STUDIES ON THE PATHOGENESIS AND THE EARLY EVENTS OF HEPADNAVIRUS REPLICATION

Ming QIAO

M.B., B.S.

Department of Microbiology and Immunology

University of Adelaide

Adelaide

South Australia

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CONTENTS

ABSTRACT

Aspects of hepadnavirus pathogenesis and the early events of hepadnavirus replication were studied in this thesis, with particular emphasis on the examination of serological profiles of DHBV infection in neonatal and adult ducks, factors determining host susceptibility and viral persistence, and hepadnavirus binding and entry into the host cell.

A radioimmunoassay was developed to detect duck hepatitis B virus surface antigen (DHBsAg) and antibody (anti-DHBs), and the results compared with those for DHBV DNA detected by dot blot hybridisation. Viraemia (DHBV DNA and/or DHBsAg) was detected in all ducks inoculated within 3 weeks post-hatch, and persistent infection developed in 93% of birds in this age group. In contrast, only 80% and 60% of ducks inoculated 4 and 6 weeks post-hatch respectively developed viraemia, and approximately 70% of the viraemic ducks became carriers. Markers of viraemia were undetected in ducks inoculated 8 weeks post-hatch and in uninoculated controls.

A typical anti-DHBs seroconversion developed subsequently in 2 of 4 birds that showed transient viraemia, and antibody also developed in 3 of 7 ducks inoculated 4-8 weeks post-hatch that showed no viraemia. However, gene amplification by the polymerase chain reaction demonstrated de novo DHBV DNA in ducks from the latter group suggesting that the antibody did not result from passive vaccination. Thus, increased resistance to infection that developed with increasing age may be related to host factors.

To examine the hypothesis that decreased susceptibility with increased age might be due to the loss of the receptor on the hepatocyte surface membrane in adult ducks, a receptor-binding study was performed using intact serum-

derived DHBV virions and purified liver plasma membrane from both young ducklings and adult ducks. These studies showed that i). DHBV was able to bind specifically to duck liver plasma membrane but not to internal membrane; ii). this binding could be inhibited by a monoclonal antibody to DHBV preS antigen, a corresponding region in hepatitis B virus that binds to human hepatocytes; and iii). there was no significant difference in the virus-binding ability between plasma membranes from ducklings and from adult ducks.

Since hepatocytes in the neonatal ducks are actively dividing, in contrast to the situation in adult ducks, the effect of liver regeneration on the level of DHBV DNA replication was then examined by partial hepatectomy in DHBV-carrier ducks. A sharp increase was noted in the level of DHBV in the serum after partial hepatectomy suggesting that DHBV replication was enhanced in dividing hepatocytes. Thus these results indicated that the age-related difference in the susceptibility of ducks to DHBV infection is not due to the loss of the receptor but may be related to an intracellular event associated with cell division.

Using a similar technique developed to examine DHBV, the HBV-receptor interaction was then examined. The study demonstrated that HBV virions bound to human liver plasma membrane and hepatoma-derived cell lines in a manner expected of a specific receptor-ligand interaction. The supporting evidence are summarised as follows: i). the reaction between HBV and the receptor is cell type- and species-specific. HBV-binding activity was detected on the hepatoma-derived cell lines HepG2 and HuH7, but not on HeLa and LTK^- cells derived from tissues other than liver. HBV was shown to bind specifically to human liver plasma membrane whereas duck liver plasma membrane showed no interaction with HBV virions; ii). HBV binding was inhibited by a synthetic peptide corresponding to the amino acid 21-47 region

of the large HBV surface antigen (L-HBsAg) and by an antibody to the large protein of the HBsAg (MA 18/7); iii). HBV binding to HepG2 cells could be saturated with excess ligand.

Using the polymerase chain reaction, the number of virus particles which bound strongly to HepG2 cells was estimated to be 55 HBV DNA containing virions per cell. This figure may actually represent approximately 500 virions bound per cell considering that the virus preparation contained approximately 45% empty particles and that strongly bound virus particles may represent only 20% of the total bound virus.

Although refractile to HBV infection, HepG2 cells were able to internalise HBV and subsequently transport the virus to the nucleus. These data suggest that HepG2 cells appear able to support many of the early events of HBV replication and consequently, the block in virus replication following infection in these cells is most likely caused by the inability to uncoat the viral DNA and/or to convert the input genome into the transcriptionally-active supercoiled DNA.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any institution. To the best of my knowledge and belief, it contains no material previously published or written by any other person, except where due reference is made in the text.

In accordance with the University of Adelaide regulations, I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Ming Qiao

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ABBREVIATIONS

aa	amino acid
anti-DHBc	antibody to duck hepatitis B virus core antigen
anti-DHBs	antibody to duck hepatitis B virus surface antigen
anti-HBc	antibody to hepatitis B virus core antigen
anti-HBe	antibody to hepatitis B virus e antigen
anti-HBs	antibody to hepatitis B virus surface antigen
anti-HBx	antibody to hepatitis B virus X antigen
AR	analytical reagent
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CAH	chronic active hepatitis
CPE	cytopathic effect
CPH	chronic persistent hepatitis
CTP	cytidine-5'-triphosphate
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DDW	deionised distilled water
dGTP	2'-deoxy-guanosine-5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease I
dNTP	2'-deoxy-nucleotide-5'-triphosphate
ds DNA	double-stranded DNA
DTT	dithiothreitol
dTTP	2'-deoxy-thymidine triphosphate
EDTA	ethylene diamine tetra acetic acid
EM	electron microscopy

FBS	foetal bovine serum
FITC	fluorescein isothiocynate
g	gravity
gp	glycoprotein
GTP	guanosine-5'-triphosphate
HCC	hepatocellular carcinoma
H & E	haematoxylin and eosin
hr	hour
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IP	intraperitoneal
IV	intravenous
kb	kilobase
kDa	kilodalton
MAb	monoclonal antibody
min	minute
ml	millilitre
mRNA	messenger RNA
MW	molecular weight
NDL	normal duck liver
NDS	normal duck serum
NHL	normal human liver
NHS	normal human serum
nm	nanometer
NMS	normal mouse serum
NRS	normal rabbit serum
nt	nucleotide
NTP	nucleotide-5'-triphosphate

o/n	overnight
p	polypeptide
PBS	phosphate buffered saline; (150mM NaCl; 6mM K_2HPO_4; 2mM KH_2PO_4 pH7.2)
PCR	polymerase chain reaction
r-HBsAg	recombinant hepatitis B surface antigen
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease A
RT	room temperature
SDS	sodium dodecyl sulphate
sec	second
SSC	standard saline citrate; (150mM NaCl, 15mM Na_3 citrate pH7.0)
ssDNA	single-stranded DNA
STET	5% Triton X-100, 50mM EDTA, 50mM Tris-HCl pH8.0, 50mM sucrose.
TAE	40mM Tris-HCl pH 8.0, 40mM acetic acid, 1mM EDTA
TCA	trichloro acetic acid
TE8	10mM Tris-HCl, 1mM EDTA, pH8.0
TN	10mM Tris-HCl pH7.4, 100mM NaCl
TNE	10mM Tris-HCl pH7.4, 100mM NaCl, 1mMEDTA
TNT	10mM Tris-HCl pH7.4, 150mM NaCl, 0.05% Tween 20
tRNA	transfer ribonucleic acid
U	unit
UTP	uridine-5'-triphosphate
UV	ultraviolet
vge	viral genome equivalent

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CHAPTER 1

INTRODUCTION

1.1 HBV EPIDEMIOLOGY AND TRANSMISSION

Although anicteric and icteric forms of serum hepatitis were recognised in man for many years, the causative agent was not identified until the 1960s, after the discovery of Australia antigen and the association of the antigen with serum hepatitis, now known as hepatitis B (Blumberg et al.,1965; Dane et al.,1970). Australia antigen is now recognised as a HBV-specific antigen in the serum of HBV-infected individuals, present as an envelope component of the HBV virion or as excess virus envelope. Thus it was designated hepatitis B surface antigen (HBsAg) replacing the term Australia antigen or any of its synonyms.

Hepatitis B is a major worldwide public health problem with over 200 million chronic carriers of the virus (Ganem & Varmus, 1987). HBV has the ability not only to cause acute and chronic infection of the liver but is also associated with the development of HCC, one of the most common cancers in the world (Szmuness, 1978; Obata et al., 1980; Beasley et al., 1981; Beasley & Hwang, 1984).

Serological surveys have revealed three basic epidemiological groupings associated with HBV infection in different geographical regions of the world. Low prevalence (<1%) is found in countries in North America, Western Europe and in certain nations in the Pacific, such as Australia and New Zealand. Intermediate prevalence (2.5-5%) occurs in countries in the Middle East, Eastern Europe and in certain countries in Asia. Hyperendemic regions are found in some developing countries including Africa, South East Asia and

the Far East where the prevalence of the HBV chronic carrier state is as high as 5-20% of the population (WHO report, 1973).

Two different patterns of HBV infection are recognised from these epidemiological surveys. The majority of HBV infections are acquired early in life in hyperendemic areas whereas in low endemic areas, infections are most common between the ages of 15-34 (Zuckerman & Howard, 1979).

Hepatitis B virus may be transmitted by several routes including:

1). Transfusion of HBV-containing blood and blood products, needlestick injuries or injections with contaminated or inadequately-sterilised syringes, needles and other medical or dental instruments, needle sharing between HBV-infected drug users (WHO report, 1973; Zuckerman, 1975).

2). Close contact with HBV-infected patients through some body fluid including saliva, semen and other genital secretions (Szmuness et al., 1975).

3). Perinatal transmission from infected mother to infant probably occurs at or shortly after delivery by exposure to blood or other body fluids of the mother, or even later, during feeding via contaminated milk, saliva etc. (Zuckerman & Howard, 1979; Tong et al., 1981; Robinson, 1982).

4). An unidentified horizontal route in children in endemic areas (Davis et al., 1989).

Infection with HBV may result in an acute self-limited disease. However, a proportion of acute infections do not resolve but result in the HBV "carrier" state, as defined by presence of HBsAg in the serum for more than 6 months, that is often lifelong (Ganem, 1982). Figure 1.1 illustrates the different outcomes of HBV infection.

Figure 1.1

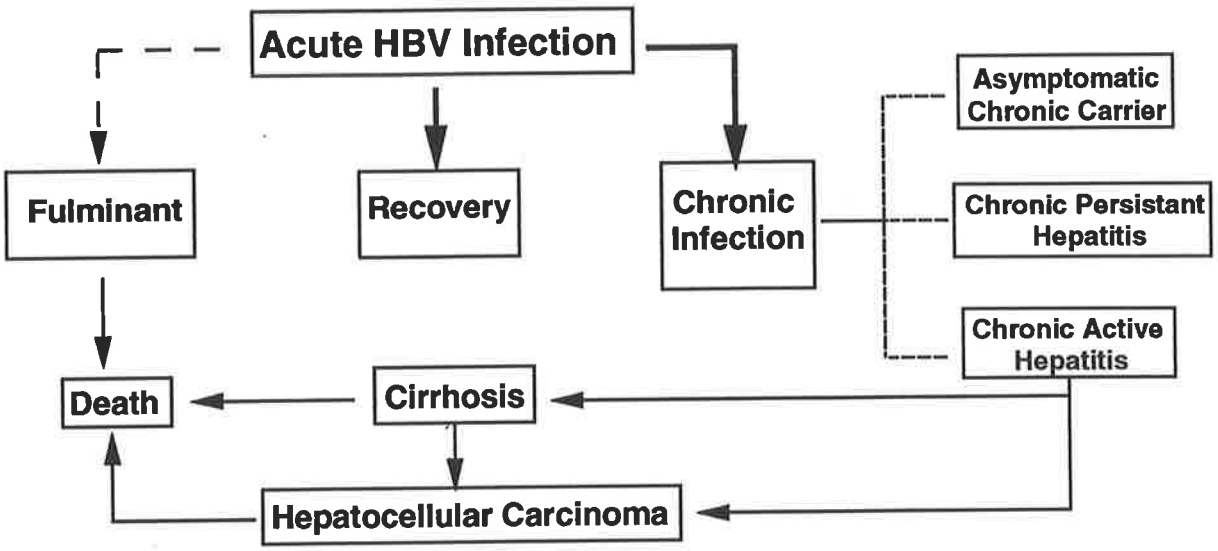


Figure 1.1 Outcomes of HBV Infection

A representation of the usual clinical and histological outcome of HBV infection in non-immunosuppressed adult patients. The prevalence of the HBV carrier state varies from 1-20% of the population in different geographical regions of the world. The proportion of patients who develop acute infection, chronic infection or fulminant hepatitis is reported to be 90-95%, 5-10% and <1% respectively in Western countries.

Two main factors are known to determine the outcome of HBV infection:
1). The age of the host at the time of exposure to HBV. Among HBV-infected individuals, large differences occur in the prevalence of HBV persistence ranging from 85-100% in neonates (Stevens et al., 1975; Beasley et al., 1981); 20-30% in children (Beasley et al., 1982) and only 5-10% in adults (Hoofnagle et al., 1978).

2). Host immunological status. Impaired immunological activity may fail to eliminate HBV and lead to viral persistence. The frequency of exposure and the level of susceptibility are both high in certain individuals with altered immune responsiveness such as chronic haemodialysis patients, patients with Down's syndrome and with leprosy (Blumberg et al., 1967; Bayer et al., 1968). The role of the host immunological response will be discussed in detail in section 1.11.

Although a majority of persistently-infected individuals are asymptomatic and clinically well, 10-50% have abnormal liver function and develop histopathological abnormalities as a result of various degrees of hepatic injury (Hoofnagle & Alter, 1984; Hollinger, 1990). Many studies have shown that HBV is not directly cytopathic in man, and that hepatic injury is mediated by a host immune response directed against virus-coded or virus-induced antigens displayed on the membrane of the hepatocyte (Thomas et al., 1982, also see Section 1.11).

1.2 THE HEPADNAVIRIDAE

For many years it was believed that HBV was a unique virus since its features were quite distinct from other known viruses. However, more recently a few other similar viruses have been identified in several different animal species.

The first of these, the woodchuck hepatitis virus (WHV), was isolated from sera of Eastern woodchucks (Maromota monax) by Summers et al. in 1978. WHV was found to be morphologically, biologically and serologically related to HBV. WHV infection was first suspected in woodchucks at the Philadelphia Zoo, since a high proportion of woodchucks which died were found to have HCC. The observation of a similar high incidence of HCC in a species of domestic ducks in the People's Republic of China (Mason et al., 1980) led to the discovery of the second HBV-like virus, the duck hepatitis B virus (DHBV). It is somewhat ironic that the incidence of HCC in the duck population was discovered later to be unrelated to DHBV infection (Sherker & Marion, 1991).

Another member of this family, the ground squirrel hepatitis virus (GSHV), was discovered as a result of the search for HBV-like viruses in Californian relatives of the Eastern woodchuck (Marion et al., 1980) and another avian virus with similar morphology, the heron hepatitis B virus (HHBV), was reported in the grey heron (Sprengel et al., 1988), but this virus is not yet well characterised. Collectively these viruses are termed the Hepadnaviridae, shortened from hepatotropic, deoxy-ribonucleic acid virus, and HBV is the prototype.

Hepadnaviruses not only have a similar virion morphology and genome structure, but also show a degree of nucleic acid homology and share many biological features including liver tropism and a tendency for persistent infection, production of excess surface antigen and a requirement for reverse transcription during virus replication. However, several properties of the avian species differ from the mammalian hepadnaviruses including a reduced level of nt sequence homology; absence of X gene; a larger core gene; absence of the filamentous form of surface antigen particles. These features are discussed below in detail. A comparison of the properties of all members of the Hepadnaviridae is shown in Table 1.1.

Table 1.1

COMPARISON OF THE PROPERTIES OF THE HEPADNAVIRIDAE

FEATURES	HBV	WHV	GSHV	DHBV	HHBV
Virion					
Diameter (nm)	42	45	47	40	40-60
Density in CsCl (g/cm^3)	1.24	1.225	1.24	1.16	?
Nucleocapsid					
Diameter (nm)	27	27	30	27	?
Density in CsCl (g/cm^3)	1.34	1.34	1.34	1.34	?
Viral Envelope					
Diameter (nm)	22	20-25	15-25	40-60	?
Density in CsCl (g/cm^3)	1.19-1.2	1.18	1.18	1.14	?
Filaments	+	+	+	-	?
Genome					
Size (bp)	3182	3308	3311	3021	3027
5' Overlap (bp)	223	212	211	46	45
5' Linked Protein	+	+	+	+	?
5' Oligo RNA	+	+	+	+	?
ORFs	4	4	4	3	3
Sequence homology (%) to HBV	100	70[a]	55[a]	<40[b]	?[b]
Miscellaneous					
Experimental host range	chimp, gibbon	marmot[c]	woodchuck, chipmunk	goose	?
Genome integration	+	+	rare	-?[d]	-
Associated HCC	+	+	+?	-?[d]	-

a. WHV & GSHV share 82% sequence homology

b. DHBV & HHBV share 78.5% sequence homology

c. Woodchucks are susceptible to GSHV but ground squirrels are not susceptible to WHV (Seeger et al., 1987)

d. Only one report available (Yokosuka et al., 1985)

References

Marion, (1988)

Schödel et al., (1989)

The discovery of these animal hepadnaviruses provided convenient models with which to examine mechanisms relating to HBV biology that are experimentally and ethically impossible to study in man. However, since the genome structure and organisation of HBV is the best studied and documented of the Hepadnaviridae, I have chosen to use this virus to discuss the major features throughout the remainder of this chapter, although I have used data from other Hepadnaviruses when HBV-specific data were unavailable or when these were more appropriate.

1.3 STRUCTURE OF HBV PARTICLES

Determination of the HBV morphology was based on EM of partially-purified HBV from infected human serum. Three types of virus associated particles were revealed in the blood of HBV-infected patients. A diagram of these particles is shown in Figure 1.2.

The HBV virion, termed "Dane particle" after Dr David Dane who first described the particle (Dane et al., 1970), represents the complete infectious mature virus. The diameter of the virion is estimated to be 42nm by EM, consistent with the results of previous ultrafiltration studies which showed that the infectious particles passed through Seitz filters with an average pore diameter of 52nm (McCollum, 1952).

The virion consists of a 7nm wide lipid-containing outer envelope, the hepatitis B surface antigen (HBsAg), which surrounds a 27nm diameter electron-dense core containing the hepatitis B core antigen (HBcAg). In addition to virions with electron-dense centres, virions with empty cores are also found in most preparations. HBsAg and HBcAg will be discussed in detail in section 1.5 of this chapter.

Figure 1.2

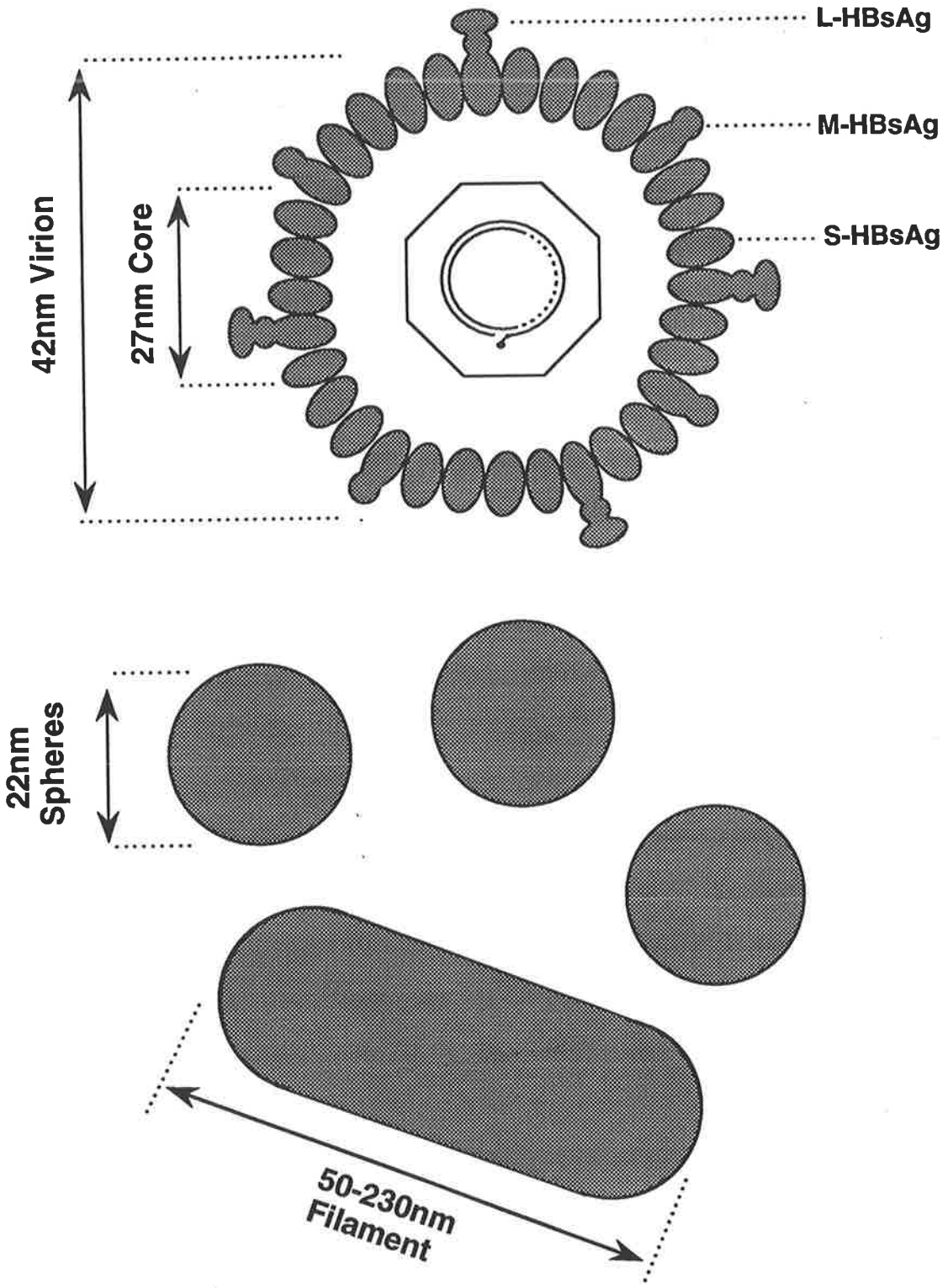


Figure 1.2 Schematic Representation of HBV Particles

The diagram of the virion particle was adapted from the model published by Schlicht et al. (1987).

The majority of hepatitis B-associated particles present in the blood of infected individuals are small spherical particles with an average diameter of 22nm. The third type of particle is a 22nm diameter filament of varying length that can be as long as several hundred nm. These particles consist of protein, carbohydrate and lipid, but because they do not contain the inner core and nucleic acid, they are non-infectious and considered to be excess viral envelopes.

The concentration of virions in infected serum varies from one individual to another, ranging from undetectable in some sera to between 10^5-10^9 particles/ml in others (Almeida, 1972). The 22nm particles, on the other hand, usually exceed the concentration of complete virions by 10^3-10^6. The reasons for the excessive production of non-infectious empty envelope particles is still not clear, although the "dummy theory" appears to be an attractive explanation (Ganem & Varmus, 1987). This theory postulated that the surface of the virion shares antigenic determinants (HBsAg) with the other particles, and these excessive particles were produced to act as "dummy" targets to adsorb neutralising antibodies that otherwise might neutralise the virions. Therefore, HBV may use this strategy to escape immunological surveillance and to persist in the infected host.

In more practical terms, the excess production of HBsAg particles can i). serve as a diagnostic marker of HBV infection; ii). provide large quantities of HBsAg particles for use as vaccine against HBV infection.

The HBsAg envelope of the virion can be removed by treatment with nonionic detergents, such as Nonidet P-40 (NP-40), to release and permit the detection of free core particles. The core particle contains a DNA polymerase activity (Kaplan et al., 1973; Robinson & Greenman, 1974), a protein kinase activity

(Albin & Robinson, 1980), and the virus genome, a partially double-stranded circular DNA molecule of 3.2 kb (Robinson et al.,1974; Summers et al., 1975), the smallest genome of any DNA animal virus yet identified that is capable of autonomous replication. Denaturation of the core particle results in conversion of HBcAg to a linear epitope-dependent antigen known as the hepatitis B e antigen (HBeAg) and this antigen is also discussed in detail in section 1.5 of this chapter.

1.4. ANTIGENIC AND BIOPHYSICAL CHARACTERISTICS OF HEPATITIS B SURFACE ANTIGEN PARTICLES

HBsAg contains a group determinant termed "a", and 2 pairs of mutually exclusive subtype determinants termed "d/y" and "r/w". The "w" subtype has also been designated w1-w4 and the following subtypes have already been identified: ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4 and adr (Vyas, 1981). Some unusual combinations of HBsAg subtype determinants have also been discovered recently, such as awr, adwr, adyw, adyr and adywr (Courouce et al., 1976). Such unusual combination of determinants may be due to phenotypic mixing or unusual genetic recombinants during mixed HBV infection (Robinson, 1982).

For chemical characterisation, the three particle types can be purified by gel filtration, rate zonal sedimentation and equilibrium centrifugation in caesium chloride (CsCl) density gradients (Gerin et al., 1971). Centrifugation in CsCl revealed that 22nm HBsAg particles have a buoyant density of 1.20g/cm^3. In contrast, HBV virions with empty cores have a density of 1.24g/cm^3 compared with 1.28g/cm^3 for complete (infectious) virions (Hruska & Robinson, 1977). These figures reflect a significant lipid content (approximately 30% by weight) of the 22nm HBsAg particles, that was further identified as a mixture of lipids consisting of phospholipids, free and esterified cholesterol, and triglycerides

in a proportion similar to that found in enveloped viruses and cell membranes (Gavilances et al., 1982), suggesting a host cell membrane origin for the lipid.

1.5 HEPADNAVIRUS GENOME ORGANISATION

The virions of all hepadnaviruses contain a small circular DNA molecule ranging from 3.0 to 3.3 kb. Each of the hepadnavirus genomes has been molecularly cloned and sequenced, e.g., HBV: Galibert et al. (1979); Pasek et al. (1979), WHV: Galibert et al. (1982), GSHV: Seeger et al. (1984), DHBV: Mandart et al. (1984), HHBV: Sprengel et al. (1988).

Sequence analyses confirmed that the genomic structure of the mammalian hepadnaviruses is similar and shows significant sequence homology of 70% between HBV and WHV, 55% between HBV and GSHV, and 82% between GSHV and WHV. In contrast, the DHBV genome shows less identity, with only scattered nt sequence homology, with the genomes of the mammalian viruses (Mandart et al., 1984; Seeger et al., 1984; Sprengel et al., 1985, Table 1.1).

1.5.1 Genome Structure

The HBV genome is a small circular partially ds DNA of 3.2 kb (Summers et al., 1975), the minus (long) strand is full length but contains a nick at a fixed position, and the plus (short) strand varies from 50-85% of the full length (Tiollais et al.,1985). See Figure 1.3.

The position of the 5' ends of both strands is fixed as is the 3' end of the minus strand, while the position of the 3' end of the plus strand is variable (Tiollais et al., 1985). The two strands form an open circular structure which is maintained by base pairing involving about 200nt between overlapping 5' ends (Sattler & Robinson, 1979). There are several key 5' terminal features, viz. a protein

Figure 1.3

The inner circles represent the two strands of the HBV DNA genome, L (-) and S (+). The dotted line of the (+) strand represents the region of the single-stranded gap. The direct repeats DR1 and DR2, the protein (●) covalently attached to the 5' ends of the (-) strand and RNA species (∧∧∧) detected at the 5' end of the (+) strand, enhancer elements Enh1 and Enh 2 and the glucocorticoid responsive element (GRE) are illustrated.

The four open reading frames (ORF-P, -C, -S and -X) are shown as arched arrowheads to indicate the position and the direction of transcription and translation. The RNA transcripts (3.5, 2.4, 2.1 and 0.7kb species) together with their common 3' polyadenylation site (AAA) are shown in the outermost portion of the figure. (This figure was adapted from a figure by Dr Thomas Macnaughton).

Figure 1.3

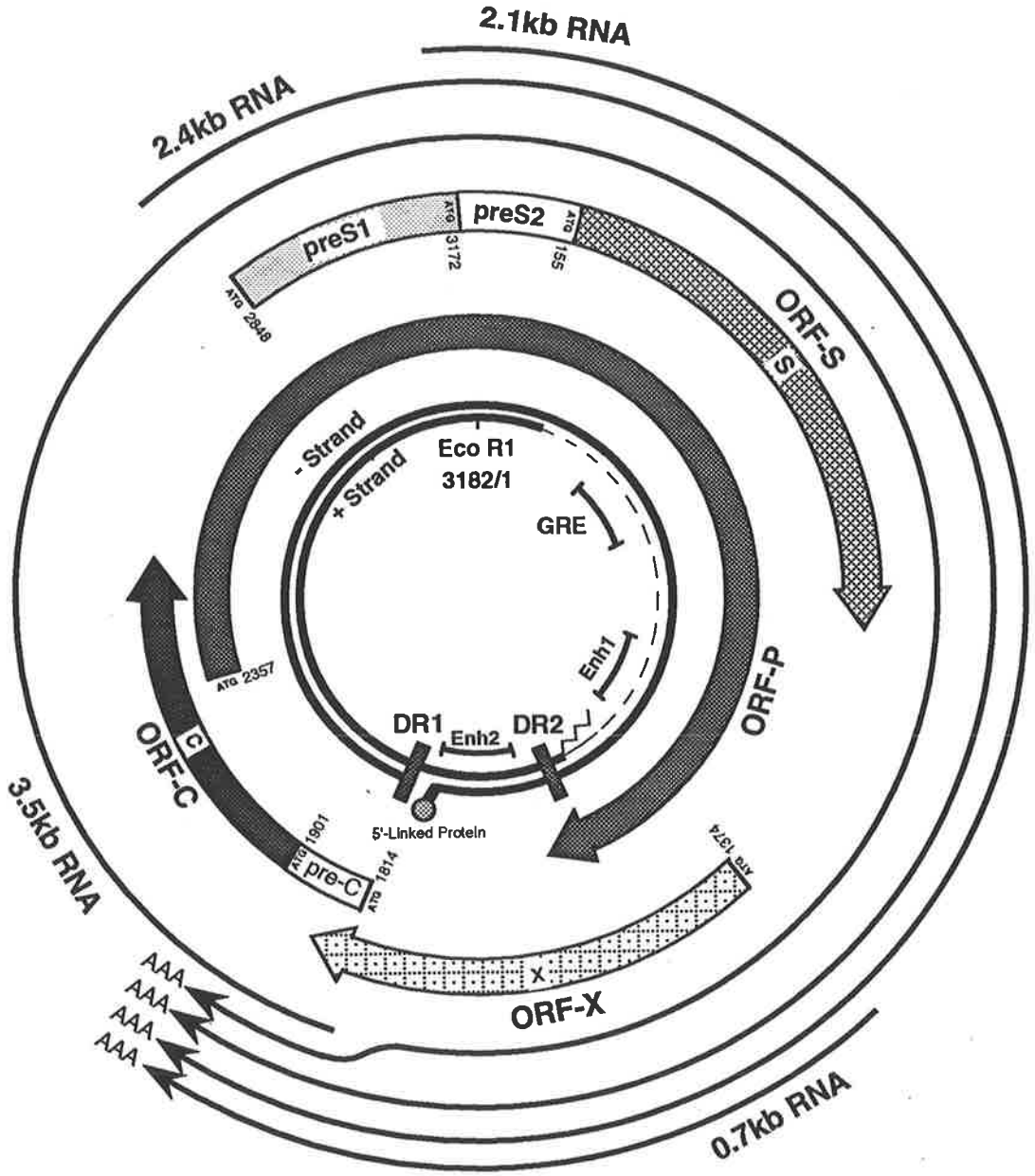


Figure 1.3 Structure and Genome Organisation of HBV DNA.

covalently linked to the 5' end of the minus strand DNA (Gerlich & Robinson, 1980) that serves as the primer for minus strand synthesis (Molnar-Kimber et al., 1983; Mason et al., 1987; Seeger & Maragos, 1990), a 9bp terminal redundancy at the 5' end of the minus strand, termed "r", (Will et al., 1987), a short oligoribonucleotide linked to the 5' end of the plus strand (Lien et al., 1986; Seeger et al., 1986; Will et al., 1987). The function of these 5' terminal features is discussed below. Two 11 bp direct repeats denoted DR1 & DR2 respectively are positioned near the 5' ends of both strands. These motifs play an important role (described below) in the initiation of viral DNA synthesis during virus replication.

Examination of the nt sequence of cloned HBV DNA revealed that there are four major overlapping open reading frames (ORF), all encoded by the minus strand DNA (Figure 1.3). They are ORF-S, ORF-C and ORF-P which encode the HBV envelope proteins, nucleocapsid and DNA polymerase/reverse transcriptase respectively. In addition, the ORF-X, which is present in mammalian but not in avian hepadnavirus genomes is likely to play some role in regulation of viral RNA transcription (Robinson, 1990).

Although two numbering systems are generally used for HBV gene sequences (Galibert et al., 1979; Pasek et al., 1979), throughout this thesis I have used the Galibert system that is based on the unique EcoRI site designated position no. 1.

1.5.2 ORF-S and Its Function

ORF-S spans ca.1200bp which encode the three different HBV surface proteins (HBsAg-large, -middle and -small proteins; L, M & S respectively), the translation of which is initiated from three different in-frame codons (Tiollais et al.,1985). In HBV, the S protein is 226 aa long and is encoded by the S region

alone (Figure 1.4). The M protein is 281 aa long and is encoded by the preS2+S region; consequently the M protein is the same as the S protein but contains an additional 55 aa at the N-terminus. Similarly the L protein is encoded by the preS1+preS2+S ORF and therefore contains 389 or 400 aa depending on different HBsAg subtypes (Heermann et al., 1984; Tiollais et al., 1985; Pfaff et al., 1986), that are coterminal with the C-terminus of the M and S proteins but unique at the N-terminus.

The above predicted proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the various HBV-associated particles (Stibbe & Gerlich, 1983). The envelope of the virus contains the three HBV-encoded polypeptides which are present as glycosylated and non-glycosylated forms. The two smallest polypeptides (p24 and gp27) represent the 226 aa products of the S region; gp33 and gp36, and p39 and gp42 represent the products of the preS2+S region and the preS1+preS2+S region respectively (Heermann et al., 1984, Figure 1.4).

The relative proportion of S, M and L proteins varies in the three particle types. Although the levels of p24/gp27 and gp33/gp36 are similar in all three types of particle, a much higher level (up to 20 times) of p39/gp42 is observed in HBV virions as compared with 22nm spherical particles (Heermann et al., 1984). The protein composition of the filamentous particles is identical to that of the virion envelope (Tiollais et al., 1985). It was estimated that one virion contains 40-80 L protein molecules whereas a 22nm particle contains only 1-2 L protein molecules (Heermann et al., 1984).

The relative proportion of S, M and L proteins in the HBV virion may vary according to the stage of the infection (Stibbe & Gerlich, 1983; Heermann et al., 1984; Pfaff et al., 1986; Petit et al., 1990). The synthesis of L and M proteins was more pronounced during active replication of HBV. The level of L

Figure 1.4

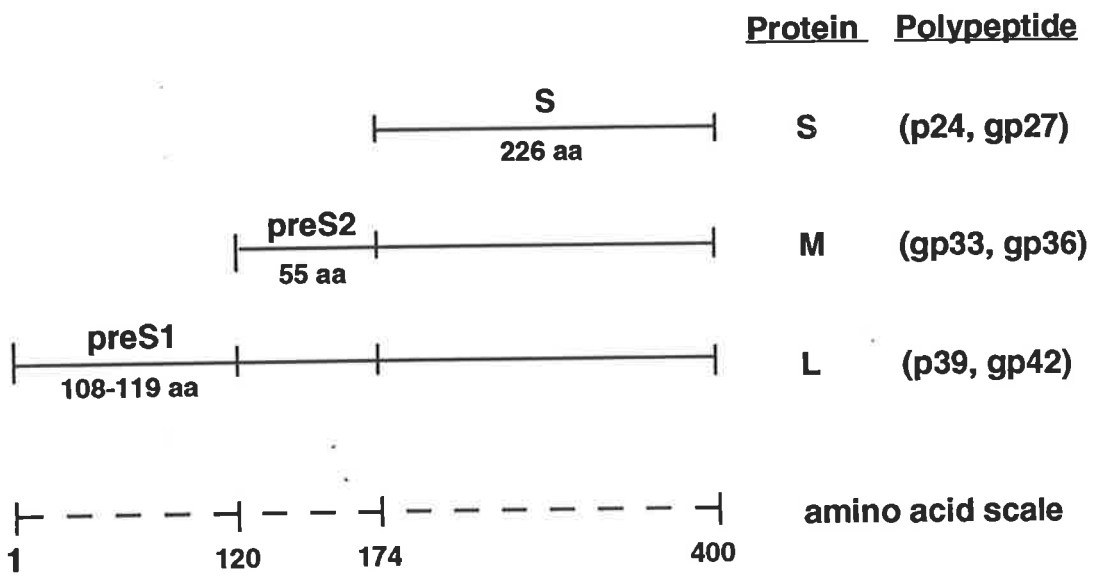


Figure 1.4 Schematic Representation of HBV Envelope Proteins

The ORF-S of HBV DNA has the capacity to code for a protein comprising 389-400 amino acids depending on the antigenic subtype of HBV. p and gp represent nonglycosylated and glycosylated forms.

protein in serum correlates well with other markers for active HBV replication viz. HBeAg and HBV DNA (See section 1.10). Therefore, the relative detection of the three proteins in sera from HBV-infected individuals may serve as important markers for the staging of infection (Lee & Vyas, 1987). However, other data show that expression of the M protein is not essential for HBV replication; HBV with mutated preS sequences unable to express the M protein can occur as a dominant or exclusive virus population in a highly viraemic chronic carrier (Fernholz et al., 1991). In contrast, expression of L protein is vital for virus assembly, secretion and infectivity (Persing et al., 1985; Ou & Rutter, 1987). The L protein also has other important biological functions including virus attachment to host hepatocytes (Neurath et al., 1985) and elucidation of virus-neutralising antibodies (Gerlich, 1991). The role of the L protein in virus replication is discussed in detail in Sections 1.7 and 1.9.

1.5.3 Virus Nucleocapsid (ORF-C)

The ORF-C of HBV is known to encode the major nucleocapsid protein (HBcAg), and a closely related secretory protein, named HBeAg. Both proteins are translated from the same reading frame (ORF-C) but from different initiation codons (Tiollais et al., 1985; Ganem & Varmus, 1987). For synthesis of HBcAg, translation initiates at the second AUG of the C gene, while HBeAg is translated from the first initiation codon in a region of the genome known as the precore (Figure 1.3).

A. HBeAg

It has been demonstrated that the first 19 aa residues of the precore region form a signal sequence which directs an HBeAg precursor (25kDa) to the endoplasmic reticulum, where this signal sequence is cotranslationally cleaved, forming a 22kDa protein derivative (p22, Bruss & Gerlich, 1988; Garcia et al., 1988). A majority of p22 is further cleaved proteolytically at the

arginine-rich C-terminus prior to secretion as the mature 18kDa HBeAg polypeptide (Garcia et al., 1988; Ou et al., 1988). Thus HBeAg is the same as HBcAg but contains an additional 10 aa at the N-terminus and lacks 34 aa at the C-terminus. A small amount of p22 is transported into the nucleus, a process mediated by the nuclear transport signal at the C-terminus (Garcia et al., 1988; Ou et al., 1989; Yeh et al., 1990).

HBV DNA with a variety of different mutations in the precore region has been described recently (Carman et al., 1989; Tong et al., 1990; Brunetto et al., 1991; Naoumov et al., 1992). These mutations (translation initiation codon mutations, mutations which create novel stop codons and frameshift mutations) develop in HBV chronic carriers during the course of HBV infection and consequently result in the lack of expression of HBeAg in the patient's serum. It is believed that the expression of HBeAg may induce immune tolerance (Milich et al., 1990) or block immunological recognition of virus-infected cells (Bonino et al., 1991). HBV mutants with a stop codon at the penultimate precore codon (see Section 1.10), that are most prevalent in anti-HBe chronic carriers, are competent in viral replication and secretion in vitro (Bonino et al., 1991).

B. HBcAg

The polypeptide product encoded from the second in-frame initiation codon of the ORF-C is the 21kDa (p21) HBcAg polypeptide (McLachlan et al., 1987; Weimer et al., 1987; Standring et al., 1988). The polypeptide is not proteolytically processed and has been localised to the nuclear and cytoplasmic cellular components. Although the mechanism of the nuclear transport of HBcAg is still unclear, it may be regulated, however, by a nuclear transport signal which is located near the C-terminus of the precore-core proteins in the arginine-rich domain (Yeh et al., 1990; Eckhardt et al., 1991). A set of two direct PRRRRSQS repeats has been identified as the sequence of

the nuclear transport signal (Yeh et al., 1990). The function of nuclear core protein is still to be determined. Interestingly, the core proteins of all other mammalian or avian hepadnaviruses are not usually transported to the nucleus.

Recently the p21 core protein has been shown to have RNA- and DNA-binding activities. The binding occurs through a C-terminal protamine-like region that contains nucleic acid-binding motifs organised into four repeats, I-IV, (Hatton et al., 1992). Packaging of the viral pregenomic RNA (see Section 1.7) into the HBV core particle is a crucial function of the p21 core protein, and requires cooperation between the core protein, the P gene product and a packaging signal in the pregenomic RNA (Hirsch et al., 1990; Junker-Niepmann et al., 1990; Chiang et al., 1992, also see Section 1.7). In addition, the C-terminal domain of the DHBV capsid protein is specifically required for DNA maturation and virus assembly (Yu & Summers, 1991).

1.5.4 The DNA Polymerase Gene and Its Product

The ORF-P covers 80% of the entire HBV genome and overlaps with all three other ORFs (Figure 1.3). Initial studies found that the P gene contained a sequence which shared homology with that of the P gene of retroviruses (Toh et al., 1983). Thus, the ORF-P was thought to encode the viral DNA polymerase which is contained within the nucleocapsid of the HBV virions and is able <u>in vitro</u> to repair the single-stranded gap present in the HBV genome (Bavand & Laub, 1988; Bavand et al., 1989). More recent studies have suggested that the ORF-P encodes several functional domains. They are; NH_2 - terminal protein (TP) - spacer region - RT/ DNA polymerase - ribonuclease H - COOH (Faruqi et al., 1991).

The TP has been proposed to serve as a primer for minus strand DNA synthesis (Gerlich & Robinson, 1980; Bosch et al., 1988). Antibody to a synthetic peptide corresponding to a N-terminal region of the theoretical P gene product of DHBV was shown to react with the polypeptide covalently attached to the 5' end of the minus DNA strand, suggesting that the P gene encodes this protein (Bosch et al., 1988).

The spacer domain does not appear to be functionally important because drastic insertion or deletion mutations can be tolerated (Chang et al., 1990; Radziwill et al., 1990). This is also supported by a recent study using oligonucleotide site-directed mutagenesis of this domain in the HBV genome which showed a reduced, yet still significant level of replication after two different missense point mutations were introduced within the spacer region (Faruqi et al., 1991).

A P gene-specific protein product of 65kDa has been identified in HBV virions by a specific antiserum (Mack et al., 1988) and DNA polymerase activity gels detected two products of 70kDa and 90kDa (Bavand & Laub, 1988). Furthermore, some weak reverse transcriptase activities have also been demonstrated in activity gels and these activities were subsequently immunoprecipitated by antibodies derived from a synthetic peptide to the P sequence (Bavand et al., 1989). It has not yet been possible to extract a functional reverse transcriptase from virions or core particles, nor has artificially expressed P protein been found to have polymerase activity in the absence of the pregenome and core protein. The P gene product was shown to be involved not only in reverse transcription but also, like HBcAg, in the selective packaging of the pregenomic RNA (Hirsch et al., 1990, see Section 1.7).

Epitope mapping studies have revealed that the RNase H domain of the P protein is an immunodominant region (Weimer et al., 1989). Screening of sera from acutely- and chronically-infected patients without HCC revealed the presence of antibodies against the RNase H domain in 73% and 87% of the sera respectively. Antibodies against the RNase H domain of the P protein may represent an early marker of infection and a novel serological marker for ongoing virus production in acute as well as chronic infection (Weimer et al., 1990).

1.5.5 THE ORF-X and Product

The X gene was so named because the function of the gene was unknown for so long. It is now known that ORF-X lies between nt position 1380-1842 overlapping the two direct repeats (Tiollais et al., 1985) and encodes a polypeptide of 154 aa with a predicted molecular weight of 17kDa. Several studies failed to detect an X gene product of this size in HBV-infected liver (Moriarty et al., 1985; Pfaff et al., 1987) although in other studies, some larger products ranging from 20-28kDa were detected (Elfassi et al., 1986; Kay et al., 1985; Meyers et al., 1986). The reasons for this are still unclear, but may be related to viral DNA integration and the production of novel chimeric proteins.

However, independent immunohistochemical studies suggested that an X protein was expressed in the liver of hepadnavirus-infected individuals (Haruna et al., 1991; Wang et al., 1991). In addition, antibody to synthetic peptide or to X-fusion proteins have also been detected in the sera of infected humans (Moriarty et al., 1985; Kay et al., 1985; Siddiqui et al., 1987). The serological responses to HBx determinants suggests that the X gene region codes for a protein that is expressed during HBV infection and that X antigenic determinants are recognised by the host immune system. However, the

correlation of anti-HBx with different stages of HBV infection is still to be determined.

The function of the X gene in the life cycle of hepadnaviruses infection or in viral pathogenesis is still unknown. However, the insertion of a translational stop codon in the ORF-X region showed no effect on the levels of viral transcription, replication, and synthesis of structural proteins suggesting that the X protein is unlikely to be central to the life cycle of HBV in vitro (Yaginuma et al., 1987; Blum et al., 1992). However, frameshift mutants near the 5' terminus of the X gene of the GSHV genome appeared to inhibit replication in the animal host suggesting that the gene may play an essential role in vivo (Ganem & Varmus, 1987).

Recently, the X protein was shown to transactivate HBV gene expression (Twu & Schloemer, 1987; Spandau & Lee, 1988) and a variety of heterologous viral and cellular promoter sequences, including the human β-interferon gene, simian virus 40 (SV40), the regulatory sequences of the long terminal repeats (LTR) of human immunodeficiency virus type 1 (HIV-1) and Rous sarcoma virus. It is still unclear whether HBV can i). affect the course of HIV or other HBx-responsive virus infections and their associated diseases during coinfection in vivo; ii). activate cellular genes which are either important for HBV replication or in the development of HCC. A recent report suggested that HBx uses a tumour promoter signalling pathway for transactivition, in which HBx transactivates through binding sites for AP-1 and other protein kinase C-dependent transcription factors, AP-2, NF-κB, (Kekulé et al., 1993).

It is possible that the X protein may play a role in the development of HCC. This is only one of a number of proposed mechanisms for hepatocyte transformation by HBV but this is attractive since i). there is a lack of specific

integration sites suggesting that insertional mutagenesis is unlikely; ii). no evidence exists for a HBV-encoded oncogene (Levrero et al., 1990); iii). direct involvement of the HBx gene in the development of liver cancer has been established in transgenic mice (Kim et al., 1991) although this result is not in agreement with an earlier report by Lee et al. (1990).

1.6 TRANSCRIPTION AND CONTROL ELEMENTS OF THE HEPADNAVIRUS GENOME

Three major species of HBV-specific transcripts have been identified in HBV-infected liver tissue and determined to be approximately 3.5, 2.4 and 2.1 kb. These unspliced transcripts have common sequences at the 3' ends, use the same polyadenylation site but differ at the 5' ends (Will et al., 1987, Figure 1.3). A fourth transcript of approximately 0.7 kb has also been detected in cells transfected with ORF-X (Saito et al., 1986; Siddiqui et al., 1987), in transgenic mice (Araki et al., 1989; Kim et al., 1991) and in liver samples from WHV-infected woodchucks (Kaneko & Miller, 1988).

Expression of these transcripts is controlled by four different promoters. The core promoter, the preS1 promoter (SPI), the S promoter (SPII) and the X promoter regulate expression of the 3.5kb, the 2.4kb, the 2.1kb and the 0.7kb transcripts respectively (Saito et al., 1986; Treinin & Laub, 1987; Yaginuma & Koike, 1989). In addition, two transcriptional enhancer elements (EnhI, EnhII) have been identified within the HBV genome (Shaul et al., 1985; Yee, 1989).

1.6.1 The 3.5kb RNA Transcript and Its Promoter

The 3.5kb transcript is the only RNA species which contains the complete genomic information of the virus and thus is able to serve as a template (the pregenome) for the reverse transcription of viral DNA in the unusual

replication strategy of HBV (see Section 1.7). The 5' end of this RNA species appears to be microheterogenous (Figure 1.5) and this is thought to reflect the dual function as pregenome and as messenger for the synthesis of viral proteins (Ganem & Varmus, 1987).

A proportion of the transcripts initiate upstream of the first AUG codon of the ORF-C sequence resulting in the synthesis of a slightly longer RNA species (precore mRNA) with the capacity to encode HBeAg, whereas the most abundant 3.5kb RNA species initiate down-stream of the first AUG codon, and thus give rise to a shorter RNA species (pregenomic RNA) which encodes HBcAg. Since the shorter RNA transcript has been found predominantly within core particles (Enders et al., 1987), it is assumed that this transcript is selected for encapsidation and used as the template for reverse transcription of the minus strand (Section 1.7). In addition, the P gene product appears to be translated from the same mRNA by internal initiation (Ou et al., 1990), which initiates at the first AUG of the P-ORF resulting in a product of 90kDa as discussed above (Chang et al., 1989; Schlicht et al., 1989; Roychoudhury & Shih, 1990). Therefore, all parts of the nucleocapsid are synthesised from a single RNA species. Such an arrangement seems to be advantageous, since it may serve to ensure a balanced production of each different component for nucleocapsid formation.

Thus the core gene promoter regulates the synthesis not only of the core and e antigens but also of the pregenomic RNA (Enders et al., 1987; Will et al., 1987). A core gene promoter sequence has been identified upstream of the ORF-C (ca. 1700-1850, Honigwachs et al., 1989; Yuh et al., 1992). However, it appeared to lack a conventional TATA-like element (Yuh et al., 1992). In addition, P protein expression by internal initiation is consistent with the lack of a P-ORF-specific promoter.

Figure 1.5

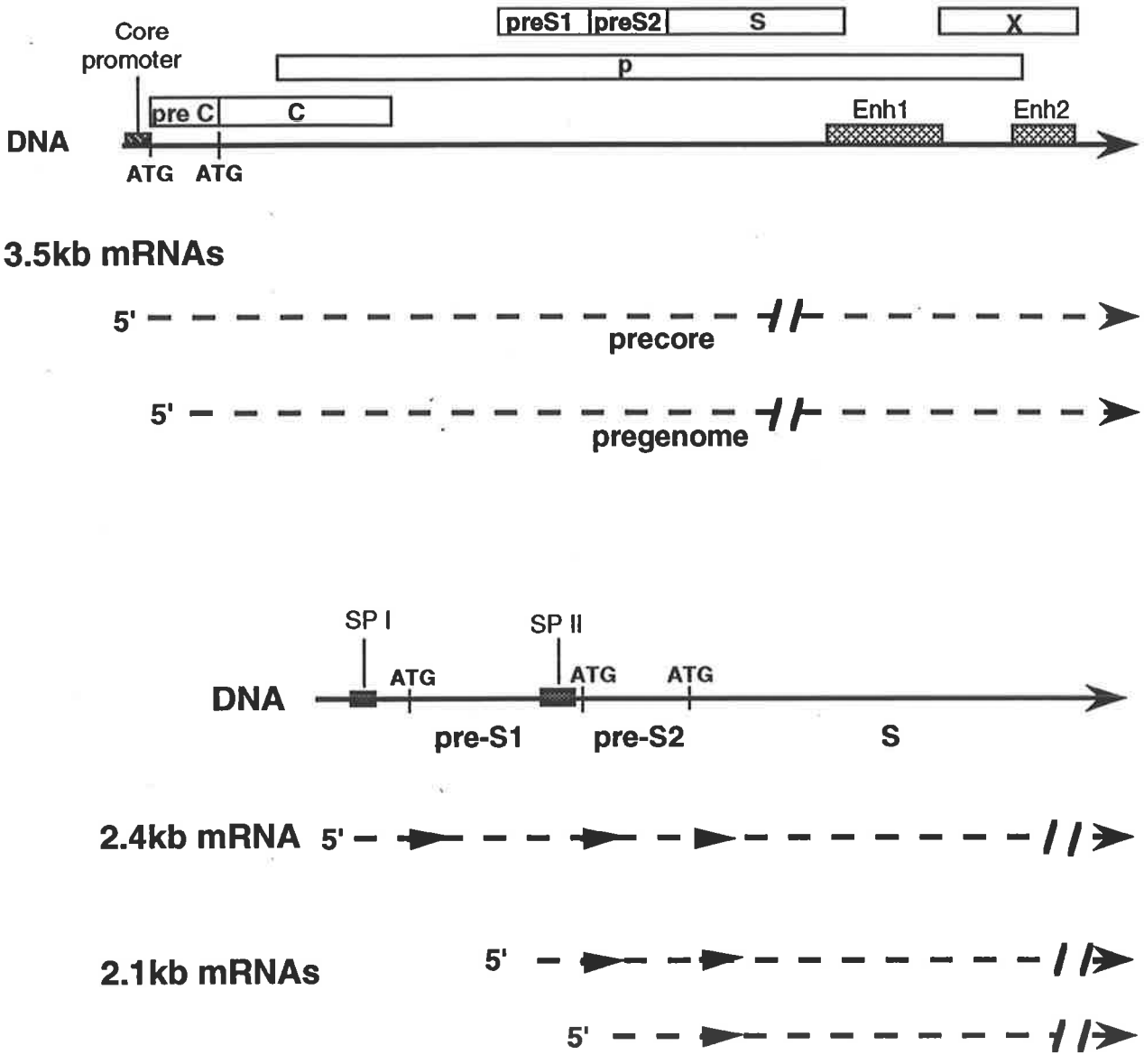


Figure 1.5 The HBV Transcripts

A schematic representation of the three major transcripts (2.1kb, 2.4kb, 3.5kb), showing the microheterogeneity at the 5' ends of the 2.1kb and 3.5kb transcripts, and the promoters and enhancers relative to coding regions.

1.6.2 The 2.1 and 2.4 RNA Transcripts and Their Promoters

The subgenomic RNAs include the two major species of transcripts of 2.1kb and 2.4kb. Sequencing of the 5' end of these transcripts from HBV-infected liver suggested that the 2.1kb species was the likely mRNA for the M and S proteins (Cattaneo et al., 1984; 1987; Standring et al., 1984) while the 2.4kb RNA was likely to serve as mRNA for the expression of the L protein (Will et al., 1987). Therefore, these subgenomic transcripts are likely to encode all three of the envelope proteins.

Like the 3.5kb RNA species, the 2.1kb RNA transcript displays microheterogeneity at the 5' end so that translation initiation from the first AUG results in the synthesis of either the M or the S protein (Figure 1.5; Cattaneo et al., 1984; Standring et al., 1984). Examination of the DNA sequence to locate a ORF-S promoter region revealed no upstream consensus TATA-like element. However, a region upstream (SPII) of the 2.1kb transcript initiation site was found to have sequence homology with the SV40 late promoter (Cattaneo et al., 1983). Since the SV 40 promoter also lacks a TATA-like element and generates transcripts with 5' end microheterogeneity (Brady et al., 1982), it is possible that the absence of a TATA-like promoter is responsible for the observed heterogeneity of the 5' end of the subgenomic RNA transcript (Ganem & Varmus, 1987). The 2.4kb transcript, however, is transcribed from the distal TATA-like promoter (SPI, Malpiece et al., 1983; Will & Rapicetta, 1988).

1.6.3 The 0.7kb RNA Transcript and Its Promoter

The transcription of the 0.7kb X mRNA is regulated by the X promoter, located in the upstream region of the X gene coding sequence (Treinin & Laub, 1987). This X promoter overlaps the enhancer element I (EnhI, see below) and the

structural relationship between the the X promoter and Enh I was recently studied. It was found that EnhI contains a site (originally termed the E element) that binds several protein factors, including AP-1, CREB (Faktor et al., 1990) and C/EBP (Pei & Shih, 1990) that appears to mediate transactivation of Enh I by the HBV X protein (Guo et al., 1991).

1.6.4 Transcriptional Enhancer Elements

The hepatotropic nature of HBV and the observations that the HBV transcripts were detected primarily in well-differentiated human hepatoma cell lines after transfection with cloned HBV DNA suggest that liver-specific factors are required for efficient transcription of the genomic transcripts from the core promoter (Sureau et al., 1986; Tsurimoto et al., 1987; Raney et al., 1989). Subsequently, two regions of the HBV genome were identified that possess properties of transcriptional enhancers (Shaul et al., 1985; Tognoni et al., 1985; Chang et al., 1987; Yee, 1989; Yuh & Ting, 1990; Su & Yee, 1992; Yen, 1993). Enh I is located between the 3' end of the S gene and the 5' end of the X gene while Enhancer II (Enh II) is located immediately upstream from the C gene (Figure 1.5). Enh I can up-regulate all four viral promoters in reporter plasmids or in recircularised genomic DNA (Antonucci & Rutter, 1989; Hu & Siddiqui, 1991). In contrast, Enh II up-regulates the S promoter in a subgenomic fragment and in recircularised genomic DNA (Yuh & Ting, 1990; Lopez-Cabrera et al., 1991). Activation of transcription by Enh I, in combination with the core promoter, is greater in hepatoma cell lines than in nonhepatic cell lines, indicating that it is responsible for liver-specific gene expression of HBV (Shaul et al., 1985). Unlike Enh I, the activity of Enh II is highly liver-specific and dependent on the state of hepatocyte differentiation (Su & Yee, 1992). However, the mechanism by which the two enhancers interact with each other to regulate liver-specific HBV gene expression remains unclear.

1.7 HEPADNAVIRUS GENOME REPLICATION

An understanding of the replication strategy of the hepadnavirus genome came after a series of experiments using DHBV-infected duck liver, that showed that the DHBV genome was replicated by an unique mechanism in which reverse transcription of an RNA intermediate (the pregenome) was involved (Summers & Mason, 1982; Summers, 1988). This mode of replication has subsequently been reported in the mammalian hepadnaviruses (Miller et al., 1984; Seeger et al., 1986), and is now accepted as a common feature for the whole hepadnavirus group.

The genome replication can be summarised in four major steps:

1. Conversion of the virion DNA to covalently closed circular DNA (CCC DNA) within the nucleus of the infected hepatocyte;

2. Transcription of pregenomic RNA from the CCC DNA template using host-derived RNA polymerase II;

3. Synthesis of the minus strand DNA from pregenomic RNA;

4. Synthesis of the plus strand DNA from the minus strand DNA.

A schematic representation of the hepadnavirus replication cycle is illustrated in Figure 1.6.

Figure 1.6

Figure 1.6a is a proposed model for Hepadnavirus replication. The replication cycle is divided into six parts: virus entry, internalisation and uncoating (I); viral DNA migrates to the nucleus, the partially single-stranded DNA is repaired resulting in a double-stranded circular molecule (II), which is supercoiled and serves as a template for transcription (III); transcribed RNA is then either used as mRNA for the production of virus-specific proteins, or encapsidated together with the virus polymerase gene product and core protein into immature core particles (IV); reverse transcription of the encapsidated RNA leads to the production of the minus strand DNA which in turn serves as the template for the synthesis of plus strand DNA. A majority of complete nucleocapsids containing this partially single-stranded genome are subsequently enveloped and secreted (V), and a small number of the core particles enter the intracellular pathway that leads to amplification of the CCC DNA within the same cell (VI). This figure was kindly provided by Dr Thomas Macnaughton. The detailed strategy of hepadnavirus DNA synthesis (step IV) is demonstrated in Figure 1.6b.

Figure 1.6 a

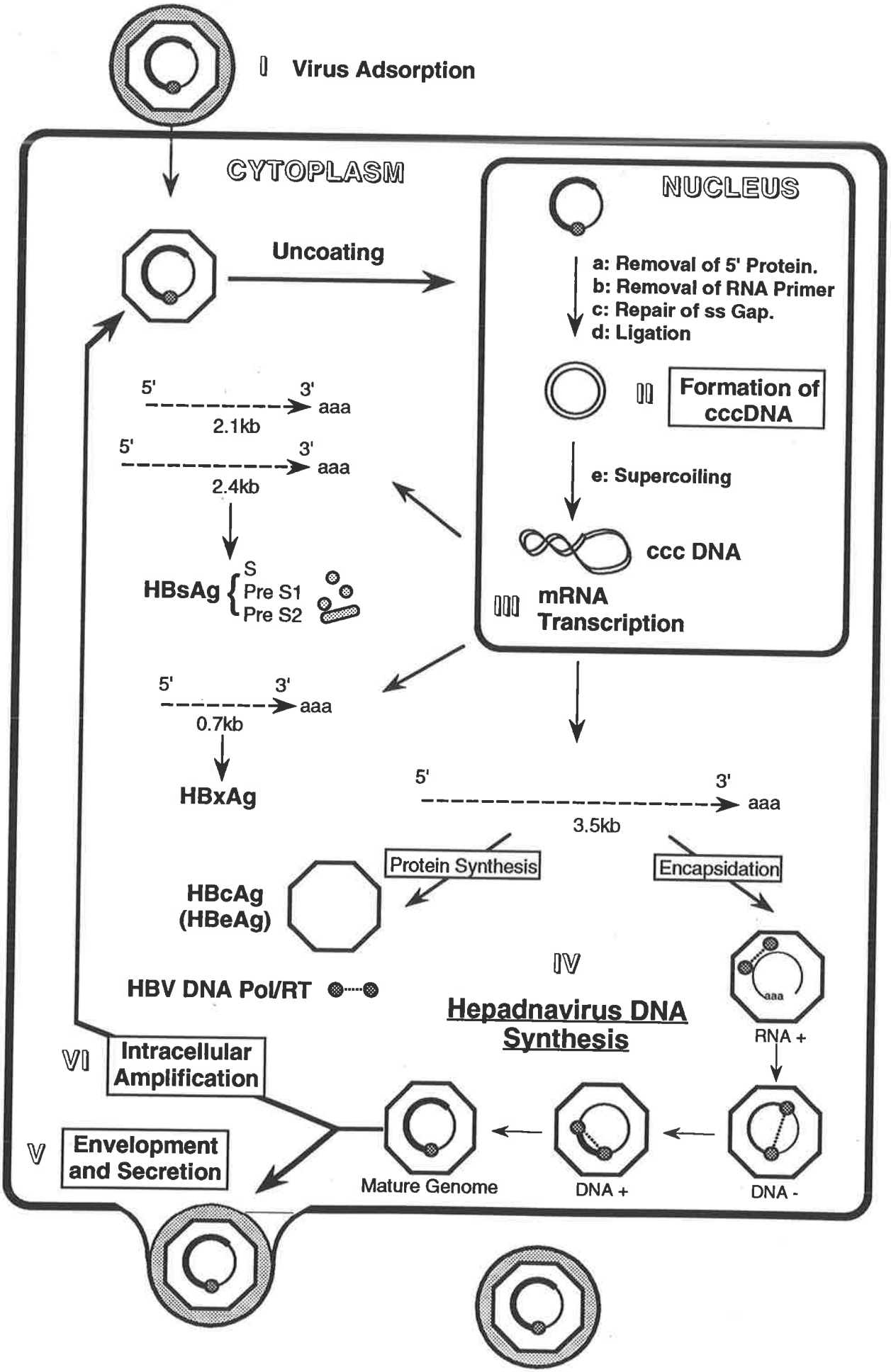
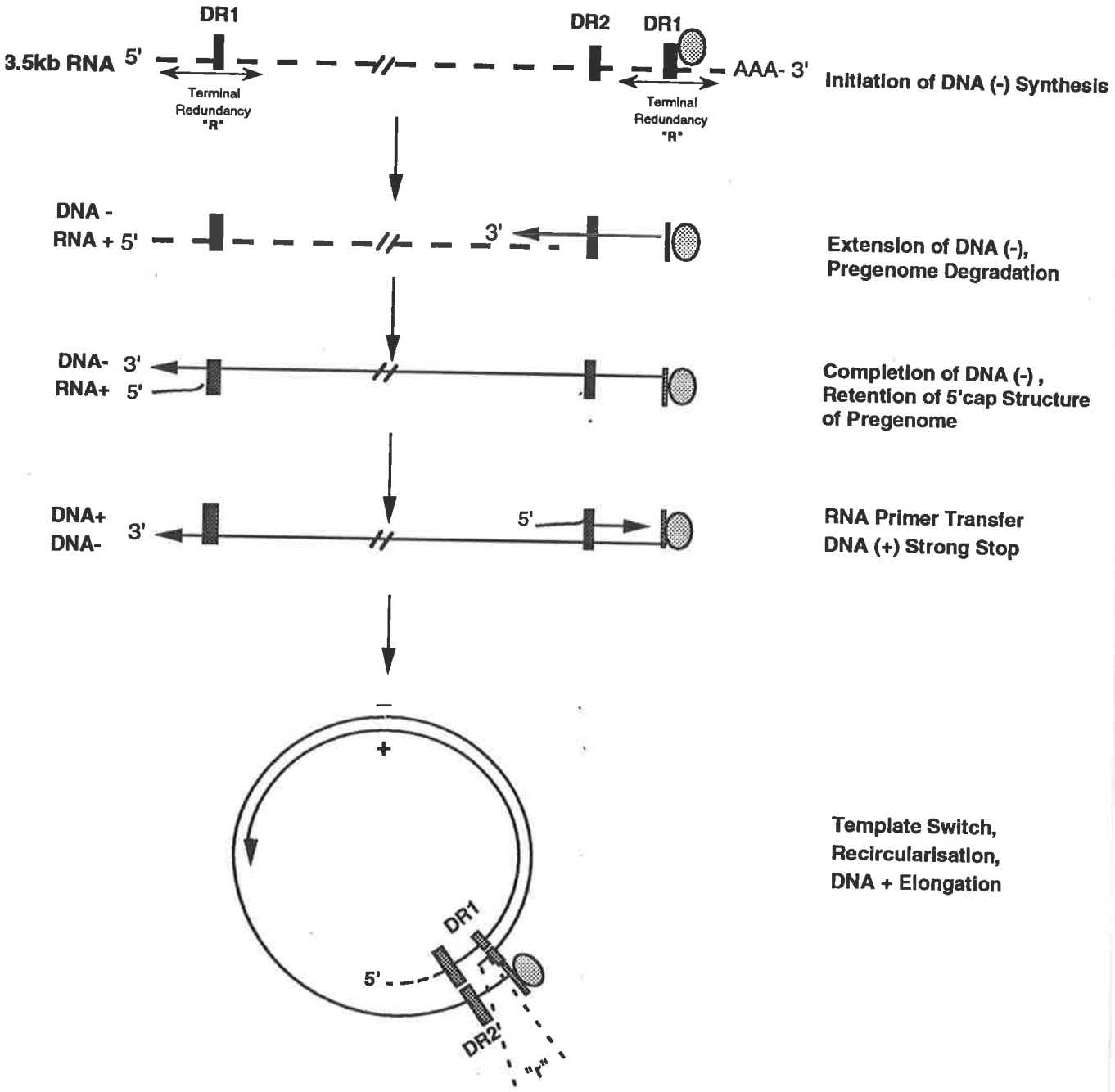

I Virus Adsorption
CYTOPLASM
NUCLEUS
Uncoating
a: Removal of 5' Protein.
b: Removal of RNA Primer
c: Repair of ss Gap.
d: Ligation
II Formation of cccDNA
e: Supercoiling
ccc DNA
III mRNA Transcription
5' 3' aaa
2.1kb
5' 3' aaa
2.4kb
HBsAg S Pre S1 Pre S2
5' 3' aaa
0.7kb
HBxAg
5' 3' aaa
3.5kb
Protein Synthesis
Encapsidation
HBcAg (HBeAg)
HBV DNA Pol/RT
IV Hepadnavirus DNA Synthesis
RNA +
DNA -
DNA +
Mature Genome
VI Intracellular Amplification
V Envelopment and Secretion

Figure 1.6 b


DR1
DR2
DR1
3.5kb RNA 5'
AAA- 3'
Terminal Redundancy "R"
Terminal Redundancy "R"
Initiation of DNA (-) Synthesis
DNA -
RNA + 5'
3'
Extension of DNA (-), Pregenome Degradation
DNA- 3'
RNA+ 5'
Completion of DNA (-), Retention of 5'cap Structure of Pregenome
DNA+
DNA-
3'
5'
RNA Primer Transfer DNA (+) Strong Stop
Template Switch, Recircularisation, DNA + Elongation
DR1
5'
DR2
"r"

Step One: Formation of CCC DNA

Following virus entry into hepatocytes (see Section 1.9) and subsequent uncoating (Figure 1.6a, part I), the single-stranded region of the plus strand is repaired, probably by host DNA polymerase (Mason et al., 1987), to form fully ds DNA which is then converted to CCC DNA (Figure 1.6a, part II). CCC DNA is the first novel, virus-specific nucleic acid detected in infected liver after infection (Mason et al., 1983, Tagawa et al., 1986), suggesting that the virion DNA is converted directly to CCC DNA.

The conversion of the relaxed circular form to CCC DNA requires a number of catalytic activities but no enzyme involvement has been proved. These events include removal of the protein covalently bound to the 5' end of the minus strand and of the 9 nt "r" sequence; removal of the oligoribonucleotide sequence at the 5' end of the plus strand; completion of the plus strand; ligation of each DNA strand and finally supercoiling. Details of these activities are not well understood.

Step Two: Synthesis and Packaging of Pregenomic RNA

It is thought that hepadnaviruses probably use host cell RNA polymerase II to transcribe viral RNA from CCC DNA, the likely template for transcription (Ganem & Varmus, 1987). As shown in Figure 1.5, several species of hepadnavirus RNA transcripts are synthesised, but only one species, the pregenomic RNA, contains all of the sequence information in the virus DNA and thus meets the criterion to serve as a template for reverse transcription (Figure 1.6a, part III). This pregenomic RNA is longer than the unit length of the viral genome bearing a region of terminal redundancy (region R) that varies from 130 to 270 nt depending on the hepadnavirus species; HBV (Yaginuma et al., 1987); WHV (Moroy et al., 1985); GSHV (Enders et al.,

1985); DHBV (Buscher et al., 1985). The redundancy appears because the pregenomic RNA is initiated near the start of the pre-C ORF, upstream of the only known polyadenylation site in the viral genome. Consequently, the signals for cleavage and polyadenylation are ignored at the first pass and obeyed only at the second pass.

Packaging of the pregenome into immature core particles along with the P gene product is highly selective as only pregenomic but not precore or subgenomic RNA molecules are encapsidated (Enders et al., 1987; Hirsch et al., 1990). The exact mechanisms by which this selection is made remain unclear. However, it has been proposed that the P protein plays a crucial role in the selective packaging of the pregenomic RNA (Hirsch et al., 1990). The P protein may bind to this RNA directly, or form a complex with core protein, that mediates the RNA recognition. Recently Bartenschlager & Schaller (1992) have shown that hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal (ε), a 137nt sequence identified near the 5' end of the pregenome, (Junker-Niepmann et al., 1990). The translational start codon of the precore mRNAs is located approximately 40nt upstream of the ε sequence and this may influence in an undefined manner why the precore mRNA is not selected for encapsidation. Furthermore, a very low copy number of the P protein, possibly only one molecule, (Bartenschlager & Schaller, 1992) was detected per core particle suggesting that the number of polymerase molecules to be encapsidated is limited by the encapsidation mechanism.

Step Three: Synthesis of Minus Strand

Newly synthesised pregenome along with the RT/pol encapsidated in immature core particles are found in the cytoplasm of the cell (Figure 1.6a, part IV). A major feature of the reaction controlling reverse transcription of

pregenomic RNA is the formation of a covalent linkage between the 5' end of minus strand DNA and the TP (Gerlich & Robinson, 1980). This protein is also encoded by the P gene and functions as the primer for reverse transcription (Molnar-Kimber et al., 1983; 1984; Lien et al., 1987). It thus substitutes for the tRNA used for DNA synthesis by all retroviral reverse transcriptases (Molnar-Kimber et al., 1983; Bartenschlager & Schaller, 1988). A recent study showed that the reverse transcriptase primed and synthesised viral DNA in a RNA-dependent reaction, which functioned independently of other viral components (Wang & Seeger, 1992). This result suggested that reverse transcription of the hepadnavirus genome can occur in the cytoplasm of the infected cell prior to packaging of the RNA pregenome into viral particles.

Reverse transcription then initiates within the DR1 region (Figure 1.6b, Molnar-Kimber et al., 1984; Seeger et al., 1986; Will et al., 1987). During synthesis of the minus strand DNA, the RNA template is degraded by an RNAse H-like activity (Summers & Mason, 1982), leaving an approximately 17nt capped oligoribonucleotide which is found in the progeny virus (DHBV: Buscher et al., 1985; Lien et al., 1986; GSHV: Seeger et al., 1986).

Step Four: Synthesis of Plus Strand

Following reverse transcription, the nascent minus strand DNA is then used as the template for the synthesis of plus strand DNA. The 17nt oligoribonucleotide derived from the 5' end of the RNA pregenome described in step 3 above contains the DR1 sequence and serves as the primer for plus strand synthesis (Lien et al., 1986; Seeger et al., 1986). In order to serve as primer, the short oligoribonucleotide must escape from degradation by RNase-H and has to be transferred from DR1 to DR2 on the nascent minus strand (Figure 1.6b). When the 3' end of the newly-synthesised plus strand reaches the 5' end of the minus strand template, a template switch to the 3'

end of the minus strand DNA must take place in order for further synthesis to proceed (Will et al., 1987). The 3' end of the new plus strand dissociates from the 5' end of the minus strand and contains a homologous sequence termed the 'r' sequence. This permits the plus strand to base-pair with "r" sequence at the 3' end of the minus strand, resulting in the formation of circularised DNA and continued elongation of the plus strand using the minus strand as template (Figure 1.6b, Will et al., 1987). The dissociation of "r" may be facilitated by the high A+T content of this region and /or by instability induced by the 5' end terminal protein.

Synthesis of plus strand DNA terminates prematurely, and results in the asymmetric DNA species present in the virion. The reason for the incomplete synthesis of the plus strand DNA is not clear, but since the DNA polymerase is unable to displace the RNA primer from DR2 (Lien et al., 1987), it is likely that the oligoribonucleotide remains firmly bound to DR2 and thereby blocks plus strand DNA completion by preventing elongation through DR2. However, this study examined DHBV in which the genome is essentially double-stranded. The asymmetric nature of HBV DNA may result from an inadequate supply of dNTPs caused by premature envelopment of the capsid by HBsAg (Marion, 1988).

A majority of complete nucleocapsids are subsequently enveloped and secreted (Figure 1.6a, part V) but a small number appear to re-enter the replication cycle to amplify the CCC DNA within the same cell, a process known as intracellular cycling (Figure 1.6a, part VI, Tuttleman et al., 1986a; Wu et al., 1990; Summers et al., 1990). It has been estimated that there are approximately 50 copies of CCC DNA molecules per cell in a steady-state infection in persistently-infected liver (Miller & Robinson, 1984) suggesting that CCC DNA must be amplified by an intracellular process. It is likely that the levels of CCC DNA in the nucleus are regulated specifically either through a

cell-directed process or through a virus-directed negative feedback mechanism to maintain levels which are sufficient for virus production but below the level of cytotoxicity.

1.8 HOST RANGE AND TISSUE TROPISM

Hepadnaviruses exhibit a narrow host range and distinct organ tropism. For HBV, the human is the only natural host, although HBV has also been transmitted experimentally to chimpanzees (Prince, 1972; Markenson et al., 1975) which are highly susceptible to infection and show disease patterns similar to those in man. Experimental infection has also been successful in gibbons and African green monkeys but this requires a higher dose indicating that these primates are less susceptible to infection than chimpanzees and man (London et al., 1972; Bancroft et al., 1977). So far, experimental infection of a subprimate species has not been achieved.

HBV has been shown to be primarily hepatotropic although some extrahepatic sites such as kidney, pancreas, spleen, endothelial- and mononuclear-cells, and lymphocytes also appear to support HBV replication less commonly and in a less productive fashion (DeJean et al., 1984; Elfassi et al., 1984; Pontisso et al., 1984a; Yoffe et al., 1986).

Although highly infectious to humans and some higher primates in vivo, HBV has not been shown reproducibly to infect continuous lines of cultured cells in vitro. So far, there is only one report claiming HBV replication in HepG2 cells after infection (Bchini et al., 1990). As a result, primary human hepatocytes are necessary to overcome the difficulty in in vitro infection. Successful infection, however, appears to be highly dependent on the differentiated status of the cells (Tuttleman et al., 1986b; Chang et al., 1987). Under normal conditions, primary hepatocyte cultures are only susceptible to hepadnavirus infection

within the first 7 days after seeding (Schlicht et al., 1987). However, supplementation of the culture medium with 2% dimethyl sulphoxide (DMSO), a solvent known to maintain cell differentiation, induced cultured hepatocytes to support DHBV replication for at least 12 days postplating (Galle et al., 1989).

In recent years, continuous cell lines were developed that secrete infectious hepadnavirus particles after stable transfection of virus DNA (Acs et al., 1987; Sells et al., 1987; Macnaughton et al., 1991). Because hepatoma-derived cell lines support HBV replication following transfection of HBV DNA but not after infection with HBV, the block in the replication may occur in an early stage of the replication cycle. It is not clear which specific factors are necessary for HBV propagation in vitro. However, since HBV replication is dependent on a well differentiated target cell, loss of differentiation during in vitro culture may largely contribute to the loss of susceptibility to HBV infection.

1.9 THE HEPADNAVIRUS RECEPTOR AND VIRUS ENTRY

The interaction between HBV and host cells, and the mechanism by which HBV enters the hepatocyte are poorly understood due to the lack of suitable hepatocyte-like cell lines capable of supporting HBV replication in vitro, and the difficulty of working with primary hepatocyte cultures.

Many studies have been carried out to examine the interaction between the envelope protein of HBV and the cell surface membranes. Firstly, polymerised human serum albumin (pHSA) was reported to bind to HBV virions and to 22nm HBsAg particles (Imai et al., 1979) and it was proposed to function as a bridge between HBV and human hepatocytes and, therefore, mediates the attachment of HBV to hepatocytes. The hypothesis is based on the observation that the M- and S-HBsAg react with glutaraldehyde-cross-linked

pHSA (GA-HSA) which may share an antigenic determinant with polymerised albumin produced in vivo by ageing (Lenkei et al., 1974).

The observation that pHSA-mediated binding occurred with HBV virions and not with HBsAg particles, which lack the pHSA binding sites (Pontisso et al., 1984b), was consistent with the observation that pHSA binds within the M-HBsAg region (Machida et al. 1983, 1984). This finding was supported by the fact that a MAb to M-HBsAg inhibited pHSA-mediated binding of virus to cells (Pontisso et al., 1989a) and also because a frameshift mutation within the preS2 region resulted in production of HBsAg particles, but eliminated pHSA binding (Persing et al., 1985).

Nevertheless, other studies are contradictory, i). only human albumin treated with glutaraldehyde bound HBsAg whereas little or no binding was observed with native albumin or albumin cross-linked by other agents or produced by ageing (Yu et al., 1985); ii). pHSA is not normally present in human liver and serum in significant levels, therefore, it is very doubtful that HBV uses this mechanism to attach to hepatocytes. Moreover, since the WHV does not use this mechanism, it is unlikely that the pHSA-mediated binding is a general feature of hepadnaviruses (Pohl et al., 1986); iii). the interaction between HBsAg linked to cellulose and HepG2 cells was not inhibited by antibodies to GA-HSA (Neurath et al., 1986).

In recent years, mounting evidence has shown that the L-HBsAg is involved in virus attachment to hepatocytes (Neurath et al., 1985). The same group showed that HepG2 cells bound to HBV- or HBsAg-linked to cellulose (Neurath et al.,1986). The reaction was considered to be specific for the L-HBsAg region because i). it was inhibited by antibodies to synthetic peptides corresponding to aa 21-47, aa 32-53 and aa 120-145 of the L- and M-HBsAg. However, only the peptide corresponding to aa 21-47 of the L-HBsAg

inhibited the direct interaction between HBsAg and HepG2 cells; ii). treatment of the HBsAg-cellulose with pepsin, known to remove only the L-HBsAg from HBsAg (Neurath et al., 1985), eliminated the binding reaction. These results also eliminated the possibility that the HBV attachment protein is present on the S protein.

More recently, these data were supported by similar studies using human liver plasma membranes and radiolabelled r-HBsAg (Peeples et al., 1987; Pontisso et al., 1989a) or serum-derived HBV virions (Pontisso et al., 1989b); these studies confirmed that only the L protein, but not the M or S proteins, bound efficiently to human liver plasma membranes. This binding was inhibited specifically by a MAb to the preS1 sequence (Pontisso et al., 1989b).

The L-HBsAg 21-47 region reveals a sequence similarity with the C region of the human IgA α1 chain (Neurath et al., 1986) and this raised the possibility that HBV uses a hepatocyte receptor which is also used by IgA. Although the aa 21-47 of the L-HBsAg was shown to contain binding sites for hepatocyte membranes which express functional and immunological cross-reactivity with binding sites on human IgA (Pontisso et al., 1992), the possibility that IgA and L-HBsAg share the same receptor has been discounted by Neurath & Strick, (1990). In recent years, several studies have reported that HBV may utilise other molecules as its receptor. These molecules include, IL-6 (Neurath et al., 1992); and transferrin receptor CD71 (Franco et al., 1992). The interaction of these factors as receptor(s) for HBV on hepatocytes is still to be determined.

1.10 SEROLOGICAL DIAGNOSIS OF HEPATITIS B

During HBV infection, although there is likely to be an immune response to most if not all viral antigens, the detection of HBsAg, HBcAg and HBeAg and the respective antibody responses are most useful in diagnosis. The patterns

which define the spectrum of serological responses to HBV have been well studied by analysis of sequential serum samples from patients with experimentally-induced hepatitis B (Lander et al., 1971a; Hoofnagle, 1978; Krugman et al., 1974).

The most common pattern of primary HBV infection is transient HBsAg positivity detected in the serum of a patient with clinical and biochemical features of acute hepatitis. In Western countries, this type of response represents 90-95% of acute, symptomatic HBV infection. Figure 1.7 shows an example of of this type of response. In a typical case of acute HBV infection, HBsAg appears 6-12 weeks after exposure to HBV, but has been detected as early as 1-2 weeks after exposure in different studies when very sensitive assays were used (Burrell, 1980). L- and M-HBsAg can also be detected very early in the incubation period (Hadziyannis et al., 1987; Budkowska et al., 1988; Petit et al., 1990). Other markers including HBeAg, DNA polymerase, and HBV DNA can usually be detected shortly after the appearance of HBsAg (Krugman et al., 1974; Nordenfelt & Andrea-Sandberg, 1976; Hindman et al., 1976). DNA polymerase and HBV DNA are direct markers of viraemia whereas HBeAg is not (see below). The appearance of HBsAg is followed 3-6 weeks later by clinical hepatitis. HBsAg remains detectable for 1-6 weeks in most patients, and clears after clinical hepatitis has resolved.

All patients who develop HBsAg also develop anti-HBc. Anti-HBc can usually be detected in the blood 3-5 weeks after the appearance of HBsAg, and before the onset of clinical symptoms of hepatic injury (Krugman et al., 1974; 1979; Hoofnagle et al., 1978). Titres of anti-HBc usually rise during the period of HBsAg-positivity, reach a plateau, then fall eventually after HBsAg becomes undetectable. However, while the titre of IgM-specific anti-HBc declines rapidly with recovery, detectable levels of IgG-specific anti-HBc generally persist for many years and may be lifelong. In cases of fulminant hepatitis in

Figure 1.7

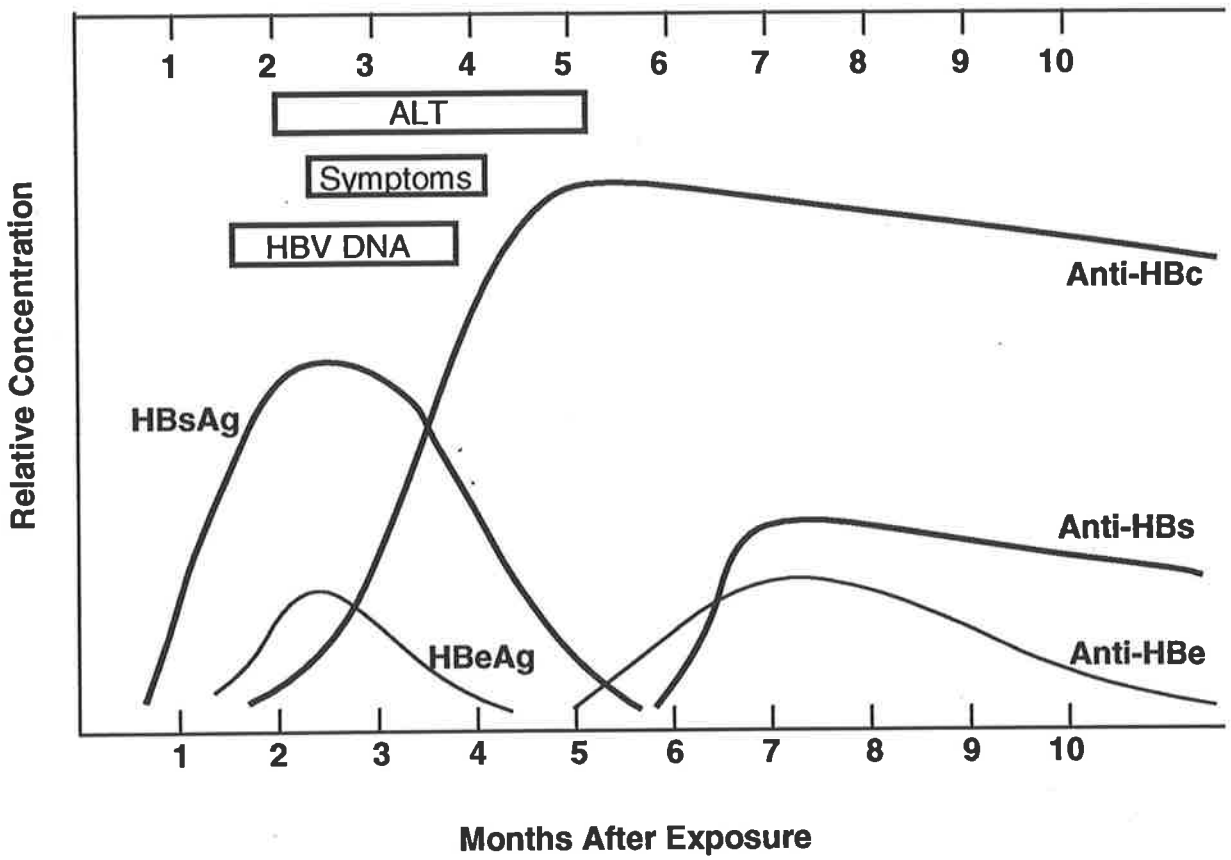


Figure 1.7 Serological and Clinical Patterns of Acute HBV Infection

which markers of viral replication are often not detectable, IgM-specific anti-HBc may be the only marker of recent infection.

In contrast to anti-HBc, anti-HBs appears in most but not all patients during convalescence after the clearance of HBsAg. However, anti-HBs cannot be detected even by the most sensitive tests in approximately 50% of patients immediately after HBsAg disappears and there is a variable time interval, the "window period", between the disappearance of HBsAg and the appearance of anti-HBs. Thus detection of anti-HBs indicates past infection and recovery from acute infection. Once developed, anti-HBs may also persist for many years after HBV infection and is considered to protect against reinfection.

The usual definition of chronic hepatitis B is HBsAg persistence for more than 6 months that is often accompanied by raised alanine aminotransferases (ALT) and necroinflammatory changes seen on histological examination of a liver biopsy sample. HBV carriers are divided into two groups: i). those in the initial "replicative" phase indicated by serum HBeAg/HBV DNA positivity accompanied by active liver disease; ii). those in a subsequent "non-replicative" phase characterised by the loss of HBeAg/HBV DNA and seroconversion to anti-HBe, and often accompanied by integration of HBV DNA, expression of HBsAg and minimal liver disease. Thus the presence of HBeAg is often accompanied by high levels of virus in serum, elevation of ALT and active liver disease. The seroconversion from HBeAg to anti-HBe, occurs spontaneously in approximately 5-10% of chronically-infected patients each year, and is followed by i). an improvement in the liver disease; ii). normalisation of ALT levels; iii). loss of HBV DNA as determined by dot blot hybridisation. Nevertheless, HBV DNA is usually still detected by PCR in this group of patients (Baker et al., 1991).

However, in some patients, the seroconversion to anti-HBe is not necessarily a sign of improvement in the liver disease, and high levels of HBV DNA persist along with active liver disease. The molecular basis for this dichotomy between HBeAg / HBV DNA in this subgroup of patients has been determined recently. A point mutation from G to A at nt 1896 in the precore region, creates a novel translational stop codon which prevents synthesis of HBeAg. However, since the core gene is unaltered, virus replication continues at similar levels to that prior to the mutation event (Carman et al., 1989; Tong et al., 1990; Brunetto et al., 1991).

1.11 PATHOGENESIS OF HBV INFECTION

1.11.1 Hepatocyte Injury and Immunological Mechanisms

Many studies have suggested that the clinical manifestations of HBV-related liver disease are caused by immunological defense mechanisms of the host, since HBV itself is not directly cytopathic to liver parenchymal cells (Thomas et al., 1982; Mondelli et al., 1988). Accordingly, HBV replicates actively in the liver of some patients without causing hepatocyte injury and death but persists in the infected host. This is also referred to as the active replication stage or immunotolerance. This category is common in patients who were infected at an early age before maturation of their immune system. In contrast, the inflammatory reaction can be severe in immunologically normal patients whose immune response to HBV infection leads to liver cell necrosis and this is described as the immunoelimination phase. This category is common in acute hepatitis and often results in the elimination of virus-infected cells due to vigorous cell-mediated responses and clearance of virus infection. Therefore, there is often an inverse relationship between the level of viral replication and the severity of liver disease. This inverse relationship is related to the state of the host immune response which may result in minimal disease on the one

hand or fulminant hepatitis due to aggressive immunological response on the other. The "HBeAg-minus" mutant virus as described above could contribute to the loss of immunotolerance and initiation of the immunoelimination phase in HBV-infected patients and therefore is associated with more severe liver disease and fulminant cases of hepatitis B infection (Bonino et al., 1991; Carman et al., 1991).

Three main types of necroinflammatory lesions, focal necrosis, piecemeal necrosis and bridging necrosis, have been observed in chronic HBV-infected liver tissues (Desmet, 1991). Focal necrosis, an intralobular accumulation of a few mononuclear cells, mainly lymphocytes, seems to represent the mechanism for clearance of virus-positive hepatocytes. Immunohistochemical and immunological evidence has suggested that the focal necrosis corresponds to killing by $CD8^+$ cytotoxic T lymphocytes (CTL), which attack HBV-infected hepatocytes under human leukocyte antigen (HLA) class 1 restriction that express HBcAg and/or HBeAg on the cell surface as target antigen (Heron et al., 1978; Moriyama et al., 1990).

Piecemeal necrosis, a distinguishing feature of CAH, is characterised by lymphocytic infiltration and destruction of hepatocytes at the connective tissue and parenchymal interface around portal tracts and along fibrous septa (Desmet, 1988). The mechanism for piecemeal necrosis also appears to involve $CD8^+$ lymphocytes which are present as the predominant cell type at sites of piecemeal necrosis. The target antigen in areas of piecemeal necrosis in chronic hepatitis B remains unclear because both HBcAg and HBsAg are detected less frequently in areas of piecemeal necrosis. However, an auto-antigen, like "liver membrane antigen" has been proposed as a target antigen (Montano et al., 1983; Thomas et al., 1984) but its role in liver cell injury is still to be determined.

Bridging necrosis is recognised by the loss of parenchymal cells, collapse of the reticulin framework and formation of fibrous passive septa (Desmet, 1988). The increase in antibody response to viral antigens (Mondelli et al., 1984) observed in fulminant viral hepatitis B has raised the possibility that the liver cell damage is induced by humoral immune mechanisms (Desmet, 1991). In addition, because an acute massive necrosis of liver can be induced by injection of antibody/complement but not by T cells (Okuda, 1989) and since liver cell damage can be caused by MAb against an organ-specific membrane antigen (Poralla et al., 1987) these studies have provided further support for this hypothesis.

1.11.2 Mechanisms of HBV Persistence

Another central question regarding the pathogenesis of hepatitis B infection is viral persistence. The mechanisms responsible for determining the course and outcome of hepatitis B are not well understood. However, several immunological mechanisms are recognised as factors that allow a virus to persist in the host.

1). Avoidance of immune surveillance could occur when HLAs are not expressed and thus fail to interact with viral antigen (Peters et al., 1991); the formation of mutant viruses, i.e., precore mutants, that fail to express a potential target for a CTL response (Barnaba & Balsano, 1992) and defects in the formation of the HLA / viral peptide complex.

2). Abrogation of lymphocyte or macrophage function (Immunosuppression) may occur if the immunocytes themselves are infected. Although there is some evidence of HBV infection in lymphocytes and macrophages (Pasquinelli et al., 1986; Yoffe et al., 1986), it is still unlikely that systematic

immunosuppression is the result of direct lymphoid cell lysis since HBV is not cytopathic.

3). Mutations within different B cell epitopes of preS1, preS2 and S sequences may permit immunological escape and therefore prevent HBV clearance (Barnaba & Balsano, 1992). A vaccine-induced escape mutant (a point mutation in the B cell epitope of the S region of HBsAg) has also been found in vaccinees, who showed evidence of HBV infection and replication in the presence of protective antibodies (Thomas & Carman, 1991).

4). Abrogation of immune reactivity may be mediated by inhibition of cytokine production e.g., by a block in α- and β-interferon production (Abb, 1985) or a functional deficiency (Peters et al., 1991). Administration of the immunosuppressive agent cyclosporine in adult woodchucks during acute experimental infection with WHV significantly increased the frequence of chronicity. It is assumed that cyclosporine inhibits interleukin-2-dependent T helper cell expansion, suppresses WHcAg- and WHsAg-specific T helper cells, which can act together under normal immunological conditions to promote anti-WHs response in the infected host and thus clear the virus infection (Cote et al., 1992).

1.12 AIMS OF THESIS

The aims of this thesis were to examine aspects of the pathogenesis of DHBV infection and to study the interaction of hepadnaviruses with their natural host cells. Although the current knowledge and understanding of the hepadnavirus have been described in this chapter as a general introduction, it was considered appropriate to outline the level of understanding in the area of interest prior to the date of commencement of this study (1989).

Prior to 1990 all four animal hepadnaviruses known to us today had been identified, the nucleotide sequence determined for all viruses except HHBV and the major details of the replication strategy determined.

It was known that hepatoma-derived cell lines could produce infectious virus after transfection with cloned hepadnaviral DNA (Sureau et al., 1986; Sells et al., 1987) and that primary duck, woodchuck and human hepatocytes were susceptible in vitro to infection by the respective hepadnaviruses (Tuttleman et al., 1986b; Gripon et al., 1988; Aldrich et al., 1989). However, the propagation of HBV in hepatoma-derived cell lines was still unsuccessful and consequently little was known of the early events of replication.

Initially, the thrust of the thesis was to investigate DHBV as a model for HBV infection. When work for the thesis began, viral-antigen and -DNA had been identified in the liver and some extrahepatic tissues of DHBV-infected neonatal ducklings (Jilbert et al., 1987). Nevertheless, the potential of the duck as a model for infection of adults with HBV had not been exploited. This was partially due to the lack of serological assays for detection of viral antigen and antibody response.

Consequently, the early chapters of the thesis were devoted to the development of serological assays to facilitate the study of DHBV infection. Later work investigated reasons for the refractile nature of adult ducks to infection. As a result of this work, because much of the desired work could be performed directly with HBV, the emphasis was changed from the use of the DHBV model to study the direct interaction of HBV with host cells.

Therefore the specific aims of this thesis were:

1). To study the serological profile of DHBV-specific antigen and antibody in neonatal- and adult-ducks.

2). To study the mechanism of host susceptibility and resistance to DHBV infection.

3). To examine the nature of the binding reaction between HBV and host cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 PURIFICATION OF VIRUS-ASSOCIATED PARTICLES

Several methods for purification of DHBV- and HBV-associated particles were performed in this thesis for several different purposes: i). to raise an antibody to purified DHBsAg for RIA (Chapter 3); ii). to establish a binding assay for DHBV in duck liver membranes (Chapter 5); iii). to study the interaction of HBV and host cells (Chapter 6). Due to their relative low titre in the infected serum, virus-associated particles were usually pelleted by ultracentrifugation (Section 2.1.2) before isolation of individual particles types either by CsCl centrifugation (Section 2.1.3A) or by column chromatography (Section 2.1.4). The latter is considered to be a more gentle procedure and thus was chosen for purification of intact HBV virions. When highly purified virus particles were required, a sucrose gradient (2.1.3B) of the HBV preparation was performed following column chromatography.

2.1.1 Source of Hepadnavirus-Positive Samples

DHBV and DHBsAg were purified from DHBV DNA-positive pooled sera (1.65×10^{10} vge/ml determined by dot blot hybridisation as described in section 2.4.2) from neonatally-infected Pekin-Aylesbury ducks.

HBV was purified from HBV-infected patients with a high level of HBV DNA (1×10^{8} -1×10^{9} vge/ml).

2.1.2 Virus Centrifugation

10ml of DHBV- or HBV-positive serum was clarified by centrifugation at 500g (IEC Centra-4X centrifuge) for 10 min at 4°C. The supernatant was overlaid on 4ml of 20% sucrose (w/v) in TNE and centrifuged in a 80Ti rotor (Beckman) at 230,000g for 4 hr at 4°C. The supernatant was removed and the side of the tube above the pellet was wiped carefully with tissue. The pellet was resuspended in 100ul of TNE at 4°C.

2.1.3 CsCl Gradient Centrifugation for Purification of DHBsAg

The above resuspended DHBV-positive pellet was diluted to 1ml with a solution of CsCl to yield a final density of 1.2g/cm^3 and overlaid on a 1ml discontinuous gradient of equal volumes of 1.4g/cm^3 and 1.25g/cm^3 CsCl in TNE. The tube was then filled with a solution of 1.1g/cm^3 CsCl in TNE, and centrifuged in a SW60 rotor (Beckman) at 270,000g for 48 hr at 10°C. 200ul fractions were collected from the bottom of the tube using a fraction collector and examined for:

a). CsCl density calculated from the refractive index (Griffith, 1979)
b). absorbance at 280nm by spectrophotometry
c). virus particle morphology by EM (Section 2.1.5)
d). DHBV DNA by dot blot hybridisation (Section 2.4.2)

Fractions containing DHBsAg particles with a density of 1.15-1.20g/cm^3 were then pooled and recentrifuged as described above. Fractions containing DHBsAg particles (1.17g/cm^3) were pooled and examined by SDS-PAGE (Section 2.3.6) to confirm the purity of the preparation. The preparations were then dialysed against PBS and stored at 4°C prior to use.

2.1.4 Column Chromatography and Sucrose Gradient Centrifugation for Purification of HBV Virions

The virus suspension described in 2.1.2 was loaded at 4°C onto a column (2.5 x 70cm) of Bio-Gel A5M (Bio-Rad) in TNE in a sample volume of 1-2% of the gel volume. TNE buffer containing 0.5% BSA and 0.02% sodium azide (NaN_3) was then pumped through the column at a rate of 4ml/hr and 1.5ml fractions were collected, assayed for HBV DNA by dot blot hybridisation (Section 2.4.2) and HBsAg by ELISA (Abbott) performed by staff from the Division of Medical Virology, IMVS.

Fractions positive for HBV DNA were combined, centrifuged through a 20% sucrose-TNE cushion as described in section 2.1.2, and the pellet resuspended in 0.5ml TNE at 4°C prior to sucrose gradient centrifugation. The preparation was overlaid on a 10.8ml of a 25-50% (w/v) continuous sucrose gradient in TNE. After 20 hr centrifugation at 150,000g in a SW41 rotor at 10°C, 200ul fractions were collected from the bottom of the tube and assayed for HBV DNA and HBsAg respectively as above.

2.1.5 Virus Particle Examination by Electron Microscopy

A carbon coated grid (provided by staff from the Division of Medical Virology, IMVS) was placed on the surface of a 5ul sample from the virus preparation for 2 min. The grid was removed and excess fluid absorbed from the edge of the grid with a filter paper. The grid was then washed in DDW for 30 sec to dissolve salts contained in the sample, excess DDW removed as before and the grid stained by 3% phosphotungstic acid (PTA) for 1 min, then dried and placed on filter paper in a petri dish. The virus preparation was examined at a magnification of 33,000x in a JEOL-100C electron microscope.

2.2 PRODUCTION OF ANTIBODIES

2.2.1 Immunisation Procedures

New Zealand White rabbits were immunised with 200ug of purified DHBsAg (Section 2.1.3) previously mixed with an equal volume of Freund's complete adjuvant (FCA). The mixture was emulsified in a syringe to a thick, creamy consistency by being repeatedly expelled through a 21 gauge needle. Primary immunisation was performed by subcutaneous injection of a total of 1ml into 6-8 sites on the back of the rabbit. Booster inoculations were given subcutaneously at 14 day intervals using 200ug of protein prepared in Freund's incomplete adjuvant (FIA) in the same manner as described above.

The animals were bled prior to immunisation and again 5-7 days after the third injection. When the relevant antibody levels were high, as determined by the intensity of immunofluorescence (IF) using DHBV-infected liver samples (Section 2.3.4), one rabbit was sacrified by intracardiac bleeding and the other rabbit was bled fortnightly for 2 months. The blood was stored at RT until it was clotted and then at 4°C o/n to allow the clot to contract. The serum was separated by centrifugation at 500g for 10 min at 4°C and stored in small aliquots at -20°C. The specificity of the resultant antibody was also determined by immunoblotting (Section 2.3.6)

2.2.2 Purification of Immunoglobulin G from Antiserum

5ml rabbit antiserum was clarified by centrifugation at 500g for 10 min and filtered through a 0.22um filter. A 50ml Protein A-Sepharose CL 4B (Pharmacia) column was washed with degassed PBS for 10-15 min at 20ml/hr and the serum sample then loaded onto the column at 15ml/hr. When

the sample had entered the gel, the column was left for 30 min then washed with PBS at 30ml/hr until the absorbance at 280nm returned to zero.

IgG was eluted from the column with 0.58% acetic acid in 0.15M NaCl pH 2.7 at a rate of 20ml/hr and 3ml fractions of elute were collected. The IgG-containing fractions were pooled, adjusted to pH 7.3 with 1M NaOH and concentrated to 2ml using a 10ml ultrafiltration cell (Amicon) with a YM30, 25mm GAR filter. The concentration of the IgG was determined by total protein assay (Bio-Rad) as described in Section 2.7.3 , adjusted to 1mg/ml with PBS and stored in small aliquots at -20°C.

2.2.3 Purification of Fab Fragment

180ug of the IgG fraction of the antibody in PBS was mixed with 1/30 volume of 1mg/ml ficin (Sigma, Mariani et al., 1991) in 50mM Tris-HCl pH7.0, 2mM EDTA. The reaction was activated with 1/10 volume of 10mM L-cysteine and incubated at 37°C for 4-5 hr. The reaction was stopped by the addition of 1/10 volume of 100mM N-ethylamaleimide, and the sample dialysed 3 times against PBS at 4°C o/n.

The sample was then loaded into the Protein A-Sepharose CL 4B column which was previously equilibrated in 0.1M citrate buffer pH 5.0 at 15ml/hr and left in the column for 30 min to permit Fc binding. The column was then washed with PBS at 20ml/hr to separate the Fab fragment from the Fc fragment. The wash solution containing the Fab fragment was collected until the absorbance at 280nm returned to zero, and concentrated to 1mg/ml using the 10ml ultrafiltration cell (Amicon). The purified Fab product was tested by SDS-PAGE (Section 2.3.6) to determine the size of the fragment and by total protein assay to determine the concentration (Section 2.7.3), and then stored in small aliquots in -20°C.

2.2.4 IgG Iodination

Iodination of purified IgG was performed essentially as described (Greenwood et al., 1963). Briefly, 20ul of purified IgG (20ug) was mixed with 20ul of reaction buffer pH 7.5 (168mM Na_2HPO_4, 32mM NaH_2PO_4), 10ul ^{125}I NaI (100uCi/ul, Amersham), 20ul chloramine T (4mg/ml in reaction buffer) and incubated for 1 min at RT. 20ul sodium metabisulphite (4mg/ml in reaction buffer) was added to terminate the reaction, and 100ul of Tris-buffered saline solution (25mM Tris-HCl saline containing 0.02% sodium azide and 0.5% BSA) then added. The protein-bound and free ^{125}I were separated by chromatography through a Sephadex G-25 column packed in a 10ml pipette and equilibrated with PBS-BSA-Azide buffer and 0.5ml fractions were collected. A sample of each fraction was counted in a Packard Gamma counter to identify the ^{125}I-labelled IgG. The first peak representing the ^{125}I-labelled IgG was usually detected in fractions 4-6, whereas the second peak, the free ^{125}I, was detected in fractions 9-13. Fractions containing ^{125}I-labelled IgG were pooled, dialysed twice at 4°C against 2 litres of PBS and stored at 4°C until use. ^{125}I-labelled-IgG prepared in this way had a specific activity of ca. 2-5x10^7 cpm/ug.

2.3 IMMUNOHISTOCHEMISTRY

2.3.1 Preparation of Tissue Sections

5um frozen sections were cut using a cryostat (IEC) and mounted on 3-aminopropyl-triethoxy-silane (APES, Sigma) coated slides. The sections were air dried for 30 min at RT then fixed in cold acetone at 4°C for 10 min and air dried again at RT for 30 min. The sections were rehydrated in PBS for 6 min at RT before staining.

Sections of formalin-fixed paraffin-embedded tissues were also mounted on APES coated slides, heated at 60°C for 30 min and dried overnight at 37°C for 16 hr to melt the paraffin and to ensure that the sections adhered to the slides. The sections were dewaxed in xylene for 2 x 10 min and rehydrated to PBS through descending grades of ethanol (i.e.,100%, 90%, 70% respectively).

2.3.2 Source of Antibodies

Anti-DHBs was raised in rabbits by immunisation with purified serum-derived DHBsAg as described above (Qiao et al., 1990); MAb to DHBV preS, and a rabbit anti-DHBc were gifts from Dr Stephen Locarnini, Virology Department, MacFarlane Burnet Centre for Medical Research, Fairfield Hospital, Melbourne.

Anti-HBsAg was purchased from Behringwerke. MAb to preS1 (MA17/8, Heermann et al., 1984) was provided by Professor Wolfgang Gerlich, Department of Medical Microbiology, University of Gottingen, Germany.

2.3.3 Adsorption of Antisera with Normal Liver Tissues

NHL or NDL homogenate was prepared by filing 50g of frozen liver tissue with a 'Surform" hollow file. The shavings were collected into ice cold PBS, centrifuged in a JA-20 rotor (Beckman) at 12,100g for 10 min at 4°C and followed by two washes in cold PBS. The homogenate was freeze-thawed twice then washed again in PBS, resuspended in sterile PBS containing 0.2% NaN_3 and incubated at 4°C for 16 hr. The homogenate was then washed 3 times with sterile PBS containing 0.08% NaN_3 and centrifuged at 12,100g in a JA-20 rotor for 5 min at 4°C.

An equal volume of the homogenate was mixed with the antiserum at one half the working dilution and incubated at 37°C for 1 hr followed by 4°C o/n. The preparation was centrifuged at 12,000g for 5 min at 4°C, the supernatant titrated by appropriate assays, diluted to the working dilution with PBS, filtered through a 0.22um filter (Millipore) and stored in small aliquots at -20°C.

2.3.4 Immunofluorescence

The rehydrated sections were overlaid with 50-100ul of the primary antibody for indirect IF or FITC-conjugated antiserum for direct IF. The antibody was diluted in 1/50 NRS and NDS (or NHS) depending on the species of the primary antibody, and incubated at 37°C for 1 hr. The sections were then washed in PBS for 2x 5 min. For indirect IF, the species-specific FITC-conjugated anti-IgG was then incubated at 37°C for 30-60 min and washed as above. All sections were mounted in glycerol saline (90% glycerol, 50mM Tris-HCl pH 8.6 in PBS) before examination under a UV microscope (Olympus).

2.3.5 Immunoblotting

A. Preparation of Polyacrylamide Gel

The gel apparatus (180 x 130 x 1.5mm) was assembled according to manufacturer's instructions (Bio-Rad). A denaturing gel system with a discontinuous buffer was used in the protein studies unless otherwise stated. The separating gel comprising 15% polyacrylamide (polyacrylamide: bisacrylamide 19:1) was made up to 40ml with DDW (Appendix 1). The solution was mixed well, degassed and poured into the apparatus until it was 3.5-4 cm from the top of the plates. The gel was then overlaid with water-saturated butanol to permit polymerisation. After polymerisation, the top of the

separating gel was washed with water, and 10ml of the 5% acrylamide stacking gel (Appendix 1) was overlaid on the separating gel. The comb was then positioned and the gel left to polymerise. The comb was removed carefully, and the gel rinsed well with water before use and inserted into the electrophoresis tank then immersed in running buffer (Appendix 1).

B. Sample Preparation

The protein samples (volume up to 35ul) were diluted 1:1 in 2x loading buffer (Appendix 1), and heated at 95°C for 5 min and immediately quenched in ice-cold water. The samples and the protein standards (Bio-Rad) were then loaded into the preformed slots in the stacking gel using a Hamilton syringe. Electrophoresis was performed at 150-200V (20-30mA) for 5-7 hr.

C. Staining of Protein Gel

The polyacrylamide gel was removed from the supporting glass plates and stained using a Bio-Rad silver stain kit. The stained gel was then placed in a gel drier (Bio-Rad) and dried according to the manufacturer's instructions.

D. Immunoblot Procedure

After electrophoresis, the gel was sandwiched in a transfer cassette in between membrane filter (Hybond-N+, Amersham), 3MM Whatman paper and supporting foam. The cassette was then placed in the transfer apparatus and the proteins transferred in TN buffer at 70V for 2 hr with a cooling system or usually o/n at 25V without cooling.

The membrane was removed from the transfer apparatus and placed in a sealed plastic bag with 100ml of TN buffer containing 3% BSA and incubated for 3 hr at RT with shaking. The membrane was then incubated with 1/200-1/500 dilution of the primary antibody in TNT buffer, 1% BSA for 4 hr at RT. Following 4x 8 min washes with TNT buffer, the membrane was incubated in

TNT, 1% BSA containing $5x10^5$-$1x10^6$ cpm/ml of ^{125}I-labelled Protein A for 3 hr at RT then washed in TNT for 4x 8 min with shaking. The membrane was then dried at RT, wrapped in plastic film, and exposed to X-ray film as described in section 2.4.

2.4 NUCLEIC ACID HYBRIDISATION

2.4.1 Sample Preparation

A. Total Cellular DNA Extraction

Cells in 24- or 48-well plates (Section 2.7.4) were washed 3 times with PBS, removed with trypsin for 5 min and centrifuged at 2000g for 2 min at RT. The cell pellet was washed with PBS, resuspended in 200ul DDW, mixed with an equal volume of 2x proteinase K buffer (Appendix 1) containing 500ug/ml proteinase K and incubated at 65°C for 15 min then 37°C o/n. The mixture was then extracted with an equal volume of phenol:chloroform and centrifuged at 12,000rpm for 10 min in a microcentrifuge (MSE). The upper aqueous phase was transferred into a fresh Eppendorf tube and the phenol:chloroform extraction step was repeated. The DNA was then ethanol precipitated by the addition of 1/10 volume of 3M NaOAc pH 5.2 and 2.5 volume of 100% ethanol at -20°C o/n. The DNA was pelleted by centrifugation at 12,000rpm for 10 min, the pellet washed with 80% ethanol, freeze dried and redissolved in 20ul of DDW. The DNA was quantitated by spectrophotometry at optical density (OD) 260nm and stored at -20°C prior to use.

B. Cytoplasmic and Nuclear DNA Extraction

After trypsinisation and subsequent washes as described above, the cell pellet was washed with PBS and redissolved in 200ul of 0.5% NP-40, 1mM EDTA, 150mM NaCl, 10mM Tris-HCl 7.6. The suspension was then vortexed and kept on ice for 10 min then pulse centrifuged for 20 sec at 4°C. The supernatant was transferred into a fresh tube and proteinase K digested as before for 1hr at 37°C. The pellet (nuclei) was then washed 5 times with PBS, resuspended in 200ul of DDW and Proteinase K (500ug/ml) digested o/n at 37°C as described above.

C. Viral DNA Extraction from Serum

Serum DNA was extracted in a similar manner to that described above. 100ul of serum was incubated for 3 hr at 70°C in equal volume of 2x Proteinase K buffer (Appendix 1) containing 100ug/ml Proteinase K and then phenol:chloroform extracted and ethanol precipitated as described above after the addition of 100ug tRNA. The DNA pellet was then redissolved in 100ul DDW.

2.4.2 Preparation of Hybridisation Membranes

A. Dot Blot Hybridisation

Nitrocellulose membranes Hybond-C extra (Amersham) were cut to fit a 96 well dot blot apparatus (Bio-Rad), wetted in DDW, then soaked in 10x SSC for 10-20 min at RT. The membrane was then assembled into the apparatus and a light vacuum applied to remove excessive fluid before the samples were loaded.

10ul of neat serum, DNA extracted from cultured cells or 200ul of neat culture fluid were mixed with an equal volume of 2M NaCl, 2 volumes of 1M NaOH and incubated for 15 min at RT. The denatured samples (in duplicate) were

filtered through individual wells of the apparatus under gentle vacuum, then followed by 100ul of neutralising solution (Appendix 1).

When used as a positive control, plasmid DNA was diluted to the required concentration to 20ul with DDW, denatured by boiling for 10 min, chilled on ice and 50ul of 20x SSC added to the sample. 50ul of 20x SSC was applied to the membrane and the DNA solution loaded directly afterwards, and the filter then washed with 100ul of 20x SSC. No vacuum was applied.

The membrane was then removed from the apparatus, rinsed briefly in 10x SSC solution, placed in between two sheets of 3MM Whatman paper and then baked at 80°C under vacuum for 2 hr.

B. Spot Blot Hybridisation

A piece of Hybond-C extra (Amersham) was marked into 1cm squares with a ballpoint pen, and 2ul of each sample spotted directly onto the dry nitrocellulose and dried at RT. The DNA was denatured for 10 min at RT by placing the membrane onto a 3MM Whatman paper soaked in denaturing buffer (Appendix 1), rinsed in 1M Tris-HCl pH 7.4 for 1 min, then neutralised for 10 min at RT in the neutralising buffer (Appendix 1). The filter was then baked as described above.

C. Southern Blot Hybridisation

a). Agarose Gel Electrophoresis of DNA

The required amount of AR grade agarose (Bio-Rad) was mixed with 1x TAE and heated until fully dissolved. The agarose was cooled to 50°C and then poured into the gel mould (Bio-Rad) and a 1-2 mm thick comb inserted. The gel was left to set for 20-30 min at RT, then placed in an electrophoresis tank,

the tank filled with 1x TAE buffer and the comb carefully removed. The DNA samples were mixed with 6x loading buffer (Appendix 1), loaded onto the gel and electrophoresised at 150V until the dye front neared the end of the gel. The gel was then stained with 0.05% ethidium bromide in DDW for 10-20 min at RT, rinsed in DDW and the DNA visualised using a UV transilluminator (Gelman Sciences, NSW) and photographed on Polaroid Type 667 or 665 film. The size of the separated fragments was determined by comparison with a λ DNA Pst 1 digest in the same gel.

b). Gel Treatment

After electrophoresis, DNA in the gel was denatured in 3 changes of 0.5M NaOH, 1.5M NaCl for 45 min with gentle agitation. The gel was then soaked in several changes of the neutralising buffer (Appendix 1) for 1hr. The nitrocellulose membrane was wetted and pre-soaked as described in Section A above and the DNA was transferred in 20x SSC in a BRL DNA blot transfer system.

2.4.3 Preparation of Radiolabelled Probes

A. Source of DNA Templates and Oligonucleotide Probes

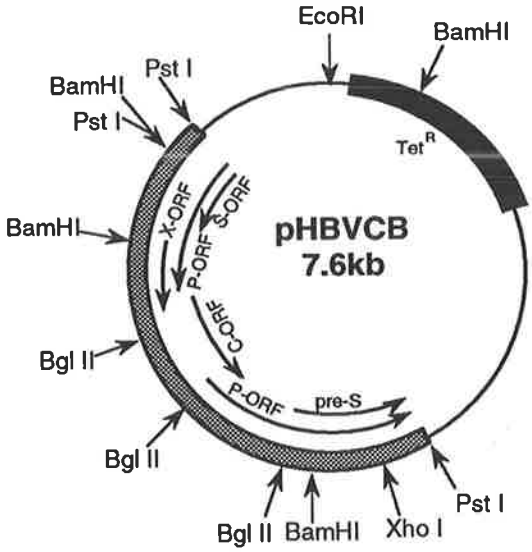
Plasmid pHBVCB (Burrell et al. 1979), obtained from Professor Ken Murray (University of Edinburgh), contains 105% of the HBV genome cloned into the Pst1 site of pBR322 (Figure 2.1a). pTKHH2 (Figure 2.1b), provided by Professor Hans Will (University of Heidelberg), contains a dimer of HBV DNA which was infectious in chimpanzees following intrahepatic inoculation. pSM4 (Figure 2.1c), provided by Ms Sue MacNamara (Dept. Microbiology and Immunology, University of Adelaide), was derived by insertion of the HBV sequences after PstI digestion of pHBVCB into the PstI site of pGEM-3. pSM4 linearised with the restriction enzymes HindIII or EcoRI gives rise to templates

Figure 2.1

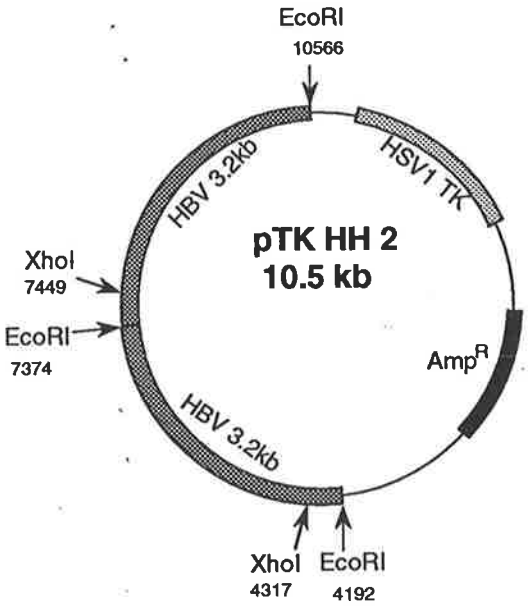
A restriction endonuclease map of pHBVCB (2.1A), pTKHH2 (2.1B) and pSM4 (2.1C). The thin line and the heavy line portion of the circle represent the vector and the HBV DNA insert or antibiotic resistance genes respectively. The location and direction of the ORFs within the HBV genome are indicated by arched arrows (2.1A) and the orientation of the RNA transcripts is demonstrated by short arrows within boxes of SP6 and T7 (2.1C). Figure 2.1A is adapted from Gough & Murray, 1982.

Figure 2.1

A.



B.



C.

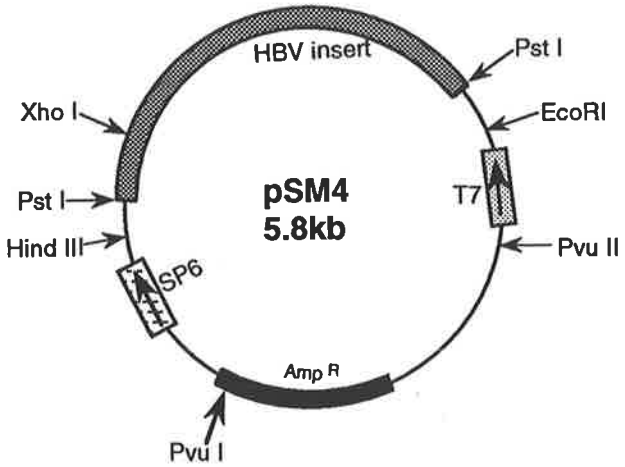


Figure 2.1 A Restriction Endonuclease Map of HBVCB, pTKHH2 and pSM4

for +ve sense RNA (HindIII digestion) and -ve sense RNA (EcoRI digestion) synthesis using T7- and Sp6-RNA polymerase respectively.

pSPDHBV 5.1 and 5.2 (Figure 2.2) were provided by Dr John Pugh of Fox Chase Cancer Center, Philadelphia, USA (Tuttleman et al., 1986b). Genome-length DHBV DNA inserted in both orientations into the EcoR1 site of pSP65 enabled both genomic and antigenomic DHBV RNA transcripts to be produced from Sac1-linearised pSPDHBV 5.1 and 5.2 templates respectively with SP6-RNA polymerase (see below in Section 2.4.3.B).

The above plasmids were constructed to contain highly specific promoter sequences (SP6 and T7) which flank the inserted template DNA to permit transcription in vitro by the DNA-dependent RNA polymerases from bacteriophages SP6 and T7 to generate strand-specific riboprobes from the plasmid DNA.

Oligonucleotide #90 (5'-CCC ACC CAG GTA GCT AGA GTC ATT AAG-3') from the core region of the HBV genome was synthesised by the staff from the Division of Haematology, IMVS.

B. Preparation of Plasmid DNA

Single colonies on antibiotic-containing agar plates were selected and cultured in 20ml 2YT broth (Appendix 1) with appropriate antibiotics (e.g., ampicillin 100ug/ml) o/n at 37°C with shaking. The culture was then transferred into 500ml of L broth (Appendix 1) with appropriate antibiotics and incubated on a cell shaker for 6 hr at 37°C. Chloramphenicol (Parke Davis) was added to a final concentration of 160ug/ml and the culture incubated o/n at 37°C.

Figure 2.2

A restriction endonuclease map of pSDHBV 5.1 and pSDHBV 5.2. Genome-length DHBV DNA was cloned in both orientations into the EcoRI site of pSP65 by Dr John Pugh. The orientation of the RNA transcripts is demonstrated by the direction of the ORF-P.

Figure 2. 2

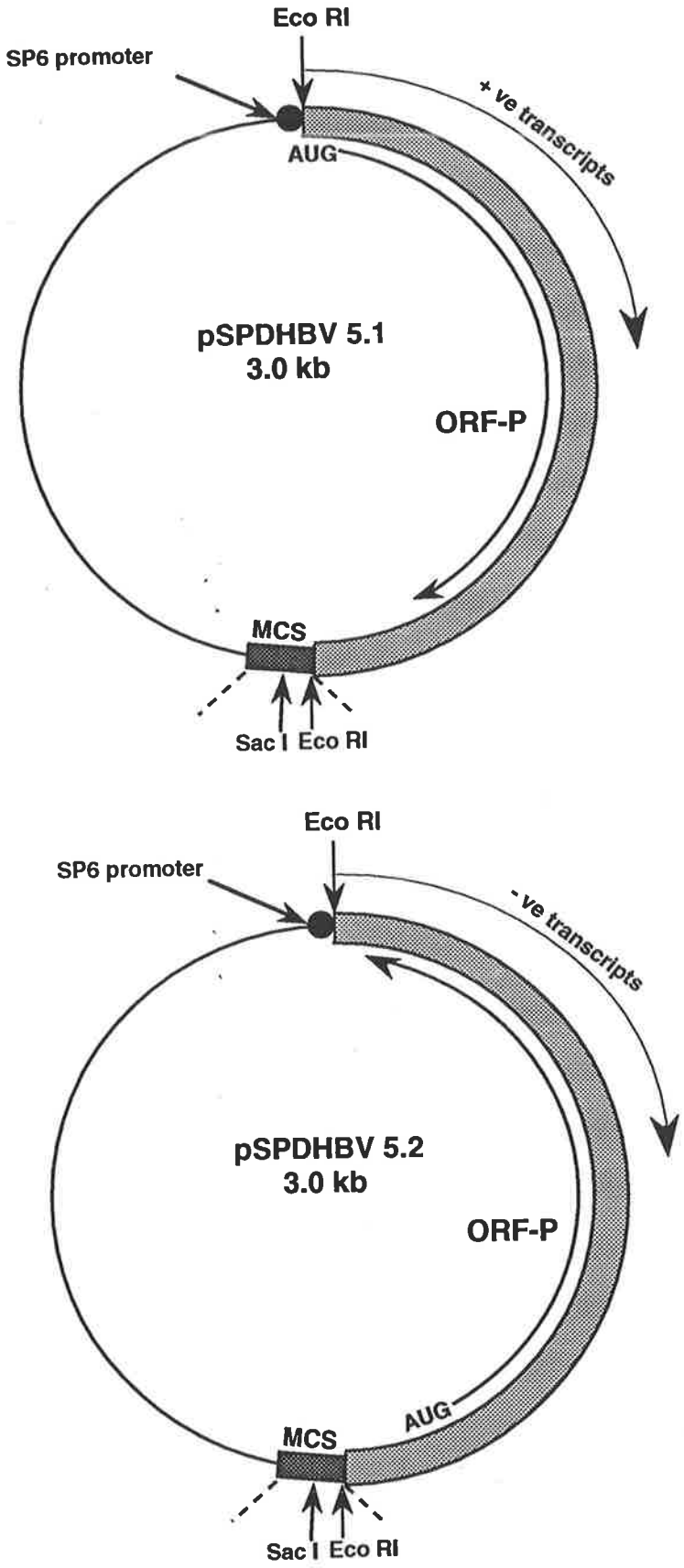


Figure 2.2 A Restriction Endonuclease Map of pSDHBV 5.1 and pSDHBV 5.2

The cells were pelleted by centrifugation at 5,520g in a Beckman JA 14 rotor for 15 min at 4°C, resuspended in 150ml STET and 1ml of 50mg/ml lysozyme in DDW added. The mixture was swirled constantly in a boiling water bath for 5 min followed by pipetting to break up the viscous mass, and centrifuged in a Beckman JA20 rotor for 15 min at 20,000g at 4°C. The supernatant was transferred to a fresh bottle, mixed with an equal volume of ice-cold isopropanol and centrifuged immediately at 20,000g (Beckman JA20 rotor) for 30 min at -5°C. The supernatant was discarded, the pellet redissolved in 17ml of TE-8 and 17.9g of CsCl added. When fully dissolved by swirling at RT for 5min, the suspension was mixed with 0.9ml of 10mg/ml ethidium bromide and centrifuged at 16,500g (Beckman JA20 rotor) for 10 min at 20°C. The supernatant was then transferred to heat seal tubes (Beckman) and centrifuged in the 80Ti rotor (Beckman L8-80 Ultracentrifuge) at 165,000g for 20 hr at 20°C.

The plasmid DNA band (lower of the two bands) was visualised under UV light and collected by using a 19 gauge needle connected to a syringe. Ethidium bromide was removed by repeated extraction with water-saturated butanol. The DNA was precipitated and pelleted as described (Section 2.4.1) and redissolved in TE-8. The DNA was then reprecipitated in ethanol, washed with 70% ethanol, freeze dried and dissolved in 0.5ml-1ml TE-8. The DNA concentration was determined by spectrophotometry at OD 260nm, the stock DNA diluted to give a final concentration of 1mg/ml, then aliquotted in small volumes and stored at -70°C.

C. RNA Probes

^{32}P-labelled RNA probes (riboprobes) were prepared using the Riboprobe Gemini System (Promega). The reaction was based on the protocol recommended by Promega but with minor modifications. The reaction mixture (Appendix 2) was incubated at 37°C for 1hr then 1U of RQ1 DNase (Promega)

was added and the reaction incubated for a further 15 min at 37°C. 1ul samples were spotted on Whatman 542 filter paper and air dried. One filter was treated in 10% TCA for 10 min and the counts compared with that of the non-TCA treated filter to determine the level of incorporation of radioactive ribonucleotides into the RNA transcripts.

The RNA probe was precipitated and pelleted as described (Section 2.4.1). The probe was then redissolved in 50ul of 0.1% SDS in DDW at 65°C for 15 min. A 1ul aliquot was used to determine the yield of RNA probe by scintillation counting. The specific activity of the probes was calculated to be 1-5x10^8 dpm/ug.

D. Preparation of DNA Probes by Nick Translation

100uCi of each ethanolic α-^{32}P-dCTP and -dATP (3000Ci/mmol, Bresatec, Adelaide, South Australia) were mixed and dried in an Eppendorf tube, then redissolved in a 25ul reaction mixture (Appendix 2), incubated at 14°C for 2 hr and the reaction terminated by the addition of EDTA to 40mM and Sarkosyl to 2%.

The incorporated and unincorporated nucleotides were either separated by gel filtration (Sepharose-G50 column, Pharmacia) or by ethanol precipitation as described in section 2.4.3B above. The probes were then freeze dried and redissolved in 50ul of 20mM DTT in DDW. Probe specific activity was determined to be 5-9x10^8 dpm/ug.

E. End-Labelling of Synthetic Oligonucleotides

The oligonucleotide was labelled at the 5' end with γ-^{32}P-ATP by T4 polynucleotide kinase. The labelling was performed by mixing the reaction mixture (Appendix 2) in an Eppendorf tube and incubated for 1hr at 37°C. A further 100ul of DDW was then added to the tube, and the incorporation of the

radioactive nucleotides was determined by TCA treatment as described above. The probes were ethanol precipitated as described above, freeze dried and finally redissolved in 50ul of DDW.

2.4.4 Hybridisation and Post-Hybridisation Procedures

A. Hybridisation Using RNA and DNA Probes

Prehybridisation was performed in a shaking water bath at 42°C for DNA probes or 47°C for RNA probes for 4 hr in a plastic bag with 10ml of hybridisation solution (Appendix 1). The ssDNA and tRNA were first denatured in formamide/DDW by boiling for 10 min, chilled on ice and the other ingredients added. The hybridisation solution contained the same ingredients with the addition of $5x10^6$-$1x10^7$ cpm/ml of the radiolabelled DNA or RNA probes, previously denatured by boiling as described above. The hybridisation was performed in a shaking water bath for 16 hr at 42°C and 47°C respectively.

After hybridisation, the membrane was removed from the plastic bag and washed 2x 15 min in 2x SSC, 0.1% SDS at RT, followed by 2x 30 min wash in 2x SSC, 0.1% SDS at 68°C. The final wash was performed for 2x 15 min in 0.1x SSC, 0.1% SDS at 68°C.

The washed membranes were air dried, wrapped in plastic film and exposed to X-ray film (X-omat RP or AR, Kodak Australasia) at -70°C in a X-ray film cassette with intensifying screens (Kronex; Dupont, USA). After exposure for a variable length of time depending on the intensity of the signal, the cassette was removed from -70°C, warmed to RT and the film processed in an Ilfospeed 2240 (Ilford) X-ray processor. Quantitation of the virus-specific signal was performed visually by comparison with plasmid DNA standards

hybridised on the same membrane and by scintillation counting of dissected membranes.

B Hybridisation Using End-Labelled Probes

Prehybridisation and hybridisation using end-labelled oligonucleotide probe were performed at 37°C for 4 hr and 16 hr respectively in 10ml of hybridisation solution (Appendix 1). End-labelled probe (1-5x10^6 cpm/ml) was added to the hybridisation solution. After hybridisation, the membranes were washed in 6x SSC, 0.1% SDS at RT for 3x 5 min, then 2xSSC / 0.1%SDS at 60°C for 2x 20 min. The membranes were then dried and exposed to X-ray film as described above.

2.5 POLYMERASE CHAIN REACTION

All reagents for the PCR were diluted in diethyl pyrocarbonate (DEPC)-treated DDW (CSL), and all reactions were performed in 0.5ml capped polypropylene microcentrifuge tubes using designated areas, hoods, pipettes and an automatic thermal cycler (Perkin Elmer Cetus). Contamination was minimised by following the recommendations of Kwok & Higuchi (1989).

In a designated laminar flow hood, a master mix containing 10mM Tris-HCl pH8.3, 50mM KCl, 1.5-3mM $MgCl_2$, 50uM of each dNTP, 100pM each of the oligonucleotide primers, 1.25U/reaction of Ampli Taq DNA polymerase (Perkin Elmer Cetus) was prepared and an aliquot of 40ul was dispensed into each PCR tube. In a separate hood, 10ul of the DNA sample prepared as described in section 2.4.1 was then added to the reaction mix in each tube and overlaid with 50ul of mineral oil (Sigma).

In the automatic thermal cycle, the DNA in the reaction tube was denatured initially at 94°C for 3-7 min prior to 25-40 cycles of amplification comprising

94°C 1 min, 50°C 1 min, 72°C 1-3 min and followed by an extension of 7 min at 72°C. An aliquot of the PCR product was analysed by Southern blot hybridisation after gel electrophoresis through 1-2% agarose essentially as described above.

2.5.1 DHBV PCR

The sequence of the primers for DHBV (a gift from Dr Stephen Locarnini, Fairfield Hospital, Melbourne) was:

Primer 1, position 2594-2615: (5'-CGT GTG TGA CTG TAC CTT TGG-3')
Primer 2, position 2978-3000: (5'-GTC GGG TTG GAA ACT TAC TATC-3')

The primers were devised from the DHBV DNA sequence described by Mandart et al. (1984) and designed to amplify a 406 base pair fragment from the core gene region. DHBV DNA immobilised to nitrocellulose as described above was then detected by hybridisation with ^{32}P-labelled RNA probes synthesised from pSP DHBV 5.1 (Section 2.4.3).

2.5.2 HBV PCR

The sequence of the primers (synthesised by staff from the Division of Haematology, IMVS) was:

DAW-1: 5'- AGT GCG AAT CCA CAC TCC -3'
DAW-2: 5'- GAA TTT GGA GCT ACT GTG GAG -3'

These primers were designed to amplify a 384bp HBV DNA from the core region of the HBV genome. The PCR product was analysed by Southern blot hybridisation or spot blot hybridisation (Section 2.4.2) using an end-labelled

oligonucleotide probe #90 (Section 2.4.3). The intensity of the HBV-specific band was compared against dilutions of HBV plasmid (pTK HH2) amplified at the same time.

2.6 DETECTION OF DHBsAg & ANTI-DHBs BY RIA

Protein A-purified anti-DHBs IgG was used as the capture and the detector antibody in direct and competitive RIA to detect DHBsAg and anti-DHBs respectively. A range of concentrations was tested to determine the optimum positive/negative (P/N) ratio, and this is discussed in results (Chapter 3).

2.6.1 DHBsAg Detection

Immunolon 1 Removawell microtitre strips (Dynatech Laboratories) were coated with 100ul of rabbit-anti-DHBs IgG (Section 2.2) in 100mM Tris-PBS pH 9.6 o/n at RT. The wells were then washed three times in PBS and incubated with 2% NRS in PBS for 2 hr at 37°C. 100ul duck serum samples diluted 1/10 in 2% NRS were then added and incubated for 4 hr at 37°C. The samples were removed from the wells and the plate washed three times with PBS prior to incubation with 100ul of ^{125}I-anti-DHBs. A range of concentrations of ^{125}I-anti-DHBs were used to determine the optimum concentration, and this is also discussed in results (Chapter 3). Following incubation at 37°C for 1 hr, the wells were washed three times with PBS containing 0.1% Tween-20 followed by three washes with PBS alone. Individual wells were counted in a Packard Gamma counter and positive samples identified as described (Chapter 3).

2.6.2 Anti-DHBs Detection

Anti-DHBs-coated plates prepared as described above were incubated with 100ul of 5ug/ml of purified DHBsAg in PBS containing 2% NRS for 2 hr at 37°C, then incubated with 100ul of a 1/5 dilution of duck serum in the same diluent as described above for 2 hr at 37°C. The ^{125}I-anti-DHBs was added, incubated, and the wells washed and counted as described above.

2.7 LIVER CELL MEMBRANE PREPARATION AND EUKARYOTIC CELL CULTURE

2.7.1 Preparation of Hepatocyte Membranes

Duck liver tissues (unperfused) were obtained from ducklings aged 5 days and from adult ducks aged 8 months. Human livers were obtained from patients who underwent abdominal surgery and at autopsy as soon as possible after death. These tissues were stored in small pieces in -70°C. Hepatocyte membranes were prepared according to the method described by Hubbard et al. (1983). All membrane isolation procedures were performed on ice and at 4°C.

A. Plasma Membrane Preparation

5g frozen liver tissue was minced and homogenised in 4 volumes of 0.25M STM buffer (0.25M Sucrose, 5mM Tris-HCl pH 7.2, 0.5mM $MgCl_2$) using a loose Dounce homogeniser. Cell debris and unbroken cells were removed from the suspension by centrifugation at 280g (Beckman JA20 rotor) for 5 min. The supernatant was removed into a fresh centrifuge tube while the pellet was homogenised again in an additional 1 volume of STM buffer, and centrifugation repeated as described above. The two supernatants were combined and centrifuged at 1,500g (Beckman JA20 rotor) for 10 min to pellet

plasma membranes. The pelleted plasma membranes were resuspended in 10ml STM buffer and adjusted to a sucrose density of 1.18g/cm^3 with 2M sucrose in TM buffer (5mM Tris-HCl pH7.2, 0.5mM $MgCl_2$). This suspension was aliquotted into Beckman ultracentrifuge tubes and overlaid with 2ml 0.25M STM. Following centrifugation at 78,000g in a SW41 rotor for 1 hr in a L8-80 Ultracentrifuge (Beckman), the plasma membrane-enriched fraction was collected from the interface of 1.18g/cm^3 sucrose and 0.25M STM, washed with STM buffer, centrifuged at 1,500g for 10 min, resuspended in PBS + 0.02% NaN_3, quantitated by Bio-Rad total protein assay (Section 2.7.3), aliquotted and stored at -70°C prior to use.

B. Internal Membrane Preparation

Internal membranes were prepared from the supernatant derived from the 1500g centrifugation step described above. This supernatant was centrifuged at 33,000g for 5 min in a SW41 rotor (Beckman). The pellet was discarded and the supernatant was centrifuged again at 78,000g in the same rotor for 100 min. The pellet was resuspended in 4ml of 57% sucrose in TM buffer, overlaid with 4ml of 37% and 4% sucrose respectively, then centrifuged at 78,000g in a SW41 rotor (Beckman) for 18 hr. The internal membrane-enriched fraction was then collected from the 4-37% sucrose interface, washed and stored as described above.

2.7.2 Enzymatic Confirmation

The separation of internal and plasma membranes was confirmed by the detection of endogenous membrane-associated enzymes: alkaline phosphatase and acid phosphatase.

Alkaline phosphatase, a marker of plasma membranes was measured as described by Ray (1970). 50ul of each sample was added to 950ul of assay

buffer (20mM Tris-HCl pH 9.8, 1mM $MgSO_4$, 2.5uM $ZnSO_4$, 0.2mg/ml BSA and 1mg/ml p-nitrophenylphosphate) and mixed thoroughly. The samples were read by spectrophotometry at OD 405nm immediately after the reaction.

Acid phosphatase activity was used as a marker to estimate lysosomal and mitochondrial contamination of the plasma membrane fraction (Allen and Crumptor, 1970; Appelmans and De Duve, 1955). 200ul of each sample was mixed with 5ml of assay buffer containing 50mM sodium acetate pH 5.5, 1.67mM $MgSO_4$, 55.5mM KCl, 5mM EDTA and 1mg/ml p-nitrophenyl-phosphate. An aliquot of 800ul was sampled and read at OD 405nm at each time interval (0'. 5', 10', 15', 20').

2.7.3 Total Protein Quantitation

Total protein quantitation was determined by a Bio-Rad protein assay. 50ul of test sample was added into 2ml of Bio-Rad reagent, mixed well and incubated for 20 min at RT then read at OD 540nm.

Human serum albumin was used as a standard and diluted to a range of concentrations i.e., 2.5mg/ml, 5mg/ml, 10mg/ml and 20mg/ml in PBS. The protein quantitation curve was plotted as absorbance at OD 540nm vs standard protein concentration.

2.7.4 Media and Culture Methods

The majority of cell culture was performed using Dulbecco's Modified Eagles Medium (DMEM; Gibco) supplemented with 5-10% non-inactivated FBS (Gibco, PA Biologicals CO), 100U/ml penicillin and 40ug/ml gentamycin. MCDB 151 medium (Sigma) containing the above supplements was used in studies of virus expression and secretion. All continuous cell lines were

maintained in flasks (Costar, Corning, Nunc) in the above DMEM supplemented with 5% FBS and antibiotics, and the medium changed every 3-4 days. The cells were subcultured every 7-10 days.

DMEM medium was supplied by the Division of Medical Virology, IMVS and MCDB 151 was prepared according to the manufacturer's instructions and sterilised by membrane filtration. Other cell culture reagents including trypsin, antibiotics and PBS were supplied by the Division of Medical Virology, IMVS.

To improve the adherence of some cell cultures, collagen (rat tail; Boehringer) was used to coat the plastic culture plates. The solution was prepared and applied in sterile 0.2% acetic acid (v/v) to give a final concentration of 2mg/ml as instructed by the supplier. The culture plate was coated with the solution (2.5ul/cm^2 surface area) by spreading it carefully on the bottom of the culture dish with a sterile rubber policeman, then air dried for about 1 hr at RT in a laminar flow cabinet. The plate was used immediately after washing the coated surface with PBS.

2.8 HEPADNAVIRIDAE RECEPTOR-BINDING ASSAYS

2.8.1 Binding Assay in Liver Membranes By Solid-Phase RIA

Binding of purified hepadnaviruses to liver membranes (duck or human) was performed by solid-phase RIA essentially as described (Pontisso et al., 1989b).

A. DHBV-Receptor Binding Assay

50ul of membrane preparation (Section 2.7) containing 15ug of protein (i.e., 300ug/ml) in PBS was coated to wells of Immunolon 1 "Removawell" microtitre strips (Dynatech Laboratories) and incubated o/n at RT. The wells

were washed 3 times with PBS, 60ul of 10% BSA in PBS was added to each well and incubated for 4 hr at RT or o/n at 4°C. 50ul of purified DHBV virions diluted to 30ug/ml in 2% BSA was added to the wells and incubated for 16-20 hr at 4°C. The wells were then washed thoroughly with cold PBS (4°C), then incubated for 4 hr with 50ul of ^{125}I-labelled anti-DHBV preS ($1x10^6$ cpm/well in 2% BSA) at 4°C. The plate was then washed 3 times with cold PBS and individual wells counted in a Packard Gamma counter.

In an experiment to block the receptor binding sites, DHBV virions were preincubated with MAb to DHBV preS for 1 hr at 37°C before incubation with plasma membranes and bound virus was detected by ^{125}I-labelled anti-DHBs in this experiment (Qiao at al.,1990).

B. HBV-Receptor Binding Assay

The binding assay of purified HBV to human liver membranes was performed in a similar manner as described above except that: i). the concentration of the plasma membranes for coating the plate was optimised to be 1ug/ml (See Chapter 5); ii). a combination of 10% NRS + 5% BSA in PBS was chosen as the blocking agent and 2% NRS + 1% BSA in PBS as the diluent; iii). purified HBV virions (Section 2.1) and ^{125}I-labelled anti-preS1 (MA18/7, Section 2.2) were used to detect the receptor binding activity; iv). a single wash with PBS+0.05% Tween-20 was included after incubation with ^{125}I-labelled antibody and before washing with PBS to reduce non-specific binding.

2.8.2 HBV-Receptor Binding in Monolayer Culture

Cells were subcultured for 2 days in 48- or 24-well plates at $1x10^5$ and $2x10^5$ cells/well respectively and cultured in DMEM supplemented with 5% FBS at 37°C in 5% CO_2 (Section 2.7.4). Before virus adsorption, the cell monolayer was washed 3 times with cold PBS (4°C) and the plates kept on ice

throughout the binding assay. 200ul (for 48-well) or 500ul (for 24-well) of purified HBV virions (2-5x10^7vge) or mock purified HBV in DMEM supplemented with 2% FBS were incubated for 4 hr. The cells were then washed 3 times with cold PBS and incubated with 200ul and 500ul respectively of ^{125}I-labelled anti-preS1 containing 2x10^6 cpm in the above medium for 2 hr. The cells were then washed once with cold PBS+0.05% Tween-20, followed by 3 times with PBS. The cells were then removed from the well in 200ul of 2% SDS in PBS, and the suspension transferred into 3DT tubes and then counted in a Packard Gamma counter.

2.8.3 Inhibition Assays

A. Using Synthetic Peptides

Synthetic peptides 12-32 and 120-145 from the preS1 and preS2 regions respectively of the HBV surface antigen gene were kindly supplied by Dr S Kent (The Scripps Research Institute, La Jolla, San Diego, USA); peptide 32-49 from the preS1 region was a gift from Professor CR Howard (Royal Veterinary College, London, UK), and peptide 21-47 was synthesised by Peptide and Protein Research Consultants (Washington Singer Laboratory, Exeter, UK). An aliquot of each of these peptides was made up to a concentration of 1mg/ml in DDW. A range of concentrations (50-500ug/ml) of each of these peptides was preincubated for 1-2 hr at 37°C with the cell monolayers or the liver membranes coated to plates. The cells or cell membranes were then washed with PBS for 3 times before the addition of HBV as described above.

B. Using HBV-Specific Antibody

To block the receptor binding site on HBV, purified HBV virions (5x10^8 vge/ml) were preincubated with 100ug/ml of the total IgG or Fab fragment (Section 2.2.3) of the HBV-specific antibody (MA 18/7) at a ratio of 1:1 (v/v) at 37°C for

20 min prior to incubation with liver membranes or cultured cells. In this assay, a ^{125}I-labelled polyclonal anti-HBs (Bohringwerke) was used as the detector.

2.8.4 Detection of HBV Internalisation

After 4 hr incubation at 4°C with HBV, the cells were washed 3 times with cold PBS then cultured at 37°C for various times (see Chapter 6) in the culture medium. At each time point after 37°C incubation, a duplicate well of each cell line was placed on ice and trypsinised for 10 min with prechilled 1% trypsin, transferred into Eppendorf tubes, washed 5 times with cold PBS and pelleted in a microcentrifuge at 6000rpm for 2 min in a cold room. The cell pellet was resuspended in 200ul of DDW, Proteinase K treated, the DNA extracted and precipitated as described (Section 2.4.1). The DNA pellet was redissolved in 20ul DDW and a 10ul aliquot was then amplified by PCR as described (Section 2.5).

2.9 USE OF ANIMAL MODEL

2.9.1 Source of Animals and Conditions of Animal Housing

1-day-old Pekin-Aylesbury DHBV-free ducklings were supplied by Tegal Duck Hatcheries Pty Ltd (Tahmoor, New South Wales, Australia). New Zealand White rabbits and Balb/c mice were obtained from the Gillies Plains Animal Resource Centre, IMVS.

All animal handling procedures were performed in the animal house and animal research theatre in accordance with the IMVS Animal Ethics Committee guidelines. The animals were maintained by the staff of the IMVS animal house.

2.9.2 Duck Inoculation

The ducks were inoculated by IV injection of the neck vein with a standard inoculum of DHBV containing $3x10^8$ vge/ml. In the time course studies, ducks aged 1, 2, 3, and 4 weeks were inoculated with 100ul, 250ul, 500ul and 1ml respectively of the standard inoculum.

2.9.3 Partial Hepatectomy

On the day of surgery, food and water were withheld for 2 hr and 0.5ml of Streptopen (Heriot Equet Pty Ltd) was given intramuscularly. Following endotracheal intubation, general anaesthesia was induced with halothane and nitrous oxide and maintained throughout the operation. The duck was laid in a recumbent position, the feathers were removed from the central and right lateral abdomen, and the surgical site was scrubbed with betadine.

An incision was made in the skin from the central and right lateral abdomen parallel to the last rib, and the subcutaneous fat layer and then the abdominal muscle layers were cut to expose the abdomen wall. This was opened to reveal part of the right lobe of the liver. Two sutures (Chromic 1) were placed from the posterior to anterior surfaces of the right lobe near the middle line below the gall bladder and were tied securely to restrict the blood flow to a portion of the right lobe. The liver below the sutures was then excised carefully. The left lobe was removed in a similar way except that one suture was adequate. The abdomen was reclosed and the birds were kept warm and quiet for a 4 hr period.

Laparotomy was performed as a control for postoperative shock and blood loss during operation. The same operating procedures were used except that

the liver was not resected. The percentage of liver excised was determined according to published data of total duck liver weight (Nickel et al., 1977).

CHAPTER 3

ESTABLISHMENT OF SEROLOGICAL ASSAYS FOR DETECTION OF DUCK HEPATITIS B INFECTON

3.1 INTRODUCTION AND AIMS

Since the next three chapters focus on studies of the pathogenesis of hepadnaviruses using DHBV as a model, a brief introduction to DHBV is presented as the basis of these studies.

3.1.1 Natural Infection of Duck Hepatitis B Virus

Duck hepatitis B virus (DHBV) was first discovered in 1980 in brown ducks from Chi-tung county of the People's Republic of China (Mason et al.,1980). These ducks were observed to have a high incidence of hepatoma, and subsequently HBV-like particles were found in the duck sera (Mason et al., 1980). Following the initial discovery of the virus, DHBV was isolated from up to 10% of some commercial flocks in the United States of America (Mason et al.,1980), 1-6% in France and the United Kingdom (Robinson et al., 1984), and up to 70% in Australia (Freiman & Cossart, 1986) and in other domestic ducks (Mason et al., 1980).

Natural transmission of the virus in flocks of Pekin ducks is considered to be predominantly vertical from mother to eggs (O'Connell et al., 1983). The evidence for this include i). DHBV was usually be detected in embryonated eggs of infected females; ii). almost all ducklings hatched from eggs obtained from DHBV-positive ducks became chronic carriers of DHBV.

It has been suggested that the primary site for DHBV replication is in the yolk sac tissue of the developing duck embryo (Urban et al., 1985). Viral replication in the yolk sac was detectable earlier and in higher levels than in the developing embryonic liver (Tagawa et al., 1987). However, the exact route of transmission from the dam to the yolk sac still has to be determined.

It has not been well established if DHBV causes liver disease in its host. However, histological studies of liver samples from DHBV-carrier ducks from Chi-tung county in China revealed a variety of liver diseases in most DHBV-infected ducks but none in two DHBV-negative flocks (Omata et al., 1983; Marion et al., 1984). There appeared to be no correlation between the degree of severity of hepatitis and levels of virus in serum or in liver, suggesting that the hepatic injuries in some of the ducks may have been caused by other non-viral factors. The liver disease seen in Chinese ducks was also observed in DHBV-infected ducks from Australia (Freiman & Cossart, 1986) but not in those from USA and thus the response of ducks to DHBV infection may vary with the species of ducks and perhaps with the diversity of the inoculum.

Horizontal transmission, a common route for transmission of HBV, has not been proven so far in ducks. The infection rate in breeding age ducks was the same as that in neonatal ducks, suggesting that horizontal transmission of the virus was unlikely (Marion et al., 1983). However, as the level of DHBV DNA usually declines as ducks grow older, horizontal transmission could occur to maintain the rate of infection in adult ducks at the same level as that found in neonatal ducklings (Robinson et al., 1984).

3.1.2 DHBV Biology:
A Comparison with the Mammalian Hepadnaviruses

DHBV shares many biological features with other mammalian hepadnaviruses, but there are some significant differences. Although it has a similar size and morphology to the mammalian hepadnaviruses, the DHBV virion is more pleomorphic, and has unique spike-like projections around the nucleocapsid that are not apparent on the core of HBV (Mason et al., 1980). In addition, the surface antigen particles are larger and more pleomorphic than those of the mammalian viruses and vary between 35-60nm in diameter (Mason et al., 1980). A filamentous form of DHBsAg particles has not been described (Mason et al., 1980; Marion & Robinson, 1983). The reason for the differences in the physical structure of DHBV from the mammalian hepadnaviruses are not yet understood.

The buoyant densities of DHBV virions and DHBV surface antigen particles also differ from those of the mammalian viruses. The buoyant density of DHBV in CsCl is 1.16g/cm^3 compared with 1.225-1.24g/cm^3 for the mammalian hepadnaviruses, and surface antigen particles of DHBV band at 1.14g/cm^3 compared with 1.18-1.2g/cm^3 for the mammalian hepadnaviruses (Werner et al., 1979; Gerlich et al., 1980; Feitelson et al., 1982; Marion, 1988).

The DHBV genome is 3021bp i.e., approximately 160bp shorter than that of HBV and is virtually completely double-stranded (Mason et al., 1981). Only three ORFs (ORF-S, -C and -P) have been identified in the DHBV minus strand (Mandart et al., 1984); the S gene of DHBV is smaller than those of the mammalian viruses, the X gene is missing and this results in one large ORF-C coding for secreted DHBeAg and structural DHBcAg (Schlicht et al., 1987). A low degree of genome homology has been found between DHBV and HBV (Sprengel et al., 1985), but the aa sequences from corresponding ORFs

revealed a clear relationship between the two genomes and thus indicated a common ancestry of the two viruses (Sprengel et al., 1985).

The DHBV envelope contains two virus-encoded proteins which result from translational initiation at two different in-phase start codons to produce proteins of 328 and 167 aa representing the preS/S and S envelope proteins respectively (Pugh et al., 1987; Schlicht et al., 1987). SDS-PAGE analysis of DHBsAg revealed only one major polypeptide of ca. 17kDa (Marion et al., 1983) compared with p24 and gp27 of HBV. One species of pre-S antigen with a molecular weight of 36kDa has also been detected (Pugh et al., 1987). In addition, a third envelope protein of 28kDa was also identified by immunoblotting of infected duck liver with rabbit anti-preS and this may represent a preS2 protein of the DHBV surface antigen (Yokosuka et al., 1988). None of these studies suggested that DHBV surface antigen polypeptides were glycosylated.

Three major species (3.2, 2.1 and 1.9kb) of polyadenylated, unspliced transcripts have been isolated from DHBV-infected liver (Mason et al., 1981; Buscher et al., 1985) The transcripts each initiate at a different site, but are polyadenylated at the unique polyadenylation site. The transcripts are thought to encode the following proteins: i). DHBcAg (p30), DHBeAg (p27, gp30, and gp33), and the multifunctional polymerase (Schlicht et al., 1987); ii). the preS+S proteins (p36 and p28); iii). the S protein (p17, Marion et al., 1983).

No serological cross-reactivity was detected between the proteins of DHBV and those of the mammalian hepadnaviruses (Marion & Robinson, 1983). However, sequence analysis has identified a region of 54 codons in the centre of the S gene of HBV that is absent in DHBV and encodes the subtype-specific antigenic determinants of HBsAg (Prince et al., 1982; Gerin et al., 1983). It is possible that the lack of this domain in DHBsAg explains the lack of

serological cross-reactivity between the two surface antigens (Sprengel et al., 1985).

3.1.3 Use of Duck Models to Study Hepatitis B Virus

Because Hepadnaviruses exhibit a limited host and tissue range and because no available cell lines are susceptible to infection, most effort has been focused on the use of the animal host, animal models and transgenic mice. Indeed, our understanding of the mechanisms of Hepadnavirus replication in the last few years has been gained almost entirely from the animal Hepadnavirus systems.

The animal Hepadnaviruses serve as convenient models for HBV. They have been used to investigate acute and persistent infection and to study pathogenesis in ways not possible with HBV infection in man. Of all the animal hepadnaviruses, DHBV has proved to be the most convenient to study hepadnavirus replication, pathogenesis and tissue tropism. The advantages of using this virus include: i). although closely related to HBV, DHBV is sufficiently different not to present a health risk to those handing infected materials and working with DHBV-infected ducks; ii). Pekin ducks are readily available commercially for experimental studies; iii). ducks are easy to house and handle; iv). DHBV virions can be isolated readily from vertically-transmitted birds before the immune system is well developed and thus, virus isolated from these ducks is free of immune complexes.

The animal Hepadnavirus infections provide ideal models for studies of the effect of therapeutic regimens on acute and chronic viral diseases. Several antiviral agents have been evaluated in live ducks (Sherker et al., 1986). In particular, the nucleoside analogue adenine arabinoside and the purine and pyrimine 2',3'-dideoxynucleosides have been tested (Jacyna &

Thomas, 1990). Recently, the guanosine analogue ganciclovir was used to treat congenitally-infected ducks and resulted in prompt and profound inhibition of viral DNA replication in serum and liver (Wang et al., 1991).

3.1.4 Background and Aims

Rapid progress in HBV research has been dependent, in part, upon the development of assays for hepatitis B antigens and the corresponding antibodies. Serological detection of viral antigens and antibodies plays an important role in the investigation of viral infection and provides a powerful tool for the diagnosis of viral infection, for determining the host susceptibility and immune response, and for evaluation of antiviral therapy. For the same reason, the potential usefulness of the ducks as a model for HBV infection of adult humans has been severely restricted due to a general lack of reagents with which to measure the serological profile of DHBV infection. Previous studies (Mason et al., 1983; Omata et al., 1984; Fukuda et al., 1987; Jilbert et al., 1987; 1988) relied solely on the detection of DHBV DNA by hybridisation analysis as a measure of viraemia. Thus the main aim of this chapter was to establish a simple and specific assay to detect DHBsAg and anti-DHBs. These assays were used to monitor DHBV infection in experimentally-infected ducks as part of my aim to refine the DHBV model and to study the pathogenesis of Hepadnavirus infection in ducks. The actual development of these assays is discussed in this chapter and applications discussed in Chapter 4.

3.2 EXPERIMENTAL DESIGN

Immunoassays use the specific interaction of antibody with antigen to provide semi-quantitative information of antigen (or antibody) concentration in test samples. Among the most sensitive and specific techniques developed for the

detection of HBV markers is RIA (Aach et al., 1971; Hollinger et al., 1971; Lander et al., 1971b). The exquisite sensitivity of the method enables as little as 1-10ng of viral antigen/ml to be detected in clinical specimens (White & Fenner, 1986). Therefore, RIA was chosen for the detection of DHBsAg and anti-DHBs in the duck model.

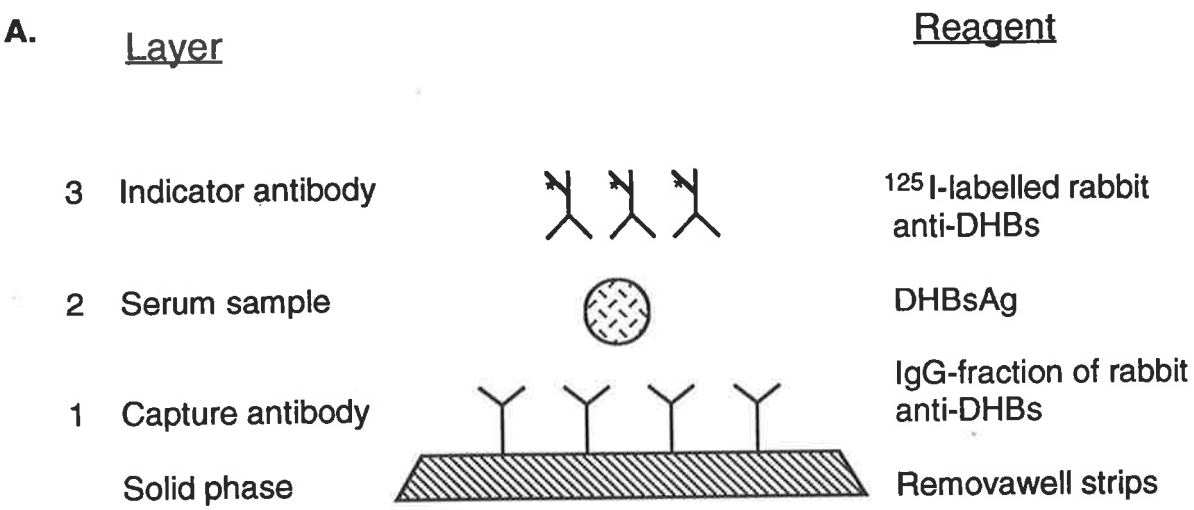
There are a number of different methods to perform RIA; direct, indirect, and reversed assays have been described (Halonen et al., 1984). Most of these are solid-phase RIAs in which the capture antibody or antigen is attached to a solid substrate, e.g., polystyrene microtitre plates. In this study, a direct solid-phase RIA to detect DHBsAg and a competitive solid-phase RIA to detect anti-DHBs were chosen for their simplicity and specificity.

In solid-phase RIA for DHBsAg detection, the capture antibody (rabbit anti-DHBs) is bound to a solid phase. The specimen is then incubated with the solid-phase, unbound material is washed off, and a radiolabelled antibody is then added. After a final wash, the bound label is measured directly by a gamma counter (Figure 3.1A). If DHBsAg is present in the sample, it will be captured and bound by the immobilised antibody (rabbit anti-HBs). The level of radioactivity binding to the solid-phase will therefore be proportional to the concentration of specific viral antigen in the test solution.

The competitive RIA to detect anti-DHBs uses a carefully-measured level of radiolabelled antibody to compete with antibody in the test serum for a finite number of antigenic sites previously bound to the solid-phase antibody. Thus if anti-DHBs is present in the test sample, the proportion of the radiolabelled rabbit anti-DHBs bound to the antigen will be reduced. The level of radioactivity bound to the solid-phase will therefore be inversely proportional to the amount of specific antibody in the test serum (Figure 3.1B).

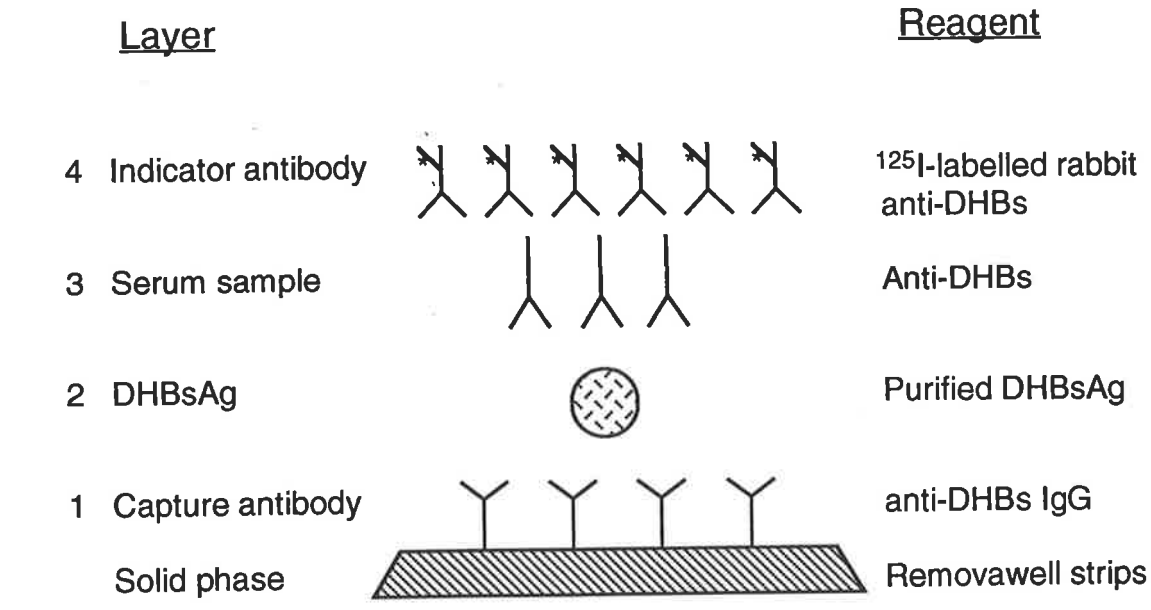
Figure 3.1

A.



Direct Radioimmunoassay (DHBsAg)

B.



Competitive Radioimmunoassay (Anti-DHBs)

Figure 3.1 Radioimmunoassay for Detection of DHBsAg by Direct RIA (A) and Anti-DHBs by Competitive RIA (B)

Since the presence of non-specific antibodies in the antisera used for capture and/or detection is most likely to give rise to false-positive reactions in a solid-phase RIA, it is necessary to use purified viral antigen as immunogen. Thus, in this study I have used a pool of DHBV-containing sera from congenitally-infected ducks as a source of DHBsAg. The purification procedure was described in Section 2.1.

Protein A purified-anti-DHBs IgG was used as the capture and detector (iodinated) antibody in direct and competitive RIA to detect DHBsAg and anti-DHBs respectively. A range of concentrations was used to determine the optimum P/N ratio. The optimal concentration of IgG in a solid-phase RIA was titrated against increasing concentrations of antigen and detector antibody. The concentrations required depend on the specific activity of the IgG preparation. This is described in results (Section 3.3.4).

3.3 RESULTS

3.3.1 Purification of DHBsAg

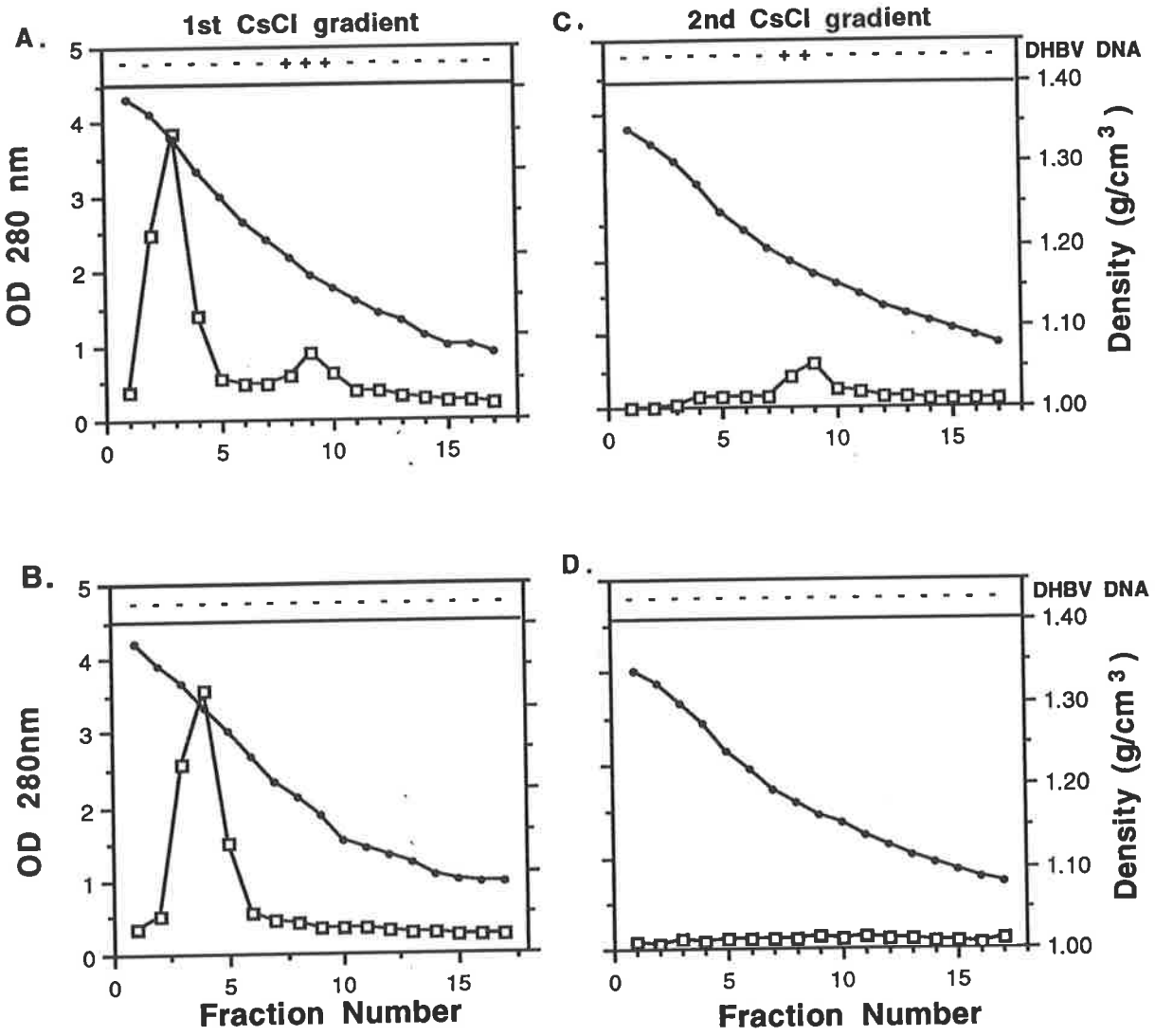
DHBsAg was purified from the serum of congenitally-infected ducks by centrifugation through a 20% sucrose cushion followed by two consecutive CsCl gradients (Section 2.1). Fractions containing DHBsAg-associated particles in each gradient were determined by absorbance at OD 280nm, by EM (Section 2.1.5), and by dot blot hybridisation of DHBV DNA (Section 2.4). The purity of the final preparation was also confirmed by polypeptide analysis (Section 2.3.6).

Figure 3.2 represents an analysis of a typical DHBsAg preparation. In the first CsCl gradient (Figure 3.2A), two peaks were observed by absorbance at OD 280nm. The first peak (major peak) was found in fractions 2-4 with buoyant

Figure 3.2

The purification of DHBsAg; two successive CsCl gradient centrifugations were carried out after viral particles were pelleted through 20% sucrose. The CsCl density was estimated from the refractive index (•). The absorbance at 280nm (□) and DHBV DNA (+ or - shown on top of the graphs) were measured in both gradients. (A) and (C), and (B) and (D) represent an example of the preparation from DHBV-positive and normal duck serum samples respectively.

Figure 3.2


A.
1st CsCl gradient
OD 280 nm
B.
OD 280nm
Fraction Number
C.
2nd CsCl gradient
DHBV DNA
Density (g/cm 3)
D.
DHBV DNA
Density (g/cm 3)
Fraction Number

densities between 1.28-1.37g/cm^3 and was present in both DHBV-infected and uninfected serum (Figure 3.2B) indicating that it was not DHBV-specific. The second peak (minor peak) with buoyant densities ranging from 1.16-1.20g/cm^3 was observed in fractions 8-10 from sera of DHBV-infected ducks only (Figure 3.2A). These fractions were pooled and centrifuged once more in CsCl as described (Section 2.1).

The second CsCl gradient, containing the above pooled fractions from the first gradient, showed a single peak of absorbance at OD 280nm between fractions 8-10, which corresponded to a buoyant density of 1.17g/cm^3 (Figure 3.2C). In contrast, mock purified DHBsAg from uninfected duck serum showed no absorbance at this density (Figure 3.2D).

DHBV DNA was quantitated by dot blot hybridisation in fractions from both CsCl gradients by comparison with a range of dilutions of a known plasmid DNA standard pSPDHBV 5.1 (Figure 2.1). A total of 1.65x10^{10} DHBV virions (assuming that all virions were complete) was present in the starting serum used for DHBsAg purification; 19.8% of the total input DHBV DNA was detected in fractions 8-10 corresponding to a density of 1.16-1.20g/cm^3 of the first gradient (Figure 3.2A). However, this was reduced to 0.05% of the total input in fraction 9 of 1.17g/cm^3 after the second gradient centrifugation (Figure 3.2C).

The level of DHBsAg was subsequently tested using the RIA developed in this study and quantitated against purified DHBsAg (Section 3.3.5 below). DHBsAg was detected in fractions 8-10 of the first gradient and fraction 9 of the second gradient and was determined to be approximately 44% and 36% of the total DHBsAg respectively in the two consecutive CsCl gradients representing approximately 1.74x10^{10} and 1.39x10^{10} DHBsAg particles/ml

respectively (Table 3.1). This figure was derived from a formula for the quantitation of HBsAg particles (Burrell, 1980).

Electron microscopic examination of the DHBsAg contained in fraction 9 of the second gradient revealed numerous empty particles. These particles were pleomorphic and ranged in size from 35-60 nm in diameter (Figure 3.3). In addition, a very limited number of DHBV virions were observed in the preparation.

The polypeptide pattern of the final DHBsAg preparation was also analysed by silver staining after SDS-PAGE. This revealed a major band at 17kDa and a minor band at 28kDa (Figure 3.4). The 36kDa polypeptide detected previously by others, and likely to correspond to the L-DHBsAg was not detected in this preparation. It also revealed that the DHBsAg was free from non-specific serum protein and other forms of DHBV-specific protein contaminants.

3.3.2 Production and Specificity of the Rabbit Anti-DHBs

After immunisation of rabbits as described in Chapter 2.2, the levels of anti-DHBs were determined by IF of DHBV-infected duck liver sections; both rabbits developed high titres (1/1000 and 1/750 respectively) of serum antibody against DHBsAg. Indirect IF (Figure 3.5A) of DHBsAg in DHBV-infected duck liver revealed intense DHBsAg staining in a majority of hepatocytes. In contrast, DHBV-infected liver sections stained with preimmune serum, and normal duck liver sections stained with the rabbit anti-DHBs showed no staining (Figure 3.5B). This result indicated that the antiserum reacted specifically with DHBsAg in DHBV-infected liver samples.

Table 3.1

QUANTITATION OF DHBV MARKERS DURING DHBsAg PURIFICATION

Step	DHBV DNA ng (%)	vge/ml	DHBsAg ng (%)	DHBsAg particle/ml
Neat Serum	500 (100)	1.65×10^{10}	1941 (100)	3.88×10^{10}
1st gradient	92 (19.8)	3.1×10^{9}	870 (44)	1.74×10^{10}
2nd gradient	0.28 (0.05)	8.2×10^{6}	698 (36)	1.39×10^{10}

Figure 3.3

Electron microscopy of purified DHBsAg particles. Purified DHBsAg particles from fraction 9 of the 2nd gradient were stained with 3% PTA then examined at a magnification of 33,000x in a JEOL-100C electron microscope. Bar = 100nm.

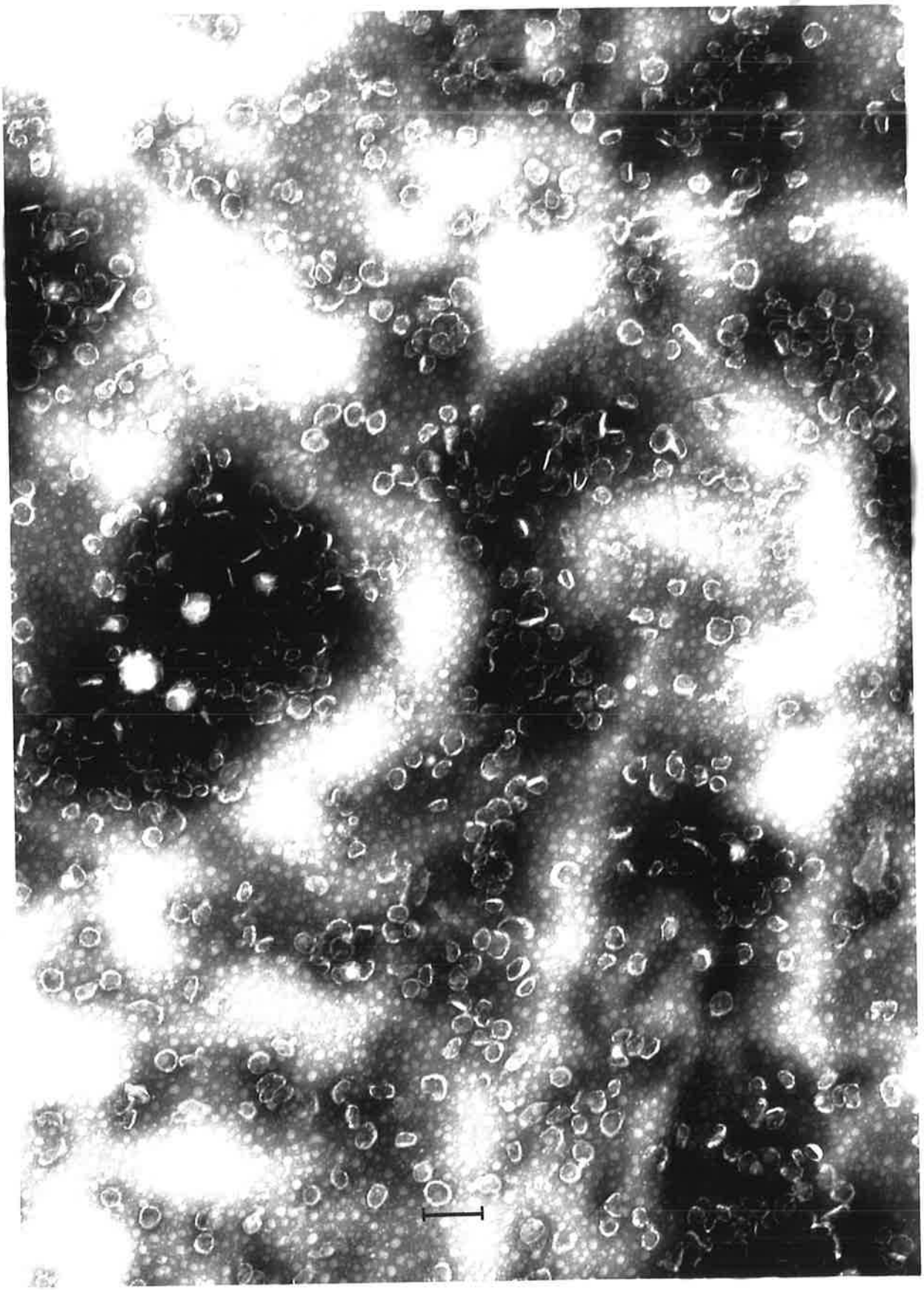


Figure 3.4

Analysis of purified DHBsAg by SDS-PAGE. The gel was stained by silver staining (Bio-Rad). Track 1 represents low molecular weight protein marker (Bio-Rad) and track 2 shows the purified DHBsAg preparation.

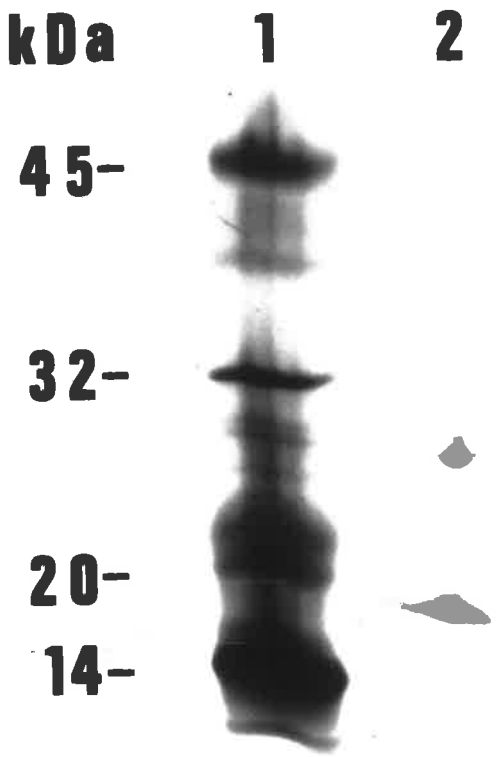
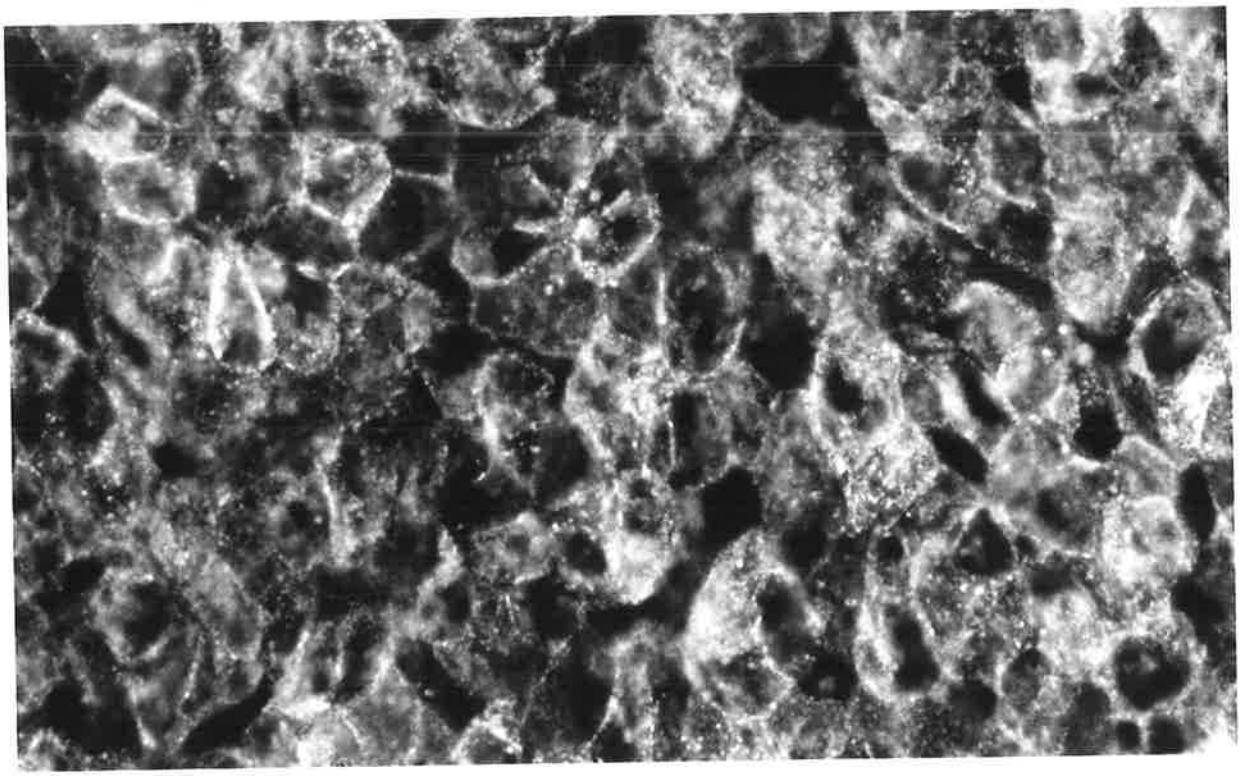
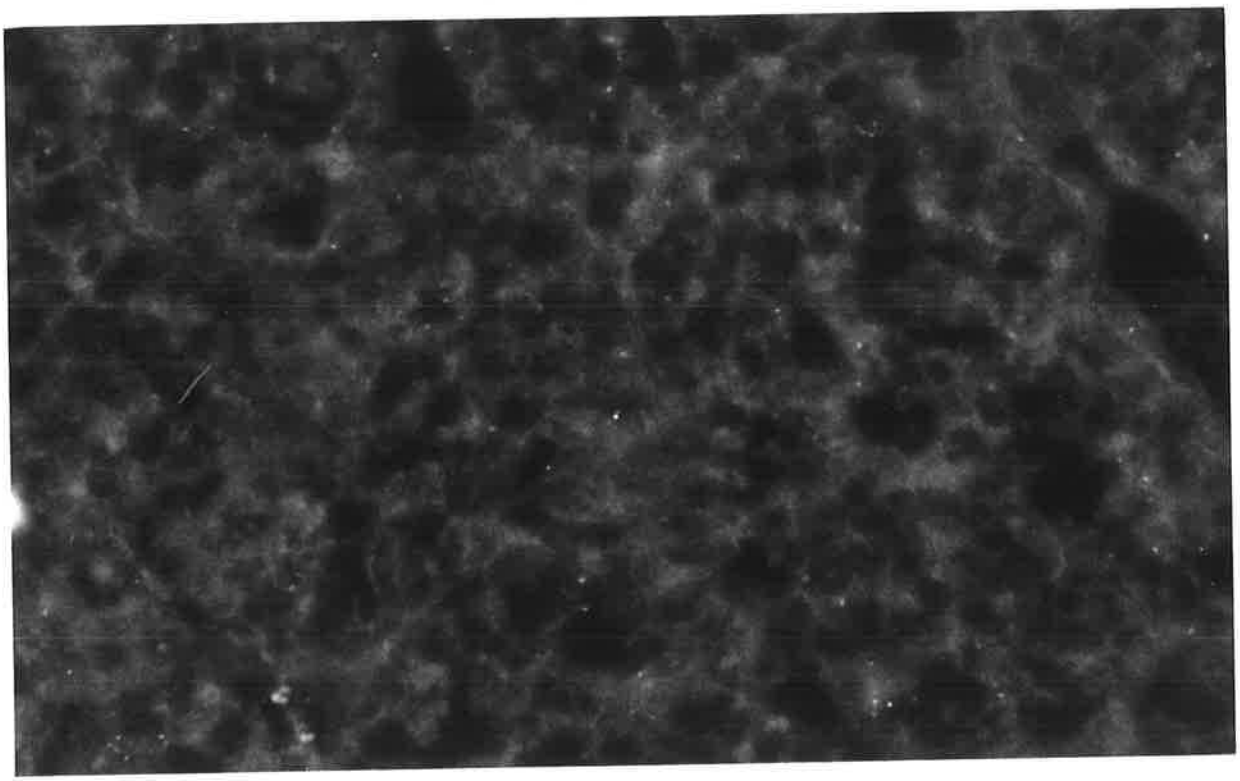

kDa
1
2
45-
32-
20-
14-

Figure 3.5

Immunofluorescence staining of DHBsAg. Frozen liver sections from a DHBV-infected duck were stained by indirect immunofluorescence using anti-DHBs raised in this study (top) and preimmune serum (bottom) respectively. Magnification x 400.





Immunoblotting of DHBsAg preparations using this specific antibody was also carried out. Two bands of 17kDa and 28kDa were detected (Figure 3.6), whereas the corresponding preimmune serum failed to detect either band. These proteins are likely to correspond to the S and M proteins respectively.

3.3.3 Optimisation of the RIA

The assays for DHBsAg and anti-DHBs were optimised by titration of both capture and detector antibodies (Figure 3.7). The capture antibody was titrated from 0.03ug/well to 4ug/well and ^{125}I-labelled anti-DHBs as the detector antibody was used from $1x10^5$ cpm/well to $5x10^6$ cpm/well. The results were calculated according to the P/N ratio which was determined by testing a DHBV DNA-positive and a DHBV DNA-negative sample in parallel.

DHBsAg was detected with concentrations of capture antibody ≥ 0.06ug/well and when the ^{125}I-labelled detector antibody concentration was $\geq 5x10^5$ cpm/well. In contrast, the P/N ratio was around the cut-off value (described in section 3.3.4) or below when the concentration of the capture antibody was 0.03ug/well irrespective of the concentrations of the detector antibody. In addition, the P/N ratio was also reduced to cut off value or below with all concentrations of the capture antibody when the concentration of the detector antibody was $<5x10^5$ cpm/well.

Concentrations of 1ug, 2ug, 4ug/well of the capture antibody gave equal P/N ratios of 2.9:1 when the detector antibody concentration was $1x10^6$ cpm/well. However, when the detector antibody concentration was $\geq 2.5x10^6$ cpm/well, the highest P/N ratios (up to 3.8:1) were actually obtained from wells with lower concentrations of the capture antibody i.e., 0.25ug, 0.5ug and 1ug/well. In contrast, high concentrations of the capture antibody (1-4ug/well) gave the highest P/N ratio when the detector antibody was reduced to $1x10^6$ cpm/well.

Figure 3.6

Immunoblot analysis of DHBsAg preparation. The DHBsAg preparation was immunoblotted using anti-DHBs raised in this study. Tracks 1 and 2 represent preimmune serum and the hyperimmune antibody raised in this study respectively.

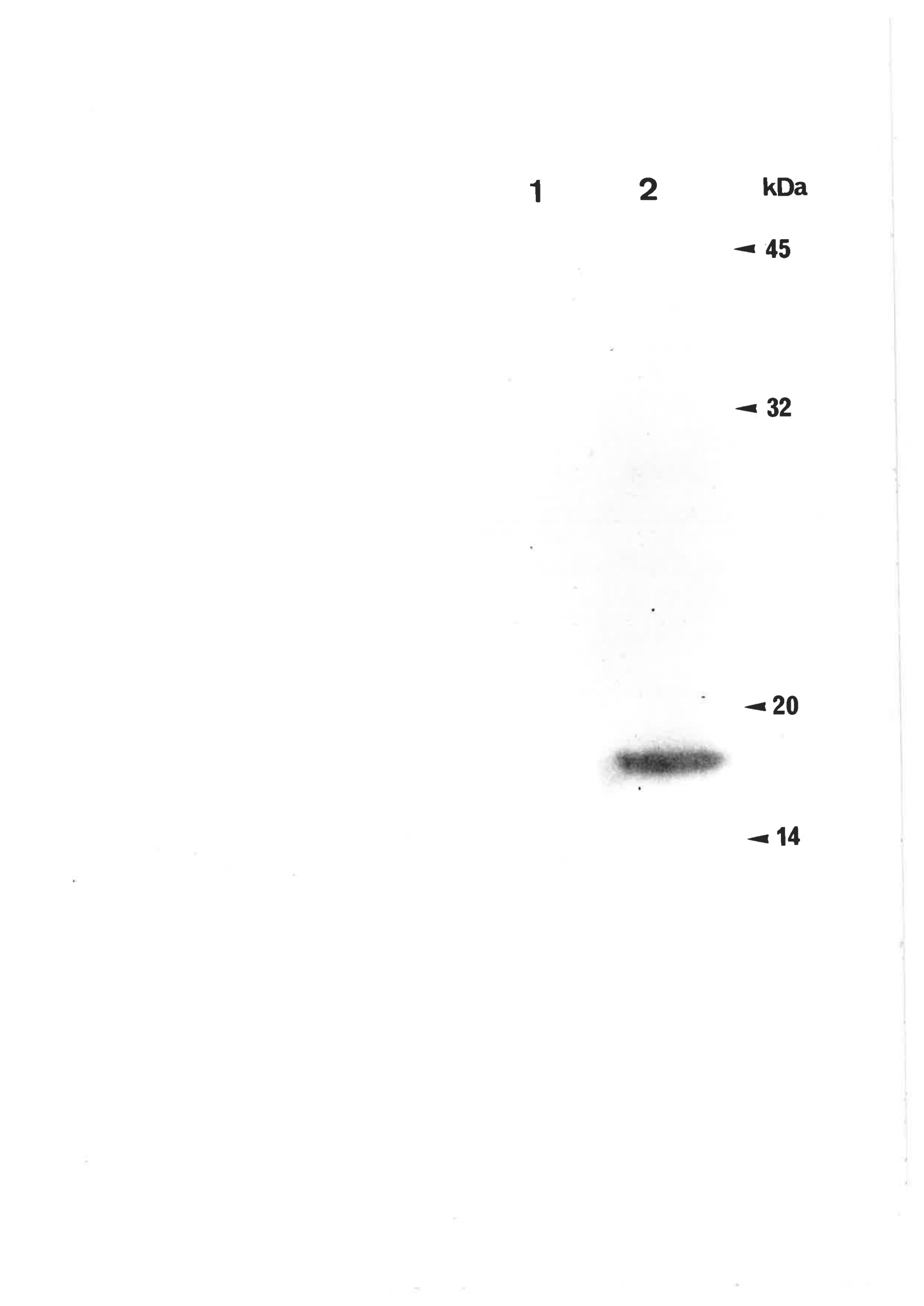
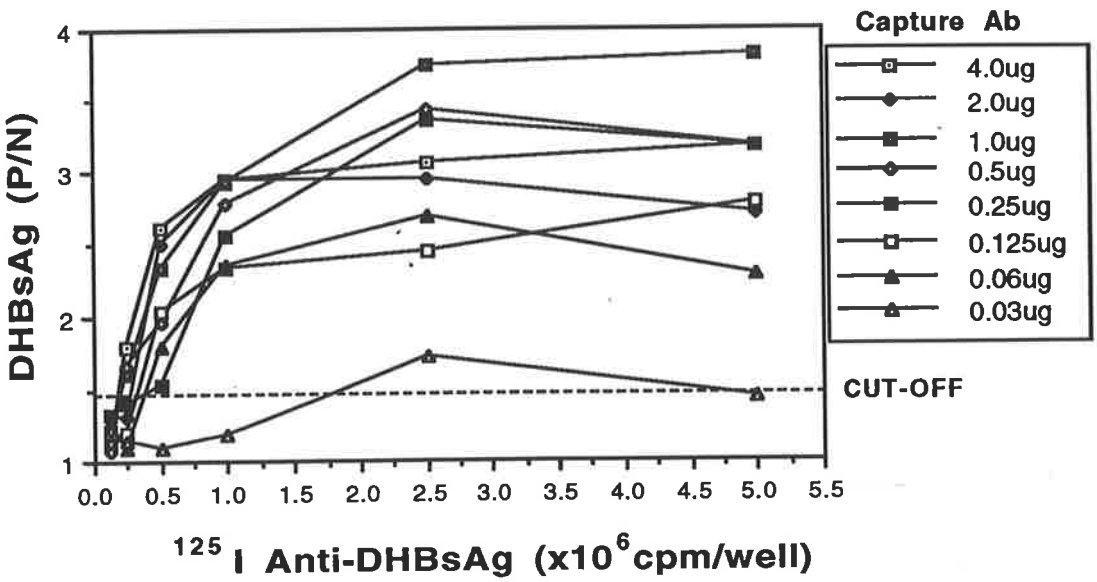

1
2
kDa
45
32
20
14

Figure 3.7

Titration of capture- and detector-anti-DHBs in RIA. The capture- and detector-anti-DHBs were titrated from 0.03-4ug/well and $1x10^5$-$5x10^6$cpm/well respectively. The results are shown as P/N ratio and the cut-off was calculated according to the formula, mean± 3SD.

Figure 3.7


Capture Ab
4.0ug
2.0ug
1.0ug
0.5ug
0.25ug
0.125ug
0.06ug
0.03ug
CUT-OFF
DHBsAg (P/N)
4
3
2
1
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
125 I Anti-DHBsAg (x10 6 cpm/well)

Thus the highest P/N ratios (3.75-3.8:1) were achieved using a combination of 1ug/well of the capture antibody and 2.5-5x10^6 cpm/well of the detector antibody. However, for reasons of economy and to minimise handling of radioisotopes, a combination of 1ug/well of capture antibody and 1x10^6 cpm/well of the detector antibody (achieved P/N of 3:1) was chosen.

Detection of anti-DHBs by competitive binding RIA was performed by using this combination of the capture and the detector antibodies. 500ng of purified DHBsAg was added as a sandwich before the addition of the serum samples (Figure 3.1B). The samples were considered to be positive for anti-DHBs if binding of the ^{125}I-labelled anti-DHBs was reduced by 50% or more compared with NDS tested at the same time.

3.3.4 Calculation of Cut-Off Value

The mean background level of ^{125}I-counts bound was determined by testing 40 DHBV DNA-negative duck serum samples in the RIA for DHBsAg. The average value was calculated to be 1011cpm±173. This permitted the cut-off value to be set as the mean value plus 3 standard deviations (Halfman, 1981). This formula was used throughout the study.

3.3.5 Sensitivity of the RIA

Using the chosen combination of the capture and the detector antibodies (1ug/well capture antibody, 1x10^6 cpm/well detector antibody) as described above, the sensitivity of the assay for the detection of DHBsAg was determined using purified DHBsAg. A range of protein concentrations (15-500ng/well) of purified DHBsAg diluted in NDS was examined and the P/N ratio calculated by comparison with dilutions of the mock-purified DHBsAg. Such quantitation was based on the assumption that the serological reactivity

was directly proportional to the quantity of DHBsAg. The results of this experiment (Figure 3.8) showed that the highest P/N ratio (5.3:1) was obtained with 500ng/well of DHBsAg. The P/N ratio decreased in a linear manner which was directly proportion to the concentration of DHBsAg. However, the P/N ratio was reduced to below the cut-off value when the DHBsAg concentration was reduced to 15ng/well or below, and thus the sensitivity of the assay was determined to be approximately 30ng of DHBsAg protein.

A comparison of virus-associated markers in the serum of DHBV-infected ducks was carried out using two detection systems viz. DHBsAg by RIA and DHBV DNA by dot blot hybridisation. A series of dilutions of a serum sample from a congenitally-infected duck was diluted from neat to 1/6400 in PBS, and both DHBsAg and DHBV DNA were detected in dilutions up to 1/800 (Figure 3.9).

The above optimisation of the DHBsAg assay was performed using pooled sera from neonatally-infected ducks. Although the initial titration (Figure 3.7) showed the highest P/N ratio to be 3.8:1, a P/N ratio of up to 10:1 was achieved in some experiments (Figure 3.9). This improvement in P/N ratio may simply be related to the titre of DHBsAg in these different samples.

3.4 DISCUSSION

3.4.1 Evaluation of Immunogen (DHBsAg)

DHBsAg particles from DHBV-infected sera were purified through two CsCl gradients and were shown to have a buoyant density of 1.17g/cm^3 in CsCl similar to that described by Marion et al. (1983), but lower than that reported by Mason et al. (1980). The reason for this differences is not clear, but may be

Figure 3.8

Titration of purified DHBsAg to determine the sensitivity of the RIA. Dilutions of purified DHBsAg were made in normal duck serum and compared with dilutions of mock-purified DHBsAg. The cut-off was determined as described in the legend to Figure 3.7.

Figure 3.8

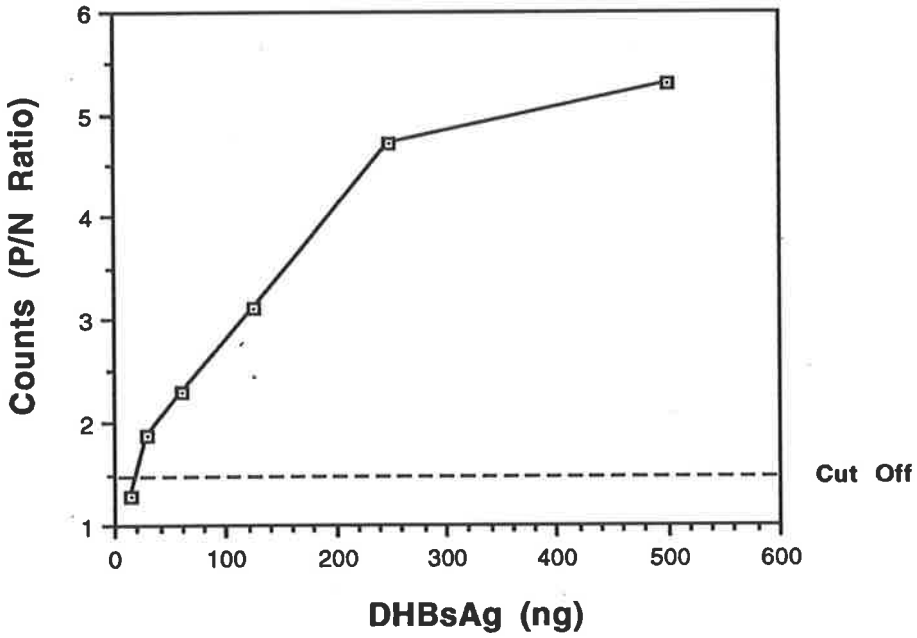
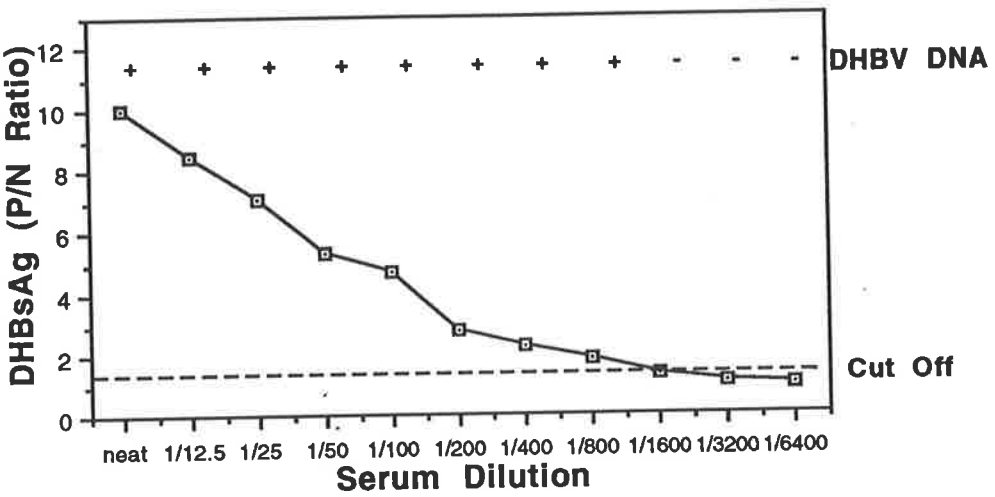

Counts (P/N Ratio)
6
5
4
3
2
1
0
100
200
300
400
500
600
DHBsAg (ng)
Cut Off

Figure 3.9

Comparison of the detection of serum DHBsAg and DHBV DNA. A range of dilutions of a DHBV-infected duck serum were tested for DHBsAg and DHBV DNA respectively to compare the relative sensitivity of these two tests. The cut off value for DHBsAg was determined as described in the legend to Figure 3.7.

Figure 3.9


DHBsAg (P/N Ratio)
12
10
8
6
4
2
0
+ + + + + + + + - - -
DHBV DNA
Cut Off
neat 1/12.5 1/25 1/50 1/100 1/200 1/400 1/800 1/1600 1/3200 1/6400
Serum Dilution

related to the purification method, as it was suggested that the presence of EDTA may affect the buoyant density of DHBsAg particles (Marion et al., 1983). EDTA was included in the CsCl solution in this, and in the Marion studies. Nevertheless, differences resulting from the use of Pekin ducks in the United States and Pekin-Aylesbury cross ducks in Australia cannot be excluded completely.

Using DHBV DNA as a measure of DHBV contamination of the DHBsAg preparation, it was noted that the pooled fractions of the first gradient corresponding to DHBsAg still contained approximately 20% of the DHBV present in the original serum. However, this was reduced to 0.05% after the second CsCl gradient (Table 3.1). Thus a large majority (99.9%) of the DHBV virions were removed from the preparation. This was confirmed by electron microscopic examination which showed minimal contamination with DHBV virions in the final DHBsAg preparation (Figure 3.4).

SDS-PAGE analysis of purified DHBsAg followed by silver staining revealed one major polypeptide of 17kDa and this is consistent with previous studies which suggested that DHBsAg polypeptides are not glycosylated (Marion et al., 1983; Schödel et al., 1989; Lambert et al., 1991). In addition, a minor polypeptide of 28kDa was observed that may represent the preS2 protein as suggested by Yokosuka et al. (1988). The 36kDa polypeptide predicted from the virus genome and detected by others in DHBV-enriched preparations (Pugh et al., 1987) was not detected in the DHBsAg preparation used in this study. This is consistent with the degree of purification of DHBsAg from DHBV as noted above. Furthermore, because 22nm spherical particles of HBV contain only one or two L-HBsAg molecules per particle compared with 40-80 L-HBsAg molecules per HBV virion (Heermann et al., 1984), it is likely that DHBsAg will also contain very low levels of the L-DHBsAg protein (36kDa). If

this is the case, then this may also be an indication of the specificity of the DHBsAg preparation.

3.4.2 Titre and Specificity of the Antisera

The success of any particular immunoassay depends largely on the quality of the antiserum used for capture and detection. The major factors are the concentration, affinity and specificity of the specific antibodies (Van Regenmortel, 1988). Titration of the rabbit anti-DHBsAg produced in this study determined that the titres were 1/1000 dilution in one rabbit and 1/750 in the other indicating that high titre antisera were obtained. The antisera detected DHBsAg in DHBV-infected liver samples, and polypeptides p17 (S) and p28 (M) in purified DHBsAg but not in uninfected samples. These results provided additional evidence for the specificity of the antisera. Although the antisera showed minimal cross reaction with normal duck tissues, the antibodies were adsorbed with homogenised NDL prior to use in RIA to ensure specificity against DHBsAg.

3.4.3 Evaluation of the RIA

The RIA developed in this chapter for the detection of DHBsAg and anti-DHBs proved to be highly reproducible and sensitive. It detected approximately 3ng/ml of DHBsAg protein (equivalent to 10^8 DHBsAg particles/ml) and this level of sensitivity is similar to that of HBsAg detection by RIA. The assay is also simple, fast to perform and represents the first report of the detection of DHBsAg and anti-DHBs in this manner (Qiao et al., 1990). More importantly, it provides an additional test for the diagnosis of DHBV infection and a unique opportunity for the analysis of the serological response in DHBV-infected ducks.

CHAPTER 4

SEROLOGICAL ANALYSIS OF DUCK HEPATITIS B VIRUS INFECTION

4.1 INTRODUCTION AND AIMS

DHBV can be transmitted experimentally to healthy ducks by IV or IP inoculation of DHBV-containing serum (Mason et al., 1983) and by intrahepatic transfection of cloned viral DNA into 1-day-old ducklings (Sprengel et al., 1984). Previous studies of experimental transmission of DHBV showed that the success of transmission depended to a large degree on the age of duck, and to a lesser degree on the dose of virus inoculated (Mason et al., 1983; Omata et al., 1983; Fukuda et al., 1987; Jilbert et al., 1987). In general terms, neonatal ducks were most susceptible to infection and virtually 100% became positive for DHBV DNA after inoculation, whereas only a varying proportion of older ducks (≥3-week-old) showed detectable levels of DHBV DNA. Furthermore, neonatal ducks usually failed to clear the infection and became persistently infected while older ducks developed a transient viraemia. These studies, however, had not examined the serological expression of DHBV-associated antigens and antibodies.

At the time this study commenced, almost nothing was known about serological responses in ducks with DHBV infection. This was mainly due to the lack of reagents. I have previously established RIA to detect DHBsAg and anti-DHBs as described in Chapter 3 and these assays allowed the examination of these markers during infection in ducks.

Previous studies (Mason et al., 1983; Omata et al., 1983; Fukuda et al., 1987; Jilbert et al., 1987) of DHBV infection examined neonatal ducks and these have provided a good model for HBV infection of human neonates. In this chapter, I wish to establish a DHBV model in post-neonatal ducks and to determine its potential usefulness as a model of adult human. The specific aims were to inoculate DHBV into ducks between the age of 1-8 weeks and to monitor the serological response of these ducks at various times after inoculation with the benefit of the RIA for DHBsAg and anti-DHBs, and to compare these markers with DHBV DNA, an established marker of DHBV infection.

4.2 EXPERIMENTAL DESIGN AND RESULTS

4.2.1 Serological Examination of Neonatal and Post-Neonatal Ducks after DHBV Infection

A. Neonatal Ducks

Samples of blood from DHBV-infected neonatal ducks were obtained from Dr Allison Jilbert who, in a previous study, inoculated neonatal ducks aged 1-7 days. All ducklings developed infection following inoculation as determined by the detection of DHBV DNA (Jilbert et al., 1988). DHBsAg was first detected at 2-3 days after inoculation and increased in concentration to a plateau by 4-5 days (Figure 4.1a). The appearance of DHBsAg in the serum of infected ducks (Figure 4.1b) showed similar kinetics to the appearance of DHBV DNA.

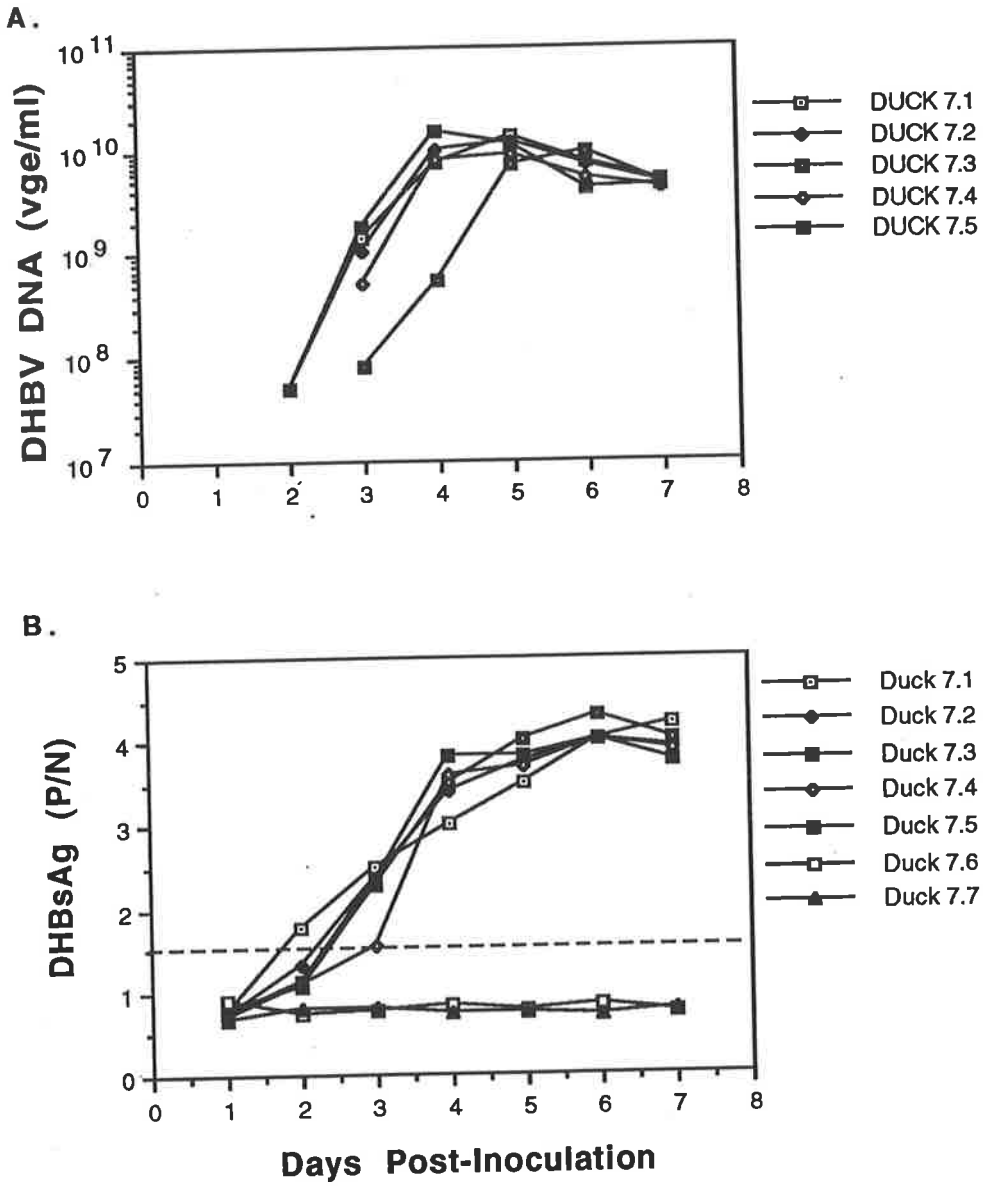
B. Post-Neonatal Ducks

To clarify the serological events after infection in ducks of different ages, six groups of five ducks aged 1, 2, 3, 4, 6, 8 weeks were inoculated by the IV route with a standard inoculum of DHBV containing $3x10^8$ vge/ml. Ducks aged

Figure 4.1

Detection of DHBV DNA and DHBsAg in neonatally-infected ducks. A 7 day study on the serological development of DHBV DNA and DHBsAg in 5 ducks (7.1-7.5) inoculated with 250ul of pooled duck serum containing $3x10^8$ vge/ml and in 2 ducks (7.6-7.7) inoculated with NDS. The initial inoculation and sampling of these ducks were performed by Dr Allison Jilbert. Serum DHBV DNA (A) was detected and quantitated by dot blot hybridisation using a ^{32}P-labelled DHBV RNA probe (Section 2.4). DHBsAg (B) was detected by RIA as described in the text.

Figure 4.1


A.
DHBV DNA (vge/ml)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
0 1 2 3 4 5 6 7 8
DUCK 7.1
DUCK 7.2
DUCK 7.3
DUCK 7.4
DUCK 7.5
B.
DHBsAg (P/N)
0 1 2 3 4 5
0 1 2 3 4 5 6 7 8
Days Post-Inoculation
Duck 7.1
Duck 7.2
Duck 7.3
Duck 7.4
Duck 7.5
Duck 7.6
Duck 7.7

1, 2, 3 and 4 weeks or older received 100ul, 250ul, 500ul and 1ml respectively of the standard inoculum. Control ducks were inoculated with NDS and caged separately. All ducks were bled at least once before inoculation, then three times in the first two weeks post-inoculation (pi), then weekly for a period of 12 weeks to one year.

All samples were tested in duplicate for DHBV DNA by dot blot hybridisation as well as DHBsAg and anti-DHBs by RIA. The tests were evaluated on the basis of 10ul of serum (neat for dot blot hybridisation and 1/10 dilution in RIA). Selected sera were also examined for DHBV DNA by PCR as described in Section 2.5.1.

4.2.2 Incubation Period and Viraemic Stage

In ducks aged between 1-3 weeks, the incubation period was 2-3 days, similar to that described above for neonatal ducks and in a previous study (Jilbert et al., 1988). In ducks aged 4-6 weeks, however, this increased to 4-7 days (Table 4.1).

The proportion of ducks that became viraemic decreased from 100% of ducks aged 1-3 weeks, to 80% of 4-week-old ducks, and 60% of 6-week-old ducks, while only 20% of the 8-week-old ducks showed detectable viraemia (Table 4.1). All of the birds inoculated 1-2 weeks post-hatch were positive for DHBV DNA and DHBsAg some 12 months pi (Figure 4.2). In contrast, in older birds, an age-related decrease in the rate of virus persistence (see below) was noted (Table 4.1).

Table 4.1

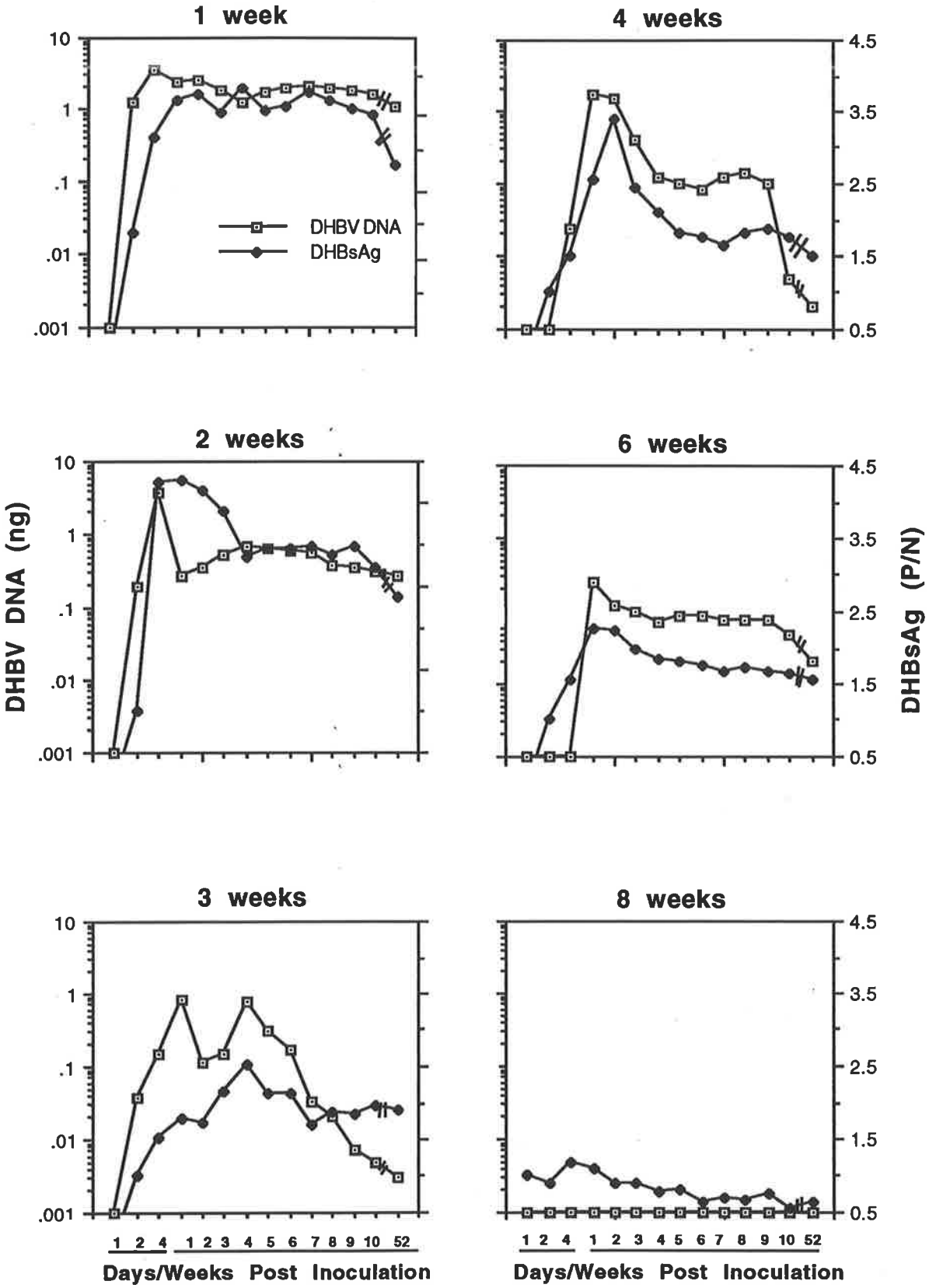
DHBV INFECTION IN DUCKS OF DIFFERENT AGE GROUPS

Age (wk) at inoculation	Incubation (Days)	Viraemia (%)		Persistence (%)		Anti-DHBs (%)
		DNA	DHBsAg	DNA	DHBsAg	
1-2	2-3	5/5(100)	5/5(100)	5/5(100)	5/5(100)	0/5 (0)
3	2-4	5/5(100)	5/5(100)	4/5 (80)	4/5 (80)	1/5 (20)
4	4-6	4/5 (80)	4/5 (80)	3/4 (75)	3/4 (75)	1/5 (20)
6	4-7	3/5 (60)	3/5 (60)	2/3 (66)	2/3 (66)	1/5 (20)
8	5-7	1/5 (20)	1/5 (20)	0/5 (0)	0/5 (0)	2/5 (40)

Figure 4.2

The time-course of the development of DHBV DNA and DHBsAg in ducks aged 1-8weeks. Six groups of five ducks aged 1, 2, 3, 4, 6, 8 weeks were inoculated with 100ul, 250ul, 500ul and 1ml respectively of pooled duck serum containing $3x10^8$ vge/ml by the IV route. Serum DHBV DNA was detected and quantitated by dot blot hybridisation using a ^{32}P-labelled DHBV RNA probe (Section 2.4) and DHBsAg detected by RIA. Each point represents an average value of DHBV DNA concentration (pg) and of DHBsAg (P/N ratio) respectively in each age group.

Figure 4.2


1 week
4 weeks
2 weeks
6 weeks
3 weeks
8 weeks
DHBV DNA
DHBsAg
DHBV DNA (ng)
DHBsAg (P/N)
10
1
.1
.01
.001
4.5
3.5
2.5
1.5
0.5
1 2 4 1 2 3 4 5 6 7 8 9 10 52
Days/Weeks Post Inoculation

4.2.3 Seroconversion Events

Only two typical seroconversion events were noted in which loss of viraemia was followed by the appearance of anti-DHBs; these occurred in ducks inoculated at 3 and 6 weeks of age. Anti-DHBs was also detected in 3 ducks which were inoculated 4 or 8 weeks post-hatch and which failed to become viraemic. Two possibilities were considered to account for this: i). silent infection; ii). passive immunisation. To differentiate these possibilities, sera from these three ducks were examined for the presence of DHBV DNA by PCR.

In the 4-week-old and in both 8-week-old ducks, PCR followed by Southern blot hybridisation detected DHBV DNA in samples taken at 11 days, and 7 and 11 days pi respectively, whereas samples taken on earlier days were negative (Figure 4.3). The PCR product was as expected, 406bp, determined by comparison with a PstI digest of λ DNA. In all cases, PCR performed on DHBV-negative serum samples, or using PCR reagents alone, were negative by Southern blot hybridisation. These results indicate that the original inoculum was not the template for PCR, and suggest very strongly that the ducks actually had a low level of transient viraemia.

4.2.4 Patterns of Serological Responses

The response of the ducks to inoculation with DHBV-positive serum varied with the age of the ducks. Three types of response were observed in these ducks (Table 4.2):

1). Persistent viraemia, defined by the presence of DHBV DNA and DHBsAg for as long as 12 months. 100% of infected ducks aged 1-2 weeks, 80% of 3-

Figure 4.3

Southern blot analysis of the product after PCR amplification of DHBV DNA-negative samples. DNA from duck serum was purified and amplified by PCR, the products separated by gel electrophoresis and transferred to nitrocellulose for hybridisation. Analysis of ducks inoculated at 4 weeks (a) and 8 weeks (b) that were subsequently negative for DHBV DNA by dot blot hybridisation and DHBsAg by RIA.

(a). track 1, 7 days pi; track 2, 11 days pi; track 3, DHBVDNA positive control (+) by dot blot hybridisation; track 4, DHBV DNA positive control (++) by dot blot hybridisation; track 5, NDS as negative control; track 6 and 7 PCR reagent only control; track 8, DHBV DNA template control.

(b). track 9, 1 day pi; track 10, 2 days pi; track 11, 4 days pi; track 12, 7 days pi; track 13, 11 days pi. The gel shown in (b) was analysed independently of (a) but showed similar reactions in the control tracks.

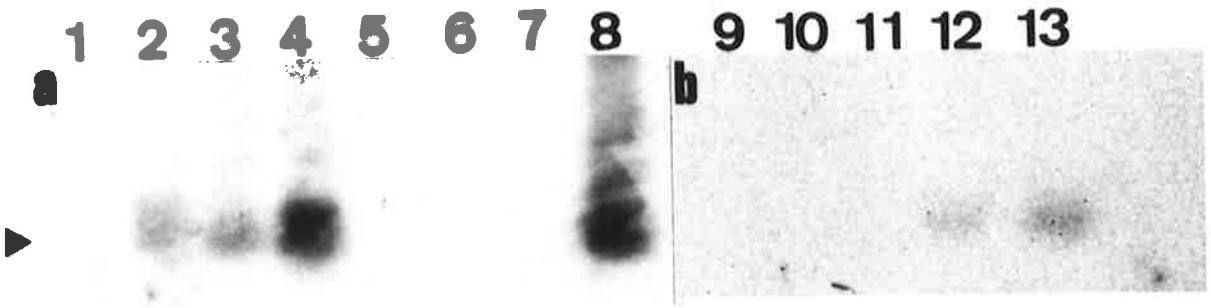

1 2 3 4 5 6 7 8 9 10 11 12 13
a
b

Table 4.2

PATTERNS OF RESPONSE IN DHBV INFECTION

Type of Response	Duck Age at Inoculation (weeks)					
	1	2	3	4	6	8
1. Persistent Viraemia	5/5	5/5	4/5	3/5	2/5	0/5
2. Transient Viraemia						
Anti-DHBs +ve	0/5	0/5	1/5	0/5	1/5	0/5
Anti-DHBs -ve	0/5	0/5	0/5	1/5	0/5	1/5
3. No Viraemia						
Anti-DHBs+ve	0/5	0/5	0/5	1/5	0/5	2/5
Anti-DHBs -ve	0/5	0/5	0/5	0/5	2/5	2/5

week-old, 75% of the 4-week-old, 66% of the 6-week-old and none of the 8-week-old fitted into this category.

2). Transient viraemia, judged by the transient appearance followed by clearance of DHBV DNA and DHBsAg with or without seroconversion to anti-DHBs. DHBV DNA and DHBsAg were cleared in 20%, 25%, 33% and 100% of the infected ducks aged 3, 4, 6 and 8 weeks respectively but a typical seroconversion to anti-DHBs only occurred in 50% of all transiently-infected ducks.

3). No detectable viraemia. 20%, 40% and 80% of the ducks aged 4, 6 and 8 weeks respectively showed no detectable levels of DHBV DNA and DHBsAg during the whole period of this study. However, anti-DHBs was detected in 40% of the ducks in this category.

Therefore, types 2) and 3) were further subdivided into ducks which were positive or negative for anti-DHBs. A representative example of each group is shown in Figure 4.4.

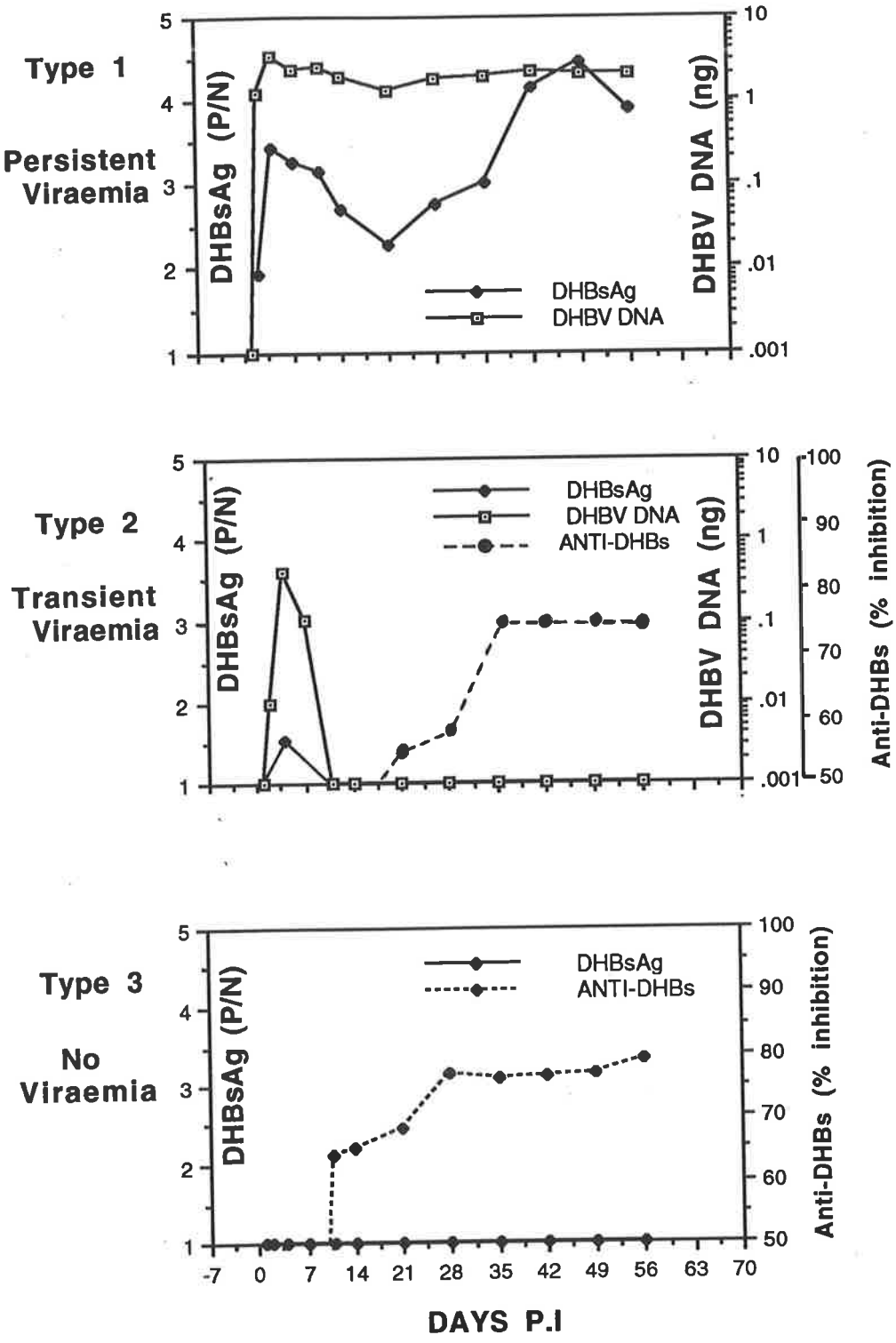
4.2.5 Correlation of Serological Markers: DHBV DNA & DHBsAg

Viraemia was determined by the presence of both DHBV DNA and DHBsAg. In a majority of infections, both markers were detected equally well. However in three cases, birds aged 4-, 6- and 8-week-old respectively, only one of the two markers was detected. Two of the three cases showed the presence of DHBV DNA alone whereas the third case detected DHBsAg alone. However, in both of the former cases very low levels of DHBV DNA (10-50pg) were detected for a maximum of 7 days and one developed anti-DHBs soon after the clearance of DHBV DNA. These results indicated that these two ducks may have shown transient expression of DHBsAg that was undetected. In the

Figure 4.4

Examples of the serological responses to DHBV infection. Serum samples were tested for DHBV DNA by dot-blot hybridisation and for DHBsAg and anti-DHBs by RIA.

Figure 4.4


Type 1
Persistent Viraemia
DHBsAg (P/N)
DHBV DNA (ng)
DHBsAg
DHBV DNA
Type 2
Transient Viraemia
ANTI-DHBs
Anti-DHBs (% inhibition)
Type 3
No Viraemia
-7 0 7 14 21 28 35 42 49 56 63 70
DAYS P.I

third case (DHBsAg positive, DHBV DNA negative), the duck also expressed DHBsAg transiently between 11-21 days pi, but did not develop anti-DHBs. However, DHBV DNA was detected 11 days pi by PCR and this indicated a genuine infection.

4.3 DISCUSSION

In this study, Pekin-Aylesbury cross-bred ducks remained highly susceptible to DHBV infection for four weeks post-hatching and even 50% of ducks aged between 6-8 weeks were infected if all three markers of infection, viz. DHBV DNA, DHBsAg and anti-DHBs, were measured. However, if DHBsAg (or DHBV DNA) was the sole marker of infection then only 20% of these 6- and 8-week-old ducks showed serological evidence of infection. In previous transmission studies which used DHBV DNA hybridisation analysis alone, birds 3 weeks or older were usually resistant to infection (Mason et al., 1983: Omata et al., 1984: Fukuda et al., 1987). The results from this study support these data when similar methods (DHBV DNA) were used to detect viraemia, but also demonstrate the need to introduce a spectrum of assays to study the evolution of DHBV infection.

DHBV DNA and DHBsAg always co-existed in ducks aged ≤ 3 weeks, but 3 ducks aged ≥4 weeks with a type 2 response (Table 4.2) i.e., transient infection showed either DHBV DNA or DHBsAg alone. These ducks are probably examples of a minor group response to infection, representing active virus replication with restricted production of excess DHBsAg, and limited replication with the production of excess DHBsAg respectively. The latter group may represent the so-called "healthy carrier" state associated with HBV infection in humans.

Thus the appearance of anti-DHBs was restricted to birds aged ≥3 weeks, but did not necessarily result from detectable viraemia, indicating that ducks in this latter category may have suffered subclinical or silent infection (see below) as has been described for HBV (Hoofnagle et al., 1978). However, two birds which showed transient infection as judged by the detection of DHBV DNA or DHBsAg (type 2, Table 4.2) remained negative for anti-DHBs indicating that additional factors could influence the humoral immune response. It is possible that the prolonged viraemic phase seen in these birds resulted from an impaired immune response. Alternative explanations for the non-appearance of antibody are: i). the level of anti-DHBs in the serum of these ducks was below the sensitivity of the assay; ii). a prolonged window period extending beyond the duration of this study.

The appearance of anti-DHBs in birds which failed to develop viraemia was due to silent infection rather than passive immunisation as suggested by the detection of DHBV DNA by PCR. Thus it is likely that these ducks have actually supported DHBV replication but the level of DHBV DNA and DHBsAg in the serum was below the sensitivity of the dot blot hybridisation and the RIA.

As described above, use of these serological markers has permitted sharper definition of the age-related transition from high susceptibility to resistance to infection, prior to a more detailed analysis of the mechanisms involved (see Chapter 5). It is believed that when this study was performed it was the first of its type and yielded unique information on the serological profile of DHBsAg/anti-DHBs and DHBV DNA in different age group ducks. The use of these assays will provide novel data on the chronicity and pathogenesis of Hepadnavirus infections.

CHAPTER 5

FACTORS INFLUENCING THE AGE-RELATED SUSCEPTIBILITY OF DHBV INFECTION OF DUCKS

5.1 INTRODUCTION AND AIMS

Studies of experimental transmission with DHBV have shown that the outcome of infection depends largely on the age of ducks at the time of inoculation (Mason et al., 1983; Omata et al., 1984; Fukuda et al., 1987). As discussed in Chapter 4, the development of viraemia following inoculation with DHBV varied from 100% in newly-hatched ducklings to 60% in 6-week-old ducks, whereas ducks aged ≥8 weeks never showed detectable viraemia when analysed by dot blot hybridisation for DHBV DNA and by RIA for DHBsAg (Qiao et al., 1990). Therefore, it is clear that the host susceptibility is age related.

The age-dependency of susceptibility to infection with DHBV is not fully understood. However, one of the key factors in determining virus susceptibility is the capacity of the host cell to bind virus through specific receptors on the cell surface (Lonberg-Holm & Philipson, 1981; Tardieu et al., 1982). The cellular receptor for DHBV has not been described so far but the HBV receptor has been extensively studied in the recent years (Section 1.9).

To determine whether the resistance to DHBV infection in adult ducks was due to the inability of adult duck hepatocytes to bind DHBV, the experiments in this chapter were designed to examine differences in neonatal and adult duck hepatocytes with regard to their ability to support the replication of DHBV. Two aspects were examined: i). the level of expression of the DHBV

receptor on hepatocytes from young ducklings and from adult ducks; ii). the effect of hepatocyte mitosis resulting from liver regeneration.

5.2 EXPERIMENTAL DESIGN

It was planned to develop a similar virus-cell membrane binding assay as described previously for other viruses (Krah & Crowell, 1982; Pontisso et al., 1989a; 1989b) to detect DHBV receptor activity on duck hepatocytes. I chose to use a RIA to take advantage of experience gained in the Chapter 3. In the work described in this chapter, duck liver tissues obtained from ducks aged 5 days and 8 months were used for the preparation of liver cell membranes (plasma and internal membranes, Section 2.7) and the levels of expression of the DHBV receptor were compared using the RIA (Section 2.8). In this assay, the purified liver membranes were bound to solid phase and then incubated in the presence of purified DHBV (Section 2.1), mock DHBV purified from NDS or PBS. The level of DHBV bound to the membrane was subsequently detected with a ^{125}I-labelled antibody specific to DHBV preS (Section 2.8) and bound radioactivity measured by a gamma counter.

Liver regeneration can occur in response to various factors, such as chemicals, toxins, infectious agents, radiation, or physical trauma (Leevy, 1973). Partial hepatectomy in the rat has been used widely as a model for study of liver regeneration (Leevy, 1973). In order to examine the effect of mitosis on the production of DHBV, it was planned to perform partial hepatectomy in DHBV-carrier ducks. 10 DHBV-carrier ducks, infected as neonates and aged between 8-18 months at the time of the experiments, were selected, six for partial hepatectomy, two for laparotomy and two were untreated. The percentage of liver excised was determined according to the published data of total duck liver weight (Nickel et al., 1977). Laparotomy was performed as a control for postoperative shock and blood loss during the

operation, in which the same operating procedures were used except that the liver was not resected. In one experiment, serial serum samples after partial hepatectomy were tested for ALT and LDH activity using commercially available kits (Merck and Trace Scientific respectively) by staff in the Department of Agriculture, Adelaide, South Australia.

5.3 RESULTS

5.3.1 Analysis of Duck Liver Membrane Preparation

Prior to the use of duck liver membranes in virus binding experiments, the authenticity of the membrane preparations was examined by EM and by endogenous enzymatic activity. Morphological examination of the membrane-enriched fractions by EM showed large connected vesicles (data not shown) which were similar to those described by Hubbard et al. (1983). Alkaline- and acid-phosphatase activity, enzymatic markers of plasma and internal membranes respectively, were also measured (Section 2.7). Table 5.1 represents the yield and specific activity of these enzymes in plasma- and internal-membrane fractions. A yield of 18.9 and 1.44mg/g wet liver of the alkaline phosphatase, and 0.1 and 1.45mg/g wet liver of the acid phosphatase was obtained in plasma- and internal-fractions respectively. The specific activities (umoles/mg protein) of the alkaline phosphatase in the plasma- and of the acid phosphatase in the internal-fractions were estimated to be 24.9 and 1.25 respectively. These data showed i). a good yield and enzymatic activity of each plasma and internal membrane fraction compared with the result described by Hubbard et al. (1983); ii). minimum contamination of each preparation indicating that good separation of plasma membranes from internal membranes was achieved.

Table 5.1

BIOCHEMICAL CHARACTERISATION OF DUCK LIVER MEMBRANE FRACTIONS

Enzymatic Marker	Yield[a] (mg/g wet liver)		Specific Activity[b] (umoles/mg protein)		Rate Activity[c] (%) in PM
	IM[d]	PM[e]	IM	PM	
Alkaline phosphatase (PM)	1.44	18.9	6.2	24.9	14.3
Acid phosphatase (IM)	1.45	0.1	1.25	0.13	4.2

a The yield of enzymes in the final membrane fractions was calculated as milligram per gram of wet liver.

b The specific activity was determined as umoles per milligram of protein.

c The rate activity of total enzymes in the final plasma membrane fraction was calculated from the percentages of recovered activity in each fractions throughout the purification of the liver membranes.

d Internal membranes

e Plasma membranes

5.3.2 Analysis of Purified DHBV Virions

It is likely that DHBV and the cellular receptor also react via the large protein of the DHBsAg as already described for HBV, and consequently care was taken to ensure that DHBV-enriched preparations contained minimal levels of DHBsAg particles. DHBV DNA and DHBsAg were purified from the serum of congenitally-infected ducks by centrifugation through a 20% sucrose cushion followed by a discontinuous CsCl gradient (Section 2.1). Fractions in the gradient were examined for DHBV DNA by dot blot hybridisation (Section 2.4) as a marker of virions and for DHBsAg by RIA (Qiao et al., 1990). As shown in Figure 5.1, a majority of DHBV DNA was detected in a fraction with a buoyant density of 1.195g/cm^3, whereas DHBsAg was detected in a broader range of fractions with a density between 1.13-1.18g/cm^3 (peak at 1.17g/cm^3). These densities of 1.195 and 1.17g/cm^3 corresponded to the density of DHBV virions and DHBsAg particles respectively indicating that separation of DHBV virions from the bulk of the DHBsAg particles was successful. Examination of the DHBV-containing fraction by EM confirmed that a DHBV virion-enriched preparation was obtained (Figure 5.2).

5.3.3 DHBV Binding Studies

Since previous studies have demonstrated that hepatocytes were the major and primary site of DHBV replication (Mason et al., 1981; Jilbert et al., 1988), I restricted the study to hepatocyte membranes and used NHL as a negative control.

The ability of internal and plasma membranes from neonatal and adult ducks to bind DHBV was examined. A solid-phase RIA (Section 2.8) was carried out at 4°C to ensure that DHBV virions remained intact throughout and to minimise subsequent release of bound virus. After binding of the DHBV to

Figure 5.1

Separation of DHBV virions and DHBsAg by CsCl gradient centrifugation. Serum containing a high titre of DHBV DNA was used as the source for purification of DHBV virions. The virus particles were pelleted through 20% sucrose then separated in a CsCl gradient. The CsCl density was estimated from the refractive index, and DHBV DNA and DHBsAg were measured in each fraction of the gradient; DHBV DNA was detected and quantitated by dot blot hybridisation and DHBsAg was detected by RIA.

Figure 5.1

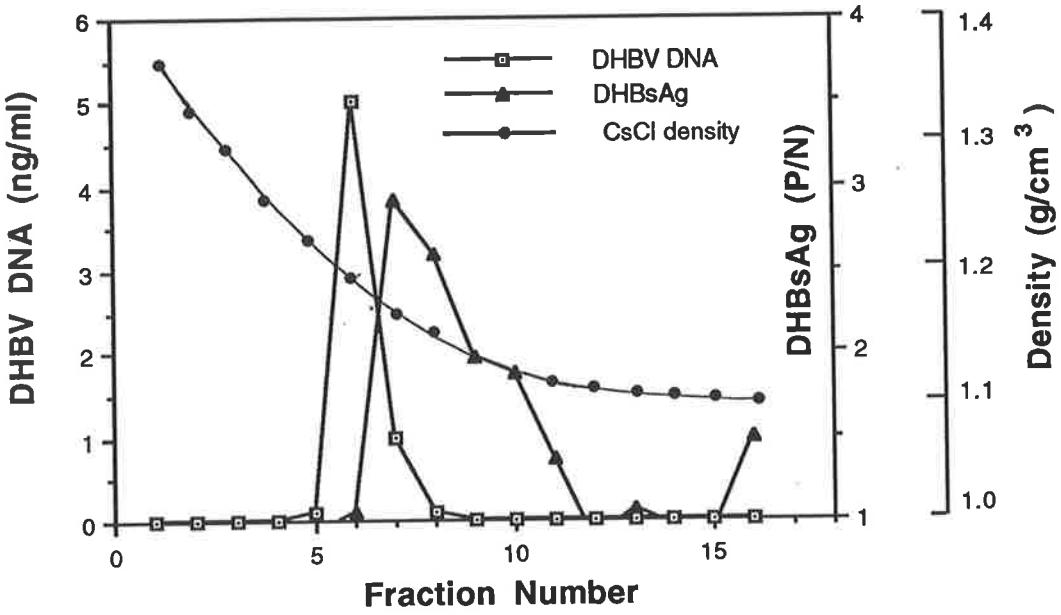
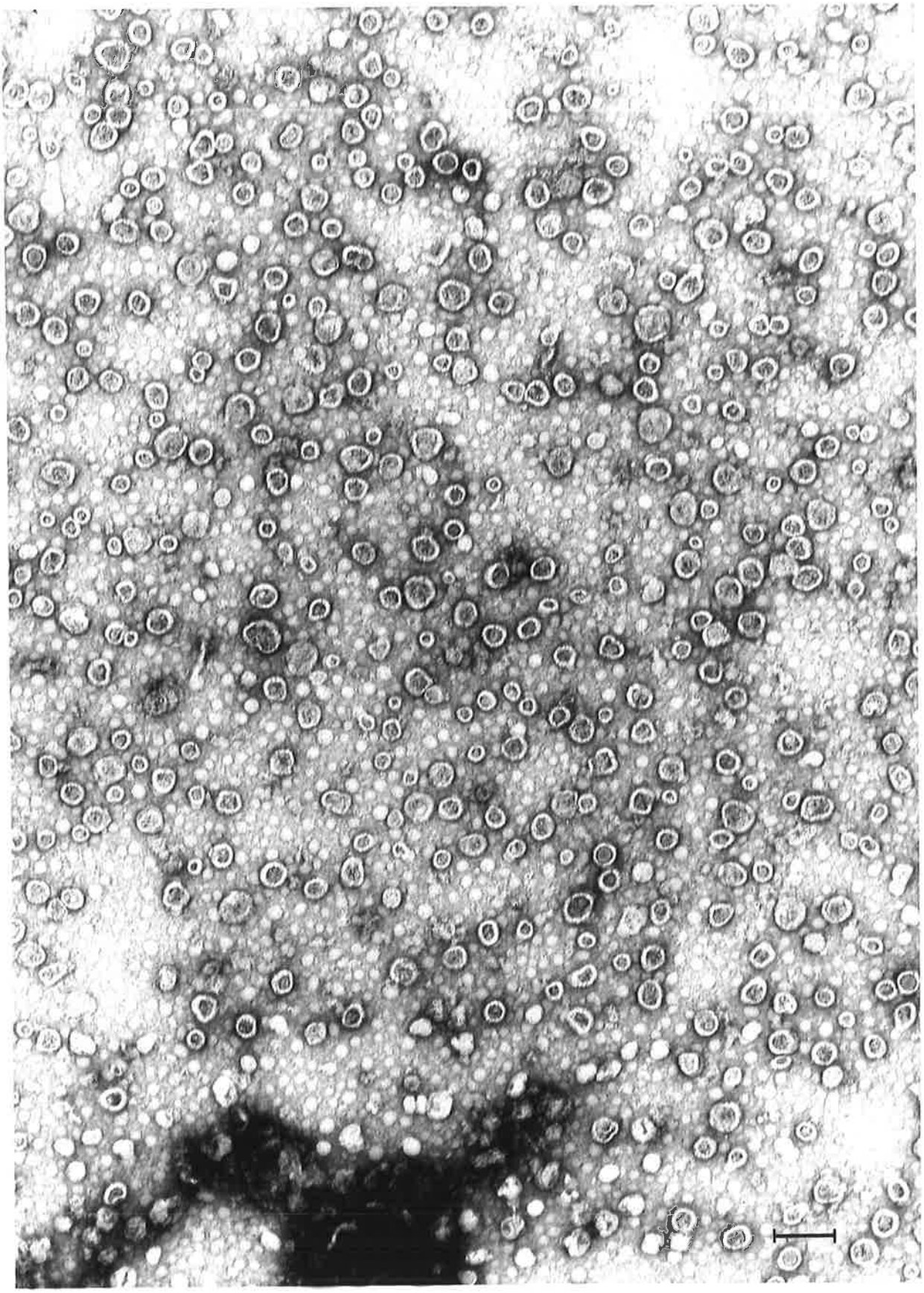

DHBV DNA
DHBsAg
CsCl density
DHBV DNA (ng/ml)
DHBsAg (P/N)
Density (g/cm 3)
Fraction Number
0
1
2
3
4
5
6
0
5
10
15
1
2
3
4
1.0
1.1
1.2
1.3
1.4

Figure 5.2

Electron microscopy of purified DHBV virions from DHBV-containing serum. Purified particles from fraction 6 of the CsCl gradient were loaded on carbon-coated grids and stained with 3% PTA. Bar = 100nm.



membranes, a ^{125}I-labelled anti-preS was then used to detect bound DHBV. As shown in Figure 5.3, liver plasma membranes from both ducklings and adult ducks were capable of binding DHBV virions, while the internal membranes from ducks and human liver plasma membranes showed no binding activity. The P/N ratio was approximately 8.5 : 1, when either binding of DHBV to internal membranes, or binding of mock purified DHBV to plasma membranes, was used as the negative control. This figure compares very favourably with previously published data using a similar method with HBV and human liver membranes (Pontisso et al., 1989b).

5.3.4 Specificity of DHBV Binding

A. Antibody Blocking

In order to confirm that the binding reaction between DHBV virions and the plasma membranes was specific, the DHBV preparation was preincubated with different antibody preparations, and bound virus was detected with ^{125}I-labelled anti-DHBs rather than anti-preS used in the above binding experiment. The binding of virus to plasma membranes was inhibited by 83% as a result of preincubation of DHBV with the MAb to DHBV preS (gift from Dr Stephen Locarnini) but no effect was observed after preincubation with either anti-DHBc or NMS (Figure 5.4). Since it is likely that the MAb to preS detected a DHBV-specific polypeptide rather than a DHBsAg-specific polypeptide, this result imparted a further level of specificity to the binding assay.

B. Receptor Saturation

The binding experiment described above suggested that plasma membranes from neonatal and adult ducks were both capable of binding DHBV. The levels of virus required to saturate the receptors were then examined, to compare to the relative abundance of receptors on neonatal and adult duck

Figure 5.3

An examination of the DHBV-binding ability of duck liver membranes. Equivalent amounts of each liver membrane preparation from neonatal, or adult ducks, or human were incubated in duplicate with purified DHBV virions, mock-purified DHBV, or PBS and bound virus detected with ^{125}I-labelled anti-DHBV preS. The results are shown as mean count (cpm) per minute bound to the membranes.

Figure 5.3

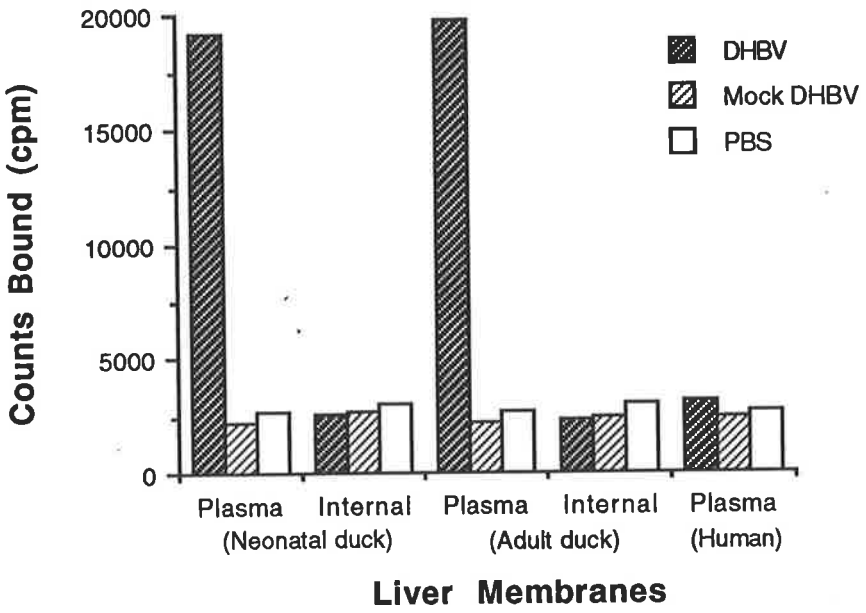
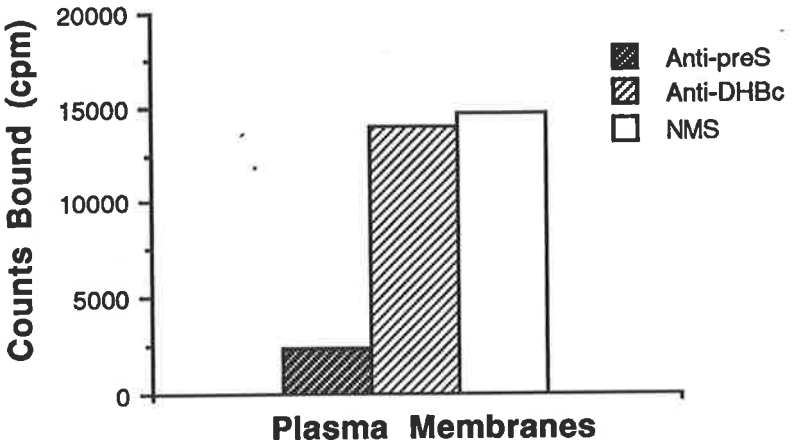

20000
15000
10000
5000
0
Counts Bound (cpm)
DHBV
Mock DHBV
PBS
Plasma
Internal
Plasma
Internal
Plasma
(Neonatal duck)
(Adult duck)
(Human)
Liver Membranes

Figure 5.4

Inhibition of DHBV-binding by DHBV preS-specific antibody. The effect of different antibodies on DHBV binding to duck liver plasma membranes was measured by preincubation of virus with an equal volume of anti-preS (50ug/ml), anti-DHBc (50ug/ml), and normal mouse serum (1/50 dilution). DHBV binding was detected by ^{125}I-labelled anti-DHBs. The results are shown as described in Figure 5.3.

Figure 5.4


Counts Bound (cpm)
20000
15000
10000
5000
0
Anti-preS
Anti-DHBc
NMS
Plasma Membranes

liver-derived membranes. In this experiment, liver membranes were used at a concentration of 50ug/ml and a wide range of virus concentrations added.

The results of this experiment showed that no bound virus was detected in membranes which were incubated in the two highest dilutions of virus (Figure 5.5). The level of non-specifically bound ^{125}I, as judged by the counts bound to membranes incubated with mock purified virus, did not change throughout. Consequently, it is likely that the counts bound after incubation with 1ug/ml of DHBV (1/300 dilution) were specific and represented the limit of detection of bound DHBV in this system. There was an increase of approximately 10 fold in the level of bound ^{125}I when the concentration of virus was increased from 1-10ug/ml, but no corresponding increase was noted when a virus concentration of 100ug/ml was used. This indicates that the plasma membranes of both neonatal and adult ducks were saturated after incubation with 10ug/ml DHBV, and suggests that both membranes expressed similar levels of the DHBV receptor.

5.3.5 The Effect of Hepatocyte Mitosis on DHBV Synthesis

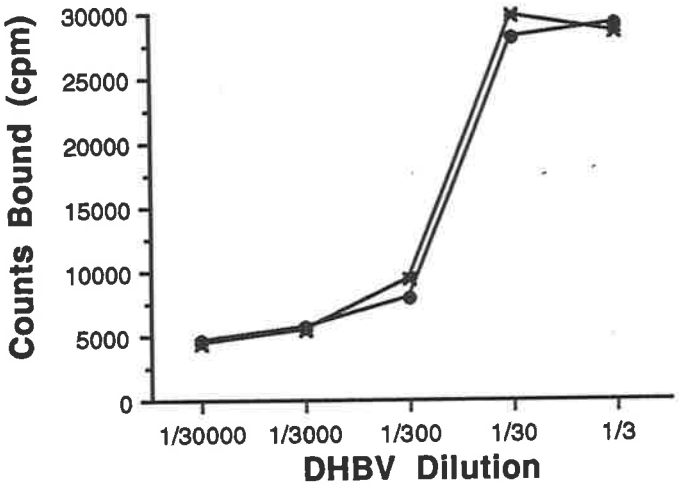
Despite the fact that adult ducks do not normally develop viraemia following DHBV inoculation, the above results showed that adult- and neonatal-duck hepatocytes expressed similar levels of the DHBV receptor. Additional factors which might influence different outcomes of infection in neonatal and adult birds include the level of maturity of the immune system, and intracellular events. Since a major difference between hepatocytes in neonate and adult ducks is that hepatocytes are dividing in the neonate, I then chose to examine the effect of hepatocyte regeneration on the expression of DHBV.

Partial hepatectomy was performed on six DHBV-carrier ducks aged 8-18 months that had been infected with DHBV as neonates. In each case, the

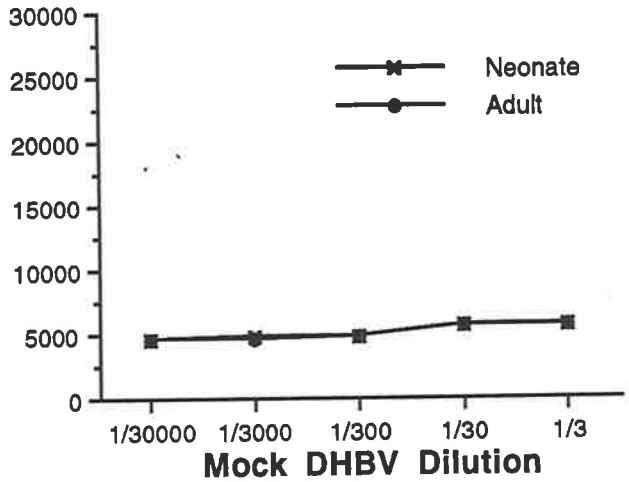
Figure 5.5

Examination of the relative levels of the DHBV receptor in plasma membranes from neonatal and adult ducks. Liver plasma membranes were incubated with increasing concentrations of (A) purified DHBV or (B) mock-purified DHBV. DHBV binding was detected by ^{125}I-labelled anti-DHBV preS and expressed as described in Figure 5.3.

Figure 5.5 A.


Counts Bound (cpm)
30000
25000
20000
15000
10000
5000
0
1/30000
1/3000
1/300
1/30
1/3
DHBV Dilution

B.


30000
25000
20000
15000
10000
5000
0
Neonate
Adult
1/30000
1/3000
1/300
1/30
1/3
Mock DHBV Dilution

level of DHBV in the serum of these birds prior to surgery was determined by dot blot hybridisation analysis (Table 5.2). After removal of approximately 50% of the liver, the level of DHBV in the serum of different birds increased between 2.6-12 fold (average 6 fold) within 96 hr of surgery but returned to pre-surgery levels 120 hr after surgery (birds 1-6, Table 5.2), whereas the control birds showed no increase (birds 7-10, Table 5.2). After 70% hepatectomy in adult rat, a peak of liver cell DNA synthesis occurs 12-48 hr later (Bucher, 1973) and it is likely that duck hepatocytes behave in a similar manner.

Consequently, the rise in serum DHBV levels was most likely to be temporally associated with hepatocyte regeneration. However, no direct measure of hepatocyte regeneration was performed, although the livers of 3 birds were found at autopsy to be restored to their original size and weight 8 weeks after partial hepatectomy. No rise was noted in the levels of DHBV in the serum of untreated ducks nor in ducks which underwent laparotomy. In one carrier bird which was subjected to laparotomy followed 6 weeks later by partial hepatectomy, a rise in DHBV levels followed partial hepatectomy only. Furthermore, the increased DHBV DNA was not accompanied by elevation of serum ALT and ADH activity (Figure 5.6) suggesting that increased DHBV DNA was not simply the result of hepatocyte lysis.

5.4 DISCUSSION

5.4.1 Expression of the Receptor for DHBV in Hepatocytes of Both Neonatal and Adult Ducks

DHBV infection of the neonatal ducks represents a good model for neonatal infection with HBV, but as described above, adult birds do not normally develop viraemia after experimental inoculation with DHBV (Qiao et al.,

Table 5.2

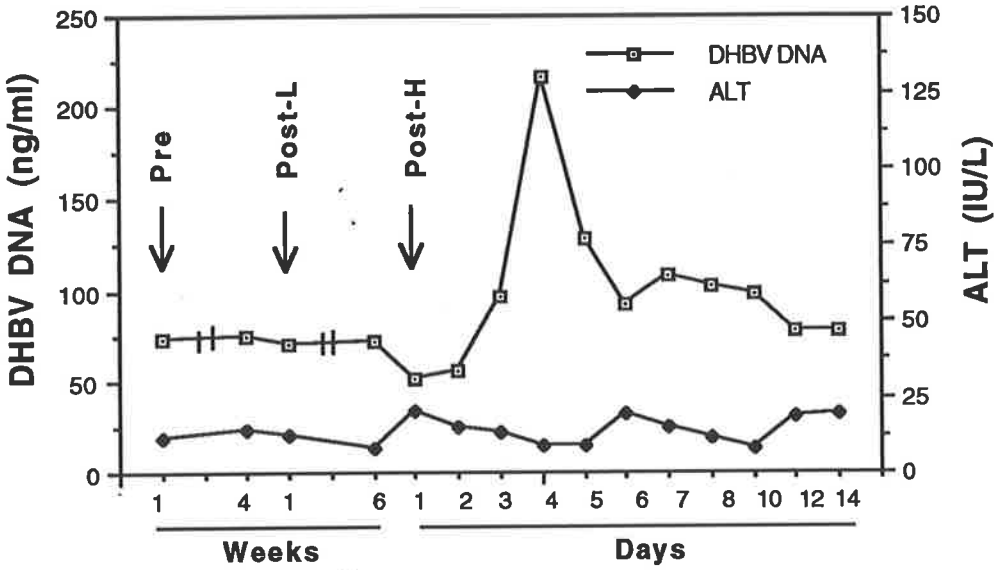
EFFECTS OF PARTIAL HEPATECTOMY ON THE LEVEL OF SERUM DHBV DNA IN ADULT CARRIER DUCKS

Duck No.	Age (Month)	Liver Excised (%)	Serum DHBV DNA (ng/ml)		Relative Increase (Times)
			Pre-Surg.	Post-Surg.	
1	8	55	1	5.5	5.5
2	9	61	1.2	9	7.5
3	13	51	0.5	6	12
4	15	55	0.75	2	2.6
5	16	46	0.25	1	4
6	18	47	0.15	0.5	3.3
7	9	0	0.5	0.75	1.5
8	16	0	0.1	0.1	0
9	9	0	0.5	0.5	0
10	10	0	0.25	0.2	0

Figure 5.6

The effect of sequential laparotomy and partial hepatectomy on the levels of DHBV and liver-specific enzymes in the serum of a DHBV-carrier duck. DHBV DNA and ALT were monitored weekly for 4 weeks presurgery (pre); 1-2 times per week for 6 weeks post-laparotomy (post-L), and daily for 14 days post-partial hepatectomy (post-H). DHBV DNA was measured by dot blot hybridisation and quantitated visually and by liquid scintillation counting. ALT levels were determined by a commercially available assay as described in the text.

Figure 5.6


DHBV DNA (ng/ml)
ALT (IU/L)
DHBV DNA
ALT
Pre
Post-L
Post-H
250
200
150
100
50
0
150
125
100
75
50
25
0
1
4
1
6
1
2
3
4
5
6
7
8
10
12
14
Weeks
Days

1990). In contrast, the adult human is highly susceptible to infection with HBV. In order to investigate possible mechanisms for this decreased susceptibility in adult birds, I examined the expression of the DHBV receptor on liver cell membranes, and found that plasma membranes, but not internal membranes, from both neonatal and adult ducks were able to bind the virus.

Furthermore, saturation binding studies suggest that adult hepatocytes express the DHBV receptor at similar levels to neonatal hepatocytes. This suggests that a later event during infection of adult ducks with DHBV represents the block in full virus replication, and the above results are consistent with a recent study (Jilbert et al., 1992) which showed that adult hepatocytes infected in vivo support many of the steps of DHBV replication but fail to secrete virus into the serum.

5.4.2 Intracellular Factors and Host Susceptibility

In vitro studies have shown that DHBV infection of primary duck hepatocytes from neonatal and older ducks can be achieved (Tuttleman et al., 1986b). Successful infection appeared to be highly dependent on the differentiated state of the cell (Section 1.8). In general, only well-differentiated hepatocytes support DHBV replication, and after 7 days in culture hepatocytes were no longer permissive. However, in the presence of 2% dimethyl sulphoxide (DMSO), the hepatocytes retained the ability to support DHBV replication for a further 7 days (Galle et al., 1989). These data suggest that intracellular factors associated with cell differentiation may play a role in determining the age-related susceptibility of DHBV infection in ducks.

This hypothesis is supported by the partial hepatectomy study which showed a distinct increase in the level of DHBV in the serum of carrier ducks after partial hepatectomy but not control surgical procedures. The increase in the

serological level of DHBV was related directly to the degree of hepatectomy, since in some experiments (data not shown) in which approximately 20% of the liver was removed, no discernible increase in DHBV levels was noted.

Although previous studies showed that a majority of hepatocytes of carrier ducks (previously infected as neonates) were supporting DHBV DNA replication (Jilbert et al., 1988), the increase in serum DHBV levels reported above are more striking still when it is considered that these birds had had approximately 50% of the liver tissue removed. The increase of DHBV DNA in the serum was not simply due to hepatocyte lysis since ALT and LTD activity remained within normal levels throughout the study. I have not examined whether hepatectomy affected primarily virus replication or virus release; in either case, the findings demonstrated that regenerating livers, which could be expected to contain dividing hepatocytes, produced DHBV more efficiently than quiescent hepatocytes of untreated duck livers.

These results might also provide an explanation for the rapid fall in the levels of DHBV in the serum of either congenitally- or neonatally-infected ducks when the ducks reach a few months of age, if this coincides with the stage at which hepatocytes stop dividing. As vertical transmission represents the normal route of DHBV transmission, then it is likely that DHBV has evolved in such a manner to utilise dividing cells which represent the natural target for the virus. This may explain why adult ducks fail to become viraemic.

5.4.3 Other Factors and Host Susceptibility

In general, very little is known about duck immunology and a mature immune system may also influence the outcome of infection. Like other lower animals, ducklings appear to be immunologically immature at the time of hatching and highly susceptible to infection with DHBV. Since the immune response to

DHBV infection may subsequently contribute to the pathogenesis, the absence of liver injury in newly-hatched birds after inoculation with DHBV is likely to be indicative of a poor cell-mediated immune response. The effects of cytokines or hormones, secreted in response to partial hepatectomy, on virus replication were not examined and thus cannot be excluded. Nevertheless, it seems unlikely that cytokines had played an important role in these circumstances since inadequate liver resection (discussed above) showed no effect on the level of viraemia. It could be expected that cytokine levels, produced after removal of 20% of the liver, would be sufficiently high to have an effect.

In contrast to DHBV infection, horizontal transmission of HBV to immunocompetent adults that results in subsequent viraemia is common. It can be speculated that the role of the HBV X gene, which is absent in the DHBV genome, may be to encode a product with transactivation activity that stimulates adult hepatocytes to facilitate HBV replication and secretion. If this is the case, then one would predict that the X gene product would be found in the virion, and evidence for this has been presented recently (Wu et al., 1990).

CHAPTER 6

INTERACTION OF HBV WITH HOST CELLS

6.1 BACKGROUND AND AIMS

As described in Chapters 3-5, the initial aims of this thesis were to study events related to DHBV infection and pathogenesis. During the study period, as a consequence of the results and expertise gained in performing the experiments of these chapters, the direction of the thesis evolved in such a manner that a logical progression of the work would be to examine the nature of the DHBV receptor. However, insofar as the DHBV is a model for HBV, and since the experimental design to identify the receptor for HBV is likely to be similar to that planned for DHBV, I decided that I would use the previous chapter as a spring board to examine details of HBV-cell binding. It was also considered that this work towards identification of the HBV receptor actually represented the ultimate goal. Accordingly, this chapter in the thesis examines details of the HBV-cell interaction, and some of the early events of HBV replication.

The virus-cell interaction which represents the initial step in virus replication, occurs at the plasma membrane of susceptible cells (Lonberg-Holm & Philipson, 1981; Tardieu et al., 1982). This attachment step is usually mediated by a virus attachment protein (VAP) expressed in multiple copies on the virion. Receptors in the plasma membrane of cells contain polar groups complementary to the VAP (Crowell & Hsu, 1989). Following virus attachment to the susceptible cell a series of intracellular events may occur, including virus internalisation, relocation to the nucleus or some other site in the cell

prior to uncoating and genome replication. The details, including the nature of the cellular receptor, of these early events remain unclear for HBV.

A major impediment to these studies is the lack of a convenient cell culture system for HBV; many attempts to establish a conventional cell culture system using hepatoma-derived lines have been unsuccessful, but as these cells can support HBV replication after transfection of HBV DNA, it is likely that the block in replication following infection is attributed to an early step in the replication cycle. However, a recent, and the only report describing infection of HepG2 cells with HBV (Bchini et al.,1990) is consistent with the HBV-binding activity described previously in these cells (Neurath et al,. 1985).

The specific aims were:

1). To examine the binding activity for HBV in human liver cells and in hepatoma-derived cell lines by a solid-phase RIA.

2). To determine the level of binding activity and to quantitate the receptor binding sites per cell.

3). To examine the possibility of HBV internalisation.

6.2 EXPERIMENTAL DESIGN

Since the envelope of the 42nm HBV particle contains the highest levels of L-HBsAg, the VAP of HBV, it was decided to use HBV virions purified from HBV-infected serum and to remove the large quantity of HBsAg particles. Column chromatography (Section 2.1), a gentle purification procedure, was used to avoid damage to the virus particles. Binding activity was examined in human liver membranes prepared as described (Section 2.7) and in monolayer

cultures of two human hepatoma-derived cell lines (HepG2 and HuH7) and two non-hepatoma lines (Hela and LTK$^-$) as described in Section 2.8. After virus adsorption, bound virus was detected by ^{125}I-labelled MA18/7 (Section 2.8). These reactions were performed at 4°C for a number of reasons: i). to prevent potential virus internalisation which usually occurs at ≥10°C; ii). to ensure that HBV virions remained intact throughout the assay; iii). to minimise any subsequent release of bound virus during the incubation and washing steps.

In some experiments, the number of bound or internalised HBV virions was measured by the detection of HBV DNA by PCR (Section 2.5). HBV DNA amplified by PCR was then analysed by gel electrophoresis followed by spot blot hybridisation using a ^{32}P-labelled oligonucleotide probe (Section 2.4) internal to the target DNA sequence, and quantitated by comparison with the counts bound to PCR products amplified at the same time from known amounts of plasmid DNA pTKHH2.

6.3 RESULTS

6.3.1 HBV Binding to Human Liver Membranes and Hepatoma-Derived Cell Lines

A. HBV Virion Purification

To separate virions from the bulk of the 22nm particles and still retain virion integrity and receptor binding activity, column chromatography followed by sucrose gradient centrifugation was chosen for purification of HBV in this study.

An example of the fractions collected during column chromatography showed that the HBV DNA was detected in fractions 42-49 with a peak between 45-46,

while HBsAg was detected in fractions 44-68 with a peak between 47-63 (Figure 6.1A). Although there was some overlap of the two markers, two separate peaks were clearly observed, that represented virions and HBsAg particles respectively. Furthermore, examination of the virus preparation by EM revealed that fractions 47-49 consisted mainly of 22nm HBsAg particles whereas fractions 44-46 consisted of virions and filaments (data not shown). The HBV-enriched fractions 44-46 were then centrifuged to their buoyant density in a continuous sucrose gradient (Section 2.1). The peak of HBV DNA was detected by spot blot hybridisation in fractions with a density of 1.24g/cm^3 whereas the peak of HBsAg was detected by ELISA in fractions with a density of 1.2g/cm^3 (Figure 6.1B). Electron microscopic examination of these fractions revealed a large majority of HBV virions and some filaments but very few spherical forms of HBsAg (data not shown) indicating that a good separation of HBV particles was achieved by this method.

An attempt to label purified HBV with ^{125}I was performed using the Bolton & Hunter radioiodination reagent according to the supplier's instruction (Amersham). However, radiolabelled particles failed to band at a density of 1.24g/cm^3 in a sucrose gradient and a majority of the radioactive material was present at a density of 1.09-1.1g/cm^3 (data not shown) suggesting that HBV virions were disrupted during the process of radiolabelling and purification. Thus, I chose to perform the HBV-receptor binding assay using a indirect system in which unlabelled HBV virions were detected by ^{125}I-labelled MA18/7, in a similar manner to the DHBV-binding experiments described in Chapter 5.

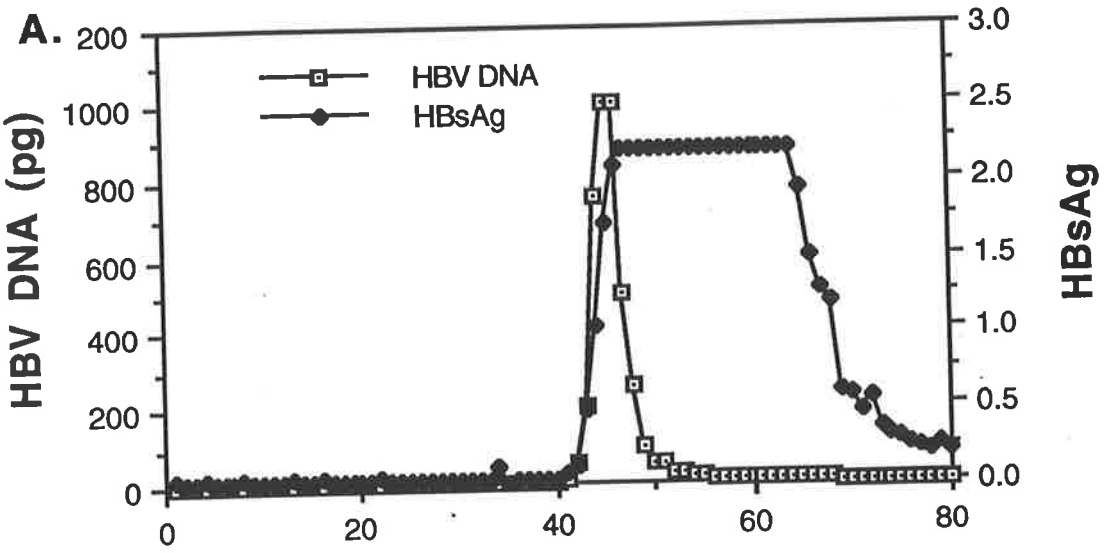
B. HBV-Receptor Binding Assays

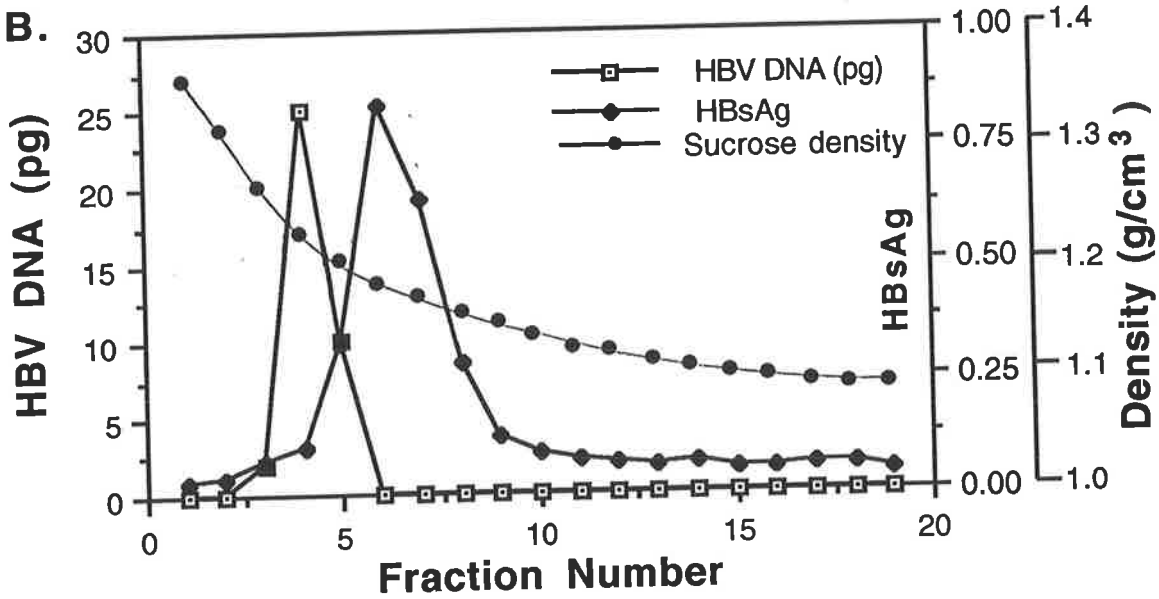
The RIA to detect binding of HBV to human liver membranes was based on experience gained with the DHBV binding assay described in Chapter 5 (Qiao et al., 1991). The internal and plasma membranes were prepared and

Figure 6.1

HBV virion purification from HBV-containing serum. High titre HBV-positive serum ($5x10^7$-$1x10^8$vge/ml) was used as the source of HBV virions. An example of HBV purification by chromatography (Bio-gel A5M) (A), followed by a sucrose gradient centrifugation (B). HBV DNA was examined by dot blot hybridisation and HBsAg by ELISA in all fractions of both purification steps. The value of HBV DNA and HBsAg are presented as pg and optical density respectively.

Figure 6.1


A.
HBV DNA
HBsAg
HBV DNA (pg)
HBsAg
200
1000
800
600
400
200
0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80


B.
HBV DNA (pg)
HBsAg
Sucrose density
HBV DNA (pg)
HBsAg
Density (g/cm3)
30
25
20
15
10
5
0
1.00
0.75
0.50
0.25
0.00
1.4
1.3
1.2
1.1
1.0
0
5
10
15
20
Fraction Number

the enzymatic activities measured as described in Chapter 5 confirmed that a good yield and separation of the membranes were obtained (data not shown). The HBV binding-assay showed that intact HBV virions were able to bind to human liver plasma membranes, but not to internal membranes nor to duck liver plasma membranes. In contrast, other ligands used as controls including mock-purified HBV, DHBV and PBS showed no binding to the membranes [although DHBV could bind to duck liver plasma membranes as described in Chapter 5, the ^{125}I-labelled MA18/7 was not expected to detect bound DHBV (Figure 6.2A)]. Furthermore, a monolayer RIA to detect binding of HBV to cultured cells was established. This was based on the liver membrane-binding assay, but was performed in tissue culture plates. The monolayer RIA showed that HBV also bound to intact cultured HepG2 cells and to a lesser extent to HuH7 cells, but not to HeLa and LTK- cells (Figure 6.2B). The ratio of HBV binding to hepatoma lines HepG2 and HuH7 cells compared with the fibroblast line LTK$^-$ was 11.5:1 and 8.3:1 respectively.

6.3.2 Specificity Assays

Since HBV binding cannot be measured by biological parameters such as infection of cells or some other biologically-measurable response, the specificity of the reaction relates to the binding assay itself. Consequently, some effect was made to ensure that the assay was specific.

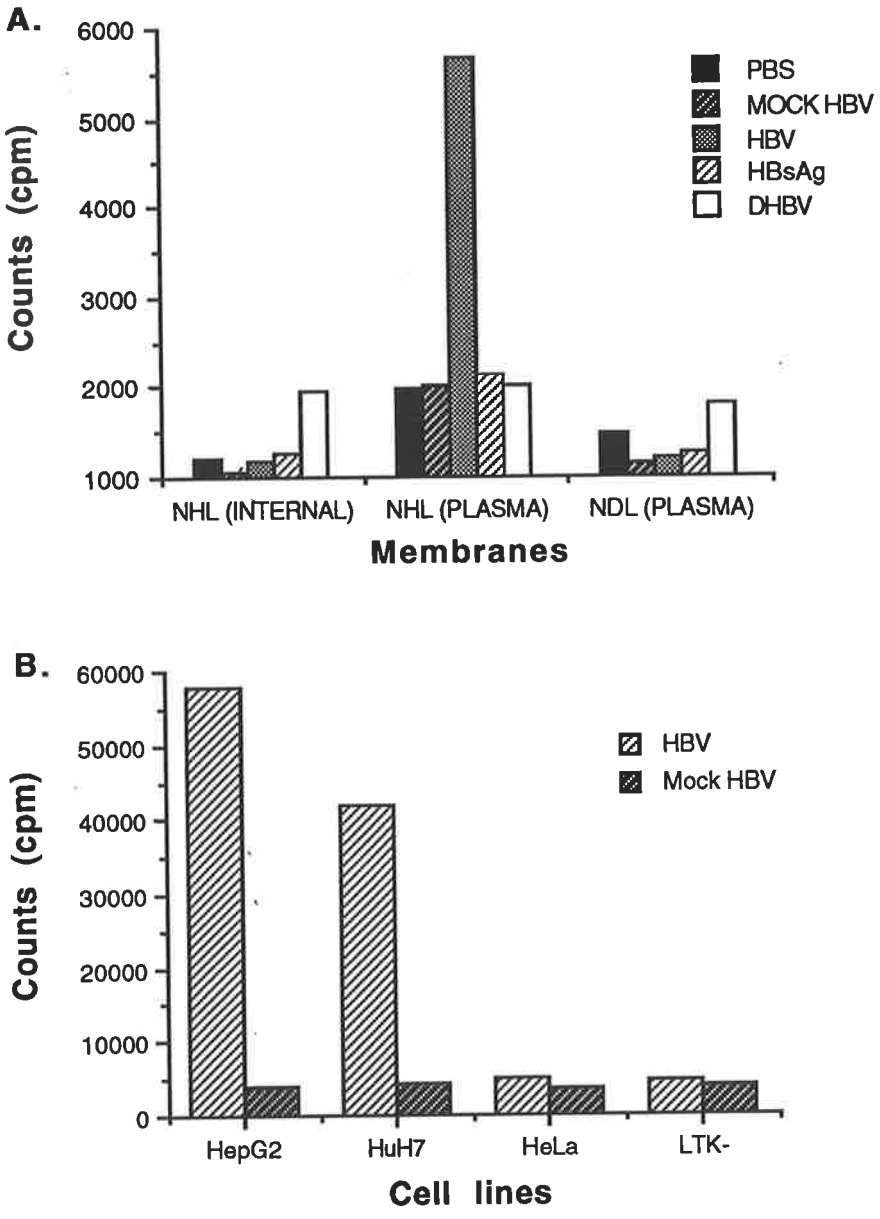
A. Competition

A range of synthetic peptides corresponding to segments of the L-HBsAg was used to inhibit HBV binding to the host cell receptors. Of the peptides used, only peptide 21-47 significantly inhibited the attachment of HBV to human liver plasma membranes and to HepG2 cells by 73% and 38% respectively. A lesser degree of inhibition (8% and 12% respectively) was also observed in both human liver plasma membranes and HepG2 cells preincubated with

Figure 6.2

The HBV-binding activity of human liver membranes and cultured cell lines detected by RIA. Purified human liver plasma- and internal-membranes (A) and monolayer cultures of HepG2, HuH7, HeLa and LTK$^-$ cells (B) were incubated with purified HBV virions, mock-purified HBV or other ligand controls and bound virus detected with ^{125}I-labelled anti-preS1(MA18/7). The results are shown as mean counts per minute bound.

Figure 6.2


A.
6000
5000
4000
3000
2000
1000
Counts (cpm)
PBS
MOCK HBV
HBV
HBsAg
DHBV
NHL (INTERNAL)
NHL (PLASMA)
NDL (PLASMA)
Membranes
B.
60000
50000
40000
30000
20000
10000
0
Counts (cpm)
HBV
Mock HBV
HepG2
HuH7
HeLa
LTK-
Cell lines

peptide 32-49, whereas peptides 12-32 and 120-145 showed no inhibition (Figure 6.3).

The specificity of the binding reaction was further confirmed by antibody inhibition studies. Whole IgG or Fab fragments of a rabbit anti-HBs and MA18/7 were preincubated with virus. In this experiment, ^{125}I-labelled-MA18/7 and a ^{125}I-labelled anti-HBs (Behringwerke) were used respectively as the detector antibody. Both antibody preparations inhibited virus binding in HepG2 cells by approximately 60%-70% when used in the form of whole IgG whereas only MA 18/7 inhibited binding when Fab fragments were used (Figure 6.4). Furthermore, total IgG of the MA18/7 was also shown to block HBV binding to human liver plasma membranes by approximately 76% (data not shown).

B. HBV Binding Kinetics and Saturability

For most viruses studied, nonspecific binding of virions to cells may occur, but these sites are nonsaturable and usually do not lead to productive infection (Tardieu et al., 1982). In general, there are only a finite number of receptor sites on the cell membrane for viral binding and thus high concentrations of virus should fully occupy or saturate them.

Consequently, the ability of excess HBV virions to saturate the receptor on HepG2 cells was examined. HBV virions were incubated with HepG2 cells at 37°C, 20°C and 4°C respectively, and at various times after adsorption, the level of bound virions was measured with the ^{125}I-labelled MA18/7 as described above. The level of bound virus increased with the incubation time and reached a peak more rapidly (2-3hr) at 37°C and 20°C than at 4°C (4hr). The binding at 4°C, however, achieved the highest ratio of bound virions to mock purified HBV (Figure 6.5). Furthermore, there appeared to be no

Figure 6.3

The effect of HBV-specific peptides on HBV-binding activity of human liver plasma membranes and HepG2 cells. Peptides corresponding to aa 12-32, 21-47, 32-49, 120-145 respectively were diluted to concentrations of 50ug/ml, 100ug/ml, 250ug/ml, 500ug/ml. Each concentration of these peptide was preincubated with purified human liver plasma membranes (A) or HepG2 cells (B) prior to adsorption with HBV virions. Bound HBV was detected by ^{125}I-labelled anti-MA18/7 and the results are shown as the percentage inhibition of total HBV-binding activity.

Figure 6.3

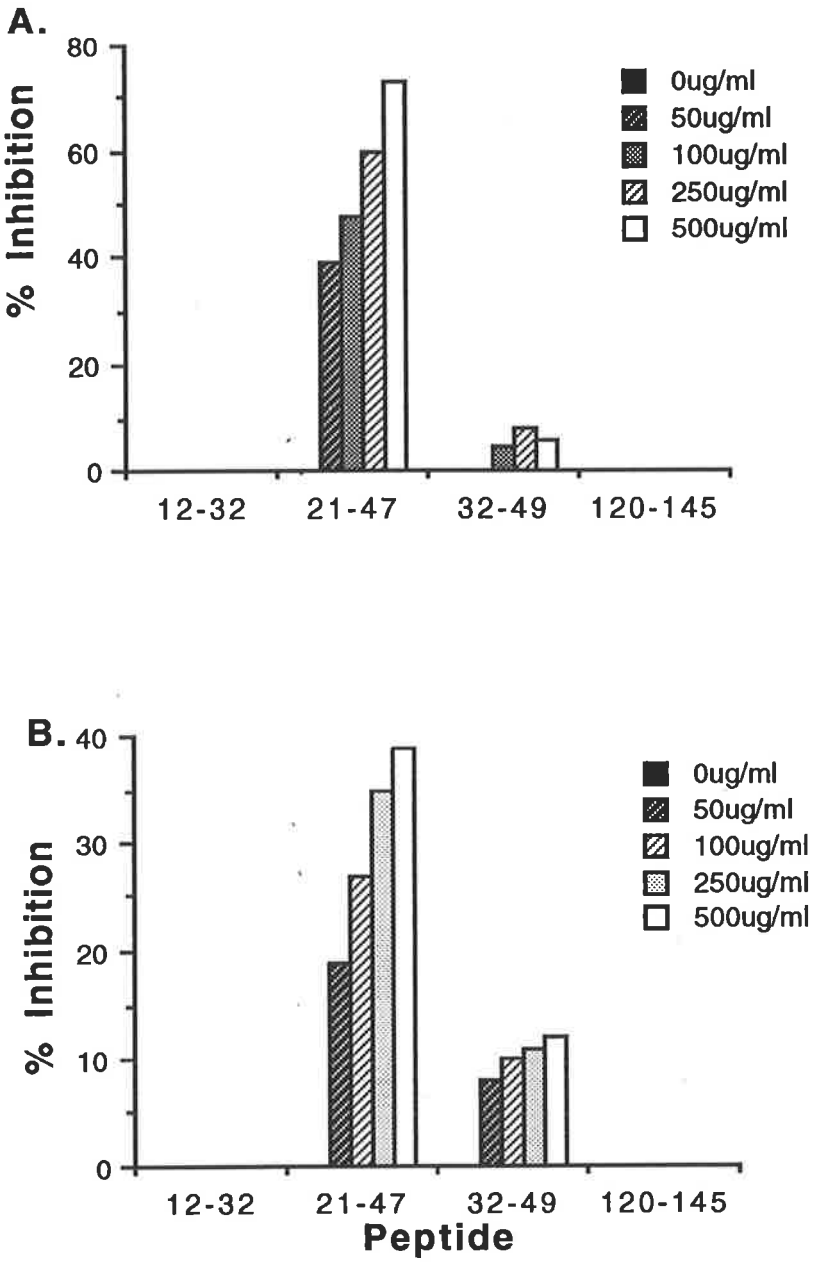

A.
% Inhibition
80
60
40
20
0
12-32
21-47
32-49
120-145
0ug/ml
50ug/ml
100ug/ml
250ug/ml
500ug/ml
B.
% Inhibition
40
30
20
10
0
12-32
21-47
32-49
120-145
Peptide
0ug/ml
50ug/ml
100ug/ml
250ug/ml
500ug/ml

Figure 6.4

The effect of HBV-specific antibodies on the HBV binding activity to HepG2 cells. Each antibody preparation (R α HBs IgG and Fab; MA 18/7 IgG and Fab; NMS) was incubated with purified HBV virions at a ratio of 1:1 (v/v) for 20min at 37°C. The mixtures were then added to the HepG2 cell monolayers and bound virions detected by ^{125}I-labelled-MA18/7 and ^{125}I-labelled RαHBs respectively. The results are shown as the percentage inhibition of the total HBV-binding activity.

Figure 6.4

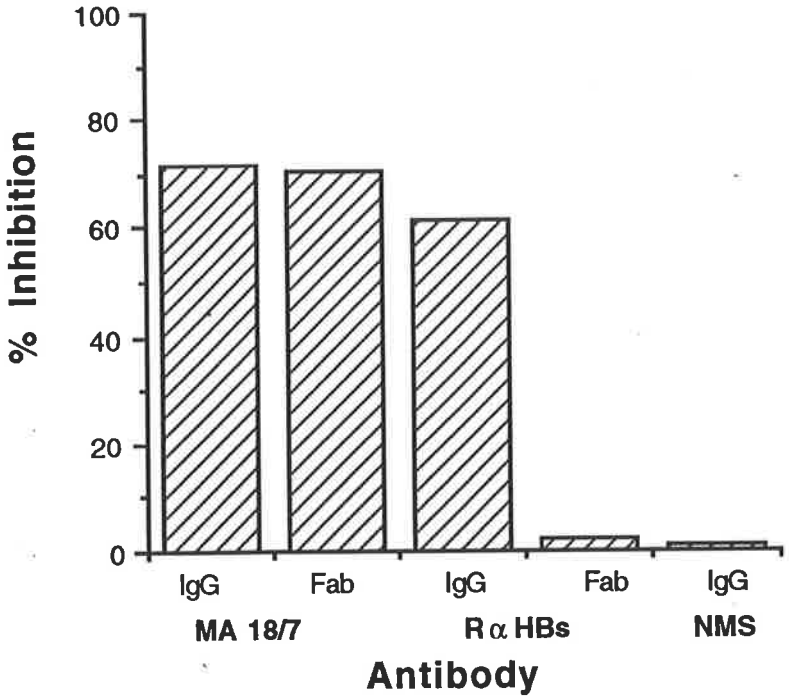
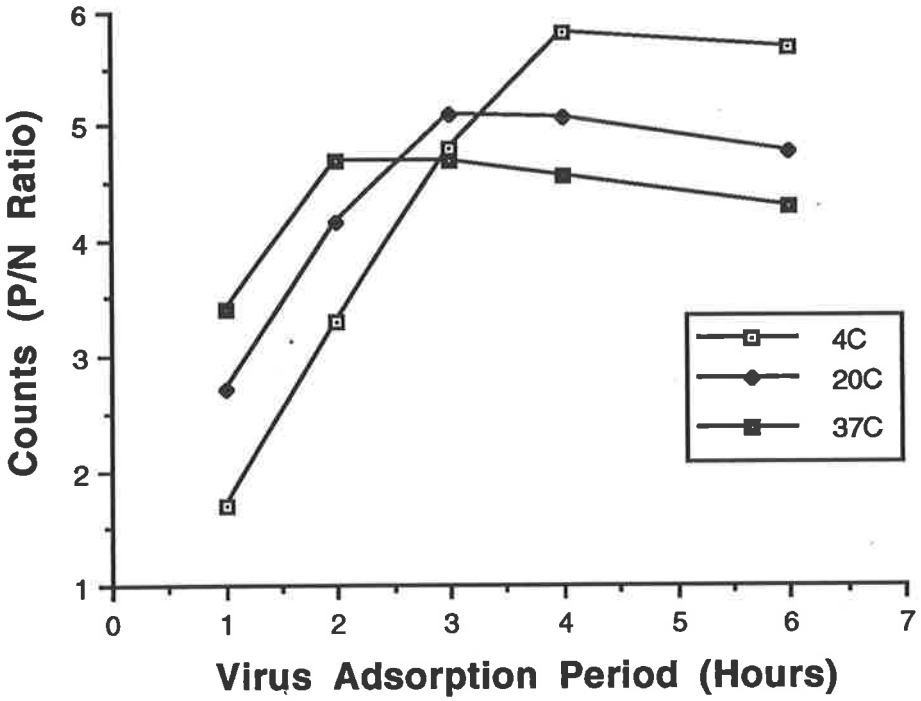

100
80
60
40
20
0
% Inhibition
IgG
Fab
IgG
Fab
IgG
MA 18/7
R α HBs
NMS
Antibody

Figure 6.5

The kinetics of HBV-binding activity in HepG2 cells. The adsorption was carried out at temperatures of 4°C, 20°C and 37°C respectively. The binding activities are presented as the ratio of HBV : mock HBV (P/N).

Figure 6.5


Counts (P/N Ratio)
6
5
4
3
2
1
0
1
2
3
4
5
6
7
Virus Adsorption Period (Hours)
4C
20C
37C

increase in the level of bound virus when the adsorption period was increased from 4-6 hr (Figure 6.5) suggesting that 4hr was optimal. Further experiments described below confirmed that excess virus was available for binding, thus indicating that virus was not limiting in this situation.

Increasing amounts of HBV virions ($1x10^6$ - $1x10^8$ vge/ml) were added to $1x10^5$/ml HepG2 cells and incubated at 4°C for 4 hr. It is likely that virus levels between $1x10^6$ - $1x10^7$ vge/ml were below the level of sensitivity of the RIA. However, the level of binding increased directly with HBV concentrations of $2.5x10^7$ vge/ml and $5x10^7$ vge/ml and appeared to reach a plateau, since no corresponding increase was observed when a virus concentration of $1x10^8$vge/ml was used (Figure 6.6A) indicating that saturation of the virus binding sites occured with virus concentration of $5x10^7$vge/ml. The background level of ^{125}I as judged by the counts bound to the control cell line LTK^- and with HepG2 cells incubated with mock-purified virus showed very little increase with increasing concentration of virus. A saturation effect was also observed in the interaction between HBV and human liver plasma membranes (10ng/ml) with $2.5x10^7$ vge/ml of HBV virions (Figure 6.6B). Thus these experiments confirmed that the HBV-cell binding reaction satisfied another criteria of a genuine receptor-ligand interaction, viz. receptor saturation.

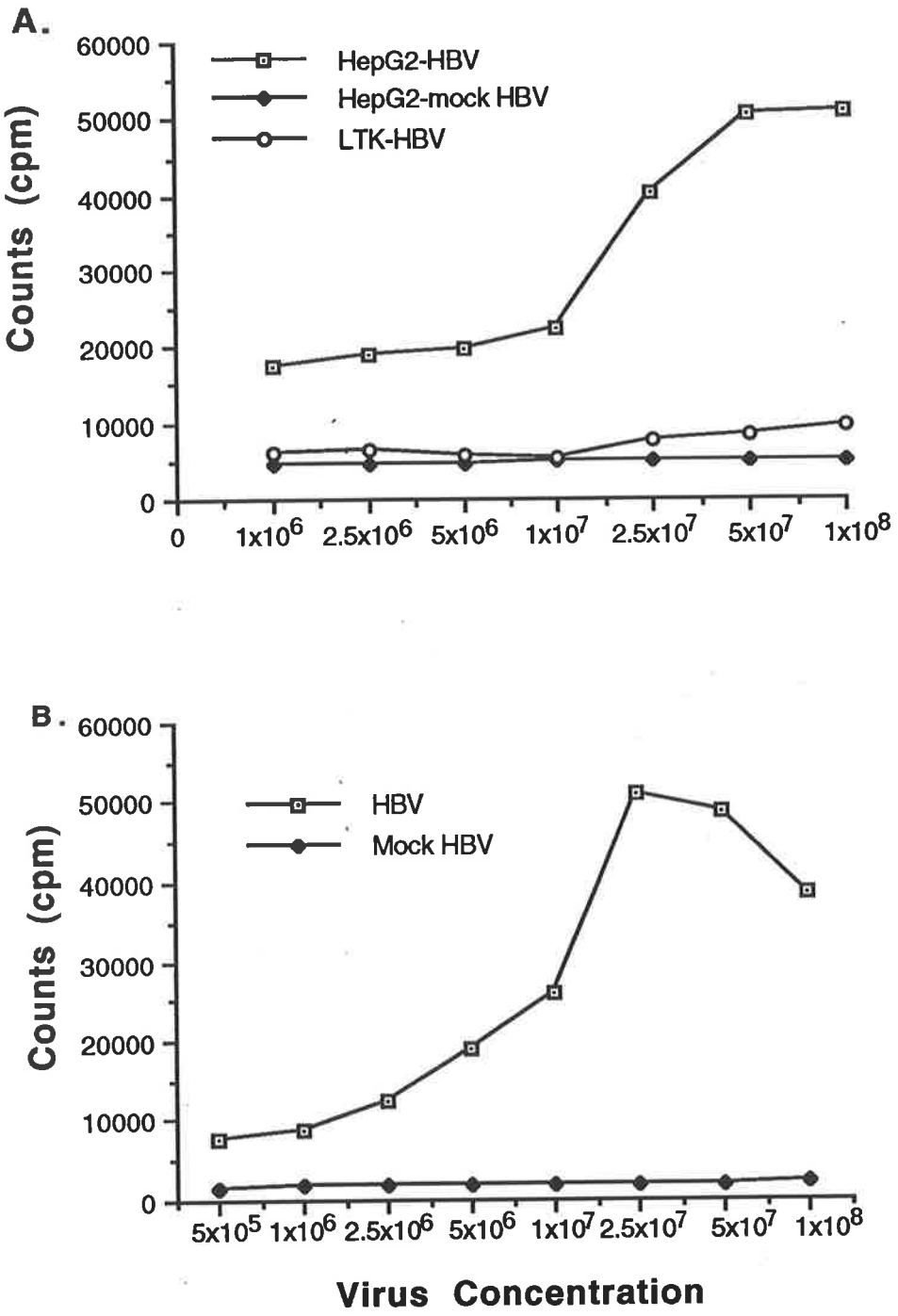
6.3.3 Quantitation of HBV DNA in Cell Binding Experiments

The above data suggested that high concentrations of HBV were able to saturate the receptor on HepG2 cells. Quantitation of the HBV DNA after saturation would then permit an indirect measurement of the number of HBV receptors expressed on HepG2 cells. However, before these experiments were performed, it was necessary to develop and quantitate the PCR for HBV DNA. The conditions for the PCR were optimised with the primers DAW-1 and

Figure 6.6

Saturation of HBV-binding sites in HepG2 cells and human liver plasma membranes. Increasing amounts of virus ($1x10^6$-$1x10^8$vge/ml) or equivalent dilutions of mock-purified HBV were incubated with a constant cell concentration ($1x10^5$/ml) or plasma membrane concentration (10ng/ml). The binding activity was detected by ^{125}I-labelled anti-MA18/7. Virus particles bound to HepG2 cells (A) or human liver plasma membrane (B) are shown as mean counts per minute.

Figure 6.6


A.
Counts (cpm)
60000
50000
40000
30000
20000
10000
0
HepG2-HBV
HepG2-mock HBV
LTK-HBV
0
1x10^6
2.5x10^6
5x10^6
1x10^7
2.5x10^7
5x10^7
1x10^8
B.
Counts (cpm)
60000
50000
40000
30000
20000
10000
0
HBV
Mock HBV
5x10^5
1x10^6
2.5x10^6
5x10^6
1x10^7
2.5x10^7
5x10^7
1x10^8
Virus Concentration

DAW-2 by titration of the $MgCl_2$ concentration (1-4mM), and 3mM shown to be optimal (data not shown). This concentration of $MgCl_2$ was used throughout this study.

To determine the PCR efficiency using primers DAW-1 and DAW-2, a range of concentrations of HBV DNA plasmid pTKHH2 (0.001pg-10pg) was amplified for a various numbers of cycles. A sample of each DNA product was taken at 10, 20, 30 and 35 cycles of amplification and measured by spot blot hybridisation using a ^{32}P-end-labelled probe #90 (Section 2.4.3). Quantitation of the amplified HBV DNA was determined by scintillation counting of dissected membranes. The sensitivity of the PCR after analysis of the PCR product by Southern or spot blot hybridisation was 0.01pg, i.e., between 10-100 fold more sensitive than direct detection of HBV DNA by spot blot hybridisation using a full-length HBV DNA probe. In addition, since the PCR products amplified from concentrations of plasmid DNA between 0.1-10pg were still on the linear part of the curve (Figure 6.7), then this permitted a standard curve to be constructed (see below).

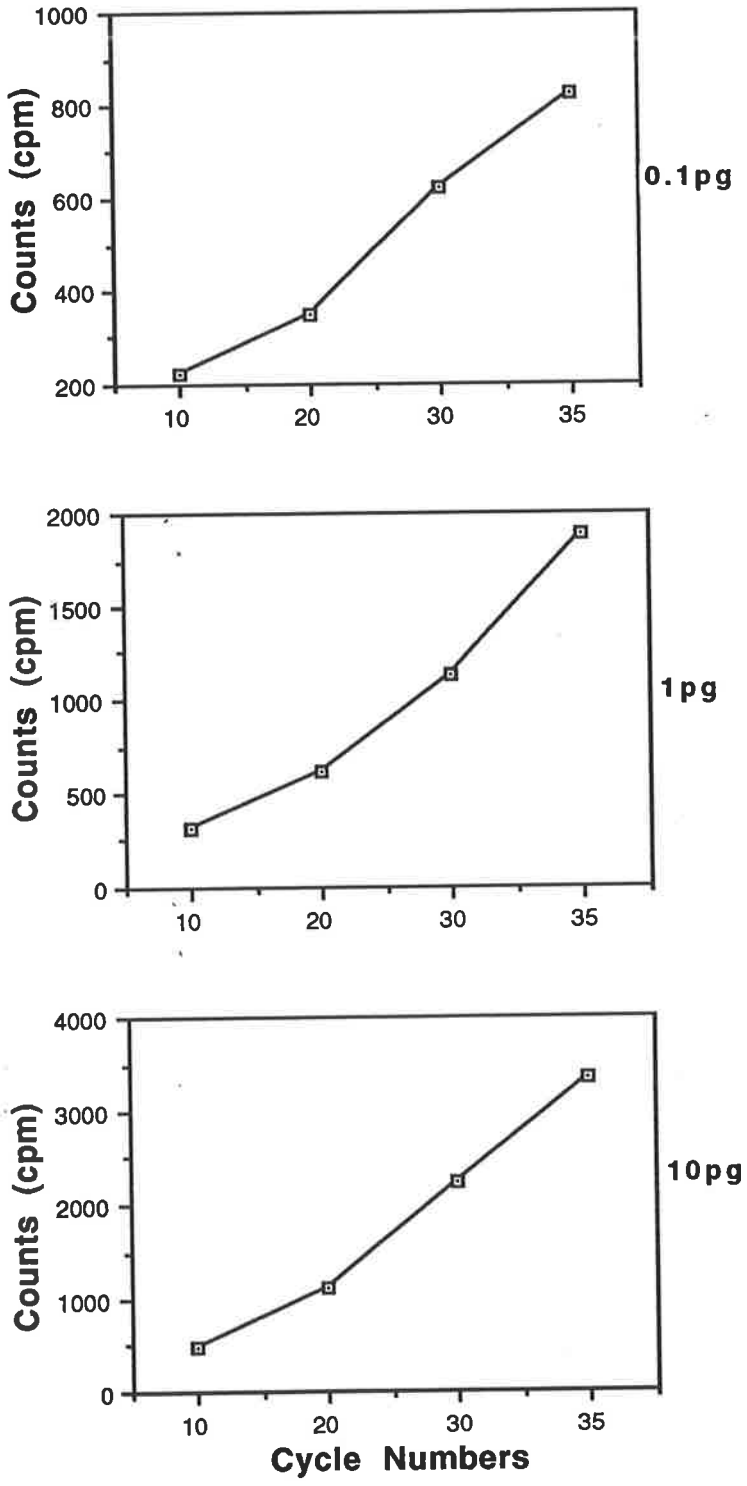
Having established a quantitative PCR, it was now possible to quantitate the number of HBV virions bound to HepG2 cells. HepG2 cells were incubated for 4 hr at 4°C with a level of virus known to saturate the virus binding sites (see Figure 6.6A). The virus solution was removed, the cells were washed as described (Section 2.8), and the HBV DNA remaining in the virus solution, present in the wash solutions or bound to the cells (after extraction of DNA) was amplified by PCR and the level determined by scintillation counting after spot blot hybridisation of the products.

Assuming that the total amount of input virus was 100%, approximately 36% and 44% of the input virus was detected in virus solution after adsorption to the HepG2 and LTK^- cells respectively, and the washes were shown to

Figure 6.7

Amplification of known amounts of HBV DNA by PCR. Plasmid DNA pTKHH2 was diluted to contain 0.1, 1 and 10pg and amplified. An aliquot of each dilution was sampled at 10, 20, 30 and 35 cycles and quantitated by spot blot hybridisation using a ^{32}P-labelled oligonucleotide probe (#90). The results are presented as counts per minute of dissected membrane.

Figure 6.7


1000
800
600
400
200
Counts (cpm)
10
20
30
35
0.1pg
2000
1500
1000
500
0
Counts (cpm)
10
20
30
35
1pg
4000
3000
2000
1000
0
Counts (cpm)
10
20
30
35
Cycle Numbers
10pg

contain approximately 35% and 53% respectively. Cell-associated virus accounted for 8% and 0% in the HepG2 and in LTK$^-$ cells respectively and thus 21% and 3% of the input virus was unaccounted for (Table 6.1).

The actual number of HBV virions bound to HepG2 cells was then estimated using a standard curve constructed by PCR amplification of known amounts of plasmid-derived HBV DNA (Figure 6.8). Comparison with the standard curve showed that approximately 19.2 pg of HBV DNA (shown as dotted line in Figure 6.8) i.e., 5.48×10^6 vge, was bound to 1×10^5 cells. Consequently, each cell was calculated to bind 55 HBV DNA-containing virions.

6.3.4 HBV Internalisation in HepG2 Cells

It was clear from the above results that HepG2 cells express a receptor for HBV and since HepG2 are generally refractile to infection, then it was logical to examine the fate of HBV virions bound during adsorption. It was considered that this information may provide further insights into the (lack of) early events of replication. To examine HBV internalisation after the binding step described above, the cells were incubated at 37°C for 30 min-48 hr then examined after trypsinisation for the presence of HBV DNA by PCR. HepG2 cells which were trypsinised after virus binding but before incubation at 37°C constituted a negative control (T0), as it was assumed that cell-surface bound virus would be digested by this treatment. Similarly, it was assumed that if HBV DNA was detected after 37°C incubation, then this would represent intracellular virus. The results of this experiment (Figure 6.9) showed that trypsinisation of the cells at T0 removed bound virus as judged by the inability of PCR to detect HBV DNA. In contrast, HBV DNA was detected in untrypsinised cells at T0 and in trypsinised cells at T1-T4, indicating that internalisation had occurred. As might be expected the level of internalised HBV remained constant 1-4 hr after adsorption although the 2 hr time point appeared to show some increase

Table 6.1

THE FATE OF HBV VIRIONS AFTER A 4HOUR ADSORPTION PERIOD

	HepG2			LTK		
	V/S[a]	Washes[b]	Cell[b]	V/S[a]	Washes[b]	Cell[c]
Count (cpm/ul)	1272	1295	664	1396	1470	0
HBV DNA (pg)	88	84	19.2	108	128	0
Proportion (%)	36	35.3	8.05	44	53.8	0

a . Virus solution

b. Washing solution

c. Cell-associated HBV

Figure 6.8

Quantitation of cell-bound HBV. Plasmid pTKHH2 was diluted to contain 0.01-10pg and amplified by PCR at the same time as the cell-bound virus DNA. An aliquot of each reaction was sampled at 35 cycles and quantitated by spot blot hybridisation using a ^{32}P-labelled oligonucleotide probe (#90). The results are presented as counts per minute of dissected membrane. The dotted line represents quantitation of PCR-amplified HBV DNA bound to HepG2 cells.

Figure 6.8

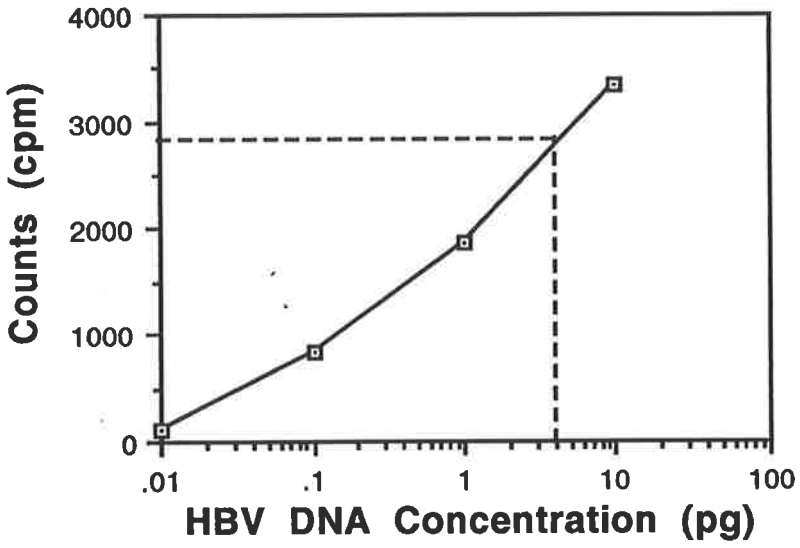
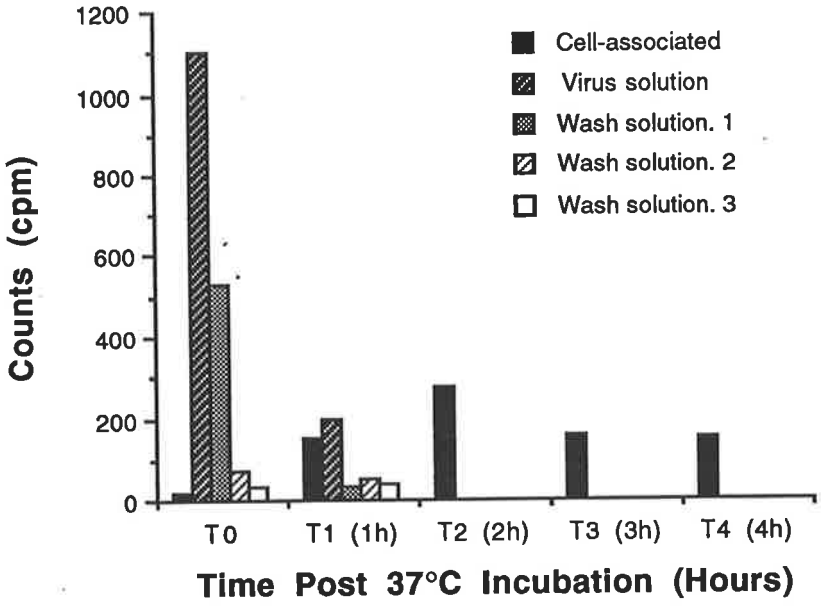

4000
3000
2000
1000
0
Counts (cpm)
.01
.1
1
10
100
HBV DNA Concentration (pg)

Figure 6.9

Relative quantitation of HBV DNA associated with cells, the virus solution (representing unbound virus after the adsorption period) or the washing solutions (collected from the three washes after virus adsortion to the cells) at various time points after adsorption. Extracted HBV DNA was amplified by PCR followed by spot blot hybridisation and quantitation as described in the legend to Figure 6.8. Only cell-associated virus was measured at T2, T3, T4.

Figure 6.9


1200
1000
800
600
400
200
0
Counts (cpm)
Cell-associated
Virus solution
Wash solution. 1
Wash solution. 2
Wash solution. 3
T0
T1 (1h)
T2 (2h)
T3 (3h)
T4 (4h)
Time Post 37°C Incubation (Hours)

which was likely to represent experimental variation. Virus internalisation was observed as early as 30 min post incubation at 37°C and virus DNA remained detectable for 48 hr (data not shown). Later time points were not examined. PCR analysis showed that only a proportion, estimated to be 33%, of cell-associated virus was internalised (Figure 6.9). Comparison with a standard curve described above determined that approximately 18 virus particles per cell were internalised.

The intracellular localisation of internalised HBV was then examined. The cells were trypsinised at various times (0, 1, 2, 4, 6 hr, T0-T4) after adsorption, then fractionated into nuclear and cytoplasmic components (Section 2.4.1B) and HBV DNA amplified by PCR as before. The results of this experiment (Figure 6.10) showed that internalised HBV DNA was detected in the cytoplasm but not in the nucleus 1-2hr after incubation at 37°C. However, the cytoplasmic fraction became negative or showed reduced levels of HBV DNA 4-6 hr after incubation at 37°C. This coincided with the appearance of HBV DNA in the nuclear fraction, suggesting that intracellular relocation of the virus DNA had occurred.

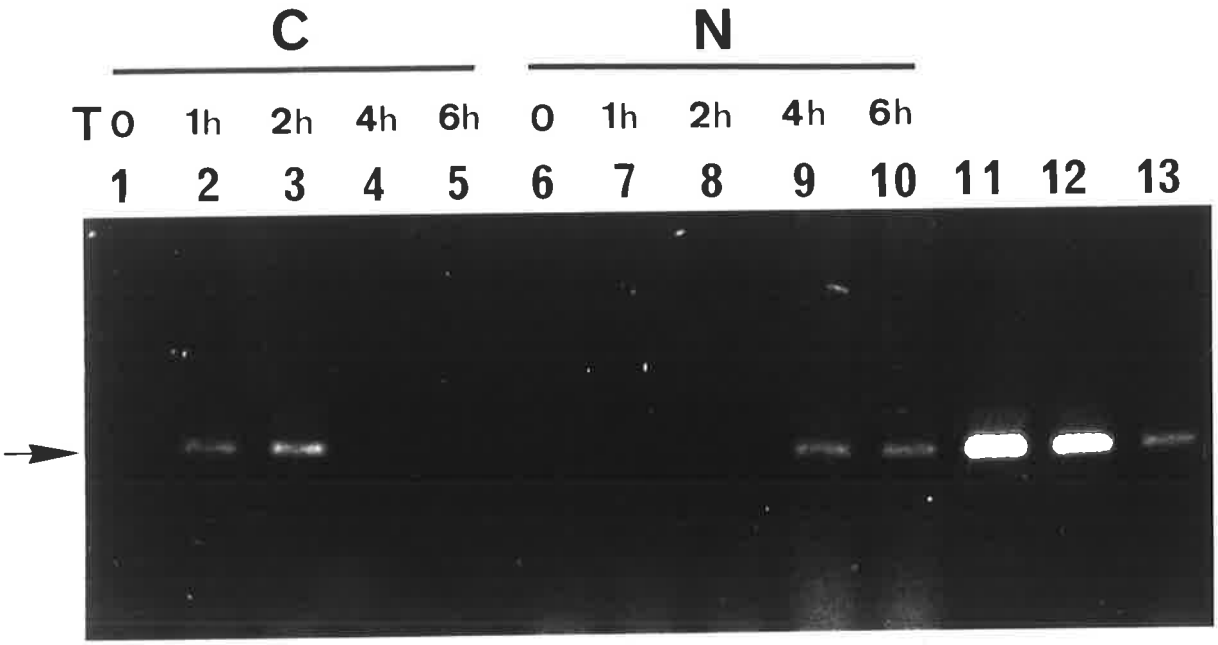
6.4 DISCUSSION

6.4.1 HBV-Receptor Interaction

The first step in virus penetration is binding to a cell surface receptor. Although the receptor for HBV has not been identified, I have described in this chapter an experimental system in which the interaction of HBV with host cells was examined. The study demonstrated that HBV virions and human liver plasma membranes or the hepatoma-derived cells reacted in a manner expected of a specific receptor-ligand interaction. The supporting evidence are summarised as follows: i). the reaction between HBV and the receptor is

Figure 6.10

Analysis by agarose gel electrophoresis of the PCR products from nuclear and cytoplasmic extracts after HBV internalisation. Tracks 1-5 and 6-10 represent cytoplasmic (C) and nuclear (N) fractions respectively at various times post incubation at 37°C. Tracks 1 and 6, 2 and 7, 3 and 8, 4 and 9, 5 and 10 represent 0, 1h, 2h, 4h and 6h post incubation at 37°C respectively. Tracks 11-13 represent the plasmid DNA control containing 10pg, 1pg and 0.1pg respectively. The position of the 384bp product is shown by an arrow and is consistent with a molecular weight marker, PstI digested λ DNA separated in the same gel.


C
N
To
1h
2h
4h
6h
0
1h
2h
4h
6h
1
2
3
4
5
6
7
8
9
10
11
12
13

cell type- and species-specific. HBV-binding activity was only detected on the human liver plasma membranes and the hepatoma-derived cell lines HepG2 and HuH7, but not on duck liver plasma membranes nor HeLa and LTK$^-$ cells derived from tissues other than liver; ii). The binding of HBV to HepG2 cell was inhibited by a peptide to the 21-47 region of the L-HBsAg and by MA 18/7; iii). HBV binding to HepG2 cells could be saturated with excess ligand.

The data suggest that the preS1 region of the L protein contains an attachment site for the receptor on human liver plasma membranes and HepG2 cells. This is consistent with the previous findings by Neurath et al. (1985) and Pontisso et al. (1989b). However, the peptide 21-47 was unable to completely block virus binding in this study but since Neurath et al. (1988) showed that the binding of heterogeneous virus subtypes were not blocked by this peptide, then this is a likely explanation. I have not attempted to subtype the virus used in this study. On the other hand, antibody to the preS1 region and to HBsAg completely inhibited the binding. However, since virus-immune complex formation is likely to account for this, the ability of the Fab fragment of these antibodies to inhibit binding was investigated. Only the Fab fragment of the MA18/7 successfully inhibited the binding, suggesting that this fragment bound to, or close to, the virus attachment site. Thus although MA18/7 and anti-HBs are both neutralising, only the MA18/7 has the ability to inhibit virus binding, whereas both antibodies may act through antibody-mediated opsonisation.

6.4.2 Receptor Quantitation and the Fate of HBV

Using quantitative PCR, I was able to determine the number of virus particles which bound to HepG2 cells at 4°C, a temperature which efficiently arrests endocytosis, thus allowing virus attachment but not penetration. This permitted an estimation of the number of receptor sites per cell at virus

saturation, and this was determined to be approximately 55 copies. However, this figure is likely to be an underestimate for two reasons: i). any HBV-positive preparation is likely to contain a proportion of particles devoid of DNA i.e., empty particles, which would be undetected by PCR; ii). weakly bound HBV virions may have been released from the cells during the wash or internalisation steps. In this regard, 66% of the adsorbed virus failed to be internalised and was released during the first hour of incubation (Figure 6.9) and this may also represent weaker or reversible binding. Using DHBV, strongly bound virus particles represented only 20% of the total bound to primary duck hepatocytes (Pugh & Summers, 1989). Thus, taking into account that the virus preparation contained approximately 45% empty particles (data not shown), and assuming that only 20% of bound particles remain bound after the washing steps i.e., are strongly bound, then it is possible that HepG2 cells actually express approximately 500 copies per cell of the receptor for HBV. Nevertheless, this figure is relatively low compared with that for poliovirus (10^3 sites/cell, Lonberg-Holm & Philipson, 1974), coxsackievirus B3 (10^5 sites/cell, Crowell, 1966) and for human rhinoviruses (10^4-10^6 sites/cell, Lonberg-Holm & Korant, 1972).

6.4.3 The Mode of HBV Entry into the Host Cell

After binding to their receptors, all enveloped animal viruses must gain entry to the host cell cytoplasm by passing through two hydrophobic barriers viz. the cell membrane and the virus membrane. This translocation is often achieved by fusion between membranes that is mediated by viral fusion proteins. Based on the pH at which fusion occurs, two groups of enveloped viruses (pH-dependent and pH-independent) have been described. The mode of entry of the hepadnaviruses to the host cell has not been elucidated, and recent studies have reported conflicting results. In one study, it was suggested that the low pH of intracellular vesicles was not important for entry of DHBV, and

that infection was initiated at neutral pH in a manner analogous to that of pH-independent viruses (Rigg & Schaller, 1992). On the other hand, Offensperger et al. (1992) showed that DHBV infection was inhibited by NH_4Cl_2 and chloroquine suggesting that infection was pH-dependent. These differences may have resulted from conditions of NH_4Cl_2 and chloroquine treatment of the cells by each researcher (Rigg & Schaller, 1992). However, the HBV binding and internalisation assays described in this chapter provide the means to examine the entry mechanism of HBV in a direct manner.

I have shown that the receptor for HBV on HepG2 cells can be saturated, and that the virus was internalised. Furthermore, the virion DNA was transported from the cytoplasm to the nucleus. Thus the HepG2 cells appear able to support many of the early events of HBV replication. Consequently, the block in replication following infection is likely to result from a failure to perform one or all of a number of steps necessary to uncoat the viral DNA and convert the input genome into the transcriptionally-active supercoiled template. Further studies are required to determine the site of this block, but in any case, these studies have provided further insight into understanding the failure of HBV replication in continuous line of hepatoma cells.

CHAPTER 7

CONCLUDING REMARKS

The experiments described in this thesis examined i). the serological profile in ducks aged 1-8 weeks following inoculation with DHBV, leading to a better understanding of the age-related susceptibility in ducks; ii). the involvement of intracellular factors but not receptor levels in determination of the host susceptibility to DHBV infection; iii). details of the binding reaction between HBV and the host cell. The significance of these studies is discussed below.

7.1 SEROLOGICAL EVENTS OF DHBV INFECTION: SIGNIFICANCE AND IMPLICATIONS

The development of the serological assays to detect DHBsAg and anti-DHBs led to a detailed examination of the serological events of DHBV infection in ducks. To my knowledge, these assays were the first available to monitor the serological response to the infection. Consequently, unique data related to the serology of DHBV infection were generated.

A positive DHBsAg result by RIA indicates DHBV infection and potential infectivity. DHBsAg (RIA) and DHBV DNA (dot blot hybridisation) were detected equally well in all the ducks inoculated at 1-8 weeks of age except for two that were positive for DHBV DNA- and one that was positive for DHBsAg-alone. The former cases may represent active viral DNA replication with restricted production of DHBsAg. The latter may result from limited replication with the production of excess DHBsAg. However, in this example it cannot be excluded that some cells may still be capable of producing infectious virus although it appeared that only the DHBsAg gene was expressed. Consequently, this was confirmed in this DHBV DNA negative,

DHBsAg positive bird by the detection of DHBV DNA by PCR indicating a low level of DHBV viraemia. The frequency of this type of infection may be greatly underestimated in HBV due to the limited use of PCR in laboratory diagnosis. One study of HBsAg carriers showed that HBV DNA was detected by PCR in a majority of patients who were negative for HBV DNA by dot blot hybridisation (Baker et al., 1991).

The experimental transmission studies in ducklings is an important model for HBV infection in neonates, to investigate mechanisms of viral pathogenesis and persistent infection. It is equally important to establish a suitable model for HBV infection in the adult human and the studies in this thesis show that DHBV infection of the adult duck mimics this more closely than suggested previously. However, susceptibility to DHBV infection in adult ducks declines significantly and the current adult model is usually derived from injection with a large dose of virus. Thus this model fails to reflect natural HBV infection which is usually acquired by transmission of a small amount of HBV positive blood. The experiments and data from this thesis represent the first step towards the eventual development of such a model.

7.2 FACTORS DETERMINING HOST SUSCEPTIBILITY

The previous findings that the success of transmission depended largely on the age of duck at the time of inoculation, promoted the view that adult ducks were resistant to infection. However, the serological events and response of ducks described in this study confirmed that although susceptibility to DHBV infection was age-related a self-limited infection took place in a proportion of the adult ducks.

Although age-related host susceptibility to HBV has also been described in man (Hoofnagle et al., 1978; Hollinger et al., 1990), the degree of resistance

to the infection in adult ducks seems to be greater than that in adult humans. The fact that liver plasma membranes from neonatal and adult ducks showed similar levels of DHBV-binding activity suggests that loss of receptor expression is not a factor in age-related susceptibility.

In contrast, a major difference (at the cellular level) between ducks of different ages relates to the state of hepatocyte division. The hepatocytes of neonatal ducks are constantly dividing, whereas those of adult ducks are generally in a quiescent state, termed G_0, (Leffert et al., 1982). Since the life span of a human hepatocyte is estimated to be 200-400 days (Fausto, 1992), and this may be similar in the adult duck, there is likely to be a low frequency of dividing hepatocytes and a balance between hepatocyte death and regeneration. In this study, the balance of dividing and quiescent hepatocytes in adult carrier ducks was altered by partial hepatectomy. This was expected to result in a marked increase in the replication of all cell types leading to the orderly replacement of hepatocytes and their supporting biliary-vascular network (Leevy, 1973), and led to increased levels of DHBV replication. This suggests that DHBV replicates or is secreted more efficiently in dividing hepatocytes and thus the decreased susceptibility in adult ducks may be related to the loss of dividing hepatocytes.

Similarly, the fluctuation in the levels of viraemia observed in HBV carriers may reflect the balance of hepatocyte necrosis and regeneration. Indirect evidence to support this hypothesis i.e., that the cell cycle may influence virus expression, was presented by Yeh et al. (1993) who showed that HBcAg expressed in cell lines accumulated in the nucleus during the G1 phase, in the cytoplasm during the S phase, and again in the nucleus when the cells became confluent indicating that nuclear transport of HBcAg <u>in vitro</u> was related to cell cycle (Yeh et al., 1993).

One stimulus for hepatocyte division may be related to hepatocyte necrosis resulting from immunoelimination of HBV-infected hepatocytes. However, there is clear evidence from a number of in vitro studies using cultured cells that the HBV X protein can activate transcription not only from HBV promoters, but also from a variety of other viral and cellular class II promoters, including the LTR of HIV-1 and Rous sarcoma virus, the SV 40 enhancer/early promoter (Seto et al., 1988, Colgrove et al., 1989), the *c-myc* promoter (Balsano et al., 1991), the β-interferon promoter, and class I MHC promoters (Zhou et al., 1990). Thus, the X protein could also influence transcription of specific cellular genes.

Although there is no direct evidence of how X protein may influence cellular factors, it has been proposed to regulate cellular gene expression by i). binding to nuclear transcription factors i.e., CREB and ATF-2 (Maguire et al., 1991) leading to increased transcriptional activity; ii). enzymatically modifying these cellular factors. Bacterial-cell derived X protein was shown to have a serine-threonine protein kinase activity (Wu et al., 1990) that may phosphorylate and activate multiple transcription factors. However, X protein does not phosphorylate either CREB or ATF-2 (Maguire et al., 1991); iii). utilising a tumour promoter signalling pathway, in which increased levels of *sn*-1,2-diacylglycerol leads to activation of protein kinase C and subsequently to activation of the transcription factor AP-1 and probably other protein kinase C-dependent transcription factors (Kekulé et al., 1993); iv). inhibiting the proteolytic digestion of cellular factors. Amino acid sequences in the X protein were found to be highly homologous to functionally essential sequences in the "Kunitz domain", characteristic of Kunitz-type serine protease inhibitors and aa substitutions in this homologous region completely abolished trans-activation (Takada & Koike, 1990). However, no protease-inhibitory function has yet been described in X protein.

In fact, many DNA viruses that replicate in the cell nucleus encode or carry a trans-activation protein (Nevins, 1989) e.g., SV 40 T antigen, papillomavirus E2 and E7 gene products, HSV αTIF. The function of these viral trans-activitors is to modify cellular factors which are normally involved in the transcription of genes in response to growth regulation. These modified cellular factors in turn stimulate the genes of the target cell resulting in the up-regulation of virus replication (Nevins, 1989). Thus, it is highly possible that the HBV X protein has a stimulatory effect on the quiescent differentiated human hepatocyte that could be vital for HBV replication.

The X gene is conserved in all mammalian hepadnaviruses (Colgrove et al., 1989), and a study of WHV suggested that the X protein has critical role in establishing infection (Ganem & Varmus, 1987), consistent with the above hypothesis.

7.3 HEPADNAVIRUS ENTRY AND EARLY VIRUS-CELL INTERACTION

Since HBV binding activity cannot be measured by biological parameters such as infection of cells or some other biologically-measurable response, binding of virus to human liver-derived plasma membranes was measured by a RIA established on the basis of the methods published by other groups (Neurath et al., 1986; Pontisso et al., 1989a; 1989b). In order to avoid any confusing results which are often caused by weak affinity binding between individual viral proteins and plasma membranes, purified serum-derived HBV virions were used. The interaction of the intact HBV virions with purified human liver plasma membranes was characteristic of a receptor-ligand reaction.

The virus-binding activity in hepatoma-derived cell lines, especially HepG2 cells was also examined using a similar RIA. This study demonstrated that HBV virions and HepG2 cells reacted in a cell type- and species-specific manner expected of a specific receptor-ligand interaction. This result is consistent with a previous report (Bchini et al., 1990) and provides more direct evidence that the refractile nature of the vast majority of HepG2 cell cultures is not due to the lack of receptor expression.

The specificity of the binding reaction was considered of paramount importance. The binding activity was inhibited by a peptide corresponding to aa 21-47 from the L-HBsAg region, and by MA18/7 and anti-HBs. It is likely that the antibodies immunoprecipitate the virus particles and thus inhibit virus binding to the cellular receptor (Nussbaum et al., 1984). However, immune complex formation can be prevented by the use of the Fab fragment of the antibody. As a result, two different potential neutralisation mechanisms were revealed.

The anti-HBs is likely to neutralise HBV by intrinsic neutralisation, i.e., the antibody induces some changes in the virion which prevent expression of genetic potential, or by virolysis resulting in irreversible damage to the envelope and destruction of virus infectivity (Mandel, 1978). However, although MA18/7 may also neutralise in a similar manner, since this antibody reacts with epitopes close to the VAP, between sequences 27-35 or 39-47 (Pontisso et al., 1989b), virion adsorption may be blocked by steric hindrance (Norrby, 1986). This mechanism, known as extrinsic neutralisation, is utilised by a neutralising antibody to Reovirus (Fields, 1984).

It is still unclear whether the binding activity detected in human liver plasma membranes and HepG2 cells represent the same receptor. This question may only be resolved by identification of the receptor in HepG2 cells and primary

human hepatocytes, and by comparison of the nature of the two proteins. Furthermore, it is still unclear if the binding activity in HepG2 cells represents any of the proposed HBV receptors described in the literature.

The assay to detect HBV-binding activity described in this thesis was a solid-phase RIA, in which purified virions were incubated with monolayer cultured cells in DMEM supplemented with 5% FBS and antibiotics. No other additives were used at any stage of the binding reaction. Since naturally occuring pHSA, unlike GA-HSA, does not bind significantly to HBsAg and as pHSA was unlikely to be present in the cell culture system this suggests that pHSA was an unlikely mechanism. Similarly, human IL-6 was unlikely to be present in the cell culture medium and, in any case, the association between IL-6 exposed on the surface of cells and the L-HBsAg has been suggested to be insufficient for entry of HBV into cells (Neurath et al., 1992).

The existence of a limited sequence similarity between the virus attachment site (21-47) in the L-HBsAg with the C region of the human IgA α1 chain (Neurath et al., 1986) raised the possibility that HBV may in fact share the same receptor with IgA. IgA has been shown to bind to human hepatocyte cell membranes as well as HepG2 cells (Neurath & Strick, 1990) suggesting that the IgA receptor is expressed in these cells. Antibodies to the preS1 (21-47) region of the L-HBsAg reacted immunologically with a peptide from the (28-55) region of the human IgA α1 C sequence and vice versa (Neurath & Strick, 1990; Pontisso et al., 1992) and this may be the consequence of sequence similarity. However, neither the IgA peptide (28-55) nor antibodies against this peptide inhibited the reaction between HepG2 cells and L-HBsAg (21-47)-immobilised on cellulose (Neurath & Strick, 1990). These data suggest that this sequence similarity is reflected in immunological mimicry but not at the level of a ligand-receptor interaction. Therefore, it seems unlikely that HBV binds to HepG2 cells through this mechanism.

The human transferrin receptor is expressed on a variety of cell types including hepatocytes, consistent with the report of HBV infection in extrahepatic sites. It is yet not known whether the human transferrin receptor is also expressed on HepG2 cells. Blocking HBV binding to HepG2 cells with soluble transferrin receptor or tranferrin itself may provide independent evidence for the role of the transferrin receptor in the HBV-binding activity of HepG2 cells. However, it cannot be excluded that the receptor for HBV may be a totally different molecule from those discussed above.

The nature of the block in HBV replication following "infection" of HepG2 cells is clearly unrelated to binding and internalisation. Since HepG2 cells support HBV replication after transfection of viral DNA, it is most likely that the block is caused by the inability to uncoat the viral DNA and/or to convert the input genome into the transcriptionally-active supercoiled DNA. However, it is unclear if these events require viral and/or cellular functions and it may only by possible to resolve these issues by careful examination of a permissive culture system i.e., primary human hepatocytes. Nevertheless, these studies have altered the view that the refractile nature of HepG2 cells is due to loss of receptor expression through passage.

7.4 FUTURE DIRECTIONS

A number of aspects of hepadnavirus pathogenesis arose in this study. Our understanding of the hepadnavirus entry mechanism has only just begun and future research should focus on:

1). Development of a adult duck model to closely mimic HBV infection in adult human. This model would be important in detailed understanding of Hepadnavirus pathogenesis, virus-cell interaction and evaluation of antiviral therapy.

2). Further identification of the receptor for HBV. The classical means of identification of the cellular receptor for viruses can be used i). production of a MAb against the receptor protein; ii). expression of a cDNA library from HepG2 cells. Both approaches require a virus-binding assay and the experiments described in this thesis have taken the first step towards receptor identification. These studies will eventually permit a detailed examination of the early events of HBV replication.

3). In vitro culture of HBV. In order to understand the block in HBV replication in HepG2 cells, it may be necessary to examine the early events of replication in primary human hepatocytes. These events may equally well be examined in primary duck hepatocytes with DHBV. This knowledge may eventually lead to an improved technique in HBV infection in HepG2 cells to increase the permissiveness, and to establish a cell line which will support HBV replication after infection. This will also permit the development of a completely novel range of anti-viral agents designed to block virus binding or uncoating (see below).

4). Development of novel vaccine and antiviral agents. Knowledge of the receptor gained from the above studies will permit the future design of novel vaccines comprising VAP to prevent the initial attachment of the HBV virion to host cells and anti-viral agents comprising soluble receptor or synthetic peptides corresponding to the sequence of the virus binding site. This approach has been demonstrated in an animal model in which a direct immunisation of chimpanzees by a peptide 12-47 from the preS region elicited antibodies protective against HBV infection (Neurath et al., 1989). In addition, in vitro cell culture of HBV will also present a convenient system for antiviral screening and possible attenuation of the virus for vaccination purposes.

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APPENDIX 1: SOLUTIONS AND MEDIA

Coating Buffer (RIA):

100mM Tris-HCl in PBS, pH9.6

Denaturing Buffer:

0.5M NaOH

1.5M NaCl

Gel Electrophoresis Running Buffer:

50xTAE (1L):

242g Tris-HCl

57.1ml glacial Acetic Acid

100ml, 0.5M EDTA pH 8.0

1xSDS-PAGE Buffer (3L):

9.06g Tris-HCl

43.2g Glycine

15ml, 20%SDS

Hybridisation Solution:

DNA and RNA Probes (10ml):

5ml, 50% deionised formamide

1.4ml, DDW

0.5ml, 10mg/ml sheared salmon sperm DNA (ssDNA, Boehringer)

50ul, 10ug/ml yeast tRNA (Boehringer)

Boil and chill the above ingredient and add the following:

2ml, 5xhybridisation buffer (20xSSC, 0.25M $NaPO_4$, 0.25M Tris-HCl pH7.2)

1ml, 80xDenhardts (16%Ficoll + 16%Polyvinylpyrrolidone)

50ul, 20% SDS

End-Labelled Probes (10ml):

3.025ml, 20xSSC

0.5ml, 1M $NaPO_4$

0.5g dextran sulphate

0.625ml, 80xDenhardts

6ml DDW

150ul, 10mg/ml ss DNA (preboiled)

Loading Buffer:

Agarose Gel (DNA), 6x:

0.25% Bromophenol Blue (BPB, BDH)

0.25% Xylene Cyanol (XC, Labchem)

15% Ficoll 400 (Sigma)

SDS-PAGE Denaturation Buffer, 2x:

50mM Tris-HCl pH6.8

2.5% SDS

12.5% Glycerol

0.0025% BPB

2.5% β-mercaptoethanol

Media:

LB (Luria Bertani) Medium

1% Tryptone (Bacto)

0.5% Yeast Extract (Difco)

171mM NaCl

(1.5% agar in LB plates)

2YT Medium

1.6% Tryptone

1% Yeast Extract

171mM NaCl

Neutralising Buffer:

1.5M NaCl

1.0M Tris-HCl pH7.4

Proteinase K Buffer (2x):

20mM Tris-HCl pH7.8

10mMEDTA

1% SDS

50ug/ml yeast tRNA

SDS-PAGE Solution:

Solution	15% Separating Gel (ml)	5% Stacking Gel (ml)
50% Acrylamide	12	1
1M Tris-HCl pH8.8	15	
1M Tris-HCl pH 6.8		1.25
20% SDS	0.2	0.1
1.5% APS	0.9	0.35
TEMED	0.025	0.01
DDW	12	7.3

Toluene-based Liquid Scintillant

2.5L AR Toluene (Analar)

10g PPO (0.4%, BDH)

1g POPOP (0.04%, Sigma)

APPENDIX 2: DATA AND FORMULAS

Low Molecular Protein Markers (kDa, Bio-Rad)

106; 80; 47; 32; 27;16

Tm of Nucleic Acid Molecules:

DNA:DNA = 16.6 log [Na^+] + 0.41 (G+C) + 81.5 - 0.72 (% FA)

RNA:RNA = 18.5 log[Na^+] + 0.584 (%G+C) + 79.8 -0.35 (%FA)

The Tm of **DNA:RNA** hybrids formed in the solution is estimated to be midway between the above equations of DNA:DNA and RNA:RNA hybrids.

Oligo= 81.5-16.6 log [Na^+] + 41 (% G+C) - 0.63 (% FA) -(600/L)

* L= length of the hybrids in bp

(Sambrook et al., 1989)

Molecules:

Protein:

1.0 (A_{280}) = 781ug/ml

1Da = 1.66×10^{-24}g

ds DNA:

1.0 (A_{260}) = 5.0ug/ml

1kb = 6.5×10^5Da

= 1.1×10^{-6}pg

ssDNA:

1.0 (A_{260}) = 4.0ug/ml

1kb = 3.3×10^5Da

= 5.5×10^{-7}pg

ssRNA:

1kb = 3.4×10^5Da

= 5.6×10^{-7}pg

1 DHBV genome:

$= 3.3 \times 10^{-6}$ pg

Radioisotopes:

1uCi= 2.2×10^{6} dpm

Riboprobe Mixture (20ul):

4ul of 5xtranscription buffer (200mM Tris-HCl pH7.5, 30mM $MgCl_2$, 10mM Spermidine, 50mM NaCl)

2ul of 100mM DTT

0.5ul of 25U/ul RNasin

1ul of each 10mM rATP, rGTP and rCTP

2.4ul of 100uM rUTP

1ul of DDW

1ul of 1ug/ul linearised DNA template

5ul of 10uCi/ul α-^{32}P UTP (3000Ci/mmol, Bresatec)

1ul of 10U/ul SP6-or T7-RNA polymerase

Nick Translation Reaction Mixture (25ul):

4ul of 600mM Tris-HCl pH7.6

4ul of 66mM $MgCl_2$

4ul of 50mM DTT

1ul of each 500uM cold dGTP and dTTP

5ul of 10pg/ul DNase 1 (Boehringer)

1ul of 10U/ul of DNA polymerase 1 (Boehringer)

4ul of DDW

1ul of 100ng of template DNA

End-Labelling Reaction Mixture (20ul):

1.5ul of 100ng/ul oligonucleotide

2.0ul of 10xkinase buffer (phor buffer, Pharmacia)

5ul of γ-^{32}P-ATP (3000Ci/mM, Bresatec)

10.5ul of DDW

1.0ul of 10U/ul T4 polynucleotide kinase (Pharmacia)

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Adsorption, Penetration and Nuclear Transport of Hepatitis B Virus in a Non-Permissive Cell Line

MING QIAO, THOMAS B. MACNAUGHTON, AND ERIC J. GOWANS*

Division of Medical Virology
Institute of Medical and Veterinary Science
P.O. Box 14 Rundle Mall
Adelaide SA 5000
and
Department of Microbiology and Immunology
University of Adelaide
North Terrace
Adelaide SA 5000
AUSTRALIA

* To whom correspondence should be addressed.
Tel: 08 228 4631
Fax: 08 224 0927

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ABSTRACT

A monolayer radioimmunoassay was established to detect hepatitis B virus (HBV)-binding activity. HBV was shown to bind to HepG2 and HuH7 cells, but not to LTK$^-$ and HeLa cells. The binding activity was inhibited by peptide 21-47 from the large hepatitis B surface antigen (L-HBsAg) region, but not by other peptides from the L- and middle (M)-HBsAg. A monoclonal antibody to L-HBsAg (MA18/7) and a polyclonal antibody to HBsAg (anti-HBs) also inhibited the binding. However, only the Fab fragment of MA18/7, but not the Fab fragment of anti-HBs was able to do so suggesting that these two antibodies may neutralize virus by different mechanisms.

Excess HBV was able to saturate the binding activity of HepG2 cells, and the number of receptors per cell was calculated to be approximately 500 by quantitative PCR. The virus was also shown to be internalized; virus DNA was detected in the cytoplasm 1-2h post adsorption and appeared in the nucleus 1-2h later. Thus the HepG2 cells appear to support many of the early events leading to HBV replication despite the fact that the cells are generally refractile to infection.

INTRODUCTION

A virus-cell interaction which represents the initial step in virus replication, occurs at the plasma membrane of susceptible cells (18). This attachment step is usually followed by virus internalization then relocation to the nucleus or some other site in the cell prior to uncoating and genome replication. Details of these early events, including the nature of the cellular receptor, remain unclear for hepatitis B virus (HBV).

However, HBV-associated particles were able to bind to the human hepatoma cell line HepG2 (10) and to purified human liver plasma membranes (13) suggesting that a functional receptor was expressed. Several lines of evidence have suggested that HBV may bind to the host cell via an intermediate molecule such as polymerised human serum albumin (13,19) or Interleukin 6 (12), that may act as a bridge for entry. Alternatively, two reports suggest that HBV binds via a direct mechanism suggested to be the transferrin receptor (3) or the IgA receptor (14). These reports are conflicting and the nature of the interaction of HBV with the host cell plasma membrane requires confirmation. The identity of the virus attachment site within the HBV envelope is better defined and has been found to be within amino acids 21-47 of the large (L)-HBsAg (10).

A major impediment to these studies is the lack of a convenient cell culture system for HBV. Many attempts to establish a conventional cell culture system using hepatoma-derived lines have been unsuccessful, but as these cells can support HBV replication after transfection of HBV DNA, it is likely that the block in replication following infection is attributed to an early step in the replication cycle. The only report describing infection of HepG2 cells with HBV (1) is consistent with the HBV-binding activity described previously in these cells (9, 10). In our hands, HepG2 cells are refractile to infection and thus bound virus

binding activity of HepG2 cells. We have examined the efficiency and details of the specificity of the binding reaction, and virus internalization.

MATERIALS AND METHODS

Preparation of HBV Virions

Purification of HBV virions was performed essentially as described by Heermann et al. (5) with some modifications. Virus from 10ml of serum with a high titer of HBV (determined by dot blot hybridization) was pelleted through a 1ml cushion of 20% sucrose using a 80Ti rotor (Beckman) at 230,000g for 4h at 4°C. The supernatant was removed and the pellet resuspended at 4°C in 50-100ul of TNE (10mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA).

To separate HBV virions from HBsAg particles, the above suspension was loaded onto a 2.5x70cm column of Bio-Gel A5M (Bio-Rad) then pumped with TNE buffer containing 0.5% BSA and 0.02% NaN_3 at a rate of 4ml/h, and 1.5ml fractions were collected. These fractions were assayed for HBV DNA by dot blot hybridization and HBsAg by ELISA (Abbott). Fractions positive for HBV DNA were combined, centrifuged through a 20% sucrose cushion as described above and the pellet resuspended in 0.5ml PBS at 4°C. The virus suspension was overlaid on a 10.8ml continuous sucrose gradient (25-50% w/v) in TNE. After 20h centrifugation at 150,000g in a SW 41 rotor at 10°C, 200ul fractions were collected from the bottom of the tube and assayed for HBV DNA and HBsAg as described above. Fractions positive for HBV DNA were combined, centrifuged through a 20% sucrose cushion as described above and the pellet resuspended in 0.5ml PBS, 0.5% BSA, 0.02% NaN_3 and stored at 4°C prior to use.

An aliquot of the virus preparations was also stained by 3% phosphotungstic

Solid-Phase HBV-Receptor Binding assay in Monolayer Culture

A monolayer radioimmunoassay to detect binding of HBV to cultured cells was established based on the liver membrane-binding assay as described previously (16). Cells were grown in 48- or 24-well plates (Costar) at $1x10^5$ and $2x10^5$cells/well respectively in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS in 5% CO_2.

Before virus adsorption, the cell monolayer was washed 3 times with cold (4°C) PBS and the plates kept on ice throughout the binding assay. 200ul (for 48-well) or 500ul (for 24-well) of purified HBV virions ($2-5x10^7$vge) or mock-purified HBV in DMEM supplemented with 2% FBS was added and incubated for 4h. The cells were then washed 3 times with cold PBS and incubated with 0.2ml of ^{125}I-labeled monoclonal antibody to L-HBsAg (MA18/7, a generous gift from Professor Wolfgang Gerlich, reference 5) containing $2x10^6$cpm in the above medium for 2h. The cells were then washed once with cold PBS containing 0.05% Tween-20, then 3 times with PBS alone. The cells were then solubilized in 0.2ml of 2% SDS in PBS, the suspension transferred into tubes and counted in a Packard Gamma counter.

i). Virus-Binding Specificity

Synthetic peptides 12-32 and 120-145 from the preS1 and preS2 regions respectively of the HBV surface antigen gene were kindly supplied by Dr S Kent (The Scripps Research Institute, La Jolla, San Diego, USA); peptide 32-49 from the preS1 region was a gift from Professor CR Howard (Royal Veterinary College, London, UK), and peptide 21-47 was synthesised by Peptide and Protein Research Consultants (Washington Singer Laboratory, Exeter, UK). A range of concentrations (50-500ug/ml) of each of these peptides was preincubated with the cell monolayers for 2h at 37°C. The cells were then

ii). Inhibition With HBV-Specific Antibodies

The ability of MA 18/7, the detector antibody in the HBV-receptor binding assay to block the receptor binding site on the HBV virion was examined. This experiment was performed using total IgG or the Fab fragment of the antibody; this was prepared as described previously (8).

To block binding, the virus preparation ($5x10^8$vge/ml) was preincubated with the IgG or Fab fragment of MA 18/7 (1:1 ratio) at 37°C for 20 min prior to incubation with cultured cells and the results compared with those using IgG or the Fab fragment of rabbit anti-HBs. In this experiment, a ^{125}I-labeled polyclonal antibody to HBsAg (anti-HBs; Behringwerke) and ^{125}I-labeled-MA18/7 were used respectively as the detector antibody .

Total Cellular DNA Extraction

Cells in 24- or 48-well plates were washed 3 times with cold PBS, removed with 1% trypsin for 10min at 4°C and transferred into an Eppendorf tube. The cells were washed with cold PBS, centrifuged at 2000g for 2 min, resuspended in 100ul DDW, then mixed with equal volume of buffer containing 20mM Tris-HCl pH7.4, 10mM EDTA, 1% SDS, 500ug/ml Proteinase K and 50ug/ml yeast tRNA, and incubated at 65°C for 15 min then 37°C o/n. The preparation was then phenol:chloroform extracted, and the DNA ethanol precipitated then redissolved in 20ul of DDW.

Extraction of DNA from Cytoplasm and Nucleus

After trypsinization as described above, cell pellets were washed three times with cold PBS and resuspended in 200ul of 0.5% NP-40, 1mM EDTA, 150mM NaCl and 10mM Tris-HCl 7.6. The suspension was vortexed and kept on ice for 10 min and then pulse centrifuged for 20 sec. The supernatant was removed and digested with proteinase K as above for 1h at 37°C. The pellet (nuclei) was

with proteinase K. The DNA in both preparations was ethanol precipitated then redissolved in 20ul DDW.

HBV DNA Detection by PCR

In some experiments, bound or internalized HBV was measured by the detection of HBV DNA by the polymerase chain reaction (PCR). The sequence of the primers was: DAW-1: 5'- AGT GCG AAT CCA CAC TCC-3'; DAW-2: 5'-GAA TTT GGA GCT ACT GTG GAG-3'. These primers were designed to amplify a 384bp region from the core gene.

10ul of the DNA samples was added to a 40ul PCR mix containing 10mM Tris-HCl pH8.3, 50mM KCl, 50uM of each dNTP, 20pM of each primer, 1.25u Ampli Taq DNA Polymerase (Perkin Elmer Cetus), 3mM $MgCl_2$, denatured at 94°C for 5min and amplified for 35 cycles at 94°C-1 min, 55°C-1 min and 72°C-1 min followed by 72°C for 7 min. The PCR product was analysed by gel electrophoresis followed by Southern- or spot blot- hybridization using a ^{32}P-labeled oligonucleotide probe (#90; 5'-CCC ACC CAG GTA GCT AGA GTC ATT AAG- 3') internal to the target DNA sequence. HBV DNA amplified by PCR was quantitated after spot blot hybridization with the same probe by comparison with the counts bound to PCR products amplified from known amounts of plasmid HBV DNA.

RESULTS

The Specificity of the HBV-Cell Binding Reaction

Initially, a monolayer radioimmunoassay was developed to measure the HBV binding activity of different cells. Two human hepatoma-derived cell lines (HepG2 and HuH7) and two fibroblast lines (HeLa and LTK^-) were chosen. Virus adsorption and the subsequent antibody detection step were performed at 4°C for a number of reasons: i) to prevent potential virus internalization which usually

during incubation and washing. The monolayer radioimmunoassay showed that HBV was able to bind to intact cultured HepG2 cells and to a lesser extent to HuH7 cells, but not to HeLa and LTK$^-$ cells (Figure 1). The positive:negative (P/N) ratio of HBV binding to hepatoma lines HepG2 and HuH7 cells compared with the fibroblast line LTK$^-$ was 11.5:1 and 8.3:1 respectively.

Since HBV binding cannot be measured by infection of cells or some other biologically-measurable response, the specificity of the reaction must be confirmed directly by competition, blocking and/or saturation of the receptor. Consequently, a range of synthetic peptides corresponding to segments of the L- and M-HBsAg was used to compete with HBV binding to the host cell receptor. Of the peptides used, only peptide 21-47 showed significant inhibition, viz 38%. In contrast, HepG2 cells preincubated with peptide 32-49 showed an inhibition of 12%, whereas peptides 12-32 and 120-145 showed no inhibition (Figure 2). These results are consistent with previous reports which support the concept that L-HBsAg region 21-47 interacts with the cell receptor. However, the level of inhibition was lower than expected, and this may be related to subtype differences (see discussion).

The specificity of the binding reaction was further confirmed by antibody inhibition. Whole antibodies or Fab fragments of anti-HBs and MA18/7 were preincubated with virus. Although both antibody preparations inhibited virus binding by approximately 60%-70% when used in the form of whole IgG, only MA 18/7 inhibited binding when Fab fragments were used (Figure 3).

Taken together, the competition and inhibition studies confirm previous reports and suggest that the HBV-cell binding reaction was specific. The studies also extend our understanding of the potential mechanisms of neutralization (see discussion).

For most viruses studied, nonspecific binding of virions to cells may occur, but these sites are nonsaturable and usually do not lead to productive infection (18). In general, only a finite number of receptor sites are present on the cell membrane that are available for virus binding and thus high concentrations of virus should be able to fully occupy or saturate them.

Consequently, we examined the ability of excess HBV virions to saturate the receptor on HepG2 cells. HBV virions were incubated with HepG2 cells at 37°C, 20°C and 4°C respectively, and after various periods of adsorption, the level of bound virions was measured by the ^{125}I-labeled MA18/7 as described above. The level of bound virus increased with the incubation time and reached a peak more rapidly (2-3h) at 37°C and 20°C than at 4°C (4h); the binding at 4°C, however, achieved the highest P/N ratio (Figure 4). Furthermore, there appeared to be no increase in the level of bound virus when the adsorption period was increased from 4-6h (Figure 4) suggesting that 4h was optimal. Further experiments described below confirmed that excess virus was available and thus this was not the limiting factor.

Using a constant cell concentration ($1x10^5$) of HepG2 cells, we then added increasing amounts of virus ($1x10^6$-$1x10^8$vge/ml) to these cells and incubated at 4°C for 4h. The level of binding increased directly with the HBV concentration except with the two highest concentrations of virus used. This suggested that a plateau was reached (Figure 5). The background level of ^{125}I, as judged by the counts bound to the control cell line LTK$^-$ and with HepG2 cells incubated with mock-purified virus, showed very little increase with increasing concentration of virus. Thus this experiment confirmed that the HBV-cell binding reaction satisfied another criteria of a genuine receptor-ligand interaction, viz. saturation.

Receptor Quantitation

saturation would then permit a direct measure of HBV receptors expressed on HepG2 cells. HepG2 cells were incubated for 4h with a level of virus known to saturate the virus binding sites (see Figure 5). The virus preparation was removed, the cells were washed as described in Materials and Methods, and the HBV DNA bound to the cells (after extraction of DNA), remaining in solution or present in the washes was amplified by PCR and the relative level determined by scintillation counting after spot blot hybridization of the products.

Assuming the input virus to represent 100%, approximately 36% and 44% of the input virus was detected in solution after adsorption to the HepG2 and LTK$^-$ cells respectively, and the washes were shown to contain approximately 35% and 53% respectively. Cell-associated virus accounted for 8% and 0% in the HepG2 and in LTK$^-$ cells respectively and thus 21% and 3% of the input virus was unaccounted for (Table 1). The actual number of HBV virions bound to HepG2 cells was then estimated from a standard curve constructed by PCR amplification of known amounts of plasmid-derived HBV DNA (Figure 6). The standard curve was constructed having previously shown that the PCR products amplified from concentrations of plasmid DNA between 0.1-10pg were still on the linear part of the curve (data not shown). This experiment showed that approximately 19.2 pg of HBV DNA i.e. 5.48×10^6vge was bound to 1×10^5 cells suggesting that 55 HBV DNA-containing virions bound per cell.

HBV Internalization in HepG2 Cells

It was clear from the above results that HepG2 cells express a receptor for HBV and since HepG2 are generally refractile to infection, we wished to examine the fate of HBV virions bound during adsorption. It was considered that this information may provide further insights into the (lack of) early events of replication. To this end, we examined HBV internalization after the binding step described above, when the cells were incubated at 37°C for 30 min-48h (T1-T8),

constituted a negative control (T0), as it was assumed that cell surface-bound virus would be digested by this treatment. Similarly, it was assumed that HBV DNA detected after 37°C incubation was protected from trypsinization and thus would represent intracellular virus. The results of this experiment (Figure 7) showed that trypsinization of the cells at T0 removed bound virus as judged by the inability to detect HBV DNA by PCR. In contrast, HBV DNA was detected in untrypsinized cells at T0 and in trypsinized cells at T1-T4. The level of internalized HBV peaked 2h post incubation at 37°C. However, PCR analysis of the cell culture medium removed prior to trypsinization showed that only a proportion, estimated to be 33%, of cell-bound virus was internalized (Figure 7). Comparison with a standard curve described above determined that approximately 18 virus particles per cell were internalized. Virus internalization was observed as early as 30min post incubation at 37°C (data not shown), and virus DNA remained detectable for 48h. Later time points were not examined.

The intracellular localization of internalized HBV was then examined. The cells were trypsinized at various times (0, 1, 2, 4, 6h, T0-T4) after adsorption, fractionated into nuclear and cytoplasmic components and HBV DNA detected by PCR as before. The results of this experiment (Figure 8) show that internalized HBV DNA was detected in the cytoplasm but not in the nucleus 1-2h after incubation at 37°C. However, the cytoplasmic fraction became negative or showed reduced levels of HBV DNA 4-6h after incubation at 37°C. This coincided with the appearance of HBV DNA in the nuclear fraction, suggesting that intracellular relocation of the virus DNA had occurred.

DISCUSSION

We have described an experimental system to examine the interaction of HBV with host cells. The study demonstrated that HBV virions and hepatoma-derived

and the receptor is cell type- and species-specific. HBV-binding activity was detected on the hepatoma-derived cell lines HepG2 and HuH7, but not on HeLa and LTK$^-$ cells derived from tissues other than liver. In a separate study, HBV was shown to bind specifically to human liver plasma membranes whereas duck liver plasma membranes showed no interaction with HBV virions (data not shown); ii) HBV-binding to HepG2 cell was inhibited by a peptide to the 21-47 region of the L-HBsAg and by an antibody to the L protein of the HBsAg (MA 18/7); iii) HBV binding to HepG2 cells could be saturated with excess ligand. Our data shows that the preS1 region of the L protein contains an attachment site for the receptor on human liver plasma membranes and HepG2 cells. This result is consistent with previous findings (9, 13). However, the peptide 21-47 was unable to block virus binding completely in our study, but since the binding of heterogeneous virus subtypes were not blocked by the 21-47 peptide (11), this is a likely explanation. We have not attempted to subtype the virus used in our studies. On the other hand, antibody to the preS1 region and to HBsAg completely inhibited the binding, but since virus-immune complex formation is likely to account for this, the ability of the Fab fragments to inhibit binding was investigated. Only the Fab fragment of the antibody to the preS region successfully inhibited binding, suggesting that this fragment bound to, or close to, the virus attachment site. Thus although anti-preS1 and anti-HBs are both neutralizing, only anti-preS1 has the ability to inhibit virus binding, whereas both antibodies may act through antibody-mediated opsonization.

We were able to determine the number of virus particles which bound to HepG2 cells at 4°C, a temperature which efficiently arrests endocytosis, thus allowing virus attachment, but not penetration. This permitted an estimation of the number of receptor sites per cell at virus saturation and this was determined to be approximately 55 per cell. However, this figure is likely to be an underestimate for two reasons: i) any HBV-positive preparation is likely to contain a proportion

during the wash or internalization steps. In this regard, 66% of the adsorbed virus failed to be internalized and was released during the first hour of incubation (Figure 7) and this may represent weaker or reversible binding. Using DHBV, strongly bound virus particles represented only 20% of the total bound to primary duck hepatocytes (15). Thus taking into account that our virus preparation contained approximately 45% empty particles (data not shown), and assuming that only 20% of bound virions remain bound after the washing steps i.e. were strongly bound, then it is possible that HepG2 cells actually express approximately 500 copies per cell of the receptor for HBV. Nevertheless, this figure is relatively low compared with that for poliovirus (10^3 sites/cell, reference 6), coxsackievirus B3 (10^5 sites/cell, reference 2) and for human rhinoviruses (10^4-10^6 sites/cell, reference 7).

Although the mode of entry for HBV to the host cell is still unclear, we have shown that the receptor for HBV on HepG2 cells can be saturated, and that the virus is internalized. Furthermore, the virion DNA was transported from the cytoplasm to the nucleus, and although we have not determined if the virus DNA was free or encapsidated during this step. Thus the HepG2 cells appear able to support many of the early events of HBV replication and consequently, the block in replication following infection is likely to result from a failure to perform one or all of a number of steps necessary to uncoat the viral DNA and/or to convert the input genome into the transcriptionally-active supercoiled template (4).

Our studies have provided further insight into the failure of HepG2 cells to consistently support HBV replication, and may ultimately lead to improved understanding of the early events which are necessary for reproducible infection of continuous cell lines.

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Figure Legends

Figure 1

The HBV-binding activity of cultured cell lines detected by monolayer radioimmunoassay. Monolayer cultures of HepG2, HuH7, HeLa and LTK$^-$ cells were incubated with purified HBV virions or mock-purified HBV and bound virus detected with ^{125}I-labeled anti-preS1(MA18/7). The results are shown as mean counts per minute bound to the cells.

Figure 2

The effect of HBV-specific peptides on HBV-binding activity to HepG2 cells. Peptides corresponding to aa 12-32, 21-47, 32-49, 120-145 respectively were diluted to concentrations of 50ug/ml, 100ug/ml, 250ug/ml, 500ug/ml. Each concentration of these peptides was preincubated with HepG2 cells for 2h at 37°C prior to adsorption with HBV virions. Bound HBV was detected by ^{125}I-labeled anti-MA18/7 and the results are shown as the percentage inhibition of total HBV-binding activity.

Figure 3

The effect of antibody preparation on the HBV binding activity to HepG2 cells. Each antibody preparation (RαHBs IgG, Fab; MA 18/7 IgG, Fab; and normal mouse serum, NMS) was incubated with purified HBV virions at a ratio of 1:1 (v/v) for 20min at 37°C. The mixtures were then added to the HepG2 cell monolayers and HBV-binding activity detected by ^{125}I-labeled anti-MA18/7 and ^{125}I-labeled RαHBs respectively. The results are shown as percentage inhibition of the total HBV-binding activity.

Figure 4

The kinetics of HBV-binding activity in HepG2 cells. The adsorption was carried

Figure 5

Saturation of HBV-binding sites in HepG2 cells. Increasing amounts of virus ($1x10^6$-$1x10^8$vge/ml) or equivalent dilutions of mock-purified HBV were incubated with a constant cell concentration ($1x10^5$/ml) of HepG2 cells and LTK$^-$ cells as a control. The binding activity was detected by ^{125}I-labeled anti-MA18/7, and shown as mean counts per minute.

Figure 6

Quantitation of HBV DNA by a standard curve constructed after PCR. Plasmid pTKHH2 was diluted to contain 0.01-10pg and amplified by PCR. An aliquot of each dilution was sampled at 35 cycles and quantitated by spot blot hybridization using a ^{32}P-labeled oligonucleotide probe (#90). The results are presented as counts per minute of dissected membrane. The dotted line represents quantitation of HBV DNA bound to HepG2 cells.

Figure 7

Relative quantitation of HBV DNA-associated with cells, supernatant or washing solutions at various time points during internalization determined by PCR followed by spot blot hybridization and quantitation as described in Figure 6 legend.

Figure 8

Analysis by agarose gel electrophoresis of the PCR products from nuclear and cytoplasmic extracts after HBV internalization. Tracks 1-5 and 6-10 represent cytoplasmic (C) and nuclear (N) fractions respectively at various times post incubation at 37°C. Tracks 1 and 6, 2 and 7, 3 and 8, 4 and 9, 5 and 10 represent 0, 1h, 2h, 4h and 6h post incubation at 37°C respectively. Tracks 11-13 represent the plasmid DNA control containing 10pg, 1pg and 0.1pg

consistent with a molecular weight marker, PstI digested λ DNA separated in the same gel.

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Serological analysis of duck hepatitis B virus infection

M. Qiao, E.J. Gowans, S.E. Bailey, A.R. Jilbert and C.J. Burrell

Division of Medical Virology, Institute of Medical and Veterinary Science, Adelaide, Australia

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Summary

A radioimmunoassay was developed to detect duck hepatitis B virus surface antigen and antibody; viraemia (DHBV DNA or DHBsAg) was detected in all ducks inoculated within 3 weeks post-hatch, and persistent infection developed in 93% of birds in this group. In contrast, only 80% and 60% of ducks inoculated 4- and 6-weeks post-hatch respectively developed viraemia, and approximately 70% of the viraemic ducks became carriers. Markers of viraemia were undetected in ducks inoculated 8 weeks post-hatch and in uninfected controls.

A typical anti-DHBs seroconversion developed subsequently in 2 of 4 birds that showed transient viraemia, and antibody also developed in 3 of 7 ducks inoculated 4–8 weeks post-hatch that showed no viraemia. However, gene amplification by the polymerase chain reaction demonstrated DHBV DNA in ducks from the latter group suggesting that the antibody did not result from passive vaccination. Thus, increased resistance to infection develops with increasing age that may be related to several factors including host immunity. This model may help elucidate similar age-related features of human hepatitis B virus infections.

Duck hepatitis B virus; Serological assay; Polymerase chain reaction; DHBsAg, anti-DHBs

Introduction

Duck hepatitis B virus (DHBV), a member of the hepadnavirus family is one of several viruses closely related to hepatitis B virus (Schodel et al., 1989). Hepad-

Correspondence to: E.J. Gowans, Division of Medical Virology, Institute of Medical and Veterinary Science, Box 14, Rundle Mall PO, Adelaide SA 5000, Australia.

naviruses share common virion morphology, genome structure and organisation, biological properties, relative hepatotropism and the ability to cause persistent infections (Marion and Robinson, 1983; Howard, 1986). The DHBV model has been used widely to investigate acute and persistent infection and to study pathogenesis in ways not possible with HBV infection.

Previous studies of experimental transmission of DHBV showed that successful transmission was dependent to a large degree on the age of the duck, and to a lesser degree upon the dose of virus inoculum (Mason et al., 1983; Omata et al., 1984; Fukuda et al., 1987; Jilbert et al., 1987). In general terms, neonatal ducks are most susceptible to infection and virtually 100% become viraemic after inoculation, whereas only a varying proportion of older ducks show detectable viraemia. Furthermore, neonatal ducks usually fail to resolve the infection and become persistently infected while older ducks develop a transient viraemia only. Thus, neonatal ducks provide a good model for HBV infection of human neonates; we have previously reported studies of the intracellular events of virus replication using this model (Jilbert et al., 1988).

However, the potential usefulness of the neonatal model and of older ducks as a model for HBV infection of adult humans has been severely restricted due to a general lack of reagents with which to measure the serological events of DHBV infection. Previous studies (Mason et al., 1983; Omata et al., 1984; Fukuda et al., 1987; Jilbert et al., 1987, 1988) have relied solely on the detection of DHBV DNA by hybridization analysis as a measure of viraemia.

To allow clarification of the serological parameters of infection using various age/dose/route of inoculation schedules, we have developed a radioimmunoassay to detect duck hepatitis B virus surface antigen (DHBsAg) and antibody (anti-DHBs), and have examined ducks for a period of eight weeks post-inoculation that were experimentally infected at times varying from 1 day to 8 weeks post hatch.

Materials and Methods

Serological detection of DHBsAg and anti-DHBs

Preparation of rabbit anti-DHBs. DHBsAg was purified essentially as described (Marion et al., 1983). Briefly, DHBV-associated particles were pelleted through 10% sucrose then banded in two consecutive caesium chloride gradients. Fractions corresponding to a density of 1.175 g/cm^3 were pooled then examined by electron microscopy and SDS-PAGE for confirmation of purity. The preparation contained a major band at 17 kilodaltons (kDa) and a minor band at 28 kDa (Fig. 1) that were interpreted as the DHBsAg small polypeptide and pre-S2 polypeptide respectively (Yokosuka et al., 1988). This preparation was emulsified in Freunds adjuvant and inoculated into rabbits. The specificity of the resultant antibody was determined by immunostaining of DHBsAg in DHBV-infected liver sections (Jilbert et al., 1988).

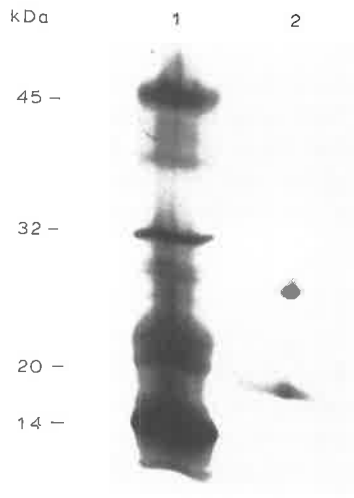

Fig. 1. Analysis by SDS-PAGE of purified DHBsAg used to inoculate rabbits and in radioimmunoassay to detect anti-DHBs. Track 1 shows molecular weight standards and track 2 shows purified DHBsAg. The tracks were stained by Coomassie blue.

Radioimmunoassay for DHBsAg and anti-DHBs

Protein A purified anti-DHBs IgG was used as the capture and detector antibody in direct and competitive radioimmunoassays (RIA) to detect DHBsAg and anti-DHBs respectively. A range of concentrations was used to determine the optimum P/N ratio, and this is described in Results. Immunolon 1 Removawell strips (Dynatech Laboratories) were coated with 100 μl of anti-DHBs in 100 mM Tris-PBS, pH 9.6, at RT overnight. The wells were then washed three times in PBS before the addition of 2% normal rabbit serum (NRS) in PBS as blocking agent for 2 h at 37 °C.

Detection of DHBsAg

Duck serum samples were diluted 1/10 in 2% NRS, 100 μl added to the anti-DHBs coated wells and incubated for 4 h at 37 °C. The sample was removed and the wells washed three times with PBS prior to the addition of 100 μl of ^{125}I-anti-DHBs, prepared by the chloramine T method (Greenwood et al., 1963) and labelled to a specific activity of ca. 1×10^7 cpm/μg. Different concentrations of ^{125}I-labelled antibody were used to determine the optimum, and this is also described in Results. The plates were incubated for 1 h at 37 °C, the wells washed three times in PBS containing 0.1% Tween 20 then three times in PBS alone. Individual wells were counted in a Packard Gamma counter, and positive samples identified as described in Results.

Detection of anti-DHBs

Antibody-coated Removawell strips prepared as described above were incubated with a 100 μl of PBS, 2% NRS containing 500 ng of purified DHBsAg for 2 h at 37 °C. The wells were washed three times in PBS and 100 μl of duck test serum diluted 1/5 in 2% NRS added and incubated for 2 h at 37 °C. Samples were considered to be positive for anti-DHBs if binding of the ^{125}I-labelled antibody was reduced by $\geq 50\%$.

Detection of DHBV DNA

For dot–blot analysis, 10 μl of test serum was mixed with 20 μl of 1 M NaOH and 10 μl of 2 M NaCl, incubated at room temperature for 15 min and the sample applied under vacuum to nitrocellulose (Schleicher and Schuell). The hybridisation steps were performed as described previously (Gowans, 1986; Jilbert et al., 1988).

Selected sera were examined for DHBV DNA by the polymerase chain reaction (PCR). One hundred microlitres of duck serum was incubated for 3 h at 70 °C in a solution containing 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% SDS, 100 μg/ml Proteinase K (Boehringer), and then phenol extracted and ethanol precipitated after the addition of 100 μg tRNA. The pellet was redissolved in 100 μl TE, and 10 μl added to a standard PCR mix comprised of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.1% gelatin, 200 μM each dNTP's, 1 μM primers and 2.5 units Amplitaq (Perkin-Elmer Cetus). The reaction volume was adjusted to 100 μl with DW and overlaid with mineral oil.

The sequence of the primers (a gift from Dr Stephen Locarnini, Fairfield Hospital, Melbourne) was: Primer 1, position 2594–2615; CGT GTG TGA CTG TAC CTT TGG: Primer 2, position 2978–3000; GTC GGG TTG GAA ACT TAC TATC. The primers were devised from the DHBV DNA sequence described by Mandart et al. (1984) and were designed to amplify a 406 base pair fragment from the core +X gene region.

After 25 cycles, 94 °C 1 min, 50 °C 1 min, 72 °C 3 min, an aliquot of the product was analysed by Southern blot hybridization after gel electrophoresis through 2.5% agarose, essentially as described (Jilbert et al., 1988).

DHBV DNA immobilised to nitrocellulose as described above was detected by hybridization with ^{32}P-labelled RNA probes transcribed from a recombinant plasmid containing full-length DHBV cloned DNA (Tuttleman et al., 1986). The plasmid (pSP65 DHBV 5.1), a gift from Dr John Pugh, was linearised by digestion with *Sac*I and ^{32}P-labelled RNA transcribed with SP6 RNA polymerase using conditions defined by the supplier (Promega Biotec).

Inoculation of ducks

The sera of seven one-day-old ducks inoculated as part of a previous study (Jilbert et al., 1988) were examined for DHBsAg, and six groups of five ducks aged between 1–8 weeks that were inoculated specifically for this study were examined

for DHBsAg and anti-DHBs by RIA, and for DHBV DNA by dot–blot hybridization analysis (Jilbert et al., 1988) or by PCR followed by Southern blot hybridization as necessary.

The ducks were inoculated by the intravenous route with a standard inoculum containing 3×10^8 virus genome equivalents/ml as described (Jilbert et al., 1988). Ducks aged 1, 2, 3 and ≥ 4 weeks received 100 μl, 250 μl, 500 μl and 1 ml respectively, of the standard inoculum.

Results

Optimisation and specificity of RIA for DHBsAg and anti-DHBs

The assays for DHBsAg and anti-DHBs were optimised by titration of both capture and detector antibodies. The positive : negative (P/N) ratio for DHBsAg was determined by testing a DHBV DNA-positive sample and a DHBV DNA-negative sample in parallel. The DHBV DNA positive serum contained approx 800 ng DHBsAg/ml by comparison with purified DHBsAg. DHBsAg was detected with concentrations of capture antibody ≥ 0.06 μg/well when the ^{125}I-labelled detector antibody concentration was greater than 5×10^5 cpm/well (Fig. 2). Although the optimal P/N was achieved with 2.5×10^6 cpm/well of detector antibody, for reasons of economy, a combination of 1 μg/well of capture antibody and 1×10^6 cpm of detector antibody was chosen.

Using this combination, the sensitivity of the assay was determined to be approx 30 ng DHBsAg by titration of the purified DHBsAg used to raise the anti-DHBs (Fig. 3).

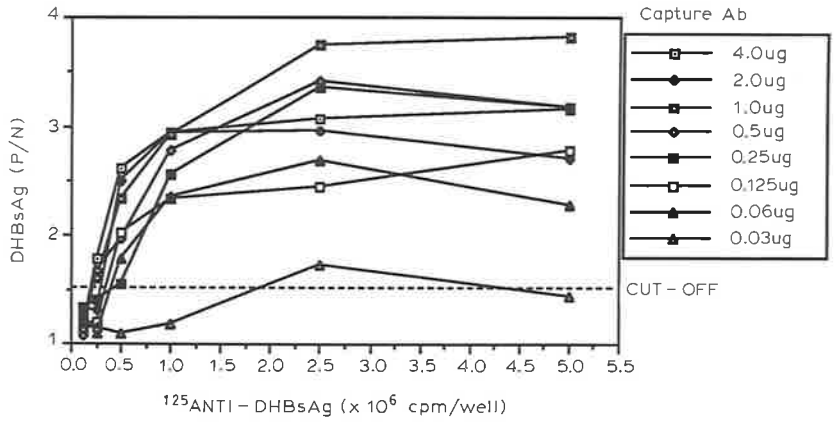

Fig. 2. Titration of capture- and ^{125}I-labelled- anti-DHBs in solid-phase radioimmunoassay to detect DHBsAg in serum. The assay was performed as described in the text and the cut-off was calculated according to the formula mean $\pm$ 3 SD.

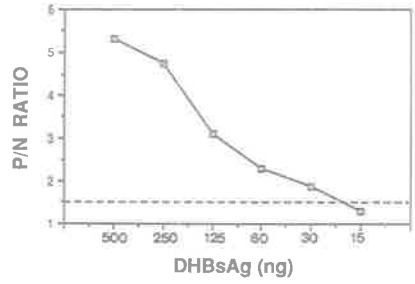

Fig. 3. Titration to determine the sensitivity of the RIA. Dilutions of purified DHBsAg were made in normal duck serum and compared with dilutions of mock-purified DHBsAg. The cut-off was determined as described in the legend to Fig. 2.

The mean background level of ^{125}I-counts bound for 40 DHBV-negative duck serum samples was then calculated (1011 cpm $\pm$ 173). This permitted the cut-off value to be set as the mean plus 3 standard deviations, and this formula was used throughout the study.

We then examined a range of serum samples from seven 1-day-old ducks for DHBsAg that were studied by hybridization analysis in a previous report (Jilbert et al., 1988). This experiment showed that DHBsAg was only detected in those ducks which were positive for DHBV DNA (Fig. 4) and furthermore, that DHBsAg followed the same kinetics as DHBV DNA. Although the P/N ratio appeared to be low, this was not improved by a reduction in the levels of detector antibody added (Fig. 2) in an effort to reduce the N value. However, the positive samples were still positive at a dilution of 1/800 (data not shown).

Analysis of DHBV serological markers

The response of the ducks to inoculation with DHBV-positive serum was followed for eight weeks post-inoculation (pi). The response varied with the age of

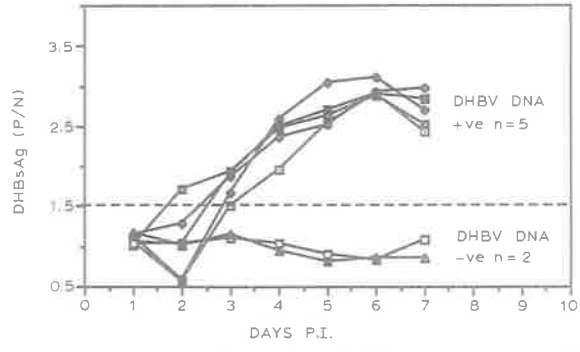

Fig. 4. Radioimmunoassay analysis of DHBsAg in 5 DHBV-positive and 2 DHBV-negative serum samples from ducks inoculated with DHBV-positive and -negative serum respectively at 1 day post-hatch.

TABLE 1

The response of ducks of differing ages to DHBV inoculation

Type of response	Duck age at inoculation (weeks)					
	1	2	3	4	6	8
1. Persistent DHBV DNA & DHBsAg	5/5	5/5	4/5	3/5	2/5	0/5
2. Transient viraemia						
Anti-DHBs + ve	0/5	0/5	1/5	0/5	1/5	0/5
Anti-DHBs–ve	0/5	0/5	0/5	1/5	0/5	1/5
3. No viraemia						
Anti-DHBs + ve	0/5	0/5	0/5	1/5	0/5	2/5
Anti-DHBs–ve	0/5	0/5	0/5	0/5	2/5	2/5

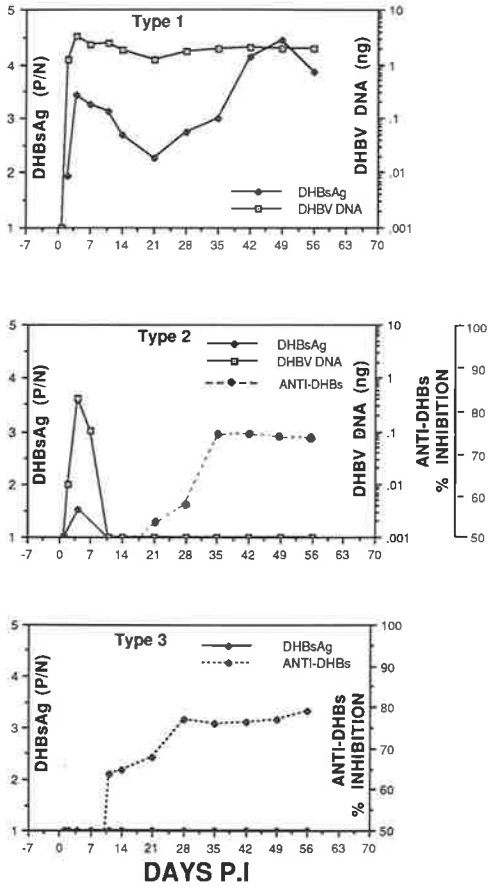

Fig. 5. Examples of the responses to DHBV-inoculation. Serum samples were tested for DHBV DNA by dot–blot hybridisation and for DHBsAg and anti-DHBs by radioimmunoassay.

the ducks and showed three main groups: (i) persistent viraemia (ii) transient viraemia (iii) no detectable viraemia (Table 1). Groups ii and iii were further subdivided into ducks which were positive or negative for anti-DHBs. Fig. 5 shows representative examples of each group.

Viraemic stage

This was measured by the detection of DHBV DNA and DHBsAg; in a majority of infections, both markers were detected equally well, but in some instances in birds aged ≥ 3 weeks, only one of the two markers was detected. In ducks aged between 1–3 weeks, the incubation period was 2–3 days, similar to that described previously for neonatal ducks (Jilbert et al., 1988), while in ducks aged 4–6 weeks, this increased to 4–7 days. The proportion of ducks that became viraemic decreased from 100% of ducks aged 1–3 weeks, to 80% of 4-week-old ducks, and 60% of 6-week-old ducks, while only one of the five 8-week-old ducks showed detectable viraemia (Table 1). All of the birds inoculated 1–2 weeks post-hatch were positive for DHBV DNA and DHBsAg some 35 days pi. In contrast, in older birds, an age-related decrease in the rates of virus persistence was noted (Table 1). In ducks aged 3 weeks, 4 weeks and 6 weeks, 80, 75 and 66%, respectively, of viraemic ducks became persistently infected.

Seroconversion events

Only two typical seroconversion events were noted in which loss of viraemia was followed by the appearance of anti-DHBs (Table 1); these occurred in ducks inoculated at 3 and 6 weeks of age. The appearance of anti-DHBs was also seen in 3 ducks which were inoculated 4 or 8 weeks post hatching and which failed to become viraemic. Thus the appearance of anti-DHBs was restricted to birds aged 3 weeks and older, but did not necessarily result from detectable viraemia, indicating that ducks in this latter category may have suffered subclinical or silent infection (see below) as has been described for HBV (Hoofnagle et al., 1978). However, two birds which showed transient infection as judged by the detection of DHBV DNA or DHBsAg (Group ii) remained negative for anti-DHBs indicating that additional factors could influence the humoral immune response. It is possible that the prolonged viraemic phase seen in these birds (17–25 days versus 7 days in normal transient infection) resulted from an impaired immune response. Alternative explanations for the non-appearance of antibody are (i) a level of viraemia below the sensitivity of the current assay (ii) sensitivity of the anti-DHBs assay (iii) a prolonged window period which extended beyond the eight weeks of this study.

Seroconversion in DHBsAg-negative ducks

The appearance of anti-DHBs in birds which failed to develop viraemia may have been due to silent infection. To confirm this interpretation and to discount the possibility that passive immunisation accounted for the antibody response, sera

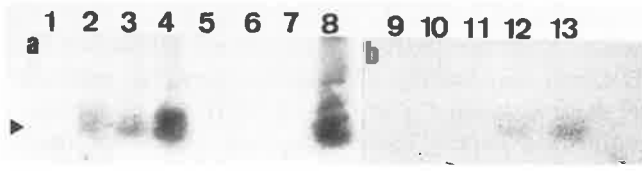

Fig. 6. Southern blot analysis of the products of PCR amplification. The products were electrophoresed through 2.5% agarose and transferred to nitrocellulose for hybridisation. Analysis of ducks inoculated at 4 weeks (a) and 8 weeks (b) that were subsequently negative for DHBV DNA- and DHBsAg: track 1, 7 days pi; track 2, 11 days pi; track 3, DHBV DNA (+) dot blot positive; track 4, DHBV DNA (++) dot blot positive, track 5 DHBV DNA negative control; tracks 6 and 7 PCR reagent only control; track 8 DHBV DNA template control. (b) Track 9, day 1 pi; track 10, day 2 pi; track 11, day 4 pi; track 12, 7 days pi; track 13, 11 days pi. The gel shown in (b) was analysed independently of (a) but showed similar reactions in the control tracks.

from three ducks in this category were examined by PCR. In the 4-week-old and in both 8-week-old ducks, PCR followed by Southern blot hybridization detected DHBV DNA in samples taken at 11 and 7 days pi respectively, whereas earlier samples were negative (Fig. 6). In all cases, PCR performed on DHBV-negative serum samples, or using PCR reagents alone, were negative by Southern blot hybridization (Fig. 6). These results suggest that the original inoculum was not the template for the PCR, and suggest very strongly that the ducks had actually supported virus replication.

Discussion

As part of our aim to refine the DHBV model for HBV infection, we report in this paper the development of serological assays to monitor DHBV infection in experimentally infected ducks. The radioimmunoassays for DHBsAg and anti-DHBs described herein can be expected to increase the usefulness of the DHBV model of hepadnavirus pathogenesis.

In our hands, Pekin-Aylesbury cross-bred ducks remained highly susceptible to DHBV infection for four weeks post hatching, and even 50% of ducks aged between 6–8 weeks were infected if all three markers of infection, viz. DHBV, DHBsAg and anti-DHBs, were measured. However, if DHBsAg (or DHBV DNA) was the sole marker of infection then only 20% of these 6–8-week-old ducks showed evidence of infection. In previous transmission studies which used DHBV DNA hybridization analysis alone, birds 3 weeks or older were usually resistant to infection (Mason et al., 1983; Omata et al., 1984; Fukuda et al., 1987). The results from this study support these data when similar methods were used to detect viraemia (DHBV DNA), but also demonstrate the need to introduce a spectrum of assays to study the evolution of DHBV infection.

DHBV DNA and DHBsAg always co-existed in ducks aged ≤ 3 weeks, but 3 ducks aged ≥ 4 weeks in group ii that were transiently infected showed either DHBV DNA (2) or DHBsAg (1) alone. These three ducks are probably examples of

minor group responses to infection, representing active virus replication with restricted production of excess DHBsAg, and limited replication with the production of excess DHBsAg respectively. The latter group may represent the so-called "healthy carrier" state associated with human HBV infection. However, confirmation of these results will require more exhaustive studies of the pathogenesis of DHBV infection.

As reported above, use of these markers has permitted sharper definition of the age-related transition from high susceptibility to resistance to infection prior to a more detailed analysis of the mechanisms involved. We believe that DHBV infection provides an authentic model for HBV infection, that will provide novel data on chronicity and pathogenesis.

Acknowledgements

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Intracellular Factors, but Not Virus Receptor Levels, Influence the Age-Related Outcome of DHBV Infection of Ducks

MING QIAO, ERIC J. GOWANS,[1] AND CHRISTOPHER J. BURRELL

Division of Medical Virology, Institute of Medical and Veterinary Science, P.O. Box 14 Rundle Mall, Adelaide SA 5000, Australia; and Department of Microbiology and Immunology, University of Adelaide, North Terrace, Adelaide SA 5000, Australia

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Previous serological studies of experimental infection with duck hepatitis B virus (DHBV) have shown that the outcome of infection depends largely on the age of the duck at the time of inoculation. To examine the hypothesis that decreased susceptibility with increased age might be due to the loss of the virus receptor on hepatocyte membranes in adult ducks, we performed receptor binding studies using intact serum-derived DHBV virions and purified liver plasma membranes from both young ducklings and adult ducks. These studies showed that (1) DHBV was able to bind specifically to duck liver plasma membranes but not to internal membranes; (2) this binding could be inhibited by a monoclonal antibody to DHBV preS, a corresponding region in hepatitis B virus that binds to human hepatocytes; and (3) there was no significant difference in the receptor binding ability between plasma membranes from ducklings and from adult ducks. Since hepatocytes in the neonatal ducks are actively dividing, in contrast to the situation in adult ducks, we examined the effect of partial hepatectomy on DHBV-carrier ducks. A sharp increase was noted in the level of DHBV in the serum after partial hepatectomy suggesting that DHBV replication was enhanced in dividing hepatocytes. Thus the age-related difference in susceptibility of ducks to DHBV infection is not due to loss of the receptor but may be related to an intracellular event associated with cell division.

INTRODUCTION

Duck hepatitis B virus (DHBV), a member of the hepadnavirus family, has been shown to resemble hepatitis B virus (HBV) in many respects such as viral genome structure, virus DNA replication, hepatotropism, and pathogenesis (Marion, 1988; Mason and Taylor, 1989; Schodel *et al.*, 1989). Both HBV and DHBV have the capacity to produce acute and chronic infections of the liver (Marion and Robinson, 1983), but unlike HBV, the natural route for infection with DHBV is vertical transmission from mother to egg. Horizontal transmission among ducks does not seem to occur through natural contact, although this is a common route for transmission of HBV (Hoofnagle *et al.*, 1978; Marion and Robinson, 1983).

Studies of experimental transmission of DHBV have shown that the outcome of infection depends largely on the age of the ducks at the time of inoculation (Mason *et al.*, 1983; Omata *et al.*, 1984; Fukuda *et al.*, 1987; Qiao *et al.*, 1990). In general, younger birds are more susceptible to infection. In one study, development of viremia following experimental transmission varied from 100% in newly hatched ducklings to 60% in 6-week-old ducks, whereas all ducks aged 8 weeks or over showed no detectable viremia when analysed by dot blot hybridization (Qiao *et al.*, 1990).

The age-dependency of susceptibility to infection with DHBV is not fully understood. However, one of the key factors in determining virus susceptibility is the capacity of the host cell to bind virus through specific receptors on the cell surface (Lonberg-Holm and Philipson, 1981; Tardieu *et al.*, 1982). To determine if the resistance to DHBV infection in adult ducks might be due to the inability of adult duck hepatocytes to bind DHBV, it is necessary to compare the level of expression of the DHBV receptor on hepatocytes from young ducklings and from adult ducks.

HBV receptor studies using naturally derived hepatitis B surface antigen (HBsAg) have shown that HBsAg was bound to HepG2 cells, a continuous line of human hepatoma cells, in a reaction which was inhibited specifically by a synthetic peptide from the preS1 (21–47) region and the corresponding antibody (Neurath *et al.*, 1986). Other studies have shown that recombinant large-HBsAg particles can bind directly to plasma membrane preparations from human liver and the binding can be prevented by the preS1 (21–47) peptide (Pontisso *et al.*, 1989). By using a similar virus–cell membrane binding assay (Krah and Crowell, 1982; Pontisso *et al.*, 1989), we have established a method to detect DHBV receptors. In this paper, we describe the use of this assay to investigate the age depen-

[1] To whom correspondence and reprint requests should be addressed.

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dency of DHBV infection in liver, although no attempt was made to examine extrahepatic sites.

MATERIALS AND METHODS

Isolation of hepatocyte membranes

Duck liver tissues (unperfused) were obtained from ducklings aged 5 days and from adult ducks aged 8 months. Hepatocyte membranes were prepared basically according to the method described by Hubbard *et al.* (1983). Briefly, 10 g of frozen liver tissue was minced and homogenized in 4 vol of STM buffer (0.25 *M* sucrose, 5 m*M* Tris–HCl, pH 7.4, 0.5 m*M* $MgCl_2$) using a loose Dounce homogenizer. Cell debris and unbroken cells were removed from the suspension by centrifugation at 280 *g* for 5 min. The supernatant was centrifuged again at 1500 *g* for 10 min to pellet plasma membranes and the internal membranes were isolated from the supernatant and prepared as described (Pontisso *et al.,* 1989). The pelleted plasma membranes were resuspended in 10 ml STM buffer and adjusted to a density of 1.18 g/cm^3 with 2 *M* sucrose in STM buffer. The above suspension was aliquoted into Beckman Ultracentrifuge tubes and overlaid with 2 ml 0.25 *M* sucrose in STM. Following centrifugation at 78,000 *g* in a SW41 rotor for 60 min, the plasma membrane-enriched fraction was collected from the interface. The final products were stored at −70° prior to use. Human liver plasma membranes were prepared by the same procedure from frozen samples.

The separation of internal and plasma membranes was measured by the detection of endogenous enzymes. Alkaline phosphatase, a marker of plasma membranes, was measured as described by Ray (1970) and acid phosphatase activity was used as a marker to estimate lysosomal and mitochondrial contamination of the plasma membrane fraction (Allen and Crumptor, 1970; Appelmans and De Duve, 1955). Total protein quantitation was determined by a Bio-Rad protein assay using human serum albumin as a standard.

Preparation of DHBV virions

High titer DHBV-positive sera were used as the source of DHBV, and DHBV virions were purified as described (Marion *et al.,* 1983); 30 ml of serum was layered over 10 ml of 20% (w/v) sucrose in TNE (10 m*M* Tris–HCl, pH 7.4, 0.1 *M* NaCl, 5 m*M* EDTA) and centrifuged at 230,000 *g* for 5 hr at 4° (Beckman L8-80). The pellet was resuspended overnight at 4° in 200 μl of TNE. The virus suspension was then layered over a 4-ml preformed linear CsCl gradient (density = 1.1–1.4 g/cm^3) and centrifuged at 280,000 *g* for 48 hr at 10°; 250-μl fractions collected from the bottom of the tube were tested for DHBV DNA and DHBsAg as described previously (Jilbert *et al.,* 1988; Qiao *et al.,* 1990).

Source of antibodies

Monoclonal antibody anti-DHBV preS (a gift from Dr. Stephen Locarnini) was prepared by immunizing mice with intact DHBV; the antibody was screened by immunofluorescence and was shown to be specific for the preS sequence by immunoblotting. The antibody was radiolabeled with ^{125}I by the chloramine T method (Greenwood *et al.,* 1963) and the specific activity was calculated to be 1×10^7 cpm/μg.

Anti-DHBs was raised in rabbits by immunization with purified serum-derived DHBsAg as described (Qiao *et al.,* 1990).

Anti-DHBc (provided by Dr. Stephen Locarnini) was raised in rabbits by immunization with a synthetic peptide comprising 13 amino acids found at the C terminus of the DHBV core protein as described (Schlicht *et al.,* 1987).

DHBV-receptor binding assay

Binding of purified DHBV virions to duck liver membranes was performed by solid-phase radioimmunoassay RIA as described (Pontisso *et al.,* 1989) with minor modifications. Fifty microliters of membrane preparation containing 15 μg of protein (i.e. 300 μg/ml) in PBS was added to wells of Immunolon 1 "Removawell" microtiter strips (Dynatech Laboratories) and incubated at 20° O/N. The wells were washed 3 times with PBS, then 60 μl of 10% BSA in PBS was added to each well and incubated for 4 hr at 20° or O/N at 4°. Following a single wash with PBS, 50 μl of DHBV virions (30 μg/ml in 2% BSA) or mock-purified DHBV from DHBV-negative duck serum was added to the wells and incubated for 20 hr at 4°. The wells were then washed thoroughly with cold PBS (4°), then 50 μl of ^{125}I-labeled anti-DHBV preS (1×10^6 cpm in 2% BSA) was added and incubated O/N at 4° then washed 3 times with cold PBS. The plates were then dried and individual wells counted in a Packard Gamma counter.

In a blocking experiment described under Results, virus was preincubated with the preS antibody, and in this experiment bound virus was detected by ^{125}I-labeled anti-DHBs (Qiao *et al.,* 1990).

Partial hepatectomy

Ten DHBV-carrier ducks infected as neonates and aged between 8–18 months at the time of the experiments were selected for this study. Of these, six were chosen at random and used for partial hepatectomy, two for laparotomy and two were untreated.

On the day of surgery, food and water were withheld for 2 hr and 0.5 ml of Streptopen (Heriot Equet Pty, Ltd) was given intramuscularly. Following endotracheal intubation, general anesthesia was induced with halothane and nitrous oxide and maintained throughout the operation. The duck was laid in recumbent position, feathers were removed from the central and right lateral abdomen, and the surgical site was scrubbed with betadine. An incision was made in the skin from the central and right lateral abdomen parallel to the last rib and the subcutaneous fat layer and then the abdominal muscle layers were cut to expose the abdomen wall. This was opened to reveal part of the right lobe of the liver. Two sutures (Chromic 1) were placed from the posterior to anterior surfaces of the right lobe near the middle line below the gall bladder and were tied securely to restrict the blood flow to a portion of the right lobe. The liver below the sutures was then excised carefully. The left lobe was removed in a similar way except that one suture was adequate. The abdomen was reclosed and the birds were kept warm and quiet for a 4-hr period. Laparotomy was performed as a control for postoperative shock and blood loss during operation. The same operating procedures were used except that the liver was not resected. The percentage of liver excised was determined according to published data of total duck liver weight (Nickel *et al.*, 1977). In one experiment, serial serum samples were tested for ALT and LDH activity using commercially available kits (Merck and Trace Scientific respectively) by the staff in the Department of Agriculture, Adelaide, South Australia.

RESULTS

Analysis of duck liver membrane preparation

The authenticity of the membrane preparations was determined in two ways: (1) by electron microscopy and (2) by endogenous enzymic activity. Morphological examination of the membrane-enriched fractions by electron microscopy showed large connected vesicles (data not shown) which were similar to those described by Hubbard *et al.* (1983).

Alkaline phosphatase and acid phosphatase activities, enzymatic markers of plasma and internal membranes, respectively, were measured. Briefly, using the enzymic markers a 14.5% final yield of plasma membrane was obtained, similar to the result described by Hubbard *et al.* (1983). In addition, contamination of the plasma membrane fraction by internal membranes was low as measured by the level of acid phosphatase activity (Table 1). Thus these experiments indicate that good separation of plasma membranes from internal membranes was achieved.

TABLE 1

BIOCHEMICAL CHARACTERIZATION OF DUCK LIVER MEMBRANE FRACTIONS

Enzymic Marker	Yield[a] (Milliunits/g wet liver)		Specific activity[b] (Milliunits/mg protein)		Rate activity in[c] plasma membrane (%)
	Internal	Plasma	Internal	Plasma	
Alkaline phosphatase (PM)	1.44	18.9	6.2	24.9	14.5
Acid phosphatase (IM)	1.45	0.1	1.25	0.13	4.2

[a] The yield of enzymes in the final membrane fractions was calculated as milliunits per gram of wet liver.

[b] The specific activity was determined as milliunits per milligram of protein.

[c] The rate activity of total enzymes in the final PM fraction was calculated from the percentages of recovered activity in each fractions throughout the purification of the liver membranes.

Analysis of purified DHBV virions

Care was taken to ensure that DHBV-enriched preparations contained minimal levels of excess DHBsAg. Both DHBV DNA and DHBsAg were examined in the fractions from the CsCl gradient. DHBV DNA was measured by dot blot hybridization as a marker of virions and was detected mainly in one fraction with a buoyant density of 1.195 g/cm^3, whereas DHBsAg measured by RIA (Qiao *et al.*, 1990) was detected in fractions with a density of 1.17 g/cm^3 (data not shown). Therefore, separation of DHBV virions from the bulk of the DHBsAg particles was successful; this was confirmed by electron microscopy, which showed a high concentration of intact virions with minimal contamination by DHBsAg particles.

DHBV–membrane interactions

Since we have demonstrated previously that hepatocytes are the major site of DHBV replication (Jilbert *et al.*, 1988), we restricted the study to hepatocyte membranes. Furthermore, because extrahepatic cells are likely to express the DHBV receptor, we used normal human liver as a negative control. The ability of both internal and plasma membranes from neonatal and adult ducks to bind DHBV was examined. A solid-phase (RIA) similar to that described by Pontisso *et al.* (1989) was carried out at 4° to ensure that DHBV virions remained intact throughout and to minimize subsequent release of bound virus. Liver plasma membranes from both young ducklings and adult ducks were capable of binding DHBV virions, while the internal membrane from ducks and human liver plasma membranes showed no binding activity (Fig. 1A). No

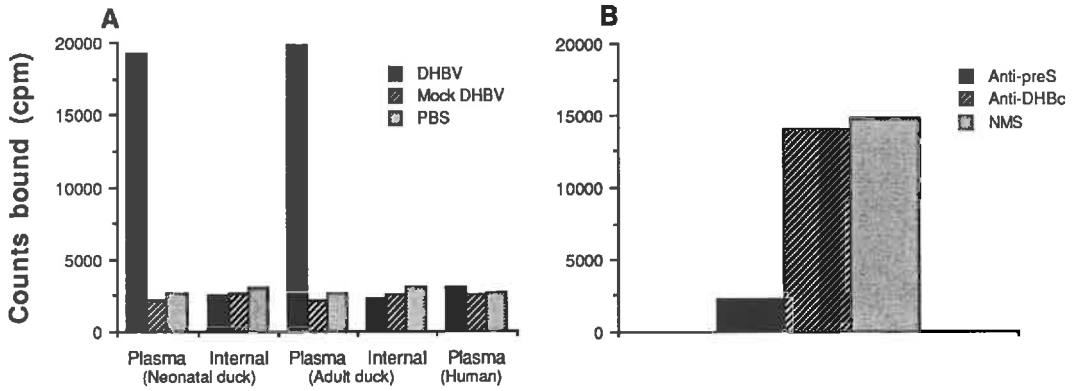

FIG. 1. (A) An examination of the DHBV-binding ability of liver membranes. Equivalent amounts of each liver membrane preparation were incubated in duplicate with purified DHBV virions (■), mock-purified DHBV virions (▨), or PBS (□) and bound virus detected with ^{125}I-labeled anti-DHBV preS. (B) The effect of prior incubation with different antibodies on DHBV binding to duck liver plasma membranes; levels of virus binding after prior incubation with an equal volume of anti-preS (50 μg/ml) (■), anti-DHBc (50 μg/ml) (▨), and normal mouse serum (1/50 dilution) (□). DHBV binding was detected by ^{125}I-labeled anti-DHBs. The results are shown as mean counts per minute bound to the membranes.

significant difference was found in the ability of liver plasma membranes from young ducklings and from adult ducks to bind DHBV (Fig. 1A). The positive:negative (P/N) ratio was approximately 8.5:1, when either binding of DHBV to internal membranes or binding of mock-purified DHBV to plasma membranes was used as negative control. This figure compares very favorably with previously published data using a similar method with HBV and human liver membranes (Pontisso *et al.*, 1989). Although the preS monoclonal antibody (MAb) has not been tested by us for virus neutralizing ability, it was chosen because it detected a DHBV-specific polypeptide rather than a DHBsAg-specific polypeptide and thus imparted a further level of specificity to the binding assay. To confirm that the binding reaction was specific, the DHBV preparation was preincubated with different antibody preparations, and bound virus was detected with antibody to the major DHBsAg. The binding of virus to the plasma membranes was inhibited by 83% as a result of preincubation of DHBV with the MAb to DHBV preS but no effect was observed after preincubation with either antibody to DHBcAg or normal mouse serum (Fig. 1B). The binding experiment described in Fig. 1A suggested that plasma membranes from neonatal and adult ducks were equally capable of binding DHBV and this was confirmed by comparing the levels of virus required to saturate the receptors in both membrane preparations. In this experiment, liver plasma membranes were used at a concentration of 50 μg/ml and a wide range of virus concentrations added. The results of this experiment are shown in Fig. 2; no bound virus was detected in membranes which were incubated in the two highest dilutions of virus. The level of nonspecifically bound ^{125}I as judged by the counts bound in membranes incubated with mock-purified virus did not change throughout. Consequently, it is likely that the counts bound after incubation with 1 μg/ml of DHBV (1/300 dilution) are specific and represent the limit of detection of bound DHBV in this system. There was an increase of approximately 10-fold in the level of bound ^{125}I when the concentration of virus was increased from 1-10 μg/ml, but no corresponding increase was noted when a virus concentration of 100 μg/ml was used. This indicates that the plasma membranes of both neonatal and adult ducks were saturated after incubation with 10 μg/ml DHBV and suggests that both membranes expressed similar levels of the DHBV receptor.

Hepatocyte mitosis stimulates DHBV synthesis

Despite the fact that adult ducks do not normally develop viremia following DHBV inoculation, the above results suggested that adult duck hepatocytes and neonatal duck hepatocytes expressed similar levels of the DHBV receptor. Additional factors that might be involved in different outcomes of infection between neonatal and adult birds include the level of maturity of the immune system and the extent of hepatocyte replication. Since a major difference between hepatocytes in neonatal and adult ducks is that hepatocytes are dividing in the neonate, we then chose to examine the effect of hepatocyte regeneration on the expression of DHBV. Partial hepatectomy was performed on six DHBV-carrier ducks aged 8–18 months that had been infected with DHBV as neonates; in each case the level of DHBV in the serum of these birds prior to surgery was determined by dot blot hybridization analysis (Ta-

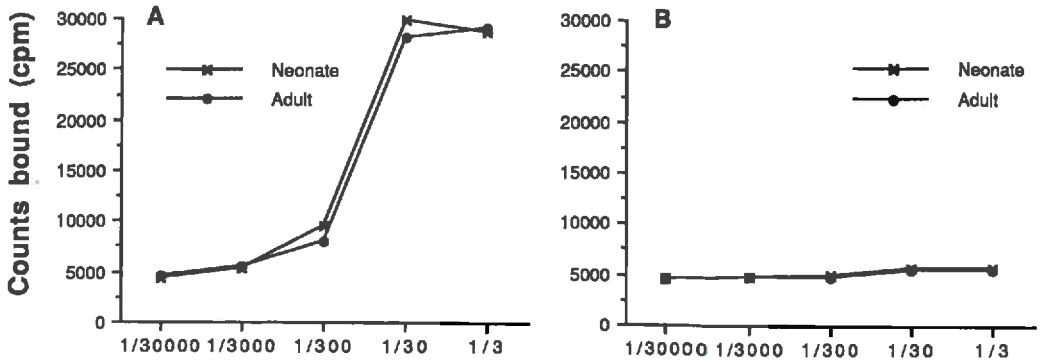

FIG. 2. Saturation of DHBV-binding sites in plasma membranes from both neonatal and adult ducks. Liver plasma membranes were incubated with increasing concentrations of (A) purified DHBV or (B) mock-purified DHBV. DHBV binding was detected by ^{125}I-labeled anti-DHBV preS and expressed as described in Fig. 1.

ble 2). After removal of approximately 50% of the liver, the level of DHBV in the serum of different birds increased between 2.6- to 12-fold (average 6-fold) within 96 hr of surgery but returned to presurgery levels 120 hr after surgery (birds 1–6, Table 2), whereas the control birds showed no increase (birds 7–10, Table 2). After 70% hepatectomy in the adult rat, a peak of liver cell DNA synthesis occurs 12–48 hr later (Bucher, 1973), and it is likely that duck hepatocytes behave in a similar manner. Consequently the rise in serum DHBV levels was most likely to be temporally associated with hepatocyte regeneration. However, no direct measure of hepatocyte regeneration was performed, although the livers of three birds were found at autopsy to be restored to their original size 8 weeks after partial hepatectomy. No rise was noted in the levels of DHBV in the serum of untreated ducks nor in ducks which underwent laparotomy. In one carrier bird which was subjected to laparotomy followed 6 weeks later by partial hepatectomy, a rise in DHBV levels followed partial hepatectomy only. Furthermore the increased DHBV DNA was not accompanied by elevation of serum ALT and LDH activity (Fig. 3).

TABLE 2

EFFECTS OF PARTIAL HEPATECTOMY ON LEVEL OF SERUM DHBV DNA IN ADULT CARRIER DUCK

Duck No.	Age (Month)	% Liver Excised	Serum DHBV DNA (ng/ml) Presurgery	Postsurgery	Relative increase
1	8	55	1	5.5	5.5
2	9	61	1.2	9	7.5
3	13	51	0.5	6	12
4	15	55	0.75	2	2.6
5	16	46	0.25	1	4
6	18	47	0.15	0.5	3.3
7	9	0	0.5	0.75	1.5
8	16	0	0.1	0.1	0
9	9	0	0.5	0.5	0
10	10	0	0.25	0.2	0

DISCUSSION

DHBV infection of the neonatal duck represents a good model for neonatal infection with HBV, but adult birds do not normally develop viremia after inoculation with DHBV (Qiao *et al.*, 1990). In contrast, the adult human is highly susceptible to infection with HBV. To investigate possible mechanisms for this decreased susceptibility in adult birds, we have examined the expression of the DHBV receptor on liver cell membranes and found that plasma membranes, but not internal

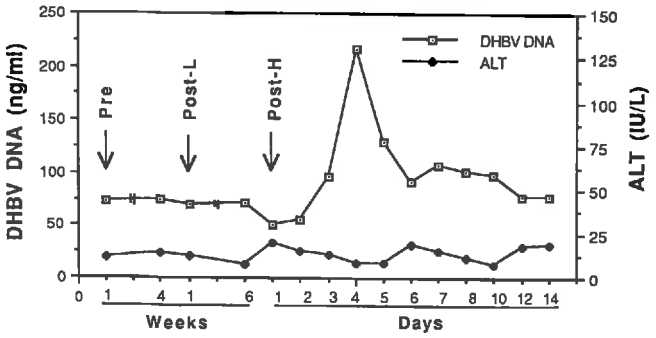

FIG. 3. An example of the effect of sequential laparotomy and partial hepatectomy on the levels of DHBV and liver-specific enzymes in the serum of the same DHBV-carrier duck. DHBV DNA and ALT were monitored weekly for 4 weeks presurgery (pre); 1–2 times per week for 6 weeks post-laparotomy (post-L), and daily for 14 days post-hepatectomy (post-H). DHBV DNA was measured by dot blot hybridization and quantitated visually and by liquid scintillation counting. ALT levels were determined by a commercially available assay.

membranes, from both neonatal and adult ducks are able to bind the virus. Furthermore, saturation binding studies suggest that adult hepatocytes express the DHBV receptor at similar levels to neonatal hepatocytes. This suggests that a later event during infection of adult ducks with DHBV represents the block in full virus replication, and our results are consistent with a recent study (Jilbert *et al.*, manuscript in preparation) which shows that adult duck hepatocytes infected *in vivo* support many of the steps of DHBV replication but fail to secrete virus into the serum.

The partial hepatectomy studies described above may help to explain these findings; our studies showed a distinct transient increase in the level of DHBV in the serum of carrier ducks that was related to partial hepatectomy but not to control surgical procedures. Furthermore, the increase in the serological level of DHBV was related directly to the degree of hepatectomy, since in some experiments (data not shown) in which approximately 20% of the liver was removed, no discernible increase in DHBV levels was noted. We have previously shown that a majority of hepatocytes of adult carrier birds are supporting DHBV DNA replication (Jilbert *et al.*, 1988), and the increases in serum DHBV levels reported above are more striking still when it is considered that these birds had had approximately 50% of liver tissue removed. The increase in DHBV DNA in the serum was not simply due to hepatocyte lysis since ALT and LDH activity remained within normal levels throughout the study. We have not examined whether hepatectomy affected primarily virus replication or virus release; in either case, our findings demonstrated that the dividing liver produced DHBV more efficiently than the quiescent liver, and partial hepatectomy restored this function to the hepatocytes of adult ducks. However, the effects on virus replication of cytokines or hormones secreted in response to the surgery were not analyzed. Thus these may have some effect on virus replication, although we favor the role of the dividing hepatocyte particularly since inadequate resection (discussed above) showed no effect on the level of viremia. Our results might also provide an explanation for the rapid fall in the levels of DHBV in the serum of either congenitally or neonatally infected ducks when the ducks reach a few months of age if this coincides with the stage at which hepatocytes stop dividing. As vertical transmission represents the normal route of DHBV transmission, then it is likely that DHBV has evolved in such a manner to utilize dividing cells which represent the natural target for the virus. This may explain why adult ducks fail to become viremic.

In contrast, horizontal transmission of HBV to adults with subsequent viremia is much more common and it can be speculated that the role of the HBV X gene, which is absent in the DHBV genome, may be to encode a product with transactivating activity that stimulates adult hepatocytes to facilitate HBV replication and secretion. If this is the case, then one would predict that the X gene product would be found in the virion, and evidence for this has been presented recently (Wu *et al.*, 1990).

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