



UNIVERSITI PUTRA MALAYSIA

**ESTABLISHMENT OF AN IN VITRO CELL CULTURE SYSTEM FOR
HUMAN HEPATITIS B VIRUS**

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**ESTABLISHMENT OF AN *IN VITRO* CELL CULTURE SYSTEM FOR
HUMAN HEPATITIS B VIRUS**

By

WANG SUK MEI

**Thesis Submitted in Fulfilment of the Requirement for the Degree of
Master of Science in the Faculty of Medicine and Health Sciences
Universiti Putra Malaysia**

July 2001



Specially dedicated to,

My beloved parents , brothers, sisters, husband and friends

for their invaluable love, support, patience and understanding.....

Abstract of thesis presented to the Senate Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

ESTABLISHMENT OF AN *IN VITRO* CELL CULTURE SYSTEM FOR HUMAN HEPATITIS B VIRUS

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July 2001

Chairman : Associate Professor Dr. Seow Heng Fong

Faculty : Medicine and Health Sciences

The major obstacle in the study of HBV has been the inability to infect either animal model system *in vivo* or continuous cell lines *in vitro* due to strict virus-host range and preferential attachment of the HBV envelope proteins onto the plasma membrane of human hepatocytes. The direct involvement of human annexin-V, a calcium dependent phospholipid - binding protein in the initial step of HBV infection has been reported. Over the last decade, various culture conditions with exogenous soluble factors have also been exploited to enhance HBV infection *in vitro*.

The aim of this study was to establish an *in vitro* cell culture system that would continuously produce sufficient HBV by episomal replication. To facilitate the penetration and internalization of HBV, the expression of annexin V (AV) was

enhanced using dexamethasone and by transfection of two liver cell lines, Chang liver and HepG2 cells. In addition, culture conditions containing (i) glucose supplement and dimethylsulphoxide (DMSO) (ii) glucose supplement and liver extract, (iii) glucose supplement, DMSO and liver extract, (iv) glucose supplement only and (v) human interleukin-6 (hIL-6) without glucose supplement, were used to determine which condition was the best for HBV infection *in vitro*.

The results indicated that 10^{-6} M dexamethasone did not significantly upregulate annexin V mRNA expression in both Chang and HepG2 cell lines and Chang cells had higher basal levels of annexin V mRNA. The Chang cell line was more susceptible to HBV infection than HepG2, before and after transfection with human annexin V (hAV), as well as in most of the culture conditions. Only culture conditions containing: (i) glucose supplement, DMSO and liver extract and (ii) glucose supplement and DMSO, inhibited the expression of viral protein probably either at transcription or translation level, without affecting the appearance of viral covalently closed circular (ccc) DNA. Glucose supplement in the presence of liver extract, and hIL-6 (without glucose supplement) supported HBV replication and enhanced viral protein expression.

The establishment of an *in vitro* cell culture system for HBV infection is very important because it is an essential tool for the development of new antiviral strategies against hepatitis B virus infection and for studying the molecular events in viral replication.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

**PENUBUHAN SISTEM SEL KULTUR SECARA *IN VITRO* UNTUK
VIRUS HEPATITIS B**

Oleh

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Penghindaran yang utama dalam penyelidikan HBV ialah ketidakupayaan menjangkiti sistem model haiwan secara *in vivo* ataupun jujukan sel berterusan secara *in vitro* disebabkan oleh lingkaran yang sempit bagi interaksi di antara virus dengan perumah serta kecenderungan perlekatan protein membran luar HBV pada membran plasma hepatosit manusia. Pembabitan annexin-V manusia, sesuatu protein-fosfolipid perlekatan yang bergantung kepada kalsium secara langsung dalam langkah awal infeksi HBV telah dilaporkan. Di sepanjang abad yang lalu, pelbagai keadaan kultur dengan faktor-faktor luaran yang terlarut telah digunakan untuk meningkatkan infeksi HBV secara *in vitro*.

Objektif utama penyelidikan ini ialah untuk menubuhkan sebuah sistem kultur sel secara *in vitro* yang akan dapat menghasilkan HBV yang mencukupi secara bereplikasi episomal. Untuk membantu penembusan dan kemasukan HBV,

ekspresi annexin-V telah dipertingkatkan dengan menggunakan dexamethasone dan transfeksi dua jujukan sel hati, sel Chang dan HepG2. Di samping itu, keadaan kultur yang mengandungi (i) glukos tambahan dan dimetilsulfoksida (DMSO), (ii) glukos tambahan dan ekstrak hati, (iii) glukos tambahan, DMSO dan ekstrak hati, (iv) glukos tambahan sahaja serta (v) interleukin 6 manusia tanpa glukos tambahan, digunakan untuk memastikan yang mana keadaan adalah paling baik untuk infeksi HBV secara *in vitro*.

Keputusan menunjukkan 10^{-6} M dexamethasone tidak meningkatkan ekspresi mRNA annexin-V secara bererti (significantly) di dalam kedua-dua jujukan sel HepG2 dan Chang, dan sel Chang mengandungi paras mRNA annexin V yang lebih tinggi. Jujukan sel Chang adalah lebih mudah dijangkiti oleh infeksi HBV daripada HepG2, sebelum dan selepas transfeksi dengan annexin V manusia, serta di dalam kebanyakan keadaan kultur. Hanya keadaan kultur yang mengandungi: (i) glukos tambahan, DMSO dan ekstrak hati dan (ii) glukos tambahan dan DMSO, telah dipamerkan dapat melarang ekspresi protein virus berkemungkinan pada langkah transkripsi atau translasi tanpa mengganggu penimbunan DNA bulat tertutup berkovalen (ccc) virus. Glukos tambahan dengan kehadiran ekstrak hati, dan IL-6 manusia (tanpa glukos tambahan) telah dipamerkan dapat menyokong replikasi HBV dan meningkatkan ekspresi protein virus.

Penubuhan sistem kultur sel secara *in vitro* bagi infeksi HBV adalah sangat penting sebab ianya merupakan alatan yang diperlukan untuk

memperkembangkan strategi antivirus yang baru bagi menentangkan infeksi HBV serta menyelidiki hal-hal molekular dalam replikasi virus.

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I certify that an Examination Committee met on 3rd July 2001 to conduct the final examination of Wang Suk Mei on her Master thesis entitled “Establishment of an *In Vitro* Cell Culture System for Human Hepatitis B Virus” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.


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TABLE OF CONTENTS

| | Page |
|----------------------------|------|
| DEDICATION..... | ii |
| ABSTRACT..... | iii |
| ABSTRAK..... | v |
| ACKNOWLEDGEMENTS..... | viii |
| APPROVAL SHEETS..... | x |
| DECLARATION FORM..... | xii |
| LIST OF TABLES..... | xvi |
| LIST OF FIGURES..... | xvii |
| LIST OF ABBREVIATIONS..... | xix |

CHAPTER

| | | |
|------------|--|-----------|
| I | INTRODUCTION..... | 1 |
| | 1.1 General View..... | 1 |
| | 1.2 Objectives..... | 5 |
| II | LITERATURE REVIEW..... | 7 |
| | 2.1 Classification..... | 7 |
| | 2.2 Viral Genome..... | 8 |
| | 2.3 Intracellular Life Cycle of HBV in Human Hepatocyte..... | 9 |
| | 2.3.1 Attachment of and Entry of HBV..... | 9 |
| | 2.3.2 Nuclear Delivery of Progeny Core DNA..... | 15 |
| | 2.3.3 Maintenance of the Nuclear ccc HBV DNA..... | 16 |
| | 2.3.4 Viral Transcription..... | 18 |
| | 2.3.5 Genome Replication..... | 20 |
| | 2.4 HBV Infection Models..... | 21 |
| | 2.4.1 Models with Non-Human Hepadnaviruses..... | 21 |
| | 2.4.2 Models with Human Hepadnaviruses..... | 25 |
| | 2.5 Human Annexin V..... | 40 |
| | 2.5.1 Overview..... | 40 |
| | 2.5.2 Structural Information..... | 40 |
| | 2.5.3 Biological Effects..... | 41 |
| | 2.5.4 HBV Infection..... | 43 |
| | 2.6 Human Interleukin 6..... | 47 |
| | 2.6.1 Biological Properties..... | 47 |
| | 2.6.2 HBV Infection..... | 48 |
| | 2.7 Antiviral Therapy of Chronic HBV Infection..... | 50 |
| | 2.7.1 Interferon Alpha and Other Cytokines Therapy..... | 51 |
| | 2.7.2 Nucleoside Analogues..... | 52 |
| III | MATERIALS AND METHODS..... | 56 |
| | 3.1 Cell Cultures..... | 56 |
| | 3.1.1 Cell Culture Media..... | 56 |



| | | |
|-----------|--|-----------|
| 3.1.2 | Growing, Freezing and Retrieval of Cells..... | 57 |
| 3.1.3 | Dexamethasone Treatment..... | 58 |
| 3.2 | RNA Extraction..... | 58 |
| 3.3 | Reverse Transcription Polymerase Chain Reaction (RT-PCR)..... | 59 |
| 3.4 | Semiquantitative Analysis of Human AV mRNA Expression..... | 61 |
| 3.5 | Plasmids Construction..... | 62 |
| 3.5.1 | Cloning of Annexin V into pTrcHis2-TOPO Vector..... | 62 |
| 3.5.2 | Cloning of Annexin V into pTARGET™ Vector..... | 62 |
| 3.5.3 | Plasmid DNA Extraction (Alkaline Lysis)..... | 63 |
| 3.5.4 | Identification of the Right Orientation of the Constructs..... | 64 |
| 3.6 | Expression of Recombinant Human Annexin V..... | 65 |
| 3.7 | Affinity Purification of Recombinant Annexin V protein..... | 66 |
| 3.8 | Purification of Plasmid DNA for Transfection..... | 67 |
| 3.9 | Transfection with Constructs Containing hAV..... | 69 |
| 3.10 | <i>In Vitro</i> HBV Infection..... | 70 |
| 3.11 | Viral DNA Extraction and Purification..... | 71 |
| 3.12 | PCR Amplification for Detection of HBV DNA and ccc DNA..... | 71 |
| 3.13 | Indirect Immunofluorescent Assay..... | 72 |
| IV | RESULTS..... | 74 |
| 4.1 | Effects of Dexamethasone on Annexin V mRNA Expression..... | 74 |
| 4.2 | Cloning and Expression of Human Annexin using pTrcHis2-TOPO Vector..... | 79 |
| 4.3 | Cloning of Human Annexin V Gene into pTARGET Vector..... | 85 |
| 4.4 | Affinity Purification of Recombinant Human Annexin V..... | 88 |
| 4.5 | Infectability of Chang Cells and HepG2 with HBV..... | 89 |
| 4.6 | Transfection of Chang Cells with pTARGET Human Annexin V Expression Plasmid (pTARGET.hAV)..... | 93 |
| 4.6.1 | Transfection Efficiency using Two Methods for Purification of Plasmid DNA..... | 93 |
| 4.7 | HBV Infection of Human Annexin V-transfected Chang Cells and Immunofluorescence Detection of Pres2 Protein..... | 96 |
| 4.7.1 | Comparison of HBV Infection In Chang Cells Cultured in PRMI versus MEM..... | 96 |
| 4.7.2 | Comparison of HBV Infection In Chang Cells Transfected with Plasmid DNA Purified by Two Methods..... | 97 |
| 4.8 | PCR Detection of HBV DNA in Infected Chang Cells..... | 100 |
| 4.9 | PCR Amplification Analysis of HBV ccc DNA..... | 103 |
| 4.10 | Immunofluorescence Analysis of PreS2 Protein in Infected Chang Cells..... | 105 |



| | | |
|-----------|--|------------|
| V | DISCUSSION..... | 114 |
| VI | CONCLUSION AND RECOMMEDATION..... | 134 |
| | 6.1 Conclusion..... | 134 |
| | 6.2 Recommendations for Future Work..... | 137 |
| | REFERENCES..... | 140 |
| | APPENDICES..... | 166 |
| | BIODATA OF THE AUTHOR..... | 180 |



LIST OF TABLES

| Table | | Page |
|--------------|---|-------------|
| 3.1 | Quantities of DNA fragments in 0.25µg GeneRuler 100 bp DNA Ladder..... | 61 |
| 4.1 | Data of semiquantitative analysis of Annexin V mRNA and GAPDH mRNA in Chang Cells..... | 77 |
| 4.2 | Summary of the expression of PreS2 protein and ccc DNA in 5 days infected Chang cell in various culture conditions..... | 113 |



LIST OF FIGURES

| Figures | Page |
|---|------|
| 2.1 Genomic organization of hepatitis B virus genome..... | 9 |
| 2.2 Life Cycle of hepatitis B virus | 19 |
| 2.3 Model for binding of human annexin V(hAV) to hepatitis B virus particles..... | 46 |
| 2.4 Mechanism of the interaction between human IL-6 and hepatitis B virus..... | 49 |
| 2.5 Potential targets for antiviral therapy on HBV replicative cycle..... | 51 |
| 2.6 Potential targets for action of lamivudine on HBV replication..... | 54 |
| 4.1 Effects of dexamethasone on human annexin V mRNA Expression in HepG2 and Chang cell lines..... | 75 |
| 4.2(a) Semiquantitative analysis of annexin V and GAPDH mRNA expression profile..... | 78 |
| 4.2(b) Expression of annexin V mRNA in relative to GAPDH mRNA with and without dexamethasone treatment..... | 78 |
| 4.3(a) SDS-PAGE analysis of protein expressed in pTrcHis2-TOPO clones after several time points of induction..... | 80 |
| 4.3(b) SDS-PAGE analysis of protein expressed in pTrcHis2-TOPO clones after 4 hours induction..... | 80 |
| 4.4 Restriction enzyme analysis of pTrcHis2-TOPO clones..... | 82 |
| 4.5(a) BLAST analysis of the sequence of the insert in clone G2 compared to the published annexin V sequence..... | 83 |
| 4.5(b) BLAST analysis of the sequence of the insert in clone 2 compared to the published annexin V sequence..... | 84 |
| 4.6(a) PCR analysis of <i>E.coli</i> transformants colonies..... | 86 |
| 4.6(b) Restriction enzyme analysis of pTARGET clones..... | 86 |
| 4.7 Confirmation of correct orientation of pTrcHis-TOPO.AV | |



| | |
|---|-----|
| and pTARGET.AV..... | 87 |
| 4.8(a) SDS-PAGE analysis of crude lysate of pTrcHis2-TOPO.AV culture expressing rhAnxV..... | 89 |
| 4.8(b) SDS-PAGE analysis of affinity-purified recombinant human annexin V..... | 89 |
| 4.9(a) PCR detection of HBV DNA in infected Chang liver and HepG2 cell lines 2 days post-infection..... | 91 |
| 4.9(b) PCR detection of HBV DNA in infected Chang liver and HepG2 5 days after infection..... | 92 |
| 4.10 Immunofluorescence detection of human annexin V in Chang cells..... | 95 |
| 4.11 Comparison of PreS2 expression in annexin V-transfected Chang cells cultured in RPMI versus MEM..... | 98 |
| 4.12 Comparison of HBV infection in Chang cells transfected with plasmid DNA purified by two methods..... | 99 |
| 4.13 PCR detection of HBV PreS2 DNA extracted from Chang liver cells cultured in MEM 2 and 5 days after infection..... | 102 |
| 4.14 PCR detection of intracellular HBV ccc DNA extracted from Chang cells cultured in MEM 2 and 5 days after infection..... | 104 |
| 4.15 Immunofluorescence detection of PreS2 in negative and positive control..... | 107 |
| 4.16 Immunofluorescence detection of PreS2 in Chang cells 2 and 5 days after infection..... | 108 |
| 4.17 Immunofluorescence detection of PreS2 in Chang cells in the presence of DMSO 2 and 5 days after infection..... | 109 |
| 4.18 Immunofluorescence detection of PreS2 in Chang cells In the presence of liver extract 2 and 5 days after infection..... | 110 |
| 4.19 Immunofluorescence detection of PreS2 in Chang cells in the presence of DMSO and liver extract 2 and 5 days after infection..... | 111 |
| 4.20 Immunofluorescence detection of PreS2 in Chang cells in the presence of human IL-6 at 2 and 5 days after infection..... | 112 |



LIST OF ABBREVIATIONS

| | |
|--------------------|---|
| ε | 5' epsilon loop of HBV pregenome |
| μ | micro |
| AMP | Adenosine monophosphate |
| Apo H | Apolipoprotein H |
| AV | Annexin V |
| bp | base pair |
| BSA | Bovine serum albumin |
| C | Core protein |
| CaCl ₂ | Calcium chloride |
| cAMP | Cyclic adenosine monophosphate |
| ccc DNA | Covalently closed circular DNA |
| CD | Cluster designation |
| cDNA | complementary DNA |
| ChHBV | Chimpanzee hepatitis B virus |
| CO ₂ | carbon dioxide |
| CTLs | Cytotoxic T lymphocytes |
| d | day(s) |
| ddH ₂ O | double-distilled water |
| DEPC | Diethyl pyrocarbonate |
| Dex | Dexamethasone |
| DHBcAg | Duck hepatitis B core antigen |
| DHBsAg | Duck hepatitis B surface antigen |
| DHBV | Duck hepatitis B virus |
| dl | Double stranded linear |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTP(s) | dideoxynucleotide triphosphate(s) (dATP, dTTP, dCTP and dGTP) |
| ds DNA | double stranded DNA |
| <i>E. coli</i> | Escherichia Coli |
| EDTA | diaminoethanetetra-acetic acid disodium salt |
| Enh | Enhancer |
| ER | endoplasmic reticulum |
| FBS | Foetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| g | gram |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| gp | glycoprotein |
| gp 180 | Glycoprotein 180 |
| GRE | glucocorticoids responding element |
| GRP94 | Glucose regulated protein 94 |
| GSHV | Ground squirrel hepatitis B virus |



| | |
|---------------------|---|
| h | hour(s) |
| hAV | Human annexin V |
| HBcAg | hepatitis B core antigen |
| HBsAg | hepatitis B e antigen |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |
| HCl | hydrochloric acid |
| HFF | Human foreskin fibroblasts cells |
| hGR | human glucocorticoid receptor |
| HHBV | Heron hepatitis B virus |
| hIL-6 | Human interleukin 6 |
| hIL-6R α | human interleukin 6 receptor alpha |
| hIL-6R β | human interleukin 6 receptor beta |
| hPXR | human pregnane X receptor |
| Hsp70 | Heat shock protein 70 |
| IFN- γ | Interferon-gamma |
| IFN- α/β | Interferon-alpha or beta |
| IFN- α | Interferon-alpha |
| IL-1 β | Interleukin-1 beta |
| IPTG | Isopropylthio- β -D-galactosidase |
| kb | kilobase |
| kDa | kilodaltons |
| L | large protein |
| LB | Luria-Bertani medium |
| M | Molar or middle protein |
| mA | milliamperes |
| MEM | Minimal essential medium |
| mg | milligram |
| MgCl ₂ | Magnesium chloride |
| MHC | Major histocompatibility complex |
| min | minute(s) |
| ml | millitre |
| mM | millimolar |
| MMLV | Moloney Murine Leukaemia Virus |
| mRNA | messenger ribonucleic acid |
| n | nano |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| NF-IL6 | Nuclear factor interleukin 6 |
| NLS | nuclear localization signal |
| NPC | Nonparenchymal cells |
| $^{\circ}$ C | degrees Centigrade |
| ORF | open reading frame |
| P/ Pol | Polymerase protein |
| p.i. | post-infection |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---------------|--|
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| PEG | Polyethylene glycol |
| pg | picogram |
| pHSA | Polymerised human serum albumin |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PreS | Hepatitis B PreS gene |
| PreS2 | Hepatitis B PreS2 gene |
| rcDNA | Relaxed circular DNA |
| rhAV | recombinant human annexin V |
| RNA | Ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| RT | reverse transcription or reverse transcriptase |
| s | seconds |
| S | Surface or small protein |
| SCID | severe combined immunodeficiency disease |
| SDS | Sodium dodecyl phosphate |
| SFM | Serum free medium |
| SHBsAg | Small hepatitis B surface antigen |
| ss DNA | Single stranded DNA |
| TAE | Tris acetate EDTA buffer |
| Taq | Thermus aquaticus thermostable DNA |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TNF- α | Tumour necrosis factor alpha |
| v/v | volume per unit volume |
| WHV | Woodchuck hepatitis B virus |

CHAPTER I

INTRODUCTION

1.1 General View

Hepatitis B virus (HBV), a small DNA virus that replicates via reverse transcription, causes acute and/or chronic hepatitis and chronically infected individuals are at high risk for developing hepatocellular carcinoma (HCC), one of the commonest malignancies in the world (Okuda, 2000). It has been estimated that more than 2 billion people today have been infected with the hepatitis B virus (HBV) (De Meyer *et al.*, 1997) and about 350 million are hepatitis B surface antigen (HBsAg) carriers, of whom over 250 000 die annually from hepatitis B associated liver disease (Lok, 2000).

The standard treatment with interferon-alpha (IFN- α) and other cytokines such as thymosin α -1 are not adequate to interfere with HBV replication as HBV covalently closed circular (ccc) DNA is not eliminated (Locarnini and Birch, 1999). Only 10% to 20% of chronically HBV infected patients with reduction in viral load (Andreas *et al.*, 1999) and limitation of dose-dependent side effects (Carithers, 1998) are observed during interferon-alpha therapy. Newer antiviral therapies using nucleoside analogues such as lamivudine, famciclovir, adefovir and lobucavir are unsatisfactory because short-term therapy is insufficient to clear viral infection but virus rebounds rapidly upon drug withdrawal, and long-term

monotherapy has been associated with resistant mutants due to the mutations in the HBV polymerase (Lai and Yuen, 2000).

Only humans, chimpanzees, gibbons, certain macaca are so far known to be susceptible to HBV infection (Walter *et al.*, 1996). Recently tree shrews (tupaia) have also been shown to be susceptible to HBV infection but with low efficiency (Ren and Nassal, 2001). The predominant site of clinical pathology for hepatitis B virus is the liver. The restricted host and tissue susceptibility of HBV infection suggests the existence of host and liver-specific viral regulatory elements for HBV replication. However, the major obstacle in the study of HBV has been the lack of an *in vitro* propagation system and the lack of animal model systems due to strict virus-host range, restraining the overall progress towards the understanding of the mechanisms that dictate the biological and tissue-tropic aspects of the HBV infection cycle.

Many aspects of HBV biology including HBV life cycle have been unravelled by studying related hepadnaviruses, such as the duck hepatitis B virus which is capable of *in vitro* infection, and the woodchuck hepatitis B virus which allows for the *in vivo* study in an animal model system. However, woodchuck hepatitis B virus and duck hepatitis B virus, are either difficult to keep or significantly different from HBV. For example, avian hepatitis B virus does not encode the X gene, the duck HBV receptor (Tong *et al.*, 1999) and coreceptor (Li *et al.*, 1999) is probably different, and the transcriptional events of woodchuck hepatitis B virus is distantly related to HBV. Convenient small animal models are

not available because rats and mice are not infectable by HBV. However, cross-species replication of HBV has been obtained in hepatocytes of transgenic mice (Guidotti *et al* , 1995) and trimera mice (Pan *et al* , 1999)

Within the last two decades, most of the *in vitro* studies of HBV replication have been performed by transfecting HBV genome into human hepatoblastoma cell line HepG2 (Sells *et al* , 1987), Huh-7 (Yaginuma *et al* , 1987) and rat hepatoma cell lines (Shih *et al* , 1989). The most widely used are the HepG2 2.2.15 cell line (Sells *et al* , 1987) derived from HepG2 hepatoblastoma cell line and HB611 derived from the HuH6 hepatoma cell line. However, there are several drawbacks which preclude the use of these cell lines in studying some aspects of HBV biology. These drawbacks are (1) Transfection with HBV genome into the cell uses constructs containing strong heterologous promoters. Viral production in this system is mainly the result of chromosomal replication and does not mimic the natural infection *in vivo* in which HBV gene expression is driven solely by endogenous HBV promoters. (2) HBV expressing cell lines contain multiple copies of integrated HBV DNA which is not an obligatory part of the HBV lifecycle and is not required for HBV replication. HBV genomes are not integrated routinely but are maintained as a pool of episomal ccc DNA molecules in the nucleus of infected cells *in vivo*. (3) HBV gene expression and replication cannot be regulated and are restricted to the fixed number of integrated HBV genomes in stable HBV expressing cell lines.

