CHAPTER III

MATERIALS AND METHODS

Study population

One hundred and one captive gibbons are kept at the Krabok Koo Wildlife Breeding Center, Royal Forest Department, Cha-Cheng-sao, Thailand. Most of them were born in the wild and all were examined in good health. The population consisted of 52 males and 49 females ranging from one to 21 years old. Gibbons were identified as *Hylobates lar* (white-handed gibbons), *H. pileatus* (pileated gibbons) and *H. agilis* (black-handed gibbon) which have their habitat in Thailand. Another species that can be found in North Vietnam and central China, *Nomascus concolar* (Tonkin black crested gibbon), was also included in the study. Two of white-handed gibbons had their offspring in house aged one and two years, respectively. General description of each animals including name, species, microchip number, cage, sex, age and color were recorded.

The 34 animal keepers of Krabok Koo Center participated to the HBV pre-vaccination screening program. They consisted of 20 males and 14 females with age ranging from 18 to 59 years.

HBV positive sera of human were obtained from the serum bank at the Viral Hepatitis research unit, Chulalongkorn University, Thailand.

Materials

1. Cell lines : Cos-7, Hep2, NKNT-3, CHO and HepG2 (kindly provided by Dr. Bart L. Haagmans)
2. *E.coli* INVαF' (Invitrogen, USA).
3. pCR2.1 vector (TA cloning® kit, Invitrogen, USA)
4. VR 1012 mammalian expression vector (Vical Incorporated, USA)
5. Antibodies and anti-HBs (kindly provided by Dr. R.A. Heijtink)

6. Enzyme immunoassay (EIA) Kit (DiaSorin s.r.l., Italy) for detecting :
   HBsAg (ETI-MAK-3)
   anti-HBs (ETI-AB-AUK-3)
   anti-HBc (ETI-AB-COREK-2)
7. Enzyme immunoassay (EIA) Kit (Abbott Laboratories, USA) for detecting :
   HBeAg (HBeAg IMX)
   anti-HBs (AUSAB)
   anti-HBc (CORZYME®)
8. HBsAg ELISA test kit (Monolisa®Ag HBs plus, Biorad, USA).
9. Blood collecting tube (Vacutainer®, Becton Dickinson, USA)
10. Saliva OraSure®Collection System (Orasure Technologie Inc., USA)
11. PCR purification kit (QIAquick®, Qiagen, Germany)
12. StrataPrep EF Plasmid Midiprep kit (Stratagene, USA)
13. PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, USA)
14. FuGENE™ 6 transfection reagent (Boehringer Mannheim,Germany)
15. Lipo-fectamine™ 2000 Reagent (Gibco BRL, USA)
16. MicroAmp PCR tube (Perkin Elemer, USA)
17. Microcentrifuge tube : 0.5 and 1.5 ml. (AxyGen®Scientific, USA)
18. Polypropylene conical tube : 50 and 15 ml. (AxyGen®Scientific, USA)
19. Pipette tip : 10 µl, 200 µl and 1000 µl (AxyGen®Scientific, USA)
20. Hematocrit capillary tube
21. Microscope slide and cover slit (Sail brand, China)
22. Glassware : Beaker, Flask , Cylinder and reagent bottles (Pyrex, USA)
23. Tissue Culture Flask, Culture plate, Sterile pipett 10, 5 and 1 ml. and High binding EIA/RIA 96-well flat bottom plates (Costar®, USA)
24. Cell strainer 100 µM nylon and polystyrene plate (Becton Dickinson, USA)

Equipments

1. Centrifuge (Beckman GS-6R, USA)
2. Refrigerated microcentrifuge (Universal 16R Hettich, USA)
3. – 70 °C freezer (Forma Scientific, USA)
4. – 20 °C freezer (Philco, USA)
5. Blood chemistry automated analyzer (912 Hitachi , Japan).
6. Light microscopy (Nikon Y52, Japan)
7. ELISA reader plate (GLR1000, Genelabs Diagnostics, USA)
8. DNA Thermal Cycler 9600 (Perkin Elmer, USA)
9. Gel electrophoresis set and power supply (Power PAC300 Biorad, USA)
10. Gel Doc 1000 (Biorad, USA)
11. Mitsubishi video copy processor (Mitsubishi, Japan)
12. SDS electrophoresis set (Biorad, USA)
13. Ultrahigh speed centrifugation (55p-72 HIMAC Centrifuge Hitachi, Japan)
14. Transmission electron microscope (JEOL MODEL JEM-1010, Tokyo Japan)
15. Spectrophotometry (Shimadzu UV-160A, Japan)
16. Perkin-Elmer 310 Sequencer (PE Biosystems, USA)
17. CO_2 incubator (TC2323 Shellab, USA)
18. FACScan analyzer (Becton Dickinson, USA)
19. Autoclave (Hydroclave MC10 Harvey, USA)
20. Hot air oven (Memmert, West Germany)
21. Multi-block heater (Lab-line, USA)
22. Balance (PB1502 Mettler Toledo, Switzerland)

Reagents

1. Proteinase K (Sigma, USA).
2. Phenol (Sigma, USA)
3. Chloroform (Merck, USA)
4. Isoamyl alcohol (Merck, USA)
5. Sodium acetate (Sigma, USA)

6. Absolute ethanol (Merck, USA)
7. Isopropanol (Merck, USA)
8. Reagents for PCR analysis

   10x PCR buffer (Finnzymes, Finland)

   Deoxynucleotide triphosphate (dNTPs) (Promega, USA)

   Taq DNA polymerase (DyNAzyme™ II DNA Polymerase, Finnzymes, Finland)
9. Restriction enzymes : *Ava* II , *Dpn* II and *Sau3A* I (New England Biolabs, USA)
10. Agarose gel (FMC Bioproducts, USA)
11. NuSieve agarose (FMC Bioproducts, USA)
12. Ethidium bromide (Sigma, USA)
13. Template suppression reagent (TSR) (Applied Biosystem, USA)
14. Polyethylene glycol 6000 (PEG-6000) (Fluka, USA)
15. Polyethylene glycol 8000 (PEG-8000) (Sigma, USA)
16. $CsCl_2$ (Sigma, USA)
17. Uranyl acetate (Sigma, USA)
18. X-gal (Biobasic Inc., Germany)
19. IPTG and ampicillin (Biobasic Inc., Germany)
20. Streptavidine (Amersham, Pharmacia Biotech, USA)
21. 3,3',5,5'-tetramethyl-benzidene (TMB) substrate (Amersham, Pharmacia Biotech, USA)
22. Amino-9-ethycarbazole (AEC) (Sigma, USA)
23. BioTrace® NT pure nitrocellulose blotting membrane (Gelman Lab. , USA)
24. Skim milk (Mission, Thailand)
25. Anti-human Ig – biotin/avidin HRP (Amersham, USA)
26. 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate (BCIP/NBT) (Biorad, USA)
27. RPMI medium and DMEM medium (Gibco BRL, USA)
28. Williams' E medium (Gibco BRL, USA)
29. Fetal bovine serum (Gibco BRL, USA)
30. Glutamine (Gibco BRL, USA)

31. Dexamethasone (Sigma, USA)
32. Insulin (Gibco BRL, USA)
33. Epidermal growth factor (Gibco BRL, USA)
34. Human growth factor (Gibco BRL, USA)
35. Normal goat serum (Gibco BRL, USA)
36. Penicillin/ Streptomycin (Gibco BRL, USA)
37. Geneticin G418 (Sigma, USA)
38. Collagenase (Sigma, USA)
39. Dnase (Gibco BRL, USA)
40. Dimethysulfoxide (DMSO) (Sigma, USA)
41. Trypsin (Gibco BRL, USA)
42. Glycine (Merck, USA)
43. Trypan blue (Sigma, USA)
44. Cell dissociation solution (1X) (Sigma, USA)
45. H_2SO_4 (Merck, USA)
46. H_2O_2 (Sigma, USA)
47. Hepes (Merck, USA)
48. NaCl (Sigma, USA)
49. KCl (Sigma, USA)
50. Na_2HPO_4 (Merck, USA)
51. Glucose (Sigma, USA)
52. EGTA (Sigma, USA)
53. $CaCl_2$ (Merck, USA)

Software and program for phylogenetic analysis

1. Clustal X program, version 1.4
2. PHYLIP package, version 3.57c (J. Felsenstein, Department of Genetics, University of Washington) : SEQBOOT program, DNADIST, NEIGHBOR and CONSENSE software
3. TREEVIEW program, version 1.5

Methods

1. Blood sample collection

Blood was obtained from gibbons by venepuncture during a brief period of ketamine anesthesia during the routine health care program. Samples were kept in blood collecting tube and sera were separated from clotted blood within 24 hours by centrifugation at 1,500 rpm for 10 minutes. Serum was kept in individual microcentrifuge tubes and stored at –70 °C until further analysis. Human sera from workers of Krabok Koo center were collected by the same procedure.

2. Saliva sample collection

Gibbon saliva was collected from 30 gibbons by OraSure® collection system following the specimen collection procedure. Briefly, saliva was collected by place the pad into the mouth between the lower cheek and gum. Stroked the pad back and forth a few times until moisture, then hold the pad in place for two minutes with the mouth closed. The pad was removed from the mouth and inserted into the preservative solution in collecting vial. To remove the specimen form the vial, the vial tip was bended over to snap off and placed into 15 ml. centrifuge tube. Centrifugation at 2,500 rpm for 15 minutes was performed to extract the pad contents into the new tube. Saliva samples were kept at –70 °C for further study.

3. Gibbon blood chemical analysis

To evaluate the health status of gibbons in Krabok Koo Center, 200 µl fresh sera of 40 random animals consisted of 12 HBV carriers and 28 normal cases were sent to Central Laboratory, Chulalongkorn University, King Chulalongkorn Memorial Hospital for clinical blood chemistry measurement including total protein, albumin, globulin and creatinine by automated analyzer. All results were compared to the standard range of normal human sample.

The level of alanine-aminotransferase (ALT) was determined by routine laboratory procedure. The correlation of ALT level and HBV infection stage was investigated. Statistical analysis was performed using t-test method and significant difference was considered if the *p* value was less than 0.05.

Percent hematocrit was estimated by centrifugation of blood capillary tube at 2000 round per minute (rpm) for 10 minutes. Total white blood cells were counted after treated with 3% glacial acetic acid and plate on Neubauer hemocytometer. Neutrophil, lymphocyte, eosinophil and basophil were identified from blood smear stained with Wright-Giemsa stain and counted under light microscopy. Chi-square test was calculated to define the relation of complete blood count and HBV infection stage.

4. HBV detection

4.1 Serological method

All sera were determined for HBsAg, anti-HBs and anti-HBc by enzyme- linked immunosorbent assay (ELISA) using a commercially available kit of DiaSorin Inc. (Voricelli, Italy) according to the manufacturer's instructions. Duplicative analysis were tested every samples. Cut off value was calculated following the recommendation of each test..

HBV seropositivity of samples was confirmed for anti-HBs, anti-HBc and HBeAg by using ELISA commercial kits from Abbott Laboratories, different from initial screening kits.

4.2 HBV DNA amplification method

4.2.1 DNA extraction

DNA was extracted from serum or saliva samples according to a previously method [101] . Two hundred μl of each sample was mixed with 100 μl lysis buffer and 20 mg/ml proteinase K (Appendix A). After incubation at 37°C for 1 hour, sample was extracted twice by phenol:chloroform:isoamyl alcohol (25:24:1) mixture and precipitated by adding 3.0 M sodium acetate and 2 volume of absolute ethanol. The mixture was kept at –20°C for at least 3 hours to allow DNA to precipitate. Sedimentation of DNA was performed by centrifugation at 12,000 rpm for 20 minutes at 4°C. DNA pellet was washed twice with 1 ml of precooled 70% ethanol. The supernatant was discarded and DNA pellet was allowed to air dry. The pellet was dissolved in 20 μl of sterile water and directly subjected to the polymerase chain reaction (PCR) method.

4.2.2 HBV DNA amplification and detection

HBV gene was amplified and detected by the PCR method. In this study, several regions of HBV genome were amplified with different primer sets. The details and sequences of primers are described in Table 2. Primers were synthesized and aliquoted by Biosynthesis inc., USA.

PCR reaction was performed following the standard condition. In general, 5 μl extracted DNA was added into the reaction tube containing 10 μl of 10x PCR buffer, 200 μM of each of dNTPs, 20 pmole of each forward and reverse primers and 1 unit of *Taq* DNA polymerase. Sterile distilled water was added to bring the final volume to 100 μl. The reaction tube was placed into DNA Thermal Cycler 9600. The PCR cycle was specific for each HBV amplified region.

After the complete PCR, ten microlitres of PCR-amplified product was throughly mixed with 6x loading buffer and loaded into the submarine 2% agarose gel (w/v) prepared in 1X Tris-borate buffer (TBE, Appendix A). Electrophoresis was carried out at constant current of 100 mA until the bromophenol blue dye reached about 1 cm. from the lower edge of the gel. The agarose gel was stained with ethidium bromide and then visualized under ultraviolet (UV) light. The gel was photographed using Gel Doc 1000.

4.2.2.1 HBV surface gene amplification

The nested PCR of *S* gene was amplified for HBV DNA screening by 2 sets of primers [102]. Primers F1 and R6 were used for the first amplification round and primers F2 and R5 in the second, respectively (Table 2). The amplification cycle required 30 cycles comprising initiation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C 1 minute, concluded by a final extension step at 72°C for 10 minutes. The expected PCR product is 1,038 bps.

4.2.2.2 *PreS1/PreS2* gene amplification

PreS1/PreS2 region was amplified for HBV genotyping analysis using primer P1 and P2 as indicated in Table 2. The PCR cycle consisted of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C, followed by 30 cycles, each of 30 seconds at 94°C (denaturation), 30 seconds at 55°C (annealing) and 1 minute at 72°C (extension), and then one cycle of 1 minute at 94°C, 2 minutes at 55°C and 10 minutes at 72°C. The 479

bps of amplified product was used as template for genotype analysis and DNA sequencing, respectively.

4.2.2.3 X gene amplification

To detect the precore/core promoter mutant (T1762/A1764) of HBV genome in this study, the primers were modified from previously reported [103] . Primers Xo1 and PC1 were used for the first amplification and primers Xi1 and Xi3 (Table 2) for the secondary round. The identical PCR cycles to the PreS1/PreS2 amplification was performed. The 596 bps amplified product was subjected to precore mutation analysis.

4.2.2.4 HBV whole genome amplification

Gibbon HBV whole genome amplification was modified from previous description [57] . Three pairs of primer were used to generate 3 overlapping regions of complete HBV genome (Table 3). The first PCR fragment covered precore/core and a part of polymerase gene, was amplified using primers HBPr108 and HBPr33. The second was amplified by primers HBPr1 and HBPr135 covering the polymerase and surface gene of HBV. The PCR of the last fragment included polymerase, surface and X gene was performed by primers HBPr 440 and HBPr 109, respectively. PCR amplified the HBV DNA over 40 cycles, with denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and elongation at 72°C for 30 seconds. The position and orientation of sequencing primers were showed in Table 3 and Figure 8.

Table 2 Primer sequences for HBV DNA detection and RFLP study.

Primer	Length	sequences (5'->3')	position	usage	amplified gene
F1	21	GGAGCGGGAGCATTCGGGCCA	3022-3042	Forward	*S*
R6	20	GGCGAGAAAGTGAAAGCCTG	1103-1084	Reverse	*S*
F2	22	CATCCTCAGGCCATGCAGTGGA	3192-3214	Forward	*S*
R5	22	AGCCCAAAAGACCCAGAAATTC	1015-995	Reverse	*S*
P1	23	TCACCATATTCTTGGGAACAAGA	2823-2845	Forward	*PreS1*
P2	20	TTCCTGAACTGGAGCCACCA	80-61	Reverse	*PreS1*
Xo1	19	CTCTGCCGATCCATACTGC	1256-1274	Forward	*X*
PC1	18	GGAAAGAAGTCAGAAGGC	1974-1957	Reverse	*X*
Xi1	19	AGCTTGTTTTGCTCGCAGC	1287-1305	Forward	*X*
Xi3	19	GGCACAGCTTGGAGGCTTG	1883-1865	Reverse	*X*

Table 3 Primer sequences for HBV whole genome sequencing (modified from Stuyver *et al.*, 2000) [57]

Primer	Length	sequences (5'->3')	position	usage
HBPr1	19	GGGTCACCATATTCTTGGG	2850-2868	Forward
HBPr3	21	CCACTGCATGGCCTGAGGATG	3226-3246	Reverse
HBPr14	17	TGGGGTGGAGCCCTCAG	3104-3120	Forward
HBPr33	17	CTGAGGGCTCCACCCCA	3104-3120	Reverse
HBPr87	22	TACTTCAAAGACTGTGTGTTTA	1704-1723	Forward
HBPr108	20	TTTTTCACCTCTGCCTAATC	1821-1840	Forward
HBPr109	20	AAAAAGTTGCATGGTGCTGG	1806-1825	Reverse
HBPr110	25	CCTCTGCCGATCCATACTGCGGAAC	1255-1279	Forward
HBPr111	25	CTGCGAGGCGAGGGAGTTCTTCTTC	2406-2430	Reverse
HBPr113	25	CCGGCAGATGAGAAGGCACAGACGG	1549-1574	Reverse
HBPr134	20	TGCTGCTATGCCTCATCTTC	414-433	Forward
HBPr135	20	CA(A/G)AGACAAAAGAAAATTGG	803-822	Reverse
HBPr374	25	GTTCCGCAGTATGGATCGGCAGAGG	1255-1279	Reverse
HBPr440	21	TATGGATGATGTGGTATTGGG	738-758	Forward
HBPr446	20	GGAGTGTGGATTCGCACTCC	2303-2323	Forward
HBPr448	21	CCCATGCTGTAGCTCTTGTTC	2868-2888	Reverse

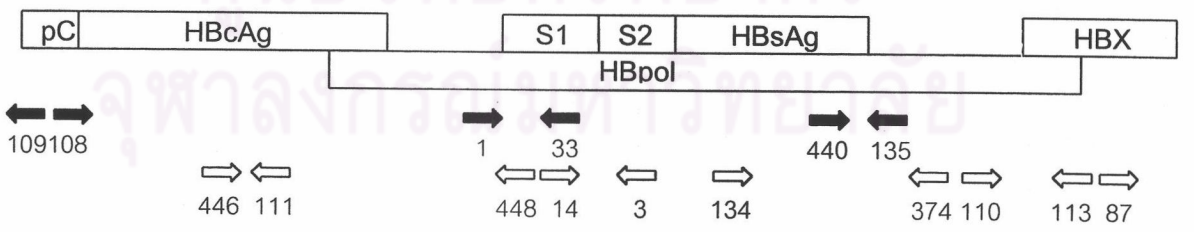

Figure 8 HBV genome organization and amplification regions using for the whole genome sequencing. Position and direction of sequencing primers are indicated with arrows [57]. Black arrows indicate three primer sets for overlapping PCR amplification of whole genome and white arrows represent the sequencing primers of each region.

5. Gibbon HBV molecular characterization method

5.1 Characteristic of gibbon HBV particles

The morphology including size and shape of human and gibbon HBV were determined by electron microscopy. HBsAg positive sera collected from gibbon and human were centrifuged at 10,000 rpm for 15 minutes at 4°C. The clarified supernatant was taken to dialysis bag and covered with PEG-6000 to absorb water from samples until the total volume was reduced to 5 ml. The samples were layered on a 15-56 % (w/v) $CsCl_2$ gradient in TBE buffer. The ultrahigh speed centrifugation was performed at 28,000 rpm for 6 hours at 4°C. The fractions were collected and determined for the HBsAg titer by Monolisa®Ag HBs plus ELISA test. The high titer of HBsAg fractions were pooled and dialyzed against PBS buffer at 4°C for 12 hours. The samples were centrifuged at 30,000 rpm for 18 hours at 4°C and the sedimented particles were resuspened in 30 μl PBS buffer. The purified particles were coded on grid and stained with 2% uranyl acetate before detected under transmission electron microscope.

5.2 Restriction fragment length polymorphism (RFLP)

5.2.1 Identification of gibbon HBV genotypes

The 479 bps amplified product of *PreS1/PreS2* gene from positive HBsAg gibbon serum was identified for HBV genotype by RFLP as previously described [104]. Two restriction enzymes *Ava* II and *Dpn* II was individually used for digestion of the amplified products. A volume of 20 μl of PCR product were mixed with 3 μl of 10x buffer, 6.5 μl of water and 0.5 μl (5 Units) of enzyme, respectively. After incubation at 37°C for 3 hours, the samples were run on a composite gel containing 2% NuSieve agarose and 1% standard agarose. The gel was electrophoresed and DNA was visualized by ethidium bromide staining. The obtained RFLP patterns were compared with the Restriction Endonuclease Analysis (REA) reference profiles for human HBV genotype classification [104].

5.2.2 HBV Precore promoter mutant (nt.1762/1764) analysis

The 596 bps PCR product of X gene was subjected to RFLP analysis using restriction enzyme *Sau3A* I to investigate the precore promoter mutation at codons 1762-1764 as published elsewhere [103]. To that end, 10 unit of *Sau3A* I were added to the reaction tube containing 1X buffer, 20 μl PCR product and water, and subsequently

incubated at 37°C for 3 hours. The RFLP products were subjected to electrophoresis on a 2 % agarose gel . The expected sizes were 596 bps for the 1762/1764 wild type, 465 and 131 bps for the 1762/1764 mutant, respectively.

5.3 HBV DNA sequencing and phylogenetic analysis

For automated DNA sequencing, PCR amplified product was purified using PCR purification kit and DNA concentration was measured at OD_{260} with the spectrophotometry . The 10-30 pmol of DNA template was mixed with 8.0 μl of Prism Terminator Mix, 3.2 pmole of primer and distilled water were added to bring the final volume to 20 μl. The sequencing reaction was performed using GeneAmp PCR System 9600 with 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. The sequencing reaction was then precipitated by adding 80 μl of 75% isopropanol, vortexed briefly and left at room temperature for 15 minutes, and then spinned at 12,000 rpm 4°C for 20 minutes. The sequencing pellet was washed once with 500 μl of 75% isopropanol and then air dried for 10 minutes before resuspened with 20 μl of Template Suppression Reagent (TSR). Sequenced product was heated at 95°C for 5 minutes before electrophoresed on a Perkin-Elmer 310 Sequencer. Results were analyzed using the Sequence Navigator Program and submitted to GenBank Database.

Nucleotide sequences were multiply aligned with the Clustal X program, version 1.4. Bootstrap analysis were performed for values representing 100 replicates using SEQBOOT program. Evolutionary distances were estimated by DNADIST, the distance was clustered into phylogenetic groupings by NEIGHBOR and CONSENSE software from the PHYLIP package, version 3.57c (J. Felsenstein, Department of Genetics, University of Washington). Genetic distances were calculated using the Kimura two-parameter method. TREEVIEW program, version 1.5, was run for phylogenetic tree construction.

6. **Surface antigen mapping of gibbon HBV**

6.1 Antibodies binding to HBsAg of gibbon HBV by ELISA method

A panel of antibodies including their descriptions and binding positions are shown in Table 4. All antibodies preparation in this study were kindly provided by Dr. R. A. Heijtink (Department of Virology, Erasmus University and Hospital, Rotterdam)

Table 4 Antibodies against HBV surface antigen and their localization

Antibodies	Description	Specificity binding site	Conjugated	Captured
F4-7b	human monoclonal anti-S	linear epitope(aa175-184)	biotin	Streptavidine-HRP
S-antiS	sheep polyclonal anti-S	HBsAg	HRP	-
F9H9	human monoclonal anti-S	"a" determinant	biotin	Streptavidine-HRP
9A	human monoclonal anti-S	first loop	biotin	Streptavidine-HRP
1Ff4	human monoclonal anti-S	second loop, close to F47b	biotin	Streptavidine-HRP
anti 127	mouse monoclonal anti S	aa.127-131	biotin	Streptavidine-HRP
anti PreS2	mouse monoclonal anti PreS2	PreS2	biotin	Streptavidine-HRP
anti PreS1*	mouse monoclonal anti-PreS1	PreS1	-	-
anti PreS2*	mouse monoclonal anti PreS2	PreS2	-	-
24 b	mouse monoclonal anti S	S	biotin	Streptavidine-HRP
28 e	mouse monoclonal anti S	S	boitin	Streptavidine-HRP

*antibodies without biotin conjugated : used as coating antibodies

HBsAg ELISA assay was performed in High binding EIA/RIA 96-well flat bottom plates. The assay plate was coated with 200 µl of 1 µg/ml F4-7b monoclonal anti-S antibodies in 0.2 M $NaHCO_3$ pH 9.6, for 12 hours at 4°C. Wells were washed three times with washing buffer and blocked with 200 µl TBS buffer for 2 hours at room temperature. After washing step, wells were incubated with 100 µl diluted gibbon or human samples in ELISA Buffer (EB, Appendix A) and incubated for 1 hour at 37°C. Subsequently, well were washed and incubations were continued with biotin-labeled anti-HBs for 1 hour at 37°C. Removing of excess antibodies were done through washing step and complex proteins were captured by streptavidine-HRP for 30 minutes at 37°C. Well were washed and color was developed by 3,3',5,5'-tetramethyl-benzidene (TMB) substrate for 10 minutes at room temperature, respectively. The reaction was stopped with 2N H_2SO_4. Absorption was read at wavelength 450/620 nm. The standard HBsAg binding assay was performed in this experiment by using human monoclonal F4-7b antibody as coated and conjugated capture reagent. Amount of gibbon and human HBsAg were estimated from the standard plate assay using F4-7b as coating and capturing antibodies.

In order to test the anibodies mapping of gibbon and human HBV, assay plate was coated by F4-7b anti-S and captured by a panel of tested antibodies. The captured reagent for antigen-antibody complex was indicated in Table 4. Most of antibodies were conjugated with biotin and captured with streptavidine-HRP, except sheep polyclonal anti-S was conjugated directly with HRP. Color development of all assay was performed by TMB substrate.

The antibodies without biotin labeling such as anti-PreS1 and anti-PreS2 (Table 4, indicated with *) were tested for binding capacity to human and gibbon HBV by another systems. These antibodies were coated on the assay plate, incubated with samples and bound to F4-7b-biotin labeling. Color developing steps are performed in the same described procedure. Efficiency of antibodies binding to gibbon HBV particles was compared to human HBV.

6.2 Inhibition binding of gibbon and human HBV by immune anti-HBs

6.2.1 HBsAg and anti-HBs positive samples

Preparation of anti-HBs was performed as previously reported [105] . Briefly, serum samples were taken from individuals immunized with the following vaccines : Engerix-B , Hepagene B and GenHevacB (Table 5). Samples were taken at least one month after the last dose in the respective immunization scheme. Pooled serum consisted of samples from five different vacinees with an average anti-HBs response for the particular vaccination study. The commercially available hepatitis immunoglobulin (Hepatect) and human monoclonal anti-HBs (F4-7b and F9H9) were used as references.

6.2.2 Inhibition binding assay of gibbon and human HBsAg by anti-HBs

In this experiment, the ELISA assay for gibbon and human HBsAg binding detection was prepared as described in 6.1. Gibbon and human HBsAg was incubated with serial dilution of tested anti-HBs. After 2 hours of incubation at room temperature, the residual amount of HBsAg was measured by HBsAg ELISA assay without removing HBsAg/anti-HBs complexes. The ELISA solid phase was human monoclonal F4-7b anti-S and conjugated capture was mixture of two anti-S antibodies, 24b and 28e antibodies (Table4). Inhibition binding of each anti-S was calculated from the reversion value of HBsAg binding to ELISA plate assay.

Table 5 Anti- HBs inhibition binding agents including in this study

Antibodies	Description
F9H9	human monoclonal anti-S (Biotest Pharma)
F4-7b	human monoclonal anti-S (Biotest Pharma)
Hepatect	human polyclonal anti-HBs (HBIg, Biotest Pharma)
Engerix-B-anti-HBs	post-vaccination anti-HBs (yeast derived recombinant DNA vaccine (adw2), SmithKilne Beecham)
HepageneB-anti-HBs	post-vaccination anti-HBs (recombinant DNA vaccine (adr) with PreS1, PreS2 and S, Medeva)
GenHevacB-anti-HBs	post-vaccination anti-HBs (CHO-cell derived recombinant DNA vaccine (ayw2), Pasteur Merieux Connaught)

7. Binding of gibbon and human HBV particles to human hepatocyte cells

7.1 Cloning and expression of complete *PreS1, PreS2* and *S* gene from human and gibbon HBV

7.1.1 HBV surface gene amplification

The 1489-1522 bps of complete HBV surface gene carried only *PreS1-PreS2-S* genes was amplified from gibbon and human positive sera using P1 and R5 primers respectively (Table 2). The PCR cycle consisted of 1 minute at 94°C, 1 minute at 55°C and 2 minute at 72°C continued for 30 cycles, and then one cycle of 1 minute at 94°C, 2 minutes at 55°C and 10 minutes at 72°C. Amplified products were analyzed by ethidium bromide staining and visualized under UV.

According to the previous paper [106], the secreting HBsAg may enhance by the cloning of downstream HBV sequence from surface gene beyond to the polyadenylation signal. Within the present study, the 2.2 Kbs of HBV surface gene and additional sequence of X gene/enhancer II, which is known to increase the level of surface gene transcripts, were amplified from gibbon and human HBV by primers HBPr1 and HBPr109 (Table 3).

7.1.2 Cloning and selection of human and gibbon HBV surface gene in pCR2.1 vector.

As shown in Figure9. Both amplified products from human and gibbon samples were ligated into a pCR2.1 vector at the TA cloning sites using T4 DNA ligase according to the manufacturer's protocol. The ligated product was transformed into *E.coli* INVαF' and the positive clones were selected by X-gal/IPTG and ampicillin resistance phenotype. Plasmids from selected clones were extracted and determined the inserted gene by rapid purification method [107] (Appendix A).

7.1.3 Subcloning of HBV surface gene into modified mammalian cell expression plasmid.

The VR1012 plasmid, mammalian cell expression vector, was particularly well in mouse skeletal muscle, lung and tumor cells. VR1012 contained CMV promoter, Intron A and kanamycin resistance gene. Due to the protein expression of stable line in mammalian cell required neomycin drug selection, modification of VR1012 vector was performed (Figure 9). Neomycin resistance gene were cut from pcDNA3.1 vector and

inserted into *Dra* I restriction sites at nucleotide 3358 and 3377 followed by selection VR1012/Neo$^+$ positive clone in *E.coli* INVαF'. Recombinant plasmid was selected and purified by Miniprep plasmid extraction method [108] . Drug resistant capacity were tested by culturing transfected cell line with 0.5 mg/ml neomycin G418 for at least 3 weeks. Viability of cells and fresh medium replacement have done every 3 days.

HBV surface gene inserted in pCR 2.1 vector was digested with the restriction enzyme *EcoR* I followed by a fill-in and dephosphorylation step (Appendix A). DNA was subcloned into a modified VR 1012 Neo$^+$ at the *EcoR* V site and transformed into *E.coli* INVαF'. Positive clones were selected by kanamycin resistant phenotype. Plasmid DNA was extracted by Miniprep plasmid extraction method and examined by restriction enzyme digestion and sequencing analysis. Recombinant DNA was purified for the transfection experiment using the StrataPrep EF Plasmid Midiprep Kit according to the manufacturer's protocol.

7.1.4 HBV surface protein expression in mammalian cells by immunostaining detection

Various cell lines such as Cos-7, Hep2 and CHO were cultured in Dulbecco's modified Eagle's medium (DMEM Medium) with 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin. Cells were seeded on 6 well plate about $5x10^5$ cells per well. Culture was kept at 37°C with 5% CO_2 for 24 hours. Two micrograms of purified vectors were transfected into mammalian cells using FuGENE™ 6 transfection reagent and Lipo-fectin® Reagent according to the manufacturers' protocols. Transfected cells were cultured for 3 days and fixed with 5% paraformaldehyde in phosphate-buffered saline (PBS). After washing with PBS, cells were incubated with 1.0 M glycine for 10 minutes followed by 0.05% H_2O_2 in 70% ethanol. Non-specific binding was blocked with 0.5% normal goat serum. The biotin-labelled human monoclonal antibody to HBsAg (F4-7b) and mouse mAb to PreS2 region (anti-PreS2) was bound to transfected cells at 4°C for 12 hours. A signal was detected by incubating with streptavidine-peroxidase reagent and color was developed by adding 3'-amino-9-ethylcarbazole (AEC) in 50 mM acetate buffer pH5.0 and examined under light microscopy.

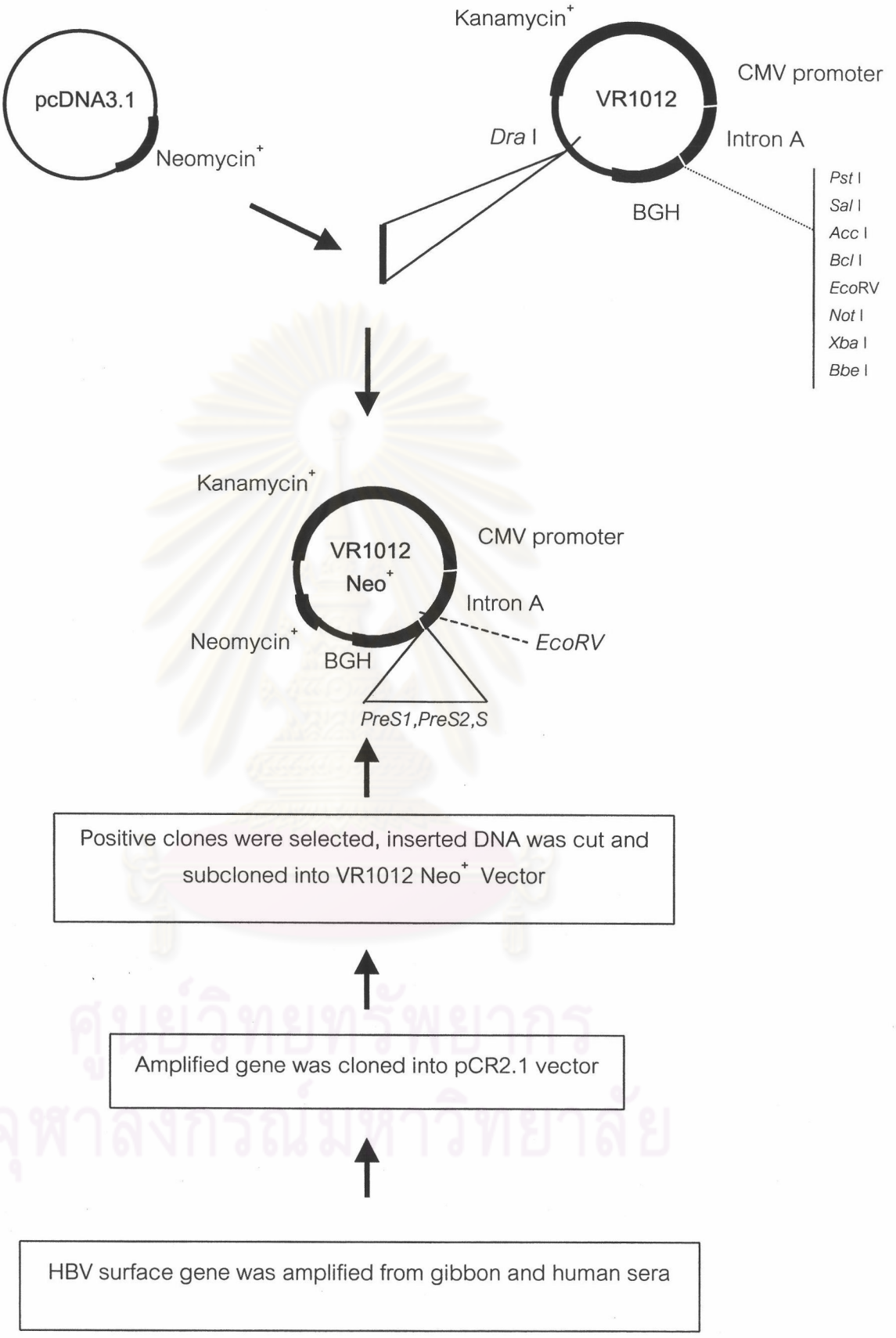

Figure 9 Diagram of VR1012 plasmid modification for neomycin resistant gene expression.

7.1.5 Stable lines selection and extracellular HBV surface protein detection in supernatant culture

Transfected cells were kept in culture with 0.5 mg/ml. G418 neomycin for 21 days. To discard the debris of unstable cells, wells were washed with PBS every 3 days and fresh medium was replaced. Nontransfected cells were performed in the same protocol as a control.

After selection, stable cell lines were detached from culture plate by trypsin. Total cells were counted and seeded onto 96 well plate at the concentration of 1-3 cells/ well. Neomycin G418 was added to medium at concentration of 0.5 mg/ml and culture was incubated at 37°C with 5% CO_2 for 7 days. Supernatant from each well was determined for HBsAg titer by ELISA method. The secreted HBV surface protein stable lines were collected and freezed in freezing medium (culture medium containing 40% FCS and 25% DMSO) and kept at –135°C refrigerator.

7.1.6 HBV surface protein concentration

HBV surface protein expressed from gibbon and human HBV were concentrated as previously described [106]. The stable cell lines were cultured in large scale for surface protein production. After incubation for 3-5 days, HBV surface protein was isolated from the pooled supernatant by precipitation with 6% PEG-8000 for 12 hours at 4°C. The precipitated was recovered by centrifugation at 10,000 g for 45 minutes at 4°C and concentrated 200 fold in phosphate buffer saline (PBS) with 25% fetal calf serum.

7.1.7 Immunoblotting for HBsAg detection

HBV surface concentrated protein was confirmed by immunoblot (dot blot). Protein was dropped on the BioTrace® NT pure nitrocellulose blotting membrane and dried at room temperature. Membrance was blocked by 5% skim milk for 1 hour and washed 3 times for 10 minutes each with PBS. Monoclonal F4-7b antibody concentration 1:1000 was added and kept overnight at 4°C. After washing step, anti-human Ig – alkaline phosphatase conjugated was used as a captured antibodies and color developed by 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) substrate.

7.1.8 Binding of HBsAg to human hepatocytes cell line (HepG2 cells)

Purified HBV particles were used as positive control for the binding assay. Particles concentrated from HBsAg positive sera by ultracentrifugation (23,000 g for 18 hours) through a sucrose-density gradient (30%wt/wt) according to the method as previously described[110].

HepG2 cells were cultured in 25 ml flask with RPMI medium containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin for 7 days. The confluent HepG2 were detached from flask by non-enzymatic cell dissociation solution and washed twice with steriled PBS . The suspended HepG2 in PBS at concentration $4x10^6$ cells/ml were incubated with concentrated HBV particles for 4 hours at 4°C. Cells were collected by centrifugation at 1,600 rpm for 6 minutes, washed twice with PBS containing 10% fetal calf serum and incubated with either human monoclonal anti-S, biotin-labeled F4-7b mAb or mouse monoclonal anti-PreS2 biotin labeling for 30 minutes at 4°C. After additional washing with 10% FCS in PBS buffer, FITC-conjugated streptavidine was added at a 1:100 concentration for 30 minutes at 4°C. As negative and positive control for the fluorescence-activated cell sorter (FACS) procedure, HepG2 cells only and HepG2 incubated with purified viral particles were processed with the same protocol. Cells were then scored using a FACScan analyzer and data were processed using CELLQuest software program. Mean log fluorescence intensity (MFI) values were obtained by subtracting the MFI of the control from the MFI of the samples.

8. *In vitro* HBV infection study

8.1 Host cell culture

8.1.1 Primary human hepatocyte isolation and culture

Section of human liver tissue was obtained from liver donor after written informed consent before transplantation process. The removed tissues were immediately kept in DMEM medium with 0.05% heparin and processed for cell culture. Isolation of hepatocytes were performed using two-step collagenase perfusion techniques[111]. Liver specimen was perfused through small vessels on the cut surface of the specimen with prewarmed buffer containing HEPES 10 mM, NaCl 137 mM, KCl 2.68 mM, Na_2HPO_4 0.7 mM, glucose 10 mM and EGTA 0.5 mM, pH 7.5 at 37°C. Using the

same buffer, except without EGTA and the addition of 5 mM $CaCl_2$, collagenase 0.05% and DNase 2 KU/ml, were added until the tissue specimen becomes sufficiently soften. The liver tissues were minced and suspended in perfusion buffer with collagenase, incubated at 37°C for 10-20 minutes. The cell suspension was filtered through cell strainer 100 μM Nylon and centrifuged at 50 g for 5 minutes, and then washed three times with PBS.

Isolated hepatocytes were checked for viability with trypan blue and resuspended in Williams' E medium supplemented with 10% FBS, glutamine 2 mM, dexamethasone 5 μM, insulin 20 μg/ml , penicillin/streptomycin 100 U/ml, epidermal growth factor 10 ng/ml and human growth factor 10 ng/ml. Hepatocytes were plated onto 35-mm collagen coated polystyrene plates. New medium was replaced after 6 hours incubation at 37°C in 5% CO_2 and renewed every 2 days.

8.1.2 NKNT-3 cell culture

NKNT-3 cell line was reversed from primary adult human hepatocyte cells [112] . As previously described, NKNT-3 cell line was chosen from primary human hepatocytes which were submitted to the reversible immortalization procedure upon transduction with SSR#69 retroviral vector. NKNT-3 cell lines expressed the simian virus 40T (SV40T) immortalizing gene and displayed morphological characteristics of liver parenchyma cells such as cytoplasmic granules and large nuclei with a few nucleoli. Procedure of the NKNT-3 cells reversible immortalization was showed in Figure10. The SV40T immortalizing gene was flanked by LoxP recombinase target. After transient expression of the Cre recombinase, precise recombination occurs between LoxP sites within the chromosomally integrated provirus. As a consequence, SV40T and Hygro-TK are premanently excised from the genome, whereas the neomycin resistance gene(NeoR), which confers resistance to G418, become activated.

NKNT-3 cells were maintained in RPMI with 10% FBS and 100 unit/ml of penicillin/streptomycin and subculture were done every 3 days.

In this experiment, the Cre/LoxP recombinase reversion was activated by pCDNA 3/Cre+ plasmid transfection into the NKNT-3 cells. The reversed cells were checked the Cre recombinase activity by anti-SV40 immunostaining method.

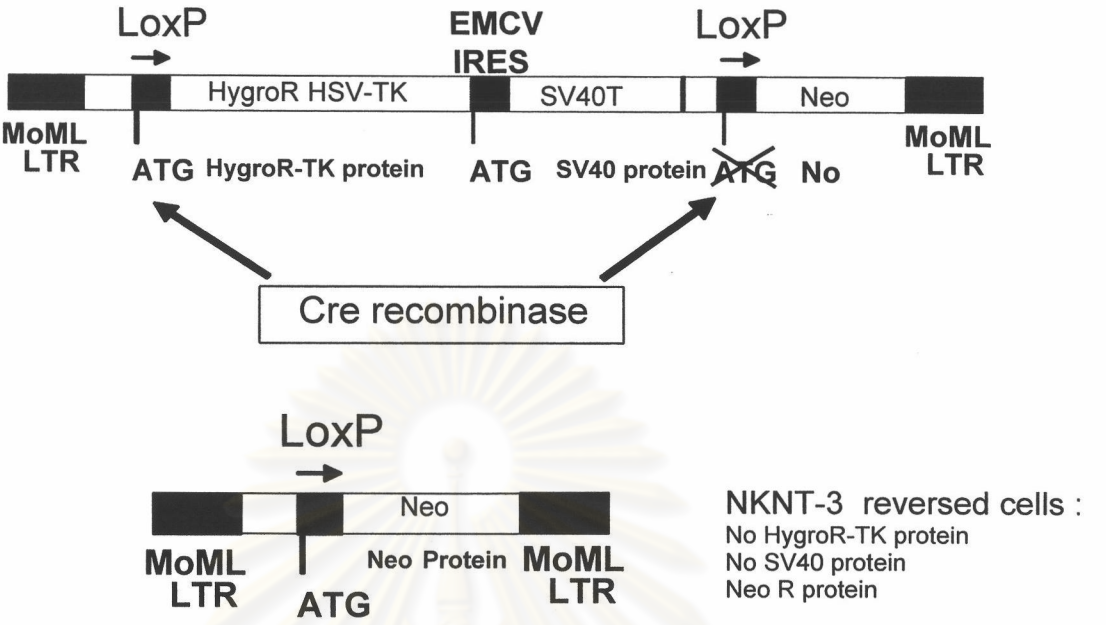

Figure 10 Principles of the procedure of NKNT-3 reversible immortalization. The retroviral vector SSR#69 comprises the following elements from 5' to 3' :

(i) Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) with packaging signal.

(ii) An initiation codon followed by a LoxP recombination target, whose overlapping open reading frame was fused to a hygromycin resistance/herpes simplex virus thymidine kinase (HygroR/HSV-TK) fusion gene.

(iii) The encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), which allows internal initiation of translation.

(iv) The supertransforming U19 mutant of SV40T from which the intron was deleted to avoid splicing of the viral transcript and prevent expression of SV 40 small t.

(v) A second LoxP in direct orientation followed in frame by the neomycin resistance (NeoR) gene, but, importantly, lacking an initiation codon.

(vi) Another LTR proceded by its polypurine track. Only HygroR/HSV-TK and SV 40T are expressed in transduction cells in the absence of Cre recombinase. After Cre/LoxP recombination, the interventing DNA segment between the two recombination target is excised, so that only cells having excised SV 40T become simultaneously G418 and ganciclovir resistant.

8.1.3 Annexin V expression cell lines

Annexin V gene was amplified from human mRNA by RT-PCR method. Human RNA was extracted from liver biopsy and Annexin V gene was amplified using primer AnV1 (5' TAAGAATTCAGTCGCCATGGCACA 3') and AnV2 (5' TTGGAATTCTTAGTCAT CTTCTCC 3'). Amplified region was cloned into pCR 2.1 vector and transformed to *E.coli* INVαF'using the previously described protocal (7.1.1 to 7.1.3). Purified positive clone was performed using the StrataPrep EF Plasmid Midiprep kit according to the manufacture's protocol. Cloning and expression of AnnexinV in mammalian cells was detected by anti-AnV antibodies.

8.1.4 HepG2 treated DMSO culture

HepG2 cells were seeded at 60% confluence ($5x10^5$ cells/ml.) in a 6-well plate for 8-12 hours, and DMSO was added to reach 2% (v/v) final concentration. Cells were kept at 37° C with 5% CO_2 for 7 days before infection experiment [78].

8.2 HBV Infection study of purified HBV particles and HBV positive serum

Purified HBV particles were concentrated from HBsAg positive sera by ultracentrifugation (23,000 g for 18 hours) through a sucrose-density gradient (30%wt/wt) according to the method as previously described[110]. One million particles of HBV or 1:5 dilution of HBV positive serum were incubated with host cells culture for 6 hours at 37°C in serum-free medium. After removal of the medium containing inoculum, cells were extensively and carefully washed at least five times with PBS. New medium was added and incubated at 37°C in 5% CO_2. Culture medium was refreshed every 2 days. Starting at day 0, cells and supernatant was collected every 7 days after infection.

8.3 HBV infectivity detection

8.3.1 Detection of HBV covalently closed circular DNA by PCR

All samples were extracted and amplified by PCR method as previously described[113]. Briefly, the extracted DNA was objected to 30 amplification cycles by 1 minute at 94 °C, 3 minute at 72 °C. To minimize self-annealing of partial elongation products, there is no annealing step. The sequences of the oligonucleotides used for amplification are as follows:

HBV 2537+: CCTCTGCCGATCCATACTGCGGAAC

HBV470- : CTGCGAGGCGAGGGAGTTCTTCTTC

HBV1966+ : TGCCATTTGTTCAGTGGTTCGTAGGGC

HBV2830-: CCGGCAGATGAGAAGGCACAGACGG

The primer pair HBV 2537+/HBV470- is approximately 100 to 1000 times less efficiency on the relaxed circular (rc) than on the ccc DNA. The primer pair HBV1966+/HBV2830- equally efficient on both templates. The estimated sized of rc and ccc DNA are 864 and 1115 bps, respectively. (Figure 11).

8.3.2 Immunostaining of HBV protein producing from hepatocyte infected cells

HBV surface protein produced inside the infected host cells were detected by mouse monoclonal antibody against PreS2 protein (anti-PreS2). Infected host cells were fixed and stained using the same procedure as described under the topic 7.1.4. The biotin-labeled anti-PreS2 antibody was bound to infected cells at 4°C for 12 hours. A signal was detected by streptavidine-peroxidase reagent and AEC substrate color development. The positive staining cells were determined under light microscopy.

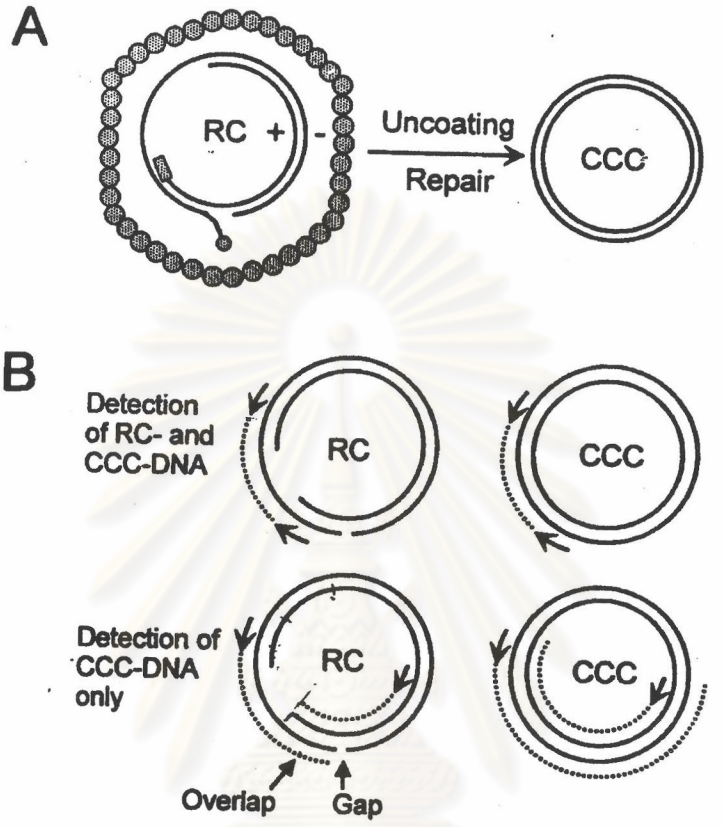

Figure11 Strategy for the selective detection of repaired cccDNA by PCR. (A) The viral genome in the nucleocapsid is a RC DNA molecule with gaps in both strands. After uncoating, the genome is transported into the nucleus, where it is converted into a ccc molecule. (B) If the PCR primer amplify across the continuous portion of the minus-strand, rc and ccc molecule can be detected with the same sensitivity. If primers are selected that amplify across the minus-strand gap, amplification of the RC DNA is only possible after self-annealing of the partial elongation products, as cccDNA form in infected cells [(113)] .