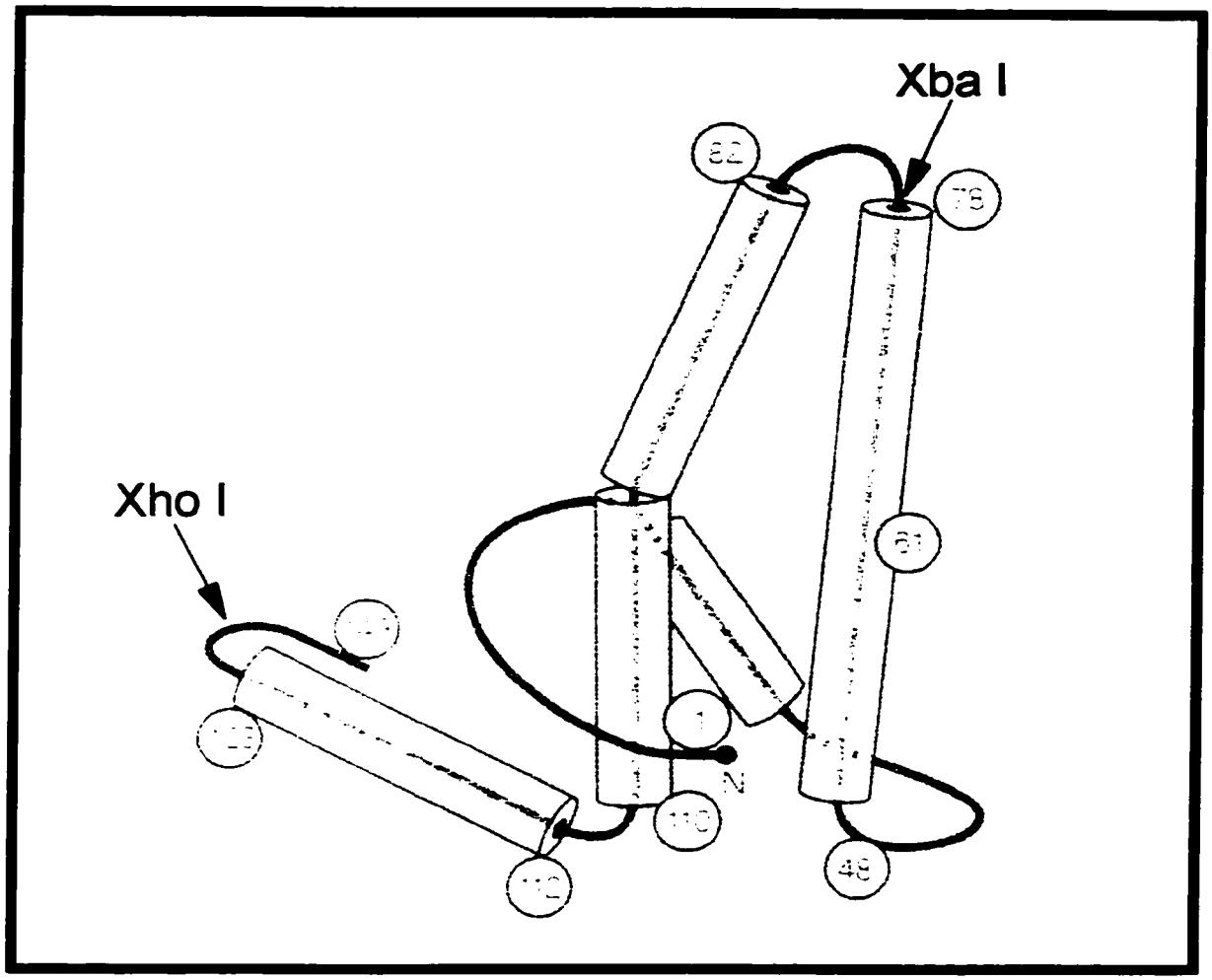


Figure 5: Hypothetical structure of the protein products of pKKWHVc150-2.3 and pKKWHVc150-8.8 constructs. The constructs carry 37 (aa 5-41) and 40 (aa 14-53) of the HCV core protein, respectively. This model was adapted from Bottcher *et al*, 1997.

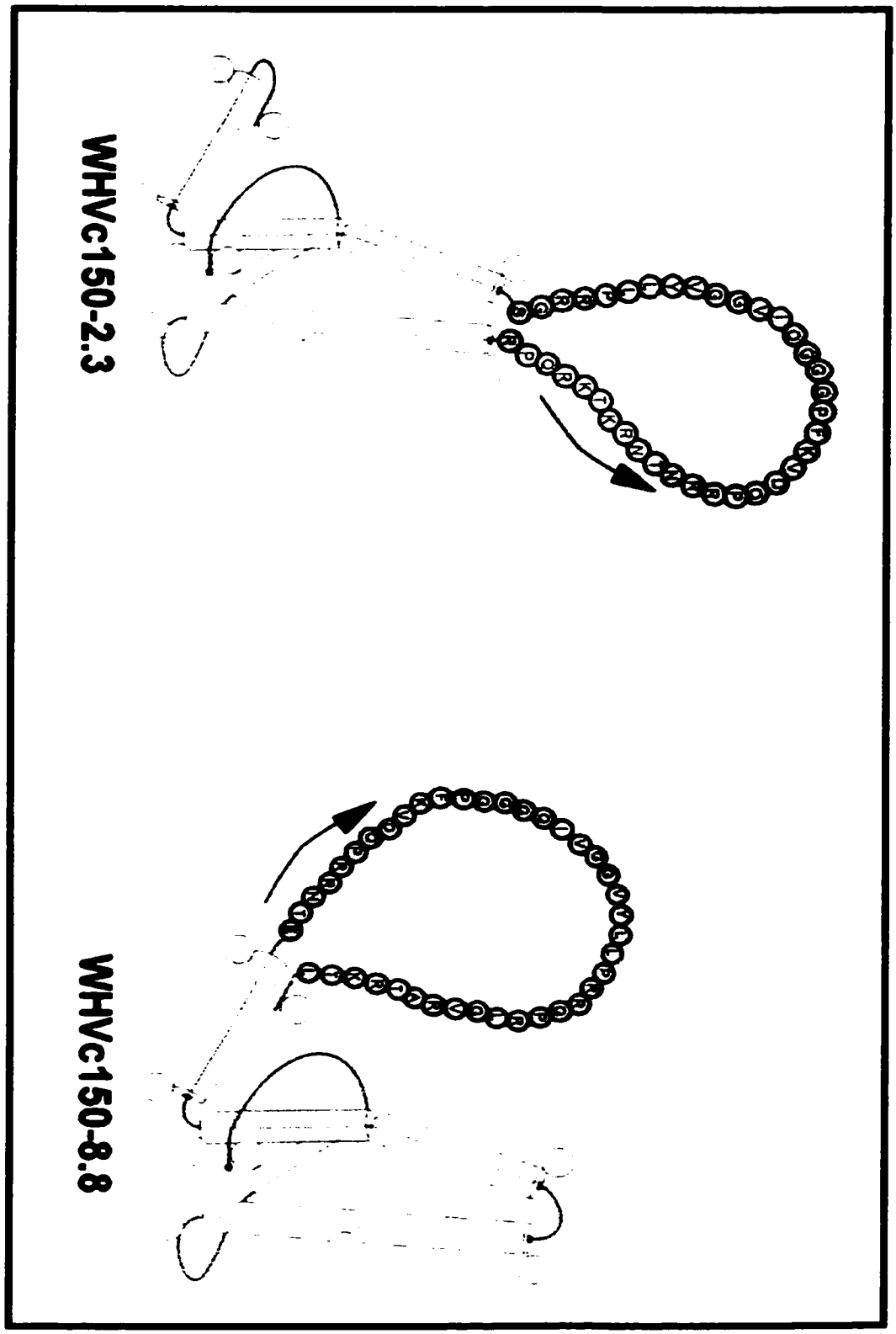
● Indicates overlapping regions of the cloned HCV core fragment in the two constructs.

● Indicates the amino acids that were modified for cloning into *Xba*I and *Xho*I sites and do not represent the primary sequence of the HCV core fragment.

● Indicates HCV core protein amino acids 42-53 present only in WHVc150-8.8 construct.

○ Indicates HCV core protein amino acids 5-13 present only in WHVc150-2.3 construct.

The loops representing HCV inserts are not drawn to scale.


WHVc150-2.3
WHVc150-8.8

Chapter 2: Materials and Methods

General Materials and Methods

Restriction endonucleases were obtained from Gibco BRL, Mississauga, Ontario, Canada, or from Promega, Madison, Wisconsin, USA. The large fragment of DNA Polymerase I (Klenow) was obtained from Gibco BRL, Mississauga, Ontario. T4 Ligase and its Ligation Buffer. T4 Polynucleotide Kinase and its Phosphorylation Buffer, and Vent Polymerase and its ThermoPol Buffer were obtained from New England Biolabs, Mississauga, Ontario, Canada. Cloning in plasmid vectors was accomplished with the Rapid DNA Ligation Kit (Cat. No. 1635379) from Boehringer Mannheim GmbH. The GENECLEAN KIT (BIO 101) for the recovery of DNA from agarose gels was obtained from BIO/CAN Scientific, Mississauga, Ontario; and Miniprep Kits for the extraction and purification of DNA were obtained from QIAGEN, Chatsworth, California, USA. Centricon columns for protein purification were obtained from Amicon, MA, USA. All reaction volumes were brought up to their final volumes with autoclaved, distilled water. Primers used for polymerase chain reaction (PCR) amplification and mutagenesis, and for DNA sequencing were obtained either from Dr. Gabriel Alvarado (Synthaid Biotechnologies, Nepean, Ontario) or from Health Canada's Laboratory Centre for Disease Control (L.C.D.C.) DNA Core facility at Tunney's Pasture, Ottawa, Canada. DNA to be sequenced was sent to the Ottawa Regional Cancer Center Sequencing Facility for analysis using the dideoxy-chain termination method with an automated DNA sequencer.

Previously described standard procedures were used for the transformation of competent *E. coli* cells, selection for uptake of plasmid DNA vectors on antibiotic-containing

LB plates, and growth of transformed colonies on either LB/ampicillin and LB/kanamycin media (Sambrook et al. 1989). Procedures for the preparation of YEP medium, preparation of competent *Agrobacterium tumefaciens* cells, and for the transformation of competent *A. tumefaciens* cells are described in this chapter under the heading "Tobacco leaf disk transformation". Standard procedures were used for the selection and growth of transformed *A. tumefaciens* colonies on YEP/kanamycin plates and YEP/kanamycin medium respectively (Cheng et al. 1998). Miniprep extractions and purification of plasmid DNA were performed using the Standard Protocol supplied by QIAGEN, Chatsworth, CA, USA. DNA digestions, dephosphorylations, and phosphorylations were performed using standard protocols and buffers supplied by the respective suppliers. Separation of digested DNA was accomplished by electrophoresis on 0.8%-1.2% agarose gels at a constant 80 volts DC. Purification of DNA fragments recovered from agarose gels was achieved by using the protocol supplied by BIO/CAN Scientific, Mississauga, ON. These fragments were phosphorylated, dephosphorylated, and/or ligated to other fragments or vectors as required.

All PCRs were performed using a DNA Thermal Cycler (Perkin-Elmer-Cetus). The reaction mixtures included the following: 30 ng of template DNA, 100 pmoles each of the forward and reverse primers, one unit of Vent Polymerase (New England Biolabs, Mississauga, ON), and 0.4 millimoles of each dNTP. The total reaction volume for each PCR was 50 µl and had a final concentration of 1X ThermoPol reaction buffer. Unless otherwise indicated, the PCR cycle consisted of one cycle of 93 °C for 3 min, 55 °C for 30 sec, and 72 °C for 90 sec, then 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 90 sec, and one cycle at 72 °C for 5 min. A negative control PCR reaction was always included where no template DNA was added to the reaction mixture.

Coding sequences (CS) for the truncated HBV and WHV core genes used in this work were provided by Dr. Anton Andonov (Bloodborne Pathogens and Hepatitis Laboratory. Bureau of Microbiology. Health Canada. Winnipeg, Manitoba).

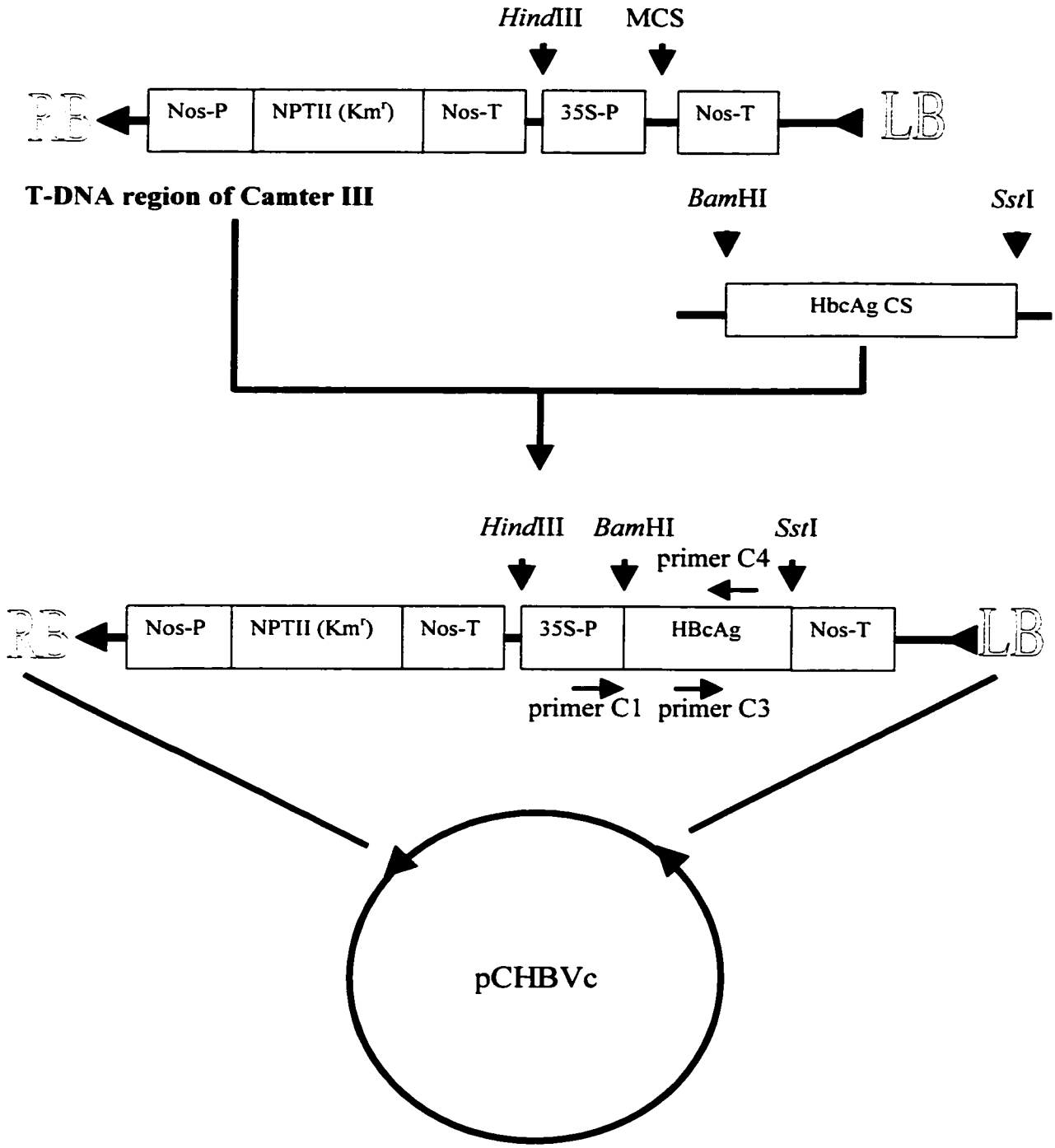
Plasmid Construction

Construction of pCHBV Plant Transformation Vector

In order to express HBV core protein in transgenic tobacco. a binary vector for *Agrobacterium*-mediated transformation was constructed using the plasmid Camter III (kind gift of Dr. Laurian Robert of Agriculture Canada) and the coding sequence for the first 150 amino acids of the HBV core protein gene (Figure 6). Camter III is a Bin19 derivative that incorporates a multicloning site between the CaMV 35S promoter and the 3' flanking region of the nopaline synthase (Nos) gene from *A. tumefaciens*. The multicloning site includes the following restriction endonuclease sites: *Xba*I. *Bam*HI. *Eco*RI. *Kpn*I. and *Sst*I (Figure 6). A coding sequence for the first 150 amino acids of the HBV core protein was provided. as previously mentioned. by Dr. Anton Andonov. This truncated gene was amplified by PCR with a pair of degenerate primers specifying a *Bam*HI restriction site at the 5' end and a *Sst*I restriction site and stop codon at the 3' end: forward primer 5'-dCTTGGGTGGATCCTGGGC**ATG**GAC (*Bam*HI site underlined. start codon in bold type and underlined): reverse primer 5'-dGACCGAGCTCT**TCA**TCTAACAAC (*Sst*I site underlined. stop codon in bold type and underlined). The plasmid Camter III and the HBV core gene PCR product were digested with *Bam*HI and *Sst*I and ligated together as described to create plasmid pCHBVc.

Figure 6: Schematic representation of the construction of plant transformation vector pCHBVc. MCS contains sites: *Xba*I, *Bam*HI, *Eco*RI, *Kpn*I, *Sst*I. Horizontal arrows show the location of sequencing primers C1, C3 and C4 designed to confirm the sequence of the HBV core protein gene coding sequence insert.

Figure 6: Schematic representation of the construction of plant transformation vector pCHBVc.



The sequence of pCHBVc was verified using primers C1 and C3 and an automated DNA sequencer. Primer C1: 5'-dTCGCAAGACCCTTCCTCTATA was designed from DNA sequence at the 3' end of the CaMV 35S promoter sequence immediately upstream of the HBV insert. Primer C3: 5'-dTTGGAAGATCCAGCATCCAG was designed from sequence data generated using primer C1 with the goal of verifying the 3' region of the HBV insert (Figure 6).

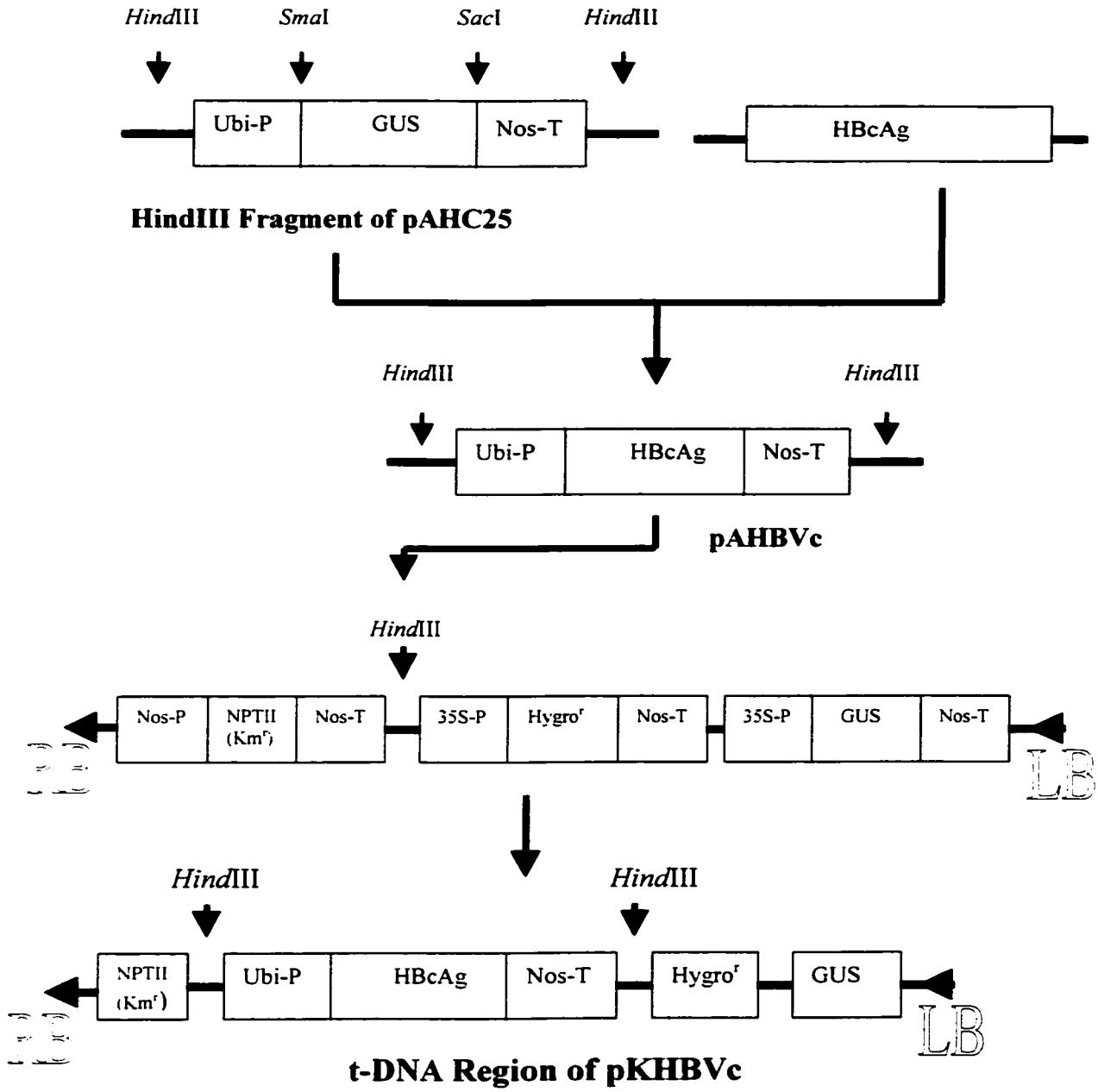
Construction of pAHBVc and pKHBVc Plant Transformation Vectors

In order to express HBV core protein in monocot plants like rice, binary vector pKHBVc for Agrobacterium-mediated transformation was constructed (Figure 7). The visible marker gene beta-glucuronidase (*Uid*) in the plasmid pAHC25 was removed by restriction digestion with endonucleases *Sma*I and *Sac*I and replaced by ligation of the HBV core protein coding sequence in the place of the *Uid* gene to create plasmid pAHBVc. pAHBVc was digested with *Hind*III to release the HBVc expression cassette for ligation into the *Hind*III-digested T-DNA region of plasmid pKHG4 to create plasmid pKHBVc. This placed the HBV core protein gene under control of the rice ubiquitin promoter and upstream of the 3' flanking region of the nopaline synthase gene. Also included in the T-DNA region of pKHBVc are the genes for kanamycin resistance, Hygromycin resistance, and *Uid* (commonly called GUS) for selection and visual identification of transformed plant cells. The sequence of pAHBVc was verified using primer C3 described above and primer C4.

Primer C4: 5'-dCTGGATGCTGGATCTTCCAA was designed from sequence data generated using primer C3 to sequence toward the 5' end of the insert (Figure 6). Plasmid pKHBVc has not been thoroughly tested in plants, however *Agrobacterium*-mediated

transformation of rice callus using this plasmid yielded kanamycin resistant callus that tested positive for expression of the *Uid* gene (results not shown).

Figure 7: Construction of pAHBVc and pKHBVc plant transformation vectors.



Construction of Plasmid pKKWHVc150-*XbaI/XhoI*

The coding sequence for the first 150 amino acid residues of the woodchuck hepatitis virus core (WHVc) gene was previously amplified by PCR using a WHV-cDNA clone as a template, a forward primer (5'-dGCCATGGACATAGATCCCTATA; *Nco*I site underlined, start codon in bold type and underlined), and a reverse primer (5-dAAGCTTCTGCAGCTCCTCT**TCA**CCT; *Pst*I site underlined, stop codon in bold type and underlined). The PCR product was digested with *Nco*I and *Pst*I and cloned into *Nco*I/*Pst*I-digested plasmid prokaryotic expression vector pKK233-2 (Clontech) to create plasmid pKKWHVc150 (Figures 3b, 8). pKK233-3 contains the IPTG (isopropylthio-beta-D-galactoside)-inducible prokaryotic *trc* promoter for high-level inducible expression in *E. coli*. The sequence of the WHVc insert was modified as follows by PCR mutagenesis to include a novel *Xba*I site corresponding to amino acid residue 78 and a novel *Xho*I site corresponding to amino acid residue 140. These sites were picked to allow insertion of sequences coding for linear epitopes from a variety of pathogens.

Primers were designed to amplify a 222 bp fragment, beginning 220 bp from the start codon, which would incorporate the natural *Sac*I site and the novel *Xba*I site at its 5' end and the novel *Xho*I site at its 3' end. The forward primer was W*SAC*F: 5'-dTGGATGAGCTCTAACATAACT**TCTAGA**CAAGTAAGA (*Sac*I site underlined, *Xba*I site in bold print), and the reverse primer was W*XHO*R: 5'-dCGGAAGAGTCTCGAGAATGGGTGCATT (*Xho*I site underlined).

Figure 8: (A) Nucleotide sequence for the truncated WHV core gene fragment cloned into vector pKKWHVc150. Upper case letters indicate the inserted sequence from the start codon to the stop codon. Start codon ATG and stop codon TGA are highlighted in bold type. Lower case letters indicate nucleotides between the coding sequence and the *Nco*I and *Pst*I restriction sites used in the cloning steps. Restriction sites are underlined. Outlined text indicates the sites targeted for PCR mutagenesis for generation of unique restriction sites (Figure 4). (B) Amino acid sequence of the truncated WHV core protein translated from the sequence in (A). The sequence includes the first 150 amino acid residues of the WHV core protein gene.

Figure 8: (A) Nucleotide sequence for the truncated WHV core gene fragment cloned into vector pKKWHVc150. (B) Amino acid sequence of the truncated WHV core protein translated from the sequence in (A).

A

*Nco*I

ccATGGACATAGATCCTTATAAAGAATTTGGTTCATCTTATCAGTTGTTGAATTTTCTTCC
TTTGGACTTCTTTCCTGATCTTAATGCTTTGGTGGACACTGCTACTGCCTTGTATGAAGAA
GAACTAACAGGTAGGGAACATTGCTCTCCGCACCATACAGCTATTAGACAAGCTTTAGTAT
GCTGGGATGAATTAACTAAATTGATAGCTTGGATGAGCTCTAACATAACTTCTGAACAAGT
AAGAACAATCATTGTAAATCATGTCAATGATACCTGGGGACTTAAGGTGAGACAAAGTTTA
TGGTTTCATTTGTCATGTCTCACTTTCGGACAACATACAGTTCAAGAATTTTTAGTAAGTT
TTGGAGTATGGATCAGGACTCCAGCTCCATATAGACCTCCTAATGCACCCATTCTCTCGAC
TCTTCCGGAACATACAGTCATTAGGTGAagaggagctgcag

*Pst*I

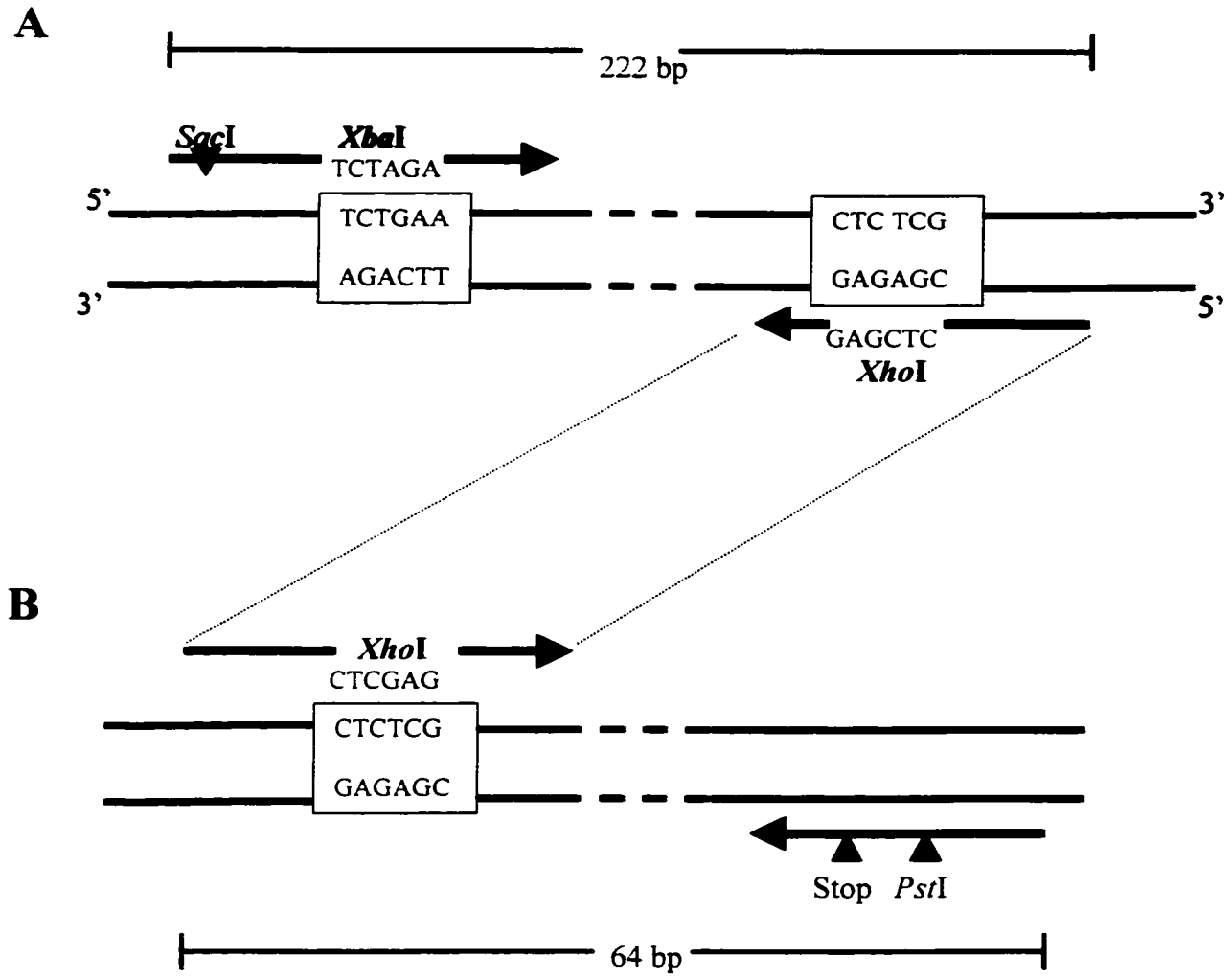
B

MDIDPYKEFGSSYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHT
AIRQALVCWDELTKLIAWMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLS
CLTFGQHTVQEFLVSFGVWIRTPAPYRPPNAPILSTLPEHTVIR

Primers were also designed to amplify a second 64 bp fragment, overlapping the 3' end of the first fragment, which would incorporate the novel *Xho*I site at its 5' end and the natural *Pst*I site at its 3' end. The forward primer was W*XHO*F: 5'-dCACCCATT**CTCGAG**ACTCTTCCGGAAC (*Xho*I site in bold print) and the reverse primer was W*PST*R: 5'-dAAGCTTGGCTGCAGCTCCTCTTCACCT (*Pst*I site underlined) (Figure 9). The *Sac*I-*Xho*I and *Xho*I-*Pst*I fragments were then subcloned into plasmid pCITE-2a(+) (Novagen) as follows. Plasmid pCITE-2a(+) was digested with *Sac*I and *Xho*I and ligated with the 222 bp PCR product from the above reaction digested with *Sac*I/*Xho*I. The successful ligation product was then digested with *Xho*I/*Pst*I and ligated with the 64 bp PCR product from the above reaction digested with *Xho*I/*Pst*I. The second ligation product was digested with *Sac*I/*Pst*I and the 286 bp *Sac*I/*Pst*I fragment was isolated by agarose gel electrophoresis. pKKWHVc150 was digested with *Sac*I/*Pst*I and separated by agarose gel electrophoresis to remove the original *Sac*I/*Pst*I fragment. Finally, the modified *Sac*I/*Pst*I fragment was ligated with the *Sac*I/*Pst*I-digested pKKWHVc150. The final product was named plasmid pKKWHVc150-*Xba*I/*Xho*I and its sequence was analyzed by manual sequencing using the dideoxy-chain termination method.

Figure 9: PCR Mutagenesis of pKKWHVc150. (A) A 222 bp PCR fragment of plasmid pKKWHVc150 was amplified which contained a native *Sac*I site, a novel *Xba*I site and a novel *Xho*I site. The novel sites corresponded to amino acid residues 78 and 140 in the expressed protein. respectively. Forward and reverse primers are described in the text. (B) A second 64 bp PCR fragment of plasmid pKKWHVc150 overlapping the 3' end of the fragment in (A) was amplified. This fragment contained the same novel *Xho*I site as in (A), but also a native *Pst*I site at its 3' end. These two fragments were cloned into *Sac*I/*Pst*I-cut pKKWHVc150 by a multistep procedure described in the text. Bold text indicates novel restriction sites. Heavy dashed lines indicate the drawing is not to scale. Fine dashed lines indicate the area of overlap of the two fragments. Boxes indicate the sites targeted for mutation.

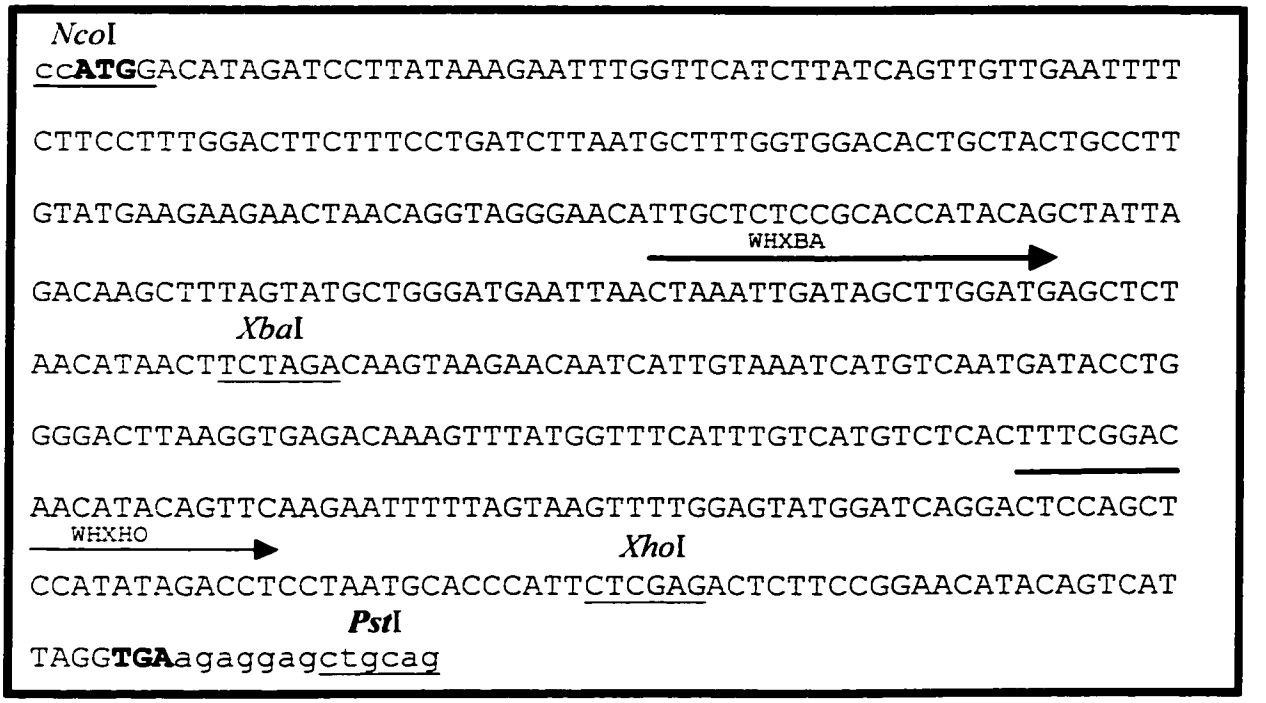
Figure 9: PCR Mutagenesis of pKKWHVc150.



Four primers were used in the sequencing of pKKWHVc150-*Xba*I/*Xho*I. Two primers were designed from regions of the plasmid vector pKK233-2 immediately flanking the inserted WHC sequence – primer KKF: 5'-dGAGCGGATAACAATTTCACAC, and primer KKR: 5'-dAATCTTCTCTCATCCGCCAA. Furthermore, two primers were designed from DNA sequence immediately 5' to the new *Xba*I and *Xho*I sites – WHXBA: 5'-dCTAAATTGATAGCTTGGATG, and WHXHO: 5'-dCTCCAGCTCCATATAGACCT (Figure 10). Primers WHXBA and WHXHO were designed to permit the sequence analysis of DNA inserts at the new *Xba*I and *Xho*I sites, respectively (Figure 10).

Figure 10: Nucleotide sequence for the modified vector pKKWHVc150: novel restriction sites *Xba*I and *Xho*I. Upper case letters indicate the truncated WHV core protein gene from the start codon to the stop codon. Start codon ATG and stop codon TGA are highlighted in bold type. Lower case letters indicate nucleotides between the coding sequence and the *Nco*I and *Pst*I restriction sites used in the cloning steps. Restriction sites are underlined. Arrows indicate location of sequencing primers WHXBA and WHXHO. Outlined text indicates novel restriction sites generated by PCR mutagenesis (Figures 4, 9).

Figure 10: Nucleotide sequence for the modified vector pKKWHVc150: novel restriction sites *Xba*I and *Xho*I.



Insertion of truncated HCV core protein coding sequences into pKKWHVc-150*Xba*I/*Xho*I

Construction of plasmids pKKWHVc150-2.3 and pKKWHVc150-8.8

To prepare pKKWHVc150*Xba*I/*Xho*I constructs carrying inserts of truncated HCV (Hepatitis C Virus) core protein. degenerate PCR primers were designed to amplify two overlapping sequences from the HCV core protein gene while incorporating either an *Xba*I site or an *Xho*I site at each end.

The first set of primers amplified a 136 bp fragment encoding 37 amino acid residues (position 5-41) of the HCV core protein between two *Xba*I sites. The forward primer was: 5`-dTGAGCTCTAGACCTAAACCT (*Xba*I site underlined), and the reverse primer was: 5`-dCGCAGGGGCTCTAGATTGGGTGTG (*Xba*I site underlined). One amino acid residue not part of the primary HCV coding sequence was added at each end of the fragment in the creation of the restriction sites (Figure 11). This fragment was called HCV-2.3.

The second set of primers amplified a 147 bp fragment encoding 39 amino acid residues (position 15-53) of the HCV core protein between two *Xho*I sites. The forward primer was: 5`-dCAAACCAAACTCGAGAACACTAACCGT (*Xho*I site underlined), and the reverse primer was: 5`-dCTGGGACCGCTCGAGAGTCTTCCT (*Xho*I site underlined). One amino acid residue not part of the primary HCV coding sequence was added at each end of the fragment in the creation of the restriction sites (Figure 12). This fragment was called HCV-8.8.

Figure 11: (A) Truncated HCV core protein coding sequence inserted into XbaI site of pKKWHVc150XX. Upper case letters indicate nucleotides that were incorporated into XbaI-digested pKKWHVc150XX to create pKKWHVc150-2.3. Underlined text indicates XbaI restriction sites generated by degenerate PCR primers. Bold text indicates the PCR primers used to amplify cloned HCV cDNA. (B) Translation of HCV core gene nucleotide sequence in pKKWHVc150-2.3. The first and last amino acids (underlined) were modified for cloning into the XbaI site of pKKWHVc150XX and do not represent the primary sequence of the HCV core fragment. The amino acids were changed from K and P, respectively.

Figure 11: (A) Truncated HCV core protein coding sequence inserted into XbaI site of pKKWHVc150XX. (B) Translation of HCV core gene nucleotide sequence in pKKWHVc150-2.3.

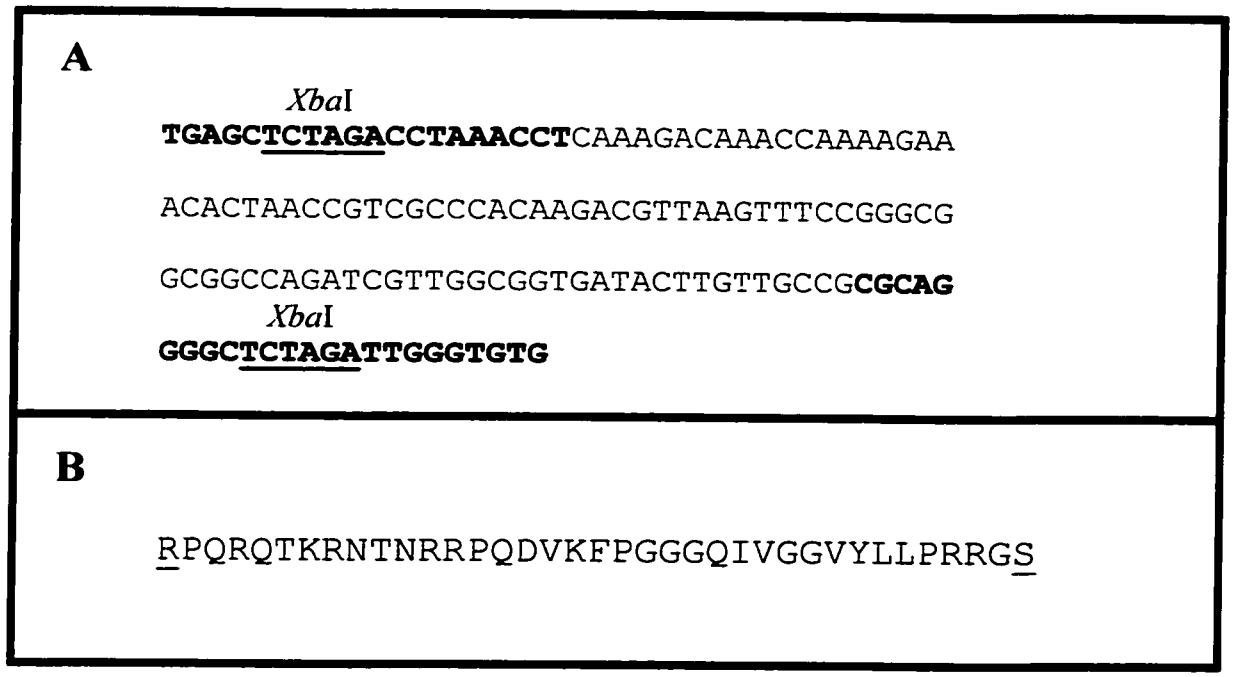
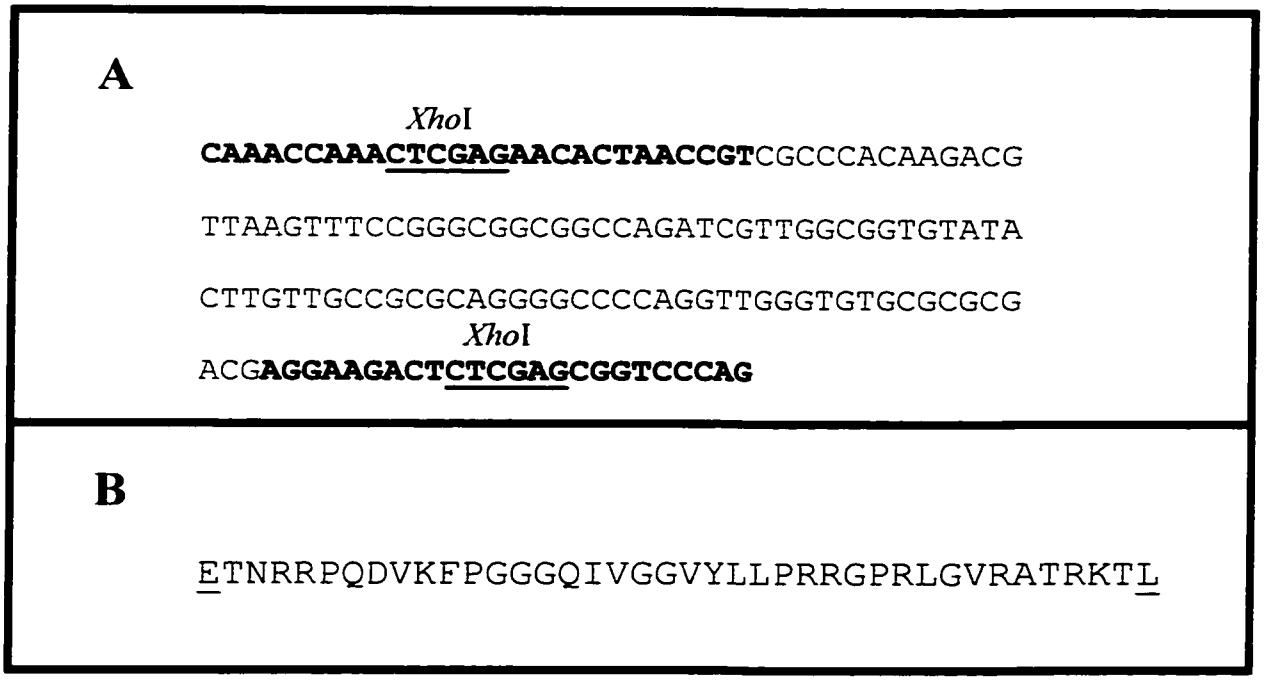


Figure 12: (A) Truncated HCV core protein coding sequence inserted into *Xho*I site of pKKWHVc150XX. Upper case letters indicate nucleotides that were incorporated into *Xho*I-digested pKKWHVc150XX to create pKKWHVc150-8.8. Underlined text indicates *Xho*I restriction sites generated by degenerate PCR primers. Bold text indicates the PCR primers used to amplify cloned HCV cDNA. (B) Translation of HCV core gene nucleotide sequence in pKKWHVc150-8.8. The first and last amino acids (underlined) were modified for cloning into the *Xho*I site of pKKWHVc150XX and do not represent the primary sequence of the HCV core fragment. The amino acids were changed from N and S, respectively.

Figure 12: (A) Truncated HCV core protein coding sequence inserted into *Xho*I site of pKKWHVc150XX. (B) Translation of HCV core gene nucleotide sequence in pKKWHVc150-8.8.



Plasmid pKKWHVc150-*Xba*I/*Xho*I was digested with *Xba*I and ligated with *Xba*I-digested HCV2.3 to create plasmid pKKWHVc150-2.3. The new plasmid was analyzed by DNA sequencing using primer WHXBA (5'-dCTAAATTGATAGCTTGGATG, described above under the heading "Construction of Plasmid pKKWHVc150-*Xba*I/*Xho*I"; see Figure 10) to verify that the HCV sequence was correctly inserted and in-frame.

Plasmid pKKWHVc150-*Xba*I/*Xho*I was digested with *Xho*I and ligated with *Xho*I-digested HCV8.8 to create plasmid pKKWHVc150-8.8. The new plasmid was analyzed by DNA sequencing using primer WHXHO (5'-dCTCCAGCTCCATATAGACCT, described above under the heading "Construction of Plasmid pKKWHVc150-*Xba*I/*Xho*I"; see Figure 10) to verify that the HCV sequence was correctly inserted and in-frame.

Tobacco Leaf Disk Transformation

This method was adapted from one developed by Laurian Robert and Anne Hermans (Eastern Cereals and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada).

Preparation of tobacco plants

Tobacco seeds (variety Wisconsin 38) were planted directly in soil (1:3:1:1 of soil:peat:sand:Perlite) and germinated. They were grown in a plant growth cabinet at 25°C for 16-hour days, at 20°C for 8-hour nights. The relative humidity was maintained at 70%. Lighting was by fluorescent and incandescent bulbs. Plants were allowed to grow for at least eight weeks to obtain sufficient leaf tissue for transformation.

Direct transformation of *Agrobacterium tumefaciens*

Obtaining competent cells of *Agrobacterium tumefaciens*

A single colony of *Agrobacterium* was grown overnight at 28°C in 5 ml of YEP media (YEP media; per liter: 10 g yeast extract, 10 g peptone, 5 g NaCl, pH 7.0, 15 g agar for solid plates). Two ml of the overnight culture was added to 50 ml of YEP media in a 250 ml flask and incubated at 28°C with shaking at 250 rpm until an OD_{600} of 0.5-1.0 was reached. The culture was chilled on ice for 10 min and centrifuged at 5000 x g for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1 ml of ice-cold 20 mM $CaCl_2$. The culture was dispensed in 100 µl aliquots into pre-chilled 1.5 ml microcentrifuge tubes. Tubes not used immediately were kept frozen at -70°C for future use.

Transformation of *Agrobacterium tumefaciens*

Approximately 1 µg of uncut plasmid DNA in less than 5 µl of water was added to an aliquot of competent cells and incubated on ice for 30 minutes. The cells were subsequently frozen in liquid nitrogen until solid, and thawed at 37°C in a water bath for 5 minutes. YEP media (1 ml) was added to the cells and they were incubated for 2 hours at 28°C with gently shaking at approximately 200 rpm. The cells were centrifuged in a microfuge for 30 sec at 3000 rpm and all but 100 µl of the supernatant was discarded. The pellet was resuspended in the remaining supernatant and spread on a YEP plate containing 50 mg/l kanamycin. The plates were incubated at 28°C for 2 days to allow transformed

colonies to become visible. The colonies were verified using the alkaline lysis miniprep method that follows.

Alkaline lysis miniprep

This protocol was derived from the Pharmacia protocol supplied with their *Agrobacterium* cells. From a single colony, a 3 ml culture was grown overnight in YEP media with 50 mg/l kanamycin. 1.5 ml of the culture was transferred to each of two microcentrifuge tubes. The tubes were centrifuged at 3000 rpm for 2 minutes at room temperature to collect the cells. The pellet was resuspended in 200 µl of filter-sterilized STE buffer with 20 mg/ml of lysozyme (STE: 25 mM Tris-HCL, pH 8; 10 mM EDTA, pH 8; 15% sucrose). Next, 200 µl of NaOH/SDS solution was added and the tubes inverted 15 times to mix (NaOH/SDS: 0.2 M NaOH, 1% SDS).

Then, 100 µl of 3M sodium acetate pH 5 was added and the tube inverted twice to mix. The tube was incubated on ice for 5 minutes before being centrifuged for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and extracted with 350 µl of phenol:chloroform. The aqueous phase was transferred to a fresh tube and the plasmid DNA precipitated overnight at -20°C with 800 µl of 100% ethanol. The plasmid DNA was recovered by centrifugation for 15 minutes at 4°C. The pellet was washed with 70% ethanol and allowed to air dry for approximately 30 minutes. The pellet was resuspended in 25 µl of standard Tris-EDTA (TE) buffer. To confirm the identity of the isolated pCHBV plasmid DNA, several restriction digests were performed as follows. Five µl of the isolated plasmid DNA was digested with *Hind*III/*Sst*I to release a 1250 bp fragment composed of the CaMV S35 promoter (800 bp) and HBVc insert (450 bp) sequences. Five µl of the same plasmid DNA

was digested with *Bam*HI/*Sst*I to release the 450 bp HBVc insert fragment. The reaction mixtures were separated by agarose gel electrophoresis to confirm that the expected fragments were present.

Tobacco leaf disk transformation

Preparation of bacteria

Medium (10 ml of Luria-Bertani medium (LB) with 50 μg/ml of kanamycin) was inoculated with a single colony selected from a plate (LB, 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, water to 1 L, pH to 7 with 5M NaOH, autoclaved). The culture was incubated at 28°C for 2 days with shaking. Media controls were included during the 2-day growth period to ensure that the media were not contaminated. 100 μl of the 2-day culture was subcultured into 10 ml of fresh LB with 50 μg/ml of kanamycin and this culture was incubated overnight at 28°C. The subculture ensured that the bacteria were fresh and healthy for the co-culture step.

Preparation of co-culture and selection media

Co-culture medium was prepared by adding to 400 ml of ddH_20: 2.287g Murashige Minimal Organics Medium (MMO) powder (Gibco BRL), 5.0 ml BA (6-benzylaminopurine, a cytokinin) stock solution (0.1 mg/ml) (Sigma), 0.5 ml NAA (alpha-napthaleneacetic acid, an auxin) stock solution (0.1 mg/ml) (Sigma), 15.0 g sucrose. The pH was checked to ensure pH 5.7-5.8 and the volume was brought to 500 ml with ddH_20. Agar (4 g) was added to produce a 0.8% agar medium (Phytoagar, Gibco BRL). The medium was

autoclaved for 23 minutes on the liquid cycle. Once cooled to approximately 60°C, the medium was poured into 100 mm disposable Petri dishes.

Selection media was prepared as for co-culture but with the addition of 0.5 ml of 300 mg/ml ticarcillin (SmithKline Beecham: 300 μg/ml final concentration) and 5 ml of 25 mg/ml kanamycin (250 μg/ml final concentration).

To prepare 0.1 mg/ml NAA, 100 mg of NAA was dissolved in 3 ml of 1 M NaOH then water was slowly added to 1 L with continuous stirring. This was stored at 4°C in an autoclaved bottle.

To prepare 0.1 mg/ml BA, 100 mg of BA was dissolved in 5 ml of 1M NaOH then water was slowly added for a total volume of 1 L with continuous stirring. The solution was stored at 4°C in an autoclaved bottle.

Co-culture

Preparation of bacteria for co-culture

The pCHBV-transformed culture of *Agrobacterium* was transferred into each of two sterile microfuge tubes. The cells in one tube were used to determine the optical density of the culture and the cells in the other were used for transformation. The OD_{600} of the culture was determined and the cells were centrifuged for 5 minutes at 13 000 rpm at room temperature and the supernatant removed. The cell pellet was resuspended in 1 ml of MMO solution (500 ml MMO: 500 ml water, 2.287 g MMO powder with no kanamycin). A dilution of the culture in MMO solution was made to provide a 15 ml solution at OD_{600}=0.1.

Preparation of tobacco leaf tissue

In a laminar flow hood, a series of five 1L beakers was prepared with the following solutions:

-Beaker 1: 70% EtOH (280 ml of 100% EtOH + 220 ml sterile water)

-Beaker 2: Javex:water in 1:3 ratio

-Beakers 3, 4 and 5: sterile water.

Young, healthy leaves were collected from greenhouse-grown tobacco plants (var. W78). Each leaf was placed in the 70% EtOH solution using flamed forceps and swirled gently for 20 seconds. The leaves were transferred to the jar containing the Javex solution and sterilized for a maximum of 5 minutes or until the cut edge of the leaf blade or petiole became white, whichever came first. The leaves were transferred aseptically to the first beaker of water for about 2 minutes with gentle occasional stirring using flamed forceps. The leaves were transferred to the second and third beakers of water in the same way. The leaves were allowed to float in the last jar in preparation for the co-culture step.

Preparation of leaf disks and co-culture

Approximately 5 ml of the diluted, resuspended bacteria was poured into the lid of a large sterile Petri dish. A leaf was transferred from the last beaker of water into the dish containing the bacterial suspension and sliced into 5 mm x 5 mm squares (leaf disks) with a sterile scalpel. The squares were cut to avoid the midvein but to include a minor vein running from one corner to the opposite corner of the leaf explant – this helped to keep the square from curling away from the regeneration medium. The leaf disks were allowed to float in the bacterial suspension while the cutting process continued. When the whole leaf

had been sectioned, the disks were transferred individually with flamed forceps to the surface of the co-culture dishes such that the upper leaf surface was in contact with the medium. Ten disks were placed in each dish. The plates were wrapped with a double layer of Parafilm and placed in a tissue culture room for 3 days. The tissue culture room conditions were as follows: 25°C, 16 hour day, 8 hour night, with fluorescent and incandescent lighting.

Transfer of co-cultivated disks to selective medium

Co-cultivated disks were transferred to dishes of selective medium containing 300 μg/ml of ticarcillin and 250 μg/ml of kanamycin as described above. Leaf disks that had not been exposed to the suspension of *Agrobacterium* were used as negative controls to verify their sensitivity to kanamycin. The dishes were sealed with two layers of Parafilm and placed in the tissue culture room for two weeks. At the end of two weeks, transformed callus began to appear. The leaf disks were quartered, ensuring that individual calli were not cut into separate pieces, and transferred to fresh dishes of selective medium. The dishes were returned to the culture room for an additional two weeks to allow transformed shoots to appear.

Rooting

Rooting medium was prepared by adding to 400 ml of ddH_20: 11.5 g Gamborg's B5 Medium powder (Gibco BRL), 500 μl of 300 mg/ml ticarcillin, and two ml of 25 mg/ml kanamycin to maintain selective pressure. The pH was checked to ensure pH 5.7-5.8 and the volume was brought to 500 ml with ddH_20. Agar (4 g) was added to produce a 0.8%

agar. The medium was autoclaved for 23 minutes on the liquid cycle. Once cooled to approximately 60°C. the medium was poured into 100 mm disposable Petri dishes.

Approximately four to five weeks post-transformation, shoots from the edges of the leaf disks became large enough to be excised for transfer to rooting medium. Shoots with a meristem and small leaves were selected. The stems were cut perpendicularly to their long axis and transferred with a forceps to rooting medium, one shoot per dish. Only one shoot per callus was selected to maximize variability of gene insertion sites. Roots began to appear in approximately two weeks.

Moving plantlets to soil

When the roots approached three to five centimetres in length, 30 plantlets were transferred to soil. 18 cm Kord pots were filled with a planting soil mixture to 2.5 cm from the top and the soil was moistened thoroughly. The plantlets were manually scooped from the rooting medium and placed in a shallow hole in the soil such that the roots were entirely covered. The plantlets were covered with a Magenta GA-7 box (Sigma) to allow for acclimation to the new growth conditions. The pots were placed in a growth chamber for five days. The Magenta boxes were propped-up slightly to allow for greater air circulation to the young plants. Seven days later, the Magenta boxes were removed entirely. When the plants were approximately 30 cm in height, the first leaves were harvested for biochemical testing.

E. coli Expression System

Expression of pKKWHVc Constructs in *E. coli* and Purification of WHVc Protein

Plasmids pKKWHVc150-*XbaI*/*XhoI*, pKKWHVc150-*Xba*I-HCV and pKKWHVc150-*Xho*I-HCV were individually transformed into cultures of *E. coli* strain JM109 (Clontech). For each construct, a five ml starting culture was grown overnight at 37°C in LB-AMP. The five ml culture was added to 500 ml of LB-AMP medium and grown to an OD of 0.8 at 600 nm. The culture was transferred to a 23°C incubator, induced with 1 mM IPTG and allowed to grow overnight. The cells were collected by centrifugation and all excess fluid was removed. All the following steps were performed on ice. The pellet of bacterial cells was quickly frozen, thawed and resuspended in 10 ml of lysis buffer (25 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (HEPES) pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM phenylmethanesulphonyl fluoride (PMSF), 2mM EDTA pH 8.0, 2mM DTT). Lysozyme was added to a concentration of 100 µg/ml. The suspension was incubated for 10 minutes with slow rotation (1.5 rotations per second) and detergent NP-40 was added to a final concentration of 0.1%. The lysate was sonicated 10 times with 40 second intervals and the insoluble debris was pelleted by centrifugation at 13 000 x g for ten minutes. The supernatant was applied to discontinuous sucrose density gradients formed in polycarbonate tubes by layering 2.2 ml each of 60%, 50%, 40%, 30% and 20% sucrose. The tubes were ultracentrifuged at 30 000 rpm for 14 hours in a Beckman SW 40 Ti rotor. Thirteen one ml fractions were collected from the bottom for ELISA and Western Blot analysis.

Molecular Analysis of Transformed Tobacco Plants

PCR Amplification of Plant Genomic DNA to Detect HBV Core Protein Coding Sequence

Genomic DNA was extracted from young leaves of 30 transformed plants using a simple method previously described (Wang *et al*, 1993). Ten mg of leaf tissue was placed in a 1.5 ml tube with 100 µl of 0.5 N NaOH solution. The leaf tissue was ground until no large pieces remained. Five µl of the resulting mixture was immediately transferred to a new tube containing 495 µl of 100 mM Tris pH 8.0 and mixed well. The samples were stored at -20°C if they were not used immediately. One µl of each sample was subjected to PCR amplification using primers designed from highly conserved areas of the HBV core gene to amplify a 250 bp fragment. The PCR products were electrophoresed through agarose. Samples containing the transgene were identified by the presence of an amplified product.

Analysis of RNA from Transformed Tobacco

Northern Blot Analysis

RNA was isolated from tobacco leaf tissue using the RNAEasy kit from Qiagen. Young leaf tissue (80 mg) from each plant was used. The RNA was fractionated on 1% agarose gels (approximately ten µg per lane), transferred to Hybond-N+ membrane and crosslinked. Two sets of RNA were crosslinked: one with Autocross crosslinker from Stratagene and one by UV illumination for 60 seconds in a transilluminator. The probe used was the PCR product from the PCR amplification of genomic DNA described above. The PCR products from samples 20 to 23 were combined, electrophoresed through low melting point agarose and eluted from the gel using the GeneClean kit (Bio101). The DNA yield was quantitated using a GeneQuant II

Spectrophotometer (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and labeled using the BrightStar Psoralen-Biotin Nonisotopic Labeling Kit (Ambion, Austin, Texas, USA). Control DNA supplied with the labeling kit was spotted in one µl aliquots of one ng, 10^{-1} ng, 10^{-2} ng, 10^{-3} ng, 10^{-4} ng and 10^{-5} ng, and crosslinked as for the sample RNA. As a second control, amplified HBV core gene DNA from the same region as the probe was also spotted in aliquots of one ng, 10^{-1} ng, 10^{-2} ng, 10^{-3} ng, 10^{-4} ng and 10^{-5} ng and transferred to the membrane as above. Hybridization of the probe to the membrane, and exposure to film was performed as recommended in the kit. Exposure time was three days.

RT-PCR

RNA was isolated from tobacco leaf tissue using the RNAEasy kit from Qiagen as above. To prepare cDNA, AMV (Avian Myeloblastosis Virus) Reverse Transcriptase (Gibco BRL) was used. A 7.5 µl aliquot of isolated RNA was added to the same volume of 2x AMV RT buffer (Gibco BRL) with dNTPs and one µl of RQ DNAse (Promega) added. The samples were incubated for 15 minutes at 37°C and 15 minutes at 95°C. The PCR was performed with the same primers as for the genomic PCR amplification described above with an expected amplification product of 350 bp in length.

Immunological Detection

Protein Extraction from pCHBV Plants for Immunodetection

Ten grams of young leaf tissue from each plant was frozen in liquid nitrogen and macerated with a mortar and pestle pre-chilled with liquid nitrogen. The leaf tissue was

transferred into 30 ml of cold extraction buffer (0°C, PBS pH 7.2, 50 mM sodium ascorbate, 2mM EDTA, 1mM phenylmethylsulfonylfluoride, 0.2% Triton X-100) in 50 ml tubes, vortexed for 30 seconds, and homogenized in a polytron at medium speed for 30 seconds. The samples were incubated on ice for one hour and vortexed for 30 seconds every 10 minutes during the incubation period. The samples were centrifuged at 3000 rpm for 5 minutes and the supernatant transferred to a fresh tube. Ammonium sulfate salt was slowly dissolved in each sample to fully saturate the solution. The samples were incubated on ice for 30 minutes and centrifuged for 30 minutes at 10 000 g and 0°C. The supernatant was discarded and each pellet dissolved in one ml of PBS pH 7.2. To remove the salt, each sample was dialyzed for 48 hours in 1L PBS pH 7.2 at 4°C. Final volume of each extract was 1.5 ml.

Immunodetection of HBV Core Protein in Extracts of Transgenic Tobacco

ELISA capture assay I

For this assay, 50 µl of 1µg/ml mouse anti-hepatitis B core antigen monoclonal IgM antibody (MAB842, Chemicon International Incorporated, Temecula, California) was coated onto the wells of a 96-well microtiter plate and incubated at 37°C for two hours. The tobacco extracts were added to the anti-HBcAg antibody-coated wells and incubated overnight at 4°C. 50 µl of polyclonal anti-HBcAg antibody labeled with horseradish peroxidase (COREZYME assay kit, Abbott Laboratories, North Chicago, IL) was added to the wells and incubated for 1 hour at 37°C. After a washing step to remove unbound antibodies, the horsesradish peroxidase substrate was added and the coloured product allowed to develop. The resultant optical signal is proportional to the amount of HBcAg present in the tobacco extract.

The negative control for this experiment was a protein extract from a wild type non-transgenic tobacco plant. The positive control was a 10^5 times dilution of a 3 ml crude protein extract from a 500 ml culture of transformed *E. coli* expressing HBV core protein. This was the required amount of HBV core protein to provide 50% inhibition of the signal in the COREZYME assay (see next section). The results were tabulated and analyzed using a statistical software program for the personal computer (SigmaStat for Windows Version 1.0, Jandel Corporation).

ELISA competition assay II

The technique used was a modification of the normal use of a commercially available detection kit for hepatitis B virus core protein. The COREZYME assay kit (Abbott Laboratories, North Chicago, IL) detects serum anti-HBcAg IgM antibodies directed against recombinant HBV core protein. The procedure was modified to assess the specific inhibition by the transgenic tobacco-expressed HBcAg of the interaction of polyclonal anti-HBcAg IgM antibodies with the HBV core recombinant protein coated onto the beads supplied with the kit. Normally, the sample to be tested is mixed with a solution of polyclonal anti-HBcAg antibody labeled with horseradish peroxidase and incubated with the HBcAg-coated beads in a microtiter plate. Any anti-HBcAg antibodies present in the serum sample compete for binding to the recombinant antigen on the beads. After washing, a substrate mixture for the horseradish peroxidase is added to the well. This generates an optical signal (absorbance) that is inversely proportional to the presence of antibodies in the sample examined. For this experiment, 50 µl of the concentrated protein extract described in the previous section was added to 50 µl of a 1:20 dilution of the horseradish peroxidase-

labeled anti-HBcAg polyclonal antibody and incubated with recombinant HBV core protein-coated beads. The rest of the protocol was followed as specified in the kit. Therefore, HBcAg present in the plant extracts competes with the HBcAg antigen coating the beads for binding with the anti-HBcAg antibody. After a washing step and the substrate reaction, the optical signal is inversely proportional to the amount of HBcAg present in the tobacco extract. The negative control for this experiment was a protein extract from a wild type non-transgenic tobacco plant. The positive control was a 10^5 times dilution of a 3 ml crude protein extract from a 500 ml culture of transformed *E. coli* expressing HBV core protein. This was the amount required to provide 50% inhibition of binding of the horseradish peroxidase-labeled anti-HBcAg polyclonal antibody to the beads.

Immunoprecipitation Assay

In microfuge tubes, 50 µl of the tobacco extracts was mixed with 50 µl of a 1:20 dilution of ^{135}I-labeled anti-HBcAg polyclonal antibody and incubated overnight at 4°C. A 1:10 000 dilution of anti-human IgG antibody was added to the mixture to precipitate the immune complexes and incubated for 1 hour at 37°C. The tubes were centrifuged at 13 000 rpm in a benchtop microfuge and the supernatant discarded. The pellet was gently washed and the radioactivity of the pellet measured by liquid scintillation counting. The negative control for this experiment was a protein extract from a wild type non-transgenic tobacco plant. The positive control was a 10^5 times dilution of a 3 ml crude protein extract from a 500 ml culture of transformed *E. coli* expressing HBV core protein. The amount of HBV core protein used was the same as for the COREZYME-based assay.

Competitive ELISA to Recognize HCV Core Epitopes in Chimeric WHVc150 Proteins

Recognition of the HCV core epitopes within WHVc150-2.3 and WHVc150-8.8 proteins expressed in *E. coli* was demonstrated using a competitive ELISA technique. The technique used was a modification of the normal use of a commercially available diagnostic kit for hepatitis C virus. The HCV EIA 2.0 kit (Abbott) detects serum anti-HCV antibodies directed against one recombinant structural protein (HCV core protein: amino acids 1-150) and two recombinant non-structural proteins (HCV proteins NS 3 and NS 4). The procedure was modified to assess the specific inhibition of the interaction of anti-HCV antibodies with the HCV core recombinant protein coated onto the beads supplied with the kit. Normally, the recombinant antigen on the beads captures the anti-HCV antibodies if present in the serum sample. After washing, a monoclonal antibody specific to HCV core protein, labeled with horseradish peroxidase and added to the microplate, competes with the antibodies, if present on the beads. After washing, the enzyme labeled monoclonal antibody present on the microplate acts on the chromogen/substrate mixture generating an optical signal that is inversely proportional to the presence of antibodies in the sample examined. For this experiment, ten µl of four sucrose gradient samples of *E. coli*-produced WHVc-2.3 and WHVc-8.8 protein were added to 10 µl of an anti-HCV positive serum from a patient with chronic hepatitis (supplied by Anton Andonov, Bloodborne Pathogens and Hepatitis Laboratory, Bureau of Microbiology, Health Canada, Winnipeg, Manitoba), diluted to 400 µl with HCV EIA 2.0 (Abbott) kit diluent and incubated with HCV recombinant protein-coated beads. The rest of the protocol was followed as specified in the kit. Therefore, any HCV antigens present in the *E.coli* extracts would react with the serum anti-HCV antibodies and prevent the antibodies from binding to the coated beads.

This generates an optical signal that is proportional to the amount of HCV antigen present in the sample of *E. coli*-produced protein. To eliminate cross reactivity with antibodies to the other two nonstructural proteins, an anti-HCV serum was selected which was strongly positive only for the HCV core protein. This was determined using the commercially available RIBA 3.0 kit (Ortho Clinical Diagnostics, USA). The RIBA 3.0 kit is designed to visualize anti-HCV antibody patterns in serum of patients with chronic HCV infection. Therefore, variability in the change in absorbance observed in the assays can be attributed largely to the presence or absence of the inserted HCV core protein in the chimeric WHVc150XX-based proteins.

SDS PAGE and Western Blot Analysis of WHVc Proteins Expressed in *E. coli*

Recombinant WHVc150 carrying an insertion of HCV core protein amino acids 6-44 at amino acid 78 was purified in a sucrose gradient (2.2 ml each of 60%, 50%, 40%, 30% and 20% sucrose) at 30 000 rpm for 14 hours in a Beckman SW 40 Ti rotor. Thirteen one ml fractions were collected from the bottom and 10 µl of each fraction was run on a denaturing SDS-PAGE gel, and stained with Coomassie Blue or transferred onto a nitrocellulose membrane for Western blot (Laemmli, 1970; Towbin *et al*, 1979). The nitrocellulose membrane was blocked with 5% non-fat dry milk powder (DM)/0.5% BSA at 4°C overnight and probed with a rabbit polyclonal anti-WHV core protein antibody (gift from B. Tennant, Cornell University) followed by a goat anti-rabbit alkaline phosphatase-labeled secondary antibody.

Dot-Blot Assay of Sucrose Gradient Purified WHVc150-2.3 and WHVc150-8.8 Produced in *E. coli*

To determine whether the results obtained in the Western Blot analysis under denaturing conditions would differ from an immunoassay performed under non-denaturing conditions, a dot-blot assay was performed as follows. Ten µl of each fraction (13 total fractions) from the sucrose gradient protein purification method described above were blotted onto a nitrocellulose membrane and immunostained with rabbit polyclonal anti-WHV core antibody.

Electron Microscopy

Electron Microscopy of Purified WHVc Proteins

Gradient fractions number two and three from WHVc150, WHVc150-2.3 and WHVc150-8.8 were selected for electron microscopy based on the expression level of the two chimeric proteins as seen by Western blot (results described in Chapter 3). Electron microscopy was performed by the electron microscopy laboratory at Health Canada's Canadian Science Centre for Human and Animal Health in Winnipeg, Manitoba. Micrographs were recorded in digital form, each of 1024 x 1024 pixels.

Chapter 3: Results

Part 1: Expression of HBV core protein in transgenic tobacco

DNA Sequencing of pCHBV and pKHBV

Figure 13 (A) shows the verified sequence of the HBV core protein gene inserted between the CaMV 35S promoter and the 3' flanking region of the nopaline synthase (Nos) gene of plasmid Camter III to create plasmid pCHBV. The same sequence was verified in plasmid pKHBV, where it was inserted between the rice ubiquitin promoter and the 3' flanking region of the nopaline synthase gene in plasmid pKHG4. It was confirmed that the expected start and stop codons were present. There were no altered nucleotides or frameshift mutations noted.

The expected product of the translation of the truncated HBV core protein gene is shown in Figure 13 (B). This sequence includes the first 150 amino acids of the HBV core protein. Deletion experiments have established that amino acid residues 145-183 are not required for particle assembly, although deletion of these residues destroys the ability of the particle to bind nucleic acids. Furthermore, deletion of these amino acids does not significantly affect the yield, size or morphology of HBV core particles expressed in *E. coli* (Gallina *et al*, 1989, Inada *et al*, 1989). Given the success of expression of similar truncated HBV core protein genes in other systems, it was hoped that this gene would be expressed to high levels in cells of transgenic tobacco.

Figure 13: (A) Nucleotide sequence for the truncated HBV core gene fragment cloned into vectors pCHBV and pAHBV. Upper case letters indicate the inserted sequence from the start codon to the stop codon. Start codon ATG and stop codon TGA are highlighted in bold type. Lower case letters indicate nucleotides between the coding sequence and the *BamH*I and *Sst*I restriction sites used in the cloning steps. Restriction sites are underlined. (B) Amino acid sequence of the truncated HBV core protein translated from the sequence in (A). The sequence includes the first 150 amino acid residues of the HBV core protein gene.

Figure 13: (A) Nucleotide sequence for the truncated HBV core gene fragment cloned into vectors pCHBV and pAHBV. (B) Amino acid sequence of the truncated HBV core protein translated from the sequence in (A).

A

*Bam*HI

ggatcctgggc**ATG**GACATTGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTC
GTTTTTGCCTTCTGACTTCTTTCCTTCCGTACGAGATCTTCTAGATACCGCCGCAGCTCTG
TATCGGGATGCCTTAGAGTCTCCTGAGCATTGTTCACCTCACCATACTGCACTCAGGCAAG
CAATTCTTTGCTGGGGAGACTTAATGACTCTAGCTACCTGGGTGGGTACTAATTTAGAAGA
TCCAGCATCTAGGGACCTAGTAGTCAGTTATGTCAACACTAATGTGGGCCTAAAGTTCAGA
CAATTATTGTGGTTTCACATTTCTTGTCTCACTTTTGGAAGAGAAACGGTTCTAGAGTATT
TGGTGTCTTTTGGAGTGTGGATTCGCACTCCTCCAGCTTATAGACCACCAAATGCCCCTAT
CCTATCAACGCTTCCGGAGACTACTGTTGTTAGA**TGA**agagctc

*Sst*I

B

MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTAAALYRDALESPEHCSPHHT
ALRQAILCWGDLMTLATWVGTNLEDPASRDLVVSYVNTNVGLKFRQLLWFHIS
CLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVR

PCR amplification of plant genomic DNA to detect HBV core protein coding sequence

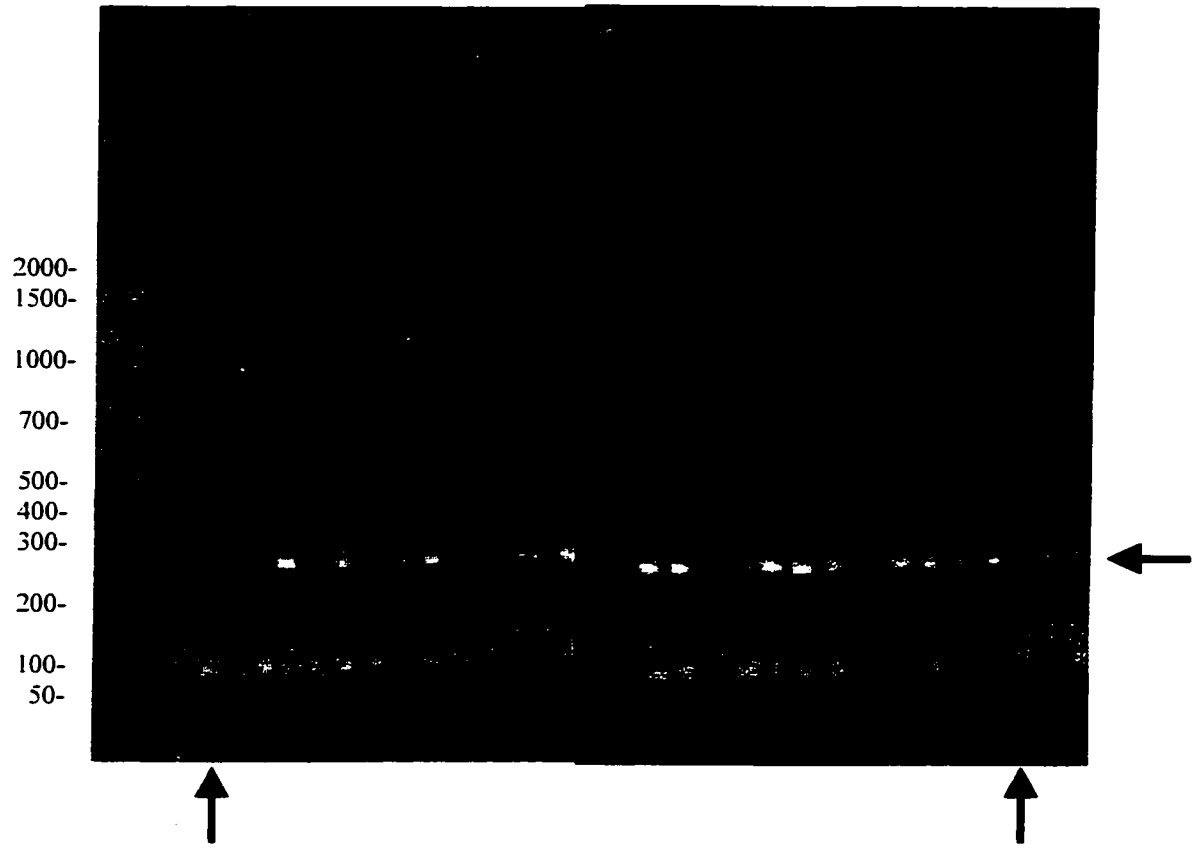
Genomic DNA was extracted from 30 transformed plants for PCR analysis as described. PCR primers were designed to amplify a 250 bp fragment of the HBV core protein gene. Figure 14 shows the results of agarose gel electrophoresis of the PCR products. An amplified product of the expected size was observed in lanes 1, 3-28, and 30. No amplified product was observed in lanes 2 and 29. Therefore, 28 of 30 (93%) of the plants tested positive for integration of the HBV core protein gene within the plant genome. Plants 2 and 29 were eliminated from further testing.

Northern Blot Analysis of HBVc-transformed tobacco RNA extracts

RNA was extracted from 13 pCHBV-transformed tobacco plants, separated by agarose gel electrophoresis, transferred to a Hybond membrane, and probed with the labeled PCR product of the genomic PCR amplification. The extracted RNA was separated by agarose gel electrophoresis and approximately 10 μg of total RNA was applied to each lane. After hybridization with the probe, the membrane was exposed to x-ray film and the film developed. No signal from the tobacco RNA extracts was detected on the film, despite multiple trials of the experiment (data not shown). The sensitivity of the assay to the positive control DNA was 10^{-4} ng for the kit-supplied DNA and 10^{-3} ng for the amplified HBV DNA. Therefore, the yield of HBV core protein mRNA was certainly less than 10^{-3} ng.

Figure 14: Genomic PCR to detect HBV core gene in transgenic tobacco plants. Plant extracts are identified by number. M = DNA strand length marker: 50-2000 bp Molecular Ruler from Bio Rad: band sizes marker in bp. Vertical arrows indicate lanes 2 and 28 where no amplification product was seen. Horizontal arrow indicates amplification product of approximately 250 bp observed in lanes 1, 3-27, and 29-30.

Figure 14: Genomic PCR to detect HBV core gene in transgenic tobacco plants.



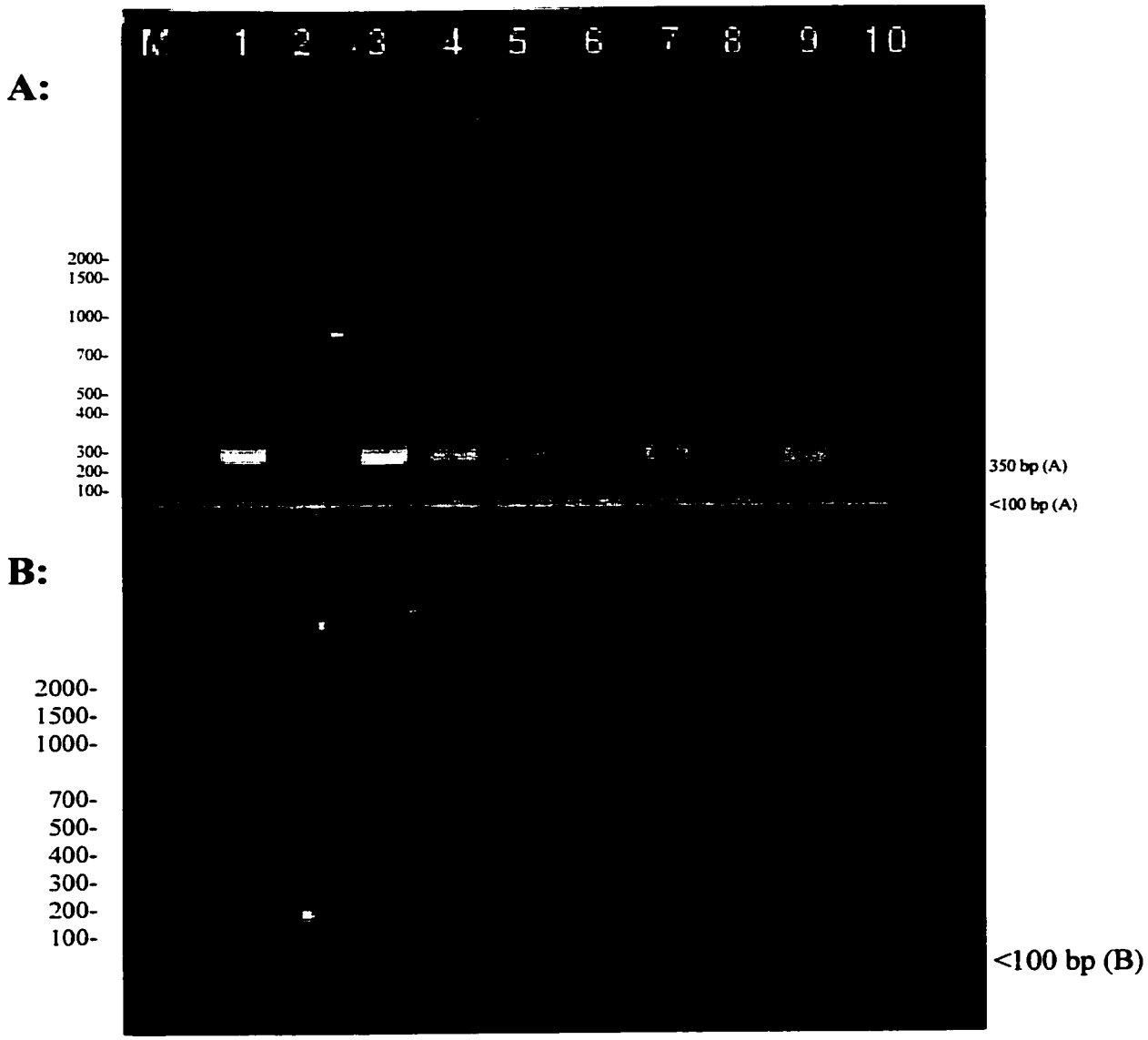
RT-PCR of HBVc-transformed tobacco

RNA was extracted from 13 pCHBV-transformed tobacco plants and subjected to a RT-PCR. The reaction mixtures were separated by agarose gel electrophoresis. The expected 250 bp fragment was not observed in any of the samples. Figure 15 shows the results of a representative RT-PCR assay on nine RNA extracts from different tobacco plants (lanes 1-9) and one negative control without RNA (lane 10). Assay (A) at the top of Figure 15 shows the RT-PCR result if DNAse is omitted from the protocol. A 250 bp band was amplified that represents contamination of the sample with genomic DNA. Assay (B) at the bottom of Figure 15 shows the RT-PCR result when DNAse is included in the protocol. No amplification product was observed, only a band smaller than 100 bp representing the reaction primers.

The procedure used was an adaptation of an in-house protocol for detection of HCV RNA that can detect as few as 6 copies of mRNA/ml of serum. The level of mRNA transcribed from the truncated HBV core protein gene in the transgenic tobacco plants was undetectable, even using this sensitive technique. It is possible that there was a problem at the level of transcription of the inserted HBV core protein gene, or that the mRNA was degraded very rapidly after transcription.

Figure 15: RT-PCR of RNA extracts of 10 tobacco plants transformed with pCHBV. (A) RT-PCR assay performed with no DNAse added. (B) RT-PCR assay performed with addition of DNAse. Lanes 1-9 indicate RNA extracts from 9 different plants. Lane 10 is the negative control sample RT-PCR with no RNA added. Lane M is the DNA length marker: 50-2000 bp Molecular Ruler from Bio Rad; band sizes marked in bp. Horizontal white lines indicate the location of the wells on the agarose gel (samples in B were electrophoresed from the midpoint of the gel).

Figure 15: RT-PCR of RNA extracts of 10 tobacco plants transformed with pCHBV. (A) RT-PCR assay performed with no DNAse added. (B) RT-PCR assay performed with addition of DNAse.



ELISA capture assay to detect HBV core protein in extracts of transgenic tobacco

Initially, ELISA capture assay I was conducted with extracts from 10 mature plants transformed with pCHBVc. Early results suggested that one of the plants tested positive for the presence of HBV core protein. However, the negative control plant in this experiment was several weeks more mature than the test samples. It was postulated that the older leaf tissue may have contributed to a higher non-specific background reading of the negative control. If this had occurred, some positive samples may have been masked by the non-specific binding in the negative control sample. Therefore, the experiment was later repeated on 20 available transformed plants and a negative control plant of the same age as the test samples, but none tested positive. It was postulated that some non-specific binding of the anti-HBcAg monoclonal antibody (coated on the microtiter plate wells) to endogenous proteins may have been occurring in the plant extracts and controls, thus making the absorbance readings non-specific to HBcAg concentration. This would also have contributed to falsely decreased readings of the amount of HBcAg. To test this hypothesis, aliquots of the plant extracts and controls were pre-incubated with a small amount of the anti-HBcAg monoclonal antibody in solution before proceeding with the ELISA. If the antibody binding were specific, then the absorbance readings were expected to fall significantly, compared to aliquots of the same sample not preincubated with the antibody. This would occur as the HBcAg-antibody complexes formed in the preincubation step would not be available for binding to the monoclonal antibodies coating the wells. Very little inhibition of binding was observed in the plant extracts while in the positive control sample, inhibition was nearly complete. This suggested that the absorbance readings in the plant extracts were not due to the presence of HBcAg. Several repeats of this assay finally suggested that none of the extracts contained

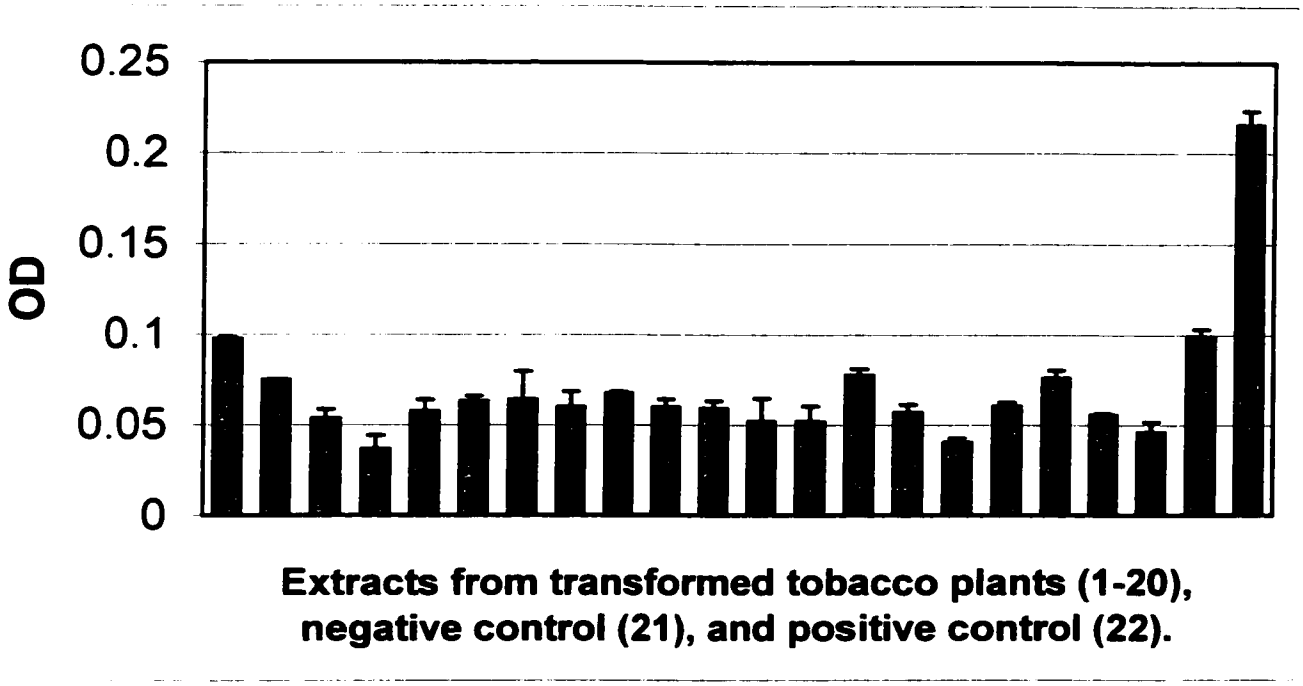
detectable amounts of HBV core protein. The ELISA competition assay II described on page 44 and the immunoprecipitation assay described on page 46 yielded essentially identical results to the first ELISA assay, therefore, only the results of the ELISA competition assay I are reported here.

Figure 16 shows the results of the ELISA competition assay I experiment. Bars 1-20 indicate the absorbance values from the transgenic tobacco protein extracts. Bar 21 indicates the absorbance value of the negative control extract, which was from a non-transgenic plant. Bar 22 indicates the absorbance value of the positive control extract, which was from a culture of *E. coli* expressing recombinant HBV core protein. In this test, the absorbance value is directly proportional to the concentration of HBV core protein in the sample, if no non-specific binding of the monoclonal antibody is occurring. However, the absorbance in the negative control sample is higher than in all the transgenic plant samples, with the exception of sample 1 where it is the same. Therefore, if the absorbance value of the negative control sample (which represents the degree of non-specific binding of the anti-HBcAg antibody to endogenous plant proteins) is subtracted from the absorbance values of the test samples, the quantity of HBV core protein in the plant samples is found to be effectively zero. This conclusion is supported by the fact that the assay was consistently able to detect *E. coli*-produced HBV core protein from crude extracts through a range of dilutions from 10^2 to 10^5 times.

Figure 16: ELISA Analysis of pCHBV-transformed tobacco leaf protein extracts.

Bars 1-20: extracts from 20 transformed tobacco plants. Bar 21: negative control sample extract from wild type tobacco plant. Bar 22: positive control: HBV core particles produced in *E. coli.* Optical density (OD) is proportional to the amount of HBV core protein in each sample.

Figure 16: ELISA Analysis of pCHBV-transformed tobacco leaf protein extracts.



Disruption of HBV core protein expression in the transgenic tobacco plants could have occurred at several levels within the biosynthetic pathway from integration of the recombinant DNA to protein folding and assembly. It is also possible that modification of the HBV core protein by glycosylation and/or phosphorylation rendered it undetectable by the antibodies. There are no asparagine residues in the sequences N-X-S or N-X-T (where X is any amino acid residue except proline) in the truncated HBV core protein that would permit N-linked glycosylation. However, two likely sites for O-linked glycosylation are predicted using the NetOGlyc 2.0 Prediction Server at the Technical University of Denmark's Center for Biological Sequence Analysis (www.cbs.dtu.dk/netOglyc/cbsnetOglyc.html). To analyze amino acid sequences, the NetOGlyc 2.0 Prediction Server uses a jury of artificial neural networks that have been trained to recognize the sequence context and surface accessibility of 299 known and verified type O-glycosylation sites extracted from a protein database (Hansen *et al*, 1995, 1997, 1998). Analysis using this method predicts that threonine 128 and serine 141 of the truncated HBV core protein are likely sites for O-linked glycosylation. Inappropriate glycosylation at these residues could possibly have prevented detection of the protein in the transgenic tobacco protein extracts.

Phosphorylation has been observed only on serine residues 155, 162 and 170 in the native HBV core protein (Pumpens and Grens, 1999). It is possible that alternative sites are phosphorylated on the truncated HBV core protein expressed in transgenic tobacco plants. The NetPhos 2.0 Protein Phosphorylation Prediction Server at the Technical University of Denmark's Center for Biological Sequence Analysis can be used to predict phosphorylation sites in amino acid sequences. The NetPhos 2.0 Protein Phosphorylation Prediction Server uses an artificial neural network method which predicts phosphorylation sites in independent

sequences with a sensitivity in the range of 69% to 96%, based on an analysis of known phosphorylation sites (Blom *et al*, 1999). Analysis using this method predicts several possible phosphorylation sites in the truncated HBV core protein including serines 26 and 44, threonines 114 and 147, and tyrosine 6. It is conceivable that inappropriate phosphorylation of the transgenic tobacco-expressed HBV core protein could have prevented its detection using antibodies directed against the native protein.

Part 2: Construction of WHVc-based epitope carrier protein and test expression in *E. coli*

DNA Sequencing of plasmids pKKWHVc150, pKKWHVc150*Xba*I/*Xho*I, pKKWHVc150-2.3 and pKKWHVc150-8.8

Figure 8 shows the verified nucleotide sequence of the truncated WHV core protein gene in plasmid pKKWHVc150. It was confirmed that the expected start and stop codons were present. There were no altered nucleotides or frameshift mutations noted. Figure 10 shows the verified nucleotide sequence of the truncated and modified WHV core protein gene in plasmid pKKWHVc150*Xba*I/*Xho*I. It was confirmed that the expected start and stop codons were present. The novel *Xba*I and *Xho*I sites were present as designed at nucleotides 234 and 430 respectively. There were no altered nucleotides or frameshift mutations noted.

Figures 11 and 17, 12 and 18 show the verified nucleotide sequence of plasmids pKKWHVc150-2.3 and pKKWHVc150-8.8 respectively. A 111 bp HCV core gene fragment was inserted into the *Xba*I site of pKKWHVc150-2.3 in frame and with no point mutations noted. A 120 bp HCV core gene fragment was also correctly inserted into the *Xho*I site of pKKWHVc150-8.8.

Figure 5 shows the hypothetical structure of the protein products of pKKWHVc150-2.3 and pKKWHVc150-8.8 constructs. These chimeric WHV core proteins carry 37 and 40 amino acid residues of the HCV core protein, respectively.

Figure 17: Sequence of modified truncated WHV core protein gene with HCV fragment in vector pKKWHVc150-2.3. Selected restriction sites are underlined. HCV core protein gene fragment is in bold type. Start and stop codons are outlined.

Figure 17: Sequence of modified truncated WHV core protein gene with HCV fragment in vector pKKWHVc150-2.3

ATGGACATAGATCCTTATAAAGAATTTGGTTCATCTTATCAGTTGTTGAATTTT

CTTCCTTTGGACTTCTTTCCTGATCTTAATGCTTTGGTGGACACTGCTACTGCC

TTGTATGAAGAAGAACTAACAGGTAGGGAACATTGCTCTCCGCACCATACAGCT

ATTAGACAAGCTTTAGTATGCTGGGATGAATTAACTAAATTGATAGCTTGGATG

*Xba*I

AGCTCTAACATAACTTCT**AGACCTAAACCTCAAAGACAAACCAAAAGAAACACT**

AACCGTCGCCCACAAGACGTTAAGTTTCCGGGCGGCGGCCAGATCGTTGGCGGT

*Xba*I

GATACTTGTTGCCGCGCAGGGGCTCTAGACAAGTAAGAACAATCATTGTAAATC

ATGTCAATGATACCTGGGGACTTAAGGTGAGACAAAGTTTATGGTTTCATTTGT

CATGTCTCACTTTCGGACAACATACAGTTCAAGAATTTTTAGTAAGTTTTGGAG

*Xho*I

TATGGATCAGGACTCCAGCTCCATATAGACCTCCTAATGCACCCATTCTCGAGA

CTCTTCCGGAACATACAGTCATTAGGTGA

Figure 18: Sequence of modified truncated WHV core protein gene with HCV fragment in vector pKKWHVc150-8.8. *Xba*I site used for cloning is underlined. HCV core protein gene fragment is in bold type. Start and stop codons are outlined.

Figure 18: Sequence of modified truncated WHV core protein gene with HCV fragment in vector pKKWHVc150-8.8.

ATGGACATAGATCCTTATAAAGAATTTGGTTCATCTTATCAGTTGTTGAATTTT

CTTCCTTTGGACTTCTTTCCTGATCTTAATGCTTTGGTGGACACTGCTACTGCC

TTGTATGAAGAAGAACTAACAGGTAGGGAACATTGCTCTCCGCACCATACAGCT

ATTAGACAAGCTTTAGTATGCTGGGATGAATTAACTAAATTGATAGCTTGGATG

*Xba*I

AGCTCTAACATAACTTCTAGACAAGTAAGAACAATCATTGTAAATCATGTCAAT

GATACCTGGGGACTTAAGGTGAGACAAAGTTTATGGTTTCATTTGTCATGTCTC

ACTTTCGGACAACATACAGTTCAAGAATTTTTAGTAAGTTTTGGAGTATGGATC

*Xho*I

AGGACTCCAGCTCCATATAGACCTCCTAATGCACCCATTCTC**GAGAACACTAAC**

CGTCGCCCACAAGACGTTAAGTTTCCGGGCGGCGGCCAGATCGTTGGCGGTGTA

TACTTGTTGCCGCGCAGGGGCCCCAGGTTGGGTGTGCGCGCGACGAGGAAGACT

*Xho*I

CTCGAGACTCTTCCGGAACATACAGTCATTAGGTGA

E. coli expression of pKKWHVc150-*XbaI/XhoI*-based vectors

Expression of the XbaI/XhoI modified truncated pKKWHVc150-*XbaI/XhoI* construct in *E. coli* was highly efficient – approximately 2 μg per ml of cell culture. The truncated core protein retained its antigenicity and was detected by ELISA and Western blot. The expressed recombinant pKKWHVc150-*XbaI/XhoI* as well as the chimeric proteins WHVc150-2.3 and WHVc150-8.8 carrying the HCV core epitopes were detected at their expected sizes. The lane marked pKK233-2 WHV 150 in Figure 19 shows a band at approximately 17 kD, the expected size of the truncated 150 amino acid-residue WHVc protein. The chimeric WHVc proteins in lanes marked pKK233-2 WHV 150-2.3 (187 aa), and pKK233-2 WHV 150-8.8 (189 aa) appear at their expected size of approximately 21.5 kD. The negative control, from a protein extract of *E. coli* transformed with unmodified plasmid pKK233-2 shows no band in this range.

In Figures 20 and 21, both chimeric proteins WHVc150-2.3 and WHVc150-8.8 were observed in fractions 2, 3, 4, and 5 of the sucrose gradient when run in SDS-PAGE and stained with Coomassie Blue. These findings were also confirmed by Western Blot. Unlike the chimeric WHVc150-2.3 and WHVc150-8.8, the bulk of the recombinant pKKWHVc150-*XbaI/XhoI* protein, a smaller protein mass, was usually found in fractions 5 and 6 (data not shown).

It appears that the truncated and modified WHV core proteins were being expressed and that the modifications did not significantly affect protein expression levels in *E. coli*..

Figure 19: SDS-PAGE and Western blot of pKK233-2, pKKWHVc150, pKKWHVc150-2.3 and pKKWHVc150-8.8 expressed in *E. coli* JM109. Insoluble portions of recombinant pKK233-2-based constructs expressed in JM109 bacterial culture were run on a SDS-PAGE gel, transferred to a nitrocellulose membrane and immunostained with a rabbit polyclonal anti-WHV core antibody followed by a goat anti-rabbit alkaline phosphatase-labeled secondary antibody.

Figure 19: SDS-PAGE and Western blot of pKK233-2, pKKWHVc150, pKKWHVc150-2.3 and pKKWHVc150-8.8 expressed in *E. coli* JM109.

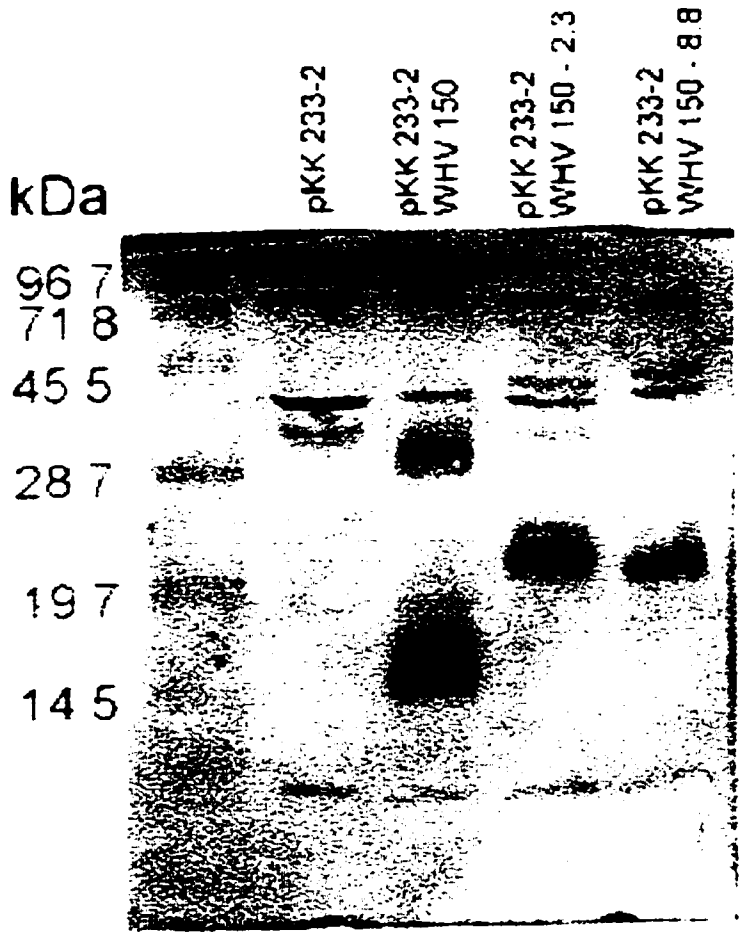


Figure 20: SDS-PAGE and Western blot of recombinant WHVc150-2.3 expressed in *E. coli* JM109. Recombinant WHVc150 carrying an insertion of HCV core protein amino acids 6-44 at amino acid 78 was purified in a sucrose gradient (2.2 ml each of 60%, 50%, 40%, 30% and 20% sucrose) at 30 000 rpm for 14 hours in a Beckman SW 40 Ti rotor. Thirteen one ml fractions were collected from the bottom and 10 µl of each fraction was run on a denaturing SDS-PAGE gel, and stained with Coomassie blue (top) or transferred onto a nitrocellulose membrane for Western blot (bottom). The nitrocellulose membrane was blocked with 5% DM/0.5% BSA at 4°C overnight and probed with a rabbit polyclonal anti-WHV core protein antibody followed by a goat anti-rabbit alkaline phosphatase-labeled secondary antibody.

Figure 20: SDS-PAGE and Western blot of recombinant WHVc150-2.3 expressed in *E. coli* JM109.

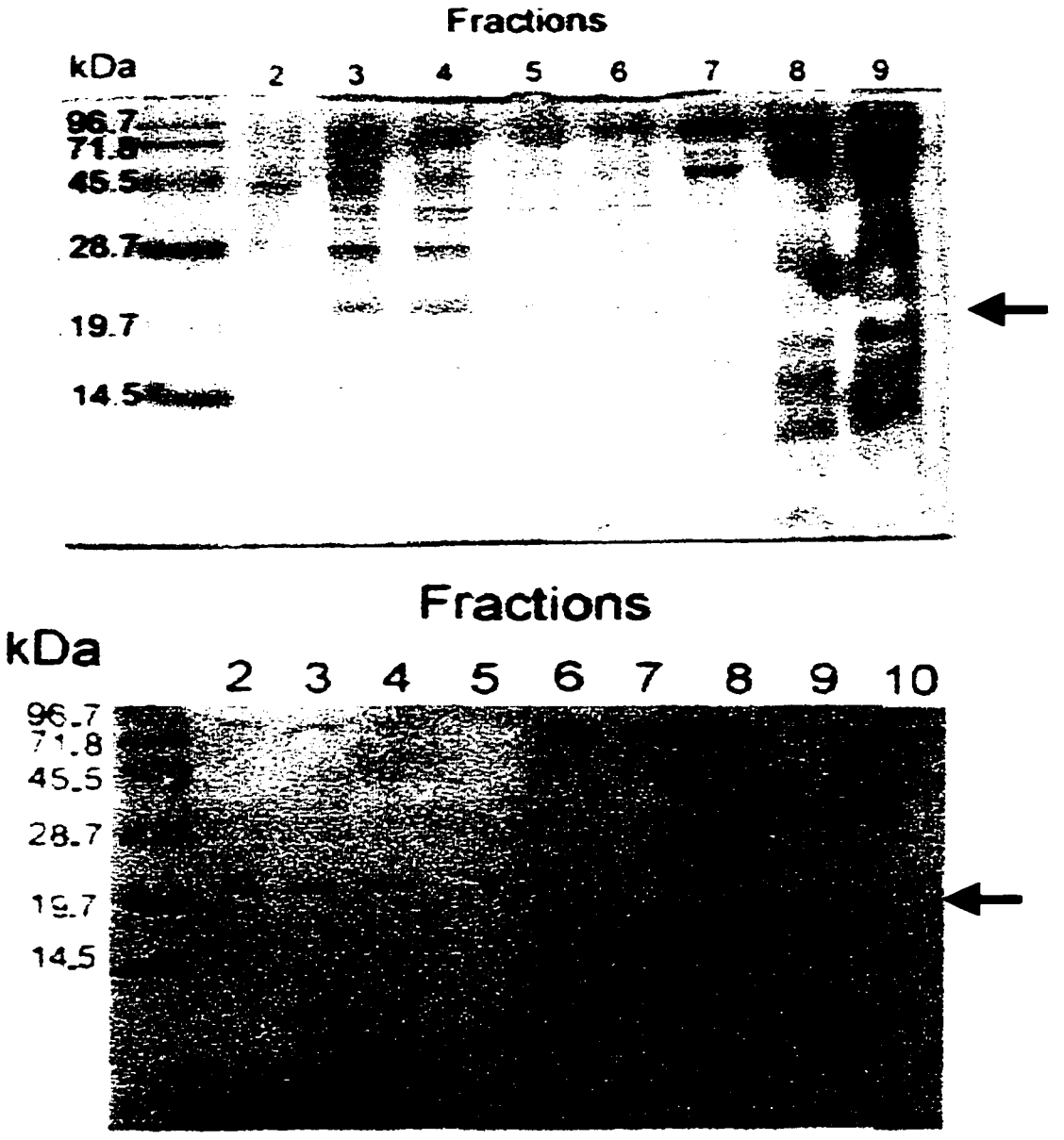
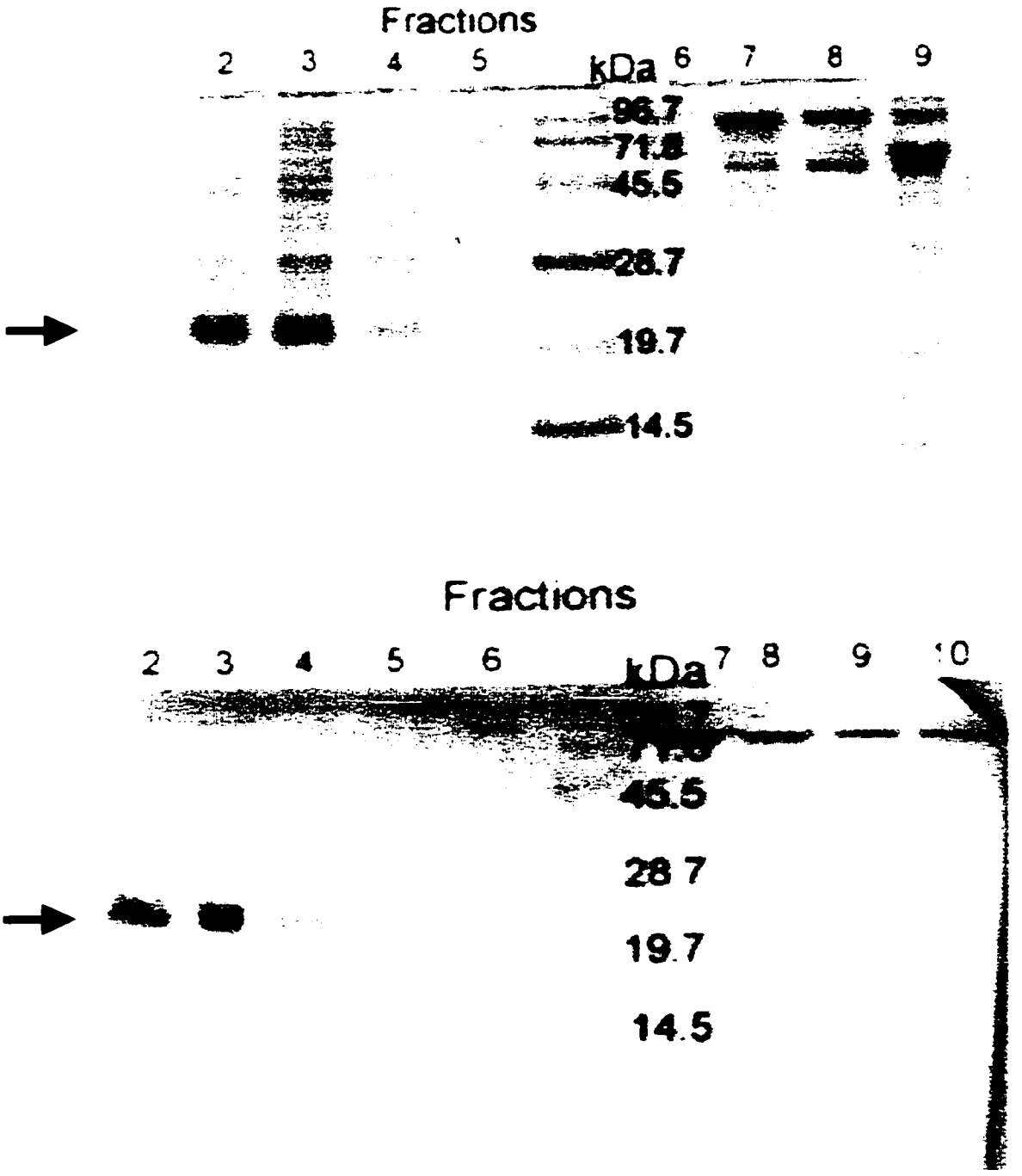


Figure 21: SDS-PAGE and Western blot of recombinant WHVc150-8.8 expressed in *E. coli* JM109. Recombinant WHVc150 carrying an insertion of HCV core protein amino acids 14-53 at amino acid 140 was purified in a sucrose gradient (2.2 ml each of 60%, 50%, 40%, 30% and 20% sucrose) at 30 000 rpm for 14 hours in a Beckman SW 40 Ti rotor. Thirteen one ml fractions were collected from the bottom and 10 µl of each fraction was run on a denaturing SDS-PAGE gel, and stained with Coomassie blue (top) or transferred onto a nitrocellulose membrane for Western blot (bottom). The nitrocellulose membrane was blocked with 5% DM/0.5% BSA at 4°C overnight and probed with a rabbit polyclonal anti-WHV core protein antibody followed by a goat anti-rabbit alkaline phosphatase-labeled secondary antibody.

Figure 21: SDS-PAGE and Western blot of recombinant WHVc150-8.8 expressed in *E. coli* JM109.



Competitive ELISA to recognize HCV core epitopes in chimeric WHVc150 proteins

Recognition of the HCV core epitopes within WHVc150-2.3 and WHVc150-8.8 proteins was demonstrated using a competitive ELISA technique. The HCV EIA 2.0 kit (Abbott) detects anti-HCV antibodies against one recombinant structural protein (HCV core) and two recombinant non-structural proteins. The procedure was modified to assess the specific inhibition (by the *E. coli*-expressed modified WHV core protein) of the interaction of anti-HCV antibodies in serum with the HCV core recombinant protein coated onto the beads supplied with the kit. To eliminate cross reactivity with antibodies to the other two nonstructural proteins, we selected an anti-HCV antibody positive serum that was strongly positive only for the HCV core peptide, as described in the Materials and Methods section. This sample was also positive for HCV RNA by PCR. Results presented in Table 1 indicate that both chimeric WHVc150-2.3 and WHVc150-8.8 proteins possess HCV antigenic activity and the observed inhibition is more than 50%, while a concentrated preparation of pKKWHVc150-*XbaI*/*Xho*I alone does not interfere with the qualitative detection of anti-HCV core antibodies. Both chimeric proteins react specifically with a patient serum previously found to be positive for anti-HCV antibodies.

Table 1: Competitive ELISA with pKKWHVc150-2.3 and pKKWHVc150-8.8. Ten µl of four gradient samples were added to 10 µl of an anti-HCV positive serum, diluted to 400 µl with HCV EIA 2.0 (Abott) kit diluent and incubated with HCV recombinant protein-coated beads (see fig. 8). HCV antigenic activity was detected in fractions 2-5 for pKKWHVc150-2.3 and fractions 2 and 3 for pKKWHVc150-8.8.

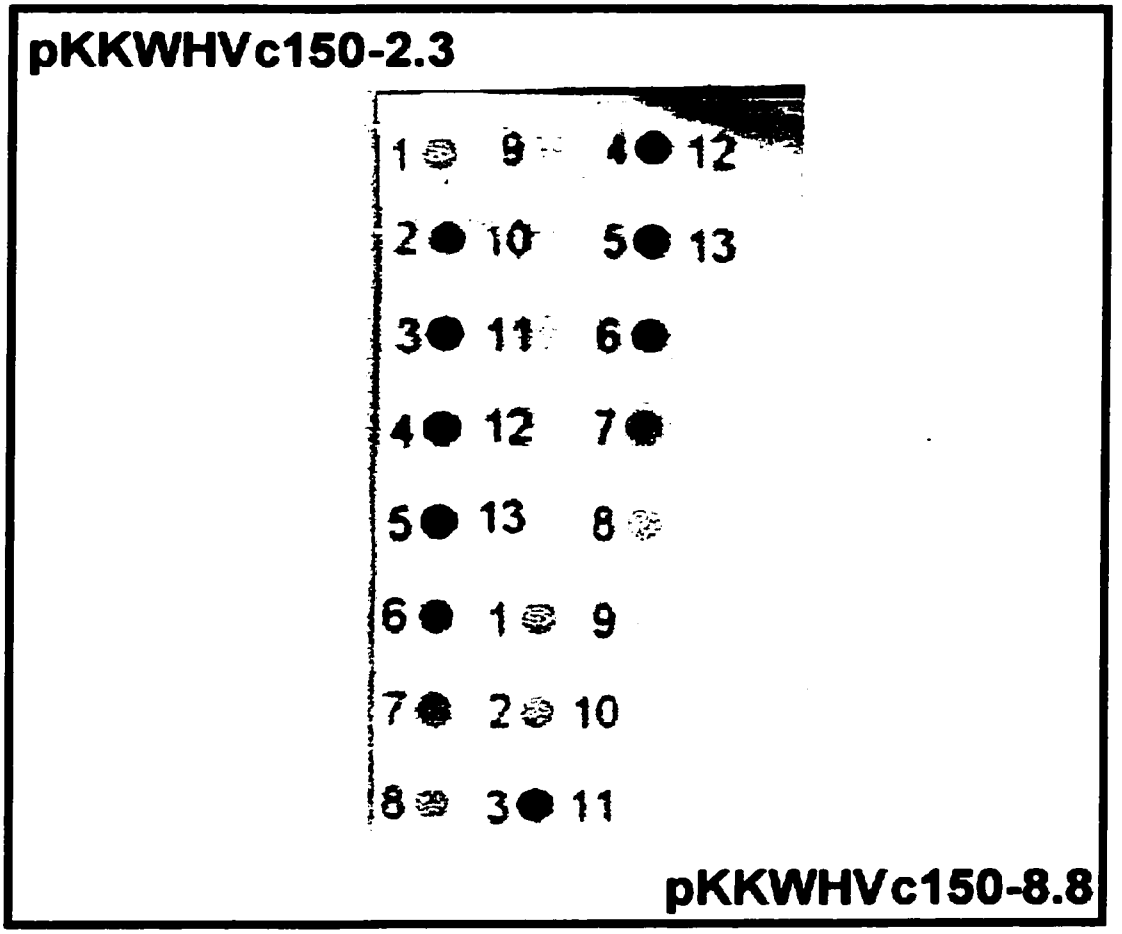
Competitive ELISA with chimeric pKKWHVc150 proteins			
WHVc construct	Gradient Fraction Number	Δ Absorbance 492:600 nm	% Inhibition
WHVc150-2.3	2	0.809	>50%
	3	0.760	>50%
	4	0.750	>50%
	5	0.878	>50%
WHVc150-8.8	2	0.987	>50%
	3	0.971	>50%
	4	1.230	
	5	1.494	
pKKWHVc150-*XbaI/XhoI*	Concentrated pKKWHVc150-*XbaI/XhoI*	1.996	
-	Anti-HCV (+) Serum	>2.000	
-	Anti-HCV (-) Serum	0.063	

Dot-blot Analysis of WHVc proteins expressed in *E. coli*

Epitopes detected in Western blot analysis are linear epitopes since conformational epitopes are largely destroyed by denaturing conditions in SDS-PAGE. To determine if a different distribution of proteins in the gradient fractions would be observed by immunological testing with a polyclonal antibody under non-denaturing conditions, a dot-blot assay was performed. In the dot-blot assay (Figure 22), the most intense labeling was observed in fractions four and five. However, the Western blot analysis of the same fractions showed the most intense labeling in fractions two and three (Figures 20 and 21). This finding may help explain the lack of observed core particles in electron microscopy of fractions two and three described below.

Figure 22: Dot-Blot Assay of Sucrose Gradient Purified WHVc150-2.3 and WHVc150-8.8 protein produced in *E. coli.* Ten µl of each fraction (13 total fractions) from the sucrose gradient protein purification method described above were blotted onto a nitrocellulose membrane and immunostained with rabbit polyclonal anti-WHV core antibody. The first series of 13 blots (upper left) shows the WHVc150-2.3 fractions. The second series of 13 blots (lower right) shows the WHVc150-8.8 fractions. Note that the strongest signal was observed from fractions 4 and 5, contrary to what was observed in the SDS-PAGE and Western Blot analysis of the same fractions.

Figure 22: Dot-Blot Assay of Sucrose Gradient Purified WHVc150-2.3 and WHVc150-8.8 protein produced in *E. coli.*



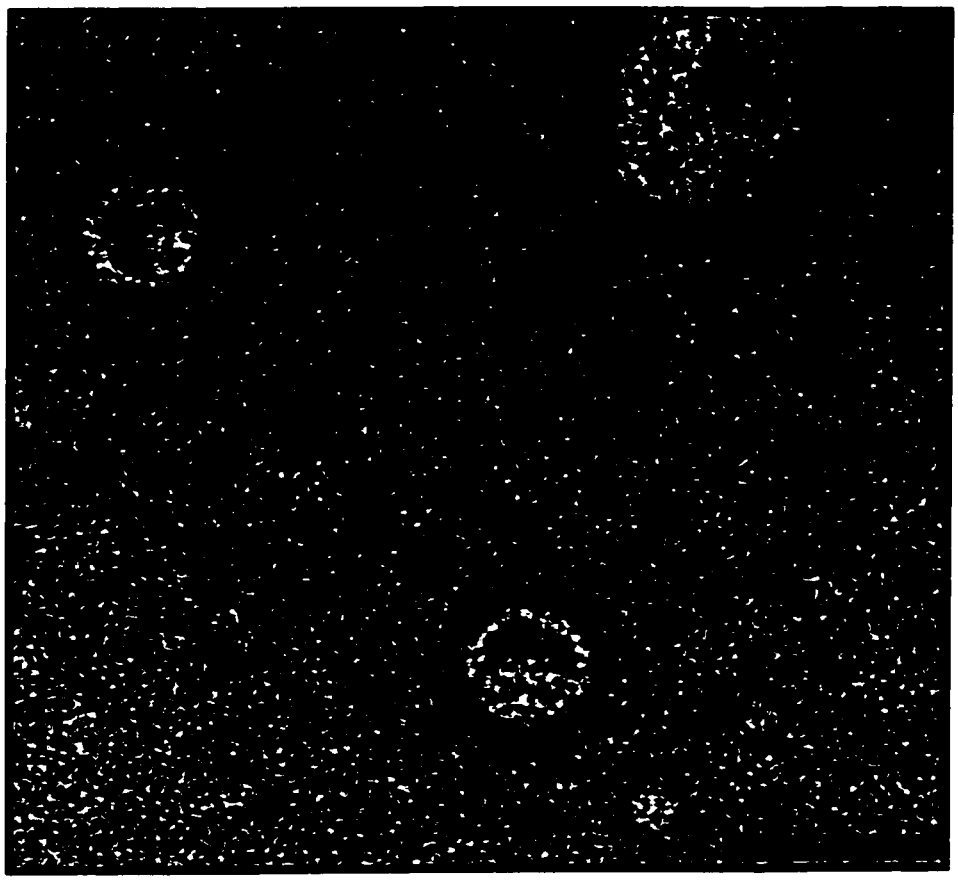
Electron microscopy of pKKWHVc proteins produced in *E. coli*

Gradient fractions numbers two and three from both WHVc150-2.3 and WHVc150-8.8 were selected for electron microscopy based on the expression level of the two chimeric proteins as detected by Western blotting (Figures 20, 21). Micrographs showed multiple aggregates of particulate nature, however no virus like particles were detected in these two fractions. Figure 23 shows an electron micrograph of unmodified WHVc150 protein expressed in *E. coli* transformed with plasmid pKKWHVc150. The image shows two virus like particles of approximately 34 nm in diameter. The upper right corner of the image shows a particulate aggregate similar to the ones observed in electron micrographs of WHVc150-2.3 and WHVc150-8.8 protein fractions. It is possible that the assembly competence of the chimeric core proteins is abolished by the insertions of the HCV core epitopes due to their length or location. The two sites for introduction of foreign antigenic determinants were selected in such a way as to mimic the insertions already known for the HBV core gene (Pumpens and Grens, 1999; Borisova *et al*, 1996; Schodel *et al*, 1996; Pumpens *et al*, 1995). Despite the similarity of the HBV and WHV core genes, it is possible that these sites do not correspond to the optimal sites for incorporation of foreign antigens in the WHV core protein. Another speculative explanation is based on results from the dot-blot analysis of the chimeric proteins (Figure 22). A correlation was not observed between the findings from the SDS-PAGE and Western blotting and the results of the dot-blot assay. The most intensive staining in the dot-blot assay was observed in fractions #4 and #5 while in the Western blot it was in fractions #2 and #3 instead. It is conceivable that different epitopes are detected by the two assays: linear in Western blot and conformational in the dot-blot by polyclonal rabbit anti-WHV core antibody. If this is true, then it is possible that

the correctly folded chimeric proteins and assembled particles were isolated in fractions #4 and #5. but not fractions #2 and #3. Electron microscopy of gradient fractions #4 and #5 may help determine if the capacity for particle assembly of the chimeric proteins was truly lost.

Figure 23: Electron micrograph of woodchuck hepatitis virus core particles isolated from a culture of E. coli transformed with plasmid pKKWHVc150. Two particles with a diameter of approximately 34 nm are visible (two-headed arrow). A particulate aggregate similar to the ones observed in electron micrographs of WHVc150-2.3 and WHVc150-8.8 protein fractions is visible in the upper right corner (arrow).

Figure 23: Electron micrograph of woodchuck hepatitis virus core particles isolated from a culture of E. coli transformed with plasmid pKKWHVc150.



Chapter 4: Discussion

Part I: Expression of HBV core protein in transgenic tobacco

Part I of this work was an attempt to express a gene coding for C-terminus truncated HBV core protein in transgenic tobacco plants. Figure 3a shows the sequence of manipulations that were accomplished toward this goal. The first step was the isolation of a DNA sequence coding for the first 150 amino acid residues of the HBV core protein and its insertion into plasmid vectors for plant transformation. Two expression vectors were designed and constructed: plasmid vector pKHBV where the truncated HBV core gene was placed under the control of the rice ubiquitin promoter, and plasmid vector pCHBV where the same coding sequence (CS) was placed under the control of the CaMV 35S promoter. Sequence analysis confirmed that the HBV core protein coding sequences were incorporated into the expression vectors in frame and with start and stop codons intact. Plasmid pKHBV was tested by *Agrobacterium*-mediated transformation of rice callus, which yielded kanamycin resistant rice callus that tested positive for expression of the marker gene *Uid* (results not shown), but no plants were regenerated from this experiment. The focus instead was on the regeneration of transgenic tobacco plants transformed with plasmid pCHBV.

Agrobacterium-mediated transformation of tobacco plants with plasmid pCHBV was accomplished and 30 regenerated plants were initially tested for incorporation of the truncated HBV core protein CS. A PCR-based approach using genomic DNA isolated from the transformed plants identified 28 plants that carried the HBV core protein CS. However, repeated immunological assays of leaf tissue from 20 of the genomic PCR-positive plants failed to identify any plants expressing detectable amounts of HBV core protein.

Furthermore, Northern blot and RT-PCR analysis of the plants failed to detect any HBV core gene mRNA. These results were discouraging until the first report of the expression of HBV core particles in transgenic plants was published while this work was in progress (Tsuda *et al*, 1998).

Tsuda *et al*'s report describes the expression of the full-length HBV core gene in transgenic tobacco plants for serological diagnosis. The approach used was similar to the one described in this report with the exception that the authors selected a full-length and not C-terminus-truncated HBV core protein CS. *Agrobacterium*-mediated transformation was used to introduce a sequence coding for the entire 183 amino acid residues of the HBV core protein into tobacco plants. The coding sequence was placed under the control of the same cauliflower mosaic virus 35S promoter as the one in plasmid CamterIII used in this work. It was found that recombinant HBV core protein accumulated in the leaves of the transformed plants. Core particle self-assembly occurred as it does in *E. coli* expression systems and the particles maintained normal HBV core protein antigenicity. The authors concluded that transgenic plants can produce reagents for serological testing and mentioned the possibility of oral vaccines. These results promised success for plant-based expression of chimeric hepadnavirus core particles for the development of oral vaccines, but it remained to be seen whether modified core proteins could be stably expressed in plants. The Epilogue of this thesis (Chapter 5) briefly cites some of the very latest unpublished results from our labs suggesting that truncated HBV and WHV core proteins can be effectively expressed in transgenic plants.

The unsuccessful expression of C-terminus truncated HBV core protein described in this report can be attributed to several possible problems. These include problems at the

level of recombinant DNA integration into the plant genome, transcription, post-transcriptional processing, translation, and post-translational processing (Kusnadi *et al*, 1997). The lack of detectable HBV core mRNA in the transgenic plants suggests the truncated HBV core protein gene was inserted into a non-transcriptionally active region of the chromatin or that it underwent transcriptional transgene silencing. Many researchers have described processes that prevent expression of transgenes including insertion of the transgene into interstitial and heterochromatic (centromeric, telomeric) regions of the plant chromosomes (Jakowitsch *et al*, 1999; Meyer and Saedler, 1996), methylation of inserted transgenes (Kooter *et al*, 1999), and condensation of transgene DNA into a dense chromatin structure (van Blokland *et al*, 1999; Meyer, 1999). RNA silencing of transgenes, whereby antisense RNA is produced either by direct transcription from the transgene DNA by a native plant promoter or from transgene sense RNA by an RNA-dependent RNA polymerase may also play a role in this case (Baulcombe, 1999). However, RNA silencing alone can not explain the complete lack of HBV core mRNA determined in the RT-PCR assays of the transformed tobacco plants.

Future attempts to express hepadnavirus core particles in transgenic plants must focus on several goals: 1) to ensure integrity of transgene DNA in transcriptionally active regions of the plant genome, 2) to increase levels of transcription using more efficient promoters, 3) to ensure efficient translation by designing transgenes with codons optimized for plant expression, and 4) to target recombinant proteins to specific tissues or organelles to prevent rapid degradation or interference with cell metabolism. The latest unpublised data described in Chapter 5 has already shown that these strategies are likely to be successful for

expression of C-terminus truncated HBV and WHV core protein genes in tobacco and other plants.

Part II: Construction of WHVc-based epitope carrier protein and test expression in *E. coli*

Part II describes the construction of a modified WHcAg protein gene designed to accept insertions of foreign epitopes, and its expression in *E. coli*. Figure 3b shows the sequence of manipulations that were accomplished toward this goal. The first step was the isolation of a DNA sequence encoding the first 150 amino acid residues of the WHV core protein and its insertion into a prokaryotic expression vector. In the pKKWHVc150-related plasmids, the truncated WHV core protein coding sequence was placed under the control of the IPTG-inducible prokaryotic *trc* promoter for high-level inducible expression in *E. coli*. Sequence analysis confirmed that the WHV core gene sequences were incorporated into the expression vectors in frame and with start and stop codons intact.

The next step was the engineering of novel restriction sites into the WHV core protein coding sequence using a PCR mutagenesis approach. Two sites were successfully introduced: *Xba*I at position 78, and *Xho*I at position 140 to create plasmid pKKWHVc150-*Xba*I/*Xho*I. Introduction of these novel restriction sites changed two codons in the WHV core protein CS. The result was a change at position 79 from glutamic acid to arginine, and at position 141 from serine to glutamic acid. In both cases, a polar amino acid residue was exchanged for another. This fact, and previous reports of expression of modified HBV core proteins suggest that these modifications alone were unlikely to affect core protein expression and particle assembly (Pumpens and Grens, 1999; Borisova *et al*, 1996; Schodel

et al, 1996). Plasmid pKKWHVc150-*XbaI*/*Xho*I represents the first report of the design and construction of a vector for expression of chimeric WHV core proteins for display of foreign epitopes.

The introduction of novel restriction sites into the WHV core protein CS was followed by insertion of gene fragments encoding HCV core protein epitopes into the modified WHV core protein CS in plasmid pKKWHVc150-*XbaI*/*Xho*I. A HCV gene fragment encoding 37 amino acid residues of the HCV core protein was inserted into the *Xba*I site at position 78 to create plasmid pKKWHVc150-2.3. A HCV gene fragment encoding 40 amino acid residues of the HCV core protein was inserted into the *Xho*I site at position 140.

Expression of the modified truncated pKKWHVc150-*XbaI*/*Xho*I construct in *E. coli* was highly efficient. As expected, the truncated core protein retained its antigenicity and was detected by Western blot (Figure 19). The chimeric proteins WHVc150-2.3 and WHVc150-8.8 carrying the HCV core epitopes were also recognized by polyclonal anti-WHV core antibodies (Figure 19). All three proteins were detected at their expected sizes, which suggests that the proteins were not subject to rapid proteolysis or degradation in *E. coli*. ELISA analysis revealed that both chimeric WHVc150-2.3 and WHVc150-8.8 proteins possess HCV antigenic activity, while the WHVc150 protein without an HCV insert did not (Table 1). These chimeric woodchuck hepatitis virus constructs, WHVc150-2.3 and WHVc150-8.8, represent novel epitope insertions into chimeric WHV core proteins. There are no reports of chimeric WHV core proteins in the literature, despite the considerable interest in chimeras of its close relative the HBV core protein. That the chimeric WHV core proteins were expressed and their foreign epitopes accurately displayed strongly suggests

that the WHV core protein can be used for presentation of foreign epitopes in vaccine technology. Immunization using chimeric WHV core particles instead of chimeric HBV core particles may reduce the chances of interfering with later serological diagnosis of HBV infection in humans. Diagnostic tests could be designed that use anti-HBV antibodies that do not react with WHV core proteins. Therefore, the chimeric WHV core particle may be a better choice for presentation of vaccine antigens via the oral route.

A requirement for optimal presentation of vaccine antigens using chimeric WHV core proteins is that the proteins self-assemble into core particles. The particle structure provides a regular arrangement of a large number of antigenic peptides on its outer surface, which is a major determinant of its ability to induce an immune response (Bachmann and Zinkernagel, 1996). When gradient fractions #2 and #3 from both WHVc150-2.3 and WHVc150-8.8 were selected for electron microscopy, multiple aggregates of a particulate nature were observed. However no virus like particles were detected in these two fractions. It is possible that the assembly competence of the chimeric WHV core proteins is abolished by the insertions of the HCV core epitopes due to their length or location. This finding could represent inability of the WHV core particles to accept insertions of this size, or it could represent a feature unique to the HCV epitopes that were selected for insertion. Previous reports of particle assembly of chimeric HBV core particles carrying larger insertions makes the first possibility unlikely. However, it is conceivable that insertion of different HCV epitopes would permit chimeric WHV core particle assembly. As described in the results section, a third possibility exists to explain the lack of core particles found by EM since a correlation was not observed between the findings from the Western blotting and those of the dot-blot assay. If different epitopes were detected by the two assays: linear in

Western blot and conformational in the dot-blot, it is possible that the correctly folded chimeric proteins and assembled particles were to be found in gradient fractions #4 and #5 instead of #2 and #3. In the future, electron microscopy of gradient fractions showing highest expression by dot-blot assay may help determine the true capacity for chimeric particle assembly.

In conclusion, the results presented here clearly demonstrate the potential of chimeric WHV core particles for vaccine delivery. In the future, a larger number of inserted epitopes must be screened to determine the range in size and type of epitope that may be used. Also, it must be conclusively demonstrated that the WHV core protein will accept peptide inserts of the necessary size without negatively affecting particle assembly. Finally, high-level plant-based expression of chimeric WHV core particles must be accomplished in order to develop effective plant-based oral vaccines.

Chapter 5: Epilogue

Recent work completed in the laboratories of Anton Andonov (Bloodborne Pathogens and Hepatitis Laboratory, Bureau of Microbiology, Health Canada, Winnipeg, Manitoba) and Illimar Altosaar (Department of Biochemistry, Microbiology and Immunology; University of Ottawa, Ottawa, Ontario) has confirmed expression of a C-terminus truncated HBV core protein gene and a C-terminus truncated WHV core protein gene in transgenic tobacco plants and in transformed carrot cells. These efforts employed HBV and WHV core protein coding sequences similar to those described in this thesis. In the continuation of this Ottawa-Winnipeg collaborative project, an expression vector containing a constitutive high-expression plant promoter cassette which incorporates the alfalfa mosaic virus translational enhancer sequence, as well as a duplicated-enhancer CaMV 35S promoter (Datla *et al*, 1993) was used. This vector has been shown to drive higher protein expression levels than the native CaMV 35S promoter employed at the outset of this thesis.

Other methods to enhance protein expression in plants are currently being tested. These methods include design and construction of hepadnavirus core protein coding sequences with codons optimized for plant expression, and inclusion of signal sequences for protein targeting to the endoplasmic reticulum.

These latest results, which confirm and advance the findings of Tsuda's group in Japan (Tsuda *et al*, 1998), suggest a promising future for the development of plant-based vaccine technology built around chimeric hepadnavirus core particles.

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