INFECTIVITY OF LYMPHOID CELL-DERIVED WOODCHUCK HEPATITIS VIRUS IN AN IN VITRO EXPERIMENTAL SYSTEM

YUAN-YEE LEW

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

INFECTIVITY OF LYMPHOID CELL-DERIVED WOODCHUCK HEPATITIS VIRUS IN AN *IN VITRO* EXPERIMENTAL SYSTEM

by

Yuan-Yee Lew

A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements for

the degree of Master of Science

Faculty of Medicine

Memorial University of Newfoundland

August 1999

St. John's Newfoundland

ABSTRACT

Hepatitis B virus (HBV), the prototypic virus of the *Hepadnaviridae* family, induces chronic liver disease in approximately 5% of the global population and is a cause of incurable hepatocellular carcinoma (HCC). The woodchuck infected with woodchuck hepatitis virus (WHV) has been validated as the most valuable natural model of HBV infection, especially in the absence of practical *in vitro* systems for HBV investigations. Although HBV and WHV are primarily hepatotropic, they also infect the host lymphatic system. The significance of this extrahepatic infection in the establishment and progression of liver disease is unknown. Therefore, it would be highly advantageous to develop an *in vitro* cell culture system in order to investigate the interaction of hepadnavirus derived from lymphoid cells with host hepatocytes. This interaction should not be complicated by host immune surveillance directed against the virus. The main objectives of this study were to design and establish an *in vitro* experimental system for the propagation of WHV in hepatocytes and lymphoid cells and to investigate the ability of virus derived from lymphatic cells and serum to replicate in cultured woodchuck hepatocytes. In this work, both cultured woodchuck hepatocytes were shown to be susceptible to WHV infection and conditions for short-term maintenance of WHV in cultured lymphoid cells were established. In addition, highly sensitive methods for the detection of WHV genes, replicative forms of the genome and virus antigens in *in vitro* infected woodchuck cells were adopted and validated.

The obtained results provide, for the first time, direct *in vitro* evidence that WHV released by naturally infected lymphoid cells is infectious to the host hepatocytes. The data indicate that lymphoid cells in the spleen constitute a site where infectious virus replicates with higher efficiency than in circulating lymphoid cells. Furthermore, it was shown that WHV originating from lymphoid cells could be passaged serially through cultured hepatocytes and remain infective to a virus-naive woodchuck. These experiments demonstrated that the virus continues to be biologically active in both *in vitro* and *in vivo* conditions. Finally, preliminary experiments indicated that replication of WHV in the hepatocyte culture system could be suppressed by the presence of antibodies to the N-terminal domain of the WHV large envelope protein or by a synthetic analogue derived from the putative WHV cell binding site 1 (CBS1). This finding suggests that WHV CBS1 plays an important role in virus-hepatocyte interaction.

The woodchuck hepatocyte culture system developed in this work will serve as an important tool to study the early events in WHV infection and replication, the effects of the cellular microenvironment on infection and genetic variation of the virus, and for evaluation of novel anti-hepadnaviral agents.

ACKNOWLEDGMENTS

Many people have helped and influenced me during the completion of my graduate degree.

First, I am indebted to my supervisor, Dr. Thomas I. Michalak, who provided me with valuable guidance and has given me the opportunity to develop my research skills. Furthermore, he has inspired me to make learning a life-long experience.

Thanks should also be extended to the other members of my supervisory committee, Drs. Vernon Richardson and Michael Grant, for their advice. A sincere thanks to Norma Churchill and Colleen Trelegan and everyone else in Dr. Michalak's laboratory, including Paul, Trish, Jinguo, Carla and Darlene for their help and the team-oriented work environment.

In addition, I wish to thank my parents, Lew Ah Yim and See Tho Wah Hap, for their love and support. Finally, I would like to thank Devin Rubia for his encouragement and unconditional love.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGMENTS iv

TABLE OF CONTENTS v

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF ABBREVIATIONS. xv

CHAPTER 1 : INTRODUCTION

1.1 VIRAL PATHOGENICITY. 1
- 1.1.1 A brief historical background 1
- 1.1.2 General structure and taxonomy of viruses 3
- 1.1.3 Virus-cell interactions. 4

1.2 VIRAL HEPATITIS. 7

1.3 HUMAN HEPATITIS VIRUSES 8

1.4 HEPATITIS B VIRUS (HBV). 11
- 1.4.1 HBV epidemiology 11
- 1.4.2 The natural course of HBV infection. 12
- 1.4.3 HBV structure. 14
- 1.4.4 Life cycle of HBV. 20
- 1.4.5 Lymphotropism of HBV. 21

1.5 HEPADNAVIRUS FAMILY 25

1.6 THE WOODCHUCK MODEL OF HEPATITIS B. 27
- 1.6.1 Woodchuck hepatitis virus (WHV). 27
- 1.6.2 Characteristics of WHV infection. 28

1.7 HEPADNAVIRUS-HOST CELL INTERACTIONS 30
- 1.7.1 Candidate receptors for the preS domain of the DHBV

envelope protein 31
1.7.2 Host cell recognition by the preS1 domain of HBV envelope ... 34
1.7.3 HBV preS2 domain-host cell interactions 37
1.7.4 Host cell binding by the HBV small envelope protein. 38
1.7.5 Host and cell-type specific recognition by WHV envelope proteins. 40

1.8 *IN VITRO* INFECTION OF CULTURED HEPATOCYTES BY HEPADNAVIRUSES. 42
1.8.1 DHBV infection of primary duck hepatocytes. 42
1.8.2 Hepadnavirus infection of mammalian hepatocytes. 42

1.9. PURPOSE OF THE STUDY. 46

CHAPTER 2 : MATERIALS AND METHODS

2.1 ANIMALS 48

2.2 SAMPLE COLLECTION 49
2.2.1 Collection of blood. 49
2.2.2 Serum isolation. 49
2.2.3 Isolation of peripheral blood mononuclear cells 49
2.2.4 Collection of specimens at autopsy. 51
2.2.5 Isolation of splenic lymphoid cells 51
2.2.6 Liver biopsies. 52

2.3 CULTURE OF WOODCHUCK HEPATOCYTES (WCM-260). 52
2.3.1 Isolation of hepatocytes 52
2.3.2 Seeding of hepatocytes for culture 54
2.3.3 Optimization of conditions for hepatocyte attachment and *in vitro* growth 54
2.3.3.1 Pre-coating of culture vessels with gelatin 54
2.3.3.2 Preparation of HepG2 cell culture supernatant 55
2.3.4 Collection of cultured woodchuck hepatocytes 56
2.3.5 Culture of hepatocytes in glucagon-free medium 57
2.3.6 Evaluation of hepatocyte viability 57
2.3.6.1 Trypan blue exclusion assay. 57
2.3.6.2 Microculture tetrazolium assay. 58

2.4 PREPARATION OF INOCULA FROM WHV-INFECTED LYMPHOID CELLS 59
2.4.1 DNase and limited trypsin digestion of isolated lymphoid cells . . 59
2.4.2 Culture of WHV-infected lymphoid cells in Hepato-STIM™ medium 61
2.4.3 Preparation of lymphoid cell culture supernatant for inoculation of cultured woodchuck hepatocytes 62

2.5 PREPARATION OF WHV INOCULA FROM SERA 63
2.5.1 Preparation of WHV virions by serum ultracentrifugation 63
2.5.2 DNase protection assay 64

2.6 EVALUATION OF WHV LOADS IN INOCULA 65

2.7 *IN VITRO* INFECTION OF HEPATOCYTES WITH WHV INOCULA 66
2.7.1 Infection of hepatocytes 66
2.7.2 Co-culture of WHV-infected lymphoid cells with virus-naive hepatocytes 70
2.7.3 Passage of WHV from *in vitro* infected hepatocytes to cultured homologous virus-naive hepatocytes 70
2.7.4 Inhibition of *in vitro* WHV infection of WCM-260 cells by anti-LP1 antibodies 71
2.7.5 Inhibition of WHV invasion of cultured woodchuck hepatocytes by JP1 peptide. 74

2.8 DETECTION OF WHV PROTEINS IN *IN VITRO* INFECTED HEPATOCYTES AND THEIR CULTURE SUPERNATANTS. 75
2.8.1 Detection of *de novo* synthesized WHV proteins. 75
2.8.1.1 Metabolic labelling of cells with ^{35}S-methionine. 75
2.8.1.2 Immunoprecipitation of ^{35}S-Methionine labelled samples 75
2.8.1.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis 76
2.8.2 Identification of hepatocyte WHV proteins by fluorescence-activated cell sorting 77

2.9 INOCULATION OF NAIVE WOODCHUCKS WITH CULTURE SUPERNATANT FROM HEPATOCYTES *IN VITRO* INFECTED WITH LYMPHOID CELL-DERIVED WHV 78

2.10 DNA ISOLATION 79
2.10.1 Isolation of total DNA 79

2.10.2 Isolation of WHV cccDNA by a potassium chloride precipitation method 80

2.11 ENZYMATIC TREATMENTS OF WHV DNA 82
2.11.1 Digestion of WHV DNA with *Eco*RI restriction enzyme 82
2.11.2 Mung bean nuclease digestion of WHV DNA 83

2.12 DETECTION OF ASIALOGLYCOPROTEIN RECEPTOR MAJOR SUBUNIT mRNA 84
2.12.1 RNA isolation 84
2.12.2 Reverse transcription reaction 85

2.13 POLYMERASE CHAIN REACTION 86
2.13.1 Design of WHV genome-specific primers 86
2.13.2 Standard PCR conditions 90
2.13.3 Modified PCR conditions 91

2.14 DETECTION OF WHV DNA 92
2.14.1 Agarose gel electrophoresis 92
2.14.2 Southern blot hybridization 93
2.14.2.1 Blotting of DNA onto nylon membrane 93
2.14.2.2 Dot-blot analysis 93
2.14.2.3 Hybridization of WHV DNA 94
2.14.2.4 Recombinant WHV DNA probe 95

2.15 CLONING OF WHV DNA 97

2.16 WHV DNA SEQUENCING 98

CHAPTER 3 : RESULTS

3.1 CHARACTERISTICS OF CULTURED WOODCHUCK HEPATOCYTES AND LYMPHOID CELLS. 102
3.1.1 Cultured normal woodchuck hepatocytes and their features ... 102
3.1.2 Effect of glucagon on cultured woodchuck hepatocytes. 107
3.1.3 Viability of WCM-260 cells in the presence of anti-LP1 antibodies 110
3.1.4 WHV-infected woodchuck lymphoid cells cultured in hepatocyte growth medium. 110
3.1.5 Survival of woodchuck lymphoid cells in the presence of mitogens 117

3.2 IDENTIFICATION OF WHV cccDNA. 118
3.2.1 The applicability of KCl precipitation method for detection of WHV cccDNA in woodchuck cell cultures. 118
3.2.2 Establishment of PCR conditions for parallel detection of total WHV DNA and WHV cccDNA sequences 125

3.3 WHV CONTENT IN INOCULA DERIVED FROM SERA AND LYMPHOID CELLS. 130

3.4 WHV REPLICATION IN *IN VITRO* INFECTED WOODCHUCK HEPATOCYTES. 136
3.4.1 Preliminary experiments evaluating susceptibility of WCM-260 hepatocytes to WHV infection. 136
3.4.2 Kinetics of WHV DNA and WHV cccDNA in WCM-260 hepatocytes after *in vitro* infection. 141
3.4.3. Examination of WHV infection in cultured hepatocytes under higher stringency conditions. 144
3.4.4 Inoculum WHV content influences the efficacy of hepatocyte *in vitro* infection. 163
3.4.5 WHV is transmittable from infected lymphoid cells to hepatocytes in the same co-culture. 166

3.5 PROPAGATION OF INFECTIOUS WHV THROUGH PASSAGES IN CULTURED WOODCHUCK HEPATOCYTES 170
3.5.1 Transmissibility of lymphoid cell-derived WHV in serial WCM-260 hepatocyte cultures 170
3.5.2 Infectivity of WHV passaged in serial hepatocyte cultures to a WHV-naive woodchuck 174

3.6 THE EXTENT OF *IN VITRO* WHV INFECTION IS MODIFIED BY INTERFERENCE WITH THE EXTREME N-TERMINAL REGION OF THE WHV LARGE ENVELOPE PROTEIN. 177

3.7 WHV cccDNA SPECIFICITY CAN BE DETECTED IN CIRCULATING VIRIONS 181

CHAPTER 4 : DISCUSSION

4.1 IDENTIFICATION OF REPLICATIVE WHV DNA SEQUENCES IN CULTURED WOODCHUCK CELLS. 191

4.2 LYMPHOID CELLS MAINTAINED IN HEPATOCYTE GROWTH MEDIUM SUPPORT WHV REPLICATION. 194

4.3 CULTURED WOODCHUCK HEPATOCYTES ARE SUSCEPTIBLE TO WHV INFECTION. 196

4.4 LYMPHOID CELL-DERIVED WHV IS INFECTIVE TO CULTURED WOODCHUCK HEPATOCYTES 203

4.5 THE LEVEL OF WHV REPLICATION *IN VITRO* DEPENDS ON THE INOCULUM WHV CONTENT BUT NOT ON ITS ORIGIN 206

4.6 WHV FROM LYMPHOID CELLS PASSAGED SERIALLY IN HEPATOCYTES INFECTS VIRUS-NAIVE ANIMAL. 207

4.7 INFECTION OF WHV IN CULTURED HEPATOCYTES IS INHIBITED BY ANTI-LP1 ANTIBODIES AND A SYNTHETIC ANALOGUE OF WHV CBS1. 209

4.8 CIRCULATING INTACT WHV VIRIONS CARRY WHV cccDNA REACTIVITY. 210

4.9 SUGGESTIONS FOR FUTURE STUDIES 214

CHAPTER 5: SUMMARY AND CONCLUSIONS 216

REFERENCES 219

LIST OF TABLES

Table 2.1	Serological status of WHV infection in chronically infected woodchucks at the time of acquisition of sera and/or lymphoid cells	60
Table 2.2	Oligonucleotide primers used for amplification of WHV DNA and cccDNA sequences in this study	87
Table 2.3	Oligonucleotide primers used for WHV DNA sequencing	100
Table 3.1	Survival of woodchuck WCM-260 hepatocytes in culture at different time points after seeding	106
Table 3.2	Survival of PBMC cultured in conditioned Hepato-STIM™ medium at different time points after seeding	113
Table 3.3	Specificity of WHV DNA fragments amplified with PCOR1-MCOR and PGAP1-MCOR primer pairs determined by direct sequence analysis of PCR products	126
Table 3.4	Preliminary experiments evaluating WHV replication markers in WCM-260 hepatocytes *in vitro* infected with PBMC and serum-derived WHV inocula	137
Table 3.5	Detection of WHV replication markers in hepatocytes *in vitro* infected with lymphoid cell and serum-derived WHV inocula	145
Table 3.6	Summary of results from *in vitro* infection of WCM-260 hepatocytes with WHV inocula derived from lymphoid cells or sera	146
Table 3.7	WHV genome contents in sequential WCM-260 hepatocyte cultures infected with lymphoid cell-derived passaged WHV	173
Table 3.8	Summary of the data on nucleotide sequence analysis of mung bean nuclease-digested DNA extracted from DNase-treated circulating WHV virions	188

LIST OF FIGURES

Figure 1.1 Schematic representation of HBV and WHV genomes..... 15

Figure 2.1 Schematic presentation of the experimental strategy applied for the *in vitro* infection of WCM-260 hepatocytes with lymphoid cell or serum-derived WHV inocula..... 67

Figure 2.2 Outline of the strategy used for multistep passage of lymphoid cell-derived WHV in cultured woodchuck hepatocytes..... 72

Figure 2.3 Schematic representation of the approximate location of direct and nested oligonucleotide primer pairs specific for the WHV C gene, X gene and the nick-spanning region superimposed on the map of the complete WHV genome..... 88

Figure 3.1 Transcription of ASGPR major subunit gene in WCM-260 hepatocytes..... 104

Figure 3.2 Presence of glucagon in culture medium did not influence the *in vitro* infection of woodchuck hepatocytes with WHV..... 108

Figure 3.3 Incubation with rabbit anti-LP1 antibodies did not affect WCM-260 hepatocyte viability..... 111

Figure 3.4 WHV DNA expression in PBMC isolated from a chronic WHV carrier cultured for different time periods in hepatocyte growth medium..... 115

Figure 3.5 Lymphoid cell WHV inocula prepared in the presence of lipopolysaccharide have comparable infectivity to inocula generated in the absence of this mitogen..... 119

Figure 3.6 Southern blot analysis of liver-derived WHV DNA isolated by standard proteinase K/phenol-chloroform procedure and by a KCl precipitation method..... 122

Figure 3.7 Determination of optimal Mg^{2+} concentration in PCR mixture for parallel amplifications of total WHV DNA and WHV cccDNA species..... 128

Figure 3.8 Southern blot evaluation of the sensitivity of PCR amplifications with primers used for detection of WHV DNA and WHV cccDNA sequences..... 131

Figure 3.9 Quantitation of WHV DNA expression in inocula prepared from PBMC, splenocytes and serum of a chronically infected woodchuck and in serial dilutions of rWHV DNA by dot-blot hybridization assay 134

Figure 3.10 SDS-PAGE analysis of anti-WHs antibody immunoprecipitated proteins from WHV-infected WCM 260 hepatocytes labelled *in vitro* with ^{35}S-methionine 139

Figure 3.11 The time course of the expression WHV DNA and WHV cccDNA in WCM-260 hepatocytes exposed to serum WHV 142

Figure 3.12 Detection of WHV DNA and WHV cccDNA in WCM-260 hepatocytes infected with WHV-positive serum from a chronically infected woodchuck (#190) 148

Figure 3.13 Southern blot detection of WHV DNA and WHV cccDNA and flow cytometry analysis of WHcAg expression in WCM-260 hepatocytes incubated with WHV produced by splenocytes isolated from a chronically infected animal (#563) 150

Figure 3.14 *In vitro* infection of WCM-260 hepatocytes with splenocyte and serum-derived WHV obtained from #563 animal 153

Figure 3.15 Analysis of WHV DNA content in serum and PBMC-derived inocula from #19109 woodchuck and in PBMC and hepatocyte washes 155

Figure 3.16 Southern blot hybridization and FACS analyses of WCM-260 hepatocytes exposed to PBMC or serum-derived WHV prepared from #19056 woodchuck 157

Figure 3.17 Detection of WHV DNA and WHV cccDNA in hepatocytes incubated with pooled PBMC culture supernatants from WHsAg-positive WHV carriers 160

Figure 3.18 Expression of WHV DNA and WHV cccDNA in *in vitro* infected hepatocytes depends on the WHV content in the inoculum 164

Figure 3.19 WHV DNA detection in hepatocytes co-cultured with naturally WHV-infected lymphoid cells 168

Figure 3.20 Expression of WHV DNA in WCM-260 hepatocytes infected with PBMC-derived WHV, hepatocyte culture supernatant, and in hepatocytes exposed to the passaged WHV..... 171

Figure 3.21 Expression of WHV DNA in sera, PBMC and liver of a woodchuck inoculated with WHV originating from circulating lymphoid cells that was passaged for six times in WCM-260 hepatocyte cultures..... 175

Figure 3.22 Anti-LP1 antibodies inhibit *in vitro* WHV infection of WCM-260 hepatocytes..... 179

Figure 3.23 JP1 peptide inhibits *in vitro* WHV infection of woodchuck hepatocytes..... 182

Figure 3.24 Detection of WHV DNA and WHV cccDNA in DNase-resistant circulating WHV particles..... 185

Figure 3.25 Circulating, DNase-resistant WHV virions carry a repaired WHV nick region..... 189

LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
anti-HBc	antibody to HBcAg
anti-HBe	antibody to HBeAg
anti-HBs	antibody to HBsAg
anti-WHc	antibody to WHcAg
anti-WHs	antibody to WHsAg
ASGPR	asialoglycoprotein receptor
bp	base pairs
CBS1	cell binding site 1
cccDNA	covalently closed circular DNA
cDNA	complementary DNA
ConA	concanavalin A
CTL	cytotoxic T lymphocyte
DLU	digital light units
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
d.p.i.	days post inoculation
DHBV	duck hepatitis B virus
EB	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FCS-HI	heat inactivated fetal calf serum
FITC	fluorescein isothiocyanate
h	hour
HBV	hepatitis B virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen

HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
Ig	immunoglobulin
kb	kilobase
kDa	kiloDaltons
LPS	lipopolysaccharide
min	minutes
mRNA	messenger RNA
MTT	microculture tetrazolium
nm	nanometer
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohemagglutinin
rcDNA	relaxed circular hepadnaviral DNA
RNA	ribonucleic acid
RT	reverse transcription
rWHV DNA	linear, complete, recombinant WHV DNA
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	seconds
ssDNA	single stranded hepadnaviral DNA
vge	virus genome equivalents
WHcAg	woodchuck hepatitis virus core antigen
WHeAg	woodchuck hepatitis virus e antigen
WHsAg	woodchuck hepatitis virus surface antigen
WHV	woodchuck hepatitis virus

CHAPTER ONE : INTRODUCTION

1.1 VIRAL PATHOGENICITY.

1.1.1 A brief historical background.

The concept that certain diseases can be transmitted between affected and healthy hosts evolved as early as the fourth century B.C. when Aristotle wrote that "...dogs suffer from madness. This causes them to become irritable and all animals they bite become diseased". This disease is now known as rabies (Steele, 1975). The role of microbial pathogens in human and animal diseases, however, was established much later in the nineteenth century. It began with Louis Pasteur, who formulated between 1857 to 1865 the germ theory saying that: "each fermentative process could be traced to a specific living microbe" (Koprowski & Plotkin, 1985). Later, when laboratory techniques allowed for the isolation of single bacterial cells, Kock and Henle devised a set of postulates to test if a specific agent caused a particular disease (Evans, 1976). The postulates stated that: "the incriminated agent can be cultured from the disease lesions, the organism can be grown in pure culture, the organism reproduces the disease when introduced into an appropriate host, and the organism can be cultured from the experimental disease".

The idea of pathogenic viruses was essentially conceived by three men who were working independently; Mayer, Ivanovsky, and Beijerink who discovered filterable agents that cause disease in plants. Beijerink discovered that the filtrates of the sap of tobacco plants infected with the tobacco mosaic disease can cause disease in other healthy plants. Thus, the name "virus" (which means "poison" in

Latin) was used to describe microbial pathogens that cannot be removed by filtration (Knight, 1974). This term was used to describe "filterable" pathogens for many following years (Levine, 1996). It was only much later, in the twentieth century, that viruses were defined as infective agents which depend on living host cells for their replication (Lycke & Norrby, 1976).

Yellow fever was the first human disease shown to be caused by a virus transmitted via mosquitoes (Reed, 1902). Since then, with the development of modern laboratory techniques, many other viruses were isolated and identified (i.e., 1400 different viruses have been classified up to 1989; Brown, 1989). One of the most important discoveries was the finding that viruses can cause malignant tumours. For example, the oncogenic potential of the Rous sarcoma virus has been shown in chickens (Rous, 1911). Among viruses afflicting humans, herpesviruses, papovaviruses (i.e., SV40 and papillomavirus), human T-cell leukemia virus-1 (HTLV-1) and hepatitis B virus (HBV) were found to be cancerous (Benjamin & Vogt, 1990). Advances over the past forty years in the field of immunology allowed for the identification of viral antigens and analysis of their contributions to the immunopathogenesis of viral infections. On the other hand, developments in molecular biological techniques enabled (*e.g.*) the analysis of viral genomic organization, virus-host interactions, and mechanisms of cell injury and oncogenicity. In recent years, there has also been an expansion in molecular genetics which led to the engineering of recombinant viruses and genetically

modified animals, providing important tools for the detailed understanding of the mechanisms of viral replication and pathogenicity.

1.1.2 General structure and taxonomy of viruses.

Viruses can either be enveloped or non-enveloped and contain DNA or RNA genomes. In comparison to non-enveloped viruses, enveloped virions have lipid bilayer membranes acquired through budding from the hosts' cell membrane. In all viruses, a capsid consisting of a protein shell surrounds the viral nucleic acid. The term nucleocapsid is used to describe the protein-genome complex of a virus (Harrison, 1990).

The International Committee on Virus Taxonomy (ICTV) has classified viruses according to descending hierarchical levels of order, family, subfamily, genus and species. Lower hierarchical levels include subspecies, strain and variant. All virus families have the *-viridae* suffix and members of a family share a distinct morphology, genomic organization, replicative strategy and a common evolutionary origin. For example, the name *Hepadnaviridae* indicates that all members of the family are hepatotropic and have a DNA genome. In 1996, there were 55 families with 166 genera of viruses. In addition, there were 23 "floating" genera that have not been assigned to a family (Pringle, 1998).

1.1.3 Virus-cell interactions.

The majority of viruses demonstrate very limited host and tissue specificity which is defined as viral tropism. In general, viral tropism is determined by two interacting factors: viral attachment site(s) and the host cellular receptor(s) that is recognized by these site(s). The viral attachment site is a molecule usually expressed on the outer surface of the virion particle, either on the envelope of enveloped viruses or on the capsid of non-enveloped viruses. It is typically composed of a single polypeptide or its fragment, or, is formed by adjacent domains of different viral polypeptides. Viral attachment sites appear to be conserved as long as the viruses maintain the same host and tissue tropism. This is even true for viruses that possess high mutation rates, such as the influenza virus (Weis *et al*, 1988). For some viruses, the specificity of such viral attachment sites also depends on the secondary and tertiary protein structures. Some viral attachment sites require activation by proteases of either host or viral origins to be able to interact with the targeted host receptor (Klenk & Garten, 1994). For example, proteolytic modification of virion surface also appears to be required for woodchuck hepatitis virus (WHV)-cell interaction (see Section 1.7.5).

In addition to viral attachment sites, many enveloped viruses possess surface glycoproteins that mediate the fusion of viral proteins with host plasma membranes. These fusion proteins often require activation by host proteases which mediate a protein conformational structure through cleavage at specific amino acid

sequences (Nagai, 1993). For example, the F_0 glycoprotein of the Newcastle disease virus is converted to the fusion protein F_1 through the proteolytic lysis of the N-terminus portion of the protein at a particular arginine located in a cluster of basic amino acid residues (Nagai, 1993). This proteolytic digestion exposes a hydrophobic stretch believed to be the fusion domain that mediates cell penetration. For some viruses, cellular endoproteases (furins; enzymes located in the Golgi apparatus) capable of the activation of virus fusion have been identified (Klenk & Garten, 1994). It is believed that their presence determines virus tropism and pathogenicity.

Viral receptors are plasma membrane molecules that usually have other important physiological functions. These receptors play a critical role in the attachment and entry of virus into host cells and determine viral infectivity. Some examples of viral receptors are: the neuronal and lymphoid cell β-adrenergic receptor (Co *et al.*, 1985) and the epidermal growth factor receptor for reoviruses (Strong *et al.*, 1993), the CD4 receptor for human immunodeficiency virus (HIV) (Dagleish *et al.*, 1984), and the CR2 receptor for Epstein-Barr virus (McClure, 1992). Although virus receptors have most often been found to be proteins, lipids and carbohydrates can also serve as cell virus binding sites, such as phosphatidylinositol, phosphatidylserine and GM3-ganglioside for vesicular stomatitis virus (Mastromarino *et al.*, 1987), and sialyloligosaccharides for the Sendai virus, Newcastle disease virus (Paulson *et al.*, 1979) and influenza virus

(Rogers, 1986). In addition, high molecular weight carbohydrates with elaborate biochemical structures, such as heparan sulfate and heparin-like glycosaminoglycans have been shown to mediate low affinity attachment of herpes simplex virus and pseudorabies virus to susceptible cells, respectively (WuDunn & Spear, 1989; Mettenleiter *et al.*, 1990). More recently, two high affinity herpes virus binding proteins, herpesvirus entry mediator (HVEM; Montgomery *et al.*, 1996) and poliovirus receptor-related protein 1 (Prr1; Geraghty *et al.*, 1998), were reported.

Viruses can also bind to a cellular receptor through an intermediate molecule (Nathanson, 1997). Antiviral antibodies are one group of such intermediate molecules that enhance virus-cell interactions through the opsonization of virions. The Fc fragments of these antibodies can bind to the Fc receptors on the target cells and facilitate virus entry. This was demonstrated for dengue virus (Halstead, 1988) and later for the West Nile virus (Peiris *et al.*, 1981). Some viruses require more than one receptor for viral attachment and entry. For example, the CXCR4 chemokine receptor and the CCR5 chemokine receptor were found to function as co-receptors along with CD4 for the fusion of T-tropic or M-tropic HIV viruses, respectively (Oberlin *et al.*, 1996; Dragic *et al.*, 1996). More recently, the coxsackievirus A21 was found to require both the decay-accelerating factor (DAF) membrane molecule and the intracellular adhesion molecule 1 (ICAM-1) for cell entry (Shafren *et al.*, 1997).

Following interaction between the viral attachment site and its receptor, pH independent virus entry into the cytoplasm is usually by direct fusion with the plasma membrane or by receptor-mediated endocytosis (Marsh & Helenius, 1989). Viruses such as HIV (Stein *et al.*, 1987) and human cytomegalovirus (Tugizov *et al.*, 1994) fuse with plasma membranes at physiological pH (pH independent fusion), while viruses such as flaviviruses (Randolph & Stollar, 1990) and influenza viruses (Stegmann *et al.*, 1985) fuse with the host plasma membrane within endosomes at acidic pH (pH dependent fusion).

1.2 VIRAL HEPATITIS.

Viral hepatitis is a systemic infection affecting predominantly the liver that is characterized by hepatocyte necrosis, lymphomononuclear cell infiltrations, and liver cell regeneration. Viral hepatitis can have distinct clinical outcomes: (1) asymptomatic infection, (2) acute hepatitis, (3) fulminant hepatitis, and (4) chronic hepatitis.

Individuals with asymptomatic infection have no or very mild manifestations, such as fatigue and flu-like symptoms. This type of infection is usually identified by coincidental blood testing. In the case of symptomatic infection, four phases can usually be identified: incubation period, preicterus, icterus and convalescence or, in some cases, chronic infection. The incubation period is often asymptomatic, although circulating viral markers (i.e., viral nucleic acid) and biochemical

indicators of liver damage (i.e., elevated alanine aminotransferase [ALT]) are detectable. In the second preicteric stage, the liver becomes tender and enlarged and is accompanied by symptoms, such as fatigue, nausea, fever and vomiting. In the icteric stage, patients have more severe symptoms, including jaundice, higher fever, shaking chills, and right upper quadrant pain. After this acute hepatitis stage, the majority of patients recover and enter the convalescent stage. The remaining individuals will develop chronic hepatitis, a condition which is caused by both hepatitis B and C viruses. Chronic hepatitis is defined as a disease lasting longer than 6 months and can be classified as persistent or active (aggressive). These forms of chronic liver necroinflammation can be diagnosed by histological examinations of liver tissue. Chronic active hepatitis frequently progresses to liver cirrhosis.

A rarer form of liver inflammation is fulminant hepatitis, which is characterized by very severe and rapidly progressing hepatocyte necrosis. This disease is often fatal. Initially, the patients suffer from symptoms similar to acute hepatitis but the condition worsens quickly, as liver damage progresses to necrosis which is followed by encephalopathy (Kumar & Pound, 1992).

1.3 HUMAN HEPATITIS VIRUSES.

Presently, there are seven known hepatotropic viruses that cause viral hepatitis in humans. These viruses are known as A, B, C, D, E, G, and TT. The

viral structure, genomic organization, routes of entry, pathogenicity, and severity of liver inflammation differ for each of these viruses.

Briefly, hepatitis A virus (HAV) is a non-enveloped virus with a single stranded plus-strand RNA genome belonging to the *Picornaviridae* family. HAV infection is usually self-limiting with different degrees of severity. For example, infection of young individuals is usually asymptomatic or mild, while infection of children under 5 years of age and adults over 50 years of age can be severe and fatal (Alter & Mast, 1994).

Hepatitis C virus (HCV) is a pathogen belonging to the *Flaviviridae* family and contains a linear, plus-strand RNA genome with a single open reading frame (Choo *et al.*, 1989). A unique feature of HCV is its high rate of chronic infection, where up to 60% of exposed individuals are persistently infected. Many of these individuals may later develop liver cirrhosis and hepatocellular carcinoma (HCC) (Koziel, 1996).

Hepatitis D virus (HDV) is an interesting and distinctive virus as its invasion requires the co-infection with HBV. The envelope of the virus has HBV surface antigen (HBsAg) reactivity. The internal nucleocapsid contains HDV specific δ antigen and a circular, viriod-like RNA genome (Rizzetto *et al.*, 1980). HDV undergoes RNA-dependent replication via a unique "double rolling circle" mechanism (Branch & Robertson, 1984). The taxonomic status of HDV has not yet been determined and currently the virus is assigned to its own genus, *Deltaviridae*.

HDV infection superimposed on HBV infection often leads to severe chronic hepatitis, which is usually more grave than liver disease induced by HBV alone (Hadler *et al.*, 1992).

Hepatitis E virus (HEV) is a non-enveloped virus with a single plus-strand 7.5 kilobase (kb) RNA genome (Reyes *et al.*, 1990) and is structurally similar to viruses of the *Calciviridae* family (Koonin, 1992). Infection with this virus is usually mild and self-limited. For unknown reasons, HEV infection in pregnant women can be serious with fatality rates of 15-25% (Tsega *et al.*, 1992).

An enteric hepatitis F virus was claimed to be identified (Deka *et al.*, 1994) but this virus was not confirmed by others (Bowden *et al.*, 1996).

Another virus associated with hepatitis in humans is the hepatitis G virus (HGV), which is a RNA virus with 25% nucleotide homology to HCV. Approximately 1-2 % of healthy blood donors in Canada may have HGV RNA. HGV appears to cause a very mild self-limiting infection. The clinical and pathological significance of HGV need to be determined (Linnen *et al.*, 1996).

Recent studies from Japan demonstrated a new hepatitis virus called the TT virus (TTV) (Nishizawa *et al.*, 1997). TTV was shown to be a non-enveloped DNA virus which is prevalent in 12% of Japanese blood donors (Okamoto *et al.*, 1998). The virus was also found in 27% of patients with fulminant hepatic failure in the United States (Charlton *et al.*, 1998). The pathogenic significance of TTV infection is still under investigation. It was recently reported that there was no correlation

between TTV and non-A to E hepatitis (Matsumoto *et al.*, 1999).

In addition to the above hepatotropic viruses, a number of other viruses can induce hepatitis, *e.g.* adenovirus, cytomegalovirus, echoviruses, Epstein-Barr virus, measles virus, rubella virus, and varicella.

1.4 HEPATITIS B VIRUS (HBV).

1.4.1 HBV epidemiology

In general, HBV is a small, enveloped DNA virus that causes liver necroinflammation and HCC in humans. There are at least 300 million HBsAg-reactive individuals chronically infected with HBV worldwide (Margolis *et al.*, 1991). In Canada, conservative estimates indicate that the prevalence of HBsAg-positive chronic HBV carriers is 0.5-1.0%, with approximately 3,000 new cases reported each year (Sherman, 1996). In western countries, the virus is mostly spread by horizontal routes, such as intravenous drug use, sexual contact or occupational exposure. However, in highly endemic regions of the world (i.e., Asia and Africa), the major route of transmission is from infected mothers to infants (Margolis *et al.*, 1991), where up to 15% of the total population are HBV carriers (Maynard *et al.*, 1989).

Patients chronically infected with HBV are at risk for significant morbidity and mortality. It was estimated that about 15-25% of them will die of cirrhosis and HCC (After & Mast, 1994). HBV is reported to be the second leading cause of cancer

worldwide, after heavy cigarette smoking (Chisari & Ferrari). Despite availability of effective, protective vaccines in the developed countries, HBV-induced diseases remain a worldwide public health problem.

1.4.2 The natural course of HBV infection.

The average incubation period of HBV is 75 days (Purcell, 1994). It is estimated that up to 70% of adults exposed to HBV will develop asymptomatic (subclinical) infection. The remaining (approximately 30%) will have clinically evident liver disease diagnosed as acute hepatitis (Hoofnagle *et al.*, 1987). About 1% of these infected adults will develop fulminant hepatitis, which often is fatal.

Most of the acutely infected patients will clinically recover from the disease and develop apparent permanent immunity (Hoofnagle *et al.*, 1987). However, recent studies have demonstrated that clinical recovery, the normalization of biochemical indicators of liver function, the disappearance of serum HBsAg, and even the rise of circulating antibodies to HBsAg (anti-HBs) do not reflect the complete elimination of the virus (Michalak *et al.*, 1994, Rehermann *et al.*, 1996). In these convalescent individuals, traces of HBV DNA and HBV particles with physicochemical properties similar to those of complete virions were identified in the circulation up to 23 years after patients' recovery. In addition, transcriptionally active HBV in peripheral blood mononuclear cells (PBMC) (Michalak *et al.*, 1994) and a strong polyclonal viral-specific cytotoxic T lymphocyte (CTL) response have

been demonstrated in these apparently completely healthy individuals (Rehermann *et al.*, 1996).

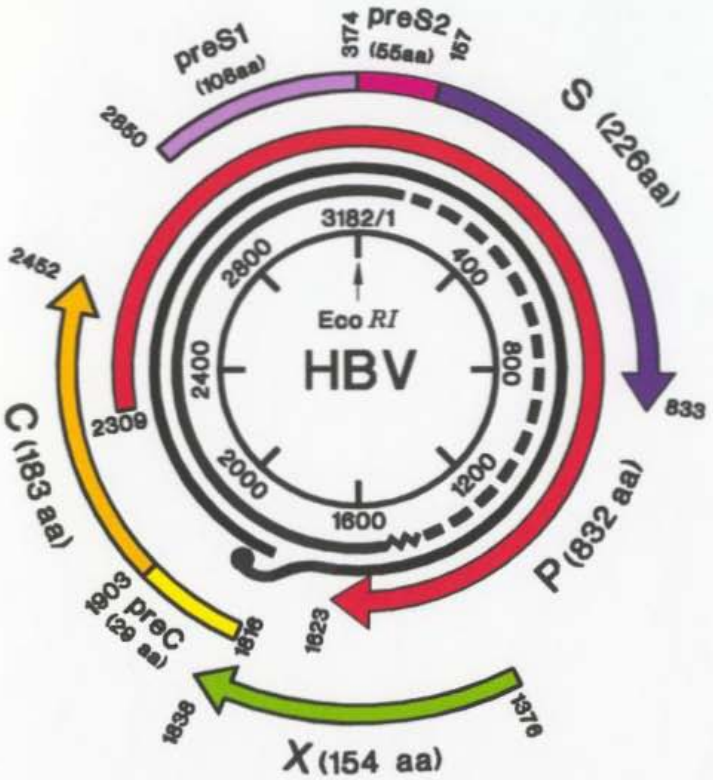
Individuals that eventually develop HBsAg-reactive chronic HBV infection may not necessarily have symptoms upon initial exposure to the virus. About 30-50% of chronic infections begin with acute hepatitis, while the remaining patients report no symptoms or just mild fatigue (Fattovich, 1991). The risk of developing chronic hepatitis B is predominantly determined by the age of the patient at the time of infection. Chronic disease occurs in 90% of infected infants, 25% to 50% of children between the ages of 1 to 5 years old, and about 5% to 10% of older individuals (Alter & Mast, 1994). Chronically infected individuals usually have progressive liver inflammation which often leads to liver cirrhosis (Fattovich *et al.*, 1991), the most common precursor of HCC (Beasley, 1988). It has been estimated that the risk of developing HCC can be approximately 200 times greater in chronic HBV carriers than in a healthy population (Beasley, 1988) and that neonates born to infected mothers are at the highest risk (Kew & Popper, 1984).

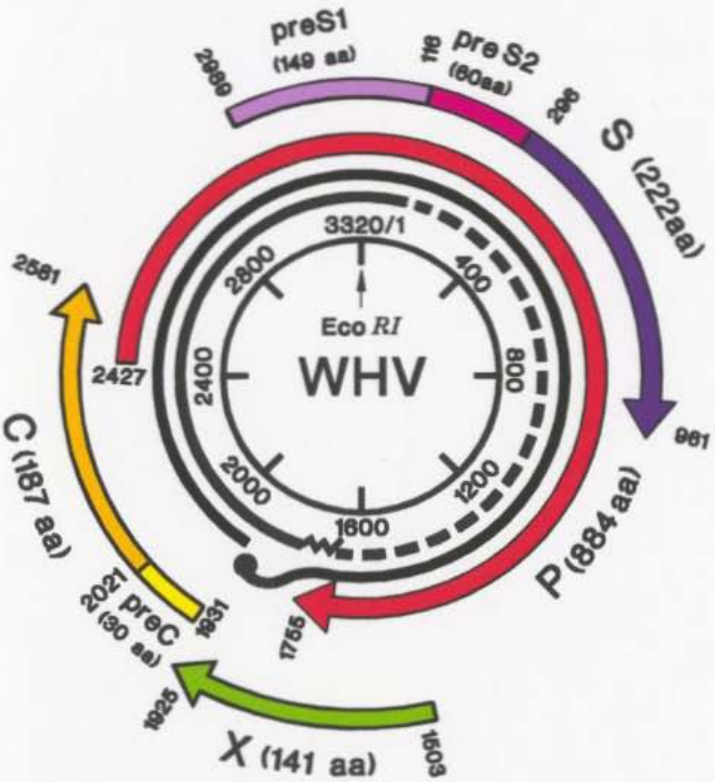
In addition to liver diseases, HBV can induce extrahepatic disorders due to the deposition of immune complexes comprised of viral antigens and specific antibodies. The pathogenic role of these immunocomplexes has been documented in glomerulonephritis (Combes *et al.*, 1971; Nowoslawski *et al.*, 1975; Slusarczyk *et al.*, 1980) and periarteritis nodosa (Gocke *et al.*, 1970; Michalak, 1978; Michalak & Krawczynski, 1981).

1.4.3 HBV structure.

The complete HBV particle or "Dane" particle is a double shelled, spherical structure that is 42 nanometer (nm) in diameter (Dane *et al.*, 1970) with a lipoprotein envelope surrounding a 22 nm electron dense icosahedral nucleocapsid (Blum *et al.*, 1989). In addition, non-infectious subviral particles, which consist of the envelope material are produced in large quantities and freely circulate in HBV-infected individuals. These particles are spheres of 20 nm in diameter or long filaments of up to 230 nm in length and they lacking capsids and viral genetic material (Hollinger, 1996). The virus capsid encloses the 3.2 kilobase (kb) circular, partially double stranded genome, known as relaxed circular DNA (rcDNA) (Fig. 1.1). This genome contains a single *EcoRI* restriction endonuclease site, which defines the first nucleotide in all hepadnaviral sequence databases. The partially double stranded viral genome is maintained in a circular form by a short cohesive overlap between the 5' ends of the two DNA strands. Each of the two HBV DNA strands is of different length. The minus strand is complete with defined 3' and 5' ends (Ganem, 1996) except for a short nick region at position 1846 (Charnay *et al.*, 1979). The plus strand is incomplete, with a variable 3'-end, creating a single-stranded gap region in the virus genome of about 200-300-bp long. At the 5' end of the minus strand, there is a covalently linked protein and at the plus strand, there is an attached 5' RNA oligoribonucleotide primer. Both of these elements are important for viral replication (see below; Ganem, 1996).

Figure 1.1. Schematic representation of HBV and WHV genomes. The inner circles represents the partially double stranded virion DNA with the first base pair marked by the *Eco*R1 endonuclease cleavage site and subsequent marking at intervals of 400 base pairs. The positive DNA strand has a dashed line to indicate a variable 3' region and a wavy line at the 5' end to indicate the covalently attached RNA oligonucleotide primer. The minus strand is complete with a 5' attached protein primer (closed circle). The broad arrows surrounding the DNA strands represent viral open reading frames: core gene (preC and C regions), surface or envelope gene (preS1, preS2 and S regions), polymerase (P) and X genes. The arrowheads indicate the direction of gene transcription. The length of the translated protein products is shown as a number of amino acids (aa).


preS1
(108aa)
3174
preS2
(55aa)
157
S (226aa)
2850
3182/1
2800
400
Eco RI
HBV
2400
800
2452
2309
2000
1200
1600
833
C (183 aa)
P (832 aa)
1903
preC
(29 aa)
1816
1623
1838
1376
X (154 aa)


preS1
(149 aa)
2999
118
preS2
(60aa)
296
S (222aa)
3320/1
2800
400
2561
Eco RI
WHV
2427
2400
800
2000
1200
961
1600
C (187 aa)
P (884 aa)
2021
preC
(30 aa)
1931
1755
1925
1503
X (141 aa)

The genome consists of 4 open reading frames (ORFs) or genes, which encode the envelope (S), nucleocapsid (C), polymerase (P), and X proteins. A unique feature of hepadnaviruses is that the ORFs' are overlapping. For example, the S ORF, C ORF, and X ORF partially overlap with the P ORF. Therefore, the virus produces substantially more protein per genome unit when compared to other viruses. In addition, hepadnaviral gene transcription regulatory elements (promoters and enhancers) are located within the protein coding regions instead of in separate regions of the genome. Thus, the HBV genome coding capability is recognized as an example of remarkable genome economy (Seeger *et al.*, 1991).

Products of the S gene derive from three co-translational regions with three different in-phase start codons. As a result, the HBV envelope proteins share a common C-terminus and have divergent N-termini and are referred to as small, middle, and large proteins. These proteins carry unique immunological specificities. However, they are collectively defined as HBsAg. These envelope polypeptides are glycosylated, giving rise to a total of six different molecular species, with and without attached carbohydrates. The most abundant polypeptide found in HBV particles is the small protein, which exists as non-glycosylated 24 kiloDaltons (kDa) and 27 kDa glycosylated proteins. The middle protein of HBV contains 55 extra N-terminal amino acids designated as the preS2 domain and occurs as 33 and 36 kDa polypeptides. The large protein is longer than the middle protein by an additional 108-119 N-terminal amino acids which is called the preS1

domain. It exists as unglycosylated 39 kDa and a glycosylated 42 kDa gene products (Tiollais *et al.*, 1985). These different polypeptide species are contained in circulating virions, subviral particles and infected hepatocytes (Robinson, 1990).

The C ORF, contains two start codons, the preC and C, which code for two overlapping proteins (Robinson, 1990). The core protein, which is translated from the C start codon, exhibits antigenic reactivity (HBcAg) and has a molecular mass of 22 kDa. Multiple copies of the core polypeptide form the capsid of HBV, which interacts with the viral DNA to form the nucleocapsid complex (Robinson, 1990). The product encoded by both the preC and C region displays e antigenic specificity (HBeAg). The variable size of this protein (from 16 to 25 kDa) results from post-translational modifications (Uy *et al.*, 1986; Robinson, 1990). Following cleavage of amino acids encoded by the preC region, as well as the 34 residues encoded by the 3'-end of the C region, serum-HBeAg has a molecular weight of 16 kDa. Although the amino acid sequences of the core and e proteins are largely identical, the e protein is immunologically distinct from HBcAg at the level of the B cell response, leading to the production of anti-HBe and anti-HBc specific antibodies (Salfeld *et al.*, 1989). Furthermore, the HBeAg can be detected as a nonstructural protein freely circulating in the blood (16 kDa) and in infected hepatocytes (16 and 25 kDa). In contrast, HBV cores do not circulate as free particles, but they occur within complete virions and in infected hepatocytes (Schlicht & Schaller, 1989). In addition, it has been established that the core protein is important in viral

replication, while the e protein is not required in virus assembly (Robinson, 1990).

The P ORF is translated into a viral polymerase enzyme from the 3.5 kb HBV RNA transcript. This protein is important in replication and encapsidation of the viral genome. It functions as a DNA-dependent DNA polymerase, reverse transcriptase, and RNase H (Bavand & Laub, 1988; Mack *et al.*, 1988). The HBV polymerase also binds to the 5' end of the 3.5 kb pregenomic RNA and serves as a packaging signal (Bartenschlager *et al.*, 1990), and acts as a primer for reverse transcription of the viral pregenome (Wang & Seeger, 1992).

The smallest ORF, the X gene, encodes a transcriptional activating protein (reviewed in Henkler & Koshy, 1996). The X gene product was found to be able to indirectly activate transcription factors such as AP-1 via mitogen-activated protein kinases (MAP kinases) (Benn *et al.*, 1996). In addition, the X protein also complexes with the p53 tumor suppressor protein and inhibits its sequence-specific DNA-binding capacity and transcriptional activation function *in vitro* (Wang *et al.*, 1994). It was also shown that the X gene product may interfere with p53 gene and inhibit apoptosis in X-transfected cells (Wang *et al.*, 1995). The X protein is postulated to be a cofactor in HCC development since this protein binds directly to a DNA repair factor protein (Lee *et al.*, 1995) and that X protein-transfected cells have impaired ability to repair ultraviolet radiation-induced DNA damage (Jia *et al.*, 1999). Thus, it is believed that the X protein may participate in one of the many mechanisms of HBV-induced hepatocarcinogenesis (reviewed in Henkler & Koshy,

1996; Feitelson, 1999).

1.4.4 Life cycle of HBV.

Hepadnaviral replication involves DNA replication through a reverse transcription mechanism that is unique for DNA viruses. The life cycle of HBV in a cell begins with viral entry. Although the initial steps of HBV cell attachment and entry are not fully understood, there is evidence of host recognition domains on the viral envelope and the involvement of one or more virus-specific receptor(s) on the host cells (see Sections 1.7.2, 1.7.3, and 1.7.4). After entry, the virion envelope is removed in the cytoplasm and the nucleocapsid containing rcDNA is transported into the nucleus. The partially double stranded species is released and repaired by host DNA polymerases and ligases to form a covalently closed circular DNA (cccDNA), which is fully double stranded. Using that cccDNA as a template, viral mRNA and RNA pregenomes are transcribed using host RNA polymerase. In HBV, there are 4 mRNA transcripts, 3.5, 2.4, 2.1, and 0.7 kb in length. The mRNA transcripts are translated into viral proteins including the three envelope proteins, the core, and X protein (reviewed in Pugh & Bassendine, 1990). Viral packaging is dependent on the 5'-proximal stem loop structure of the HBV pregenomic RNA (the ε), which binds to the hepadnaviral polymerase (Nassal & Schaller, 1995). This interaction causes capsid formation around the RNA-polymerase complex. Subsequently, these encapsidated particles are transported into the cytoplasm.

The ε structure also serves as a template for the synthesis of a short DNA primer by the viral polymerase (Nassal & Schaller, 1995). Then, reverse transcriptase uses the viral RNA pregenome as a template for the synthesis of the minus DNA strand. After cleavage of RNA-DNA hybrids by RNaseH, the plus strand of DNA is synthesized from the minus strand using a 5' RNA oligoribonucleotide as a primer, forming rcDNA. The mature nucleocapsid particles, containing rcDNA, are then packaged in the endoplasmic reticulum into envelope proteins along with host cell lipids to form complete virions. The complete Dane particle is then transported through the endoplasmic reticulum and exported from the cell (reviewed in Pugh & Bassendine, 1990). The rcDNA species can also be transported back into the nucleus where they can serve as the precursor for the formation of a pool of viral cccDNA. Since hepadnaviral cccDNA is a renewable template, there is no need for a large pool of cccDNA in the nucleus (Tuttleman *et al.*, 1986b).

The conversion of cccDNA from rcDNA is considered to be the first step of hepadnavirus replication. Thus, detection of cccDNA within a cell is used as an indicator of active viral replication (Tuttleman *et al.*, 1986b).

1.4.5 Lymphotropism of HBV.

Although the main site of HBV replication is the liver, HBV is also lymphotropic and is known to replicate in the lymphatic system. Earlier studies using nucleic acid hybridization techniques demonstrated the presence of HBV

DNA sequences in lymphoid cells, such as PBMC, obtained from patients with chronic HBV infection (Pontisso *et al.*, 1984). Later, it was documented that lymphoid cells can be sites of active viral replication since HBV DNA and RNA were found in some patients using Southern and Northern hybridization, respectively (Lobbiani *et al.*, 1990). This was also confirmed by *in situ* hybridization technique (Hadchouel *et al.*, 1988). When more sensitive polymerase chain reaction (PCR) assays were utilized, HBV nucleic acids were found in all chronically infected patients (Pasquinelli *et al.*, 1990). More significantly, HBV transcriptional products also detected in PBMC of patients with chronic hepatitis B infection using PCR with reverse transcription step (RT-PCR) (Baginski *et al.*, 1991). More recently, the presence of HBV cccDNA and HBV mRNA in circulating lymphoid cells from patients with high viremia were identified using PCR with primers that span the HBV nick region and with quantitative RT-PCR, respectively (Stoll-Becker *et al.*, 1997). In this work, all HBV mRNA transcripts (i.e., 3.5, 2.4, 2.1 and 0.7-kb species) were found in circulating lymphoid cells. Interestingly, the X mRNA (0.7 kb) was detected in the highest amount.

The detection of HBV nucleic acid sequences in lymphoid cells was supported by identification of viral translational products in or on the cells. In one of the studies, most of the individuals with chronic hepatitis B tested were reported to express HBsAg and HBcAg in their PBMC (Parvaz *et al.*, 1987). In another report, HBV large envelope proteins were shown to exist in PBMC lysates prepared

from chronic HBV carriers using Western blot analysis (Zoulim *et al.*, 1991).

The PBMC subsets frequently found to carry replicative HBV DNA intermediates in chronically infected individuals are still not clearly established. One laboratory showed that CD4+, as well as CD8+ T cells, B cells and monocytes contain HBV DNA sequences, as detected by Southern hybridization (Calmus *et al.*, 1994). In another report, monocytes were apparently found to contain the highest levels of HBV DNA compared to T and B cells (Yoffe *et al.*, 1986).

In *in vitro* conditions, HBV production was demonstrated in cells derived from a bone marrow aspirate of a patient with acute hepatitis B. After 10 months in culture, these cells were found to be HBsAg positive, as detected by immunofluorescence, and secreted HBsAg-containing viral particles that had similar buoyant densities as intact virions (Dane particles), suggesting that the cells were able to synthesize complete, enveloped virus (Romet-Lemonne *et al.*, 1983). In addition, extrachromosomal forms of HBV DNA with migrational characteristics similar to rcDNA and cccDNA, were detected in these cells by Southern blot hybridization (Elfassi *et al.*, 1984). It was also shown that HBV-infected lymphocytes can transmit virus to non-infected lymphoid cells *in vitro*. This was demonstrated through the use of a HBV-infected lymphocyte hybridoma co-cultivated with HBV-negative T and B cells from a healthy individual (Colucci, *et al.*, 1988).

Furthermore, it was shown that culturing PBMC derived from chronically

infected patients with phytohemagglutinin (PHA) and concanavalin A (ConA) increased the levels of intracellular HBV DNA and HBV replicative intermediates (Bouffard *et al.*, 1992). It was also found that HBV can persist in the PBMC in the absence of hepatic HBV replication. This was observed in patients with chronic hepatitis B that underwent orthotopic liver transplantation. During the follow-up of 2-24 months, 23 out of 24 patients had no HBV DNA in their livers and remained serum HBsAg-negative. However, PCR analysis identified HBV DNA in some PBMC samples from these patients indicating HBV persistence in the lymphatic system in the absence of virus in the liver (Feray *et al.*, 1990). HBV persistence was also observed in two patients with end-stage liver disease following chronic HBV infection who accepted baboon liver transplants. In these instances, HBV DNA sequences were detected post-transplantation in the PBMC, spleen, and bone marrow, but not in a liver biopsy of the transplanted baboon liver (Lanford, *et al.*, 1995). These findings strongly suggest that cells of the lymphatic system can serve as a site of HBV replication.

HBV-specific DNA and RNA sequences were also identified in PBMC from patients years after complete clinical and serological recovery from acute hepatitis B, indicating the persistence of replicating virus in lymphoid cells in convalescent, anti-HBs positive individuals (Michalak *et al.*, 1994; Reherman *et al.*, 1996; Penna *et al.*, 1996; Cabrerizo *et al.*, 1997; Yotsuyanagi *et al.*, 1998). It was also reported that HBV DNA sequences can persist in PBMC of patients with either spontaneous

or therapy-induced recovery from chronic hepatitis B (Trippler *et al.*, 1996; Oesterreicher *et al.*, 1995). These data indicate that the lymphoid system plays an important role in the establishment and maintenance of hepadnaviral persistence.

In general, the findings summarized above clearly demonstrate that HBV is not only hepatotropic, but also can infect lymphoid cells and that these cells can support persistent virus replication. The pathogenic implications of HBV lymphotropism are not yet fully understood.

1.5 HEPADNAVIRUS FAMILY.

HBV is the prototypic virus of *Hepadnaviridae*. These DNA viruses characteristically have a very narrow host range. For example, HBV infects only humans, chimpanzees and some other higher primates (Barker *et al.*, 1975).

The hepadnaviral family is divided into two genera: Orthohepadnaviridae (mammalian) and Avihepadnaviridae (avian). The mammalian genus include hepadnaviruses replicating in the following hosts: HBV in humans, WHV in eastern North American woodchucks (*Marmota monax*) (Summers *et al.*, 1978), and ground squirrel hepatitis virus (GSHV) in *Spermophilus beecheyi* (Marion *et al.*, 1980) and in *Spermophilus richardsonii* (Tennant *et al.*, 1991). To this genera are also included related viruses; such as the tree squirrel hepatitis virus (TSHV) infecting *Sciurus carolinesis* (Feitelson *et al.*, 1986), arctic ground squirrel hepatitis virus (ASHV) in *Spermophylus parryi kennicotti* (Testut *et al.*, 1996), and woolly monkey

hepatitis B virus (WMHBV) in *Lagothrix lagotricha* (Lanford *et al.*, 1998). The avian hepadnaviruses include: duck hepatitis B virus (DHBV) found in *Anas domesticus* (Mason *et al.*, 1980), heron hepatitis virus (HHBV) in *Ardea cinerea* (Sprengel *et al.*, 1988), and the more recently discovered maned duck hepatitis B virus (MDHBV) in *Chenonetta jubata* (Li *et al.*, 1997), grey teal hepatitis B virus (GTHBV) in *Anas gibberifrons gracilis* (Li *et al.*, 1997), stork hepatitis B virus (STHBV) in *Ciconia ciconia* (Pult *et al.*, 1998), and the snow goose hepatitis B virus (SGHBV) in *Anser caerulescens* (Chang *et al.*, 1998).

These viruses, particularly mammalian viruses, have common molecular, structural, antigenic, and pathogenic properties. The shared features are: (1) virion ultrastructure characterized by an envelope surrounding a spherical inner nucleocapsid; (2) small, partially double-stranded DNA genome ranging from 3 to 3.3 kb; (3) genomic organization and nucleotide sequence homology; (4) similar replication strategy; (5) identical number of structural and non-structural proteins which have homologous sequences; (6) antigenic cross-reactivity and comparable patterns of antiviral antibody responses; and (7) similar profile of liver disease (Tiollais *et al.*, 1985).

In contrast to mammalian viruses, avian hepadnaviruses do not have the X gene, they have a longer C gene sequence, and the S gene does not contain a preS1 region. Avian hepadnaviral envelope proteins do not form filaments in circulation. Furthermore, avian hepadnaviruses have no association with the

development of HCC (Cova *et al.*, 1993).

1.6 THE WOODCHUCK MODEL OF HEPATITIS B.

1.6.1 Woodchuck hepatitis virus (WHV).

The woodchuck hepatitis virus (WHV) was first identified in 1977 in a colony of eastern North American woodchucks (*Marmota monax*) in the Philadelphia Zoological Garden. In these animals, a high incidence of chronic hepatitis and HCC were observed (Summers *et al.*, 1978). Among all the members of the hepadnavirus family, WHV is accepted as the most suitable model to study HBV as the viruses share significant similarities with regards to ultrastructure, genomic organization, antigenic cross-reactivity of gene translation products, range of targeted organs (liver and lymphatic system), course of infection, the immunovirological and pathological features of the virus-induced liver disease, and the development of HCC (Michalak, 1998).

The ultrastructure of WHV is similar to HBV. Mature virions of both viruses are spherical enveloped particles with diameters of 45 nm for WHV and 42 nm for HBV. The circular WHV genome is 3.3 kb and is slightly longer than the HBV DNA (Fig. 1.1). It has a similar genomic organization as HBV and shares approximately 70% nucleotide sequence homology with HBV (Galibert *et al.*, 1982). In WHV, the molecular sizes of WHV envelope large proteins are 42 and 39 kDa, middle proteins are 36 and 33 kDa, and the small proteins are 26, 23, and 19 kDa

(Michalak & Lin, 1994). These proteins demonstrate significant antigenic cross-reactivity with the equivalent proteins of HBV found in humans. This enables the use of cross-reactive commercial assays designed for detection of HBsAg and antibodies to HBsAg (anti-HBs) (*e.g.* AusRIA and AUSAB; Abbott Laboratories, North Chicago, Illinois) to identify WHV surface antigens (WHsAg) and antibodies to WHsAg (anti-WHs), respectively. HBcAg and WHV core antigens (WHcAg) have also been shown to have common antigenic determinants (*e.g.* Werner *et al.*, 1979).

1.6.2 Characteristics of WHV infection.

The epidemiology of WHV infection also bears significant similarities with HBV-induced disease. WHV is transmitted by blood or body fluids and is spread vertically from infected mothers to their offspring (Kulonen & Millman, 1988); including mothers convalescent from hepatitis (Coffin & Michalak , 1999). In up to 90% of animals born to WHsAg-positive, chronically infected mothers or those which were infected during the first few days of life, serologically evident (*i.e.*, WHsAg positive with antibodies against WHcAg [anti-WHc]) hepatitis will develop. Inevitably, most of these animals will subsequently progress to HCC within 2-3 years, a situation that occurs at a much higher frequency than that observed in humans (Cova *et al.*, 1993).

Adult woodchucks infected with WHV have courses of infection and liver

disease comparable to adult humans afflicted with HBV. Both WHV and HBV induce acute hepatitis, which 10-15% of the time progresses to serologically evident chronic liver disease. Analogous to HBV-infected humans, woodchucks persistently infected with WHV exhibit chronic liver inflammation with different degrees of hepatic injury and lymphomononuclear cell infiltrations (Michalak, 1998). However, in contrast to humans, woodchucks chronically infected with WHV do not develop liver cirrhosis (Summers, 1981). Among hepadnaviruses, WHV has the highest oncogenic potential, which is believed to be caused by *cis*-activation of cellular oncogenes, particularly *c-myc* and *N-myc*, through the integration of viral promoter sequences in the vicinity of these host oncogenes (Hsu *et al.*, 1988; Fourel *et al.*, 1990). In contrast, HBV appears to integrate into host DNA in random patterns, thus, *cis*-activation mechanisms are probably not involved in hepatocarcinogenesis in humans (reviewed in Feitelson & Duan, 1997). Current evidence suggest that both viral and host-related factors contribute to divergent tumorigenicity of hepadnaviruses in different species (Feitelson, 1999).

At the cellular level, hepatocyte plasma membranes purified from WHV infected woodchucks were shown to express WHsAg, WHcAg, and WHeAg. Both envelope and core specific polypeptides were found to be associated with the membrane lipid bilayer as peripheral and integral membrane proteins (Michalak & Churchill, 1988; Michalak *et al.*, 1990; Michalak & Lin, 1994).

WHV is highly hepatotropic, although the host lymphoid system is also

involved from the earliest stages of virus infection (Korba *et al.*, 1989a; Chemin *et al.*, 1993; Michalak, 1998). WHV DNA, replicative DNA intermediates, and virus-specific mRNA sequences were detected in circulating and in organ lymphoid cells. Increased expression of these WHV nucleic acid sequences was demonstrated in PBMC cultured in the presence of mitogens, such as lipopolysaccharide (LPS) (Korba *et al.*, 1988). In addition, WHV DNA reactive particles with physicochemical properties of complete virions and abilities to induce classical acute hepatitis in naive woodchucks can be generated in cultured PBMC derived from convalescent animals (Michalak *et al.*, 1999; Coffin & Michalak, 1999). These results indicate that even transient exposure to WHV can result in persistent infection of the lymphatic system and the production of trace amounts of pathogenic virus.

Currently, the woodchuck model is accepted as the most valuable natural system to study hepadnavirus-host interactions, pathogenicity of liver injury and HCC, mechanisms of hepadnaviral persistence, and to test the efficacy of anti-HBV drugs.

1.7 HEPADNAVIRUS-HOST CELL INTERACTIONS.

Several different hepadnaviral envelope protein epitopes have been proposed as sites which interact with host cell receptor molecules. Also, several putative cellular receptors have been proposed which bind to hepadnavirus (HBV and DHBV) envelope proteins. However, not all of these cellular molecules fulfill

the criteria of receptor interaction, such as ligand specificity, saturation kinetics and competitive binding.

1.7.1 Candidate receptors for the preS domain of the DHBV envelope protein.

In contrast to the three envelope proteins present in mammalian hepadnaviruses (see Section 1.4.3), DHBV has only two structurally related envelope proteins, called the large and small envelope protein (preS/S and S domains, respectively). The preS domain appears to play an important role in host cell recognition. This was demonstrated with recombinant subviral particles containing only the large preS/S envelope protein that were able to compete and inhibit *in vitro* infection of cultured duck hepatocytes by DHBV. Recombinant subviral particles containing just the small protein failed to inhibit infection of the cells (Klingmuller & Schaller, 1993). In addition, binding of radiolabelled subviral particles prepared from serum to primary duck hepatocytes was inhibited by the recombinant particles containing large proteins. These results confirmed that the preS domain of the DHBV envelope was essential for virus attachment and entry. PreS domain-mediated entry did not require low pH activation, since inhibition of the endosomal function in duck hepatocytes failed to block DHBV uptake and viral cccDNA synthesis (Kock *et al.*, 1996b). Thus, the attachment and host cell penetration occurs at the plasma membrane at neutral pH. It is believed that the preS host binding domain relies upon quaternary structure because complexes of

recombinant large polypeptide were 30-fold more efficient in blocking DHBV infection when compared to monomeric recombinant large proteins (Urban *et al.*, 1998).

Carboxypeptidases are one group of the proteins that have been suggested to be involved in DHBV binding. A 170-kDa protein (p170) was identified in duck hepatocyte lysates and found to interact with the DHBV envelope preS domain (Tong *et al.*, 1995). However, this p170 protein was also present in extrahepatic tissues, such as pancreas, kidneys, and spleen. Furthermore, in competitive binding studies, the attachment of hepatocytes to DHBV abolished the interaction between the virus and p170, which may suggest that p170 is the binding site for DHBV. Purification and sequencing of isolated p170 revealed four different peptide sequences that have homologies to human and animal carboxypeptidases H, M, and N (Tong *et al.*, 1995).

Another candidate receptor for DHBV is carboxypeptidase D (Kuroki *et al.*, 1994). Co-precipitation of labelled duck hepatocyte proteins and DHBV virions using monoclonal antibodies against DHBV envelope proteins identified a 180-kDa hepatocyte glycoprotein (gp180) whose interaction was dependent on the preS domain of the DHBV large protein. In addition, gp180 was able to compete and inhibit infection of primary duck hepatocytes by DHBV (Breiner *et al.*, 1998). Transfection of a human hepatoma cell line (HuH7) with gp180 allowed binding of DHBV and internalization of viral particles, although no evidence of productive

DHBV infection was obtained. It was suggested that virus-gp180 interaction likely requires the presence of a cell membrane-associated co-receptor, since gp180 was found in the trans-Golgi network but not on the cell surface (Eng *et al.*, 1999). Although gp 180 has been identified as a homolog of the mammalian metallocarboxypeptidase D, the role of carboxypeptidases in HBV receptor binding has not yet been demonstrated.

In another experiment, purified DHBV large protein conjugated to Sepharose beads was used to precipitate a 120 kDa protein (p120) from duck hepatocyte lysates. This molecule was found to be expressed on duck liver, kidney and pancreas. Site-directed mutagenesis identified three amino acids located between residues 100 to 102 of the preS domain as being critical for the binding to p120 and substitutions of these residues abolished the preS-p120 interaction (Li *et al.*, 1996). Thus, analogous to mammalian hepadnaviruses (see Sections 1.7.2, 1.7.3, and 1.7.5) a short amino acid sequence of the DHBV envelope protein appears to constitute a host cell binding site.

Another putative DHBV binding molecule has been identified which has a molecular mass of 55 kDa. This protein was detected using a series of monoclonal antibodies which were generated against duck hepatocytes and screened for their ability to inhibit DHBV-host cell interaction in duck hepatocyte cultures (Guo & Pugh, 1997). Two monoclonal antibodies were identified. Subsequent immunoprecipitation using these antibodies resulted in the isolation of a 55 kDa

protein that appears to be expressed on duck hepatocytes and tissues such as (*e.g.*) kidney, pancreas and spleen.

1.7.2 Host cell recognition by the preS1 domain of HBV envelope.

Since the preS1 domain of the large envelope protein appears to be located most distal on the outside of the viral membrane (Schaeffer *et al.*, 1986; Jin *et al.*, 1996), this region is naturally predisposed to be involved in the host cell receptor recognition.

It has been proposed that the main HBV binding site for hepatocytes (HepG2 cells) is located between amino acid residues 21-47 of the preS1 domain, since this peptide and antibodies against preS1(21-47) competed or inhibited the binding between the cells and immobilized HBsAg (Neurath *et al.*, 1986b). In addition, a monoclonal antibody generated against this preS1(21-47) sequence was able to inhibit the binding of HepG2 cells to both HBsAg and preS1(21-47) peptide (Petit *et al.*, 1991). It was suggested that this monoclonal antibody could be useful for the generation of anti-idiotypic antibodies for the study of possible HBV receptors. Using this strategy, it was demonstrated that HBV preS1(21-47) sequence was able to recognize more than one protein species from HepG2 cells (Petit *et al.*, 1992). These antibodies reacted with a main protein species of 35 kDa and minor proteins with sizes of 40, 43 and 50 kDa. All the proteins were V8 protease sensitive and endoglycosidase H resistant. It was postulated that the identified HBV preS1

binding proteins may form a 3 dimensional structure composed of several proteins that can serve as a macro-receptor for HBV.

Another group also demonstrated that interleukin 6 (IL-6) can bind to the HBV preS1(21-47) sequence which in turn could bind to the IL-6 receptor (Neurath *et al.*, 1992a). This was revealed by studies demonstrating that the binding of synthetic preS1(21-47) peptide to Chinese hamster ovary cells transfected with human IL-6 cDNA (Neurath *et al.*, 1992b).

The same group also suggested a role for the immunoglobulin (Ig) A receptor in HBV-hepatocyte interaction. Amino acid sequence analysis revealed that a fragment of human IgA is homologous to HBV preS1(21-47) (Neurath & Strick, 1990) and that human IgA competed with the preS1(21-32) peptide for binding to human hepatocyte plasma membranes (Pontisso *et al.*, 1992). Moreover, when antibodies directed against HBV and IgA were used to map common epitopes, it was found that both the preS1 domain of the HBV large protein and IgA shared antigenic cross-reactivity. This raised the possibility that both HBV and IgA can utilize the IgA receptor to enter human liver cells (Pontisso *et al.*, 1992). However, results from another laboratory showed no direct role for IgA receptor in the binding of HBV to HepG2 cells, as IgA did not inhibit the interaction between HBV and the cells (Dash *et al.*, 1992). Instead, a 31 kDa protein was suggested to be involved in HBV attachment to HepG2 cells.

Another host molecule which may possibly be involved in HBV uptake is the

hepatic asialoglyoprotein receptor (ASGPR). Its possible contribution to virus attachment was shown by using two methods, a radioimmunoassay with HBV particles and solid phase-bound ASGPR and another enzyme immunoassay using biotin-labelled ASGPR and immobilized HBV (Treichel *et al.*, 1994). HBV binding to ASGPR was inhibited by ligands containing D-galactose (ASGPR ligand) and with anti-ASGPR antibodies. Furthermore, a monoclonal antibody against HBV large protein inhibited HBV binding to immobilized ASGPR. This observation was further confirmed when interaction between HBsAg and ASGPR was shown to be inhibited by asialofetuin, an ASGPR-specific ligand (Treichel *et al.*, 1997).

In addition to primary human hepatocytes and HepG2 cells, cells of extrahepatic origin, such as peripheral B lymphocytes and cultured B-cells, neuroblastoma, amnion, and embryonic carcinoma cell lines, were found to be able to bind HBsAg and preS1(21-47) peptide (Neurath *et al.*, 1990). Later, a shorter preS1(27-49) sequence of the HBV large protein was identified as a PBMC recognition site (Pontisso *et al.*, 1991).

Furthermore, the preS1(28-36) and preS2(120-145) synthetic polypeptides were shown to interact with a 50 kDa HBV binding factor (HBV-BF) found in human serum and on hepatocytes (Budkowska *et al.*, 1993). This HBV-BF was subsequently isolated, characterized, and found to be a neutral metalloproteinase and was used to digest purified HBV particles. This treatment cleaved HBV in the preS2(136-141) region and allowed HBV to bind and enter cultured T lymphocytes.

The result suggests that protease treatment of HBV can result in the generation of lymphotropic virus (Budkowska *et al.*, 1997).

It was also demonstrated that V8 protease-treated HBV virions were able to infect HepG2 cells and these cells displayed molecular markers of viral replication, such as HBV cccDNA and pregenomic RNA (Lu *et al.*, 1996). Immunofluorescent analysis of HBcAg and middle protein expression showed that 10-30% of cells were stained. This indicated that proteolytic treatment may facilitate the uptake of HBV by HepG2 cells.

1.7.3 HBV preS2 domain-host cell interactions.

Some reports have implicated the preS2 domain of the middle HBV envelope protein in host cell recognition. It was shown that antibodies against the preS2 region were able to neutralize a HBV inoculum that was subsequently injected into chimpanzees (Neurath *et al.*, 1986a). These findings raised the possibility that the preS2 region could be important in the establishment of infection. In addition, anti-idiotypic antibodies that contained an internal image of the preS2(120-126) amino acid sequence were found to interact with the extracellular matrix fibronectin of human liver sinusoids (Budkowska *et al.*, 1995). The authors suggested that fibronectin which is present on the liver sinusoidal endothelium may facilitate HBV binding to hepatocytes.

There have also been many reports suggesting the interaction between the

middle envelope protein of HBV and polymerized human serum albumin (pHSA) which would in mediate recognition by the hepatocyte (Machida *et al.*, 1983). For example, it was shown that albumin, with antigenicity similar to that of pHSA, is a component of the HBV envelope and is likely incorporated into the viral membrane during HBsAg synthesis in the cytoplasm of infected hepatocytes (Michalak *et al.*, 1980). Other studies have shown that pHSA binds to human liver cells (Trevisan *et al.*, 1982) and that receptors specific for serum albumin polymerized by treatment with glutaraldehyde exist on the surface of rabbit (Lenkei *et al.*,1977), human (Thung & Gerber, 1983) and woodchuck hepatocytes (Michalak & Bolger, 1989). In addition to pHSA, a monomeric form of HSA was found to interact with the preS2 domain of HBV envelope (Krone *et al.*, 1990), suggesting that both monomeric, native albumin and its polymer may facilitate HBV entry into hepatocytes.

1.7.4 Host cell binding by the HBV small envelope protein.

Endonexin II (E-II) or annexin V (molecular mass 34 kDa) was purified from human hepatocytes and was identified as a hepatocyte protein that specifically bound the small protein of HBV envelope in a calcium dependent manner (Hertogs *et al.*, 1993). In addition, native E-II successfully competed with isolated human hepatocytes and purified human liver plasma membranes for the binding of HBsAg particles formed by the major envelope protein. Similar results were also obtained when recombinant E-II was used (Leenders & de Bruin, 1994). In addition, anti-

idiotypic antibodies developed in chickens immunized with IgG of rabbit antibodies to human liver E-II were able to compete with liver-derived E-II for binding to the virus small envelope protein (Hertogs *et al.*, 1994). This observation was supported by inhibition of the interaction between the HBsAg particles and human hepatocytes with both the anti-E-II and the anti-idiotypic antibodies (de Bruin *et al.*, 1995). More recently, non-permissive rat hepatocytes that were transfected with human annexin V DNA and subsequently exposed to HBV, exhibited active viral replication (Gong *et al.*, 1999). These cells expressed HBV mRNA and cccDNA and secreted HBV DNA-reactive molecules into the culture medium. In addition, approximately 10-15% of these cells were positive for HBsAg and HBcAg when tested by immunocytochemical staining. These data indicated that the host specificity exhibited by HBV is most likely determined at the level of the initial virus-host interaction and is mediated by annexin V.

In another study, a 46-kDa protein of hepatocyte plasma membranes was identified as a HBsAg binding molecule (Mehdi *et al.*, 1994). It was reported that this 46 kDa protein is apolipoprotein H (apoH). Since this molecule can be co-purified with HBsAg, and lipoproteins are taken up by hepatocytes during normal lipid trafficking, it was suggested that interaction of HBV envelope with apoH may be a mechanism of viral entry into host hepatocytes.

Extrahepatic binding sites for the small protein were also found on cells other than hepatocytes, such as fibroblasts (Leenders *et al.*, 1990), PBMC (Leenders *et*

al., 1990), and primate kidney cells (Vero and CV-1) (Peeples et al., 1987).

1.7.5 Host and cell-type specific recognition by WHV envelope proteins.

As in the case of HBV, the nature of WHV-host cell interactions and their contribution to virus host interactions and cell tropism are not yet completely understood. In this laboratory, albumin is consistently detectable in highly purified WHsAg particle preparations (Lew & Michalak, unpublished data). It was also reported that normal woodchuck hepatocyte plasma membranes exhibited receptors for glutaraldehyde-polymerized woodchuck serum albumin (pWSA) (Michalak & Bolger, 1989). Two classes of biochemically distinct binding sites on the host cell for pWSA were identified. A high-affinity, low-density receptor was found to be glycoprotein in nature and behaved as an integral plasma membrane protein. A low-affinity, high-density binding site, which behaved as a peripheral membrane protein, was activated by lipase, suggesting that the binding site activity was constrained by membrane lipids.

In addition, the involvement of a woodchuck-specific 330-kDa proteoglycan in the binding of WHsAg particles to woodchuck hepatocytes was identified (Desousa & Michalak, 1990). The glycan interacted with WHV envelope via its N-linked polymannose and O-linked heparan sulfate. The binding of WHsAg particles to both the isolated 330-kDa proteoglycan and purified woodchuck hepatocytes plasma membranes was inhibited by heparin (Michalak, 1998).

In another series of studies, the extreme N-terminus of WHV large envelope protein was determined to carry a host and cell-type specific recognition site (Michalak *et al.*, 1991; Jin *et al.*, 1996). The site was mapped to residues 10-13 of the preS1 domain of the protein and named cell binding site 1 (CBS1). Synthetic analogues containing the sequence Asn-Pro-Asp-Lys (NPDK) were shown to bind to woodchuck hepatocytes and lymphoid cells in a species-restricted manner which followed characteristics of a typical ligand-receptor binding, such as ligand specificity, saturability and competition.

Interestingly, this sequence consistently demonstrated much greater ability to interact with woodchuck splenic and circulating lymphoid cells than with hepatocytes, suggesting that the lymphatic system could be the preferred target of WHV. Antibodies raised against synthetic N-terminal preS1(1-25) sequence recognized the CBS1 and proteolytic or acidic-treated subviral WHsAg particles (Jin *et al.*, 1996). Interactions between antibodies against preS1(1-25) sequence and protease-modified WHV envelope particles or synthetic analogues of the CBS1 were inhibited by extracts from woodchuck hepatocytes, splenocytes and PBMC, but not those from woodchuck kidney or from human or rat cells. These findings implied that proteolytic cleavage could be required to activate the CBS1 site prior to its interaction with targeted host molecules. Preliminary affinity chromatography experiments with woodchuck PBMC lysates revealed that an 89-kDa molecule on woodchuck lymphoid cells is recognized by WHV CBS1 (Michalak, 1998).

Although some of the above results are promising, receptors for HBV and WHV have yet to be convincingly identified. This important issue will require further studies when appropriate *in vitro* infection systems become available.

1.8 *IN VITRO* INFECTION OF CULTURED HEPATOCYTES BY HEPADNAVIRUSES.

1.8.1 DHBV infection of primary duck hepatocytes.

In vitro infection of primary duck hepatocytes with DHBV has been well-established since 1986. Active replication of DHBV in these hepatocytes was documented by identification of viral DNA, viral replicative DNA intermediates, including cccDNA and virus-specific mRNA (Tuttleman *et al.*, 1986a). This *in vitro* infection system has been validated in several laboratories and contributed to the identification of putative DHBV receptors (as summarized in Section 1.7.1, see Section 4.3).

1.8.2 Hepadnavirus infection of mammalian hepatocytes.

Establishment of equivalent culture systems for the propagation of mammalian hepadnaviruses has proven to be much more difficult. This is mainly because of technical obstacles in maintaining differentiated mammalian hepatocytes in culture.

In general, using primary human hepatocytes and infectious HBV inocula

derived from sera of chronically infected patients, only very limited HBV propagation has been documented in some studies (see below). It has been shown that chemical agents that increase cell membrane permeability, such as dimethyl sulfoxide (DMSO) (Gripon *et al.*, 1988; Rumin *et al.*, 1996) and a mixture of DMSO and polyethylene glycol (PEG) (Gripon *et al.*, 1993), can enhance HBV synthesis in cultured primary human hepatocytes. Although the above *in vitro* infection systems could be useful for the study of events downstream of viral entry, they have not yet meaningfully contributed to the understanding of virus-cell attachment and virus penetration.

There are also limited reports on low-level HBV replication in naive (chemically untreated) primary human hepatocytes and a human hepatoma cell line (HepG2 cells) exposed *in vitro* to the serum-derived virus (see below and Section 4.3). These systems should more accurately imitate the natural virus-host interactions and can be potentially used to identify cell surface receptors for HBV.

Primary cultures of cryopreserved human hepatocytes were also reported to be permissive for *in vitro* HBV infection (Rijntjes *et al.*, 1988). Productive infection was shown by the detection of HBcAg in the nuclei and HBsAg in the cytoplasm by immunostaining with specific antibodies and by identification of HBV DNA by *in situ* hybridization and Southern blot analysis. Furthermore, 42-nm virion-like particles were revealed by electron microscopy in the culture supernatants of these cells.

In yet another report, HBV virions derived from a human hepatoma cell line

which stably supports HBV replication (HB 611 cells), were used to infect primary human fetal hepatocytes (Ochiya *et al.*, 1989). It was demonstrated that the hepatocytes inoculated with virus expressed HBV-specific RNA and secreted both HBsAg and HBeAg, 3-4 days after infection. Moreover, virus DNA replicative intermediates accumulated progressively in the cells during the 16-day observation period.

In a separate study, HBV inocula derived from sera of chronic carriers were used for infection of primary human hepatocytes (Mabit *et al.*, 1996). It was found that serum-derived HBV induced viral replication and that the HBV cccDNA load increased between 4 and 8 days post-inoculation (d.p.i). The authors suggested that events downstream from viral uptake, such as the amplification of HBV cccDNA, may be the limiting step in the viral replicative cycle. This may be due to host cell factors that are involved in the regulation of virus reverse transcription and translation of viral proteins.

Other experiments, using human hepatoma HepG2 cells as targets, demonstrated also that this cell line is susceptible, to some degree, to infection with serum derived-HBV (Bchini *et al.*, 1990). These cells were kept for several months after inoculation and were able to express low levels of HBV cccDNA and mRNA specific sequences, as well as HBsAg. Furthermore, complete viral particles containing HBV DNA and DNA polymerase activity were secreted by these cells. Infection of HepG2 cells with serum-derived HBV was also reported to be able to

generate a cell line that grew out of infected HepG2 cells following a massive cell death that occurred at 35 d.p.i. These cells have integrated HBV DNA sequences and secrete HBV envelope proteins (Mabit *et al.*, 1994).

Recently, productive HBV infection of primary hepatocytes from tree shrews (*tupaia belangeri*) was reported. It was shown that the infection resulted in viral DNA and RNA synthesis and the secretion of HBsAg and HBeAg (Walter *et al.*, 1996).

In the case of other hepadnaviruses, WHV and GSHV were found to infect, with limited success, primary woodchuck hepatocytes in culture (Aldrich *et al.*, 1989). Infection with these viruses was demonstrated by showing the presence of viral cccDNA sequences at 2 d.p.i and an accumulation of intracellular viral DNA intermediates at 7-10 days after infection.

Infectivity of lymphoid cell-derived hepadnaviruses to homologous hepatocytes and characteristics of this infection have not yet been investigated in *in vitro* conditions.

1.9. PURPOSE OF THE STUDY.

Although the lymphotropism of WHV has been well documented in this and other laboratories, the significance of the lymphatic system in infection, pathogenicity, and the progression of WHV-induced disease remains to be established. It is expected that lymphoid cells can serve as a site of persistent hepadnavirus replication and as a reservoir of infectious virus. Thus, it would be advantageous to develop an *in vitro* model to study the hepatotropism and infectivity of virus derived from lymphoid cells. This *in vitro* system would be valuable to study hepadnavirus-host cell interactions, and also for testing potential therapeutic agents which interfere with the virus life cycle. Since there is no host immunological pressure to prompt viral adaptive mutations, virus propagated in culture could be more genetically stable and, thereafter, more desirable for *in vitro* and *in vivo* studies.

The main objectives of this work were to design and establish an *in vitro* experimental system allowing WHV propagation in woodchuck lymphoid cells and hepatocytes and to investigate the ability of lymphoid cell and serum-derived WHV to invade cultured woodchuck hepatocytes. In addition, WHV-hepatocyte interactions and infectivity of the virus originating from *in vitro* infected hepatocytes were studied.

The specific aims of this study were as follows:

1. to design cell culture conditions enabling *in vitro* infection of cultured woodchuck hepatocytes with WHV and to co-culture WHV-infected woodchuck lymphoid cells with virus-naive homologous hepatocytes.

2. to adopt molecular and immunological methods for the detection of WHV replication and identification of its gene translation products in cultured cells.

3. to test the *in vitro* and *in vivo* infectivity of WHV propagated in hepatocyte cultures.

4. to investigate the initial WHV-cell interaction using cultured woodchuck hepatocytes as targets.

CHAPTER TWO : MATERIALS AND METHODS

2.1 ANIMALS.

All woodchucks used in the study were kept in a colony maintained by the Molecular Virology and Hepatology Research Laboratory at the Health Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland. The animals were housed under environmental and biosafety conditions established specifically for this species and fed with a herbivore diet *ad libitum* supplemented with fresh vegetables twice weekly (Michalak & Churchill, 1988; Michalak & Bolger, 1989; Michalak *et al.*, 1990; Michalak & Lin, 1994; Michalak, 1998).

Animals infected with WHV were kept separately from healthy woodchucks. Chronically infected animals were defined as those with continuous presence of circulating WHsAg, anti-WHc antibodies, and WHV DNA for at least 6 months prior to the start of the experiment. Diagnosis of chronic hepatitis was supported by histological examination of liver biopsies that demonstrated typical features of protracted necroinflammatory liver injury. Healthy woodchucks had no serological markers of current or past WHV infection, i.e., they were negative for WHsAg, anti-WHc and anti-WHs antibodies. In addition, DNA extracted from sera, PBMC, and liver biopsies from these animals were WHV DNA non-reactive, as tested by nested PCR with WHV genome specific primers (Section 2.13.2) and Southern blot analysis of the amplified PCR products (Section 2.14.2).

2.2 SAMPLE COLLECTION.

2.2.1 Collection of blood.

Blood was obtained under general inhalant anaesthesia with isofluorane (CDMV Inc., St. Hyacinthe, Quebec) from the digitalis vein using a butterfly catheter. Approximately 5 ml of blood was collected into a tube with no additives (red top Vacutainer®; Becton Dickinson, Rutherford, New Jersey) and used for isolation of serum, as described below. An additional 5 to 10 ml of blood was collected into tubes containing sodium ethylenediamine tetra-acetic acid (EDTA; lavender top Vacutainer®; Becton Dickinson) from which plasma and PBMC were isolated, as described in Section 2.2.3.

2.2.2 Serum isolation.

Blood was allowed to clot at room temperature for approximately 1 hour (h). The tube was then centrifuged at 720 *x* g for 10 minutes (min). The isolated serum was aseptically aliquoted to small volumes and stored at -70°C until use.

2.2.3 Isolation of peripheral blood mononuclear cells.

Approximately 5 ml of uncoagulated blood was overlaid on 4 ml of Ficoll-Paque® (Pharmacia Biotech, Baie d'Urfé, Quebec) and spun at 330 *x* g for 30 min. The top layer consisting of plasma was collected and stored at -70°C. The interface containing peripheral blood mononuclear cells (PBMC) was transferred to a new

tube, diluted with 10 ml of sterile phosphate buffered saline with EDTA (PBS-EDTA; 0.15 M NaCl, 2 mM NaH_2PO_4, 8 mM Na_2HPO_4, pH 7.4, with 1 mM EDTA), and centrifuged at 330 *x* g for 5 min. The resulting pellet was suspended in 3 ml of ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, pH 7.3, with 0.1 mM EDTA) and red blood cells were lysed at room temperature for 10 min. Then, 10 ml of PBS-EDTA was added to the tube and PBMC were washed by pelleting under the same conditions as indicated above. The final cell pellet was suspended in 1 ml of Hanks' balanced salt solution (HBSS; Gibco BRL, Burlington, Ontario). A 4-µl sample of the suspension was diluted 1:50 with 0.1% trypan blue and the number of viable cells counted using a hematocytometer, as described for hepatocytes in Section 2.3.6.1. In the case of PBMC derived from WHV-infected animals, approximately 3 x 10^7 cells were used immediately for DNase and limited trypsin digestion (Section 2.4.1) to remove any possible cell surface-attached WHV virions and free WHV DNA fragments and then, PBMC were cultured as described below in Sections 2.4.2. The remaining cells were frozen at approximately 1 x 10^7 cells/ml in a mixture of 90% heat-inactivated (at 56°C for 1 h) fetal calf serum (FCS-HI; Gibco BRL) and 10% DMSO (Sigma Chemical Co., St. Louis, Missouri) at -70°C and transferred to liquid nitrogen the next day for long-term storage. Frozen cells were used for analysis of WHV nucleic acid expression, as described in Sections 2.13 & 2.14.

2.2.4 Collection of specimens at autopsy.

For autopsy, animals were injected with a xylazine and ketamine mixture. Then, 50-100 ml of blood was collected by heart puncture and used for isolation of serum, plasma, and PBMC, as described in Sections 2.2.2 and 2.2.3. After opening the abdominal cavity, organs were removed under aseptic conditions. Typically, the following tissues were collected: liver, spleen, kidneys, small intestine, pancreas, lymph nodes, bone marrow, and skeletal muscle and frozen promptly in liquid nitrogen for subsequent nucleic acid analyses. Approximately two-thirds of the removed spleen was used immediately for isolation of splenic lymphoid cells (splenocytes), as described below (Section 2.2.5).

2.2.5 Isolation of splenic lymphoid cells.

In selected cases, splenocytes were isolated from animals chronically infected with WHV using a previously reported procedure (Michalak *et al.*, 1995). For this purpose, the spleen was cut into small pieces under sterile conditions. The tissue fragments were suspended in 10 ml of PBS-EDTA and passed through a cell sieve fitted with a 50-mesh wire screen (Sigma Chemical Co.). The sieve was rinsed with another 10 ml of PBS-EDTA and 20 ml of this cell suspension was aliquoted into 5-ml samples and overlaid onto 4 ml of Ficoll-Paque®. The gradients were spun and splenocytes recovered from the gradients as described for PBMC in Section 2.2.3. Prior to culture, the cells were treated with DNase and trypsin to

remove possibly attached WHV virions and WHV DNA fragments, as presented in Section 2.4.1.

2.2.6 Liver biopsies.

Liver biopsies were obtained by surgical laparotomy under aseptic conditions. Each animal was sedated by an intramuscular injection of a mixture of ketamine (23 mg/kg; Ketaset; CDMV Inc.) and xylazine (10 mg/kg; Lloyd Laboratories, Shenandoah, Iowa) and then anaesthetised with isofluorane (CDMV Inc.). Laparotomies were performed by Dr. T.I. Michalak with assistance from Ms. C.L. Trelegan and Mr. L. Grenning in the Animal Surgery Unit, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland. In the case of woodchuck #260 (WHV-naive), a portion (approximately 0.5 cm^3) of the liver sample was used immediately for hepatocyte isolation, as described in Section 2.3.1. The remaining tissue fragments were frozen in liquid nitrogen for analysis of WHV nucleic acid expression. Other fragments were fixed in 10% buffered formalin (Fisher Scientific Ltd., Nepean, Ontario) for histological examination.

2.3 CULTURE OF WOODCHUCK HEPATOCYTES (WCM-260).

2.3.1 Isolation of hepatocytes.

The WCM-260 hepatocyte cell line used in this study was established following the procedure described previously (Diao *et al.*, 1998). A fragment of liver

tissue obtained from a healthy, WHV-naive woodchuck (#260) was immediately micro-perfused at multiple sites under aseptic conditions with pre-warmed (37°C) and oxygenated calcium-free perfusion buffer (140 mM NaCl and 2.7 mM KCl in10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.4). Perfusion was carried out using a 27-gauge syringe needle at a flow rate of approximately 10 ml/min for a total of 15 min with the use of a peristaltic pump (Sorvall, Norwalk, Connecticut). The perfused tissue was then sliced and fragments incubated at 37°C for 45 min with 10 ml of pre-warmed, continuously oxygenated calcium-containing buffer (140 mM NaCl, 2.7 mM KCl and 5 mM $CaCl_2$ in 10 mM HEPES, pH 7.6) supplemented with 0.05% (wt/vol) collagenase (type IV; Sigma Chemical Co.). The subsequent steps were carried out in a laminar flow cabinet (Nuaire Inc., Plymouth, Minnesota) to limit the possibility of microbiological contamination. The enzyme-treated tissue was passed gently through a sterile cell sieve fitted with a 50-mesh wire screen. The resultant cell suspension was pelleted at 45 *x* g for 5 min. The recovered cells were washed twice by centrifugation at 45 *x g* for 5 min in 10 ml of pre-warmed (37°C) complete Hepato-STIM™ medium (Becton Dickinson) supplemented with 10 ng/ml epidermal growth factor (EGF; Becton Dickinson), 2 mM L-glutamine (ICN Pharmaceuticals, Montreal, Quebec), 100 nM glucagon (Sigma Chemical Co.), and with 50 units/ml penicillin (Gibco BRL) and 50 µg/ml streptomycin sulfate (Gibco BRL). Isolated hepatocytes were used for culture as described below (Section 2.3.2).

2.3.2 Seeding of hepatocytes for culture.

The WCM-260 hepatocytes (~2 x 10^5 cells), with viability of 70% by trypan blue exclusion (Section 2.3.6.1), were resuspended in 5 ml of conditioned Hepato-STIM™ medium which consisted of 80% complete Hepato-STIM™ and 20% (vol/vol) of HepG2 culture supernatant (Section 2.3.3.2). Then, the hepatocyte suspension was poured into a 25-cm^2 tissue culture flask (Corning®; Corning Costar Corp., Cambridge, Massachusetts) pre-coated with 0.1% gelatin, as outlined in Section 2.3.3.1. The cells were allowed to attach during a 3-h incubation at 37°C in a humidified tissue culture incubator with 5% CO_2 (Forma Scientific, Marietta, Ohio). The culture supernatant containing non-adherent cells was removed by careful pipetting and 5 ml of fresh, pre-warmed, conditioned Hepato-STIM™ medium was added. The medium was replaced every 2 to 3 days for at least a week after seeding. The hepatocytes were cultured for another week, harvested by trypsinization (see Section 2.3.4), and then replated in new gelatin-coated culture flasks.

2.3.3 Optimization of conditions for hepatocyte attachment and *in vitro* growth.

2.3.3.1 Pre-coating of culture vessels with gelatin.

All flasks and plates used for the culture of woodchuck hepatocytes used in this study were routinely pre-coated at 0.2 ml/cm^2 of vessel surface with 0.1%

(wt/vol) gelatin (Bacto© Gelatin; Difco Laboratories, Detroit, Michigan) in PBS at 37°C for 3 h or at 4°C for 16 h. The excess gelatin solution was poured off immediately before the seeding of hepatocytes.

2.3.3.2 Preparation of HepG2 cell culture supernatant.

It was found that complete Hepato-STIM™ medium when supplemented with 20% (vol/vol) of supernatant from culture of human HepG2 cells (Diao *et al.*, 1998) promotes growth of woodchuck liver parenchymal cells. Of note is that the HepG2 cell line (ATCC Number HB-8065; American Tissue Culture Collection, Rockville, Maryland) was originally derived from a human hepatoblastoma and that these cells resemble normal human hepatocytes in regard to the expression of many hepatocyte-specific proteins, including albumin, α2-macroglobulin, transferrin, ceruloplasmin, and beta lipoprotein (Knowles *et al.*, 1980). These HepG2 cells were cultured in Dulbecco's-modified Eagle medium (D-MEM; Gibco BRL) supplemented with 1.5 g/L $Na(CO_3)_2$, 1 mM sodium pyruvate (Gibco BRL), 0.1 mM non-essential amino acids (Gibco BRL), 50 U/ml penicillin, 50 µg/ml streptomycin sulfate, and 10 % FCS-HI, under conditions recommended by the supplier (ATCC). The culture supernatant used to enrich complete Hepato-STIM™ medium was prepared when the HepG2 cell monolayer became 90% confluent in a 75 cm^2 culture flask. At that time, the medium was replaced with fresh D-MEM and HepG2 cells incubated for an additional 24 h. The culture medium was then removed,

sterilized by filtration through a 0.2 µm filter (Acrodisc®; Gelman Sciences, Ann Arbor, Michigan), and added to complete Hepato-STIM™ medium. In contrast, it was observed that supplementation of Hepato-STIM™ medium with fresh D-MEM alone did not promote growth of woodchuck hepatocytes.

2.3.4 Collection of cultured woodchuck hepatocytes.

The plated woodchuck hepatocytes were allowed to grow until they became confluent (approximately 6-8 days) and then harvested by trypsinization. Trypsinization was done using the following procedure. The complete Hepato-STIM™ medium was removed gently from the culture flask, attached cells rinsed with 5 ml of HBSS, and 1 ml of 0.25% (wt/vol) trypsin solution with 0.02% (wt/vol) EDTA (Trypsin-EDTA; Gibco BRL) was added. Hepatocytes were incubated with the trypsin solution in a 5% CO_2 atmosphere at 37°C for 15 min. Then, 5 ml of HBSS was added and the cells were transferred to a 15-ml centrifuge tube and pelleted at 45 *x* g for 5 min. Recovered hepatocytes were resuspended in 1 ml of fresh, conditioned Hepato-STIM™ medium. Ten µl of this mixture was diluted 1:2 with 0.1% trypan blue and cells counted in a hematocytometer (Section 2.3.6.1). Occasionally, approximately 1 x 10^6 hepatocytes in 1 ml of 90% FCS-HI/10% DMSO were frozen in liquid nitrogen for long-term storage. Hepatocytes were maintained in culture by repeated trypsinization and seeding at 6 x 10^4 cells/ml in new gelatin-coated flasks at approximately weekly intervals.

2.3.5 Culture of hepatocytes in glucagon-free medium.

After approximately 2 years in culture, prompted by an observation that the presence of 100 nM of glucagon inhibited DHBV replication in cultured duck hepatocytes (Hild *et al.*, 1998), a separate WCM-260 cell line was established. These cells maintained without glucagon supplementation under conditions described in Section 2.3.4. These hepatocytes were used as targets in the majority of the *in vitro* infection experiments described in this study. This new WCM-260 cell line had the same characteristics as WCM-260 hepatocytes maintained in glucagon-containing complete Hepato-STIM™ medium, as it is described in Section 3.1.2.

2.3.6 Evaluation of hepatocyte viability.

2.3.6.1 Trypan blue exclusion assay.

Typically, viability of the hepatocytes maintained in culture was determined by staining with trypan blue. Trypan blue is a dye that is rapidly excreted by viable cells, while dead cells are unable to remove the dye and thus, they appear blue under a light microscope. Therefore, the number of viable cells in a test sample can be determined by counting the number of colourless cells. For this purpose, a 10-µl sample of suspended hepatocytes was diluted 1:2 in 0.1% trypan blue (Gibco BRL) in PBS, and total cell number and number of viable cells were counted using a hematocytometer. The concentration of viable cells in 1 ml was calculated by

multiplying the number of colourless cells accounted in a 16-square array by 10^4 and by the dilution factor. The percentage viability was determined by dividing the viable cell number with the total cell number and multiplied by 100. Usually, the viability of cultured hepatocytes harvested at weekly intervals was greater than 95%.

2.3.6.2 Microculture tetrazolium assay.

The microculture tetrazolium (MTT) assay for the test of cell viability is based on the mitochondrial oxidation of a colourless compound, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolil blue (MTT reagent), to a blue metabolite, formazan-blue (Alley *et al.*, 1988). This metabolite can be quantitated spectrophotometrically. Using this assay, cytotoxicity of rabbit anti-LP1 antibodies directed against the synthetic peptide containing the N-terminal sequence of WHV large protein (LP1; preS1[1-25]) were examined. For this purpose, approximately 2.4 x 10^4 WCM-260 hepatocytes were seeded in each well of a 96-well, flat-bottomed plate (Linbro®; ICN Pharmaceuticals) along with 200 µl/well of 1%, 5% or 10% (vol/vol) of heat-inactivated rabbit preimmune serum or anti-LP1 antibodies diluted in conditioned Hepato-STIM™ medium. Each serum or antiserum concentration was tested in triplicate. The cells were incubated in a CO_2 incubator for 24 h. Subsequently, the supernatant was carefully removed and substituted with 100 µl/well of MTT reagent (2 mg/ml; Sigma Chemical Co.) and the cells allowed

to metabolize the dye for 3 h in a CO_2/O_2 incubator at 37°C. The supernatant was then removed and 100 µl/well of DMSO was added to solubilize the formazan blue metabolite. Results were read at wavelength 594 nm in a microplate reader (Bio-Rad Laboratories, Mississauga, Ontario). Results were calculated based on the average of three absorbance readings.

2.4 PREPARATION OF INOCULA FROM WHV-INFECTED LYMPHOID CELLS

PBMC and splenocytes isolated from WHsAg-positive woodchucks chronically infected with WHV (more than 6 months; Section 2.1) were used to generate inocula for *in vitro* infection of woodchuck hepatocytes (Table 2.1). To ensure that infectious virus was derived from lymphoid cells but not from WHV virions attached to the surface, and to allow culture of WHV-infected lymphoid cells in conditioned Hepato-STIM™ medium designed for hepatocytes, specific procedures described below were developed.

2.4.1 DNase and limited trypsin digestion of isolated lymphoid cells.

To exclude the possibility of carry-over of WHV virions or WHV DNA molecules on the surface of PBMC and splenocytes used to generate WHV inocula, DNase and trypsin digestions of viable lymphoid cells were performed. For this purpose, approximately 3 x 10^7 cells isolated on Ficoll-Paque® density gradients (Section 2.2.3) were suspended in 900 µl of HBSS and supplemented with 100 µl

Table 2.1. Serological status of WHV infection in chronically infected woodchucks at the time of acquisition of sera and/or lymphoid cells.

Woodchuck No.	WHsAg (cpm/NC$\bar{x}$)[a]	anti-WHc[b]	WHV DNA (vge/ml)[c]
184	17.8	+	+[d]
190	13.0	+	3.6×10^6
191	3.8	+	+
270	5.6	+	+
563	12.0	+	1×10^8
591	10.3	+	+
1252	3.4	+	8.5×10^9
1255	18.1	+	+
1256	12.6	+	+
19056	12.9	+	9.2×10^{10}
19109	5.7	+	2×10^8

[a] WHsAg was identified by a "sandwich" radioimmunoassay (RIA) using cross-reactive AUSRIA-II kit for detection of HBsAg (Abbott Laboratories, N. Chicago, IL) and expressed in cpm/NC$\bar{x}$ units (test sample counts per minute [cpm]/ mean of negative control cpm). Based on detection of purified WHsAg, the assay sensitivity was estimated to be 3.25 ng protein/ml (Michalak *et al.*, 1989).

[b] Anti-WHc was detected using a specific competition ELISA developed in this laboratory (Churchill and Michalak; unpublished). Assay results were accepted when the positive controls inhibited ≥95% of the anti-WHc antibody binding to WHcAg and the negative controls gave no inhibition. Samples that produced ≥50% inhibition were considered positive for anti-WHc.

[c] Serum WHV DNA content was evaluated by dot-blot hybridization as described in Section 2.14.2 and represented as vge/ml (viral genome equivalents/ml).

[d] WHV DNA reactive but vge level not determined.

of DNase buffer (100 mM $MgCl_2$ in 500 mM Tris-HCl buffer, pH 8.0) and 10 µl of DNase I (1 mg/ml; activity 2000 U/mg; Type IV from bovine pancreas; Sigma Chemical Co.). Digestion was carried out in a tissue culture incubator at 37°C for 30 min. The cells were subsequently digested with 10 µl of trypsin (10 mg/ml; 7300 U/mg; Type XI from bovine pancreas; Sigma Chemical Co.) in the presence of 10 µl of 0.1 M $CaCl_2$. This limited trypsin digestion was conducted on ice for 30 min. The enzymatic treatment was terminated with the addition of 20 µl of trypsin inhibitor (10 mg/ml; Type II-O from chicken egg white; Sigma Chemical Co.). In the following step, WHV DNA possibly released from digested extracellular virions was eliminated by digestion with DNase I for 1 h under the same conditions as described above. Then, the lymphoid cells were washed twice with a total of 20 ml of PBS-EDTA by pelleting the cells each time at 330 *x* g for 5 min. The final cell wash was decanted and saved for testing of WHV DNA reactivity. Number and viability of the cells were determined using trypan blue exclusion assay (Section 2.3.6.1). The treated cells were immediately suspended in conditioned Hepato-STIM™ medium, with or without glucagon supplementation, and cultured as described below in Section 2.4.2. Approximately 2 x 10^6 cells were kept at -20°C for subsequent WHV nucleic acid analysis.

2.4.2 Culture of WHV-infected lymphoid cells in Hepato-STIM™ medium.

To directly use lymphoid cell derived WHV for inoculation of cultured

hepatocytes without any further manipulation, lymphoid cells were cultured in conditioned Hepato-STIM™ medium. Thus, to establish the survival of PBMC in this medium, cells were seeded in 25 cm^2 flasks at 1.3 x 10^6 cells/ml in 7.5 ml of conditioned Hepato-STIM™ medium containing 100 nM glucagon and incubated for 3, 6 or 9 days. At each time point, the cell number and their viability were evaluated using trypan blue exclusion (Section 2.3.6.1) and the extracted DNA was analysed for WHV genome expression by PCR (Section 2.13.2) followed by Southern blot hybridization (Section 2.14).

2.4.3 Preparation of lymphoid cell culture supernatant for inoculation of cultured woodchuck hepatocytes.

Isolated, washed and DNase and trypsin-digested PBMC or splenocytes from chronically infected woodchucks (Table 2.1.) were cultured for 72 h in 25-cm^2 flasks at 2 x 10^6 cells/ml in 10 ml of conditioned Hepato-STIM™ medium with or without glucagon supplementation. Then, the cell suspension was centrifuged at 650 *x* g for 10 min, and the culture supernatant was collected and filtered through a 0.2 µm syringe filter to remove cellular debris. The resultant supernatant (about 9 ml) was immediately used for *in vitro* infection of cultured WCM-260 hepatocytes or kept at 4°C. A one-ml aliquot of this supernatant was also preserved at -20°C for analysis of WHV DNA expression.

2.5 PREPARATION OF WHV INOCULA FROM SERA.

Sera used as a source of WHV for *in vitro* infection of hepatocytes were obtained from woodchucks chronically infected with WHV (Table 2.1) and were prepared in two different ways. In the majority of the experiments, to mimic natural conditions, whole sera, either freshly isolated or stored at -70°C, were passed through a 0.2 µm pyrogen-free filter and immediately used for inoculation of cultured woodchuck hepatocytes.

2.5.1 Preparation of WHV virions by serum ultracentrifugation.

Using a previously established method (Michalak & Lin, 1994), sera from selected woodchucks chronically infected with WHV were used to obtain preparations enriched in complete WHV virions. These preparations were subsequently treated with DNase to eliminate any WHV DNA not protected by the viral envelope.

Five ml of serum from a chronic WHV carrier, prepared as described in Section 2.2.2, was centrifuged at 10,000 *x* g for 4 min at 4°C to remove any protein complexes or precipitates possibly formed during storage. A 2.5-ml aliquot of this pre-cleared serum was overlaid onto 2.5 ml of 30% (wt/vol) sucrose in TNE buffer (140 mM NaCl, 10 mM EDTA in 10 mM Tris-HCl buffer, pH 7.2) and centrifuged at 200,000 *x* g for 24 h in a SW 50.1 rotor (Beckman Instruments, Palo Alto, California). The supernatant was carefully removed leaving approximately 300 µl

of aliquot at the bottom of the tube. The pellet together with the remaining aliquot was then resuspended in 5 ml of TNE buffer and washed by centrifugation under the same conditions as described above. The final pellet was allowed to dissolve in 1 ml of TNE buffer overnight at 4°C. Isolated virion particles were stored at -20°C until use.

2.5.2 DNase protection assay.

WHV DNA in complete enveloped virions is resistant to DNase digestion due to protection provided by a lipoprotein envelope. Therefore, the extraction of virion DNA and subsequent PCR analysis were preceded by DNase digestion of virions to identify virus-specific DNA sequences enclosed in complete virions. This approach eliminates a possible detection of viral DNA originating from free WHV DNA or that present within damaged virions (Michalak *et al.*, 1994).

To ensure exclusive identification of complete virions, triple digestion of tested preparations was conducted using a 50 µl aliquot of virion preparation (Section 2.5.1), 20 µl DNase I (1 mg/ml), 12 µl of DNase digestion buffer (Section 2.4.1), and 13 µl of water. The samples were incubated for 1 h at 37°C in a water bath. Then, a further 20 µl of DNase I was added to each tube and incubated for another hour. Digestion was repeated for a third time by adding 20 µl of DNase to each sample and tubes were incubated again for 1 h. Finally, the DNase was inactivated by digestion with 200 µg of proteinase K (fungal; activity >20 U/mg;

Gibco BRL) in lysis buffer (10 mM NaCl, 1 mM EDTA, and 0.5% [wt/vol] sodium dodecyl sulfate [SDS] in 10 mM Tris-HCl buffer, pH 8.0). DNA was then extracted under standard conditions described in Section 2.10.1. Extracted samples were resuspended in 10 µl of double distilled water, mung bean nuclease digested (Section 2.11.2), and divided into 2 aliquots predestined for PCR with WHV core and WHV cccDNA-specific primers (Section 2.13.3). A mock sample consisted of 50 µl of serum from a WHV-naive animal which was DNase treated in parallel. As a negative control, a recombinant plasmid containing the full length WHV genome (see Section 2.14.2.4) was digested with DNase to ascertain that the digestion was complete. This control sample was treated exactly as the virion preparation by using 3 µg (20 µl) of plasmid DNA suspended in 43 µl of serum from a WHV-naive woodchuck and supplemented with 20 µl of DNase I and 12 µl of DNase buffer. Additional controls were prepared with WHV virions, WHV-negative serum or recombinant WHV DNA suspended in the same reagent mixtures, but which were not digested with DNase.

2.6 EVALUATION OF WHV LOADS IN INOCULA.

Number of WHV genome copies (virus genome equivalents; vge) in each WHV inocula used in our experiments were determined, in the first step, by dot blot hybridization, as described in Section 2.14.2.2. If this method was not sufficiently sensitive, DNA extracted from WHV inocula and 50 fg of recombinant, linear,

complete WHV DNA (rWHV DNA; see Section 2.14.2.4) used as a quantitation standard, were amplified using WHV core gene-specific primers. These PCR products were subjected to Southern blot analysis with ^{32}P-labelled rWHV DNA probe and then, to densitometric analysis using a phosphorimager (Cyclone™, Canberra Packard, Montreal, Quebec).

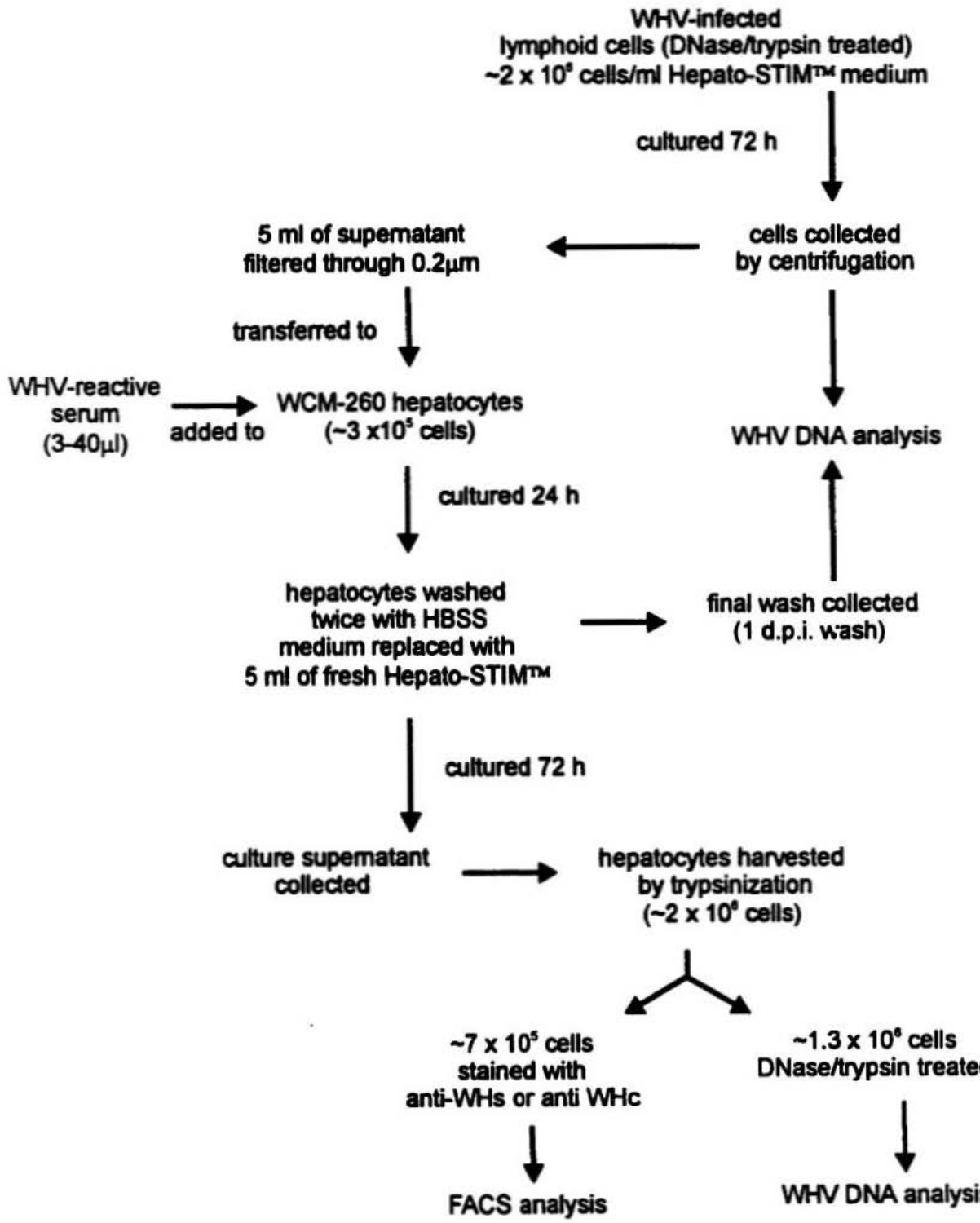
In both dot-blots and PCR/Southern hybridization blots, WHV vge/ml in the tested samples were calculated based on the digital light units (DLU) emitted by the rWHV DNA standard.

2.7 *IN VITRO* INFECTION OF HEPATOCYTES WITH WHV INOCULA.

2.7.1 Infection of hepatocytes.

Figure 2.1 shows a flow-chart outlining the experimental conditions used in this study for inoculation of cultured woodchuck hepatocytes with WHV. Approximately 1 x 10^6 WCM-260 hepatocytes were removed from liquid nitrogen storage, thawed at 37°C, and grown until confluent before trypsinization. Hepatocytes were seeded at a concentration of 6 x 10^4 cells/ml in 5 ml of conditioned Hepato-STIM™ medium in gelatin-precoated 25 cm^2 flasks (Section 2.3.3.1). The cells were incubated for 16 h at 37°C in a humidified CO_2/O_2 atmosphere and then the culture medium was removed. Subsequently, culture supernatant derived from WHV-infected PBMC or splenocytes was added at a volume of 0.2 ml/cm^2 of vessel surface. For hepatocyte infection with WHV-positive

Figure 2.1. Schematic presentation of the experimental strategy used for the *in vitro* infection of WCM-260 hepatocytes with lymphoid cell or serum-derived WHV inocula.


WHV-infected
lymphoid cells (DNase/trypsin treated)
~2 x 10⁶ cells/ml Hepato-STIM™ medium
cultured 72 h
cells collected
by centrifugation
5 ml of supernatant
filtered through 0.2µm
transferred to
WHV-reactive
serum
(3-40µl)
added to
WCM-260 hepatocytes
(~3 x10⁵ cells)
WHV DNA analysis
cultured 24 h
hepatocytes washed
twice with HBSS
medium replaced with
5 ml of fresh Hepato-STIM™
final wash collected
(1 d.p.i. wash)
cultured 72 h
culture supernatant
collected
hepatocytes harvested
by trypsinization
(~2 x 10⁶ cells)
~7 x 10⁵ cells
stained with
anti-WHs or anti WHc
~1.3 x 10⁶ cells
DNase/trypsin treated
FACS analysis
WHV DNA analysis

sera, conditioned Hepato-STIM™ (with or without 100 nM glucagon) was supplemented with whole or concentrated serum at volumes ranging between 3 to 40 µl/ml of medium. The hepatocytes were exposed to inoculum for 24 h at 37°C. The inoculum was then removed, cells washed 2 times with HBSS, and fresh conditioned Hepato-STIM™ medium was added. The final HBSS wash was saved for subsequent analysis of WHV DNA presence. Since it was determined that WHV cccDNA can be detected when the cells reached confluence at 4 d.p.i. (Section 3.4.2), the cells were allowed to grow for 72 h (4 d.p.i.). Then, the cells in the flask were collected, washed with HBSS, and trypsinized (as described in Section 2.3.4). The cells recovered from the flasks were divided into two parts. One part (~7 x 10^5 cells) was predestined for analysis of expression of WHV-specific proteins by fluorescence-activated cell sorting (FACS; Section 2.8.2). The second part (~1.3 x 10^6 cells) was DNase/trypsin digested (Section 2.4.1), washed, and used for WHV DNA evaluation (Sections 2.13 & 2.14). In some experiments, to reduce the amount of reagents used, WCM-260 cells were seeded in 6-well plates at 6 x 10^4 cells/ml in 2 ml of medium per well. These hepatocytes were cultured under standard conditions described above. At 4 d.p.i., the cells were harvested by trypsinization, DNase/trypsin digested, and used for DNA isolation and WHV DNA analysis. In each experiment, a negative control consisting of cells cultured in the absence of WHV inoculum (non-infected hepatocytes) was used.

2.7.2 Co-culture of WHV-infected lymphoid cells with virus-naive hepatocytes.

PBMC or splenocytes from a chronic WHV carrier were isolated as described in 2.2 and approximately 4 x 10^6 cells in 1 ml of conditioned Hepato-STIM™ were seeded in each cell culture insert mounted with 0.4 µm permeable membrane (Falcon; Becton Dickinson). Each insert was placed into a well of a 12-well companion plate (Falcon) containing 1 ml of 6 x 10^5 hepatocytes seeded 16 h prior to the initiation of the co-culture. The lymphoid cells and hepatocytes were co-cultured at 37°C in a humidified CO_2/O_2 atmosphere for 4 days. Then, the lymphoid cells were carefully removed from the inserts and used for DNA isolation (Section 2.10.1). The attached hepatocytes were collected by trypsinization (Section 2.3.4), treated with DNase and trypsin (Section 2.4.1), and evaluated for presence of WHV DNA (Sections 2.13.3 & 2.14).

2.7.3 Passage of WHV from *in vitro* infected hepatocytes to cultured homologous virus-naive hepatocytes

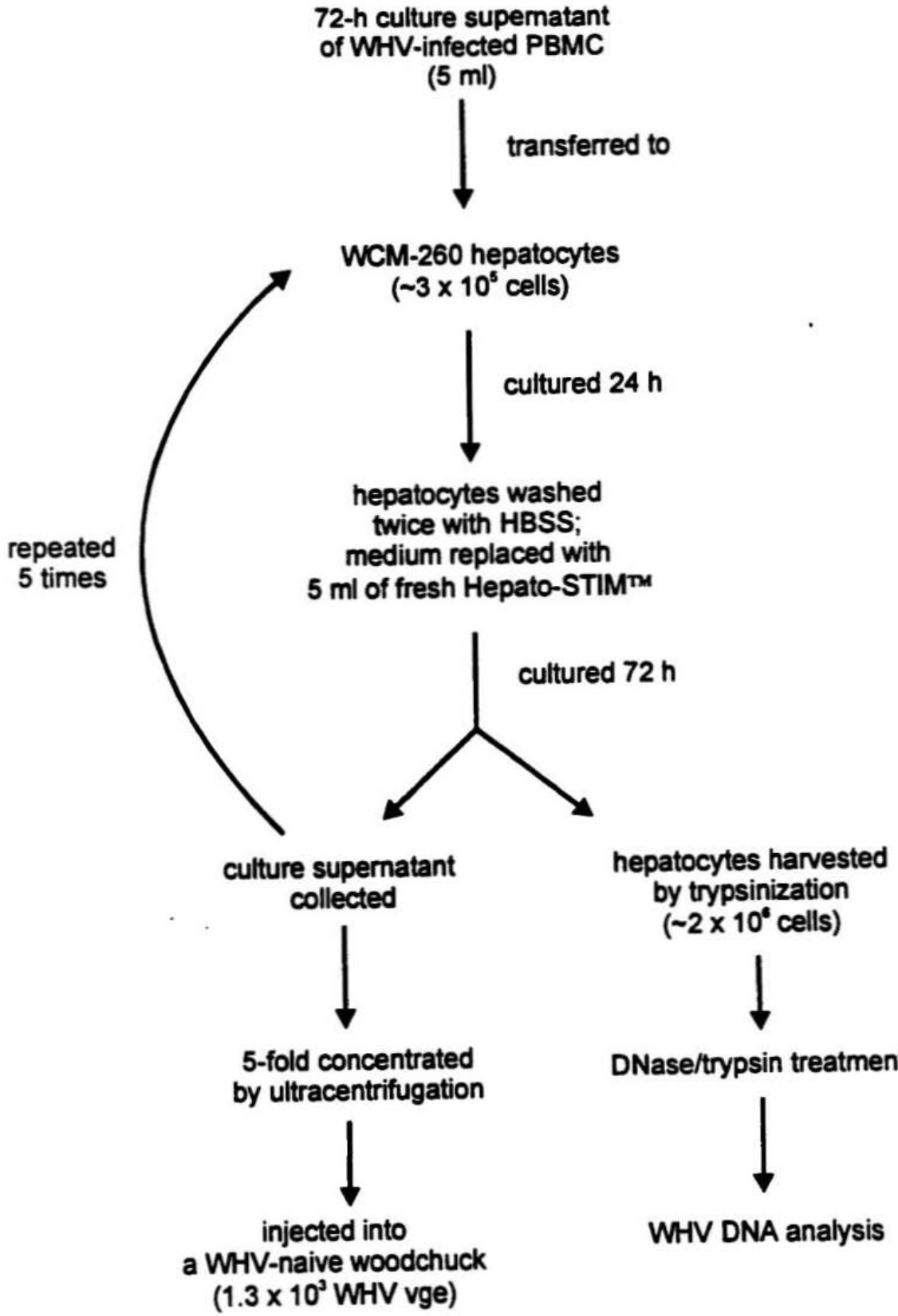
To document that cultured woodchuck hepatocytes are able to support replication and secrete infectious WHV, 5 ml of culture supernatant recovered from woodchuck hepatocyte cultures which were infected *in vitro* with WHV derived from PBMC of woodchuck #19109 (experiment #6, Table 3.5), was transferred to a culture of virus-naive WCM-260 hepatocytes. These hepatocytes were seeded 16 h prior to WHV inoculation at 6 x 10^4 cells/ml in a gelatin-coated 25 cm^2 flask (see

Fig 2.2). After 24 h incubation, the culture medium was removed, the cells were washed thoroughly with HBSS, and supplemented with 5 ml of fresh conditioned Hepato-STIM™ medium. The cells were maintained for 72 h (4 d.p.i.) under standard conditions. Then, hepatocytes were harvested by trypsinization and were treated with DNase/trypsin prior to WHV DNA analysis by PCR (Section 2.13). In addition, the culture supernatant collected at 4 d.p.i. was used for subsequent inoculation of newly seeded woodchuck WCM-260 hepatocytes as described above. This procedure was repeated 5 times. The final culture supernatant was concentrated by ultracentrifugation at 200,000 *x* g for 16 h in a SW 50.1 rotor (Beckman Instruments), the pellet resuspended in 1 ml of sterile PBS and used for the analysis of expression of WHV DNA and for inoculation of a WHV-naive woodchuck.

2.7.4 Inhibition of *in vitro* WHV infection of WCM-260 cells by anti-LP1 antibodies.

To investigate the initial WHV-hepatocyte interaction, the effect of rabbit anti-LP1 antibodies on the *in vitro* WHV invasion of hepatocytes was examined. For this purpose, inocula containing 10% (vol/vol) of heat-inactivated anti-LP1 was prepared by adding 400 µl of anti-LP1, 2 ml of splenocyte culture supernatant prepared from #563 animal (1 x 10^7 vge/ml) and 1.6 ml of conditioned Hepato-STIM™ medium. The mixture was rotated for 2 h at room temperature and then for 16 h at 4°C to

Figure 2.2. Outline of the strategy used for multistep passage of lymphoid cell-derived WHV in cultured woodchuck hepatocytes. Culture supernatant used as inoculum was prepared from WHV-infected PBMC as presented in Section 2.7.1.


72-h culture supernatant
of WHV-infected PBMC
(5 ml)
transferred to
WCM-260 hepatocytes
(~3 x 10^5 cells)
cultured 24 h
hepatocytes washed
twice with HBSS;
medium replaced with
5 ml of fresh Hepato-STIM™
cultured 72 h
repeated
5 times
culture supernatant
collected
hepatocytes harvested
by trypsinization
(~2 x 10^6 cells)
5-fold concentrated
by ultracentrifugation
DNase/trypsin treatment
injected into
a WHV-naive woodchuck
(1.3 x 10^3 WHV vge)
WHV DNA analysis

facilitate antibody-WHV interaction. Subsequently, 2 ml/well of this preparation was added to 6-well plates containing WCM-260 hepatocytes that were seeded 16 h prior to this assay. The cells were cultured under standard conditions (Section 2.7.1), collected by trypsinization, DNase/trypsin treated, and tested for WHV DNA presence. As controls, an inoculum with 10% (vol/vol) of heat-inactivated rabbit preimmune serum and an inoculum without rabbit serum supplementation were included.

2.7.5 Inhibition of WHV invasion of cultured woodchuck hepatocytes by JP1 peptide.

To study if the synthetic analogue of WHV preS1(1-13), JP1, can compete with natural WHV virions for the binding of host receptor molecules, the following experiment was done. One ml/well of conditioned Hepato-STIM™ medium containing 15 µg/ml of JP1 was added to WCM-260 hepatocytes seeded in 6-well plates 16 h prior to the experiment. Then, 1 ml/well of splenocyte inoculum (2×10^7 WHV vge/ml) was added and cells were cultured for 24 h. Inoculum was removed, hepatocytes were extensively washed in HBSS, and medium was replaced with fresh conditioned Hepato-STIM™ containing 15 µg/ml of JP1. The cells were cultured and collected for DNA analysis under standard conditions described in Section 2.7.1. As a control, an inoculum without peptide supplementation was included.

2.8 DETECTION OF WHV PROTEINS IN *IN VITRO* INFECTED HEPATOCYTES AND THEIR CULTURE SUPERNATANTS.

2.8.1 Detection of *de novo* synthesized WHV proteins.

2.8.1.1 Metabolic labelling of cells with ^{35}S-methionine.

In selected experiments (Table 3.4), infected hepatocytes were labelled with ^{35}S-methionine for detection of *de novo* synthesized WHsAg. For this purpose, cells predestined for WHV inoculation were seeded in parallel in 25 cm^2 flasks and 6-well plates (see Section 2.7.1). At 4 d.p.i., hepatocytes cultured in the flasks were used for flow cytometry and WHV DNA analysis. In contrast, cells in 6-well plates were incubated in medium which contained 20% conditioned Hepato-STIM™ medium and 80% methionine-free RPMI 1640 medium (ICN Pharmaceuticals) with 20 nM insulin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin sulfate, 100 nM glucagon, and 10 µCi/ml ^{35}S-methionine (ICN Pharmaceuticals). The cells were cultured for an additional 24 h. Then, WCM-260 cells and culture supernatants containing newly synthesized WHsAg that have incorporated the radioisotope were detected by immunoprecipitation with specific antibodies and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.8.1.2 Immunoprecipitation of ^{35}S-Methionine labelled samples

Labelled hepatocytes were lysed in 2 ml of triple detergent lysis buffer

containing 1% (vol/vol) NP-40, 0.5% (wt/vol) deoxycholate, 0.05% (wt/vol) SDS and 0.2 U/ml aprotinin (Sigma Chemical Co.) in PBS at ambient temperature for 1 h. Culture supernatant and cell lysates were pre-cleared by incubation with 20 µl of rabbit preimmune serum at room temperature for 1 h. Then, 100 µl of 10% (wt/vol) protein A beads (purified protein A immobilized to Sepharose CL-4B beads; Sigma Chemical Co.) suspended in loading buffer (0.3 M NaCl, 0.4 M KCl, 5 mM EDTA, 1% [vol/vol] Triton X-100 in 50 mM Tris-HCl buffer, pH 7.6) was added and rocked for 1 h at ambient temperature. Protein A beads with bound proteins were removed by pelleting at 210 *x* g for 1 min, the supernatant was then transferred to a new tube containing 40 µl of rabbit antibodies against WHsAg (anti-WHs), and the mixture was incubated for 1 h at room temperature. Then, 100 µl of protein A beads was added and the mixture was rotated for another hour. Protein A and associated proteins were pelleted and washed 3 times in a total of 3 ml of loading buffer. The pellet was suspended in 28 µl of a SDS-PAGE sample buffer (10% [vol/vol] glycerol, 5% [vol/vol] β-mercaptoethanol, 3% [wt/vol] SDS in 62.5 mM Tris-HCl buffer, pH 6.8), treated at 92°C for 15 min, and centrifuged at 210 *x* g for 3 min. The resultant supernatant (24 µl) was loaded onto a 12% SDS-PAGE mini-gel as described below.

2.8.1.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

A 12% acrylamide gel in 37.5 mM Tris-HCl buffer, pH 8.8 with 0.1% (wt/vol)

SDS along with a 3% acrylamide upper gel in 12.5 mM Tris-HCl buffer, pH 6.8 with 0.1% (wt/vol) SDS was prepared in a mini-gel apparatus (Mini-Protean II™ dual slab cell; Bio-Rad Laboratories). Subsequently, samples with volumes ranging from 5 µl to 24 µl were loaded and the gel was run at constant voltage (200 V) for about 45 min in reservoir buffer (20 mM glycine, 2.5 mM Tris base). In addition, 25 nCi of ^{14}C-methylated molecular weight markers (Amersham Life Science, Oakville, Ontario) was electrophoresed in parallel. Then, the gel was dried in a gel dryer apparatus (Bio-Rad Laboratories) and exposed on a phosphor screen (Cyclone™; Canberra-Packard), followed by autoradiography with X-ray film (XRP-1; Kodak, Eastman Kodak Company, Rochester, New York).

2.8.2 Identification of hepatocyte WHV proteins by fluorescence-activated cell sorting.

Hepatocytes infected *in vitro* with WHV (as described in Section 2.7.1) were harvested by trypsinization and allowed to recover at a concentration of 1.1 x 10^6 cells/ml in complete Hepato-STIM™ medium for 30 min at 37°C. These cells were divided into 100 µl aliquots and placed in 12 x 75 mm tubes (Fisher Scientific) kept on ice. Rabbit anti-WHs or rabbit antibodies against WHcAg (anti-WHc) were added at a 1:10 ratio and cells left on ice for 20 min. Cells were then washed twice with 1 ml of HBSS by pelleting at 45 *x* g for 5 min. The washed cell pellets were resuspended in 100 µl of HBSS and stained for 20 min on ice with goat anti-rabbit

IgG (heavy and light chains) conjugated to fluorescein isothiocyanate (FITC; Organon Teknika Cappel, Durham, North Carolina). As controls, unstained cells and cells incubated with HBSS or rabbit pre-immune sera were included. All cells in the second antibody layer were incubated with FITC-conjugated goat anti-rabbit IgG. Hepatocytes were washed twice by centrifugation and cell pellets suspended in 200 µl of 1% (wt/vol) paraformaldehyde (Marivac Limited, Halifax, Nova Scotia) in PBS. Fixed hepatocytes were analysed with a FACS Star™-Plus flow cytometer (Becton Dickinson).

The FACS data were analysed using the CellQuest® software (Becton Dickinson) and presented as histograms. FACS profiles of test samples were overlaid onto negative controls (i.e., WHV- nonexposed hepatocytes stained with the same antibodies). Gating was done to determine the number of cells that have more than a selected amount of fluorescence. The gated cells were expressed as a percentage of the total number of events detected. The percentage of the test sample was subtracted from the percentage of its negative control and a difference of more than 10% was considered positive.

2.9 INOCULATION OF NAIVE WOODCHUCKS WITH CULTURE SUPERNATANT FROM HEPATOCYTES *IN VITRO* INFECTED WITH LYMPHOID CELL-DERIVED WHV.

Approximately 0.6 ml concentrated inocula prepared as described in Section

2.7.3 was injected intravenously into a naive woodchuck. The woodchuck was bled at 0, 3, 5, and 7 d.p.i. and then weekly. In addition, PBMC were isolated at 21, 28, 35, 42, and 49 d.p.i., and a liver biopsy was obtained at 42 d.p.i. These sequential sera, PBMC and liver biopsy material was used for analysis of WHV genome expression.

2.10 DNA ISOLATION.

2.10.1 Isolation of total DNA.

DNA was extracted from approximately 1 x 10^6 cells (PBMC, splenocytes or cultured hepatocytes), from 100 µl of serum, cell culture supernatant or cell washes, or from 50 mg of homogenized tissues. All tissue fragments predestined for DNA isolation were washed twice in 1 ml of HBSS by centrifugation at 1,000 *x* g prior to homogenization in a final volume of 100 µl of HBSS. Cells used for DNA extraction were resuspended in 100 µl of HBSS. Then, the samples were incubated with 200 µl of lysis buffer (Section 2.5.2) containing 50 µg of proteinase K at 42°C for 16 h. The digest was supplemented with 300 µl of phenol equilibrated in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1% (wt/vol) hydroxyquinilone (Sigma Chemical Co.), mixed for 15 min at ambient temperature, and centrifuged at 10,000 *x* g for 3 min to separate the aqueous and organic phases. The upper aqueous layer was removed and transferred to a new 1.5-ml tube containing 300 µl chloroform:isoamyl alcohol (24:1, vol/vol; Sigma Chemical Co.). The mixture was rotated for 10 min

and then centrifuged for 3 min at 10,000 *x* g at room temperature. The aqueous layer containing DNA was transferred to a 1.5-ml Eppendorf tube and precipitated with 750 µl of cold 100% ethanol (Sigma Chemical Co.) with 30 µl of 3 M sodium acetate (pH 5.2) and kept for 16 h at -20°C. DNA was recovered by centrifugation at 10,000 *x* g for 20 min at 4°C. The resultant pellet was washed by centrifugation with 600 µl of cold 70% ethanol. The DNA was then dried under vacuum for 5 min and resuspended in 40 µl (cell or tissue DNA) or in 5 µl (DNA from serum, culture supernatant or cell washes) of either TE buffer (1 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0) or autoclaved double distilled water.

DNA was quantitated based on the ultraviolet absorbance at 260 nm and the purity of the DNA preparation was determined based on the ratio of the absorbances at 260 nm and 280 nm (OD_{260}/OD_{280}) using the Warburg-Christian equation in a DU 530 spectrophotometer (Beckman Instruments, Inc., Fullerton, California). The DNA preparations used in this study had OD_{260}/OD_{280} values between 1.8 and 2.0.

2.10.2 Isolation of WHV cccDNA by a potassium chloride precipitation method

It was shown in experiments performed in other laboratories (Aldrich *et al.*, 1989; Wu *et al.*, 1990, Jilbert *et al.*, 1992) that hepadnaviral cccDNA species can be isolated from livers containing high levels of replicating viral genomes by precipitation with high molar potassium chloride (KCl) (see below). In this method,

cellular proteins are precipitated allowing separation of protein-associated viral DNA and host DNA species from nucleic acids that are not covalently attached to proteins (e.g. viral cccDNA). The procedure used in the present study was adopted from those described in previous works of Wu *et al.*, 1990.

For this purpose, approximately 100 mg of liver or spleen tissue was homogenized in a total volume of 7.5 ml of lysis solution (0.5% SDS [wt/vol], 10 mM EDTA in 10 mM Tris-HCl buffer; pH 7.5), mixed briefly, and supplemented with 2.5 ml of 2.5 M KCl (BDH, Toronto, Ontario). The suspension was left at ambient temperature for 30 min and then centrifuged at 12,000 *x* g for 5 min. The resultant supernatant was collected and extracted twice with 10 ml of phenol, for 15 min each. The mixture was centrifuged for 5 min at 720 *x* g to separate the aqueous and the organic phases. The aqueous layer was added to 10 ml of chloroform:isoamyl alcohol mixture (24:1, vol/vol) and mixed for 15 min. Supernatant obtained after centrifugation at 720 *x* g for 5 min was pipetted into 20 ml of 100% ethanol and kept at room temperature for 16 h. The resultant DNA precipitate was isolated by centrifugation at 13,500 *x* g for 30 min. The pellet was washed in 2 ml of 70% ethanol and centrifuged at the same conditions as described above. The resultant DNA was resuspended in 40 µl of TE buffer and its concentration evaluated by spectrophotometric analysis (Section 2.10.1). For analysis of the isolated WHV DNA species, samples were loaded at 10 µg DNA per well onto 1% (wt/vol) agarose (Gibco BRL) gel in TAE buffer (1 mM EDTA in 40 mM

Tris-HCl buffer, pH 8.0) containing 0.5 µg/ml ethidium bromide (EB), separated by electrophoresis (Section 2.14.1), transferred onto nylon membrane, and hybridized to a WHV DNA probe (Section 2.14.2).

2.11 ENZYMATIC TREATMENTS OF WHV DNA.

2.11.1 Digestion of WHV DNA with *Eco*RI restriction enzyme .

Since the WHV genome contains a single *Eco*RI restriction site (Galibert *et al.*, 1982), this restriction enzyme can be used to confirm the existence of circular WHV DNA molecules by Southern blot analysis of electrophoretically separated DNA species. *Eco*RI cleavage of circular, partially or fully double-stranded WHV DNA molecules will linearize these 3.3 kb WHV DNA molecules, modifying the migrational properties of the digested WHV DNA. The digestion of WHV DNA isolated by KCl precipitation was done using 30 µg of DNA, 120 units *Eco*RI (10 units/µl; Gibco BRL), 8 µl of REact® 3 buffer (100 mM $MgCl_2$, 1 M NaCl in 500 mM Tris-HCl buffer, pH 8.0; Gibco BRL), in a total volume of 80 µl. Twenty-five µl samples were removed after 1 h, 3 h, and 16 h incubation at 37°C in a water bath. These aliquots were analysed by electrophoresis on 1% EB-agarose gel (Section 2.10.2) along with 50 pg of rWHV DNA. The separated DNA species were then transferred onto a nylon membrane, and analysed by Southern blot hybridization (Section 2.14.2).

2.11.2 Mung bean nuclease digestion of WHV DNA.

As the rcDNA species of HBV and WHV are partially double stranded, they are susceptible to digestion by single strand-specific nucleases, such as mung bean nuclease. This nuclease digests single stranded DNA at the 3'-end of adenine (A; A↓pN) and thymine (T; T↓pN) (Kowalski *et al.*, 1976). Thus, this enzyme was employed in the present work to treat DNA samples containing WHV rcDNA and cccDNA prior to PCR amplification in order to discriminate WHV cccDNA sequences from rcDNA. The following method was modified from a procedure established for HBV cccDNA detection, which was developed in another laboratory (Stoll-Becker *et al.*, 1997).

Approximately 2 -10 µg of tissue or cell-derived DNA or DNA extracted from 50 µl isolated WHV virions or from 200 µl cell washes or culture supernatants, was supplemented with 2 µl of 10x mung bean digestion buffer (500 mM NaCl, 10 mM L-cysteine, 100 mM sodium acetate, 1 mM zinc acetate, pH 5.0 with 50% [vol/vol] glycerol; Gibco BRL), 1 µl of mung bean nuclease (40 U/µl; Gibco BRL), and water to a total volume of 20 µl. This mixture was incubated for 30 min at 37°C in a water bath. The reaction was terminated by the addition of 3 µl of 0.1 M EDTA. A 10-µl aliquot of each digested sample was used for PCR amplifications with either WHV cccDNA or core gene specific primers (see Section 2.13).

2.12 DETECTION OF ASIALOGLYCOPROTEIN RECEPTOR MAJOR SUBUNIT mRNA.

2.12.1 RNA isolation.

Origin of the cultured woodchuck liver cells was confirmed by identification of RNA transcripts known to be specifically synthesized by hepatocytes. Among these proteins, expression of the asialoglycoprotein receptor (ASGPR) is considered to be unique to parenchymal liver cells (Hubbard & Stukenbrok, 1979; Hubbard *et al.*, 1979).

For this purpose, total RNA was isolated from 1 x 10^6 cultured cells using TRIzol® reagent (Gibco BRL) following the manufacturer's instructions. Briefly, hepatocytes in 1 ml of TRIzol® reagent were incubated at room temperature for 30 min. Then, 200 µl of chloroform was added and the tubes were shaken vigorously for 30 sec and samples centrifuged at 12,000 *x* g for 15 min at 4°C. Approximately 550 µl of the upper phase was transferred to new tubes and 500 µl of isopropanol (Sigma Chemical Co.) was added to precipitate RNA. Samples were incubated for 10 min at ambient temperature and centrifuged at 12,000 *x* g for 10 min at 4°C. The RNA precipitate was then vortexed in 1 ml of 75% ethanol and spun at 7,500 *x* g for 5 min at 4°C. The RNA pellet was air dried and resuspended in RNase-free water prepared by treatment with diethyl pyrocarbonate (DEPC; Sigma Chemical Co.), and quantitated spectrophotometrically based on the ultraviolet absorbance at 260 nm. Prepared RNA was used immediately for reverse transcription, as described

below.

2.12.2 Reverse transcription reaction.

Reverse transcription (RT) reaction was done on selected RNA samples for conversion to cDNA prior to PCR amplification. Two µg of RNA in 10 µl of RNase-free water was added to 1.5 µl of random primers (100 ng/µl) and 4 µl of 5x RT buffer (375 mM KCl, 15 mM $MgCl_2$ in 250 mM Tris-HCl buffer, pH 8.3; Gibco BRL). The mixture was incubated for 4 min at 75°C, chilled on ice and aliquoted into two portions. One of these samples was mixed with 1 µl of dithiothreitol (DTT; 0.1 M; Gibco BRL), 1 µl of deoxynucleotide triphosphate (dNTP) mixture (10mM of each nucleotide; Promega Corp., Madison, Wisconsin), 10 units of RNase inhibitor (RNasin®; 40 units/µl; Promega Corp.) and 200 units of reverse transcriptase (from Moloney murine leukemia virus; 200 units/µl; Gibco BRL), whereas the second portion (negative control) contained all the above components without reverse transcriptase. After incubation at 37°C for 1 h and at 95°C for 5 min, the samples underwent PCR amplification using primers specific for the major subunit of ASGPR (Slaney, 1999). Subsequently, PCR products were analysed by Southern blot hybridization using a cloned woodchuck ASGPR major subunit cDNA prepared in this laboratory (Slaney, 1999) as a probe, which was radiolabelled with ^{32}P-dCTP using the *redi*prime method (Section 2.14.2.4).

2.13 POLYMERASE CHAIN REACTION.

2.13.1 Design of WHV genome-specific primers.

All PCR primers utilized in the study were designed in the consensus regions of 4 published WHV genome sequences (Galibert *et al.*, 1982; Kodama *et al.*, 1985; Cohen *et al.*, 1988; Girones *et al.*, 1989). All primer sequences were selected to exclude non-complementarity to other regions of the WHV genome and to the opposite WHV DNA strand. Pairs of primers were matched based on primer melting temperature (less than 5°C difference) and primer length (20-27 bases) to ensure the most efficient and specific amplification. Primers were created with the assistance of the PC/Gene software (Intelligenetics, Geneva, Switzerland) and were synthesized by Gibco BRL Custom Primers laboratory (Burlington, Ontario).

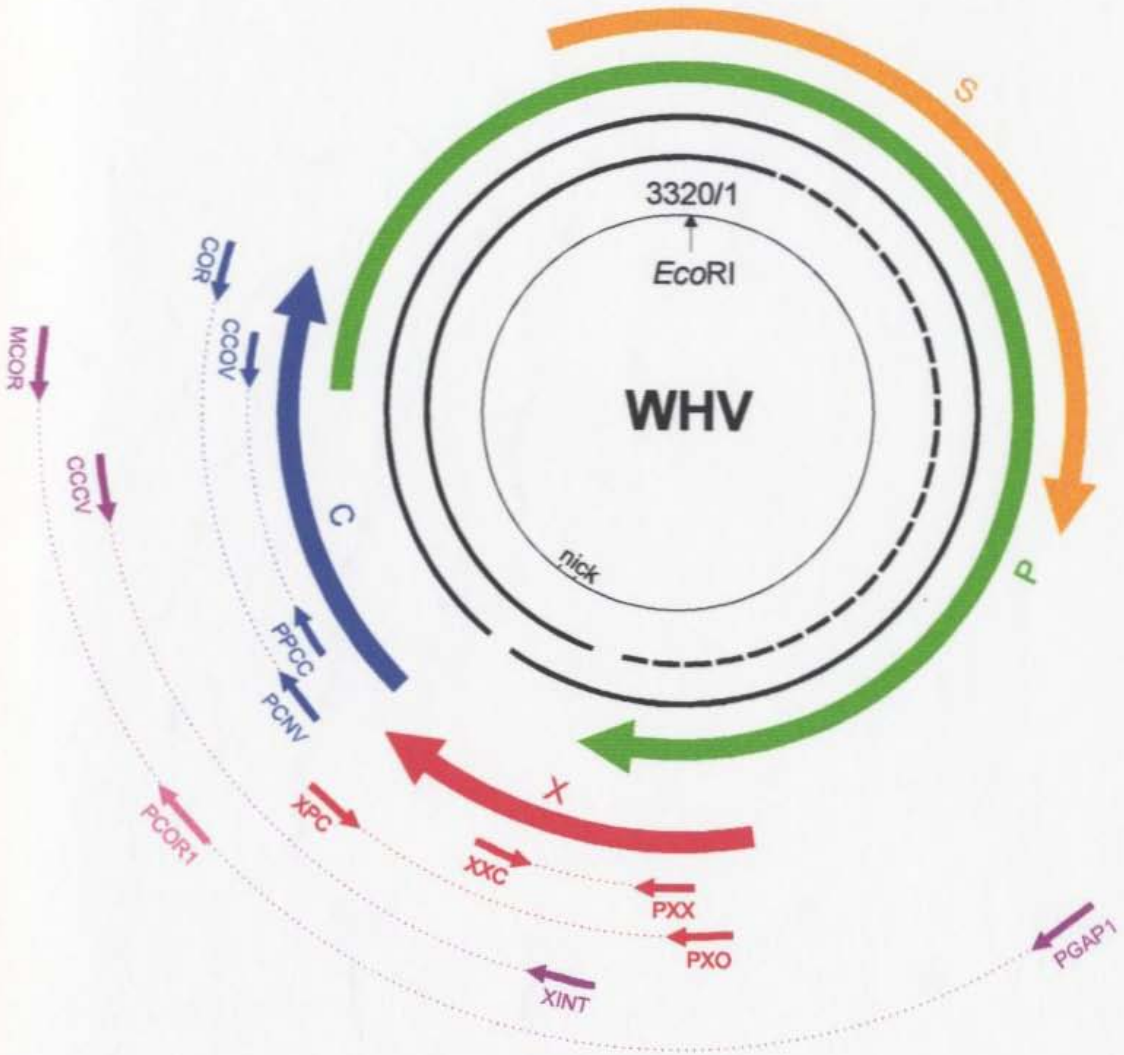
In this study, the standard PCR conditions employed primers specific for two separate, non-overlapping subgenomic regions of WHV DNA, *i.e.*, core (C) and X genes (Table 2.2, see also Fig. 2.3). In the modified PCR conditions, amplifications were employed both to identify and discriminate the WHV replicative (cccDNA) species from the core gene sequence (i.e., total WHV DNA content). For this last purpose, two sets of primers were designed using an approach described by others (Köck *et al.*, 1996a, Stoll-Becker *et al.*, 1997). Thus, PCOR1-MCOR was used for identification of WHV core gene sequences (Table 2.2 and Fig. 2.3). In a parallel reaction, the detection of WHV cccDNA was conducted with the nick-spanning primer pair (i.e., PGAP1-MCOR; Table 2.2 and Fig. 2.3).

Table 2.2. Oligonucleotide primers used for amplification of WHV DNA and cccDNA sequences in this study.

Description	Primer pairs	Sequence 5' - 3'	Position[a]	Target sequence (bp)
Primers specific for WHV subgenomic sequences	**C gene**			
	External primers			
	PCNV (sense)	5'-TTCAAGCCTCCAAGCTGTGCCTTGG	1983-2007	
	COR (antisense)	5'-CTCGAATTCTTATGTACCCATTGAAG	2589-2605	620
	Internal primers			
	PPCC (sense)	5'-CTCGGATCCCTATAAAGAATTTGG	2033-2049	
	CCOV (antisense)	5'-GTATGTTCCGGAAGAGTCGAGA	2439-2460	428
	X gene			
	External primers			
	PXO (sense)	5'-GCCAACTGGATCCTGCGCGGGACGTC	1522-1547	
	XPC (antisense)	5'-TAGGAGGCTGTAGGCAT	1891-1907	386
	Internal primers			
	PXX (sense)	5'-CCTCAATCCAGCGGAC	1569-1584	
	XXC (antisense)	5'-ATGGATTCCACCGTGAAC	1743-1760	192
Primers that discriminate between WHV DNA and cccDNA	**total WHV DNA (core gene)**			
	External primers			
	PCOR1 (sense)	5'-TGTGTTCCATGTCCTACTGTTCAAGCC	1964-1990	
	MCOR (antisense)	5'-CCGGAAGAGTCGAGAGAATGGGTGC	2453-2429	490
	WHV cccDNA (nick-spanning)			
	External primers			
	PGAP1 (sense)	5'-TGGTGTGCTCTGTGTTTGCTGACGC	1298-1322	
	MCOR (antisense)	5'-CCGGAAGAGTCGAGAGAATGGGTGC	2453-2429	1156
	Internal primers			
	XINT (sense)	5'-CTTCGCCTTCGCCCTCAGACGAGT	1630-1653	
	CCCV (antisense)	5'-GTCCCCAGGTGTCAGTGACA	2303-2284	674

[a] Numbers denote nucleotide positions in the WHV genome sequence based on Galibert *et al.*, 1982 (GenBank accession number J02442).

Figure 2.3. Schematic representation of the approximate location of direct and nested oligonucleotide primer pairs specific for the WHV C gene, X gene and the nick-spanning region superimposed on the map of the complete WHV genome.


3320/1
EcoRI
WHV
nick
S
P
X
C
COR
CCOV
MCOR
CCCV
PPCC
PCNV
XPC
PCOR1
XXC
PXX
PXO
XINT
PGAP1

For some PCR fragments, a set of internal oligonucleotide primers were selected (Table 2.2). In these reactions, the external primer pair was used for the first (direct) round of PCR amplification. If the direct PCR product was not detectable following Southern blot analysis, an aliquot after the first reaction was amplified with an internal primer pair by nested PCR.

2.13.2 Standard PCR conditions.

Typical PCR amplification of subgenomic WHV DNA sequences was performed using 1-5 µg of total DNA derived from tissue or cells or with DNA isolated from 100 µl of serum, culture supernatant or cell washes. Reaction was performed in a total volume of 100 µl containing 200 µmol/L of each dNTP (Gibco BRL), 300 ng of each primer, 2 units of *Taq* polymerase (Gibco BRL), 1.5 mM $MgCl_2$, and 50 mM KCl in 20 mM Tris-HCl buffer, pH 8.4 (Gibco BRL). The reaction was overlaid with 100 µl of mineral oil (Sigma Chemical Co.) and incubated in a programmable thermal cycler (TwinBlock System; Ericomp Inc., San Diego, California). The following program was used: 92°C for 5 min, 52°C for 2 min, 70°C for 3 min in the first cycle and 92°C for 30 seconds (sec), 52°C for 30 sec, 70°C for 30 sec for 30 cycles, followed by a final extension step at 70°C for 5 min. For each thermocycling reaction, 1 µg of liver DNA extracted from a WHV chronic carrier was used as a positive control and a mock sample, containing all reagents used during the DNA extraction and PCR, was included as a negative control. For nested PCR

with internal primers, 10 µl of direct PCR product was amplified using the above PCR reagents (total volume of 100 µl). Thermocycling was conducted as described above. As controls, 10 µl of WHV-positive liver DNA direct PCR product (positive control) and 10 µl of the mock PCR product (negative control) were amplified in parallel.

2.13.3 Modified PCR conditions.

To determine the optimal Mg^{2+} concentration in the PCR mixture after mung bean nuclease inactivation with EDTA, a preliminary PCR was done using 1.5, 2.0, 2.5, 3.0, 3.5, or 4.0 mM of $MgCl_2$, along with the mixture components described above (Section 2.13.3). Thermocycling was performed using the program described below. Based on this experiment (Section 3.2.2), subsequent reactions were conducted with 3 mM of $MgCl_2$.

For detection of WHV DNA and WHV cccDNA, PCR was carried out with 10 µl of mung bean nuclease-digested DNA (1 - 8 µg or DNA extracted from 100 µl of serum, culture supernatant or cell washes) in a total volume of 100 µl with the reaction components described above, except for 3 mM $MgCl_2$. The PCR was done using the following program: 1 cycle of 95°C for 5 min, 56°C for 2 min, 72°C for 3 min, followed by 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and, finally, incubation at 72°C for 15 min. In addition, 1 µg of mung bean nuclease-treated DNA extracted from the liver of a WHsAg-reactive WHV carrier and 50 fg

of rWHV DNA were used as positive controls and a mock sample, containing all reagents used in the DNA extraction and PCR, was included as a negative control. In selected cases, nested PCR was used with internal primers for further amplification of cccDNA sequences (Table 2.2). For this reaction, 10 µl of direct PCR product was amplified in a total volume of 100 µl, with 200 µmol/l of each dNTP, 300 ng of each internal primer, 2 units of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 50 mM KCl in 20 mM Tris-HCl buffer, pH 8.4. Thermocycling was conducted as described above in this paragraph. As controls, 10 µl of rWHV DNA direct PCR product (positive control) and 10 µl of the mock PCR product (negative control) was amplified in parallel.

2.14 DETECTION OF WHV DNA.

2.14.1 Agarose gel electrophoresis.

Each 20 µl DNA sample (extracted DNA or PCR product) was mixed with 2 µl of 6x blue/orange loading dye (Promega Corp.) prior to loading on a 1% EB-agarose gel (Section 2.10.2). In addition, 0.25 µg of ϕX 174 DNA/*Hae* III molecular weight standards (Promega Corp.) was also loaded in a parallel well. The gel was run at 80 V for 60 min. DNA bands were visualized by ultraviolet light and photographed with a transilluminator camera (Fotodyne Inc., Bio/Can Scientific, Mississauga, Ontario).

2.14.2 Southern blot hybridization.

2.14.2.1 Blotting of DNA onto nylon membrane.

Following electrophoresis and photography, the gel predestined for Southern blot analysis was soaked in denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 30 min and then rocked in neutralizing buffer (1.5 M NaCl in 1 M Tris-HCl buffer, pH 8.0) for 1 h.

Transfer of DNA from the gel to a nylon membrane was done using capillary transfer. A glass platform was set up in a dish containing 10x standard saline citrate (SSC, diluted from 20x SSC; 3 M NaCl in 0.3 M $Na_2Cit._2H_2O$, pH 7.0). A 3MM Whatman filter paper wick was soaked in 10x SSC and mounted on this platform. The gel was placed upside down on the wick and parafilm was used to surround the gel. A piece of nylon membrane (Hybond-N; Amersham) and 2 pieces of filter paper cut to size and briefly soaked in 6x SSC were laid on top of the gel, followed by 5 cm of paper towels, trimmed to size. A glass weight of approximately 500 g was placed on top to facilitate the 16 h-capillary transfer. The set-up was dismantled the next day and the membrane was dried in a filter paper envelope for 30 min at room temperature, and then baked at 80°C in a vacuum oven for 1.5 h.

2.14.2.2 Dot-blot analysis.

Two-fold serial dilutions beginning with 600 µl of lymphoid cell culture supernatant or 1 µl of serum was blotted onto a nylon membrane (Hybond-N) by

vacuum suction using a Bio-Dot microfiltration apparatus (BioRad Laboratories). After the sample aliquot had completely passed through, the membrane was sequentially transferred onto 2 sheets of 3MM Whatman filter paper, for 5 min each. The first sheet was saturated with denaturing solution and the second wetted with neutralizing buffer. Then, the blot was air-dried, placed between two pieces of dry 3MM paper and baked for 2 h at 80°C in a vacuum oven. The nylon membrane was hybridized to a ^{32}P-labelled rWHV DNA probe, as described in Section 2.14.2.3. To quantitate the number of WHV genome equivalents (vge) in each sample tested, two-fold dilutions of rWHV DNA from 1 ng to 7.8 pg were immobilized onto the membrane in parallel.

2.14.2.3 Hybridization of WHV DNA.

The baked nylon membrane containing WHV DNA was pre-wetted with 6x SSC and heat sealed in a plastic bag with 6 ml of hybridization buffer (6x SSC, 0.5% SDS, 5x Denhardts' solution [diluted from 50x Denhardts' solution; 1% {wt/vol} Ficoll, 1% {wt/vol} polyvinylpyrrolidone, 1% {wt/vol} bovine serum albumin], and 100 µg/ml sonicated salmon sperm DNA). The membrane was prehybridized in a 65°C shaking water bath for 1.5 h. Subsequently, the buffer was discarded and fresh hybridization buffer was added to the bag along with 6 x 10^6 counts per minute (cpm) of ^{32}P-labelled rWHV DNA probe (Section 2.14.2.4), which was previously boiled for 5 min and chilled on ice for 2 min. The bag was hybridized in a 65°C

shaking water bath for 16 h. Then, the blot was washed twice at 65°C in 50 ml of pre-warmed 2x SSC, 0.1% SDS for 10 min each. This was followed by 2 washes at ambient temperature with 50 ml of 0.1x SSC, 0.05% SDS for 15 min each. The membrane was then rinsed in 0.1x SSC and dried briefly on paper towels. It was then covered in plastic wrap and exposed on a phosphor screen for phosphorimaging (Cyclone™; Canberra-Packard) or on an X-ray film (XRP-1; Kodak) in cassettes equipped with intensifying screens at -70°C for autoradiography. In selected cases, semi-quantitation of WHV genome copies was conducted on dot-blots and Southern blots using the method described in Section 2.6.

2.14.2.4 Recombinant WHV DNA probe.

Recombinant WHV DNA was derived from the pSP65 plasmid vector containing the *Eco*RI fragment of the full WHV genome (Galibert *et al.*, 1982). This plasmid was kindly provided by Dr. J. Summers from the University of New Mexico, Albuquerque, New Mexico (Pardoe & Michalak, 1995). The construct was digested with *Eco*RI and separated by electrophoresis in 1% low melting point EB-agarose (Gibco BRL). The band containing the linear, cloned WHV DNA (rWHV DNA) was excised from the gel and isolated using the Wizard™ PCR Prep DNA Purification System (Promega Corp.), as per manufacturer's instruction. DNA concentration was determined based on its absorbance at 260 nm, as described above (Section

2.10.1).

This rWHV DNA was labelled with ^{32}P-dCTP using the *redi*prime™ DNA labelling system (Pharmacia Biotech) following manufacturer's instructions. Briefly, 20 ng of rWHV DNA was made up to 45 µl in autoclaved double distilled water, boiled for 5 min, and chilled on ice for about 2 min. The DNA mixture was added to the *redi*prime™ reaction tube and the tube was flicked gently several times to resuspend the lyophilized mixture which consisted of dATP, dGTP, dTTP, Klenow enzyme, and 9-mer random primers. Then, 5 µl of α-^{32}P-dCTP (10 µCi/µl; Amersham) was added and the tube was flicked a few times. The labelling reaction was incubated for 1 h at 37°C in a water bath.

The ^{32}P-labelled probe was isolated from unincorporated ^{32}P-dCTP by fractionation on a NICK™ column (Pharmacia Biotech), as described in the manufacturer's instruction sheet. The column was washed with 3 ml of TE buffer and inserted in a 15-ml disposable centrifuge tube. Fifty µl of the probe mixture was applied onto the column, followed by 400 µl of TE buffer. The eluent was discarded and 450 µl of TE buffer was applied to the column. This eluted fraction containing the labelled probe was collected and a 2-µl sample was used for radioactivity counting in a scintillation counter. The cpm per µl of the aliquot was determined. The probe was stored at 4°C prior to being used for Southern blot hybridization (Section 2.14.2.3).

2.15 CLONING OF WHV DNA.

Selected samples that were PCR amplified with PGAP1-MCOR (cccDNA specific) primers were purified with Wizard™ PCR Prep kit and inserted into plasmids using the TOPO TA Cloning© Kit (Invitrogen, Carlsbad, California). Briefly, 5 µl of purified PCR product (25 ng) and 1 µl of the TOPO TA cloning vector (pCR® II) were incubated for 8 min at 37°C. Four µl of the above mixture was added to TOPO One Shot™ cells containing 20 mM β-mercaptoethanol and the mixture was kept for 30 min on ice. The cells were then heat shocked at 42°C for 30 sec and placed on ice for 2 min. Subsequently, 250 µl of SOC medium (2% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10mM $MgSO_4$, and 20 mM [wt/vol] glucose) was added to the cells and the tube was shaken horizontally at 37°C for 60 min. One hundred µl of this cell mixture was spread on a 50 µg/ml kanamycin-agar plate containing 40 mg/ml of X-gal (Sigma Chemical Co.). The plate was incubated upside down at 37°C for 16 h. Two white and 2 blue colonies were selected and plasmid DNA was prepared from them using the Mini-Prep method.

Each bacterial colony selected was grown to saturation at 37°C for 16 h in 5 ml of sterile LB-kanamycin medium. Subsequently, 1.5 ml of cells was pelleted at 15,000 *x* g for 20 sec. The cells were resuspended in 100 µl of GTE solution (50 mM glucose, 10 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0) and allowed to sit at ambient temperature for 5 min. The cells in GTE were lysed by mixing with 200 µl

of NaOH/SDS solution (0.2M NaOH and 1% [wt/vol] SDS) and placed on ice for 5 min. Then, 250 µl of potassium acetate (3 M, pH 5.5) was added, the tube was vortexed for 2 sec and placed on ice for 5 min. The mixture was spun for 3 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to a new tube and 1 ml of 95% ethanol was added to precipitate plasmid DNA and RNA. After 2 min at room temperature, the tube was centrifuged at 15,000 *x* g for 1 min. The pellet was washed with 1 ml of 70% ethanol, pellet dried under vacuum and resuspended in 30 µl of TE buffer. One µl of this plasmid DNA was used for each sequencing cocktail.

2.16 WHV DNA SEQUENCING.

In selected cases, WHV DNA fragments which were amplified by PCR with WHV cccDNA or WHV core gene-specific primers were purified and sequenced to confirm their nucleotide sequence specificity. This was done either after cloning (Section 2.15) or directly after PCR amplification. In the latter situation, 40 µl of a PCR product was separated on a 0.8% low melting point EB-agarose gel and the visualized band was excised and purified using Wizard® PCR Prep. (Section 2.14.2.4). Subsequently, the recovered DNA was quantitated spectrophotometrically (see Section 2.10.1).

Isolated WHV DNA cloned or purified directly from PCR mixture was used as a template for cycle sequencing with the *fmol*® DNA sequencing system

(Promega Corp.), using manufacturer's procedure. Briefly, 17 µl of a sequencing cocktail, containing 40 fmol of template DNA or 1 µl of plasmid nucleic acid, 1.5 pmol of γ-^{32}P-ATP (Amersham) end-labelled W0HV DNA-specific sequencing primers (QC02, QX12 or QP11; Table 2.3), 5 units of sequencing grade *Taq* DNA polymerase, 2 mM of $MgCl_2$ in 50 mM Tris-HCl buffer, pH 9.0, was prepared. Four µl of the cocktail was aliquoted into 4 tubes containing 2 µl of G (guanine), A (adenine), T (thymine) or C (cytosine) terminating nucleotide/dNTP mixture. Cycle sequencing was conducted using the following program: 95°C for 5 min for 1 cycle and 95°C for 30 sec, 42°C for 30 sec, and 70°C for 1 min, for 30 cycles. Upon completion of the cycle sequencing, 3 µl of sequencing stop solution (10 mM NaOH, 95% [vol/vol] formamide, 0.05% [wt/vol] bromophenol blue, and 0.05% [wt/vol] xylene cyanole) was added to each tube and samples were kept at -20°C until application on a sequencing gel. Prior to loading on the gel, each sample tube was heated to 70°C for 2 min and 3 µl/lane of the sequencing product was loaded in the order of G, A, T, C. The DNA fragments were separated on an 8% polyacrylamide gel containing 7 M urea (Gibco BRL) in a Sequi-Gen® GT Nucleic Acid electrophoresis cell (Bio-Rad Laboratories) equipped with a temperature probe and a power supply (Power Pac 3000; Bio-Rad Laboratories). After electrophoresis at 50°C for 2.5 h, the gel was fixed with a 10% (vol/vol) methanol and 10% (vol/vol) acetic acid solution and dried in a gel dryer for 2 h. The gel was exposed on an X-ray film (Kodak) or on a phosphor screen and analysed by a phosphorimager.

Table 2.3. Oligonucleotide primers used for WHV DNA sequencing.

Primer	Sequence 5' - 3'	Position[a]
QCO2	5'-CTCGGATCCCTATAAAGAATTTGG	2030-2049
QP11	5'-CCATGGCTGCTCGCCTGTGTTGCC	1501-1524
QX12	5'-GGCATGCCAAGTAAGGACC	1791-1809

[a] Numbers denote nucleotide positions in the WHV genome sequence based on Galibert *et al.*, 1982 (GenBank accession number J02442).

The WHV specificity of the nucleotide sequences determined in this study was confirmed using the GeneBank BLAST search (world wide web service; http://www.ncbi.nlm.nih.gov/BLAST/).

CHAPTER THREE : RESULTS

3.1 CHARACTERISTICS OF CULTURED WOODCHUCK HEPATOCYTES AND LYMPHOID CELLS.

3.1.1 Cultured normal woodchuck hepatocytes and their features.

Our previous attempts to grow normal, differentiated, woodchuck hepatocytes for extended periods on plastic surfaces or in suspension were not successful. It was found that hepatocytes can attach to commercially available basement membrane matrices, such as Matrigel® (Becton Dickinson), but they do not proliferate (Churchill & Michalak, unpublished). Subsequent experiments (Diao *et al.*, 1998), prompted by an observation made in human hepatocyte cultures (Roberts *et al.*, 1993), showed that the coating of culture vessels with 0.1% gelatin allows woodchuck hepatocytes to attach and propagate. Furthermore, it was established that complete Hepato-STIM™ formulation recommended by the manufacturer (Becton Dickinson) for the culturing of rat hepatocytes did not support the growth of woodchuck hepatocytes (Churchill & Michalak, unpublished). However, it was found that the same Hepato-STIM™ medium when supplemented with 20% of supernatant from the culture of human HepG2 liver cells promotes growth of woodchuck hepatocytes (Diao *et al.*, 1998). These woodchuck cells synthesized hepatocyte-specific proteins, such as ASGPR and albumin, as was shown previously by Western blot analysis and flow cytometry (Diao *et al.*, 1998).

In the present study, normal differentiated woodchuck hepatocytes (WCM-260) have been maintained by weekly passages for more than 3 years without loss

of expression of hepatocyte-specific gene transcripts (i.e., ASGPR). Transcriptional activity of the ASGPR major subunit gene was demonstrated by using PCR with an RT step on RNA samples from WCM-260 hepatocytes which were either continuously kept in culture for more than 3 years or recently re-established from a frozen stock of the cells (Fig. 3.1). Specificity of the detected ASGPR cDNA amplification signals was confirmed in parallel reactions in which RNA extracted from human HepG2 cells was used as a positive control (Fig. 3.1, lane 5) and by PCR amplifications of WCM-260 RNAs without the preceding reverse transcription step, as negative controls (Fig. 3.1, lanes 2, 4, & 6). In addition, specificity was ascertained by Southern blot analysis of the PCR products with radiolabelled, cloned, woodchuck ASGPR major subunit fragment prepared recently in this laboratory (Slaney, 1999). These results demonstrate that WCM-260 cells invariably displayed transcriptional activity of hepatocyte-specific ASGPR gene.

To determine the dynamics of WCM-260 hepatocyte growth in conditioned Hepato-STIM™ medium, the number and viability of these cells were determined by trypan blue exclusion at 2, 4, and 6 days after seeding. It was found that the cells multiply with approximate doubling time of 2 days (Table 3.1). The yield of hepatocytes after a 6-8 day culture was in the range of 1.9×10^6 to 3×10^6 cells per 25-cm^2 flask. Hepatocytes subjected to multiple passages displayed a stable morphology of elongated cells with single nuclei, survived storage in liquid nitrogen for a prolonged period of time, and re-establish a confluent monolayer culture a few

Figure 3.1. Transcription of ASGPR major subunit gene in WCM-260 hepatocytes. One μg of RNA extracted from WCM-260 cells was converted to cDNA by RT (RT+) and PCR amplified with ASGPR major subunit-specific primers. As a positive control, cDNA prepared from HepG2 cells was amplified in parallel. A mock sample containing all reagents used but not RNA and RNA samples treated in the absence of reverse transcriptase (RT-) were used as negative controls. Electrophoretically separated PCR products were hybridized by Southern blotting with a ^{32}P-labelled, cloned woodchuck ASGPR major subunit probe. The expected size of the PCR product is indicated on the right side of the panel.

RT
+
-
WCM-260 (3 years)
+
-
WCM-260 (from stock)
+
-
HepG2
Mock
<426 bp

Table 3.1. Survival of woodchuck WCM-260 hepatocytes in culture at different time points after seeding[a].

Days post seeding	Cells recovered (10^5/well)	Viability (%)
2	2.6 ± 0.49	98
4	6.7 ± 0.06	98
6	13.5 ± 0.98	99

[a] Cells were seeded at 1.2 x 10^5/well in 6-well culture plates under conditions described in Materials and Methods. Harvested hepatocytes were evaluated by trypan blue exclusion at the time points indicated. Results are presented as the mean ± standard deviation (SD) from three separate determinations.

days after seeding.

3.1.2 Effect of glucagon on cultured woodchuck hepatocytes.

Following a recent observation that the presence of 100 nM glucagon in a culture medium inhibited DHBV replication in primary duck hepatocytes (Hild *et al.*, 1998), we aimed to establish whether a similar phenomenon may occur in woodchuck hepatocytes exposed to WHV in our culture system. Thus, WCM-260 cells were cultured in conditioned Hepato-STIM™ medium in the presence or absence of 100 nM glucagon (Section 2.3.5). It was found that removal of glucagon from the culture medium did not affect the growth, long-term (more than 10 months) survival of the cells, as well as to their morphology. In addition, woodchuck hepatocytes maintained in glucagon-free, conditioned Hepato-STIM™ medium demonstrated approximately the same susceptibility to WHV infection and ability to support virus replication as WCM-260 hepatocytes maintained in glucagon-containing medium, as illustrated in Figure 3.2. In general, the data obtained revealed that the presence or absence of glucagon in the culture medium does not affect growth of woodchuck hepatocytes or has an inhibitory effect on WHV infectivity and replication. Thus, unless indicated, the data presented in this study were obtained by using cells cultured in the absence of glucagon.

Figure 3.2. Presence of glucagon in culture medium did not influence the *in vitro* infection of woodchuck hepatocytes with WHV. Approximately 1 µg of mung bean nuclease-digested DNA from WCM-260 cells, which were exposed to serum-derived WHV and then cultured with (WHV/260 [+ glucagon]) or without glucagon (WHV/260 [- glucagon]), were amplified by PCR with WHV core gene primers (WHV DNA) as described in Materials and Methods. As a positive control, 1 µg of mung bean nuclease-digested DNA extracted from the liver of a chronic WHV carrier was included, whereas mung bean nuclease-treated DNA from non-infected WCM-260 cells maintained in the presence (+g) or absence (-g) of glucagon were used as negative controls. PCR products were probed with ^{32}P-labelled rWHV DNA. The expected size of the amplified PCR fragment is shown on the right side of the panel.

Positive control
WHV/260 (+ glucagon)
WHV/260 (- glucagon)
Negative controls
+g
-g
<490 bp

3.1.3 Viability of WCM-260 cells in the presence of anti-LP1 antibodies.

Since the effect of rabbit antibodies against a synthetic analogue of the N-terminal sequence of WHV large protein (anti-LP1) on the *in vitro* WHV infectivity was examined, it was necessary to establish whether these antibodies are or are not cytotoxic for WCM-260 cells. For this purpose, cultured hepatocytes were incubated with increasing concentrations of anti-LP1 serum and their viability was evaluated by MTT assay, as described in Section 2.3.6.2 of Materials and Methods. It was found that 24 h incubation with heat-inactivated anti-LP1 serum at a concentration as high as 10% (vol/vol) in culture medium did not affect survival of the hepatocytes (Fig. 3.3).

3.1.4 WHV-infected woodchuck lymphoid cells cultured in hepatocyte growth medium.

To assess survival of isolated woodchuck lymphoid cells in culture conditions established to support growth of WCM-260 cells, PBMC from a chronically infected WHV carrier were incubated in conditioned Hepato-STIM™ medium for 3, 6, and 9 days (see Section 2.4.2). It was observed that PBMC can survive for up to 3 days without significant loss of viability and change in cell number. When cultured for longer than 3 days, there was substantial and progressive decrease in the number of recovered cells and in their viability (Table 3.2). Thus, approximately half of the cells were lost at day 6 and up to two thirds at day 9 in comparison to PBMC

Figure 3.3. Incubation with rabbit anti-LP1 antibodies did not affect WCM-260 hepatocyte viability. Hepatocytes were incubated with increasing concentration (0-10% [vol/vol]) of rabbit anti-LP1 serum or preimmune serum (negative control) for 24 h prior to MTT assay as described in Materials and Methods. Data are represented as absorbance of solubilized intracellular MTT metabolite at 595 nm (A_{595}) and expressed as the mean ± SD from three separate determinations.

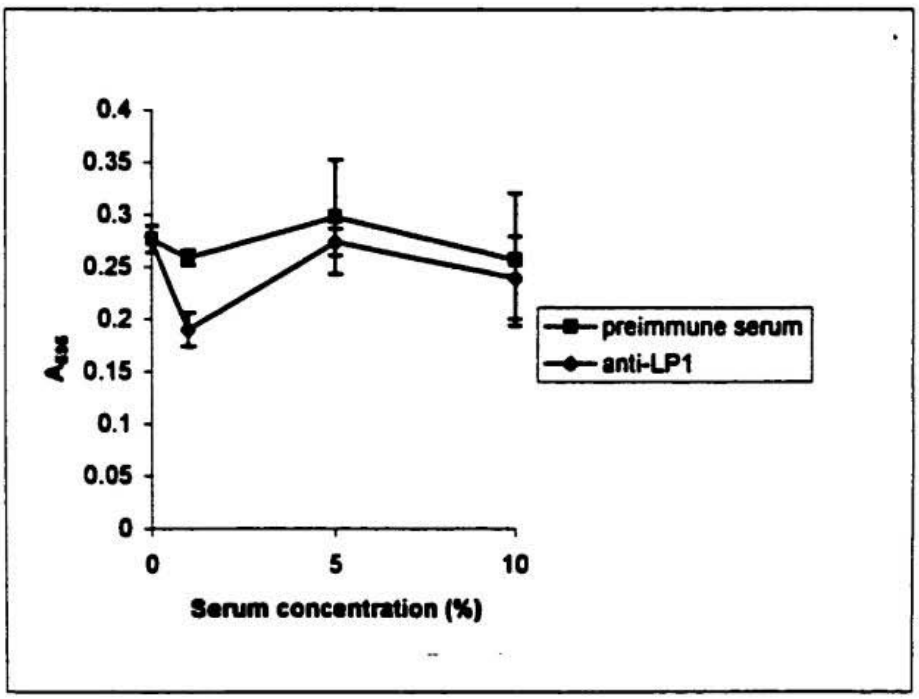

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
A_{595}
0
5
10
Serum concentration (%)
preimmune serum
anti-LP1

Table 3.2. Survival of PBMC cultured in conditioned Hepato-STIM™ medium at different time points after seeding[a].

Days in culture	Recovered cells (10^5/ml)	Cell viability (%)
3	13.0	92
6	6.7	66
9	4.3	50

[a] WHV-infected PBMC isolated from a chronic WHV carrier were seeded at 1.3 x 10^6 cells/ml in 7.5 ml of conditioned Hepato-STIM™ medium supplemented with 100 nM glucagon and incubated for 3, 6 or 9 days under conditions presented in Materials and Methods. At each of the above time points, PBMC number and their viability were evaluated by trypan blue exclusion test.

recovered on the third day after seeding. Under test conditions, cell viability decreased from 92% at day 3 to 66% and 50% at days 6 and 9, respectively (Table 3.2). This indicates that the conditions used for culture of woodchuck hepatocytes were also appropriate for a short-term (up to 3 days) maintenance of woodchuck PBMC. Of note is that the rate of woodchuck PBMC survival was similar for cells either maintained in conditioned Hepato-STIM™ medium or in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.05% β-mercaptoethanol and antibiotics. This may suggest that the relatively short survival of these cells in culture was unlikely due to the adverse effect of the hepatocyte culture medium, but rather a consequence of an intrinsic property of the isolated woodchuck PBMC. Lymphoid cells isolated from spleens of WHV-infected woodchucks cultured in conditioned Hepato-STIM™ medium survived at the same rates as woodchuck PBMC.

Analysis of WHV genome expression in PBMC cultured for different time periods in conditioned Hepato-STIM™ medium showed that WHV DNA was detectable by direct PCR/Southern blot hybridization (sensitivity of 3×10^3 vge/ml) until 6 days (Fig. 3.4, lane 4), but not in the cells kept for 9 days (Fig. 3.4, lane 5). However, WHV DNA sequences were demonstrable in the latter cells by nested PCR assay and EB-agarose (sensitivity of 3×10^2 vge/ml; data not shown). In addition, further experiments showed that PBMC and splenocytes cultured for up to 4 days expressed WHV cccDNA (see example in Section 3.4.5). This finding

Figure 3.4. Presence of WHV DNA in PBMC isolated from a chronic WHV carrier cultured for different time periods in hepatocyte growth medium. One µg of DNA extracted from PBMC cultured for 0, 3, 6 or 9 days in conditioned Hepato-STIM™ supplemented with 100 nM glucagon was amplified by direct PCR using WHV C gene primers. DNA isolated from the liver of a WHsAg-positive chronic carrier was used as a positive control and a mock sample treated as DNA but not containing nucleic acid was used as a negative control. Electrophoretically separated PCR products were probed with ^{32}P-labelled rWHV DNA by Southern blot hybridization. The expected size of the WHV DNA-specific PCR product is indicated on the right side of the panel.


Positive control
Days in culture
0
3
6
9
Negative control
<620 bp

indicates that the culture conditions established for maintenance of woodchuck hepatocytes were also adequate to support WHV in the naturally infected PBMC for at least 6 days. Therefore, considering survival of infected PBMC *in vitro* (as described above) and the presence of WHV genome, woodchuck lymphoid cells were cultured for 72 h in conditioned Hepato-STIM™ medium in all subsequent experiments.

3.1.5 Survival of woodchuck lymphoid cells in the presence of mitogens.

In an attempt the prolong the survival of woodchuck lymphoid cells in *in vitro* conditions, we cultured PBMC in conditioned Hepato-STIM™ medium supplemented with 5 µg/ml of PHA (Sigma Chemical Co.) or 10 µg/ml of LPS (Sigma Chemical Co.). It was found that stimulation with these two mitogens did not improve viability of woodchuck lymphoid cells in the used culture conditions (data not shown).

Previous studies have shown that the culture of WHV-infected PBMC with LPS or ConA increased viral replication (Korba *et al.*, 1988; Coffin & Michalak, 1999) and that culture supernatants from these cells were infectious to virus-naive woodchucks (Korba *et al.*, 1989; Michalak *et al.*, 1999; Coffin & Michalak, 1999). However, in the present study, the results of a dot-blot hybridization assay showed that WHV DNA content in culture supernatant of splenocytes cultured in the presence of hepatocyte growth medium supplemented with LPS was not different

from that in culture supernatant of the cells cultured without LPS (Fig. 3.5A).

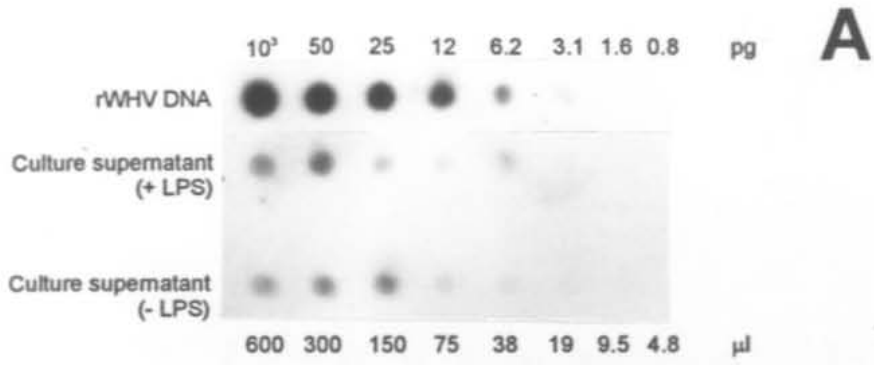
In addition, WHV inocula generated from lymphoid cells cultured with and without LPS showed a comparable ability to induce infection of WCM-260 hepatocytes (Fig. 3.5B & C). Thus, DNase/trypsin treated WCM-260 cells which were infected with WHV originating from either PBMC (Fig. 3.5B) or splenocytes (Fig. 3.5C) cultured in the presence or absence of LPS contained WHV DNA at comparable quantities, as revealed by similar density of the PCR product bands. In addition, WCM-260 hepatocytes exposed to WHV derived from splenocytes cultured in the presence or absence of LPS contained comparable amounts of WHV cccDNA (Fig. 3.5C). The lack of an increase in WHV nucleic acid expression in hepatocytes exposed to inoculum prepared in the presence of LPS was not due to the mitogen's cytotoxic effect on hepatocytes, since it was previously established that the incubation of WCM-260 cells with 10 μg/ml of LPS for 24 h did not affect their viability in an MTT assay (data not shown).

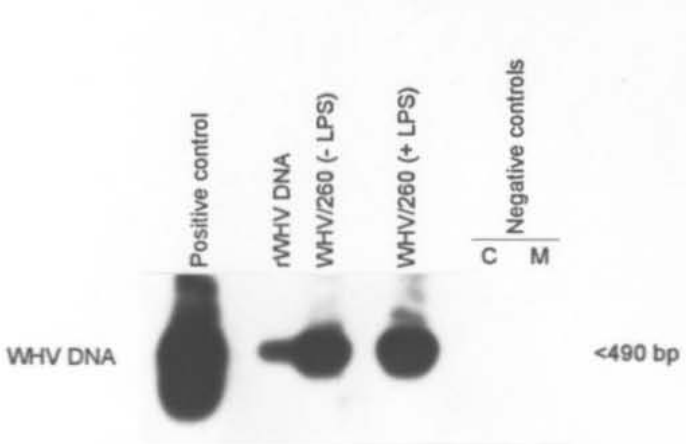
3.2 IDENTIFICATION OF WHV cccDNA.

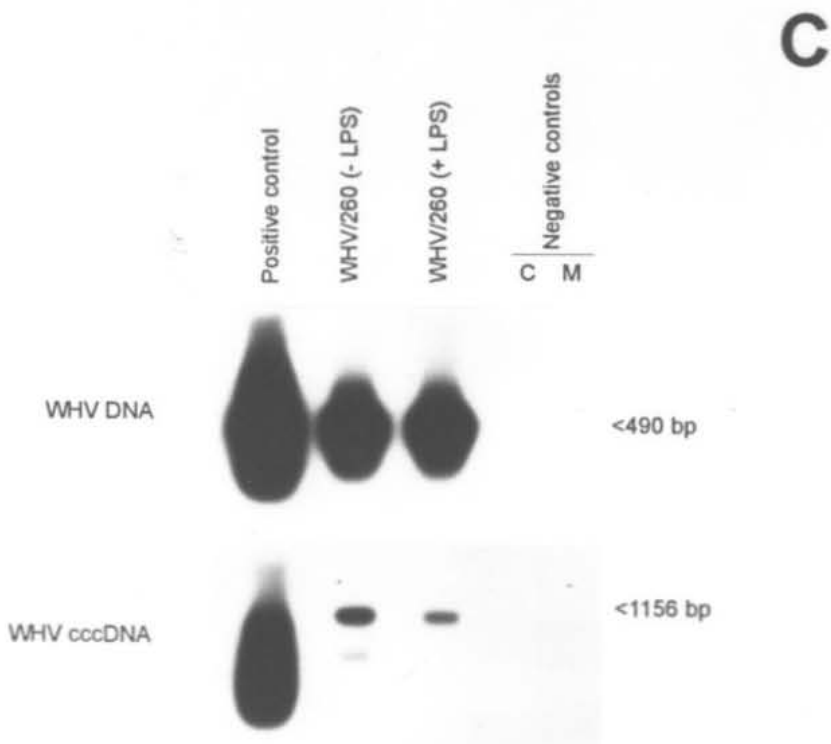
3.2.1 The applicability of KCl precipitation method for detection of WHV cccDNA in woodchuck cell cultures.

The different forms of non-protein associated WHV DNA were isolated using KCl precipitation and identified by Southern blot hybridization. Since the electrophoretic properties of the various WHV DNA species are different, they can

Figure 3.5. Lymphoid cell WHV inocula prepared in the presence of lipopolysaccharide have comparable infectivity to inocula generated in the absence of this mitogen. (A) Supernatants from WHV-infected splenocytes cultured with (+ LPS) or without (- LPS) 10 μg/ml of LPS were evaluated for WHV DNA content by dot-blot hybridization. The supernatants were loaded in serial two-fold dilutions (beginning from 600 μl/dot) in parallel with samples of rWHV DNA standard (beginning from 10^3 pg of DNA/dot). (B) DNA (2.5 μg/sample) from WCM-260 hepatocytes exposed to inocula prepared from WHV-infected PBMC cultured in the absence (WHV/260 [- LPS]) or presence (WHV/260 [+ LPS]) of 10 μg/ml LPS were tested for WHV DNA expression by PCR with primers specific for WHV core gene (PCOR1-MCOR) and then the PCR products analysed by Southern blot hybridization. (C) DNA (3.8 μg/sample) from WCM-260 hepatocytes exposed to inocula prepared from WHV-infected splenocytes cultured in the absence (WHV/260 [- LPS]) or presence (WHV/260 [+ LPS]) of 10 μg/ml LPS were evaluated for WHV DNA and WHV cccDNA by PCR and then analysed by Southern blot hybridization. Inocula were prepared simultaneously from PBMC and splenocytes of #19056 animal. One μg mung bean nuclease-digested DNA extracted from WHV-positive liver and 1 fg of rWHV DNA were used as positive controls. As negative controls, DNA from virus naive WCM-260 hepatocytes (C) and mock (M) samples were used. All blots were probed with a ^{32}P-labelled rWHV DNA probe. The expected sizes of the amplified PCR products are marked on the right.

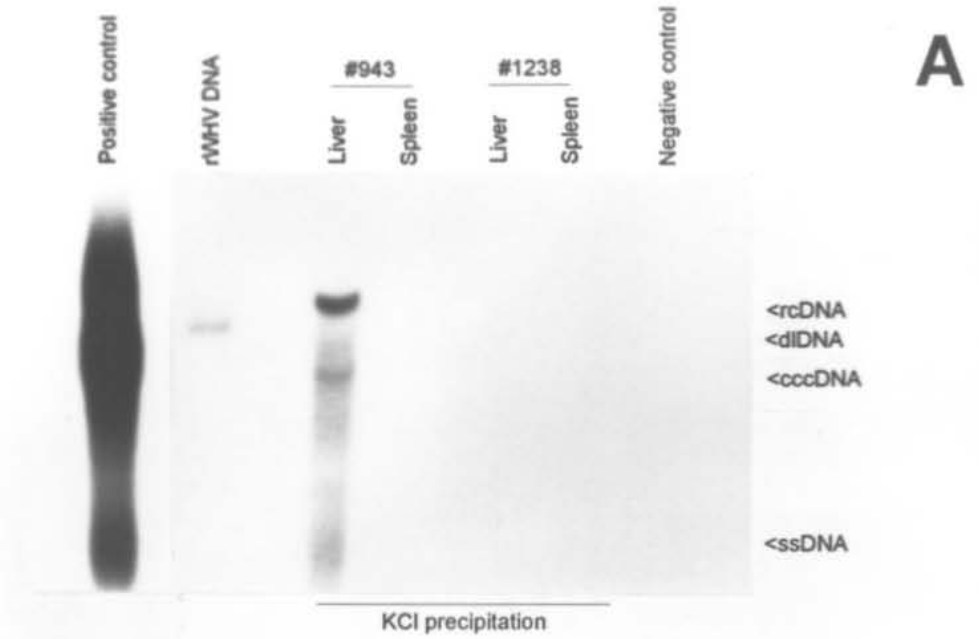

A
10³ 50 25 12 6.2 3.1 1.6 0.8 pg
rWHV DNA
Culture supernatant (+ LPS)
Culture supernatant (- LPS)
600 300 150 75 38 19 9.5 4.8 µl

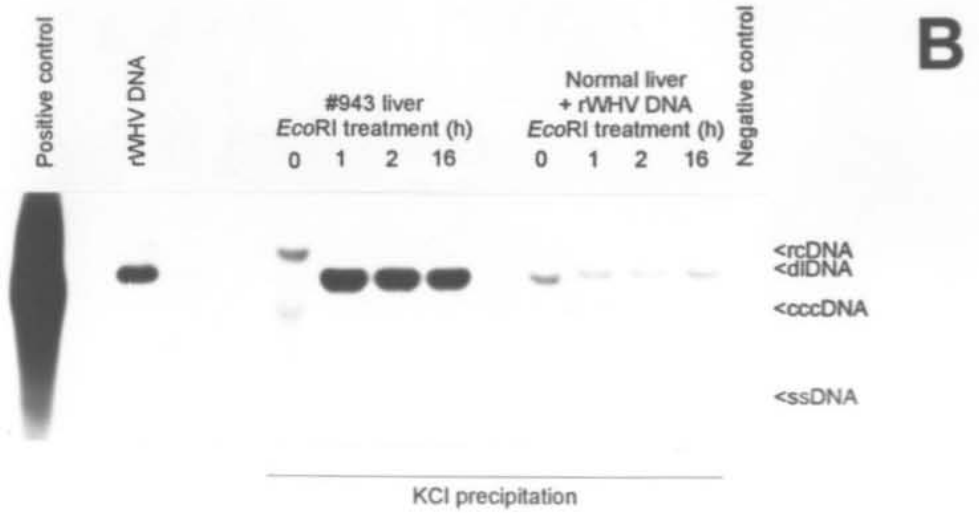

B
Positive control
rWHV DNA
WHV/260 (- LPS)
WHV/260 (+ LPS)
Negative controls
C M
WHV DNA
<490 bp


C
Positive control
WHV/260 (- LPS)
WHV/260 (+ LPS)
Negative controls
C M
WHV DNA
<490 bp
WHV cccDNA
<1156 bp

be discriminated based on their relative migration in agarose gel. WHV DNA extracted by KCl precipitation have 3 distinguishable circular DNA species: rcDNA, cccDNA and single-stranded DNA (ssDNA) (Fig. 3.6A, lane 3). This is in contrast to WHV DNA extracted using the standard proteinase K/phenol-chloroform protocol which yields viral DNA fragments appearing as smears on Southern blots (Fig. 3.6A, lane 1). Based on *Eco*RI enzyme-linearized ~3.3 kb recombinant WHV DNA (Fig. 3.6A, lane 2) and a standard curve plotted using *Hae*III molecular weight markers electrophoresed in parallel, WHV rcDNA and cccDNA isolated by KCl precipitation showed apparent molecular weights of 3.8 and 2.3 kb, respectively (Fig. 3.6A, lane 3). These species were identified in the liver of a chronically infected woodchuck (#943) that carried high levels of WHV DNA in hepatic tissue (1×10^8 vge/μg of total DNA) and in serum (1×10^{11} vge/ml). However, the same WHV DNA species were not detectable in the spleen from the same animal or in the liver of another woodchuck (#1238) with chronic WHV infection carrying lower levels of viral DNA in hepatic tissue (1×10^6 vge/μg of total DNA) and in serum (1×10^7 vge/ml). This finding indicates that Southern blot analysis of DNA extracted by KCl precipitation does not have sufficient sensitivity to identify replicative WHV DNA intermediates either in the lymphoid cells or in the liver of animals with lower levels of WHV replication. It was concluded this method cannot be employed to detect WHV cccDNA sequence in *in vitro* infected hepatocytes which, as we have found, displayed usually less than 1×10^3 vge/μg of genomic DNA.

Figure 3.6. Southern blot analysis of liver-derived WHV DNA isolated by standard proteinase K/phenol-chloroform procedure and by a KCl precipitation method. DNA obtained by a standard extraction method (5 µg/lane) and DNA isolated by a KCl precipitation protocol (10 µg/lane) were electrophoresed on EB-agarose, transferred on nylon membranes, and hybridized with a ^{32}P-labelled rWHV DNA probe. (A) DNA extracted by a KCl precipitation method from livers and spleens of a chronically infected woodchuck (#943) with high levels of circulating WHV and from a woodchuck (#1238) with low virus load. (B) DNA extracted by a KCl-precipitation protocol from liver of a chronic WHsAg-carrier (#943) and from normal woodchuck liver spiked with rWHV DNA were subjected to *Eco*RI digestion for 1, 2, and 16 h. Five µg of DNA isolated by a standard DNA extraction protocol from the livers of #943 and a normal woodchuck were used as positive and negative controls, respectively. As references, 50 pg of rWHV DNA was included as a DNA migration control. The predicted positions of WHV rcDNA, cccDNA, ssDNA, and double stranded linearized WHV DNA (dlDNA) are indicated on the right side of the panel.


A
Positive control
rWHV DNA
#943
Liver
Spleen
#1238
Liver
Spleen
Negative control
<rcDNA
<dlDNA
<cccDNA
<ssDNA
KCl precipitation


B
Positive control
rWHV DNA
#943 liver
EcoRI treatment (h)
0 1 2 16
Normal liver
+ rWHV DNA
EcoRI treatment (h)
0 1 2 16
Negative control
<rcDNA
<dlDNA
<cccDNA
<ssDNA
KCl precipitation

However, to complete the evaluation of the KCl precipitation method's applicability for detection of WHV cccDNA, isolated DNA from the liver of a #943 woodchuck was digested with *Eco*RI restriction endonuclease (Section 2.11.1) and then analysed by Southern blot hybridization to confirm that the WHV DNA molecular forms detected were in fact circular and contained a single *Eco*RI restriction endonuclease site. It was found that all WHV DNA species (i.e., rcDNA, cccDNA, and ssDNA), which displayed different mobilities prior to digestion (Fig. 3.6B, lane 3), became linearized and migrated with the same velocity after treatment with *Eco*RI for 1, 3 or 16 h (Fig. 3.6B, lanes 4-6). Of note is that the band densities of the *Eco*RI-digested WHV DNA (Fig. 3.6B, lanes 4-6) were greater than that of undigested circular WHV DNA (Fig. 3.6B, lane 3). This was most likely due to the convergence of the heterogenous circular WHV DNA species into a single band of linearized WHV DNA. In addition, the linearized WHV DNA molecules had migrational characteristics similar to the control rWHV DNA spiked into a normal liver homogenate, which was extracted by KCl precipitation and digested with *Eco*RI enzyme for the same periods of time as test samples (Fig. 3.6B, lanes 7-10). These findings indicate that the KCl precipitation method allows for the separation of circular, non-integrated WHV DNA molecules when the virus occurs in large quantities (more than 10^8 vge/µg total DNA).

3.2.2 Establishment of PCR conditions for parallel detection of total WHV DNA and WHV cccDNA sequences

Since the KCl precipitation method was insensitive for identification of WHV cccDNA in samples with a low virus load, a PCR procedure specifically amplifying the fragment spanning the WHV nick region was designed. In parallel, to evaluate total content of WHV DNA in the same test DNA samples, the uninterrupted region located in the virus core gene was amplified (Table 2.2). For this purpose, two sets of oligonucleotide primers PCOR1-MCOR and PGAP1-MCOR specific for core gene and nick-spanning region, respectively, were designed as described in Materials and Methods (Section 2.13). In a preliminary experiment, it was established that the modified PCR conditions, which included the increase in annealing temperature from 52°C to 56°C and extending the reaction times from 30 sec to 1 min per step, improved the stringency and efficiency of WHV cccDNA and core gene detections in comparison to our standard PCR amplification method employing C gene-specific primers (Table 2.2). Nucleotide sequence analysis of the PCR products amplified with PCOR1-MCOR and PGAP1-MCOR primer pairs confirmed that the amplified fragments in fact represented the core gene sequence and the nick-spanning region of WHV DNA, respectively, and that they were homologous to the equivalent WHV DNA sequences reported (Table 3.3, also see Fig. 3.23).

Since EDTA has to be used to inactivate mung bean nuclease (which was routinely used to eliminate WHV DNA molecules with single stranded regions prior

Table 3.3. Specificity of WHV DNA fragments amplified with PCOR1-MCOR and PGAP1-MCOR primer pairs determined by direct sequence analysis of PCR products[a].

Primer pair	Amplified WHV DNA fragment	WHV DNA sequence amplified[b] (nt position)	Sequencing primer	WHV DNA fragment sequenced (nt position)[b,c]	% homology with reported WHV DNA sequences[c]
PCOR1-MCOR	core gene	1964-2429	QCO2	2105-2224	96
PGAP1-MCOR	nick region	1298-2429	QP11	1591-1723	94
			QX12	1870-2073	97

[a] PCR amplification was done using 1 µg of mung bean nuclease-digested DNA extracted from the liver of a woodchuck with chronic WHV infection under thermocycling and sequencing conditions described in Materials and Methods.

[b] nt; nucleotide position according to WHV DNA sequence reported by Galibert *et al.*, 1982 (GenBank accession number J02442).

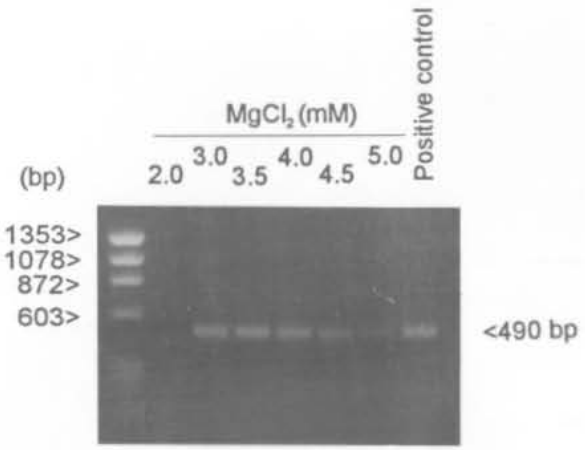
[c] Location of the amplified and sequenced WHV fragments and their homology to the reported WHV DNA sequence (Galibert *et al.*, 1981).

to PCR for WHV cccDNA identification) and it interferes with the activity of Mg^{2+} ions in the PCR mixture, the concentration of the latter has to be adjusted to compensate for the EDTA presence. To determine the appropriate Mg^{2+} concentration in the PCR mixture, 1 pg of rWHV DNA was amplified in the presence of increasing amounts of $MgCl_2$ (Section 2.13.3). Analysis of the obtained PCR products by agarose gel electrophoresis showed that WHV DNA was not visualized when 2 mM of Mg^{2+} was used, while concentrations from 3 mM to 5 mM gave DNA fragments of the expected molecular sizes (Fig. 3.7). Of note is that the band intensity decreased slightly as Mg^{2+} concentration increased beyond 3 mM. This was probably due to the declining stringency of the amplification reaction due to the excess of Mg^{2+} ions. Therefore, all subsequent PCR amplification of mung bean nuclease-digested DNA samples were done in the presence of 3 mM of Mg^{2+}. For the nested PCR amplification of fragments spanning the nick region (using XINT-CCCV primer pair; Table 2.2), it was found that the standard concentration of 1.5 mM $MgCl_2$ was sufficient.

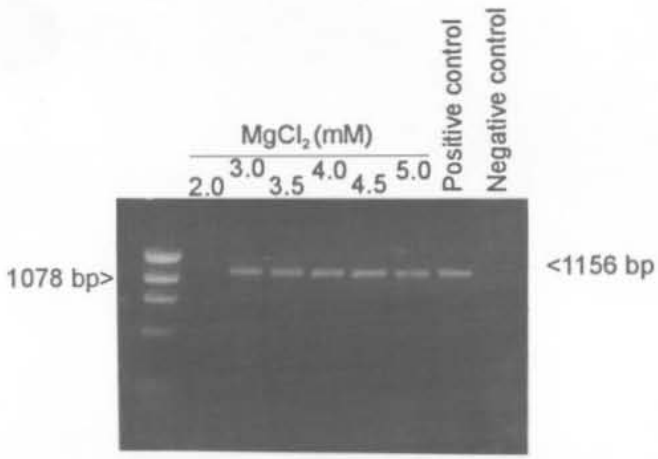
To achieve the most comparable conditions for WHV core gene and cccDNA sequence amplifications, both primer pairs (i.e., PCOR1-MCOR and PGAP1-MCOR; Table 2.2) shared a common antisense primer (i.e., MCOR). In addition, to further equalize the reaction milieu, all DNA preparations predestined for both core gene and cccDNA-specific amplifications were digested with mung bean nuclease and treated with EDTA (Section 2.11.2). To establish the sensitivity of the PCR

Figure 3.7. Determination of optimal Mg^{2+} concentration in PCR mixture for parallel amplifications of total WHV DNA and WHV cccDNA species. One pg of rWHV DNA was used in a series of reactions containing increasing concentrations of $MgCl_2$ (2 - 5 mM). PCR was performed in the presence of 130 mM of EDTA with PCOR1-MCOR primers (panel A) or with PGAP1-MCOR primers (panel B), designed for identification of WHV core gene or WHV cccDNA, respectively. In each set of reactions, 1 pg of rWHV DNA in the presence of 1.5 mM $MgCl_2$ (without EDTA) was amplified as a positive control. As negative control, PCR mixture without rWHV DNA was included. Aliquots of PCR products were electrophoresed on an EB-agarose gel and visualized by UV light. The molecular sizes (bp) of the markers are indicated on the left and the positions and sizes of the expected WHV fragments are shown on the right side of the panels.

A


MgCl2 (mM)
2.0
3.0
3.5
4.0
4.5
5.0
Positive control
(bp)
1353>
1078>
872>
603>
<490 bp

B


MgCl2 (mM)
2.0
3.0
3.5
4.0
4.5
5.0
Positive control
Negative control
1078 bp>
<1156 bp

performed under these modified conditions, 10-fold dilutions of recombinant WHV DNA, containing from 1 pg to 100 ag, were used as templates and amplified using both PGAP1-MCOR and PCOR1-MCOR primer pairs. EB-agarose gel evaluation of the resulting PCR products showed that the sensitivity for detection of WHV core gene and WHV cccDNA fragments was 10 fg WHV DNA (or 3×10^4 vge/ml) and 100 fg WHV DNA (or 3×10^5 vge/ml), respectively (data not shown). Subsequent Southern blot analysis of the same PCR products increased the sensitivity of the detection by 10-fold (Fig. 3.8). Sensitivity of nested PCR/Southern blot hybridization for detection of WHV cccDNA sequences with XINT-CCCV primers was 1 fg or 3×10^3 WHV vge/ml (data not shown). To increase the sensitivity and specificity of the WHV DNA detection, Southern blot hybridization of PCR products was routinely employed.

3.3 WHV CONTENT IN INOCULA DERIVED FROM SERA AND LYMPHOID CELLS.

To determine the virus load in the WHV inocula used for *in vitro* cell infections, the WHV DNA content was examined in the first instance by dot-blot hybridization, as described in Section 2.6 & 2.14.2. In the preliminary dot-blot experiment, when the rWHV DNA were loaded in two-fold serial dilutions beginning from 1 ng (3×10^6 WHV vge)/dot, a linear relationship between WHV DNA amount and densitometric digital light units (DLU) values was observed, as illustrated in

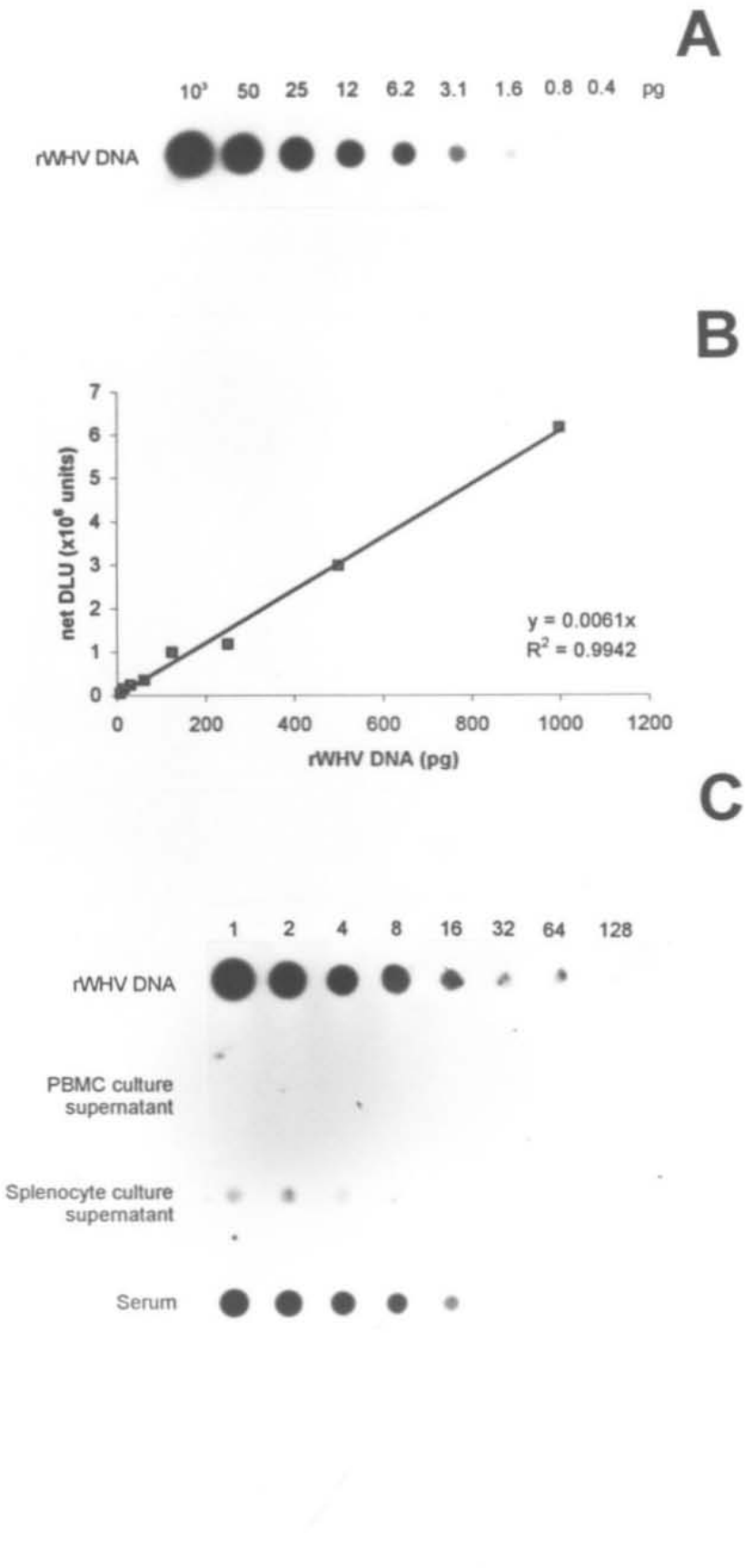
Figure 3.8. Southern blot evaluation of the sensitivity of PCR amplifications with primers used for detection of WHV DNA and WHV cccDNA sequences. Ten-fold dilutions of rWHV DNA, ranging from 1 pg to 100 ag, was amplified in the presence of 130 mM EDTA and 3 mM Mg^{2+} with PCOR1-MCOR primers (for total WHV DNA identification) (A) or with PGAP1-MCOR primers (for WHV cccDNA detection) (B). As negative controls, PCR mixtures without rWHV DNA were included. The positions and sizes of the PCR products are indicated on the right side of the panel.

A
1 pg
100 fg
10 fg
1 fg
100 ag
Negative control
WHV DNA
<490 bp
B
1 pg
100 fg
10 fg
1 fg
100 ag
Negative control
WHV cccDNA
<1156 bp

Figure 3.9B. It was established that sensitivity of the assay is approximately 2.0 x 10^6 WHV vge /dot (Fig. 3.9A). Using this assay, WHV DNA was detected in inocula prepared from cultured splenocytes and in sera, but not in PBMC, which were used in culture infection experiments shown in Table 3.5. An example of the dot-blot evaluations of WHV DNA content in inocula prepared from splenocytes and serum of #563 animal is shown in Figure 3.9C. Densitometric analysis of this blot revealed the presence of 2 x 10^7 and 4.7 x 10^{10} WHV genome copies/ml in splenocyte culture supernatant and serum, respectively. In contrast, the dot-blot hybridization assay did not have sufficient sensitivity to detect WHV DNA in the supernatant from cultured PBMC examined in this study, as it is illustrated for #563 animal in Figure 3.9C. In general, the WHV genome content in splenocyte culture media and sera used as inocula in the described experiments ranged from 1 to 2 x 10^7/ml and 0.8 to 9.2 x 10^{10} vge/ml, respectively.

When WHV DNA was not detectable by dot-blot hybridization assay, its content was estimated by direct PCR using PCOR1-MCOR primer pair (Table 2.2). which was followed by Southern blot analysis of the amplified PCR products. Since WHV DNA sequences originating from PBMC culture supernatants cannot be visualized after direct PCR on EB-agarose, but can be identified by direct PCR/Southern blot hybridization, and knowing that sensitivity of EB-agarose detection for WHV DNA amplified by direct PCR is at least 3 x 10^4 WHV copies/ml and that of direct PCR/Southern blot analysis is 3 x 10^3 WHV genome

Figure 3.9. Quantitation of WHV DNA expression in inocula prepared from PBMC, splenocytes and serum of a chronically infected woodchuck and in serial dilutions of rWHV DNA by dot-blot hybridization assay. (A) Two-fold dilutions of linearized, recombinant, complete WHV DNA (rWHV DNA) were blotted onto a nylon membrane by microfiltration. The first dot (far left) contained 10^3 pg of WHV DNA (3×10^8 vge). (B) Plot of net digital light units (net DLU) of the hybridization signals shown in panel A analysed by densitometric scanning. (C) Dot-blot hybridization analysis of serial two-fold dilutions of culture supernatants from cultured PBMC and splenocytes and serum obtained from #563 animal. The dilution rates are presented as reciprocal values on the top of the panel. The most far left dots contained 10^3 pg of rWHV DNA, 600 µl of PBMC or splenocyte culture supernatants or 1 µl of serum. The blots were probed with ^{32}P-labelled rWHV DNA as described in Materials and Methods.


A
10³ 50 25 12 6.2 3.1 1.6 0.8 0.4 pg
rWHV DNA
B
net DLU (x10⁶ units)
7
6
5
4
3
2
1
0
0 200 400 600 800 1000 1200
rWHV DNA (pg)
y = 0.0061x
R² = 0.9942
C
1 2 4 8 16 32 64 128
rWHV DNA
PBMC culture supernatant
Splenocyte culture supernatant
Serum

equivalents/ml, it was estimated that PBMC inocula used in this study contained between 3 x 10^3 to 3 x 10^4 genome equivalents/ml.

3.4 WHV REPLICATION IN *IN VITRO* INFECTED WOODCHUCK HEPATOCYTES.

3.4.1 Preliminary experiments evaluating susceptibility of WCM-260 hepatocytes to WHV infection.

In the initial experiments, WHV DNA sequences and, in selected situations, the expression of WHV core and surface antigens were evaluated in WCM-260 hepatocytes incubated with sera or PBMC culture supernatants obtained from chronic carriers of WHV (Table 3.4). In this early phase of our study, infected hepatocytes were not subjected to DNase/trypsin treatment prior to DNA extraction. Therefore, the possibility of WHV DNA contamination originating from virions or WHV DNA fragments attached to hepatocyte surfaces cannot be completely excluded. Nevertheless, WHV DNA was detected in hepatocytes from all experiments completed in this phase one study (Table 3.4).

In addition, it was found that WHV antigens could be identified in infected WCM-260 cells, i.e., experiment #5 (Table 3.4). In this particular trial, culture supernatant from PBMC of a chronic WHV carrier (#270) was used as inoculum and *de novo* synthesized WHsAg was detected after 24-h incubation with ^{35}S-methionine, followed by immunoprecipitation with anti-WHs antibodies and

Table 3.4. Preliminary experiments evaluating WHV replication markers in WCM-260 hepatocytes *in vitro* infected with PBMC and serum-derived WHV inocula[a].

Experiment No.	Animal No.	Inoculum source	Hepatocytes				
			WHV DNA expression[b]		WHV antigen detection		
			direct PCR	nested PCR	^{35}S-WHsAg labelling[c]	FACS (% of positive cells)[d]	
						WHsAg	WHcAg
1	191	Serum	+	n.t.	n.t.		
2	1252	Serum	-	+	n.t.		
3	591	PBMC	+	+	-	n.t.	
4	1256	PBMC	+	+	-	n.t.	
5	270	PBMC	-	+	+	-	19

[a] Experiments were conducted in the presence of 100 nM glucagon.

[b] Tested by direct or nested PCR with WHV core gene-specific primers (PCNV-COR and PPCC-CCOV) using 1 µg of total DNA per reaction. The amplified viral sequences were detected by Southern blot hybridization with rWHV DNA as a probe.

[c] Cells were labelled with ^{35}S-methionine that was followed by immunoprecipitation with anti-WHs and protein A beads, and proteins eluted from beads were subjected to SDS-PAGE.

[d] Represents % of tested cells with greater fluorescence than control cell population.

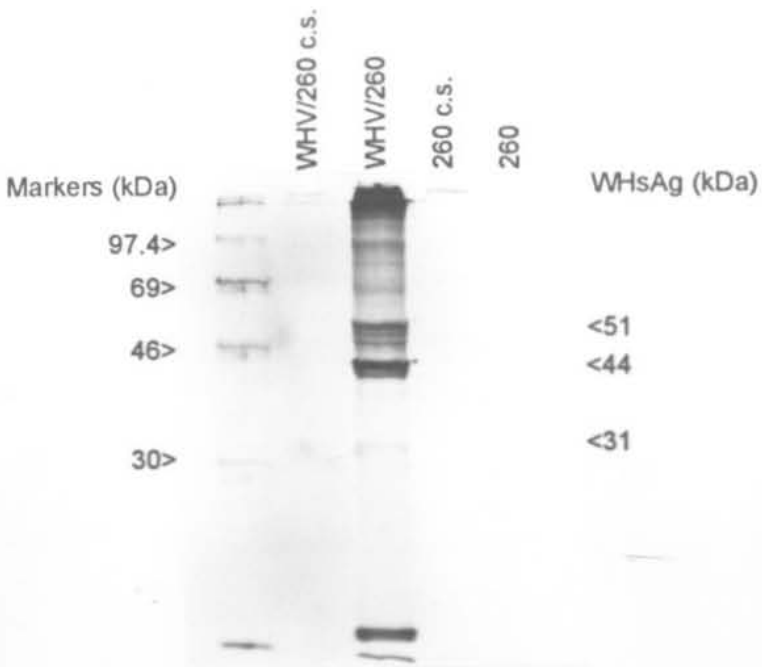
n.t., not tested.

autoradiography of a gel after SDS-PAGE (Fig. 3.10). However, the use of the same detection procedure with both anti-WHs and anti-WHc antibodies at other occasions (Table 3.4 and data not shown) failed to yield positive results. This showed that ^{35}S-methionine labelling assay is not sensitive enough for routine detection of WHV antigens in *in vitro* infected hepatocytes in our system.

Alternatively, flow cytometry analysis of WCM-260 hepatocytes exposed to culture supernatant from WHV-positive PBMC, which were then incubated with anti-WHs and anti-WHc antibodies (experiment #5; Table 3.4), revealed that although WHsAg was not identified, WHcAg can be detected. It was subsequently found (Table 3.5) that this detection procedure allowed for demonstration of WHV antigens in *in vitro* infected hepatocytes in approximately half of the trials. Since the FACS analysis of the hepatocytes was performed at 4 d.p.i., while the ^{35}S-methionine-based detection utilized proteins *de novo* synthesized within 24 h, it was not surprising that flow cytometry technique was more sensitive than ^{35}S-methionine labelling/immunoprecipitation method. Based on the above results, WHV antigen expression was routinely evaluated by flow cytometry analysis but not by ^{35}S-methionine labelling assay.

Other main differences between the first (Table 3.4) and second (Table 3.5) phase of experiments were that in the former, WHV cccDNA expression was not examined and that for detection of WHV core gene sequences, primer pairs PCNV-COR and PPCC-CCOV (Table 2.2) were used. Moreover, the time between

Figure 3.10. SDS-PAGE analysis of anti-WHs antibody immunoprecipitated proteins from WHV-infected WCM 260 hepatocytes labelled *in vitro* with ^{35}S-methionine. One ml of culture supernatant (WHV/260 c.s.) from infected hepatocytes and approximately 3 x 10^5 of the hepatocytes (WHV/260) were immunoprecipitated with rabbit anti-WHs and protein A-Sepharose beads. Samples eluted from protein A beads were loaded onto a 12% gel for SDS-PAGE analysis. Electrophoresed gel was dried and exposed for 7 days on Kodak X-OMAT film at -70°C. As negative controls, non-infected hepatocytes (260) labelled with ^{35}S-methionine and their culture supernatant (260 c.s.) were immunoprecipitated with anti-WHs and protein A beads. The molecular weights (kDa) of the protein markers are shown on the left of the panel and the sizes of the identified WHsAg polypeptides (51, 44, and 31 kDa proteins); are shown on the right side of the panel.

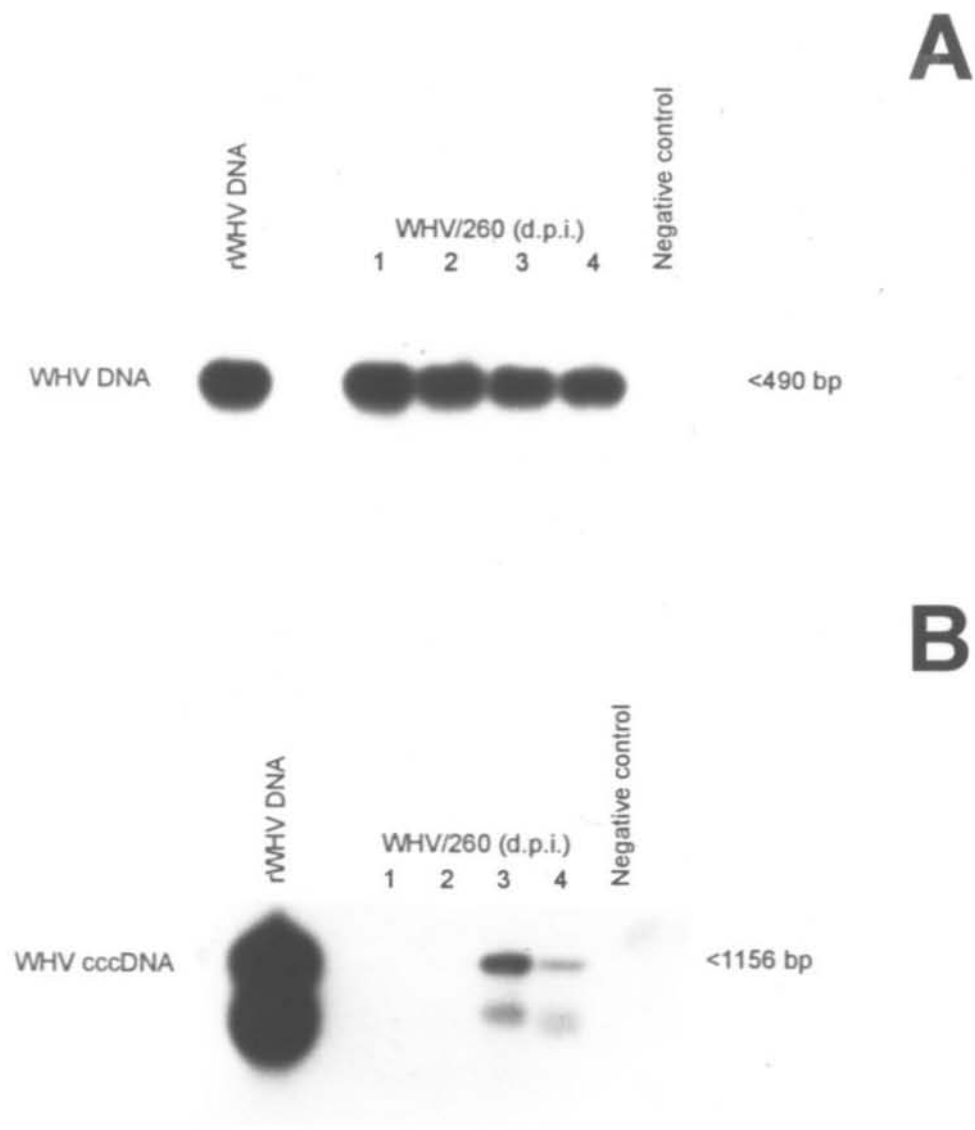

WHV/260 c.s.
WHV/260
260 c.s.
260
Markers (kDa)
97.4>
69>
46>
30>
WHsAg (kDa)
<51
<44
<31

hepatocyte seeding and their inoculation with WHV was reduced from 72 h in the former set of experiments to 24 h in later experiments. This was introduced to ensure that the cells were exposed to the virus during an early phase of active growth. Furthermore, instead of ending the experiments at 5 d.p.i., experiments in the second phase were terminated when the cells reached confluence at 4 d.p.i. as it was established that WHV cccDNA can be identified beginning from 3 d.p.i. (see below).

3.4.2 Kinetics of WHV DNA and WHV cccDNA in WCM-260 hepatocytes after *in vitro* infection.

In an experiment designed to determine the time course of WHV DNA and cccDNA accumulation in *in vitro* infected WCM-260 hepatocytes, cells exposed to 1×10^7 WHV vge/ml of serum WHV were harvested at 1, 2, 3, and 4 d.p.i. and analysed by direct PCR. It was found that WHV DNA was present in the hepatocytes from 1 d.p.i. and its expression was unchanged at each of the time point examined (Fig. 3.11A). Based on the densitometric analysis, it was estimated that 1 in 40 hepatocytes contained a copy of WHV DNA. WHV cccDNA was identified beginning at 3 d.p.i., with a decrease (30%) in expression at 4 d.p.i. (Fig. 3.11B). Densitometric analysis of the Southern blot hybridization signals revealed that at 3 d.p.i. and 4 d.p.i., approximately 1 in 300 and 1 in 430 cells contained a copy of WHV cccDNA, respectively. This suggests that although WHV DNA is

Figure 3.11. The time course of the expression WHV DNA and WHV cccDNA in WCM-260 hepatocytes exposed to serum WHV. Cells were seeded at 6 x 10^4/ml in 6-well culture plates for 24 h prior to incubation in WHV inoculum. At 1 d.p.i., the hepatocytes were extensively washed with HBSS following standard procedures described in Materials and Methods. A portion the these cells were harvested at 1 d.p.i. and DNase/trypsin treated before being subjected to DNA analysis. The remainder of the hepatocytes were supplemented with fresh hepatocyte growth medium and allowed to grow until 2, 3 or 4 d.p.i. At each of these time points, WCM-260 cells were harvested and DNase/trypsin treated prior to DNA extraction. Subsequently, approximately 4 µg of mung bean nuclease-digested DNA isolated from the hepatocytes (WHV/260) at the above time points were evaluated for WHV DNA (panel A) or WHV cccDNA (panel B) expression by PCR/Southern blot hybridization. As a positive control, 50 fg of rWHV DNA and as a negative control, 4 µg of mung bean nuclease-treated DNA from non-infected WCM-260 hepatocytes were used. The sizes of the amplified PCR products are marked on the right.


A
rWHV DNA
WHV/260 (d.p.i.)
1 2 3 4
Negative control
WHV DNA
<490 bp
B
rWHV DNA
WHV/260 (d.p.i.)
1 2 3 4
Negative control
WHV cccDNA
<1156 bp

found a day after exposure to virus, WHV replication occurs a few days later and rapidly decreased when cells reach confluence.

3.4.3. Examination of WHV infection in cultured hepatocytes under higher stringency conditions.

Table 3.5 summarises the results on evaluation of WHV DNA and WHV cccDNA expression in WCM-260 hepatocytes exposed to inocula derived from lymphoid cell culture supernatants or from sera obtained from woodchucks with chronic WHV infection. In all experiments, lymphoid cells used as sources of inocula and target hepatocytes were subjected to limited digestion with DNase and trypsin (Section 2.4.1) to ensure that only intracellular viral sequences were being examined (for more information, see Fig. 3.15). Expression of WHsAg and WHcAg in *in vitro* infected hepatocytes was identified by staining with anti-WHs or anti-WHc and by FACS analysis.

Overall, the data from these experiments revealed that intracellular WHV DNA was detected in all (14/14) trials in which cultured woodchuck hepatocytes were exposed to WHV inocula prepared from either lymphoid cells or sera (Table 3.6). Further, WHV cccDNA sequences, reflecting existence of replicating virus, were identified in half (7/14) of the experiments. In addition, WHV antigens were found in approximately half (8/14) of hepatocyte cultures incubated with test inocula. The results from representative experiments in which inocula originating from WHV-

Table 3.5. Detection of WHV replication markers in hepatocytes *in vitro* infected with lymphoid cell and serum-derived WHV inocula.

Animal No.	Experiment No.	Inoculum		Infected hepatocytes					
		Source	WHV vge/ml	WHV DNA expression[a]				WHV antigen detected (% of positive cells)[b]	
				DNA tested (µg)	total WHV DNA	WHV cccDNA		WHsAg	WHcAg
					direct PCR	direct PCR	nested PCR		
190	1	Serum	7.2×10^8	1	+	+	n.t.	0	0
19056	2	Serum PBMC	1.2×10^9 $<3 \times 10^3$	2.5 5	+ +	+ -	+ -	53 0	0 0
	3	PBMC	$<3 \times 10^4$	2.6	+	-	-	20	12
	4	Serum	8.4×10^8	1	+	-	-	0	16
	5	Splenocytes	1.9×10^7	6.2	+	+	n.t.	26	0
19109	6	Serum PBMC	4×10^8 $<3 \times 10^4$	0.1 0.1	+[c] +	- -	- -	0 0	0 19
563	7	Serum Splenocytes	1.4×10^8 2×10^7	5 5	+ +	- -	- -	0 0	0 0
	8	Serum Splenocytes	1.4×10^8 2×10^7	5 5	+ +	+ +	n.t. n.t.	0 0	13 0
	9	Splenocytes	2×10^7	5	+	+	n.t.	0	26
184, 270, 1255, 1256	10	PBMC (pool)	$<3 \times 10^4$	0.2	+	-	+	0	20

[a] Tested by direct or nested PCR followed by Southern blot hybridization with rWHV DNA as a probe.
[b] Represents % of tested cells with greater fluorescence than control cell population.
[c] 72-h culture supernatant was positive when tested by direct PCR followed by Southern blot hybridization.
n.t., not tested.

Table 3.6. Summary of results from *in vitro* infection of WCM-260 hepatocytes with WHV inocula derived from lymphoid cells or sera.

Source of WHV inoculum	Total no. of experiments	Markers of viral replication in hepatocytes		
		WHV DNA expression		WHV antigen expression (WHsAg and/or WHcAg)
		total WHV DNA	WHV cccDNA	
PBMC	4	4/4	1/4	3/4
Splenocytes	4	4/4	3/4	2/4
Serum	6	6/6	3/6	3/6
Total	**14**	**14/14**	**7/14**	**8/14**

reactive PBMC, splenocytes or sera were used are described below.

The data from experiment #1 (Table 3.5) illustrate detection of WHV DNA and WHV cccDNA sequences in WCM-260 cells exposed to highly WHV DNA positive serum (3.6×10^{10} vge/ml) which was used as WHV inoculum at a final concentration of 7.2×10^{8} vge/ml. After direct PCR amplification and Southern blotting, a strong WHV DNA signal and a weaker, but clearly identifiable WHV cccDNA, were seen in the hepatocytes (Fig. 3.12). However, the inoculated WCM-260 cells were found to be negative when tested for WHV antigens (Table 3.5).

When splenocyte culture supernatant containing 2×10^{7} WHV vge/ml was used as a source of virus (experiment #9, Table 3.5), both WHV DNA and WHV cccDNA, as well as WHcAg, were detected in the exposed WCM-260 cells. Based on the densitometric analysis (Fig. 3.13A), it was estimated that at least 1 in 30 and 1 in 300 hepatocytes contained a copy of WHV DNA and WHV cccDNA, respectively. When virus core antigen expression was evaluated by FACS, about 26% of the analysed hepatocytes were found positive (Fig. 3.13B). Taken together, these results indicate that splenocyte-derived WHV was capable of establishing infection in cultured woodchuck hepatocytes.

In another experiment (experiment #8,Table 3.5), WCM-260 hepatocytes were incubated with either splenocyte culture supernatant (2×10^{7} WHV vge/ml) or serum (1.4×10^{8} WHV vge/ml), which originated from the same #563 woodchuck. WCM-260 cells exposed to these inocula and subsequently digested with

Figure 3.12. Detection of WHV DNA and WHV cccDNA in WCM-260 hepatocytes infected with WHV-positive serum from a chronically infected woodchuck (#190). Five µg of mung bean nuclease-digested DNA obtained from 4 d.p.i.-WCM-260 hepatocytes (WHV/260) which were exposed to serum WHV and from a 100-µl sample of the hepatocyte culture supernatant (WHV/260 c.s.) were amplified by direct PCR with WHV core gene or nick-spanning primer pairs to detect total WHV DNA or WHV cccDNA. One µg of mung bean nuclease-treated DNA isolated from the liver of a chronic WHV carrier and 100 ng of rWHV DNA were used as positive controls, whereas 5 µg of mung bean nuclease-treated DNA isolated from non-infected WCM-260 hepatocytes was used as a negative control. The products were identified by Southern blot hybridization with ^{32}P-labelled rWHV DNA. The expected sizes of PCR fragments are indicated on the right.

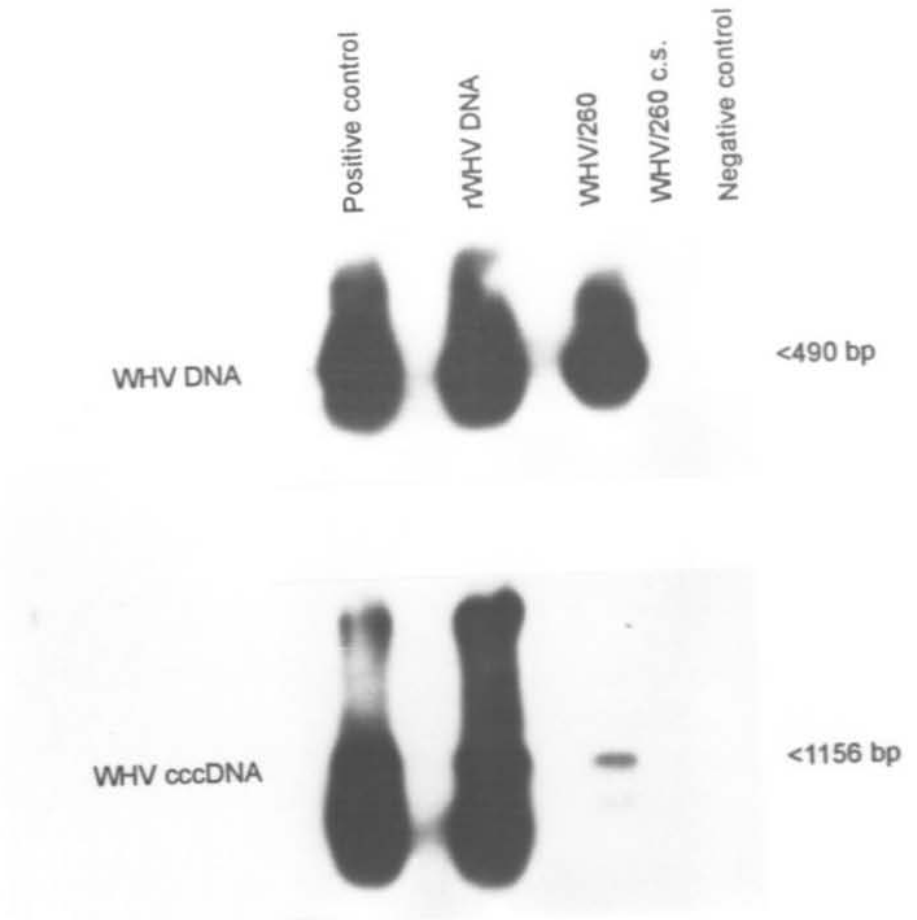
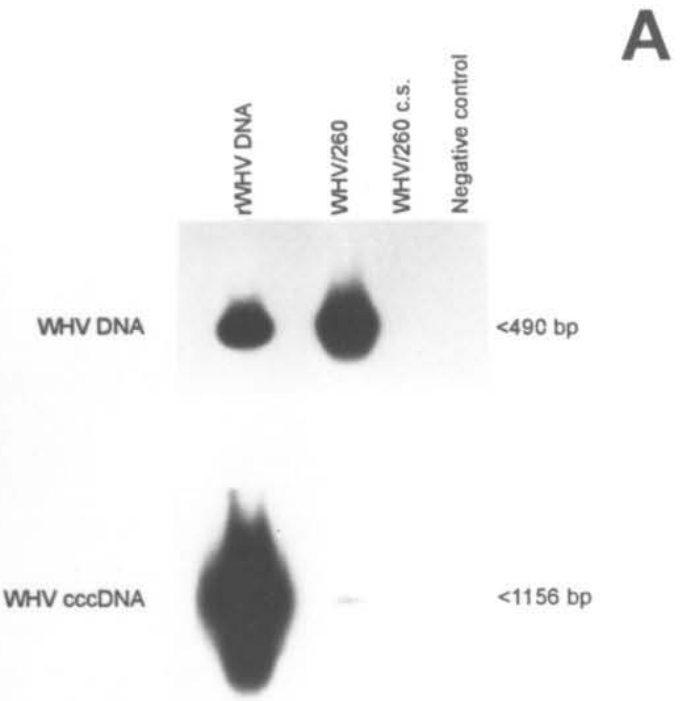
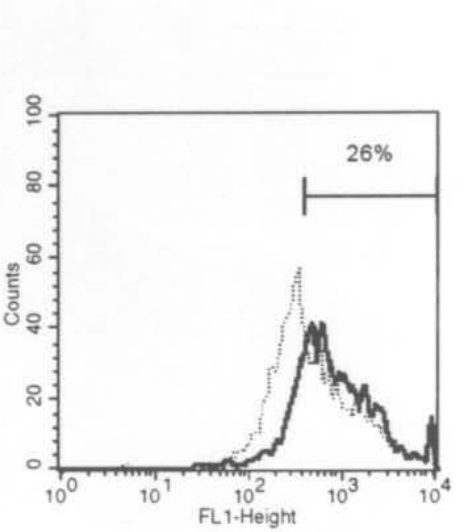

Positive control
rWHV DNA
WHV/260
WHV/260 c.s.
Negative control
WHV DNA
<490 bp
WHV cccDNA
<1156 bp

Figure 3.13. Southern blot detection of WHV DNA and WHV cccDNA and flow cytometry analysis of WHcAg expression in WCM-260 hepatocytes incubated with WHV produced by splenocytes isolated from a chronically infected animal (#563). (A) Five µg of mung bean nuclease-digested DNA extracted from WCM-260 hepatocytes exposed to splenocyte inoculum (WHV/260) and from 100 µl of the hepatocyte culture supernatant (WHV/260 c.s.) were amplified by direct PCR to detect total WHV DNA and WHV cccDNA sequences. Fifty fg of rWHV DNA was used as a positive control, while 5 µg of mung bean nuclease-digested DNA from non-infected WCM-260 hepatocytes was used as a negative control. The PCR amplified fragments were hybridized with ^{32}P-labelled rWHV DNA. The sizes of the identified PCR products are marked on the right. (B) FACS histogram of test WCM-260 hepatocytes stained with anti-WHc antibodies. WHV-exposed (solid line) and non-exposed (dotted line) hepatocytes were incubated with rabbit antibodies against WHcAg and then antibodies against rabbit immunoglobulins conjugated with FITC. Cell numbers (counts) are plotted against the log of fluorescence intensity (FL1-Height). The number inside the panel indicates percentage of WHcAg-positive cells.


A
rWHV DNA
WHV/260
WHV/260 c.s.
Negative control
WHV DNA
<490 bp
WHV cccDNA
<1156 bp

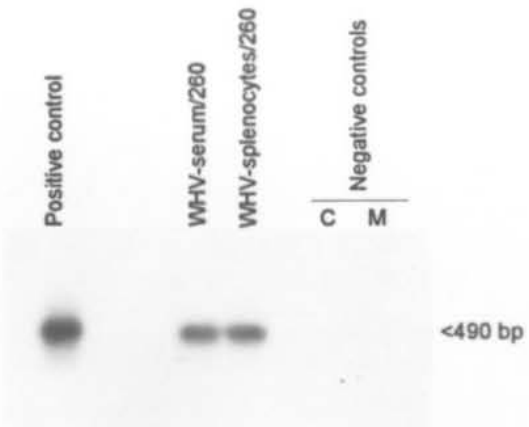

B
26%
Counts
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4
FL1-Height

DNase/trypsin to eliminate WHV material potentially attached to the cell surface were found to be reactive for both WHV DNA (Fig. 3.14A) and WHV cccDNA (Table 3.5) by direct PCR/Southern blot hybridization. WHcAg was detected only in 13% of the hepatocytes exposed to serum-derived WHV (Fig. 3.14B), whereas the cells incubated with splenocyte-derived inoculum were both WHcAg and WHsAg non-reactive (data not shown). Although inoculum originating from serum had 7-fold greater WHV vge numbers per ml than that from splenocytes, densitometric analysis of the Southern blot signals (Fig. 3.14A) showed that hepatocytes infected with these inocula had approximately equal WHV DNA content after 96-h culture.

In experiment #2 (Table 3.5), cultured hepatocytes were exposed to culture supernatant from PBMC, as well as to serum of #19056 woodchuck which contained $< 3 \times 10^3$ WHV vge/ml and 1.2×10^9 WHV vge/ml, respectively. In this experiment, DNase/trypsin-treated PBMC, obtained before and after a 72-h culture to produce cell supernatant (WHV inoculum), were examined for WHV DNA expression and both samples were found to be WHV DNA-reactive (Fig. 3.15A). Further, it was found that the final PBMC wash obtained after DNase/trypsin digestion was negative for WHV DNA, indicating that the treated PBMC were free of any extracellular WHV material when tested using a PCR/Southern blot hybridization assay (Fig. 3.15B). Despite the fact that WHV DNA was not detectable in 72-h culture supernatant from these PBMC (Fig. 3.15A), WCM-260 hepatocytes exposed to this inoculum were evidently WHV DNA positive (Fig. 3.16A). The final

Figure 3.14. *In vitro* infection of WCM-260 hepatocytes with splenocyte and serum-derived WHV obtained from #563 animal. (A) Five µg of mung bean nuclease-digested DNA from WCM-260 cells exposed to either serum (WHV-serum/260) or splenocyte (WHV-splenocytes/260) inoculum were amplified by direct PCR with PCOR1-MCOR primer pair to detect total WHV DNA. One µg of mung bean nuclease-digested DNA derived from a WHV chronic carrier was included as a positive control and 5 µg of mung bean nuclease-treated DNA from non-infected WCM-260 hepatocytes (C), as well as a mock (M) sample were included as negative controls. The PCR amplified fragments were identified by Southern blot hybridization with a ^{32}P-labelled rWHV DNA probe. The size of the amplified PCR product is shown on the right side of the panel. (B) FACS histogram shows WHV inoculum-exposed (solid line) and virus naive (dotted line) hepatocytes stained with anti-WHc antibodies. Cell numbers (counts) are plotted against the log of fluorescence intensity (FITC). The number inside the panel indicates percentage of WHcAg-positive cells.

A


Positive control
WHV-serum/260
WHV-splenocytes/260
Negative controls
C
M
<490 bp

B

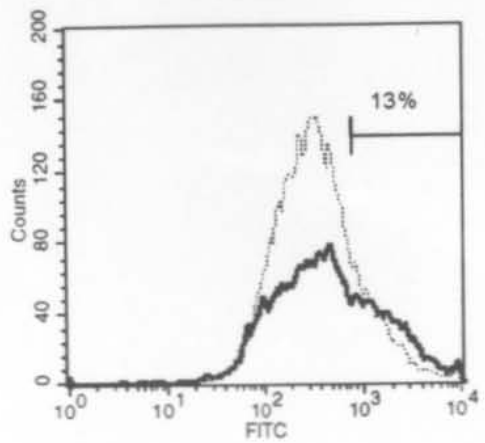

13%
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FITC

Figure 3.15. Analysis of WHV DNA content in serum and PBMC-derived inocula from #19056 woodchuck and in PBMC and hepatocyte washes obtained during the course of experiment #2. (A) DNA from 50 µl of serum, 1 µg of mung bean nuclease-digested DNA from DNase/trypsin-treated PBMC, collected before (PBMC) and after 3-day culture.(cultured PBMC), and DNA from 100 µl of PBMC culture supernatant (PBMC c.s.) were analysed for WHV DNA expression by direct PCR using WHV core gene-specific primers. (B) Mung bean nuclease-treated DNA were extracted from 100-µl samples of the final PBMC wash after DNase/trypsin digestion (DNase wash [PBMC]), from hepatocyte wash collected at 1 d.p.i. from WCM-260 cells exposed to either serum inoculum (1 d.p.i. wash [WHV-serum/260), or PBMC inoculum (1 d.p.i. wash [WHV-PBMC/260]), and from the wash obtained at 4 d.p.i. after DNase/trypsin treatment of WCM-260 hepatocytes exposed to WHV from serum (DNase wash [WHV-serum/260]) or to WHV from PBMC (DNase wash [WHV-PBMC/260]). The DNA preparations were subjected to WHV DNA testing by direct PCR with WHV core gene-specific primers. Mung bean nuclease-digested DNA derived from a WHV chronic carrier was used as a positive control, whereas a mock sample was included as a negative control. PCR products were probed with ^{32}P-labelled rWHV DNA. The size of the amplified fragment is shown on the right.

Positive control
DNase wash (PBMC)
1 d.p.i. wash (WHV-serum/260)
DNase wash (WHV-serum/260)
1 d.p.i. wash (WHV-PBMC/260)
DNase wash (WHV-PBMC/260)
Negative control
<490 bp

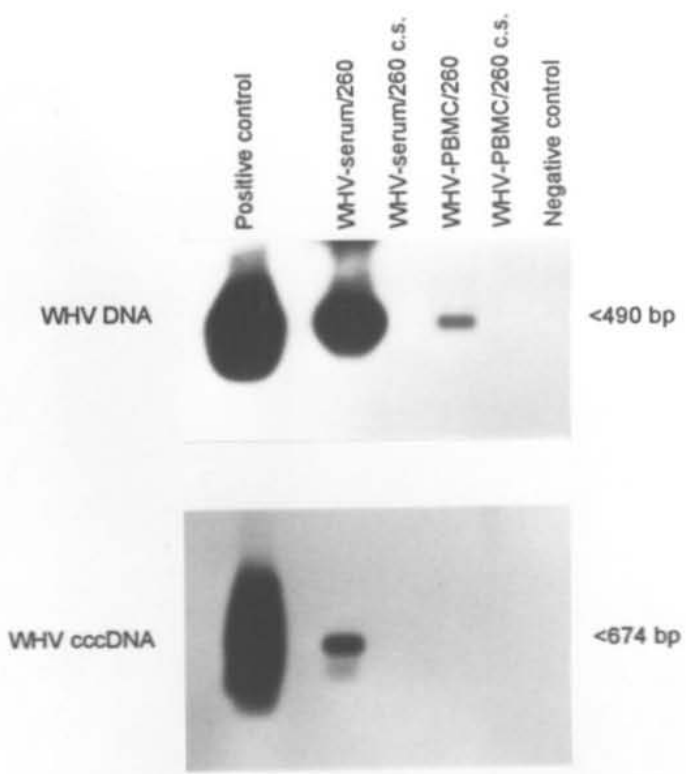
B

Positive control
Serum
PBMC
Cultured PBMC
PBMC c.s.
Negative control
<490 bp

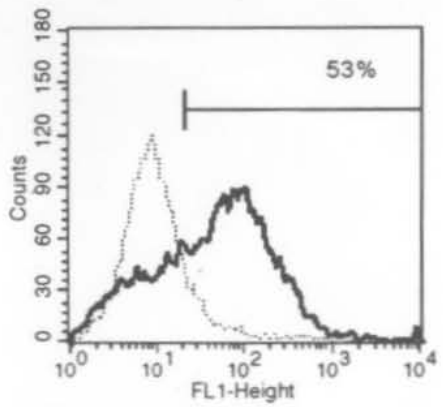
A

Figure 3.16. Southern blot hybridization and FACS analyses of WCM-260 hepatocytes exposed to PBMC or serum-derived WHV prepared from #19056 woodchuck. (A) Approximately 2.5 µg of DNA from hepatocytes incubated with serum WHV (WHV-serum/260), 5 µg of DNA from WCM-260 cells exposed to PBMC-derived inoculum (WHV-PBMC/260), and DNA extracted from 100 µl of 4 d.p.i. culture supernatants of hepatocytes incubated with WHV inoculum from serum (WHV-serum/260 c.s.) or PBMC (WHV-PBMC/260 c.s.) were amplified by direct PCR with WHV core gene-specific primers and by nested PCR with WHV nick-spanning primers to detect WHV DNA and WHV cccDNA sequences, respectively. As a positive control, mung bean nuclease-digested DNA derived from a WHV chronic carrier was used, whereas as a negative control, a mock sample was included. (B) Cells exposed to #19056 woodchuck serum were stained with anti-WHs antibodies and analysed by flow cytometry. Histogram of hepatocytes exposed to inoculum (solid line) was overlaid on histogram of non-exposed, virus naive cells (dotted line). Cell numbers (counts) are plotted against the log of fluorescence intensity (FL1-Height). The number inside the panel indicates the percentage of the hepatocytes exposed to inoculum that were reactive with anti-WHs antibody.

A


Positive control
WHV-serum/260
WHV-serum/260 c.s.
WHV-PBMC/260
WHV-PBMC/260 c.s.
Negative control
WHV DNA
<490 bp
WHV cccDNA
<674 bp

B


53%
Counts
0
30
60
90
120
150
180
10^0
10^1
10^2
10^3
10^4
FL1-Height

hepatocyte wash obtained after 24 h exposure (1 d.p.i.) to PBMC-derived inoculum was WHV DNA negative, demonstrating that WHV used for inoculation was removed from the hepatocyte culture (Fig. 3.15B). In addition, the final hepatocyte wash obtained following DNAse/trypsin digestion after 4 d.p.i. culture (see Fig. 2.1) was WHV DNA non-reactive. The above results convincingly document that the WHV DNA signal identified in the hepatocytes exposed to WHV released by WHV-infected PBMC was of intracellular origin. In the same experiment, WCM-260 cells were also incubated in serum WHV. It was found that after exposure to this inoculum, hepatocytes became both WHV DNA and WHV cccDNA positive (Fig. 3.16A). Since the hepatocyte wash after 24-h exposure to serum WHV and the final wash collected after DNase/trypsin digestion of these cells at 4 d.p.i. were WHV DNA negative (Fig. 3.15B), this clearly demonstrates that the WHV DNA detected was of intracellular origin. In addition, approximately 50% of the hepatocytes incubated with serum WHV expressed WHsAg when tested by FACS (Fig. 3.16B).

In another experiment, culture supernatants prepared from PBMC isolated from 4 WHV chronic carriers (#184, #270, #1255 and #1256) were pooled and used as a source of WHV (experiment #10, Table 3.5). This inoculum contained less than 3×10^4 WHV vge/ml. Southern blot hybridization of PCR products amplified using primers specific for WHV core gene or nick-spanning region revealed that the inoculum-exposed hepatocytes contained intracellular WHV DNA (Fig. 3.17A) and WHV cccDNA sequences were detectable after nested PCR/Southern blot

Figure 3.17. Detection of WHV DNA and WHV cccDNA in hepatocytes incubated with pooled PBMC culture supernatants from WHsAg-positive WHV carriers. One µg of mung bean nuclease-treated DNA from inoculum-exposed WCM-260 cells (WHV/260) and DNA from 100 µl of culture supernatant (WHV/260 c.s.) were analysed for WHV DNA expression by direct PCR (panel A) and by nested PCR (panel B), for identification of WHV DNA and WHV cccDNA, respectively. As positive controls, 100 pg of rWHV DNA and 1 µg of mung bean nuclease-digested DNA isolated from the liver of a chronically infected woodchuck were used, while non-infected hepatocytes (C) and a mock (M) sample were included as negative controls. The size of the predicted PCR products is shown on the right side of the panel.

A

Positive control
rWHV DNA
WHV/260
WHV/260 c.s.
Negative controls
C
M
WHV DNA
<490 bp

B

Positive control
rWHV DNA
WHV/260
WHV/260 c.s.
Negative controls
C
M
WHV cccDNA
<674 bp

hybridization (Fig. 3.17B; band not obviously visible on photograph). This was the only experiment in our study in which WHV cccDNA was detected in hepatocytes inoculated with PBMC-derived WHV.

In all the remaining experiments (n = 5; Table 3.5), which included incubation of WCM-260 cells with WHV-positive sera (experiments #4, #6, and #7), WHV from PBMC (experiments #3 and #6) and WHV from splenocytes (experiments #5 and #7), WHV DNA was detected after DNase/trypsin treatment of the cells by direct PCR/Southern blot hybridization. WHV cccDNA was found in one of the two trials in which splenocyte inoculum was used. WHV surface and/or core antigen-positive hepatocytes were identified in 4 of the experiments (#3, #4, #5, and #6). Interestingly, hepatocytes inoculated with PBMC-derived WHV were reactive for WHsAg and/or WHcAg in 2 of the trials despite the fact that WHV cccDNA sequences were not detected by nested PCR/Southern blot hybridization, suggesting that replicating virus in fact existed in these cells.

In summary, although serum-derived inocula contained 10-fold greater amounts of WHV vge/ml (mean $3.3 \pm 3.5 \times 10^8$ WHV vge/ml) than those from splenocytes (mean $1.97 \pm 0.05 \times 10^7$ WHV vge/ml), WHV cccDNA was detectable in the hepatocytes with approximately the same proportion of the trials (Table 3.6). Furthermore, WHsAg and/or WHcAg was identified in 50% of the experiments in which serum or splenocyte WHV was used. This may suggest that virus produced by infected splenocytes was somehow more effective in the induction of WHV

replication than that from serum.

Inocula obtained from cultured PBMC, which contained less than 3×10^4 WHV vge/ml, were evidently less effective in the establishment of WHV replication in WCM-260 hepatocytes, when assessed by WHV cccDNA expression (Table 3.6). The observed discrepancy between the apparent infectivity of splenocyte and PBMC-derived WHV was most likely due to the low levels of virions secreted in culture by naturally-infected PBMC, although the same numbers of splenocytes and PBMC were used to generate this inocula (see Section 2.4.3). This observation may suggest that the number of splenocytes infected with WHV or the rate of WHV propagation in these cells is greater than that in circulating lymphoid cells.

3.4.4 Inoculum WHV content influences the efficacy of hepatocyte *in vitro* infection.

To examine the effect of WHV content on the inoculum infectivity in WCM-260 cultures, splenocyte-derived inoculum was prepared from a chronically infected woodchuck (#563) and two-fold diluted with conditioned Hepato-STIM™ to obtain 1×10^7, 5×10^6, and 2.5×10^6 WHV vge/ml. DNA samples isolated from inoculated hepatocytes after 4-day cultures and analysed by PCR/Southern blot analysis showed a decrease in WHV DNA expression in hepatocytes as the dilution rate of WHV inoculum increased (Fig. 3.18A). Based on densitometric analysis, it was estimated that approximately 1 in 40 hepatocytes exposed to 2.5×10^6 WHV vge/ml

Figure 3.18. Expression of WHV DNA and WHV cccDNA in *in vitro* infected hepatocytes depends on the WHV content in the inoculum. Approximately 8 µg of mung bean nuclease-digested DNA from hepatocytes exposed to splenocyte culture supernatant containing WHV at 2.5×10^6, 5×10^6 and 1×10^7 vge/ml were amplified by direct PCR with PCOR1-MCOR primer pair (A) and by nested PCR with PGAP1-MCOR and then XINT-CCCV primers (B). One µg of mung bean nuclease-treated DNA from WHV-positive liver and 50 fg of rWHV DNA were used as positive controls, while 8 µg of mung bean nuclease-digested DNA from non-infected WCM-260 hepatocytes (C) and a mock (M) sample were used as negative controls. The PCR-amplified fragments were hybridized with ^{32}P-labelled rWHV DNA. The expected sizes of PCR products are marked on the right.

A
Positive control
rWHV DNA
vge/ml
2.5 x 10^6
5 x 10^6
1 x 10^7
Negative controls
C
M
WHV DNA
<490 bp
B
Positive control
vge/ml
2.5 x 10^6
5 x 10^6
1 x 10^7
Negative controls
C
M
WHV cccDNA
<674 bp

contained a single WHV genome copy (Fig. 3.18A, lane 3). In addition, Southern blot analysis of nested PCR products amplified with primers spanning the virus nick region revealed that WHV cccDNA was present in hepatocytes exposed to 1×10^7 WHV vge/ml (Fig. 3.18B, lane 4), as well as in the cells incubated with greater dilutions of the same inoculum, although these signals were only identifiable after prolonged exposure of same blot (data not shown). These results suggest that the WHV inocula should contain at least 1×10^7 vge/ml to induce infection in cultured woodchuck hepatocytes with readily identifiable WHV cccDNA levels. Furthermore, this experiment supported our hypothesis that WHV content in an inoculum is the main determinant of the level of viral replication in WHV-exposed WCM-260 hepatocytes.

3.4.5 WHV is transmittable from infected lymphoid cells to hepatocytes in the same co-culture.

To investigate whether WHV from lymphoid cells can be transmitted directly to WCM-260 hepatocytes and if this approach can improve the infection of hepatocytes and create a micro-environment in which the virus can have access to two types of target cell (i.e., non-infected lymphoid cells or hepatocytes), PBMC isolated from a WHsAg-carrier were co-cultured with WCM-260 hepatocytes, as described in Section 2.7.2 of Materials and Methods. Southern blot analysis of PCR products amplified with WHV core gene-specific primers revealed the presence of

intracellular viral DNA in the co-cultured, DNase/trypsin treated hepatocytes (Fig. 3.19A). Based on the densitometric analysis, it was estimated that 1 in 30 infected hepatocytes contained a copy of viral genome. WHV cccDNA was not detected in the hepatocytes even after nested PCR followed by Southern blot hybridization. This could be due to the low amounts of virus generated by co-cultured PBMC, as previous experiments have shown that when hepatocytes were incubated with inocula containing less than 10^7 WHV vge/ml, WHV cccDNA was usually not readily detectable (see Section 3.4.4).

In another experiment, using the same approach as above, naturally infected splenocytes were co-cultured with WCM-260 hepatocytes. WHV DNA was identified in DNase/trypsin-treated splenocytes before and after culture (Fig. 3.19B, lanes 2 - 4) and in the co-cultured WHV-exposed hepatocytes (Fig. 3.19B, lane 5). It was estimated that approximately 1 in 5 of the co-cultured WCM-260 cells contained a copy of WHV DNA. Thus, co-culture of WHV-positive splenocytes with hepatocytes yielded greater (approximately 6-fold) numbers of WHV DNA-positive target cells than those co-cultured with PBMC. This phenomenon is most likely a consequence of the higher amounts of virus that is released by splenocytes compared to PBMC.

Figure 3.19. WHV DNA detection in hepatocytes co-cultured with naturally WHV-infected lymphoid cells. (A) Mung bean nuclease-digested DNA (2.5 µg/reaction) extracted from hepatocytes (WHV/260) co-cultured with PBMC isolated from a chronic WHV carrier was amplified by direct PCR using core gene primers (PCOR1-MCOR) to determine WHV DNA expression. (B) DNA (0.4 µg/reaction) samples extracted from WHV-positive non-cultured splenocytes (not cultured), splenocytes cultured without hepatocytes for 96 h (cultured) or co-cultured with WCM-260 hepatocytes (co-cultured), and from hepatocytes (WHV/260) co-cultured with these splenocytes were analysed by PCR with PCOR1-MCOR primers to detect WHV DNA. In each blot, 50 fg of rWHV DNA was included as a positive control, whereas non-infected cells (C) and a mock (M) sample were used as negative controls. Electrophoretically separated PCR products were probed with ^{32}P-labelled rWHV DNA by Southern blot hybridization. The positions and sizes of the WHV DNA-specific PCR product are indicated on the right side of the panels.

A
rWHV DNA
WHV/260
Negative controls
C
M
<490 bp
B
rWHV DNA
splenocytes
not cultured
cultured
co-cultured
WHV/260
Negative controls
C
M
<490 bp

3.5 PROPAGATION OF INFECTIOUS WHV THROUGH PASSAGES IN CULTURED WOODCHUCK HEPATOCYTES

3.5.1 Transmissibility of lymphoid cell-derived WHV in serial WCM-260 hepatocyte cultures

WHV-naive WCM-260 hepatocytes were exposed to culture supernatant derived from PBMC of #19109 woodchuck (Experiment #6, Table 3.5; see also Fig. 2.2). WHV DNA was found in these hepatocytes (Fig. 3.20A, lane 2) and in their concentrated culture supernatant collected at 4 d.p.i. (Fig. 3.20B). In addition, flow cytometry analysis revealed that at least 19% of the hepatocytes expressed WHcAg (Fig. 3.20D) It was estimated that the original (primary) hepatocyte culture supernatant (i.e., obtained at 4 d.p.i. following exposure to WHV), contained 1.3 x 10^4 vge/ml of medium. Subsequently, this culture supernatant was used as inoculum to passage WHV to virus-naive WCM-260 cells.

Hepatocytes that were inoculated with the primary hepatocyte culture supernatant were collected after each of the five passages, DNase/trypsin treated, and examined by nested PCR/Southern blot analysis with WHV DNA C gene primers (see Fig. 2.2). These WCM-260 cells were found to be WHV DNA reactive (Fig. 3.20C). The number of WHV vge detected in the cells recovered after each passage was lower than that in the second passage, but not significantly different between passages 3 to 6 (Table 3.7). This indicates that although PBMC-derived virus was evidently transmitted from one WCM-260 hepatocyte culture to another,

Figure 3.20. Expression of WHV DNA in WCM-260 hepatocytes infected with PBMC-derived WHV, hepatocyte culture supernatant, and in hepatocytes exposed to the passaged WHV. (A) Approximately 0.2 µg of DNA isolated from WCM-260 cells exposed to either serum (WHV-serum/260) or PBMC (WHV-PBMC/260) inoculum and their 4 d.p.i. culture supernatants (WHV-serum/260 c.s. or WHV-PBMC/260 c.s.) were evaluated for WHV DNA by direct PCR with WHV core gene-specific primers. (B) DNA extracted from 50 µl of concentrated hepatocyte culture supernatants obtained 4 days after infection with WHV originating from serum (WHV-serum/260 c.s.) or PBMC (WHV-PBMC/260 c.s.) were amplified by direct PCR to identify WHV DNA. (C) Different amounts of DNA (see Table 3.7) isolated from WCM-260 hepatocytes exposed to passaged WHV (Passage No. 2 - 6) and DNA from 100 µl of concentrated 6th passage culture supernatant (6th passage c.s.) were analysed.for WHV DNA by nested PCR. One µg of DNA from the liver of a chronic WHV carrier and 1 fg of rWHV DNA were included as positive controls while non-infected hepatocytes (C) and a mock (M) sample were used as negative controls. PCR products were hybridized to a rWHV DNA probe. The sizes of the identified PCR products are shown on the right. (D) Flow cytometry histogram of WCM-260 cells exposed to PBMC-derived WHV (solid line) and virus naive WCM-260 cells (dotted line) stained with rabbit antibodies against WHcAg. Cell numbers (counts) are plotted against the log of fluorescence intensity (FITC). The number in the panel is the percentage of WHcAg-positive cells.

A
Positive control
WHV-serum/260
WHV-serum/260 c.s.
WHV-PBMC/260
WHV-PBMC/260 c.s.
C
M
Negative controls
<490 bp
B
Positive control
rWHV DNA
WHV-serum/260 c.s.
WHV-PBMC/260 c.s.
Negative control (M)
<490 bp
C
rWHV DNA
Passage No.
2 3 4 5 6
6th passage c.s.
Negative control (C)
<428 bp
D
Counts
0 50 100 150 200 250
19%
10^0 10^1 10^2 10^3 10^4
FITC

Table 3.7. WHV genome contents in sequential WCM-260 hepatocyte cultures infected with lymphoid cell-derived passaged WHV[a].

Passage No.	Total DNA used in PCR[b] (µg)	WHV vge per µg DNA[c]
2	0.3	683
3	1.12	200
4	1.8	132
5	1.8	104
6	0.85	172

[a] Based on densitometric analysis of hybridization signals presented in Figure 3.20C.

[b] Calculated from the absorbance at 260 nm.

[c] WHV vge content was determined by extrapolation from the vge number contained in 1 fg of rWHV DNA standard which was amplified by PCR in parallel.

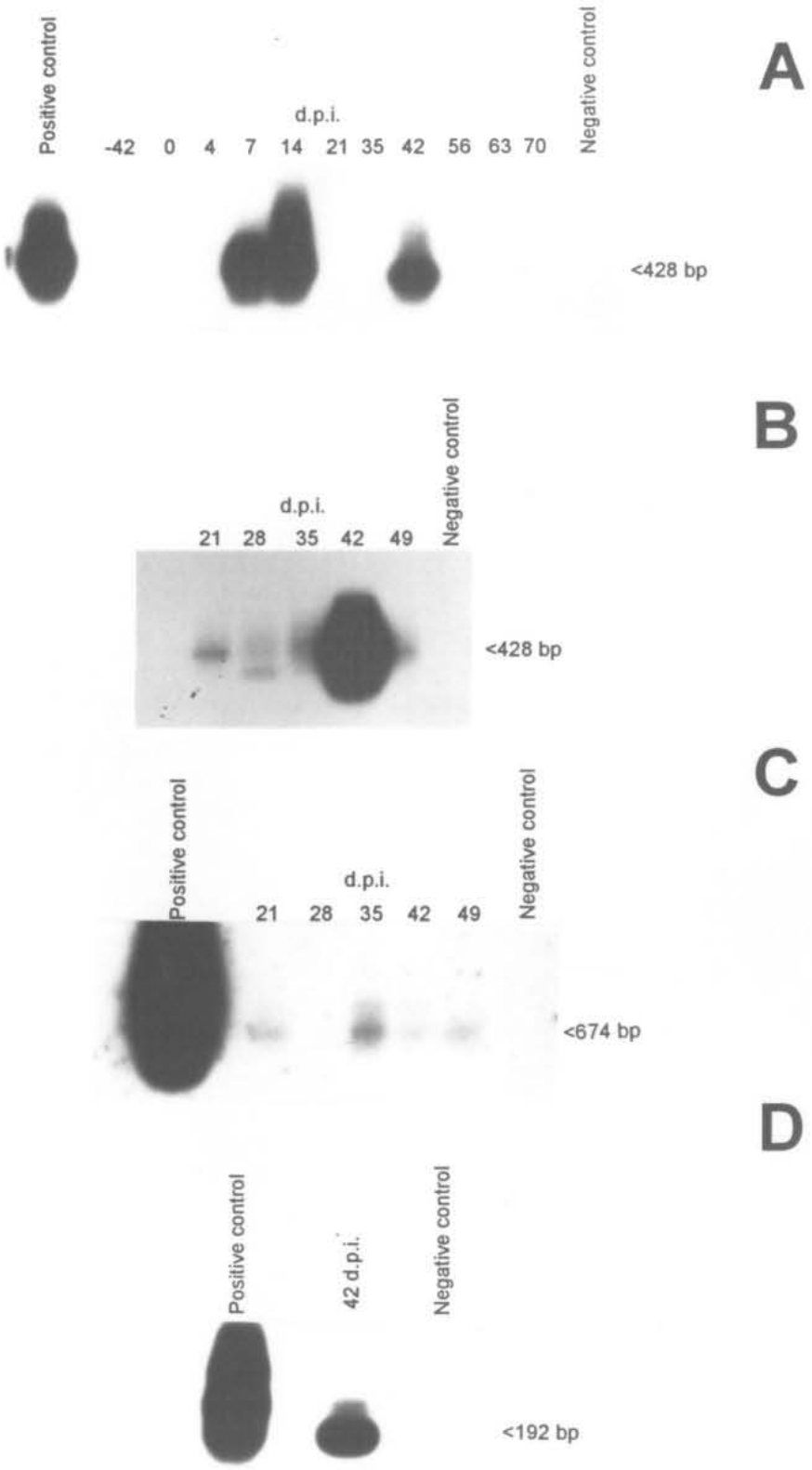
the virus did not multiply exponentially in the culture system.

In addition, the culture supernatant from the final sixth passage was evaluated for WHV content after concentration by ultracentrifugation. It was found that this concentrated culture supernatant (1 ml) contained 2.2×10^4 WHV vge (Fig. 3.20C, lane 2). Approximately 1.3×10^3 WHV vge was injected intravenously into a WHV-naive woodchuck.

3.5.2 Infectivity of WHV passaged in serial hepatocyte cultures to a WHV-naive woodchuck

Analysis of sequential serum samples collected from a woodchuck inoculated with hepatocyte culture supernatant obtained after sixth passage in WCM-260 cells of WHV derived from infected PBMC showed the appearance of WHV DNA sequences beginning at 7 d.p.i. (Fig. 3.21A). WHV DNA persisted in the circulation of the woodchuck until the end of the follow-up (70 d.p.i.), however, its levels varied, sometimes falling below the detection limit of the nested PCR/Southern blot hybridization assay. Similar results were obtained when sequential PBMC samples were analysed for WHV core gene sequence expression (Fig. 3.21B). Analysis of WHV cccDNA in the peripheral lymphoid cells showed a low but persistent presence of this replicating DNA species (Fig. 3.21C). Liver biopsy obtained at 42 d.p.i. was found to be positive for WHV C gene (data not shown) and X gene (Fig. 3.21D, lane 2). Although molecular evidence of virus infection was detected, anti-

Figure 3.21. Expression of WHV DNA in sera, PBMC and liver of a woodchuck inoculated with WHV originating from circulating lymphoid cells that was passaged for six times in WCM-260 hepatocyte cultures. Sequential samples obtained at the indicated days post-inoculation (d.p.i.) were analysed by nested PCR using primers specific for different WHV genes. Subsequently, PCR products were probed with ^{32}P-labelled rWHV DNA by Southern blot hybridization. (A) Detection of WHV C gene sequences in DNA extracted from 100 µl of serum samples taken before and after inoculation. (B) Identification of WHV C gene expression in DNA samples (1 µg/reaction) isolated from PBMC of the inoculated woodchuck. (C) Mung bean nuclease-digested DNA (5 µg/reaction) from PBMC amplified by PCR with WHV cccDNA-specific primers. (D) Five µg of DNA obtained from a liver sample taken at 42 d.p.i. was analysed using WHV X gene-specific primers. One µg of DNA from WHV-positive liver was used as a positive control and a mock sample was included as a negative control. The position and size of the WHV DNA-specific PCR products are marked on the right side of the panels.


A
Positive control
d.p.i.
-42 0 4 7 14 21 35 42 56 63 70
Negative control
<428 bp
B
d.p.i.
21 28 35 42 49
Negative control
<428 bp
C
Positive control
d.p.i.
21 28 35 42 49
Negative control
<674 bp
D
Positive control
42 d.p.i.
Negative control
<192 bp

WHc antibodies and WHsAg were not present and the woodchuck did not exhibit histological evidence of viral hepatitis. This pattern of WHV infection is usually observed when WHV doses containing 10^3-10^4 vge are administered into adult, virus-naive woodchucks (Coffin & Michalak, 1999). The significance of the above lies in that WHV originating from WHV-infected lymphoid cells and passaged sequentially in woodchuck hepatocyte cultures remains infectious to the virus-naive host. Therefore, these results fully support our findings presented in the previous section (Section 3.4) where the established *in vitro* experiments demonstrated that lymphoid cell-derived virus is biologically competent and capable of establishing infection in hepatocytes.

3.6 THE EXTENT OF *IN VITRO* WHV INFECTION IS MODIFIED BY INTERFERENCE WITH THE EXTREME N-TERMINAL REGION OF THE WHV LARGE ENVELOPE PROTEIN.

In regard to recognition of the nature of WHV-cell interaction, previous studies from our laboratory demonstrated that the binding of anti-WHV preS1(1-25) antibodies (anti-LP1) to synthetic analogues bearing a 4-amino acid host cell recognition motif (NPDK; designated as WHV CBS1) was inhibited by extracts from woodchuck hepatocytes, splenocytes and PBMC, but not those from other woodchuck tissues (Michalak *et al.*, 1991; Jin *et al.*, 1996) (see also Section 1.7.5). This suggested that the WHV-host interaction and subsequent entry could be

mediated by the N-terminal domain of the WHV large envelope protein. To assess whether this preS1(1-25) region is involved in *in vitro* WHV-hepatocyte binding and subsequent viral invasion, the effect of rabbit anti-LP1 antibodies or the WHV preS1(1-13) synthetic peptide (JP1) on the *in vitro* WHV infection of WCM-260 hepatocytes was investigated.

WCM-260 cells exposed to splenocyte-derived inoculum containing 1×10^7 WHV vge/ml in the presence of 10% anti-LP1, showed approximately 50% reduction in intracellular WHV DNA content when compared with the signal detected in the cells incubated with the same inoculum supplemented with 10% rabbit preimmune serum (Fig. 3.22). Densitometric analysis of the Southern blots revealed that 1 in 40 hepatocytes which were incubated with WHV in the presence of anti-LP1 contained a copy of WHV DNA and 1 in 20 WCM-260 cells which were exposed to WHV and preimmune serum have at least a single WHV vge. This finding indicates that by interaction with WHV CBS1, anti-LP1 antibodies are able to diminish virus binding and its uptake by host hepatocytes and subsequently lower the viral replication in these cells.

In a supplementary experiment, the effect of JP1 peptide, which represent the WHV preS1(1-13) sequence, on WHV-host cell interaction was tested. For this purpose, WCM-260 cells were exposed to splenocyte-derived WHV inoculum containing 1×10^7 vge/ml in the presence of 15 µg/ml of JP1. It was found that JP1 at this concentration decreased the intracellular WHV DNA signal by 90% when

Figure 3.22. Anti-LP1 antibodies inhibit *in vitro* WHV infection of WCM-260 hepatocytes. WCM-260 cells were exposed to WHV (1×10^7 vge/ml) derived from splenocytes of a chronically infected woodchuck in the absence (WHV/260) or presence of 10% (vol/vol) of rabbit preimmune serum (WHV/260 + preimmune serum) or presence of 10% (vol/vol) of anti-LP1 serum (WHV/260 + anti-LP1). (A) Five µg of mung bean nuclease-treated DNA isolated from the test cells were evaluated for WHV DNA content by direct PCR using PCOR1-MCOR primer pair. The PCR products were then probed with ^{32}P-labelled rWHV DNA. As positive controls, 1 µg of mung bean nuclease-digested DNA from liver a chronic WHsAg carrier and 50 fg of rWHV DNA were included, whereas DNA from non-infected hepatocytes (C) and a mock (M) sample were used as negative controls. The size of the PCR amplified fragment is shown on the right of the panel. (B) Plot of net digital light units (net DLU) of the WHV DNA hybridization signals shown in panel A analysed by densitometric scanning.

A
Positive control
rWHV DNA
WHV/260
WHV/260 + preimmune serum
WHV/260 + anti-LP1
C
M
Negative controls
<490 bp
B
net DLU (x 10^5)
0
1
2
3
4
5
WHV/260
WHV/260 + preimmune serum
WHV/260 + anti-LP1

compared with that detected in the cells incubated with the same inoculum alone (Fig. 3.23). Densitometric analysis of the Southern blot revealed that 1 in 230 hepatocytes which were incubated with WHV in the presence of JP1 contained a single WHV vge and that 1 in 28 WCM-260 cells which were exposed to WHV alone have at least a copy of WHV DNA. Since JP1 peptide was apparently able to inhibit the WHV invasion of host hepatocytes *in vitro*, this result suggests that the synthetic preS1(1-13) amino acid sequence efficiently competed with natural, infectious WHV particles for binding to the hepatocyte surface.

Taken together, these findings support previous data (Jin *et al.*, 1996) indicating that the WHV preS1(1-13) sequence containing the predicted WHV CBS1 is in fact involved in WHV-hepatocyte binding and subsequent internalization of the virus.

3.7 WHV cccDNA SPECIFICITY CAN BE DETECTED IN CIRCULATING VIRIONS

In the course of developing a WHV cccDNA-specific PCR assay, it was found that mung bean nuclease-treated DNA extracted from sera of WHsAg-positive chronic WHV carriers contained WHV DNA molecules with a repaired nick region. To confirm this unexpected observation, a series of experiments were done to determine whether this WHV cccDNA species exists as intact virions or occurs in a free form in circulation. For this purpose, WHV virions was enriched from sera of woodchucks #191 and #943 by ultracentrifugation through a 30% sucrose cushion

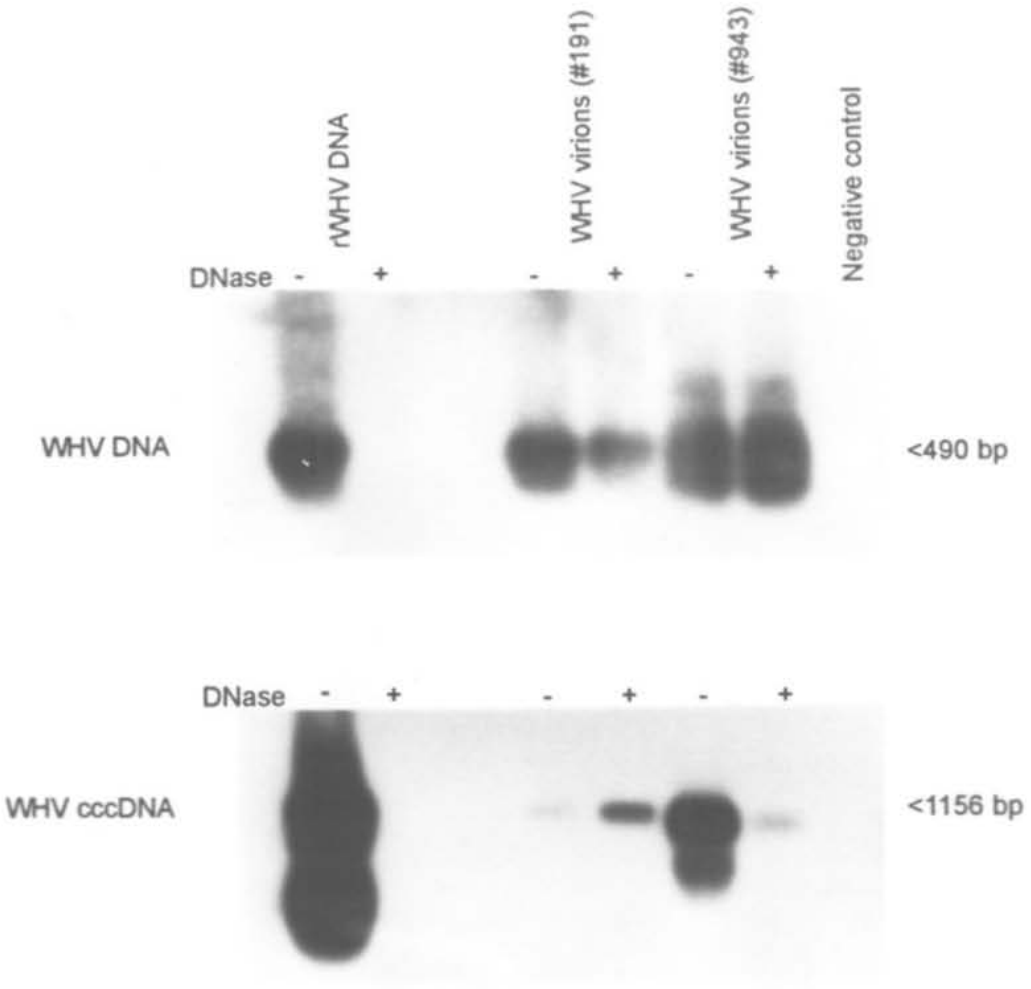
Figure 3.23. JP1 peptide inhibits *in vitro* WHV infection of woodchuck hepatocytes. WCM-260 cells were exposed to WHV (1×10^7 vge/ml) derived from naturally infected splenocytes in the presence (WHV/260 + JP1) or absence (WHV/260) of 15 µg/ml of JP1 synthetic peptide. One µg of mung bean nuclease-treated DNA isolated from the cells was tested for WHV DNA content by direct PCR using PCOR1-MCOR primers. The PCR products were then probed with ^{32}P-labelled rWHV DNA by Southern blot hybridization. As positive controls, 1 µg of mung bean nuclease-digested DNA from the liver of a chronic WHsAg carrier and 50 fg of rWHV DNA were included, whereas DNA from non-infected hepatocytes (C) and a mock (M) sample were used as negative controls. The size of the PCR amplified fragment is shown on the right of the panel.

Positive control
rWHV DNA
WHV/260 + JP1
WHV/260
Negative controls
C
M
<490 bp

(Section 2.5.1) and subjected to triple DNase digestion (Section 2.5.2) to completely remove non-enveloped WHV DNA molecules. Subsequently, proteinase K/phenol-chloroform extracted DNA was digested with mung bean nuclease and used as a template for PCR amplification with PGAP1-MCOR or PCOR1-MCOR primers. Southern blot examination of the PCR products obtained by amplification with PGAP1-MCOR primer pair revealed the presence of WHV cccDNA specific-sequences in DNase-resistant virions (Fig. 3.24), indicating that WHV DNA with repaired nick region existed in the circulation. Furthermore, Southern blot analysis of the PCR products amplified with WHV core gene primers demonstrated that most of the WHV DNA extracted from serum pellets originated from enveloped virion particles but not from freely circulating WHV DNA, as DNase digestion did not significantly diminish the detection of WHV DNA (Fig. 3.24). To confirm that DNase-digestion completely removed non-enveloped WHV DNA molecules from tested serum samples, a plasmid containing complete WHV genome suspended in serum from a healthy animal underwent the same triple DNase digestion as virions, which was followed by DNA extraction, PCR amplification with either PCOR1-MCOR or PGAP1-MCOR primers, and Southern blot analysis of the amplified products. The results showed that DNase treatment eliminated WHV DNA reactivity from the plasmid sample, while the undigested plasmid (control) remained positive (Fig. 3.24).

Subsequently, to further confirm the validity of the WHV cccDNA detection

Figure 3.24. Detection of WHV DNA and WHV cccDNA in DNase-resistant circulating WHV particles. Fifty µl of WHV virions prepared from the sera of two chronically infected #191 and #943 woodchucks were either not treated (DNase -) or extensively treated with DNase (DNase +) as described in Materials and Methods. DNA extracted from the above samples was subjected to mung bean nuclease digestion and analysed for WHV core gene and WHV cccDNA expression by direct PCR with WHV core gene and WHV nick-spanning primers, respectively. This was followed by Southern blot hybridization of the PCR products with a ^{32}P-labelled rWHV DNA probe. The following controls were included: rWHV DNA incubated in the absence (DNase -) or presence (DNase +) of DNase and a mock sample (negative control). The identified sizes of the PCR products are shown on the right.


rWHV DNA
WHV virions (#191)
WHV virions (#943)
Negative control
DNase - + - + - +
WHV DNA
<490 bp
DNase - + - + - +
WHV cccDNA
<1156 bp

in circulating viral particles, the PCR fragments generated from DNase-digested virions that were amplified across the nick region (Fig. 3.24) or from a control WHV-positive liver were sequenced either directly or after molecular cloning. In the samples analysed, the existence of the WHV nick region sequence was confirmed. The nucleotide sequence detected in DNase-resistant virions isolated from serum of #943 animal was identical with the published WHV DNA sequences and contained nucleotides located in position 1878-2043 of the WHV genome (Table 3.8, see also Fig. 3.25). This sequence includes the nick region beginning at position 2011 (Galibert *et al.*, 1982). These results strongly suggest that WHV DNA sequences containing a repaired nick region (i.e., cccDNA) exist in circulating enveloped virions.

Table 3.8. Summary of the data on nucleotide sequence analysis of mung bean nuclease-digested DNA extracted from DNase-treated circulating WHV virions.

Sequencing primers (position)[a]	Position of identified WHV DNA sequence[a]		
	# 943 serum[b]	# 191 serum[c]	# 1232 Liver[d]
QP11 (1501-1524)	n.t.	1588-1633	1591-1723
QX12 (1791-1809)	1878-2043	1875-1955	1870-2073

[a] Nucleotide positions aligned according to Galibert *et al.*,1982 (GenBank accession number J02442).

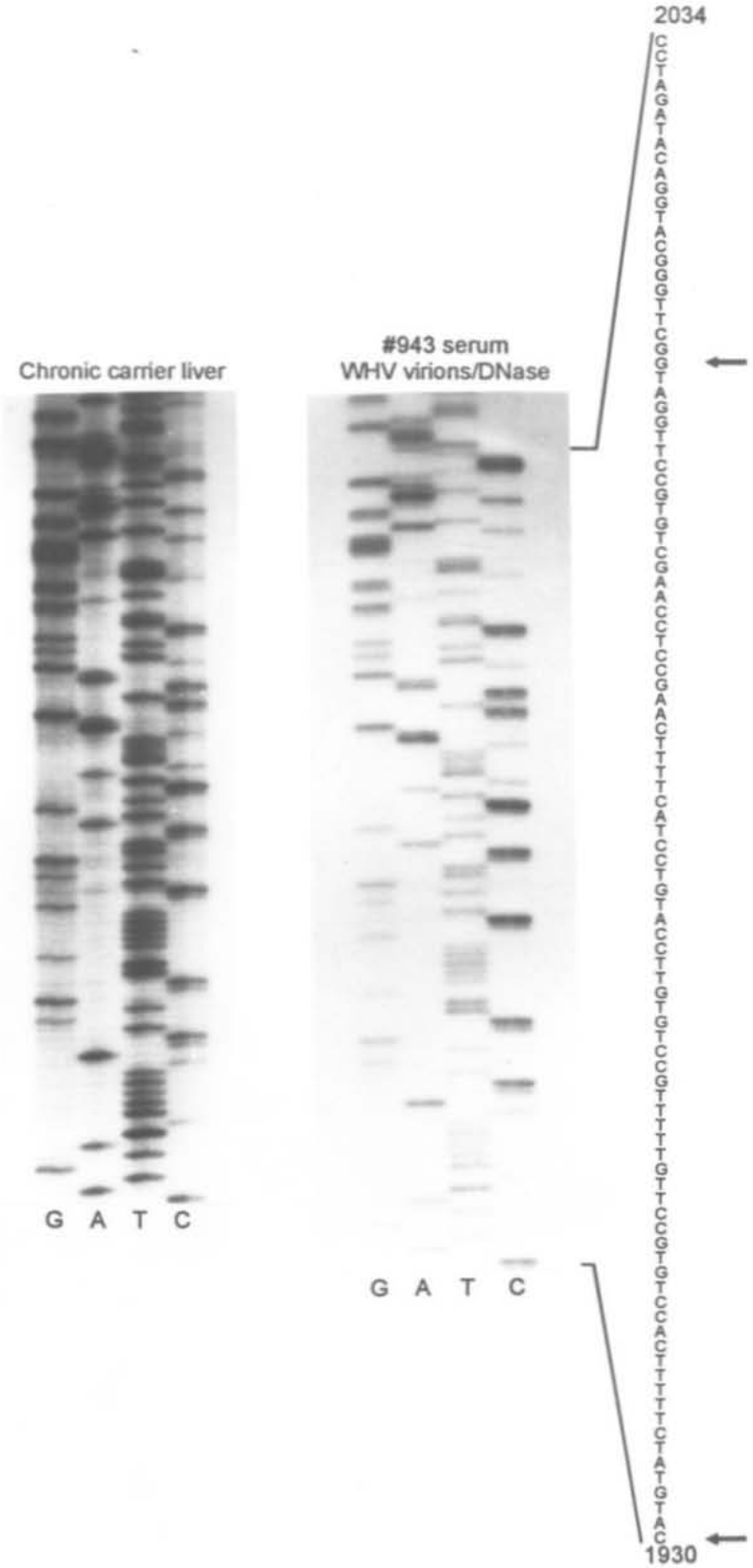
[b] DNA fragment amplified by PGAP1-MCOR primers was cloned and sequenced as described in Materials and Methods.

[c] Sequence obtained by direct sequencing of the excised PCR product.

[d] As a positive control, PCR fragment amplified from DNA extracted from the liver of a chronic WHsAg carrier was directly sequenced in parallel.

n.t., not tested.

Figure 3.25. Circulating, DNase-resistant WHV virions carry a repaired WHV nick region. One µl of cloned PCR product amplified with PGAP1-MCOR primers, which originated from DNase-digested WHV virions prepared from serum of #943 woodchuck was used as the template for cycle sequencing. As a positive control, nick-spanning PCR product amplified from DNA of a WHsAg-carrier's liver was also sequenced under the same conditions. Nucleotide sequencing was done as described in Materials and Methods using ^{32}P-labelled QX12 primer. The nucleotide positions relative to the published WHV genome (Galibert *et al.*, 1982; GenBank accession number J02442) is marked on the right and the nucleotide sequence is also shown on the right. Arrows mark the location the putative WHV nick region (1930-2011).


2034
CCTAGATACAGGTACGGGTTCGGTAGGTTCCGTGTCGAACCTCCGAACTTTTCATCCTGTACCTTGTGTCCGTTTTTGTTCCGTGTCCACTTTTTCTATGTAC
1930
Chronic carrier liver
G A T C
#943 serum
WHV virions/DNase
G A T C

CHAPTER FOUR : DISCUSSION

4.1 IDENTIFICATION OF REPLICATIVE WHV DNA SEQUENCES IN CULTURED WOODCHUCK CELLS.

It is generally acknowledged that detection of hepadnavirus cccDNA within host cells is indicative of active virus replication and therefore, it is considered to be the most reliable marker of active infection (Tuttleman *et al.*, 1986b). In this study, a significant effort was made to assure uncompromised identification of WHV cccDNA in cultured woodchuck hepatocytes and lymphoid cells. As revealed by our initial experiments, the detection of WHV cccDNA in the *in vitro* WHV infection system developed in this study was hindered by two main obstacles: (1) WHV cccDNA occurred in cultured cells at quantities below the detection limit of the commonly used procedure; i.e., the Southern blot analysis of virus DNA species precipitated by potassium chloride methods, and (2) unexpected detection of WHV cccDNA sequences in sera of woodchucks chronically infected with WHV which were used as WHV inocula (for further discussion of this issue see Section 4.8). To circumvent these problems: (1) a more sensitive PCR method combined with mung bean nuclease digestion of test DNA was adopted for detection of WHV cccDNA and (2) all cells examined for WHV cccDNA presence by the above PCR assay were subjected to extensive DNase/trypsin digestion and washing to eliminate any virions or WHV DNA fragments possibly attached to the cell surface. As revealed by the experimental data obtained in the course of this study, these steps ensured the full authenticity of the identified WHV cccDNA signals.

Using a KCl-precipitation/Southern blot hybridization procedure, the various WHV DNA replicating species can be distinguished based on their electrophoretic migrations (Aldrich *et al.*, 1989). In an agarose gel, the supercoiled hepadnaviral cccDNA migrates faster, while the uncoiled rcDNA species has slower mobility when compared with linear double stranded hepadnaviral DNA. The KCl precipitation method allows for the isolation of non-protein associated DNA from host DNA and integrated WHV DNA sequences, giving better resolution of the various non-integrated WHV DNA forms after Southern blot hybridization. Therefore, this method is superior to the conventional proteinase K/phenol-chloroform DNA extraction procedure, giving more readily distinguishable WHV rcDNA, cccDNA and ssDNA species. However, the sensitivity of this method, as estimated in this study, was only ~10^5 WHV vge/sample (data not shown) and it was not sufficient to detect small amounts of WHV cccDNA which typically occur in lymphoid cells and lymphatic tissues even in chronic carriers with high WHV loads and in *in vitro* infected WCM-260 hepatocytes.

In this study, the relative electrophoretic migration of WHV rcDNA and cccDNA species isolated by the KCl precipitation method was found to be 3.8 and 2.3 kb, respectively. This result agrees with a previous report where WHV cccDNA was found to have relative electrophoretic mobility of 2.3 kb (Moraleda *et al.*, 1997). The migratory pattern of these WHV DNA species was also similar to that demonstrated for HBV DNA and cccDNA, which migrate at 3.2 and 2.0 kb,

respectively (Miller & Robinson, 1984). Our results also showed that when digested with *Eco*RI endonuclease, the various WHV DNA species (i.e., rcDNA, cccDNA and ssDNA) gave a single band consisting of linear 3.3 kb WHV DNA. This is in agreement with that reported for HBV DNA by other investigators (Miller & Robinson, 1984). Therefore, although we succeeded in detecting WHV cccDNA by the KCl precipitation/Southern blot hybridization method, this technique was not utilized in our studies due to its intrinsic low sensitivity.

Subsequently, the PCR method of WHV cccDNA identification was adopted from published procedures used for detection of HBV cccDNA sequences. The strategy applied in this PCR assay is to allow for amplification across the nick region of hepadnaviral rcDNA. Thus, *Taq* DNA polymerase should generate the expected PCR product from double-stranded hepadnaviral cccDNA template, while amplification from virus rcDNA template will prematurely terminate at the single-stranded nick and gap regions existing in the hepadnaviral genome. Originally, the method was developed without the preceding mung bean nuclease digestion (Kock *et al.*,1996a). More recently, another group used mung bean nuclease to digest the single-stranded nick and the gap regions of the HBV genome to further improve the stringency of the cccDNA detection (Stoll-Becker *et al.*, 1997). In our laboratory, to increase the likelihood of WHV cccDNA identification by nick-spanning PCR, the amounts of DNA and also mung bean nuclease used in each reaction were increased. Correspondingly, the $MgCl_2$ concentration supplementing the PCR

mixture was changed to compensate for the larger quantities of EDTA employed to inactivate mung bean nuclease. In addition, since the WHV-specific primers used differed from the published HBV-specific primer sequences, the thermocycling program was also modified to achieve optimal amplification conditions. In this study, the primers that spanned the nick region of the WHV DNA minus strand were designed to be at least 200 bases away, in both directions, from the nick, since the location of the nick in different WHV isolates has not yet been as extensively characterized as in HBV. The specificity of our WHV cccDNA detection procedure, which combined mung bean nuclease digestion of test DNA with subsequent PCR amplification of the DNA digest with the virus nick region-specific primers, was determined by nucleotide sequence analysis of the amplified WHV DNA fragments. This analysis revealed complete homology between the amplified products and the previously reported WHV nick sequences.

4.2 LYMPHOID CELLS MAINTAINED IN HEPATOCYTE GROWTH MEDIUM SUPPORT WHV REPLICATION.

It was found that cells derived from the woodchuck lymphatic system, such as PBMC and splenocytes, were able to survive in hepatocyte medium for up to 3 days without changes in cell viability and expression of WHV DNA. It was also found that WHV cccDNA expression in lymphoid cells cultured for 3 days was the same as in cells which were not cultured. These findings demonstrated that the

nutritional supplementation in conditioned Hepato-STIM™ medium were adequate for short-term culture of WHV-infected lymphoid cells and to support WHV replication in these cells.

It was observed that WHV genome expression in cultured woodchuck PBMC substantially decreased after 6 days of culture. This was most likely due to the rapid decrease in the cell survival as the culture progressed, which led to the loss of lymphoid cells carrying the virus. Overall, the study established that woodchuck lymphoid cells and hepatocytes can be maintained in the same culture conditions (at least for 72 h) eliminating a need for intermediate manipulation on the lymphoid cell-derived WHV inoculum. This constitutes a significant experimental advantage which allows a direct transfer of the virus to fragile woodchuck hepatocyte cultures for *in vitro* infection studies.

Although previous studies have shown that the presence of 10 µg/ml of LPS induced the production of WHV transcripts and secretion of WHV virions in naturally infected PBMC cultured in Dulbecco's minimum essential medium (Korba *et al.*, 1988) or in RPMI 1640 medium (Michalak *et al.*, 1999; Coffin & Michalak 1999), an increase in viral replication (i.e., WHV DNA and cccDNA expression) was not observed when WHV-positive PBMC were incubated in conditioned Hepato-STIM™ medium containing LPS. One main difference between the previous and the present studies is that the current culture system utilized hepatocyte medium supplemented with dexamethasone. This corticosteroid has been shown to

increase HBV DNA expression and HBV protein production in transfected hepatoblastoma cells (Tur-Kaspa & Laub, 1990; Chou *et al.*, 1992) and in HBV infected-PLC/PRF/5 hepatoma cell line (Saito *et al.*, 1992). Thus, it is possible that WHV replication in woodchuck lymphoid cells cultured in the presence of Hepato-STIM™ medium was already upregulated by this drug and, therefore, remained unresponsive to additional mitogen stimulation. In summary, our data showed that the mitogen stimulation of WHV-infected lymphoid cells did not increase WHV production or virus infectivity toward woodchuck hepatocytes in our *in vitro* system.

4.3 CULTURED WOODCHUCK HEPATOCYTES ARE SUSCEPTIBLE TO WHV INFECTION.

Most *in vitro* infection experiments for hepadnaviruses utilized freshly seeded primary hepatocytes as targets. In our system, we used a well established, normal woodchuck hepatocyte cell line. Thus, we secured a consistent source as a virus cell target that allowed for repetition of experiments under precisely the same culture conditions. These hepatocytes were isolated from liver biopsy of a healthy, WHV-naive animal and maintained in culture for more than 3 years without losing their ability to synthesize hepatocyte-specific ASGPR transcripts. The expression of ASGPR and albumin in these cells, which are distinctive markers of hepatocytes, have been documented previously (Diao *et al.*, 1998). As we have shown in this work, these liver cells were susceptible to WHV invasion and were competent to

support WHV replication and virion assembly.

Analysis of the WHV DNA and WHV cccDNA expression at different days post-inoculation of WCM-260 hepatocytes revealed that although WHV DNA was found from the first day after exposure to virus, WHV cccDNA was detected beginning from 3 d.p.i. This result may suggest that the viral genome repair machinery becomes operational a few days after viral invasion or that WHV cccDNA needs to accumulate within infected cells before it reaches detectable levels. Similarly, in one culture system reported by another laboratory, WHV DNA was detectable in primary woodchuck hepatocytes from the first day post-infection and progressively accumulated in the cells until the termination of the experiment at 10 d.p.i. (Aldrich *et al.*, 1989). In the same cells, WHV cccDNA appeared at 2 d.p.i., was greater at 4 d.p.i., and remained at the 4 d.p.i.-level until 10 d.p.i. In a following study, WHV cccDNA was detected in infected hepatocytes for as long as 24 d.p.i., when the experiment was terminated (Moraleda *et al.*, 1997). Comparable results were also obtained for HBV where *in vitro* infected primary human hepatocytes demonstrated detectable levels of HBV cccDNA beginning from 4 d.p.i. (Mabit *et al.*, 1996).

In our system, hepatocytes exposed to WHV were maintained in culture for 4 d.p.i., i.e., to the moment when WCM-260 cells have reached confluence. Therefore, the kinetics of WHV infection were not studied after 4 d.p.i. As we have found, the highest expression of WHV cccDNA occurred at 3 d.p.i. and decreased

slightly at 4 d.p.i. This suggests that WHV infection in our experimental system should not be extended beyond 4 d.p.i., as continued culture of WCM-260 hepatocytes may decrease the amount of WHV cccDNA due to its relatively short half-life. It is of note that the half-life of DHBV cccDNA has been estimated to be 3-5 days (Civito & Locarnini, 1994). In addition, experimental evidence have shown that DHBV replication took place only when host cells underwent division (Turin *et al.*, 1996). In the above study, it was demonstrated that incubation of primary duck hepatocytes with *n*-butyrate, a drug that suppresses cell division at the G1 phase, inhibited the accumulation of DHBV cccDNA. This inhibitory effect was reversible, as removal of *n*-butyrate from the culture medium caused an increase in DHBV cccDNA expression. It is possible that the dependence of hepadnaviral cccDNA formation on cell cycle is due to the transport of viral nucleocapsids to the nucleus, which is upregulated during the G1 phase of cell division (Yeh *et al.*, 1998). Taken together, it would be reasonable to assume that the accumulated intracellular WHV cccDNA will be quickly degraded and, since there is very little cell division when a hepatocyte culture is confluent, there will be very little WHV cccDNA production. Subsequently, the WHV cccDNA content will quickly diminish. This decrease in WHV cccDNA pool was observed to begin as early as 4 d.p.i. in our culture system. Following this reasoning, *in vitro* infected WCM-260 hepatocytes were harvested immediately after they reached confluence to optimize the number of cells harvested and to ensure that there was no excessive breakdown of synthesized

viral cccDNA.

Moreover, based on the same argument, WCM-260 hepatocytes were inoculated with WHV a day after seeding to maximize cell proliferation in the presence of virus and thus, to allow for the utmost production of WHV cccDNA and WHV proteins and to facilitate the most efficient detection of these viral replicative markers. Therefore, summarizing the data from experiments performed in our and other laboratories, it can be assumed that the active cell growth is corelated with an increase in cell susceptibility to hepadnavirus infection.

In the majority of the experiments, WHV DNA and cccDNA from WHV-exposed hepatocytes were detectable after Southern blot hybridization of the PCR products, indicating that relatively low copy numbers of WHV nucleic acids were present. In fact, when densitometry was employed, it was estimated that about 1 in 30 and 1 in 300 WCM-260 hepatocytes exposed to inocula containing at least 10^7 WHV vge/ml carried a copy of WHV DNA and WHV cccDNA, respectively. This estimation could be the result of a small number of hepatocytes containing a large amount of WHV DNA or a large proportion of WCM-260 cells with a small viral DNA load. The observed copy numbers in WCM-260 hepatocytes are lower than those reported for primary woodchuck hepatocyte cultures in which it was estimated that each cell contained a copy of WHV cccDNA (Aldrich *et al.*, 1989). The major difference between the mentioned study and ours is that they were using freshly isolated and seeded hepatocytes which were incubated with 10^9 WHV virions/ml of

culture medium. It is possible that the relatively limited ability of WCM-260 cells to support WHV replication was due to a certain rate-limiting step in WHV replication *in vitro*, e.g. the rate of formation of viral cccDNA. Hence, a limited copy number of this viral template will lead to decrease in downstream events, such as synthesis of WHV DNA and viral proteins, and formation of complete virions.

The results presented in this report demonstrated that WHV replication in *in vitro* infected WCM-260 hepatocytes was not affected by the presence of glucagon and that elimination of glucagon from the culture medium did not influence the rate of woodchuck hepatocyte growth. These results are in contrast to those reported for the *in vitro* DHBV infection of primary duck hepatocytes where glucagon at a concentration as low as 100 nM inhibited the establishment of virus infection (i.e., formation of DHBV cccDNA; Hild *et al.*, 1998). This process was shown to be mediated through the up-regulation of cyclic AMP (cAMP), which probably modifies the host and viral proteins involved in DHBV replication. Therefore, the results of our investigation indicate that the rate of WHV replication in cultured woodchuck hepatocytes is not modulated by glucagon, as in the case of avian hepadnavirus. This may suggest that different cellular mechanisms might be involved in the establishment and progression of WHV and DHBV infections *in vitro*.

In our study, 12-53% of tested cells were found to be positive for WHV antigens (WHcAg and/or WHsAg) when tested by flow cytometry. The finding that only a small fraction of cultured hepatocytes expressed hepadnaviral antigens is a

common observation (Ochiya *et al.*, 1989; Rijntjes *et al.*, 1988; Tuttleman *et al.*, 1986a). For example, 0.1% and 5% primary human hepatocytes infected with HBV DNA and HBeAg-positive serum were found reactive for HBcAg and HBsAg, respectively (Rijntjes *et al.*, 1988). In a separate study, where primary human fetal hepatocytes were co-cultured with HBV-transfected HB 611 cells (human HCC-derived cells), about 12% of the hepatocytes were positive for HBcAg (Ochiya *et al.*, 1989). Comparable results were obtained in primary duck hepatocytes infected *in vitro* with DHBV where 10% of the DHBV-infected duck hepatocytes were positive for viral surface and core antigens (Tuttleman *et al.*, 1986a). Therefore, our findings on WHV antigen expression in WCM-260 hepatocytes are closely comparable to those reported for HBV and DHBV infections in respective cell culture systems.

In this study, the estimated number of cells carrying WHV DNA and cccDNA did not corelate with the number of hepatocytes positive for WHV antigens. Since there is no direct relationship between the intracellular viral DNA expression and the rate of hepadnavirus protein synthesis, the above observation is not surprising. In addition, this apparent discrepancy could be influenced by different detection methods employed for WHV DNA and antigen identification. In this regard, it is also of interest to mention that in so called healthy chronic HBV carriers, there are abundant hepatocellular deposits of HBsAg in the context of low HBV DNA expression (Hadziyannis *et al.*, 1983).

It was estimated that WHV-infected WCM-260 hepatocytes secreted approximately 10^4 WHV vge/ml of culture medium when exposed to inocula containing 10^4 - 10^8 WHV vge/ml which originated from either lymphoid cells or serum. This appears to be 100-fold less than the amount of HBV reported to be released by primary human fetal hepatocytes after exposure to 10^6 HBV vge/ml derived from HBV-transfected HCC cells (Ochiya *et al.*, 1989). Six days after exposure to HBV inoculum, these fetal human hepatocytes secreted approximately 5×10^6 HBV vge/ml. Although this system differs in many ways from ours, the main variations are the virus origin and the fact that WCM-260 cells were derived from an adult animal and maintained for a long time in culture prior to WHV infection, whereas freshly seeded fetal liver cells were used in the above mentioned work.

The observed low levels of hepadnaviral replication in *in vitro* infected hepatocytes are in contrast to typical findings in acutely or chronically, naturally infected hosts where much greater percentages of hepatocytes display evident molecular and antigenic markers of viral replication. For example, it was estimated that each hepatocyte nucleus contains approximately 6 to 30 copies of WHV cccDNA and between 50 to 75% of hepatocytes become infected with WHV in woodchucks with acute hepatitis (Kajino *et al.*, 1994). In the duck hepatitis model, DHBV DNA was estimated to be between 160 and 1600 vge/cell, while DHBV cccDNA occur at 6 to 30 copies per cell, and DHBV antigens were found in 27 to 80% of infected hepatocytes (Jilbert *et al.*, 1992). The present data are more

compatible with the levels of WHV DNA expression observed in woodchucks convalescent from acute infection where an estimated 0.02 to 2 WHV vge per 10^4 hepatocytes were detected (Michalak *et al.*, 1999). This clearly indicates that all *in vitro* hepadnaviral infection systems established so far, including our model, are not as efficient in supporting hepadnavirus propagation as naturally infected hepatocytes in actively progressing infection. However, they appear to support virus replication to a greater degree than hepatocytes in occult (serologically silent) hepadnavirus infection *in vivo*.

4.4 LYMPHOID CELL-DERIVED WHV IS INFECTIVE TO CULTURED WOODCHUCK HEPATOCYTES.

For the first time, this study documents that WHV produced by infected lymphoid cells is directly infectious to the host hepatocytes. This was demonstrated by two independent experimental approaches: (1) exposure of WCM-260 hepatocytes to supernatants from cultures of PBMC or splenocytes isolated from WHV-infected animals and (2) co-culture of WHV-infected lymphoid cells with virus-naive WCM-260 hepatocytes in the same medium but separated from each other by a cell impermeable membrane.

In the first system, when WCM-260 hepatocytes were incubated with culture supernatants from WHV-positive lymphoid cells, WHV DNA was detected within hepatocytes in all experiments (8/8), while WHV cccDNA was found in half of the

trials (4/8). In addition, WHV antigens (WHcAg and/or WHsAg) were identified in approximately two-thirds of these experiments (5/8). These results document that virus generated by lymphoid cells is hepatotropic and infectious in *in vitro* conditions. In addition, using culture supernatants from WHV-infected lymphoid cells as inocula, we have found that WHV produced by splenocytes had greater infectivity toward hepatocytes when compared to virus secreted by comparable numbers of WHV-infected PBMC. This observation could indicate that splenocyte-derived virus has somehow a greater potential to infect host hepatocytes than that from PBMC. A more likely explanation for this observation is that supernatants from splenic lymphoid cells contained greater amounts of WHV. Indeed, it was found that splenocyte culture supernatants had usually ~10^7 WHV vge/ml, whereas PBMC-derived inocula typically contained <10^4 WHV vge/ml, indicating that splenocytes generate a larger amount of virus per lymphoid cell than PBMC. Since the spleen contains a larger population of less mature lymphoid cells which undergo more cell divisions than the mature cells in the circulation, splenocytes have probably greater capacities to support virus production and secretion into the culture medium and be more susceptible to infection through contact with other infected lymphoid cells or blood.

In co-culture experiments, naturally infected PBMC or splenocytes were incubated with WCM-260 cells. This culture system used a cell-impermeable membrane with pore size of 0.4 μm to ensure that both types of cells share the

same culture medium and can exchange secreted microparticles, but do not have any direct contact. As we have found, hepatocytes co-cultured with WHV-positive lymphoid cells became WHV infected and WHV DNA reactive at 4 d.p.i. We observed that the co-culture of WHV-positive PBMC and WCM-260 cells did not improve the extent of viral replication in hepatocytes in comparison to hepatocytes exposed to culture supernatant derived from a comparable number of PBMC obtained from the same animal. However, interestingly, co-culture of WHV-infected splenocytes with WCM-260 cells gave a much higher number of infected liver cells compared to the usual inoculation utilizing splenocyte culture supernatant as a virus source. This may suggest that WHV freshly released by splenocytes have a greater invasive potential and/or is able to more readily establish virus replication in cultured WCM-260 hepatocytes than WHV from the same cells, but stored prior to incubation with the hepatocytes. There is only one study in literature where a comparable experimental approach was used (Ochiya *et al.*, 1989). In this work, co-culture of HBV-infected hepatoblastoma cells with virus-negative primary hepatocytes contained within a cell culture insert resulted in detection of HBV-specific DNA and RNA, as well as HBV cccDNA and HBeAg in hepatocytes after a 72-h co-culture (Ochiya *et al.*, 1989; see Section 1.7.2). However, the cells were not subjected to DNase/trypsin digestion prior to nucleic acid analysis, raising a possibility that not all detected virus-specific signals resulted from *de novo* HBV replication.

Previous works have clearly demonstrated that woodchucks inoculated with culture supernatants from WHV-positive PBMC develop classical acute hepatitis (Korba *et al.*, 1989b; Michalak *et al.*, 1999; Coffin & Michalak, 1999). The current study suggests that inocula derived from WHV-infected splenic lymphoid cells may constitute an even better source of infectious virus and cause more severe viral hepatitis in challenged woodchucks.

As WHV and HBV are closely related, our *in vitro* study demonstrating the capacity of lymphoid-cell derived WHV to infect hepatocytes has some clinical relevance. The data presented here provide direct evidence that lymphoid cells are reservoirs of virus which is capable of infecting liver cells and that they support the notion that lymphoid tissue is an important compartment for the maintenance of hepadnaviral persistence. This can have serious implications for antiviral therapies against HBV as both the liver and the lymphatic system have to be targeted to eliminate the virus. In addition, this does not provide a good prognosis for HBV-infected patients with liver transplants, since the transplanted organ can be (and they are; O'Grady *et al.*, 1992) rapidly infected by HBV from viral stores in the lymphatic system.

4.5 THE LEVEL OF WHV REPLICATION *IN VITRO* DEPENDS ON THE INOCULUM WHV CONTENT BUT NOT ON ITS ORIGIN.

In this study, we have repeatedly found that WHV inocula should contain at

least 10^7 vge/ml in order to induce infection in WCM-260 hepatocytes accompanied by relatively readily (50%) identifiable WHV cccDNA. Thus, PBMC-derived inocula appeared to be less infective, as determined by WHV cccDNA expression, compared to inocula derived from splenocytes or sera. This was most likely due to much lower WHV content in culture supernatants from PBMC, which typically was $<10^4$ WHV vge/ml. In contrast, the amounts of virus in splenocyte (~10^7 WHV vge/ml) and serum (~10^8 - 10^9 WHV vge/ml) derived inocula were comparable and both inocula types induced WHV cccDNA-positive infection in half of the trials. These data clearly indicate that the ability of WHV to establish productive infection in cultured hepatocytes depends primarily on the virus content, but not on whether the virus originates from lymphoid cells or serum. They also demonstrated that the virus released by splenocytes is as biologically competent as that in the serum.

4.6 WHV FROM LYMPHOID CELLS PASSAGED SERIALLY IN HEPATOCYTES INFECTS VIRUS-NAIVE ANIMAL.

The experiment in which WHV secreted by naturally infected PBMC were serially passaged 6 times in WCM-260 hepatocytes showed that the virus was transmittable through all cell passages. This was demonstrated by detection of intracellular WHV DNA in DNase/trypsin-treated cells. In addition, culture supernatant obtained after the sixth passage was found to contain at least 4.4×10^2 WHV vge/ml. In a much less extensive and remotely related study from another

laboratory, HBV produced by *in vitro* infected primary human fetal hepatocytes (not lymphoid cells) infected naive fetal hepatocytes (Ochiya *et al.*, 1989). These newly exposed cells produced detectable amounts of HBsAg and HBeAg beginning from 4 d.p.i. Our investigation of lymphoid cell-derived WHV went a few steps further, since the number of passages was greater and the final culture supernatant was used as inoculum to infect a virus-naive woodchuck. Using this approach, we demonstrated not only that the passaged WHV originating from lymphoid cells remained infectious *in vitro*, but also that it was able to induce infection in its natural host. These results convincingly documented that virus released by the lymphoid cells continued to be biologically competent in spite of its multistep transfer throughout cultured hepatocytes. They also provide evidence that our WCM-260 cell culture system does not alter WHV functional capabilities.

In the inoculated animal, WHV DNA sequences were detected both in sera, PBMC and the liver, although their levels were relatively low and identifiable by nested PCR/Southern blot hybridization. WHV cccDNA signals were seen in PBMC but not in the liver, suggesting that virus might predominantly replicate in lymphoid cells. In addition, no serological markers of WHV infection (i.e., WHsAg and anti-WHc) were detected. This serologically silent infection most likely resulted from the small amounts of the virus in the administered inoculum. The molecular and immunovirological patterns of this infection were closely comparable to the profiles observed in woodchucks infected with viable lymphoid cells isolated from animals

convalescent from WHV hepatitis (Michalak *et al.*, 1999) and offsprings born to dams recovered from acute WHV infection (Coffin & Michalak, 1999). In the above mentioned studies, infection was induced by traces of WHV and was frequently restricted to the host lymphatic system.

4.7 INFECTION OF WHV IN CULTURED HEPATOCYTES IS INHIBITED BY ANTI-LP1 ANTIBODIES AND A SYNTHETIC ANALOGUE OF WHV CBS1.

Previously, the specificity of rabbit antibodies directed against amino acids 1-25 (anti-LP1) of the N-terminal domain of the large WHV envelope protein was characterized in detail (Michalak *et al.*, 1991; Jin *et al.*, 1996). These polyclonal antibodies were shown to be strictly specific for WHV preS1(1-25) domain and able to inhibit the binding of the synthetic analogues of the WHV CBS1 and proteolytically modified WHsAg particles to woodchuck hepatocytes and lymphoid cells. Although these previous findings provided much evidence for the interaction of WHV CBS1 with host receptors (see Section 1.7.5), it did not directly demonstrate the involvement of this region in the mediation of virus uptake by host hepatocytes. The experiments completed in this study indicate that the anti-LP1 antibodies may have the ability to inhibit, at least partially, invasion of woodchuck hepatocytes by WHV. Preincubation of splenocyte-derived WHV with 10% anti-LP1 led to ~50% decrease in the WHV DNA content within hepatocytes. This may suggest that higher concentrations of anti-LP1 have to be examined in future

experiments to completely prevent virus uptake or that, although less likely (see below), yet another epitope of the WHV envelope contributes to recognition and uptake of WHV by WCM-260 hepatocytes. In a related study, it has been reported that antibodies against the preS1(20-120) region of the HBV large envelope protein (10% [vol/vol]) were able to completely inhibit HBsAg production when the antiserum was added to HBV inoculum used for infection of primary human hepatocyte cultures (Galle *et al.*, 1994). However, the effect of the antiserum on HBV DNA replication has not been evaluated in these cells.

In addition, the results from the parallel set of experiments also demonstrated that a synthetic analogue to the WHV CBS1 was able to compete with and inhibit the uptake of WHV into cultured WCM-260 hepatocytes. This provides additional evidence that this extreme N-terminal region of the WHV large envelope protein is intimately involved in host hepatocyte specific recognition. Further extensive experiments will be required to fully explain the role of the WHV CBS1 in productive WHV infection. The *in vitro* infection system developed in this study will certainly contribute to these investigations.

4.8 CIRCULATING INTACT WHV VIRIONS CARRY WHV cccDNA REACTIVITY.

In this study, we have shown that DNase-resistant, WHV cccDNA-reactive particles can be detected in the circulation of woodchucks chronically infected with WHV. These particles were separated from sera of woodchucks with high virus

loads and were subjected to extensive DNase digestion, which is known to eliminate hepadnaviral sequences not contained in the protective envelope (Michalak *et al.*, 1994, Coffin & Michalak, 1999). In spite of the fact that the identification of hepadnaviral cccDNA has been considered as an exclusive feature of intracellular virus replication, there are reports demonstrating the presence of hepadnaviral cccDNA in infected sera. However, these studies did not fully establish whether the identified HBV cccDNA sequences originated from intact virions, as the samples were not subjected to DNase treatment prior to testing. In one study, HBV cccDNA was found in sera of 18 (42%) out of 43 HBsAg-positive chronic HBV carriers when tested by PCR with primers amplifying a fragment that spans the nick region (Maillard & Pillot, 1996). Among these 43 patients, different proportions of HBV molecular forms (i.e., HBV rcDNA and cccDNA) were seen, e.g., HBV rcDNA existed at high levels without any detectable HBV cccDNA or HBV cccDNA levels were as high as HBV rcDNA. In addition, the amount of HBV cccDNA cannot be corelated with the amount of total HBV DNA in serum, suggesting that there is little or no relationship between the synthesis of the fully double stranded HBV genome (cccDNA) and HBV rcDNA. In another study applying an oligonucleotide probe specific for the plus strand 5'-fixed end, which is present in HBV cccDNA but not in rcDNA, HBV cccDNA was detected in 53 (51%) of 104 serum samples (Lin *et al.*, 1989). It was estimated that HBV cccDNA consists of less than 1% of total HBV DNA in circulation (Lin *et al.*, 1989). In a

study where a HBV-infected chimpanzee was examined, it was found that DNA extracted from circulating Dane particles contained supercoiled HBV DNA and that these HBV cccDNA sequences constituted less than 1% of total HBV DNA (Ruiz-Opazo *et al.*, 1982). The authors suggested that HBV cccDNA-containing particles may be infectious forms of the virus, while HBV rcDNA-reactive virions may be non-infectious. The latter form may exist in large amounts in the circulation as a mechanism to vanquish the host's anti-virus specific immunological responses. Similar observations were also made in the duck hepatitis model where a 2 kbp DHBV cccDNA form was detected in intracellularly derived complete virions (Tong *et al.*, 1998). Thus, when DHBV-positive serum was used as inoculum to *in vitro* infect carboxypeptidase D-(a candidate DHBV receptor; Section 1.6.1) transfected COS cells, DHBV cccDNA was found in core and complete viral particles within the infected cells. The above findings are consistent with our results that only a minority of WHV virions contain WHV cccDNA. This conclusion was based on the observation that the PCR/Southern blot hybridization of DNA extracted from WHV-positive sera gave significantly weaker signals (~10^2-fold) after PCR with nick-spanning primers compared to those generated by PCR using core gene-specific primers.

In the study mentioned above (Maillard & Pillot, 1996), not all sera samples were positive for HBV cccDNA. In our studies, DNase-treated pellets from sera of all 5 woodchucks examined contained WHV cccDNA sequences when tested by

PCR after mung bean nuclease digestion of DNA. Many factors can contribute to this difference. One major reason could be that although HBV and WHV are closely related, they are two distinct viruses which pathways of replication and virion assembly may have some unique features. Therefore, the difference noticed could be due to the different inherent characteristics of each virus.

Since DNase digestion of serum samples analysed was very extensive and complete (as revealed by control digestion of recombinant WHV DNA), it is unlikely that the PCR signals detected after amplification with nick-spanning primers could be the result of DNA contamination from freely circulating WHV DNA molecules. Moreover, nucleotide sequence analysis of WHV virion-derived DNA amplified by PCR confirmed that the PCR fragment contained the sequence spanning the nick region and was homologous to the sequences published. These nick sequences contained no deletions or mutations when compared to the previously reported sequences and to the sequence of WHV DNA extracted from liver of a chronic WHV carrier residing in our colony. This indicates that cccDNA detected within WHV virions was wild type viral genome.

Although the existence of circulating WHV particles containing WHV cccDNA was demonstrated in chronically infected animals in this study, their biological significance remain to be established. It is possible that these viral particles may play a significant role in the pathogenesis of WHV infection, since this form of WHV virion can bypass the genome repair step mediated by WHV DNA polymerase and

migrate directly to the nucleus, where it can serve as the template for the first step of viral replication. Another issue to consider is the origin of the WHV cccDNA in virions. These cccDNA-reactive virions could be the consequence of premature packaging of WHV cccDNA during viral replication within the host cells or the product of reactions mediated by virion-associated enzyme(s) that are responsible for the formation of hepadnaviral cccDNA from the rcDNA precursor. Since hepadnaviral cccDNA do not contain the ε hairpin structure (Section 1.4.4), which is a signal for encapsidation, it is more likely that WHV cccDNA were formed after formation of nucleocapsids.

4.9 SUGGESTIONS FOR FUTURE STUDIES

The results obtained in this study raised several points worthy of further investigation. Some of these future experiments may include:

1. The development of a quantitative PCR with primers spanning the nick region for a more accurate determination of WHV cccDNA content. This could be especially important for determination of WHV cccDNA expression in hepatocytes employed for *in vitro* evaluation of antiviral effects of agents acting on virus-cell initial interactions or virus replication.
2. Further improvement of the efficiency of WHV propagation in the woodchuck hepatocyte cultures allowing upscale generation of a homogeneous, well characterized pool of infectious virus for *in vitro* and *in vivo* investigations.

3. By taking advantage of the absence of virus-specific immunological pressure in *in vitro* environment, examination of WHV mutational changes during long-term culture in naturally-infected and *in vitro* infected woodchuck hepatocytes and lymphoid cells, and after multiple hepatocyte to hepatocyte and/or lymphoid cell to lymphoid cell passages.

4. *In vitro* analysis of the susceptibility of lymphoid cells to WHV and dynamics of the virus transmission from infected to virus-naive cells within the lymphatic system.

5. Detailed characterization of the physicochemical properties of circulating WHV particles containing WHV cccDNA sequences and determination of their infectivity and pathogenic role in hepadnavirus infection.

CHAPTER FIVE : SUMMARY AND CONCLUSIONS

In this study, infectivity of lymphoid cell-derived hepadnavirus to homologous hepatocytes has been documented for the first time in *in vitro* conditions. For this purpose, a unique experimental system employing virus-naive, well-characterized and continuously propagating woodchuck hepatocytes as WHV targets was established. Also, highly discriminatory and sensitive methods enabling evaluation of the expression of molecular indicators of WHV replication and virus antigens in *in vitro* infected hepatocytes were developed and routinely used. The results obtained in the course of our investigation can be summarized as follows:

1. Woodchuck hepatocytes can be successfully maintained as a cell line for prolonged periods of time in culture without losing expression of hepatocyte-specific molecules and susceptibility to WHV infection. These cells can provide an important model for further studies on the early events of WHV infection and replication, and for pre-clinical evaluation of the potency and action of novel antihepadnaviral agents under *in vitro* conditions.

2. Cultured woodchuck hepatocytes used in this study were prone to WHV infection and supported its replication regardless of whether the virus originated from serum or from lymphoid cells. However, the rate of virus propagation was dependent on the amount of virus present in the inoculum. This suggests that the quantity of the

virus but not its origin (serum or lymphoid cells) is the limiting factor in the induction of effective WHV replication in cultured hepatocytes.

3. Lymphoid cells naturally infected with WHV released the virus which was infectious to cultured hepatocytes, serially transmittable from infected to non-infected woodchuck liver cells and infectious to a virus-naive woodchuck. These data document that virus propagating within the lymphatic system is biologically competent in regard to its ability to establish active infection and support the notion that lymphoid cells are an important reservoir of the virus from which it can spread through the host.

4. Splenic lymphoid cells released at least 100-fold greater amounts of WHV than comparable numbers of circulating lymphoid cells. The above findings remain in agreement with previous observations that organ lymphoid cells are sites of more active hepadnavirus propagation than PBMC and that they could be the major extrahepatic reservoir of infectious virus.

5. The observed inhibition of WHV DNA expression in cultured woodchuck hepatocytes by anti-LP1 antibodies and JP1 synthetic analogue of WHV cell binding site 1 (CBS1) support our previous findings that the preS1(1-13) sequence of the WHV large envelope protein is critical for initial recognition of host

hepatocytes leading to productive virus infection.

6. Finally, in the process of evaluating methods for detection of WHV cccDNA, it was demonstrated that woodchucks with chronic WHV infection and a high virus load in serum had circulating WHV cccDNA molecules that were packaged into DNase-resistant particles, suggesting that they were present within intact WHV virions. Subsequent DNA sequence analysis of the PCR-amplified WHV cccDNA fragments revealed that neither the nick region nor flanking sequences of the virus minus DNA strand had altered nucleotide sequences, suggesting that wild type sequence was carried in these freely circulating particles. This raises the hypothetical possibility that serum WHV particles containing cccDNA molecules could be a typical consequence of virion assembly.

REFERENCES

Aldrich, C.E., L. Coates, T.T. Wu, J. Newbold, B.C. Tennant, J. Summers, C. Seeger, & W.S. Mason. 1989. *In vitro* infection of woodchuck hepatocytes with woodchuck hepatitis virus and ground squirrel hepatitis virus. *Virology* **172**:247-252.

Alley, M.C., D.A. Scudiero, A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, & M.R. Boyd. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Research* **48**:589-601.

Alter, M.J. & E.E. Mast. 1994. The epidemiology of viral hepatitis in the United States. *Gastroenterology Clinics of North America* **23**:437-455.

Baginski, I., I. Chemin, P. Bouffard, O. Hantz, & C. Trépo. 1991. Detection of polyadenylated RNA in hepatitis B virus-infected peripheral blood mononuclear cells by polymerase chain reaction. *Journal of Infectious Diseases* **163**:996-1000.

Barker, L.F., J.E. Maynard, R.H. Purcell, J.H. Hoofnagle, K.R.. Berquist, & W.T. London. 1975. Viral hepatitis, type B, in experimental animals. *American Journal of Medical Science* **270**:189-195.

Bartenschlager, R., M. Junker-Niepmann, & H. Schaller. 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. *Journal of Virology* **64**:5324-5332.

Bavand, M.R., & O. Laub. 1988. Two proteins with reverse transcriptase activities associated with hepatitis B virus-like particles. *Journal of Virology* **62**:626-628.

Bchini, R., F. Capel, C. Dauguet, S. Dubanchet, & M.A. Petit. 1990. *In vitro* infection of human hepatoma (HepG2) cells with hepatitis B virus. *Journal of Virology* **64**:3025-3032.

Beasley, R.P. 1988. Hepatitis B virus: the major etiology of hepatocellular carcinoma. *Cancer* **61**:1942-1956.

Benjamin, T. & P.K. Vogt. 1990. Cell transformation and viruses, pp. 317-367. In B.N. Fields & D.M. Knipe (eds.), *Fields Virology* Raven Press, New York.

Benn, J., F. Su, M. Doria, & R.J. Schneider. 1996. Hepatitis B virus HBx protein induces transcription factor AP-1 by activation of extracellular signal-regulated and

c-Jun N-terminal mitogen-activated protein kinases. *Journal of Virology* **70**:4978-4985.

Blum, H.E., W. Gerok, & G.N. Vyas. 1989. The molecular biology of hepatitis B virus. *Trends in Genetics* **5**:154-158.

Bouffard, P., J.P. Lamelin, F. Zoulim, D. Lepot, & C. Trepo. 1992. Phytohemagglutinin and concanavalin A activate hepatitis B virus in peripheral blood mononuclear cells of patients with chronic hepatitis B virus infection. *Journal of Medical Virology* **37**:255-262.

Bowden, D.S., L.D. Moaven, & S.A. Locarnini. 1996. New hepatitis viruses: are there enough letters in the alphabet? *Medical Journal of Australia* **164**:87-89.

Bowers, D.F., B.A. Abell, & D.T. Brown. 1995. Replication and tissue tropism of the alphavirus Sindbis in the mosquito *Aedes albopictus*. *Virology* **212**:1-12.

Branch, A.D. & H.D. Robertson. 1984. A replication cycle for viroids and other small infectious RNA's. *Science* **223**:450-455.

Breiner, K.M., S. Urban, & H. Schaller. 1998. Carboxypeptidase D (gp180), a Golgi-resident protein, functions in the attachment and entry of avian hepatitis B viruses. *Journal of Virology* **72**:8098-8104.

Brown, F. 1989. The classification and nomenclature of viruses: summary of results of meetings of the International Committee on Taxonomy of Viruses in Edmonton, Canada 1987. *Intervirology* **30**:181-186.

Bruss, V., J. Hagelstein, E. Gerhardt, & P.R. Galle. 1996. Myristylation of the large surface protein is required for hepatitis B virus *in vitro* infectivity. *Virology* **218**:396-399.

Budkowska, A., C. Quan, F. Groh, P. Bedossa, P. Dubreuil, J.P. Bouvet, & J. Pillot. 1993. Hepatitis B virus (HBV) binding factor in human serum: candidate for a soluble form of hepatocyte HBV receptor. *Journal of Virology* **67**:4316-4322.

Budkowska, A., P. Bedossa, F. Groh, A. Louise, & J. Pillot. 1995. Fibronectin of human liver sinusoids binds hepatitis B virus: identification by an anti-idiotypic antibody bearing the internal image of the pre-S2 domain. *Journal of Virology* **69**:840-848.

Budkowska, A., P. Maillard, N. Theret, F. Groh, C. Possehl, A. Topilko, R. Crainic. 1997. Activation of the envelope proteins by a metalloproteinase enables attachment and entry of the hepatitis B virus into T-lymphocyte. *Virology* **237**:10-22.

Cabrerizo, M., J. Bartolome, P. De Sequera, C. Caramelo, & V. Carreno. 1997. Hepatitis B virus DNA in serum and blood cells of hepatitis B surface antigen-negative hemodialysis patients and staff. *Journal of the American Society of Nephrology* **8**:1443-1447.

Calmus, Y., P. Marcellin, G. Beaurain, L. Chatenoud, C. Brechot. 1994. Distribution of hepatitis B virus DNA sequences in different peripheral blood mononuclear cell subsets in HBs antigen-positive and negative patients. *European Journal of Clinical Investigation* **24**:548-552.

Casjens, S. & J. King. 1975. Virus assembly. *Annual Review of Biochemistry* **44**:555-611.

Chang, S.F., H.J. Netter, M. Bruns, K. Frolich, & H. Will. 1998. Identification and characterization of a new avian hepadnavirus derived from snow goose. *Molecular Biology of Hepatitis B viruses* meeting; p. 3 (Abstract).

Charlton, M., P. Adjei, J. Poterucha, N. Zein, B. Moore, T. Therneau, R. Krom, & R. Wiesner. 1998. TT-virus infection in North American blood donors, patients with fulminant hepatic failure, and cryptogenic cirrhosis. *Hepatology* **28**:839-842.

Charnay, P., C. Pourcel, A. Louise, A. Fritsh, & P. Tiollais. 1979. Cloning in *Eschericia coli* and physical structure of hepatitis B virion DNA. *Proceedings of the National Academy of Science U.S.A.* **76**:2222-2226.

Chemin, I., C. Vermot-Desroches, I. Baginski, J.P. Lamelin, O. Hantz, C. Jacquet, D. Rigal, & C. Trepo. 1993. Monitoring of early events of experimental woodchuck hepatitis infection: studies of peripheral blood mononuclear cells by cytofluorometry and PCR. *FEMS Immunology and Medical Microbiology* **7**:241-249.

Chisari, F.V., & C. Ferrari. 1997. Viral Hepatitis, pp. 745-778. In. N. Nathanson (ed.), *Viral Pathogenesis* Lippincott-Raven Publishers, Philadelphia.

Choo, Q., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, & M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**:359-362.

Chou, C.K., L.H. Wang, H.M. Lin, & C.W. Chi. 1992. Glucocorticoid stimulates hepatitis B viral gene expression in cultured human hepatoma cells. *Hepatology* **16**:13-18.

Civitico, G.M. & S.A. Locarnini. 1994. The half-life of duck hepatitis B virus supercoiled DNA in congenitally infected primary hepatocyte cultures. *Virology* **203**:81-89.

Co, M.S., G.N. Gaulton, B.N. Fields & M.I. Greene. 1985. Isolation and biochemical characterization of the mammalian reovirus type 3 cell-surface receptor. *Proceedings of the National Academy of Science U.S.A.* **82**:1494-1498.

Coffin, C. & T.I. Michalak. 1999. Persistence of infectious hepadnavirus in the offspring of woodchuck mothers recovered from viral hepatitis. *Journal of Clinical Investigation* **104**:203-212.

Cohen, J.I., R.H. Miller, B. Rosenblum, K. Denniston, J.L. Gerin, & R.H. Purcell. 1988. Sequence comparison of woodchuck hepatitis virus replicative forms shows conservation of the genome. *Virology* **162**:12-20.

Colucci, G., P. Lyons, Y. Beazer, & S.D. Waksal. 1988. Production of hepatitis B virus-infected human B-cell hybridomas: transmission of the viral genome to normal lymphocytes in cocultures. *Virology* **164**:238-244.

Combes, B., J. Shorey, A. Barrera, P. Stastny, E.H. Eigenbrodt, A.R. Hull, & N.W. Carter. 1971. Glomerulonephritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. *Lancet* **31**:234-237.

Cova, L., I. Fourel, L. Vitvitski, V. Lambert, S. Chassot, O. Hantz, & C. Trepo. 1993. Animal models for the understanding and control of HBV and HDV infections. *Journal of Hepatology* **17 Suppl 3**:S143-148.

Dalgleish, A.G., P.C.L. Beverley, P.R. Clapham, D.H. Crawford, M.F. Greaves, & R.A. Weiss. 1984. The CD4(T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**:763-767.

Dane, D.S., C.H. Cameron, & M. Briggs. 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* **1**:695-698.

Dash, S., K.V. Rao, & S.K. Panda. 1992. Receptor for pre-S1(21-47) component

of hepatitis B virus on the liver cell: role in virus cell interaction. *Journal of Medical Virology* **37**:116-121.

de Bruin, W.C., K. Hertogs, W.P. Leenders, E. Depla, & S.H. Yap. 1995. Hepatitis B virus: specific binding and internalization of small HBsAg by human hepatocytes. *Journal of General Virology* **76**:1047-1050.

Deka, N., M.D. Sharma, & R. Mukerjee. 1994. Isolation of the novel agent from human stool samples that is associated with sporadic non-A, non-B hepatitis. *Journal of Virology* **68**:7810-7815.

Desousa, J.R.B. & T.I. Michalak. 1990. Cell-surface sulfated proteoglycan mediates binding of woodchuck hepatitis virus envelope to host hepatocytes. *Molecular Biology of Hepatitis B Viruses* meeting; p. 81 (Abstract).

Diao, J., N.D. Churchill, & T.I. Michalak. 1998. Complement-mediated cytotoxicity and inhibition of ligand binding to hepatocytes by woodchuck hepatitis virus-induced autoantibodies to asialoglycoprotein receptor. *Hepatology* **27**:1623-1631.

Dragic, T., V. Litwin, G.P. Allaway, S.R. Martin, Y. Huang Y, K.A. Nagashima, C. Cayanan, P.J. Maddon, R.A. Koup, J.P Moore, & W.A. Paxton. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**:667-673.

Elfassi, E., J.L. Romet-Lemonne, M. Essex, M. Frances-McLane, & W.A. Haseltine. 1984. Evidence of extrachromosomal forms of hepatitis B viral DNA in a bone marrow culture obtained from a patient recently infected with hepatitis B virus. *Proceedings of the National Academy of Sciences U.S.A.* **81**:3526-3528.

Eng, F.J., O. Varlamov, & L.D. Fricker. 1999. Sequences within the cytoplasmic domain of gp180/carboxypeptidase D mediate localization to the trans-Golgi network. *Molecular Biology of the Cell* **10**:35-46.

Evans, A.S. 1976. Causation and disease: the Henle-Koch postulates revisited. *Yale Journal of Biological Medicine* **49**:175-195.

Fattovich, G., L. Brollo, G. Giustina, F. Noventa, P. Pontisso, A. Alberti, G. Realdi, & A. Ruo. 1991. Natural history and prognostic factors for chronic hepatitis type B. *Gut* **32**:294-298.

Feray, C., A.L. Zignego, D. Samuel, A. Bismuth, M. Reynes, P. Tiollais, H. Bismuth, & C. Brechot. 1990. Persistent hepatitis B virus infection of mononuclear blood cells without concomitant liver infection. The liver transplantation model. *Transplantation* **49:**1155-1158.

Feitelson, M. A., I. Millman, T. Halbherr, H. Simmons, & B. S. Blumberg. 1986. A newly identified hepatitis B type virus in tree squirrels. *Proceedings of National Academy of Science U.S.A.* **83:**2233-2237.

Feitelson, M.A. & L.X. Duan. 1997. Hepatitis B virus X antigen in the pathogenesis of chronic infections and the development of hepatocellular carcinoma. *American Journal of Pathology* **150:**1141-1157.

Feitelson, M.A. 1999. Hepatitis B virus in hepatocarcinogenesis. *Journal of Cellular Physiology* **181:**188-202.

Fung, Y. Nakatsuji, J.W. Shih, L. Young, M. Piatak Jr., C. Hoover, J. Fernandez, S. Chen, J.C. Zou, T. Morris, K.C. Hyams, S. Ismay, J.D. Lifson, J.P Kim, *et al.* 1996. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science* **271:**505-508.

Galibert, F., T. N. Chen, & E. Mandart. 1982. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: comparison with the hepatitis B virus sequence. *Journal of Virology* **41**:51-65.

Galle, P.R., J. Hagelstein, B. Kommerell, M. Volkmann, P. Schranz, & H. Zentgraf. 1994. In vitro experimental infection of primary human hepatocytes with hepatitis B virus. *Gastroenterology* **106:**664-673.

Ganem, D. 1996. *Hepadnaviridae* and their replication, pp. 2703-2737. In B. N. Fields & D. M. Knipe (eds.), *Fields Virology* Raven Press, New York.

Gocke, D.J., K. Hsu, C. Morgan, S. Bombardieri, M. Lockshin, & C.L. Christian. 1970. Association between polyarteritis and Australia antigen. *Lancet* **2:**1149-1153.

Gong, Z.J., S. De Meyer, J. van Pelt, K. Hertogs, E. Depla, A. Soumillion, J. Fevery, & S.H. Yap. 1999. Transfection of a rat hepatoma cell line with a construct expressing human liver annexin V confers susceptibility to hepatitis B virus infection. *Hepatology* **29:**576-584.

Geraghty, R.J., C. Krummenacher, G.H. Cohen, R.J. Eisenberg, & P.G. Spear. 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**:1618-1620.

Girones, R., P.J. Cote, W.E. Hornbuckle, B.C. Tennant, J.L. Gerin, R.H. Purcell, & R.H. Miller. 1989. Complete nucleotide sequence of a molecular clone of woodchuck hepatitis virus that is infectious in the natural host. *Proceedings of the National Academy of Science U.S.A.* **86**:1846-1849.

Gripon, P., C. Diot, N. Theze, I. Fourel, O. Loreal, C. Brechot, & C. Guguen-Guillouzo. 1988. Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. *Journal of Virology* **62**:4136-4143.

Gripon, P., C. Diot, & C. Guguen-Guillouzo. 1993. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology* **192**:534-540.

Gripon, P., J. Le Seyec, S. Rumin, C. Guguen-Guillouzo. 1995. Myristylation of the hepatitis B virus large surface protein is essential for viral infectivity. *Virology* **213**:292-299.

Guo, J.T. & J.C. Pugh. 1997. Monoclonal antibodies to a 55-kilodalton protein present in duck liver inhibit infection of primary duck hepatocytes with duck hepatitis B virus. *Journal of Virology* **71**:4829-4831.

Hadchouel, M., C. Pasquinelli, J.G. Fournier, R.N. Hugon, J. Scotto, O. Bernard, & C. Brechot. 1988. Detection of mononuclear cells expressing hepatitis B virus in peripheral blood from HBsAg positive and negative patients by *in situ* hybridisation. *Journal of Medical of Virology* **24**:27-32.

Hadler, S.C., M. Alcala de Monzon, D. Rivero, A. Perez, A. Bracho, & H. Fields. 1992. Epidemiology and long-term consequences of hepatitis delta virus infection in the Yuca Indians of Venezuela. *American Journal of Epidemiology.* **136**:1507-1516.

Hadziyannis, S.J., H.M. Lieberman, G.G. Karvountzis, & D.A. Shafritz. 1983. Analysis of liver disease, nuclear HBcAg, viral replication, and hepatitis B virus DNA in liver and serum of HBeAg vs. anti-HBe positive carriers of hepatitis B virus. *Hepatology* **3**:656-662.

Halstead, S.B. 1988. Pathogenesis of dengue: challenges to molecular biology.

Science **239**:476-481.

Harrison, S.C. 1990. Principles of Virus Structure, pp. 37-63. In B.N. Fields & D.M. Knipe (eds.), *Fields Virology*. Raven Press, New York.

Henkler, F.F. & R. Koshy. 1996. Hepatitis B virus transcriptional activators: mechanisms and possible role in oncogenesis. *Journal of Viral Hepatitis* **3**:109-121.

Hertogs, K., W.P. Leenders, E. Depla, W.C. De Bruin, L. Meheus, J. Raymackers, H. Moshage, & S.H. Yap. 1993. Endonexin II, present on human liver plasma membranes, is a specific binding protein of small hepatitis B virus (HBV) envelope protein. *Virology* **197**:549-557.

Hertogs, K., E. Depla, T. Crabbe, W. De Bruin, W. Leenders, H. Moshage, & S.H. Yap. 1994. Spontaneous development of anti-hepatitis B virus envelope (anti-idiotypic) antibodies in animals immunized with human liver endonexin II or with the F(ab')2 fragment of anti-human liver endonexin II immunoglobulin G: evidence for a receptor-ligand-like relationship between small hepatitis B surface antigen and endonexin II. *Journal of Virology* **68**:1516-1521.

Hild, M., O. Weber, & H. Schaller. 1998. Glucagon treatment interferes with an early step of duck hepatitis B virus infection. *Journal of Virology* **72**:2600-26006.

Hsu, T., T. Moroy, J. Etiemble, A. Louise, C. Trepo, P. Tiollais, & MA. Buendia. 1988. Activation of *c-myc* by woodchuck hepatitis virus insertion in hepatocellular carcinoma. *Cell* **55**:627-635.

Hollinger, F.B. 1996. Hepatitis B virus, pp. 2739-2807. In B.N. Fields & D.M. Knipe (eds.), *Fields Virology* Lippincott-Raven Publishers, Philadelphia.

Hoofnagle, J.H., D.A. Shafritz, & H. Popper. 1987. Chronic type B hepatitis and the "healthy" HBsAg carrier state. *Hepatology* **7**:758-763.

Hubbard, A.L. & H. Stukenbrok. 1979. An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. II. Intracellular fates of the ^{125}I-ligands. *Journal of Cell Biology* **83**:65-81.

Hubbard, A.L., G. Wilson, G. Ashwell, & H. Stukenbrok. 1979. An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-ligands among the liver cell types. *Journal of Cell Biology*

83:47-64.

Jia, L., X.W. Wang, & C.C. Harris. 1999. Hepatitis X protein inhibits nucleotide excision repair. *International Journal of Cancer* **80**:875-879.

Jilbert, A.R., T.T. Wu, J.M. England, P.M. Hall, N.Z. Carp, A.P. O'Connell, & W.S. Mason. 1992. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvement. *Journal of Virology* **66**:1377-1388.

Jilbert, A.R., J.A. Botten, D.S. Miller, E.M. Bertram, P.M. Hall, J. Kotlarski, & C.J. Burrell. 1998. Characterization of age- and dose-related outcomes of duck hepatitis B virus infection. *Virology* **244**:273-282.

Jin, Y.M., N.D. Churchill, & T.I. Michalak. 1996. Protease-activated lymphoid cell and hepatocyte recognition site in the preS1 domain of the large woodchuck hepatitis virus envelope protein. *Journal of General Virology* **77**:1837-1846.

Kajino, K., A.R. Jilbert, J. Saputelli, C.E. Aldrich, J. Cullen, & W.S. Mason. 1994. Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. *Journal of Virology* **68**:5792-5803.

Kew, M.C. & H. Popper. 1984. Relationship between hepatocellular carcinoma and cirrhosis. *Seminars in Liver Diseases* **4**:136-146.

Klenk, H. -D. & W. Garten. 1994. Host cell proteases controlling virus pathogenicity. *Trends in Microbiology* **2**:39-43.

Klingmuller, U. & H. Schaller. 1993. Hepadnavirus infection requires interaction between the viral pre-S domain and a specific hepatocellular receptor. *Journal of Virology* **67**:7414-7422.

Knight, C.A. 1974. Molecular Virology, pp. 1-33. In *Molecular Virology*. McGraw-Hill, New York.

Knowles, B.B., C.C. Howe, & D.P. Aden. 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **209**:497-499.

Kock, J., L. Theilmann, P. Galle, & H. Schlicht. 1996a. Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate

from replicating virus. *Hepatology* **23**:405-413.

Kock, J., E.M. Borst, & H.J. Schlicht. 1996b. Uptake of duck hepatitis B virus into hepatocytes occurs by endocytosis but does not require passage of the virus through an acidic intracellular compartment. *Journal of Virology* **70**:5827-5831.

Kodama, K., N. Ogasawara, H. Yoshikawa, S. Murakami. 1985. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: evolutional relationship between hepadnaviruses. *Journal of Virology* **56**:978-986.

Koonin, E.V., A.E. Gorbalenya, M.A. Purdy, M.N. Rozanov, G.R. Reyes, & D.W. Bradley. 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proceedings of the National Academy of Science U.S.A.* **89**:8259-8263.

Koprowski, H. & S.A. Plotkin (eds.) 1985. *World's debt to Pastuer.* Alan R Liss, New York.

Korba, B.E., P.J. Cote, & J.L. Gerin. 1988. Mitogen-induced replication of woodchuck hepatitis virus in cultured peripheral blood lymphocytes. *Science* **241**:1213-1216.

Korba, B.E., P.J. Cote, F.V. Wells, B. Baldwin, H. Popper, R.H. Purcell, B.C Tennant, & J.L. Gerin. 1989a. Natural history of woodchuck hepatitis virus infections during the course of experimental viral infection: molecular virologic features of the liver and lymphoid tissues. *Journal of Virology* **63**:1360-1370.

Korba, B.E., P.J. Cote, M. Shapiro, R.H. Purcell, & J.L. Gerin. 1989b. *In vitro* production of infectious woodchuck hepatitis virus by lipopolysaccharide-stimulated peripheral blood lymphocytes. *Journal of Infectious Disease* **160**:572-576.

Korba, B.E., T.L. Brown, F.V. Wells, B. Baldwin, P.J. Cote, H. Steinberg, B.C. Tennant, J.L. Gerin. 1990. Natural history of experimental woodchuck hepatitis virus infection: molecular virologic features of the pancreas, kidney, ovary, and testis. *Journal of Virology* **64**:4499-4506.

Koziel, M.J. 1996. Immunology of Viral Hepatitis. *American Journal of Medicine* **100**:98-109.

Krone, B., A. Lenz, K.H. Heermann, M. Seifer, X.Y. Lu, & W.H. Gerlich. 1990.

Interaction between hepatitis B surface proteins and monomeric human serum albumin. *Hepatology* **11**:1050-1056.

Kulonen, K. & I. Millman. 1988. Vertical transmission of woodchuck hepatitis virus. *Journal of Medical Virology* **26**:233-242.

Kumar, S. & D.C. Pound. 1992. Serologic diagnosis of viral hepatitis. *Postgraduate Medicine* **92**:55-62, 65, 68.

Kuroki, K., R. Cheung, P.L. Marion, & D. Ganem. 1994. A cell surface protein that binds avian hepatitis B virus particles. *Journal of Virology* **68**:2091-2096.

Lanford, R.E., M.G. Michaels, D. Chavez, K. Brasky, J. Fung, & T.E. Starzl. 1995. Persistence of extrahepatic hepatitis B virus DNA in the absence of detectable hepatic replication in patients with baboon liver transplants. *Journal of Medical Virology* **46**:207-212.

Lanford, R.E., D. Chavez, K.M. Brasky, R.B. Burns 3rd, & R. Rico-Hesse. 1998. Isolation of a hepadnavirus from the woolly monkey, a New World pimate. *Proceedings of the National Academy of Science U.S.A.* **95**:5757-5761.

Lee, T.H., S.J. Elledge, & J.S. Butel. 1995. Hepatitis B virus X protein interacts with a probable cellular DNA repair protein. *Journal of Virology* **69**:1107-1114.

Leenders, W.P., H.L. Glansbeek, W.C. de Bruin, & S.H. Yap. 1990. Binding of the major and large HBsAg to human hepatocytes and liver plasma membranes: putative external and internal receptors for infection and secretion of hepatitis B virus. *Hepatology* **12**:141-147.

Leenders, W.P. & W.C. de Bruin. 1994. Cloning and production of functional active recombinant hepatitis B virus surface antigen binding protein. *Biochemical Biophysical Research Communications* **205**:52-59.

Lenkei, R., D. Onica, & V. Ghetie. 1977. Receptors for polymerized albumin on liver cells. *Experientia* **33**:1046-1047.

Levine, A.J. 1996. In *The origins of virology*. Lippincott-Raven Publishers, Philadephia.

Li, J.S., S.P. Tong, & J.R. Wands. 1996. Characterization of a 120-Kilodalton pre-S-binding protein as a candidate duck hepatitis B virus receptor. *Journal of*

Virology **70**:6029-6035.

Li, L., R.J. Dixon, & D.A. Anderson. 1997. Comparative *in vitro* studies of the australian wild duck hepatitis B viruses with duck hepatitis B virus. *Molecular biology of hepatitis B viruses* meeting; p. 93 (Abstract).

Lieberman, H.M., W.W. Tung, & D.A. Shafritz. 1987. Splenic replication of hepatitis B virus in the chimpanzee chronic carrier. *Journal of Medical Virology* **21**:347-59.

Lin, H.J., H.T. Chung, C.L. Lai, S. Leong, & O.S. Tam. 1989. Detection of supercoiled hepatitis B virus DNA and related forms by means of molecular hybridization to an oligonucleotide probe. *Journal of Medical Virology* **29**:284-288.

Linnen, J., J. Wages Jr., Z.Y. Zhang-Keck, K.E. Fry, K.Z. Krawczynski, H. Alter, E. Koonin, M. Gallagher, M. Alter, S. Hadziyannis, P. Karayiannis, K. Lobbiani, A.F. Lalatta, F. Lugo, & G. Colucci. 1990. Hepatitis B virus transcripts and surface antigen in human peripheral blood lymphocytes. *Journal of Medical Virology* **31**:190-194.

Lu, X., T.M. Block, & W.H. Gerlich. 1996. Protease-induced infectivity of hepatitis B virus for a human hepatoblastoma cell line. *Journal of Virology* **70**:2277-2285.

Lycke, E. & E. Norrby. 1976. Viruses-a unique kind of infectious agent, pp. 1-3. In: *Textbook in Medical Virology* Butterworth, London.

Mabit, H., S. Dubanchet, F. Capel, C. Dauguet, M.A. Petit. 1994. *In vitro* infection of human hepatoma cells (HepG2) with hepatitis B virus (HBV): spontaneous selection of a stable HBV surface antigen-producing HepG2 cell line containing integrated HBV DNA sequences. *Journal of General Virology* **75**:2681-2689.

Mabit, H., C. Vons, S. Dubanchet, F. Capel, D. Franco, M.A. Petit. 1996. Primary cultured normal human hepatocytes for hepatitis B virus receptor studies. *Journal of Hepatology* **24**:403-412.

Machida, A., S. Kishimoto, H. Ohnuma, H. Miyamoto, K. Baba, K. Oda, T. Nakamura , Y. Miyakawa, & M. Mayumi. 1983. A hepatitis B surface antigen polypeptide (P31) with the receptor for polymerized human as well as chimpanzee albumins. *Gastroenterology* **85**:268-274.

Mack, D.H., W. Bloch, N. Nath, & J.J. Sninsky. 1988. Hepatitis B virus particles contain a polypeptide encoded by the largest open reading frame: a putative reverse transcriptase. *Journal of Virology* **62:**4786-4790.

Maillard, P. & J. Pillot. 1996. Polymerase chain reaction to monitor repair of the HBV genome, the first step in viral replication. *Research in Virology* **147:**5-16.

Margolis, H.S., M.J. Alter, & S.C. Hadler. 1991. Hepatitis B: evolving epidemiology and implications. *Seminars in Liver Disease* **11:**84-92.

Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard, & W. S. Robinson. 1980. A virus in Beechey ground squirrels that is related to hepatitis B virus in humans. *Proceedings of National Academy Science U.S.A.* **77:**2941-2945.

Marsh, M. & A. Helenius. 1989. Virus entry into animal cells. *Advances in Virus Research* **36:**107-151.

Mason, W.S., G. Seal, & J. Summers. 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *Journal of Virology* **36:**829-836.

Mastromarino, P., C. Conti, P. Goldoni, B. Hauttecoeur, & N. Orsi. 1987. Characterization of membrane components of the erythrocyte involved in vesicular stomatitis virus attachment and fusion at acidic pH. *Journal of General Virology* **68:**2359-2369.

Matsumoto, A., A.E. Yeo, J.W. Shih, E. Tanaka, K. Kiyosawa, & H.J. Alter. 1999. Transfusion-associated TT Virus Infection and its relationship to liver disease. *Hepatology* **30:**283-288.

Maynard, J.E., M.A. Kane, & S.C. Hadler. 1989. Global control of hepatitis B through vaccination: Role of hepatitis B vaccine in the Expanded Programme on Immunization. *Reviews in Infectious Diseases* **11(Suppl 3):**s574-578.

McClure, J.E. 1992. Cellular receptor for Epstein-Barr virus. *Progress Medical Virology* **39:**116-138.

McNeill, W.H. 1977. In *Plagues and peoples*. Doubleday,New York.

Mehdi, H., M.J. Kaplan, F.Y. Anlar, X. Yang, R. Bayer, K. Sutherland, & M.E. Peeples. 1994. Hepatitis B virus surface antigen binds to apolipoprotein H.

Journal of Virology **68**:2415-2424.

Mettenleiter, T.C., L. Zsak, F. Zuckerman, N. Sugg, H. Kern, & T. Ben-Porat. 1990. Interaction of glycoprotein gII with a cellular heparin-like substance mediates adsorption of pseudorabies virus. *Journal of Virology* **64**:278-286.

Michalak, T.I. 1978. Immune complexes of hepatitis B surface antigen in the pathogenesis of periarteritis nodosa. A study of seven necropsy cases. *American Journal of Pathology*. **90**:619-632.

Michalak, T., K. Krawczynski, J. Ostrowski, & A. Nowoslawski. 1980. Hepatitis B surface antigen and albumin in human hepatocytes. An immunofluorescent and immunoelectron microscopic study. *Gastroenterology* **79**:1151-1158.

Michalak, T. & K. Krawczynski. 1981. Vascular lesions in hepatitis B virus infection, pp. 39-58. In E. Bartoli, L. Chiandussi, & S. Sherlock (eds.), *Systemic effects of HBsAg immune complexes* Piccin Medical Books, Padova, Italy.

Michalak, T.I. & N.D. Churchill. 1988. Interaction of woodchuck hepatitis virus surface antigen with hepatocyte plasma membrane in woodchuck chronic hepatitis. *Hepatology* **8**:499-506.

Michalak, T.I. & G.T. Bolger. 1989. Characterization of the binding sites for glutaraldehyde-polymerized albumin on purified woodchuck hepatocyte plasma membranes. *Gastroenterology* **96**:153-66.

Michalak, T.I., R.L. Snyder, & N.D. Churchill. 1989. Characterization of the incorporation of woodchuck hepatitis virus surface antigen into hepatocyte plasma membrane in woodchuck hepatitis and in the virus-induced hepatocellular carcinoma. *Hepatology* **10**:44-55.

Michalak, T.I., B. Lin, N.D. Churchill, P. Dzwonkowski, & J.R. Desousa. 1990. Hepadnavirus nucleocapsid and surface antigens and the antigen-specific antibodies associated with hepatocyte plasma membranes in experimental woodchuck acute hepatitis. *Laboratory Investigation* **62**:680-689.

Michalak, T.I., J.R.B. Desousa, N.D. Churchill, N.S. Rangraju, & V.S. Ananthanarayanan. 1991. Host and organ specific recognition site within preS1-encoded sequence of woodchuck hepatitis virus envelope, pp. 262-266. In F.B. Hollinger, S.M. Lemon, & H. Margolis (eds.), *Viral Hepatitis and Liver Disease* Williams & Willkins, Baltimore.

Michalak, T. I. & B. Lin. 1994. Molecular species of hepadnavirus core and envelope polypeptides in hepatocyte plasma membrane of woodchucks with acute and chronic viral hepatitis. *Hepatology* **20:**275-286.

Michalak, T.I., C. Pasquinelli, S. Guilhot, & F.V. Chisari. 1994. Hepatitis B virus persistence after recovery from acute viral hepatitis. *Journal of Clinical Investigation.* 93:230-239.

Michalak, T.I., N.D. Churchill, D. Codner, S. Drover, & W.H. Marshall. 1995. Identification of woodchuck class I MHC antigens using monoclonal antibodies. *Tissue Antigens* **45:**333-342.

Michalak, T.I. 1998. The woodchuck animal model of hepatitis B. *Viral Hepatitis Reviews* **4:**139 -165.

Michalak, T.I., I.U. Pardoe, C.S. Coffin, N.D. Churchill, D.S. Freake, P. Smith, C.L. Trelegan. 1999. Occult lifelong persistence of infectious hepadnavirus and residual liver inflammation in woodchucks convalescent from acute viral hepatitis. *Hepatology* **29:**928-938.

Miller, R.H. & W.S. Robinson. 1984. Hepatitis B virus DNA forms in nuclear and cytoplasmic fractions of infected human liver. *Virology* **137:**390-399.

Moraleda, G., J. Saputelli, C.E. Aldrich, D. Averett, L. Condreay, & W.S. Mason. 1997. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. *Journal of Virology* **71:**9392-9399.

Moroy, T., A. Marchio, J. Etiemble, C. Trepo, P. Tiollais, & M.A. Buendia. 1986. Rearrangement and enhanced expression of c-myc in hepatocellular carcinoma of hepatitis virus infected woodchucks. *Nature* **324:**276-2879.

Montgomery, R.I., M.S. Warner, B.J. Lum, & P.G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87:**427-436.

Murphy, F.A. & D.W. Kingsbury. 1990. Virus Taxonomy, pp. 9-35. In B.N. Fields & D.M. Knipe (eds.), *Fields Virology*. Raven Press, New York.

Nagai, Y. 1993. Protease-dependent virus tropism and pathogenicity. *Trends in Microbiology* **1:**81-87.

Nassal, M. & H. Schaller. 1996. Hepatitis B virus replication--an update. *Journal of Viral Hepatitis* **3**:217-226.

Nathanson, N. 1997. Introduction and history, pp.1-11. In Nathanson, N (ed.), *Viral Pathogenesis.* Lippincott-Raven Publishers, Philadelphia.

Neurath, A.R., S.B. Kent, K. Parker, A.M. Prince, N. Strick, B. Brotman, & P. Sproul. 1986a. Antibodies to a synthetic peptide from the preS 120-145 region of the hepatitis B virus envelope are virus neutralizing. *Vaccine* **4**:35-37.

Neurath, A.R., S.B. Kent, N. Strick, & K. Parker. 1986b. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. *Cell* **46**:429-436.

Neurath, A.R., N. Strick, P. Sproul, H.E. Ralph, & J. Valinsky. 1990. Detection of receptors for hepatitis B virus on cells of extrahepatic origin. *Virology* **176**:448-457.

Neurath, A.R. & N. Strick. 1990. Antigenic mimicry of an immunoglobulin A epitope by a hepatitis B virus cell attachment site. *Virology* **178**:631-634.

Neurath, A.R., N. Strick, & P. Sproul. 1992a. Search for hepatitis B virus cell receptors reveals binding sites for interleukin 6 on the virus envelope protein. *Journal of Experimental Medicine* **175**:461-469.

Neurath, A.R., N. Strick, & Y.Y. Li. 1992b. Cells transfected with human interleukin 6 cDNA acquire binding sites for the hepatitis B virus envelope protein. *Journal of Experimental Medicine* **176**:1561-1569.

Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshizawa, Y. Miyakawa, & M. Mayumi. 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochemical Biophysical Research Communications* **241**:92-97.

Nowoslawski, A., K. Krawczynski, T. Nazarewicz, J. Slusarczyk. 1975. Immunopathological aspects of hepatitis type B. *American Journal of Medical Science* **270**:229-39.

Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, J.L. Virelizier, F. Arenza-Seisdedos, O. Schwartz, J.M. Heard, I. Clark-Lewis, D.F. Leghler, M. Loetscher, M. Baggiolini, & B. Moser. 1996. The CXC chemokine SDF-1 is the ligand for

LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**:833-835.

Ochiya, T., T. Tsurimoto, K. Ueda, K. Okubo, M. Shiozawa, & K. Matsubara. 1989. An *in vitro* system for infection with hepatitis B virus that uses primary human fetal hepatocytes. *Proceedings of the National Academy of Sciences of U.S.A.* **86**:1875-1879.

O'Grady, J.G., H.M. Smith, S.E. Davies, H.M. Daniels, P.T. Donaldson, K.C. Tan, B. Portmann, G.J. Alexander, & R. Williams. 1992. Hepatitis B virus reinfection after orthotopic liver transplantation. Serological and clinical implications. *Journal of Hepatology* **14**:104-111.

Okamoto, H., T. Nishizawa, N. Kato, M. Ukita, K. Ikeda, Y. Miyakawa, & M. Mayumi. 1998. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. *Hepatology Research* **10**:1-16.

Oesterreicher, C., J. Hammer, U. Koch, F. Pfeffel, G. Sunder-Plassmann, D. Petermann, & C. Muller. 1995. HBV and HCV genome in peripheral blood mononuclear cells in patients undergoing chronic hemodialysis. *Kidney International* **48**:1967-1971.

Pardoe, I.U. & T.I. Michalak. 1995. Detection of hepatitis B and woodchuck hepatitis viral DNA in plasma and mononuclear cells from heparinized blood by the polymerase chain reaction. *Journal of Virological Methods* **51**:277-288.

Parvaz, P., J.P. Lamelin, L. Vitvitski, P. Bouffard, C. Pichoud, L. Cova, & C. Trepo. 1987. Prevalence and significance of hepatitis B virus antigens: expression in peripheral blood mononuclear cells in chronic active hepatitis. *Clinical Immunology & Immunopathology* **43**:1-8.

Pasquinelli, C., M. Melegari, E. Villa, P.P. Scaglioni, M. Seidenari, N. Mongiardo, B. De Rienzo, & F. Manenti. 1990. Hepatitis B virus infection of peripheral blood mononuclear cells is common in acute and chronic hepatitis. *Journal of Medical Virology* **31**:135-140.

Paulson J.C., J.E. Sadler, & R.L. Hill. 1979. Restoration of specific myxovirus receptors to asialoerythrocytes by incorporation of sialic acidwith pure sialyltransferases. *Journal of Biological Chemistry* **254**:2120-2124.

Peeples, M.E., K. Komai, R. Radek, & M.J. Bankowski. 1987. A cultured cell receptor for the small S protein of hepatitis B virus. *Virology* **160**:135-142.

Peiris, J.S.M., S. Gordon, J.C. Unkeless, & J.S. Porterfield. 1981. Monoclonal anti-Fc receptor IgG blocks antibody enhancement of viral replication in macrophages. *Nature* **289**:189-191.

Petit, M.A., N. Strick, S. Dubanchet, F. Capel, & A.R. Neurath. 1991. Inhibitory activity of monoclonal antibody F35.25 on the interaction between hepatocytes (HepG2 cells) and preS1-specific ligands. *Molecular Immunology* **28**:517-521.

Petit, M.A., F. Capel, S. Dubanchet, & H. Mabit. 1992. PreS1-specific binding proteins as potential receptors for hepatitis B virus in human hepatocytes. *Virology* **187**:211-222.

Pontisso, P., M.C. Poon, P. Tiollais, & C. Brechot. 1984. Detection of hepatitis B virus DNA in mononuclear blood cells. *British Medical Journal (Clinical Research Edition)* **288**:1563-1566.

Pontisso, P., G. Morsica, M.G. Ruvoletto, R. Zambello, C. Colletta, L. Chemello, & A. Alberti. 1991. Hepatitis B virus binds to peripheral blood mononuclear cells via the pre S1 protein. *Journal of Hepatology* **12**:203-206.

Pontisso, P., M.G. Ruvoletto, C. Tiribelli, W.H. Gerlich, A. Ruol, & A. Alberti. 1992. The preS1 domain of hepatitis B virus and IgA cross-react in their binding to the hepatocyte surface. *Journal of General Virology* **73**:2041-2045.

Pringle, C.R. 1998. The universal system of virus taxonomy of the International Committee on Virus Taxonomy (ICTV), including new proposals ratified since publication of the Sixth ICTV Report in 1995. *Archives of Virology* **143**:203-210.

Pugh, J.C. & M.F. Bassendine. 1990. Molecular biology of hepadnavirus replication. *British Medical Bulletin* **46**:329-353.

Pult, I., H.J. Netter, K. Frohlich, E.F. Kaleta, & H. Will. 1998. Identification, structural and functional analysis of a new avian hepadnavirus from storks (STHBV). *Molecular Biology of Hepatitis B viruses* meeting; p. 2 (Abstract).

Purcell, R.H. 1994. Hepatitis viruses: changing patterns of human disease. *Proceedings of the National Academy of Science U.S.A.* **91**:2401-2406.

Reed, W. 1902. Recent researches concerning etiology, propagation and prevention of yellow fever, by the United States Army Commission. *Journal of Hygience* **2**:101-119.

Rehermann, B., C. Ferrari, C. Pasquinelli, &. F. V. Chisari. 1996. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nature Medicine* **2**:1104-1108.

Reyes, G.R., M.A. Purdy, J.P. Kim, K.C. Luk, L.M. Young, K.E. Fry, & D.W. Bradley. 1990. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* **247**:1335-1339.

Randolph V.B. & V. Stollar. 1990. Low pH-induced cell fusion in flavivirus-infected Aedes albopictus cell cultures. *Journal of General Virology* **71**:1845-1850.

Rijntjes, P.J., H.J. Moshage, & S.H. Yap. 1988. *In vitro* infection of primary cultures of cryopreserved adult human hepatocytes with hepatitis B virus. *Virus Research* **10**:95-109.

Rizzetto, M., B. Hoyer, M.G. Canese, J.W.K. Shih, R.H. Purcell, & J.L Gerin. 1980. delta Agent: association of delta antigen with hepatitis B surface antigen and RNA in serum of delta-infected chimpanzees. *Proceedings of the National Academy of Science U.S.A.* **77**:6124-6128.

Roberts, E.A., Z.W. Xie, S. Yang, & J. Lipa. 1993. Inducibility of enzyme activities associated with the cytochrome P-450 1A family, ethoxyresorufin O-deethylase, and methoxyresorufin O-demethylase in human hepatocyte lines derived from normal liver tissue. *Drug Metabolism Disposition* **21**:56-61.

Robinson, W.S. 1990. Hepadnaviridae and their replication, pp. 2137-2169. In B.N. Fields & D.M. Knipe (eds.), *Fields Virology*. Raven Press, New York.

Rogers, G.N., G. Herrier, J.C. Paulson, & H.D. Klenk. 1986. Influenza C virus uses 9-O-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. *Journal of Biological Chemistry* **261**:5947-5951.

Romet-Lemonne, J.L., M.F. McLane, E. Elfassi, W.A. Haseltine, J. Azocar, M. Essex. 1983. Hepatitis B virus infection in cultured human lymphoblastoid cells. *Science* **221**:667-669.

Rous, R. 1911. Transmission of a malignant new growth by means of a cell-free filtrate. *Journal of American Medical Association* **56**:198.

Ruiz-Opazo, N., P.R. Chakraborty, & D.A. Shafritz. 1982. Evidence for supercoiled hepatitis B virus DNA in chimpanzee liver and serum Dane particles: possible implications in persistent HBV infection. *Cell* **29**:129-136.

Rumin, S., P. Gripon, J. Le Seyec, M. Corral-Debrinski, & C. Guguen-Guillouzo. 1996. Long-term productive episomal hepatitis B virus replication in primary cultures of adult human hepatocytes infected *in vitro*. *Journal of Viral Hepatitis* **3**:227-238.

Saito, H., T. Kagawa, S. Tada, S. Tsunematsu, F.M. Guevara, T. Watanabe, T. Morizane, & M. Tsuchiya. 1992. Effect of dexamethasone, dimethylsulfoxide and sodium butyrate on a human hepatoma cell line PLC/PRF/5. *Cancer Biochemistry and Biophysics* **13**:75-84.

Salfeld, J., E. Pfaff, M. Noah, & H. Schaller. 1989. Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. *Journal of Virology* **163**:798-808.

Schaeffer, E., R.L. Snyder, & J.J. Sninsky. 1986. Identification and localization of pre-s-encoded polypeptides from woodchuck and ground squirrel hepatitis viruses. *Journal of Virology* **57**:173-182.

Schlicht, H.J. & H. Schaller. 1989. The secretory core protein of human hepatitis B virus is expressed on the cell surface. *Journal of Virology* **63**:5399-5404.

Seeger, C., J. Summers, & W.S. Mason. 1991. Viral DNA synthesis. *Current Topics in Microbiology and Immunology* **168**:41-60.

Shafren, D.R., D.J. Dorahy, R.A. Ingham, G.F. Burns, & R.D. Barry. 1997. Coxsackievirus A21 binds to decay-accelerating factor but requires intercellular adhesion molecule 1 for cell entry. *Journal of Virology* **71**:4736-4743.

Sherman, M. 1996. The epidemiology of hepatitis B in Canada. *Canadian Journal of Gastroenterology Hepatitis Update* **10**:(issue 5).

Slaney, D. 1999. Evaluation of the expression of selected cellular genes as potential markers of hepatocellular carcinoma. Honors dissertation, Memorial University of Newfoundland, St. John's.

Slusarczyk, J., T. Michalak, T. Nazarewicz-de Mezer, K. Krawczynski, & A. Nowoslawski. 1980. Membranous glomerulopathy associated with hepatitis B core antigen immune complexes in children. *American Journal of Pathology* **98**:29-43.

Sprengel, R., E. F. Kaleta, & H. Will. 1988. Isolation and characterization of a hepatitis B virus endemic in herons. *Journal of Virology* **62**:3832-3839.

Steele, J.H. 1975. History of rabies, pp. 1-32. In: G.M. Baer (ed.), *The natural history of rabies* Academic Press, New York.

Stegmann, T., D. Hoekstra, G. Scherphof, & J. Wilschut. 1985. Kinetics of pH-dependent fusion between influenza virus and liposomes. *Biochemistry* **24**:3107-3113.

Stoll-Becker, S., R. Repp, D. Glebe, S. Schaefer, J. Kreuder, M. Kann, F. Lampert, & W.H. Gerlich. 1997. Transcription of hepatitis B virus in peripheral blood mononuclear cells from persistently infected patients. *Journal of Virology* **71**:5399-5407.

Stein, B.S., S.D. Gowda, J.D. Lifson, R.C. Penhallow, K.G. Bensch, & E.G. Engleman. 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell* **49**:659-668.

Strong, J.E., D. Tang, & P.W. Lee. 1993. Evidence that the epidermal growth factor receptor on host cells confers reovirus infection efficiency. *Virology* **197**:405-411.

Summers, J., J.M. Smolec, & R. Snyder. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proceedings of the National Academy of Science U.S.A.* **75**:4533-4537.

Summers, J. 1981. Three recently described animal virus models for human hepatitis B virus. *Hepatology* **1**:179-183.

Tennant, B. C., N. Mrosovsky, K. McLean, P. J. Cote, B. E. Korba, R. E. Engle, J. L. Gerin, J. Wright, G. R. Michenner, E. Uhl, & J. M. King. 1991. Hepatocellular carcinoma in Richardson's ground squirrel (*Spermophilus richardsonii*): evidence for association with hepatitis B-like virus infection. *Hepatology* **13**:1215-1221.

Testut, P., C.A. Renard, O. Terradillos, L. Vitvitski-Trepo, F. Tekaia, C. Degott, J. Blake, B. Boyer, & M.A. Buendia. 1996. A new hepadnavirus endemic in arctic ground squirrels in Alaska. *Journal of Virology* **70**:4210-4219.

Thung, S.N. & M.A. Gerber. 1983. Albumin binding sites of human hepatocytes. *Liver* **3**:290-294.

Tiollais, P., C. Pourcel, & A. Dejean. 1985. The hepatitis B virus. *Nature* **317**:489-495.

Tong, S., J. Li, & J.R. Wands. 1995. Interaction between duck hepatitis B virus and a 170-kilodalton cellular protein is mediated through a neutralizing epitope of the pre-S region and occurs during viral infection. *Journal of Virology* **69**:7106-7112.

Tong, S., J. Li & J.R. Wands. 1998. DHBV entry into carboxypeptidase D reconstituted cells is followed by the formation of cccDNA inside viral particles. *Molecular Biology of Hepatitis B viruses* meeting; p. 10 (Abstract).

Treichel, U., K.H. Meyer zum Buschenfelde, R.J. Stockert, T. Poralla, & G. Gerken. 1994. The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. *Journal of General Virology* **75**:3021-3029.

Treichel, U., K.H. Meyer zum Buschenfelde, H.P. Dienes, & G. Gerken. 1997. Receptor-mediated entry of hepatitis B virus particles into liver cells. *Archives of Virology* **142**:493-498.

Trevisan, A., F. Gudat, R. Guggenheim, G. Krey, U. Durmuller, G. Luond, M. Duggelin , `J. Landmann, P. Tondelli, & L. Bianchi. 1982. Demonstration of albumin receptors on isolated human hepatocytes by light and scanning electron microscopy. *Hepatology* **2**:832-835.

Trippler, M., H. Hampl, B. Goergen, U. Spies, P. Knolle, B. Grimm, K.H. Meyer zum Buschenfelde, & G. Gerken. 1996. Ligase chain reaction (LCR) assay for semi-quantitative detection of HBV DNA in mononuclear leukocytes of patients with chronic hepatitis B. *Journal of Viral Hepatitis* **3**:267-272.

Tsega, E., B.G. Hansson, K. Krawczynski, & E. Nordenfelt. 1992. Acute sporadic viral hepatitis in Ethiopia: causes, risk factors, and effects on pregnancy. *Clinical Infectious Disease* **14**:961-965.

Tugizov, S., D. Navarro, P. Paz, Y. Wang, I. Qadri, & L. Pereira. 1994. Function of human cytomegalovirus glycoprotein B: syncytium formation in cells constitutively expressing gB is blocked by virus-neutralizing antibodies. *Virology* **201**:263-276.

Turin, F., C. Borel, M. Benchaib, A. Kay, C. Jamard, C. Guguen-Guillouzo, C. Trepo, & O. Hantz. 1996. *n*-Butyrate, a cell cycle blocker, inhibits early amplification of duck hepatitis B virus covalently closed circular DNA after in vitro infection of duck hepatocytes. *Journal of Virology* **70**:2691-2696.

Tur-Kaspa, R & O. Laub. 1990. Corticosteroids stimulate hepatitis B virus DNA, mRNA and protein production in a stable expression system. *Journal of Hepatology* **11**:34-36.

Tuttleman, J.S., J.C. Pugh, & J.W. Summers. 1986a. *In vitro* experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. *Journal of Virology* **58**:17-25.

Tuttleman, J.S., C. Pourcell, & J. Summers. 1986b. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* **47**:451-460.

Urban, S., K.M. Breiner, F. Fehler, U. Klingmuller, & H. Schaller. 1998. Avian hepatitis B virus infection is initiated by the interaction of a distinct pre-S subdomain with the cellular receptor gp180. *Journal of Virology* **72**:8089-8097.

Uy, A., V. Bruss, W.H. Gerlich, H.G. Kochel, & R. Thomsen. 1986. Precore sequence of hepatitis B virus inducing e antigen and membrane association of the viral core protein. *Virology* **155**:89-96.

Walter, E., R. Keist, B. Niederost, I. Pult, & H.E. Blum. 1996. Hepatitis B virus infection of tupaia hepatocytes *in vitro* and *in vivo*. *Hepatology* **24**:1-5.

Wang, G.H. & C. Seeger. 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell* **71**:663-670.

Wang, X.W., K. Forrester, H. Yeh, M.A. Feitelson, J.R. Gu, & C.C. Harris. 1994. Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. *Proceedings of the National Academy of Science U.S.A.* **91**:2230-2234.

Wang, X.W., M.K. Gibson, W. Vermeulen, H. Yeh, K. Forrester, H.W.

Sturzbecher, J.H. Hoeijmakers, & C.C. Harris. 1995. Abrogation of p53-induced apoptosis by the hepatitis B virus X gene. *Cancer Research* **55**:6012-6016.

Wei, Y., G. Fourel, A. Ponzetto, M. Silvestro, P. Tiollais, & M-A. Buendia. 1992. Hepadnavirus integration: mechanisms of activation of the N-*myc*2 retroposon in woodchuck liver tumours. *Journal of Virology* **66**:5265-5276.

Weis, W., J.H. Brown, S. Cusack, J.C. Paulson, J.J. Skehel, & D.C. Wiley. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* **333**:426-431.

Werner, B.G., J.M. Smolec, R. Snyder, & J. Summers. 1979. Serological relationship of woodchuck hepatitis virus to human hepatitis B virus. *Journal of Virology* **32**:314-322.

Wu, T.T., L. Coates, C.E. Aldrich, J. Summers, & W.S. Mason. 1990. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. *Virology* **175**:255-261.

WuDunn, D. & P.G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *Journal of Virology* **63**:52-58.

Yamamoto, T., K. Kajino, M. Ogawa, I. Gotoh, S. Matsuoka, K. Suzuki, M. Moriyama, H. Okubo, M. Kudo, Y. Arakawa, & O. Hino. 1998. Hepatocellular carcinomas infected with the novel TT DNA virus lack viral integration. *Biochemical Biophysical Research Communications* **251**:339-343.

Yeh, C.T., H.T. Chiu, C.M. Chu, & Y.F. Liaw. 1998. G1 phase dependent nuclear localization of relaxed-circular hepatitis B virus DNA and aphidicolin-induced accumulation of covalently closed circular DNA. *Journal of Medical Virology* **55**:42-50.

Yoffe, B., C.A. Noonan, J.L Melnick, & F.B. Hollinger. 1986. Hepatitis B virus DNA in mononuclear cells and analysis of cell subsets for the presence of replicative intermediates of viral DNA. *Journal of Infectious Disease* **153**:471-477.

Yotsuyanagi, H., K. Yasuda, S. Iino, K. Moriya, Y. Shintani, H. Fujie, T. Tsutsumi, S. Kimura, & K. Koike. 1998. Persistent viremia after recovery from self-limited acute hepatitis B. *Hepatology* **27**:1377-1382.

Zoulim, F., L. Vitvitski, P. Bouffard, C. Pichoud, P. Rougier, J.P. Lamelin, C.

Trepo. 1991. Detection of pre-S1 proteins in peripheral blood mononuclear cells from patients with HBV infection. *Journal of Hepatology* **12**:150-156.