Studies on the protective and therapeutic efficacy of duck hepatitis B virus vaccines

Miriam Triyatni

M.B., B.S.

Department of Microbiology and Immunology

The University of Adelaide

Adelaide, South Australia

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

October 1998

To the bravest and the most sincere person I've ever known,
who taught me to pursue my dreams,
whose love and strength will always inspire me for a life time.

In dedication to my mother

ABSTRACT

The existence of safe and efficacious HBV vaccines has given the opportunity to eradicate HBV infection worldwide. Nonetheless, problems encountered with the current vaccines such as the failure of 5-10% of vaccine recipients to develop protective levels of anti-HBs antibody responses, and the necessity for booster injection remains unresolved. Thus, development of more effective HBV vaccines is still desirable. In addition, the possibility of vaccination which could also be used to treat chronic HBV infection that at present affect ~350 million people worldwide would be another advantage. Second generation recombinant HBV vaccines have included the pre-S proteins in addition to the S protein. Studies on mice and limited number of human trials have shown that pre-S containing HBV vaccines can overcome non-responsiveness to the current vaccines. However, the long term significance of pre-S containing HBV vaccines in increasing the seroconversion rate and reducing the need of booster injection has not been established.

Recently, DNA vaccines have been regarded as a possible next-generation of HBV vaccines. This approach offers vaccination not only as a means of prevention, but also as therapeutic agent for chronic HBV infection. So far, studies on DNA vaccines encoding HBV surface proteins have been performed in mice and in a very limited number of chimpanzees, the only higher primates that can be infected with HBV. Using DHBV infection in Pekin ducks as model for HBV infection in human, this study aimed to evaluate the protective and therapeutic efficacy of DNA vaccines encoding DHBV pre-S/S and S proteins against DHBV infection. In addition, comparison of the protective efficacy of DNA vaccines to the yeast-derived DHBV surface proteins was also performed.

As the initial step in designing DHBV vaccines, a full-length genome of an Australian DHBV (AusDHBV) isolate was cloned from a pool of serum from congenitally DHBV-infected ducks. Comparison of the nucleotide sequence of this clone indicated that the AusDHBV isolate is a new member of the Chinese DHBV branch in the phylogenetic tree of the avian hepadnaviruses.

Yeast-derived DHBV pre-S/S and S proteins were produced in *S. cerevisiae*; analysis of the S values and the buoyant density of both proteins suggested that they were assembled as intracellular particles or aggregates. Purification of yeast-derived proteins was carried out through several sucrose gradient steps and revealed that the yeast-derived DHBV proteins were closely associated with the yeast membrane proteins. Three injections of 40 μg of yeast-derived proteins elicited moderate levels of anti-DHBs antibody responses in ducks; no difference was found in the antibody levels between the pre-S/S and S vaccinated ducks. The antibody levels were >3 log lower than those produced by age matched ducks injected three times with 250 μg of pre-S/S and S DNA vaccines. Possible explanations for the strong immunogenicity of DNA vaccines included the prolonged and the mode of antigen presentation to the host immune system, in addition to the adjuvant effect of plasmid DNA.

The protective efficacy of both yeast-derived and DNA vaccines was determined by: (i) the rate of virus removal from the bloodstream following i.v. challenge with high dose of inoculum, (ii) the extent of virus replication at 4 days post-challenge (p.c.) and (iii) the development of viremia. Following challenge, ducks vaccinated with yeast-derived pre-S/S protein and S proteins removed the inoculum from the bloodstream between 60-110 min and 30-45 min, respectively; these rates were not significantly different to those shown by non-vaccinated control ducks (70 min). Both yeast-derived vaccines did protect ducks, albeit partially, against DHBV infection since virus infection in the liver at 4 days p.c. was restricted to ~40% of

hepatocytes compared to the widespread infection (>95% hepatocytes) observed in the non-vaccinated ducks. An interesting observation was that the S DNA vaccine was more effective than the pre-S/S DNA vaccine based on: (i) the rate of virus removal from the bloodstream p.c. (5-15 min versus 60-100 min), (ii) the absence of virus replication in the liver at 4 days p.c. (DHBsAg-positive were found in 10-40% of hepatocytes of the pre-S/S vaccinated ducks), and (iii) the ability of anti-S serum to neutralize viral infectivity *in vitro* and *in vivo*. Both vaccines, however, offered protection as no viremia was observed in any of the ducks during 8 weeks of monitoring. The lower efficacy of pre-S/S DNA vaccine was likely due to the biological properties of anti-pre-S and anti-S antibodies produced; this hypothesis was inferred from an analysis of the *in vitro* transient transfection products, whereby in contrast to the S protein, the pre-S/S protein was not secreted.

The therapeutic potential of pre-S/S and S DNA vaccines was also assessed in congenitally DHBV-infected ducks and in 3 other more favourable vaccination settings (pre-infection, at the time of challenge, and 7 days post-infection). DNA vaccines were effective only in the pre-infection setting; in the other settings, vaccinated ducks showed persistent viremia up to 24 weeks of age, albeit at reduced levels of viremia and lower levels of DHBsAg expression in the liver, compared to the non-vaccinated ducks.

Altogether, this study has confirmed the value of DHBV infection in ducks as a model to evaluate the protective and therapeutic efficacy of DNA vaccines against hepadnavirus infection. The possibility that this model could be explored further to evaluate various combinations of antigens and cytokines 'cocktail' DNA vaccines that elicit the most effective humoral and effective CMI responses for prevention and treatment of HBV infection is discussed.

DECLARATION OF ORIGINALITY

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

I give my consent to this copy of my thesis, when deposited into the University Library, being made available for loan and photocopying.

MIRIAM TRIYATNI Date: 6 October 1998

PUBLICATIONS ARISING FROM THIS THESIS RESEARCH.

INTERNATIONAL CONFERENCE PRESENTATIONS:

Triyatni, M., Jilbert, A.R., Qiao, M., and Burrell, C.J. Protective efficacy of both yeast-derived and DNA-based vaccine against DHBV infection. (*Oral presentation*). "Molecular Biology of Hepatitis B Viruses Meeting", September 18-22, 1996, Cold Spring Harbor Laboratory, New York, USA.

Triyatni, M., Jilbert, A.R., Qiao, M., and Burrell, C.J. Cloning and sequencing of an Australian DHBV strain: Comparison with two other DHBV strain. (*Poster*). "Molecular Biology of Hepatitis B Viruses Meeting", September 18-22, 1996, Cold Spring Harbor Laboratory, New York, USA.

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. Protective efficacy of DNA vaccines against DHBV infection in neonatal ducklings. (*Oral presentation*). "Molecular Biology of Hepatitis B Viruses Meeting", September 22-25, 1997, Institute Pasteur, Paris.

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. Protective and therapeutic efficacy of DNA vaccines against DHBV infection in newly hatched ducks. (*Oral presentation*). "The Molecular Biology of Hepatitis B Viruses", August 30-September 3, 1998, University of California, San Diego, USA.

NATIONAL CONFERENCES PRESENTATIONS:

Triyatni, M., Jilbert, A.R., Qiao, M., and Burrell, C.J. Studies of immune escape mutants of DHBV infection. (*Oral presentation*). "Australian Viral Hepatitis Workshop", March 21-23, 1995, Sydney, Australia.

Triyatni, M., Jilbert, A.R., Qiao, M., and Burrell, C.J. Production of yeast-derived DHBV surface proteins and their use as vaccines. (*Oral presentation*). "Australian Viral Hepatitis Workshop", March 21-23, 1996, Sydney, Australia.

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. Protective efficacy of DNA-based vaccine against DHBV infection. (*Oral presentation*). "Australian Viral Hepatitis Workshop", March 20-22, 1997, Sydney, Australia.

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. Protective efficacy of DNA-based vaccine against DHBV infection. (*Oral presentation*). "Australian Society for Microbiology Annual Scientific Meeting and Exhibition", September 28-October 3, 1997, Adelaide, Australia.

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. Protective and therapeutic efficacy of DNA vaccines against DHBV infection in newly hatched ducks. (*Oral presentation*). "Australian Viral Hepatitis Workshop", March 20-22, 1998, Sydney, Australia.

JOURNAL ARTICLES:

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. Protective efficacy of DNA vaccines against DHBV infection. (1998). *J. Virol.* **72:**84-94.

Triyatni, M., Qiao, M., Ey, P., Burrell, C.J., and Jilbert, A.R. (1998). Complete nucleotide sequencing of an Australian duck hepatitis B virus. *EMBL AJ006350.*

MANUSCRIPT IN PREPARATION:

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. Protective and therapeutic efficacy of DNA vaccines against DHBV infection in newly hatched ducks. In preparation.

ACKNOWLEDGEMENTS

The work for this thesis was performed in the Department of Microbiology and Immunology, University of Adelaide, and in the Infectious Diseases Laboratories (IDL), Institute of Medical and Veterinary Science (IMVS), Adelaide. I wish to thank the staff of IDL and the Department of Microbiology and Immunology for their help, and the Australian Agency for International Development (AusAID) for support.

I would like to express my sincere gratitude to my supervisors Dr. Allison Jilbert, Dr. Ming Qiao and Prof. Christopher Burrell for their continuous guidance, support, editorial comments and encouragement throughout my Ph.D. candidature. I wish to make special acknowledgment to Prof. Ieva Kotlarski for her concentrated efforts in the critical review of this thesis and excellent suggestions.

I would like to thank the previous and present members of the Hepatitis Research Laboratory, Dr. Keril Blight, Dr. Mark Arens, and Dr. Edward Bertram for their advice and friendship. Cathy Scougall, Ailong Huang, and Darren 'Duckman' Miller, for his continuous help with the animal experiments, also for their friendship and making the laboratory a pleasant place to work.

I would like to thank Dr. Peter Ey for his excellent expertise with the Molecular Evolutionary Genetics Analysis software program. Thanks also to Dr. John Pugh for provision of the anti-pre-S specific monoclonal antibodies 1H.1.

Thanks to Gary Penney, John Mackrill, Gail Bezuidenhoudt, Shelley Pezy and Tony Richardson, for their help in many different areas and pleasant conversation over the years.

Special thanks to Dr. Budiman Bela, Dr. David Ogunniyi and Joseph Ogierman for their bountiful support and friendship. Likewise, to Kerrilyn Diener, Dr. Lothar Staendner and Dr. Nicholas Coates for their support and close, cherished friendship through the good and tough times especially in the last year of my study.

I would also like to thank all the members of the Department of Microbiology and Immunology for their help, advice and friendliness; all of which have made my time in the department enjoyable throughout the years.

Further thanks to Mark Fitz-Gerald and Peta Grants of the Photographic Unit, IMVS for their excellent assistance and photography. Thanks also to the staff members of the Animal House, IMVS for their assistance and the Division of Tissue Pathology, IMVS for provision of wax-embedded tissue sections.

I would like to thank my parents for giving me every opportunity to be successful in life with continual love and encouragement. Special thanks to my sisters and brothers for their bountiful love, support and understanding. I wish to express my deepest gratitude to my family, Rino and Adam, for their love, support and patience throughout my study. Finally, I am thankful to God for giving me faith and courage to pursuit my dreams.

LIST OF ABBREVIATIONS

β-ME	β-mercaptoethanol
Ω	ohms
μCi	microcurie
μg	microgram
μF	microfaraday
μl	microliter
μm	micrometer
μmol	micromole
x g	relative centrifugal force
%	percentage
ALT	alanine aminotransferase
Amp	Ampicillin
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
APC	antigen presenting cell
APS	ammonium persulfate
AGSHV	arctic ground squirrel hepatitis virus
AST	aspartate aminotransferase
ATP	adenosine 5'-triphosphate
AusDHBV	Australian isolate of duck hepatitis B virus
bp	base-pair
BPB	bromo phenol blue
BSA	bovine serum albumin
°C	degrees Celcius
CAH	chronic active hepatitis
cccDNA	covalently closed circular deoxyribonucleic acid

CID_{50}	chimpanzee infectious dose
CIP	calf intestinal phosphatase
cm	centimeter
CMI	cell mediated immunity
CpG motifs	5'-Purine Purine CG Pyrimidine Pyrimidine-3'
CPH	chronic persistent hepatitis
cpm	counts per minute
CsCl	caesium chloride
CTL	cytotoxic T lymphocyte
DAB	3,3-diaminobenzidine tetrahydrochloride
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytosine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
DHBcAg	duck hepatitis B virus core antigen
DHBsAg	duck hepatitis B virus surface antigen
DHBV	duck hepatitis B virus
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOTAP	N-[1-(2,3,-Dioleoyloxy) propyl]-N,N,N-trimethyl-ammonium methylsulfate
DTT	dithiothreitol
dTTP	deoxythymidine-5'-triphosphate
DW	distilled water
EAA	ethanol:acetic acid (3:1)
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme linked immunosorbant assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gravity
GSHV	ground squirrel hepatitis virus

H&E	haematoxylin and eosin
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HHBV	heron hepatitis B virus
hr	hour
HRP	horseradish peroxidase
IFN-α,-β,-γ	interferon-alfa, -beta, -gamma
Ig	immunoglobulin
i.m.	intramuscular
IPTG	isopropylthio-β-D-galactoside
IMF	immunofluorescence
ISS	immunostimulatory sequence
ISS-ODN	immunostimulatory sequence-oligonucleotide
i.v.	intravenous
kDa	kilodalton
kV	kilovolt
L protein	large surface protein
M	molar
mA	milliampere
mAb	monoclonal antibody
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	milliliter
mM	millimolar
M protein	middle surface protein
mRNA	messenger ribonucleic acid
MW	molecular weight
NDS	normal duck serum

ng	nanogram
nm	nanometer
NP-40	nonidet-P40 detergent
NRS	normal rabbit serum
OD	optical density
OPD	*o*-phenylenediamine
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	PBS with 0.05% Tween 20
p.c.	post-challenge
PCR	polymerase chain reaction
p.i.	post-inoculation
PEG	poly ethylene glycol
PHA	passive hemagglutination
pM	picomoles
PMSF	phenyl methyl sulfonyl fluoride
PVP	polyvinyl pyrolodone
QA	quadriceps anterior
rDHBcAg	recombinant duck hepatitis B virus core antigen
RE	restriction enzyme
RPHA	reverse passive hemagglutination
RT	room temperature
RNA	ribonucleic acid
rpm	revolution per minute
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
sec	second
S protein	small surface protein
S value	Svedberg value
TBS	Tris buffered saline (10 mM Tris-HCl pH 7.5, 0.9% NaCl)
TBS-T	Tris buffered saline with 0.05% Tween-20

TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA buffer
TEMED	N,N,N',N'-tetramethylenediamine
Th	helper T lymphocyte
Tm	melting temperature
Tris	3,3,5,5-tetramethylbenzidine
WHC	woodchuck hepatitis B virus
WMHBV	woolly monkey hepatitis B virus
V	volt
v	volume
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

TABLE OF CONTENTS

Chapter 1

Introduction

1.1. BACKGROUND AND EPIDEMIOLOGY.

Hepatitis B virus (HBV) infection is a global public health problem with more than 350 million chronic carriers worldwide (Kane, 1996). The prevalence of HBV carriage differs from region to region across the world: 0.1-1% in North America, Scandinavia, Western Europe, Australia, and New Zealand; 2-7% in South and Central America, the Middle East, North Africa and Eastern Europe; and 5-20% in China, Southeast Asia, Africa, sub-Saharan Africa, and Alaska (Kane, 1996; Hollinger, 1990). The main route of spread of HBV infection is through parenteral or percutaneous transmission and through sexual contact. High risk groups have included infants born to HBV-carrier mothers, recipients of unscreened blood or blood products, hemodialysis patients, staff of hemodialysis units and mentally-handicapped institutions, patients for organ transplantation, intravenous drug abusers, male homosexuals and promiscuous individuals. Transmission from chronic HBV carriers to others living in the same household has also been demonstrated, possibly via shared razors or toothbrushes (Hollinger, 1990).

In the areas with high prevalence of HBV, infection most commonly occurs during the perinatal period from HBV-carrier mothers, or during early childhood, possibly by unrecognized blood-blood contact. A long-term study in Taiwan showed that the HBV carriage rate was inversely related to the age at which the infection occurred: 90% in perinatal transmission, 25% in pre-school children, and less than 3% in adolescents and young adults (Beasley *et al.*, 1983; Chen, 1993). Once infection became chronic, HBsAg carriage was long-lived with an overall incidence of HBsAg clearance in only 0.6% of carrier children per year (Chen, 1993). Chronic HBV infections in infants and children, although usually asymptomatic (so-called 'healthy' HBsAg carrier state), serve as a

reservoir of HBV and increase the risk of chronic liver disease and/or hepatocellular carcinoma (HCC) later in life. An effective measure to prevent perinatal transmission has been demonstrated by HBV vaccination of infants born to HBV-carrier mothers. More than >95% of these infants that received hepatitis B immunoglobulin (HBIG) and HBV vaccination at birth, followed by vaccination at 1 and 6 month, were prevented from becoming chronic carriers (Beasley *et al.,* 1983). Therefore, vaccination of all infants born to HBV carrier-mothers within the areas with high prevalence of HBV is extremely important to reduce the number of new HBV carriers and, subsequently, to reduce the mortality rates associated with chronic liver disease and HCC.

The effect of universal HBV vaccination on the incidence of HCC in a country with high prevalence rate of HBV has recently been demonstrated in Taiwan (Chang *et al.,* 1997). Since 1984, HBV vaccination was given to all infants, and in 10 years, this approach has reduced the HBsAg carrier rate in children from 10% to less than 1%. At the same time, the incidence of HCC among children aged between 6 to 14 years also declined significantly, from 0.7/100,000 children in 1981-1986 to 0.36 between 1990-1994. This result demonstrated an early effect of universal vaccination in reducing the rate of HCC. Although, it should be noted that the long term effects may even be more impressive since the incidence of HCC in Taiwan peaks in the sixth decade of life.

In the areas with low prevalence of HBV, most infections occur in adult life where more than 90% of infections are transient and only 5-10% become persistent (Fig. 1.1). In these areas, HBV vaccination was initially recommended only for high risk individuals. However, this selective vaccination approach has not been effective in lowering the overall incidence of disease; for example, in the United States of America, a 37% increase

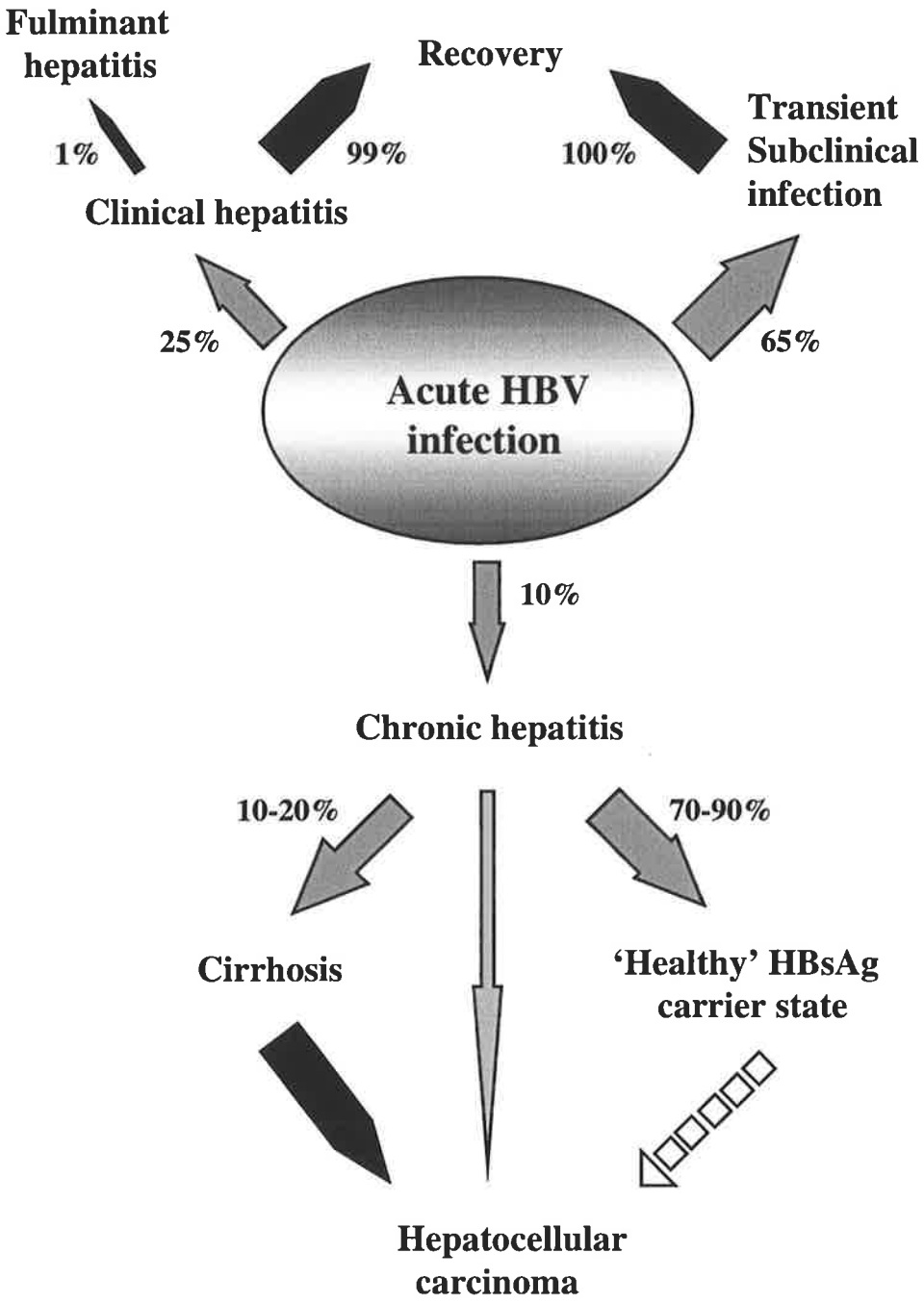


Figure 1.1. The outcome of HBV infection in adults.
Adapted from Hoofnagle, J.H. (1987).

of the HBV incidence in the overall population was documented between 1979 and 1989 despite the introduction of HBV vaccination for high risk groups during this period (Centers for Disease Control, 1991). It is increasingly acknowledged that universal vaccination of all infants is the only certain way to eliminate HBV infection, since it would minimize not only vertical but also later, the horizontal modes of transmission.

1.2. HEPADNAVIRUS FAMILY.

HBV belongs to the virus family hepadnaviridae that is divided into two subfamilies, the mammalian and avian hepadnaviruses, each with restricted host specificity. The mammalian hepadnaviruses include HBV, which infects only humans and higher primates and is the prototype hepadnavirus (Galibert *et al.,* 1979). Other members include woodchuck hepatitis virus (WHV; Summers *et al.,* 1978), ground squirrel hepatitis virus (GSHV; Marion *et al.,* 1980), arctic ground squirrel hepatitis virus (ASHV; Testut *et al.,* 1996), and woolly monkey hepatitis B virus (WMHBV; Lanford *et al.,* 1998). The avian hepadnaviruses include duck hepatitis B virus (DHBV; Mason *et al.,* 1980), heron hepatitis B virus (HHBV; Sprengel *et al.,* 1988), and Ross goose hepatitis virus (RGHV; Shi *et al.,* 1993). This chapter will mainly focus on the molecular biology and immunological aspects of HBV infection, and their relevance to DHBV infection in ducks that is used as a model for HBV infection in human.

1.2.1. Genomic Organization of HBV.

Hepadnaviruses have a partially double-stranded (ds) DNA genome and replicate through an RNA intermediate by the use of reverse transcription. The HBV DNA genome is

~3200 base pairs (bp) in length; it carries four (three for avian hepadnaviruses) overlapping open reading frames (ORFs), and shows an extremely compact organization: all of the DNA encodes protein, more than half of the nucleotides are used for more than one ORF and all regulatory signals overlap with coding regions (Fig. 1.2). For instance, the S ORF is entirely overlapped by the P ORF; any nucleotide changes that lead to a loss of function of either protein will therefore be lethal (Nassal & Schaller, 1993).

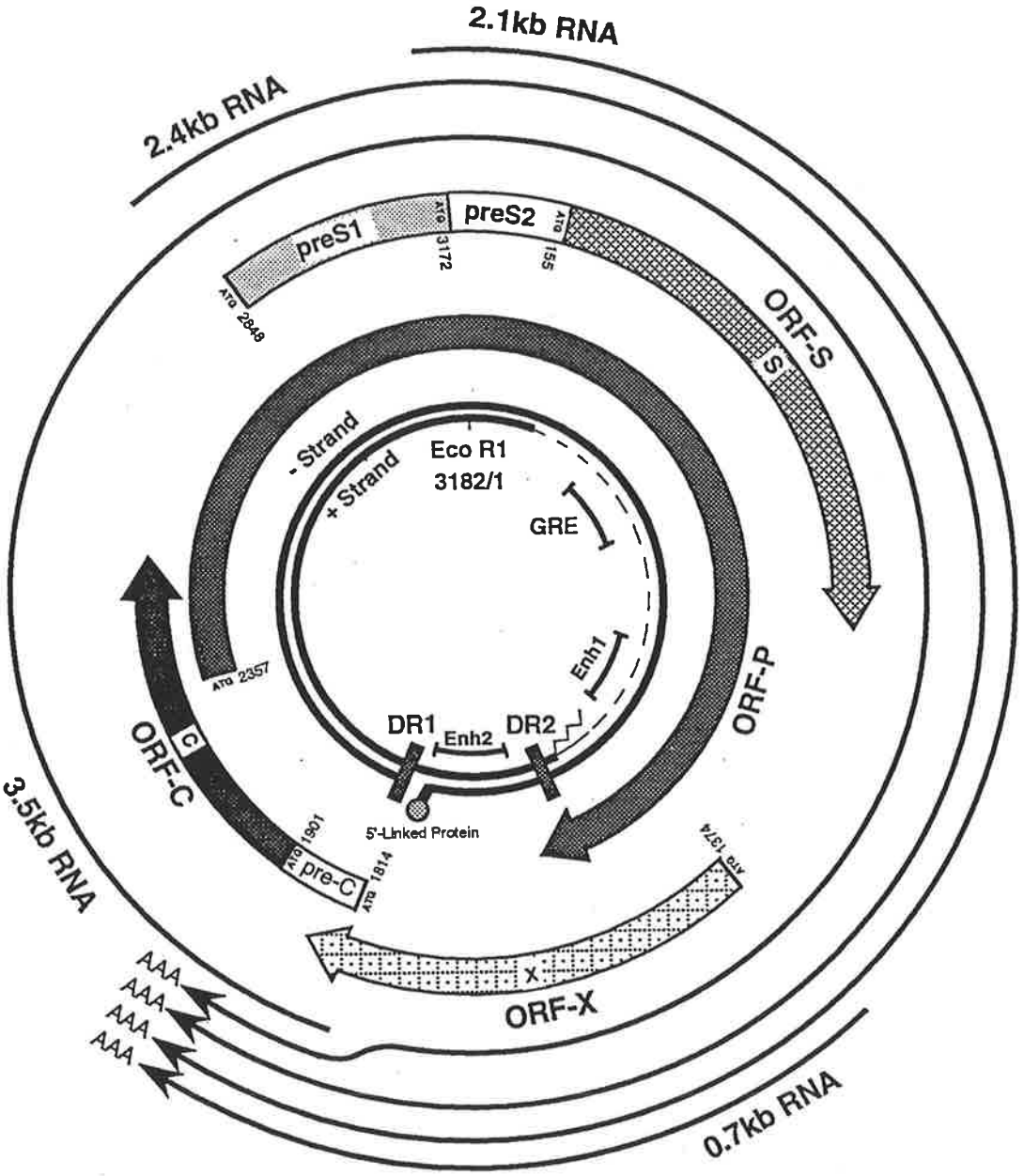
The four ORFs found within the negative strand of the HBV DNA genome are termed the P, S, pre-C/C, and X genes, respectively. The P gene covers approximately 80% of the entire genome and encodes the viral RNA- and DNA-directed DNA polymerase (P protein). The P protein is translated from a 3.5 kb mRNA transcript which is greater than genomic length, that is also used to synthesize core protein and serves as the RNA pregenome (Nassal, 1996). The central and C-terminal region of the P protein contains the DNA polymerase and RNase H domains which share significant homology with the corresponding domains of other viral polymerases with reverse transcriptase functions. The N-terminal domain is separated by a spacer from the polymerase domain and bears a tyrosine residue at position 96 (Tyr-96). Binding of P protein via this Tyr-96 residue to the epsilon (ε) region of the RNA pregenome will initiate the synthesis of negative strand DNA (see below). In addition to its role in DNA synthesis, the P protein is also essential for packaging the RNA pregenome into viral nucleocapsid particles (Hu and Seeger, 1996).

The S gene contains three different in-frame start codons, namely pre-S1, pre-S2, and S, all of which share a common carboxyl terminus. Translation of the entire pre-S1/pre-S2/S gene yields the large (L) viral surface protein, while translation of pre-S2/S and of S

Figure 1.2. Genomic structure of HBV.

Inner circles represent the partially double-stranded circular viral DNA genome. *Grey hatched boxes marked DR1 and DR2* represent the direct repeats, *grey hatched circle* is the P protein covalently attached to the 5' end of the negative-strand DNA. *Wavy line* is the RNA primer at the 5' end of positive-strand DNA. Open reading frames (ORFs) are indicated by *arrows*. *Numbers* are nt positions, the numbering system is according to (Tiollais *et al.*, 1985). *Outer lines* represent the viral transcripts, shown with their common 3' polyadenylation sites (AAA). *Enh1* and *Enh2, enhancer elements. GRE,* glucocorticoid responsive element.

From (Ming Qiao, 1994, Ph.D thesis) with permission.


2.1kb RNA
2.4kb RNA
preS1
preS2
ATG
3172
155
2849
ORF-S
S
- Strand
+ Strand
Eco R1
3182/1
GRE
Enh1
ATG 2357
DR1
Enh2
DR2
ORF-P
ORF-C
C
ATG 1901
pre-C
ATG 1814
5'-Linked Protein
3.5kb RNA
ATG 1374
X
ORF-X
AAA
AAA
AAA
AAA
0.7kb RNA

produce the middle (M), and the small or major (S) proteins, respectively. Thus all three forms of surface proteins contain the S domain, also known as HBsAg. The surface proteins are translated from two distinct mRNA species: L protein is translated from a 2.4 kb transcript, while M and S proteins are translated from a 2.1 kb transcript by separate start codons. All surface proteins produced by the mammalian hepadnaviruses are also present in glycosylated forms; accordingly, the molecular masses of the HBV surface proteins are p39/gp42 (L), p30/gp33/gp36 (M), and p24/gp27 (S) (Heerman *et al.,* 1984; Stibbe & Gerlich, 1983).

The pre-C/C gene has two in-frame start codons that initiate two distinct products: the secreted e protein, so-called hepatitis B e antigen (HBeAg), and the core protein which forms the viral nucleocapsid, also known as hepatitis B core antigen (HBcAg). Both products are translated from greater than genomic length, 3.5 kb mRNA transcripts that have different 5'-ends. The first ATG (pre-C) is the translation start codon for HBeAg. A signal sequence at the amino terminal of the pre-C sequence allows translocation into the lumen of the endoplasmic reticulum where 19 of the 29 pre-C amino acids are cleaved off by a signal peptidase (Bruss & Gerlich, 1988). A portion of the HBeAg molecules are then transported to the plasma membrane (Schlicht & Schaller, 1989) as membrane-bound HBeAg (23 kDa). The remaining molecules are further cleaved by a Golgi protease, and the protein released is then secreted into the plasma as soluble HBeAg with a size which varies between 16-20 kDa. The second ATG (C) encodes the 21 kDa HBcAg that assembles during replication to form the viral nucleocapsid.

The size of the X gene varies among different HBV isolates (the largest is found in HBV *adr* subtype) and encodes an X protein (pX) of 154 AA (Hollinger, 1990). The precise

role of this protein in HBV infection is not known, although it has been detected in some HBV-infected liver and HCC tissues (Su *et al.,* 1998). The X gene is capable of *trans*-activating many viral and cellular promoters, including those of genes involved in cell growth regulation, i.e. c-*fos*, c-*jun*, and c-*myc* (Chirillo *et al.,* 1997; Twu *et al.,* 1993). In a recent study using the NIH 3T3 polyclonal cell line, transfection of the HBV X gene induced cell cycle progression and programmed cell death (apoptosis). Both effects were exerted by pX and required the presence of the wild-type p53 protein; a mutation or conformational inactivation of p53 protein abolished the effect of pX on apoptosis (Chirillo *et al.,* 1997). Nevertheless, it remains a controversial issue whether *trans*-acting activity of the pX alone is responsible for HBV-associated liver carcinogenesis.

1.3. REPLICATION OF THE HEPADNAVIRUS GENOME.

The infectious virus particle attaches to its target cell through interaction of the L protein, especially its pre-S1 domain, with a specific receptor(s) that is yet to be identified. Uncoating and transport of the viral genome into the nucleus then occurs. Viral replication then proceeds in four major stages (Fig. 1.3 and Fig. 1.4).

First, the partially double-stranded DNA found in virions is converted to covalently closed circular DNA (cccDNA). This cccDNA serves as template for the host RNA polymerase to synthesize subgenomic and genomic RNAs. These are then exported into the cytoplasm and used for translation of the various gene products mentioned above. The surface proteins are targeted to the endoplasmic reticulum (ER) where they are inserted into membrane structures, and are either secreted via the Golgi as subviral

Figure 1.3. Schematic diagram of the life cycle of the hepadnaviruses.

Infectious virions bind via the pre-S1 domain of L protein to an yet as unknown receptor on target cells. The nucleocapsid enters the cytoplasm and directs the partially ds DNA genome to the host nucleus where it is converted to cccDNA. The cccDNA serves as a template for transcription of genomic and subgenomic RNAs that are translated in the cytoplasm. Core and P proteins interact with the RNA pregenome, forming new nucleocapsids. The RNA is reverse transcribed, and the mature capsids either recycle the DNA back to the nucleus, or are exported via interaction with the surface proteins at the membrane of the ER, or intermediate compartment (IC). Subviral particles are secreted in excess compared to virions. Adapted from Nassal, M. & Schaller, H. (1996).

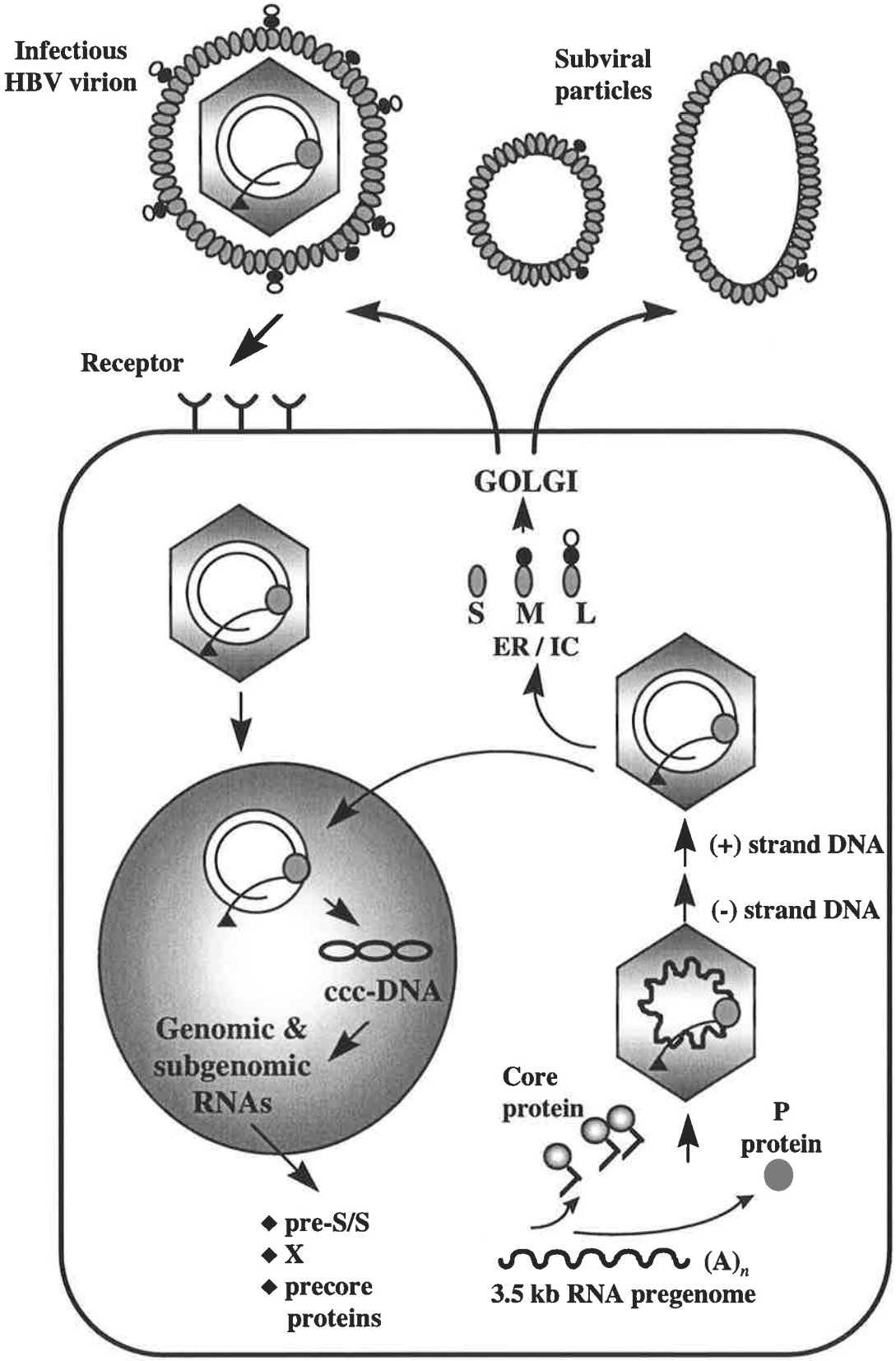
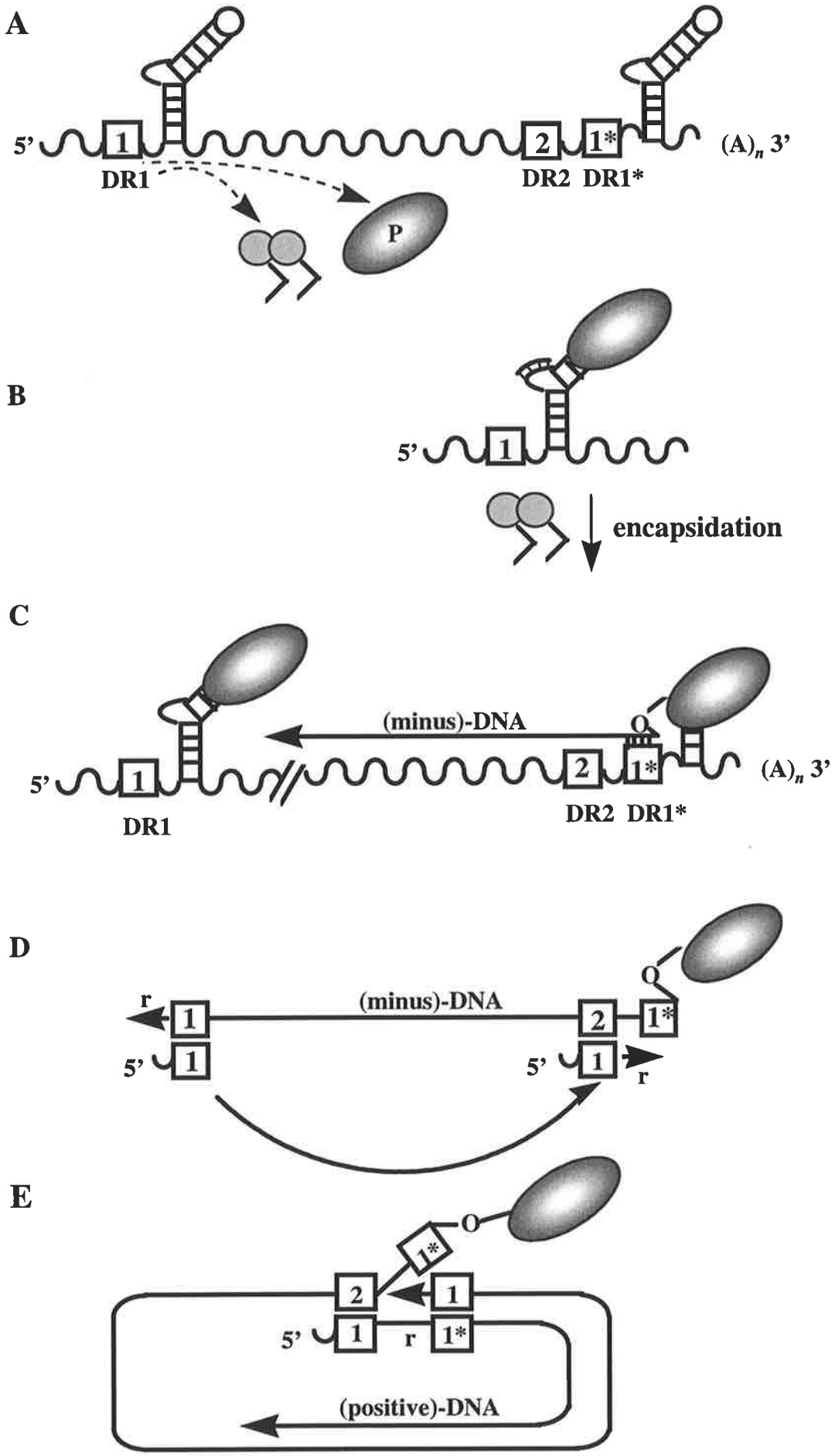

Infectious
HBV virion
Subviral
particles
Receptor
GOLGI
S M L
ER / IC
(+) strand DNA
(-) strand DNA
ccc-DNA
Genomic &
subgenomic
RNAs
Core
protein
P
protein
◆ pre-S/S
◆ X
◆ precore
proteins
(A)n
3.5 kb RNA pregenome

Figure 1.4. Replication strategy of the hepadnaviruses.

(A) The wavy line represents the terminally redundant RNA pregenome, which also serves as mRNA for both the core and the P proteins. The ε sequence is shown as a symbolic hairpin, and the direct repeat elements DR1, DR2, and DR1* are shown as boxes. **(B)** Binding of P protein via its Tyr-96 residue to the 5' copy of ε triggers the following events: (i) addition of core protein dimers and hence nucleocapsid assembly, and (ii) initiation of synthesis of three to four DNA nucleotides using the bulge region of ε as template. **(C)** The P protein-DNA complex is then translocated to DR1* to initiate synthesis of the negative-strand of DNA that is extended to the 5' end of RNA pregenome. **(D)** The RNA template is degraded, except for a short oligonucleotide at the 5' end containing the DR1 sequence. The RNA oligonucleotide is then transferred to DR2 and used as a primer for synthesis of the positive-strand DNA. **(E)** Due to a short terminal redundancy (r), the 3' end of the positive-strand DNA can use the 3' end of negative-strand DNA to circularize the genome and to continue positive-strand synthesis. Adapted from Beck, J. & Nassal, M. (1997).


A
5'
1
DR1
2
1*
DR2 DR1*
(A)n 3'
P
B
5'
1
encapsidation
C
(minus)-DNA
O
5'
1
DR1
2
1*
DR2 DR1*
(A)n 3'
D
r
1
5'
1
(minus)-DNA
O
2
1*
5'
1
r
E
O
1*
2
1
5'
1
r
1*
(positive)-DNA

particles, or used for envelopment of viral nucleocapsids. The core and P proteins remain in the cytoplasm where they play essential roles in virus replication.

Second, the RNA pregenome is produced by host RNA polymerase from the cccDNA template. This RNA is greater than genomic length (3.5 kb) due to terminally redundant sequences: The 5' end contains the epsilon (ε) and 'direct repeat' (DR1) sequence, while the 3' end contains ε, DR1*, and DR2 sequences (Junker-Niepman *et al.,* 1990). The sequence of ε forms a highly conserved stem-loop structure with an internal bulge which is used both as the template for initiation of viral negative strand DNA synthesis and as a packaging signal. A specific UUAC motif located in the bulge region of ε acts as a template for these reactions. Encapsidation of the RNA pregenome is initiated by the binding of P protein (via its Tyr-96 residue) to ε; this event triggers the self assembly of dimers of core protein that stabilize the complex and form the nucleocapsid (approximately 240 subunits of core protein per nucleocapsid; Nassal & Schaller, 1996; Böttcher *et al.,* 1997; Conway *et al.,* 1997). The core protein is 183 amino acids (AA) long and contains a very basic carboxy-terminal region that is believed to interact with the viral genome. Removal of this region, provided that the truncation is before AA 140, still allows the core protein to self-assemble via dimeric intermediates into empty icosahedral nucleocapsid particles (Nassal & Schaller, 1993; Böttcher *et al.,* 1997). It has been shown that hepadnaviruses have evolved an unique strategy for replication which ensures that encapsidation proceeds only when both the RNA pregenome and the P protein are present. The precise mechanism(s) of how hepadnaviruses regulate the functions of the 3.5 kb RNA transcript as the pregenome or as the mRNA for both core and P proteins, remains unclear. However, it has been suggested that the alternative use of the 3.5 kb

RNA as pregenome or as mRNA for both proteins might be regulated by ε whose position overlaps with the translation initiation signal for core protein. The demonstration that the ratio of RNA pregenomes being encapsidated or serving as mRNA depended on the concentration of the gene products is consistent with this suggestion (Junker-Niepman *et al.*, 1990).

Third, the RNA pregenome within the nucleocapsid is reverse transcribed into the first (negative) strand of DNA by P protein. Following incorporation of three to four DNA nucleotides by P protein, the nascent DNA strand is then translocated to the DR1* sequence at the 3' end of the RNA pregenome, where negative strand DNA synthesis continues to the 5' end of the pregenome. During this process, degradation of the RNA template occurs by the RNase H activity of P protein. All of the RNA pregenome is degraded except for the last 15-18 nucleotides at the 5' end containing the DR1 sequence, which serve as the primer for synthesis of the second DNA strand.

Fourth, the second (positive) strand of DNA is synthesized by copying the first DNA strand. For circularization, the primer molecule is transferred to the DR2 region and extends to the 5' end of the negative DNA strand. Due to a short terminal redundancy ("r"), the 3' end of the positive strand can use the 3' end of the negative strand to initiate DNA synthesis, yielding the characteristic circular, partially double-stranded DNA molecule with P protein covalently attached to the 5' end of the first (negative) DNA strand. These replication-competent nucleocapsids either leave the cell after envelopment with the surface proteins present in internal membranes, or they may cycle back into the nucleus to maintain the pool of cccDNA (Ganem & Varmus, 1987; Nassal & Schaller, 1993; Nassal, 1996). Following budding through the ER, the infectious virion consists of

viral DNA within a nucleocapsid that is enveloped by all three surface proteins in association with host-derived lipid.

1.4. ASSEMBLY OF VIRAL AND SUBVIRAL PARTICLES.

HBV shows three morphologically distinct particle types, namely the 42nm double-shelled virion or Dane particle, and two forms of subviral particles consisting of (i) spheres of 22nm in diameter, and (ii) filamentous structures with similar diameter but of variable length. Typical particle numbers in the serum of patients with chronic HBV infection are in the range of 10^{13}/ml for the spheres, 10^{10}/ml for filaments, and up to 10^{9}/ml for virions (Nassal, 1996); these values, however, are extremely variable from patients to patients (Heermann and Gerlich, 1991).

The hallmark of HBV infection is overproduction of the 22nm subviral particles. These particles are non-infectious as they do not contain a viral genome or nucleocapsid; they are composed mostly of S protein and host-derived lipid, with variable amounts of M protein and only trace amounts of L protein.

S protein alone is able to form 22nm particles that are efficiently secreted from cells of higher eukaryotes, but not from insect or yeast cells (Nassal, 1996; Standring *et al.,* 1986). The same is true for M protein; in contrast, L protein is retained in the ER unless S protein is also present. Previous studies in a number of expression systems have demonstrated that over-expression of the L protein inhibits the release of subviral particles or virions in a dose-dependent fashion (Bruss & Ganem, 1991). Likewise, the L protein also inhibits M and S production and secretion (Chisari *et al.,* 1986; Chow *et al.,*

1997; Molnar-Kimber *et al.,* 1988; Persing *et al.,* 1986; Standring *et al.,* 1986). This phenomenon has led to the hypothesis that one function of the L protein in virion assembly might be to generate patches of HBV surface proteins retained in the ER membrane by forming transmembrane aggregates with M and S proteins. It has been suggested that these patches could become sites for viral budding (Bruss & Ganem, 1991).

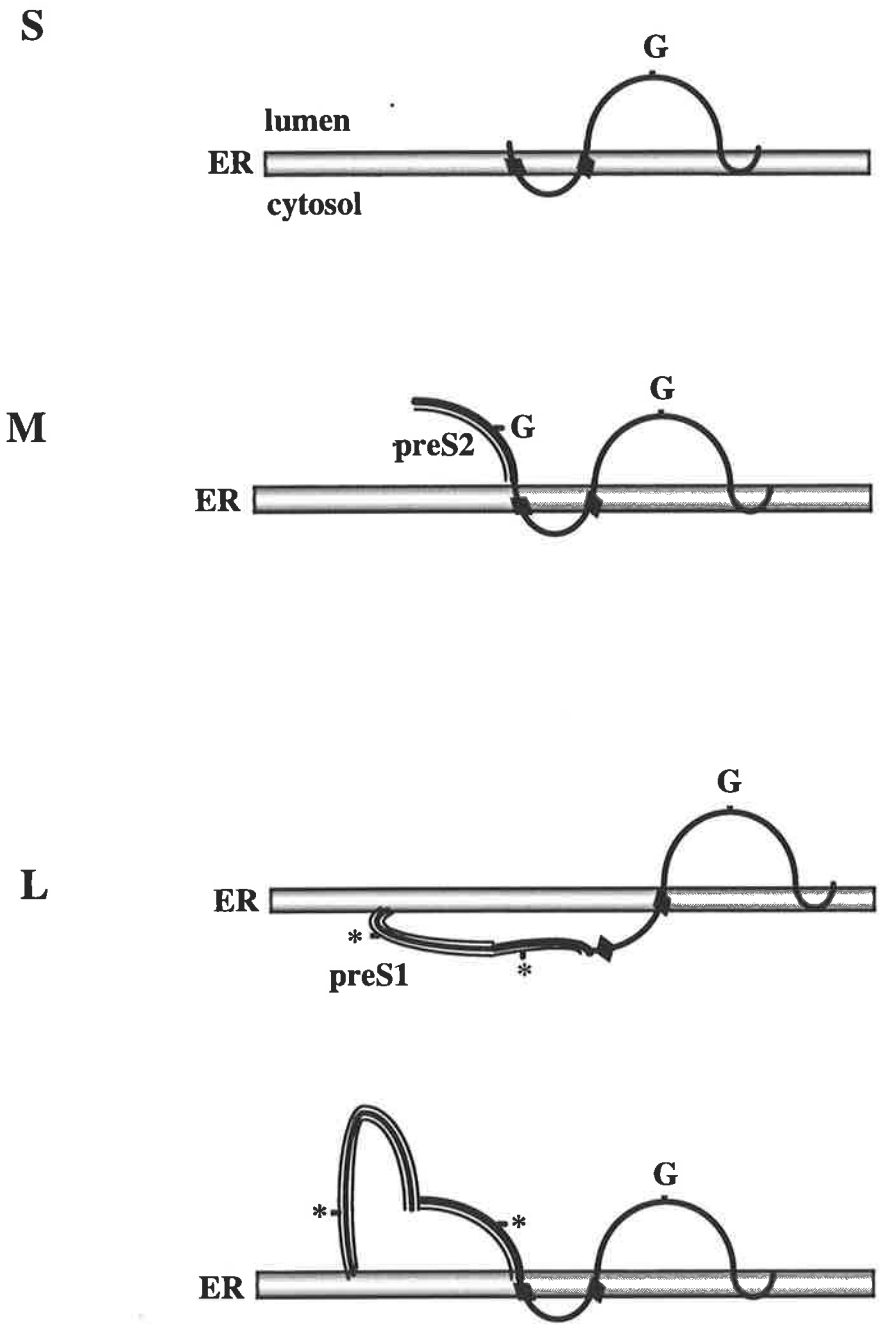
1.4.1. Membrane topology of the HBV surface proteins.

All hepadnaviral surface proteins are integral membrane proteins, they are initially targeted to the ER membrane and are subsequently exposed on the luminal side (Fig. 1.5). The N terminus of S protein contains signals that direct the protein to traverse the ER membrane and to form a cytosolic loop as well as a luminal domain (Bruss & Vieluf, 1995). The luminal domain contains the major antigenic epitopes of HBsAg and an N-glycosylation site. The C terminus of S protein is very hydrophobic and is embedded in the lipid bilayer. The M protein has a similar transmembrane topology to the S protein and exposes the pre-S2 domain in the ER lumen. In contrast to the L protein (see below), the transmembrane topology of the S and M proteins is retained during virus assembly, thereby exposing the pre-S2 domain of M and the major epitopes of the S protein on the surface of viral particles (Bruss & Vieluf, 1995).

The L protein displays an unique transmembrane topology; it is initially synthesized so that the pre-S1 domain has a cytoplasmic disposition. Subsequently, in a fraction of the L protein molecules, the pre-S1 domain is post-translationally translocated across the membrane (Bruss & Vieluf, 1995; Nassal, 1996). Although there are three potential

Figure 1.5. Transmembrane topology of the HBV surface proteins.

The S protein traverses the membrane twice and exposes the hydrophilic domain on the luminal side. This domain carries the major HBsAg antigenic epitopes and a glycosylation site (G). The M protein adopts a similar topology to the S protein and has an additional glycosylation site (G) at the N-terminal of the pre-S2 domain. The L protein is initially synthesized with cytoplasmic disposition of the pre-S1 domain (*top*), and is then post-translationally translocated across the membrane (*below*). * denotes the potential glycosylation sites in the pre-S1 and pre-S2 domains of L protein (neither of which are used). Adapted from Bruss & Vieluf (1995).


S
G
lumen
ER
cytosol
M
G
preS2
G
ER
L
G
ER
*
preS1
*
*
*
G
ER
virion

glycosylation sites, the L protein is only glycosylated at its S site; neither the pre-S2 nor pre-S1 specific sites are used.

1.4.2. The roles of HBV surface proteins.

The fact that approximately 20 times more L protein is found in virions than in the empty subviral particles (Heermann *et al.*, 1984), may be related to the essential roles of this protein, especially its pre-S1 domain, in the viral life cycle. These include (i) virus attachment to the target cell, (ii) formation of complete virions, i.e. envelopment of the nucleocapsid, and (iii) viral release from the cell. The viral attachment site for HBV has been shown to reside within AA 21-47 of the pre-S1 domain (Neurath *et al.,* 1986). The latter two functions require the pre-S1 domain to be located once in the interior and once on the exterior of the virion, respectively, which probably explains why the L protein has the unique topology as described above. In comparison to HBV, a recent study has revealed that both external and internal forms of the L protein of DHBV are found in mature viral particles (Swameye & Schaller, 1997) (see also Section 3.3.7).

While the M protein has no major role in viral morphogenesis or infectivity, the S protein contains the major antigenic epitope of HBsAg, including the so-called *a* determinant, to which neutralizing antibodies are raised that are able to confer immunity against HBV infection (Waters *et al*, 1987a; Waters *et al.,* 1987b) (see also Section 1.5.3). During infection, the presence of a vast excess of subviral particles, which consist mainly of the S protein, presumably act as a decoy and bind neutralizing antibodies, thereby facilitating the progression of viral infection.

1.5. IMMUNE RESPONSES TO HBV INFECTION.

1.5.1. Cell-mediated immune responses.

It has been known that HBV is a non-cytopathic virus. A classic observation that supports this notion is that full expression of HBV proteins and the release of virions may occur without biochemical evidence of liver disease. For instance, in a typical case of acute HBV infection, HBsAg appears 2-6 weeks before the onset of symptoms or the elevation of serum alanine (ALT) and aspartate (AST) aminotransferases which are indicative of hepatocellular damage (Fig. 1.6; Scheuer *et al.,* 1996). Therefore, the varying degrees of histopathological change seen in the livers of HBV-infected patients are believed to be due to lysis of virus-infected hepatocytes e.g. by cytotoxic T cells (CTLs). HBV-specific CTLs that bind to HBcAg and HBeAg displayed on the cell surface in association with the major histocompatibility complex (MHC) class I molecules play a major role in this process (Bertoletti *et al.*, 1991). Antigen-specific CTLs may also suppress HBV gene expression without liver cell damage, through cytokines including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) that are produced by these cells in response to antigen stimulation (Guidotti *et al.*, 1996). The dual mechanisms of CTL cytotoxicity (requiring cell-to-cell contact) and the anti-viral effect of cytokines (especially IFN-γ and TNF-α) may act synergistically to ensure viral clearance. This phenomenon might also operate in ducks and woodchucks infected with their respective hepadnaviruses, which resolve acute hepatitis without evidence of massive necrosis of hepatocytes (Jilbert *et al.*, 1992; Kajino *et al.*, 1994).

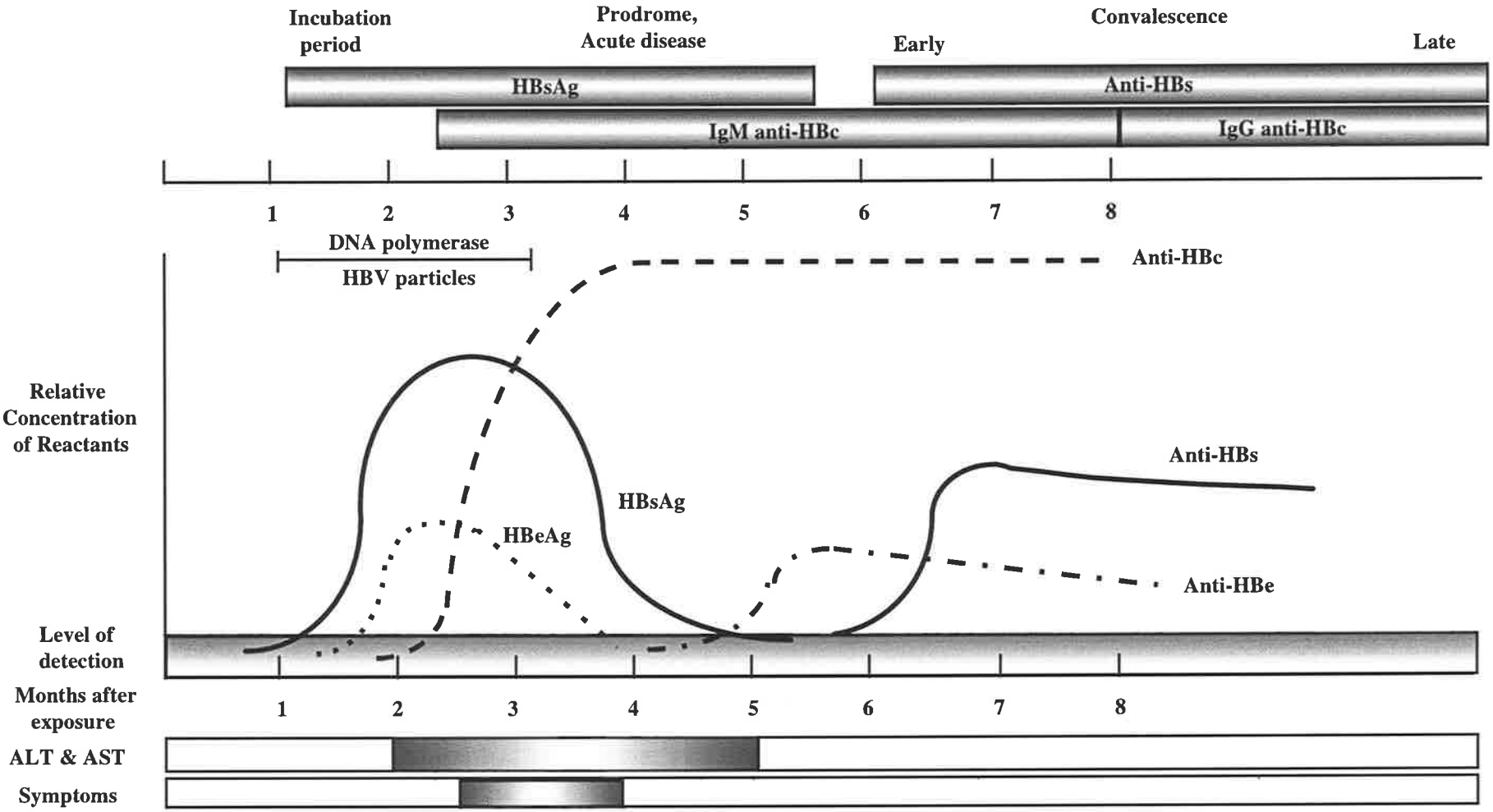


Figure 1.6. Serological and clinical patterns observed during acute HBV infection. Adapted from Hollinger (1990).

1.5.2. Humoral immune responses against HBsAg.

The S protein is highly immunogenic and contains several antigenic epitopes. Antibodies produced against these epitopes are called anti-HBs. Antibodies are also elicited against epitopes located within the pre-S2 and pre-S1 domains (anti-pre-S2 and anti-pre-S1 antibodies, respectively). S protein carries a group-specific determinant, *a*, common to all HBV subtypes and two additional subtypic determinants, *d* or *y* and *w* or *r* (LeBouvier, 1971; Bancroft *et al.*, 1972). Therefore, the major HBV subtypes are *adw, ayw, adr* or *ayr*. Based on indirect evidence from structural-prediction work (Stirk *et al.,* 1992) and from experiments using linear and cyclical peptides (Brown *et al.,* 1984), the *a* determinant is thought to consist mainly of conformationally dependent epitopes. In all serotypes of HBV, the *a* determinant is located within domains bordered by AA 120-147 which forms a double-loop structure (Ashton-Rickardt & Murray, 1989). Some of the anti-HBs antibodies are specific for a certain subtype, but anti-HBs/*a* are the most prominent antibodies in convalescent sera or sera from vaccinees (Waters *et al.,* 1987a; Waters *et al.,* 1987b).

The presence of HBsAg in serum is a classical indicator of ongoing infection (either transient or persistent). Disappearance of HBsAg and appearance of anti-HBs antibodies indicate resolution of infection and development of immunity to reinfection. In general, the presence of HBsAg and anti-HBs antibodies in a patient's serum are mutually exclusive i.e. anti-HBs antibodies are detected in serum after the disappearance of HBsAg. Nevertheless, the concurrence of both markers is not a rare serological event. It may be found in up to 25% of patients with either acute or chronic hepatitis, and more commonly in chronic active hepatitis (Shiels *et al.*, 1987). The co-detection of HBsAg

and anti-HBs antibodies in the same serum sample could be due to the utilization of highly sensitive radioimmunoassays which may shorten or eliminate the serologic 'window period' that usually separates the disappearance of HBsAg and the appearance of anti-HBs. Other circumstances of concurrence may include: (i) the presence of HBsAg-anti-HBs immune complexes when neither species is in large excess, and (ii) the commercial anti-HBs antibody test may detect a variety of subtypic antibodies (anti-*y*, -*d* and -*w*) in addition to anti-*a*. In most cases of concurrence, HBsAg detected was of one subtype while anti-HBs antibodies were exclusively specific for HBsAg determinant of an alternate subtype (Shiels *et al.*, 1987).

1.5.3. Humoral immune responses against other viral antigens.

During hepadnavirus infection, humoral immune responses are seen against individual viral antigens, viz: surface (pre-S1, pre-S2 and S domains), nucleocapsid (HBcAg) and e (HBeAg) antigens. Free HBcAg has not been detected in serum during HBV infection, although the corresponding anti-HBc antibody response is an important marker of HBV infection. IgM anti-HBc antibodies appear early in the course of HBV infection and decline within approximately 6 months. IgG anti-HBc antibodies then become the predominant class of antibodies which may remain detectable for long periods. HBeAg is detected both as membrane-bound antigen and in soluble form in the serum (Ou *et al.*, 1986; Schlicht & Schaller, 1989). Soluble HBeAg is a 'classical' marker of active viral replication and is associated with higher levels of infectivity. In chronic HBV infection, HBeAg tends to persist for many months or even years. Disappearance of HBeAg and appearance of anti-HBe antibodies indicates that the infection has progressed to a phase with less viral replication and less hepatocyte damage. Neither anti-HBc nor anti-HBe

antibodies are regarded as neutralizing antibodies since both antibodies can be detected in the presence of viremia. Furthermore, it has been shown that passive immunization of chimpanzees with monoclonal anti-HBc and anti-HBe antibodies did not offer protection against HBV infection (Pignatelli *et al.,* 1987).

1.6. CHRONIC HBV INFECTION AND NEONATAL TOLERANCE.

1.6.1. Chronic HBV infection.

Chronic HBV infection is defined as the persistence of HBsAg in the serum for more than 6 months (Hoofnagle *et al.*, 1987). Chronically infected individuals may show relatively normal liver histology, chronic persistent hepatitis (CPH) with little hepatocyte damage, or chronic active hepatitis (CAH) with alternating periods of remission and exacerbation of inflammatory liver disease (Hoofnagle *et al.*, 1987).

The course of chronic HBV infection following neonatal exposure can be divided into three successive phases (Chen, 1993). The first is the immune tolerance phase that occurs during early childhood. Despite the presence of continuing active viral replication indicated by high HBV DNA and HBeAg levels during this phase, clinical symptoms are not apparent and liver histology only shows mild and non-specific abnormalities. The second is the viral clearance phase which occurs during adolescent or adult life. The earlier immune tolerance no longer exists, possibly because the level of HBV replication declines with time and allows the emergence from the thymus of HBV-antigen specific T lymphocytes. These T cells mount specific responses to viral antigens, especially to HBcAg and HBeAg. This period is characterized by clinical symptoms of hepatitis, and

elevation of serum ALT. Liver histology may range from CPH to CAH and to cirrhosis. After development of anti-HBe antibodies, if cirrhosis does not supervene, the liver histology may recover with only minimal histologic abnormalities. The final stage is the viral DNA integration phase. At this stage, active HBV replication ceases, although HBsAg is continuously produced by liver cells that contain integrated HBV genomes, as seen in patients with HCC (Chen *et al.,* 1982). It remains unclear when the HBV DNA starts to integrate into the host chromosomal DNA.

The inverse relationship between the rate of developing chronic infection and the age of host at the time of infection is believed to reflect the progressive maturation of the immune system with age. The inability of individuals infected as neonates to clear viral infection was suggested to be due to neonatal tolerance (Lok, 1992; Milich *et al.*, 1993). Nevertheless, >90% of vaccinated infants born to HBV-carrier mothers develop neutralizing anti-HBs antibody responses, demonstrating that infants can develop humoral responses to HBsAg under certain circumstances. Therefore, inability to clear infection acquired at a young age may be due to tolerance to the viral antigens that are involved in inducing cell-mediated immune clearance of infection. The rarity of intrauterine infection suggests that intact virions do not traverse the placenta and induce immune tolerance, but HBeAg from HBV-carrier mothers could gain access to the foetal thymus through the circulation.

1.6.2. The role of HBeAg as an 'immunotolerant' antigen.

The possibility that HBeAg induces immune tolerance to both itself and HBcAg has been suggested as a mechanism for impaired viral clearance following neonatal infection.

Support for this mechanism came from observations that HBeAg is a secreted protein and it has been detected in umbilical cord blood of 88% of infants born to HBeAg-positive mothers. Together with HBcAg, HBeAg can induce very effective cell-mediated immune responses. Both antigens share 160 AA homology, a part of which has been mapped as CTL epitopes (Milich *et al.*, 1990). However, HBcAg and HBeAg elicit distinct antibody responses (Milich *et al.,* 1997).

In contrast to HBcAg, HBeAg is not essential for viral replication and is not part of the virion (Chang *et al.,* 1987; Schlicht *et al.,* 1987b). However, HBeAg is highly conserved among HBV isolates. It has been suggested that, by crossing the placenta, HBeAg induces immunotolerance through clonal deletion of MHC class II, HBeAg-specific T helper (Th) cells, in the fetal thymus, resulting in a state of non-responsiveness or anergy (Milich *et al.*, 1990). The fetal T cells might be anergized by vast amounts of maternal HBeAg present in the circulation, rendering them tolerant to subsequent virus infection occurring during the perinatal or postnatal period (Milich *et al.,* 1993). This hypothesis was proposed from studies with HBeAg-expressing transgenic mice, whose T cells, but not B cells, were shown to be tolerant to HBcAg and HBeAg. HBcAg/HBeAg-specific T cell tolerance was reversible following regression of the thymus and only persisted for 12-16 weeks in transgenic mice, after which the host could develop CTL responses. Transgenic mice could produce anti-HBc, but not anti-HBe antibodies following vaccination with the respective antigens, supporting the notion that HBcAg can act as a T cell-independent antigen, whilst HBeAg is a strictly T-cell dependent antigen (Milich & McLachlan, 1986).

The results above mimic the phenomenon seen in non-vaccinated neonates born to HBV-carrier mothers: the majority of infants do become asymptomatic chronic carriers with minimum liver damage, and develop anti-HBc, but not anti-HBe or anti-HBs antibodies (Milich *et al.,* 1997). In contrast, neonatal infection with an HBeAg-negative mutant of HBV can result in fulminant rather than chronic hepatitis (Terazawa *et al.,* 1991).

Nonetheless, the concept of thymic education to discriminate between 'self' and 'non-self' in the fetal or early neonatal periods might not be the only mechanism responsible for neonatal tolerance against HBV or other viral infections (Matzinger, 1994; Ridge *et al.,* 1996).

1.6.3. Neonatal tolerance.

The established paradigm of neonatal tolerance, i.e. that antigenic challenge in the neonatal period commonly results in immune tolerance rather than immune activation raised two main mechanisms: (i) a 'passive' model which suggests that neonatal tolerance occurs by negative selection, i.e. the clonal deletion of antigen specific Th cells in the thymus, and (ii) an 'active' model which suggests that the newborn T cells generate predominantly 'biased' T helper cell type 2 (Th_2) immune responses which suppress CMI responses (Ridge *et al.,* 1996). The latter model is supported by studies in newborn mice that preferentially developed immune responses of the Th_2 phenotype (which produce interleukin-4 (IL-4), IL-5, and IL-10, and promote humoral immunity) than of Th_1 phenotype (which produce IL-2, IFN-γ, and TNF-α and promote cell-mediated immunity) (Barrios *et al.,* 1996). It is possible that, once established, a Th_2 bias may persist later in life in spite of the maturation of the immune system and persistence of HBV infection

after neonatal exposure may reflect such an outcome (Barrios *et al.,* 1996). As a consequence of developing immune responses of the Th_2 phenotype, the mechanisms responsible for the destruction of infected cells and the clearance of intracellular pathogens have been shown to be significantly impaired.

Recent studies, however, demonstrated that the immune system of neonates can respond to vaccination, can be made tolerant, or be switched to Th_1 or Th_2 type responses depending on the antigen dose, the type of adjuvant used, and the form of antigen presentation (Ridge *et al.,* 1996; Sarzotti *et al.,* 1996; Forsthuber *et al.,* 1996). Taken together, these studies provided new insights about antigen recognition by the immune system of the newborn. First, neonatal T cells could either be activated if the antigens were presented by professional antigen presenting cells (APC) such as dendritic cells, that constitutively release 'co-stimulatory' signals necessary for T cell activation, or be tolerized if the antigens were associated with B cells which were less effective in providing such signals. Second, switching to Th_2 type responses following vaccination was favoured by high doses of antigen, as well as the presence of incomplete Freund's adjuvant (IFA), whereas low doses of antigen or antigen in complete Freund's adjuvant (CFA) would induce Th_1 type responses (Sarzotti *et al.,* 1996; Forsthuber *et al.,* 1996).

The notion that antigen dose might influence the ability of newborns to clear infection was also suggested for perinatal transmission of HBV. An earlier study showed that the outcome of infection was directly related to the quantity of maternal viral DNA to which the infant was exposed: chronic infection was established in only 3.2% of infants born to HBsAg-positive mothers with no detectable HBV DNA in their serum, compared to

97.2% of infants born to mothers with levels >1.4 ng/ml of HBV DNA (Burk *et al.,* 1994).

1.7. HBV IMMUNE-ESCAPE MUTANTS.

HBV and the related animal hepadnaviruses have a mutation rate which is intermediate between DNA and RNA viruses because they replicate through reverse transcription. The rate of emergence of new mutations for HBV has been estimated to be 4.57×10^{-5} substitution/site/year (Orito *et al.,* 1989), and in the case of WHC, the rate is around $<2 \times 10^{-4}$ substitution/site/year (Girones & Miller, 1989). Nevertheless, nucleotide substitutions in chronic HBV infection can accumulate over years or decades, and may eventually result in a significant number of mutations that alter viral infectivity. In this regard, selection pressure would permit the emergence of HBV mutants which might be 'fitter' than the wild-type to survive under host immune surveillance. Indeed, viral evasion of host immune responses by mutation of immunodominant epitopes has been demonstrated in numerous viral infections. This phenomenon has also been demonstrated *in vitro,* by the addition of monoclonal antibodies of restricted specificity to infected cell cultures, or *in vivo,* by inoculation of ducklings with DHBV pre-incubated with monoclonal antibodies (Sunyach *et al.,* 1997). In chronic HBV infection, mutants of both B-cell and CTL epitopes have been identified; it has been suggested that these mutations contribute to viral latency (Okamoto *et al.*, 1990), the severity of liver disease (Omata *et al.*, 1991) and vaccine escape (Carman *et al.*, 1990).

1.7.1. HBeAg-negative mutants.

Point mutations within the pre-C region of HBV that result in novel translational stop codons, produce HBeAg negative-mutants that have been implicated in some cases of fulminant hepatitis (Carman *et al.*, 1991; Liang *et al.*, 1991). In line with the immunotolerogenic function of HBeAg (as mentioned above), the emergence of HBeAg-negative mutants in chronic hepatitis can be seen as the mechanism of virus to escape HBcAg/HBeAg-specific CTL recognition. The link between pre-core (HBeAg)-negative mutants and the severity of liver damage, however, is far from clear since pre-core mutants have not always been seen in cases of fulminant hepatitis (Liang *et al.*, 1994).

1.7.2. HBsAg 'escape' mutants.

'Escape' mutants also occur with mutations in other highly antigenic regions of viral proteins such as the *a* determinant of HBsAg. HBV 'escape' mutants within the *a* determinant have been reported in vaccinated infants of HBV-carrier mothers from various countries (Carman *et al.*, 1990; Okamoto *et al.*, 1992; Harrison *et al.*, 1991). The classic observation showed that the infants had initially produced protective levels of anti-HBs antibodies but then became HBsAg- and HBeAg-positive and developed chronic hepatitis. Genomic sequencing of HBV isolates from these infants, but not their mothers, revealed a G (guanosine) to A (adenosine) mutation at nucleotide position 587 of the S gene, resulting in a glycine (Gly) to arginine (Arg) substitution at AA 145 in the second loop of the *a* determinant of HBsAg. This finding suggested that the mutant might have arisen *de novo* in the infants, or was selected from a minor population in the mother and became predominant in the infants under the selective pressure of HBIG which is given at

birth together with the HBsAg vaccine (Carman *et al.*, 1990). Similar mutations have also been described in liver transplant patients receiving therapy with monoclonal antibodies specific for the *a* determinant (anti-*a* antibodies) (McMahon *et al.*, 1992), in a patient with lymphoma after successful cytotoxic therapy (Carman *et al.*, 1993), and during the course of a natural chronic HBV infection (Yamamoto *et al.*, 1994). Altogether, these phenomenon imply that mutations within the *a* determinant emerge as a result of immune selection directed against the immunodominant epitopes of HBsAg. Although the Gly-145 to Arg-145 mutation is the most commonly reported one in HBV vaccine-escape mutant cases, other mutations within the *a* determinant have also been documented such as Lys-141 to Glu-141 (Karthigesu *et al.*, 1994), and Ile-126 or Thr-126 to Ser-126 or Asn-126, respectively (Yamamoto *et al.*, 1994).

It is conceivable that mutations within the *a* determinant alter the antigenicity of HBsAg and allow the virus to 'escape' from neutralization by anti-HBs antibodies. In relation to diagnostic assays, this is particularly important as some mutants might not be detected using current enzyme immunoassays which are mostly based on anti-*a* monoclonal antibodies. Patients who harbour HBV escape mutants might also be seropositive for both HBsAg and anti-HBs antibodies (Yamamoto *et al.*, 1994). It is possible that HBsAg escape mutants might be present in cases with high levels of HBsAg coincident with high anti-*a* antibodies or in patients seronegative for HBsAg but seropositive for HBeAg and other HBV markers.

1.8. HEPATITIS B VACCINES.

1.8.1. 'Classical' HBsAg vaccines.

The observation that HBsAg elicits neutralizing antibodies during the course of HBV infection, and that infected hepatocytes secret vast numbers of subviral HBsAg particles into blood plasma, has allowed the development of HBsAg subunit vaccines. 'Classical' HBsAg vaccines contain HBsAg either purified from the serum of chronically HBV-infected patients (plasma-derived) or produced by recombinant DNA technology (mammalian cell- and yeast-derived). These vaccines have proved to be highly protective against HBV infection in the 80-95% of vaccinees who develop anti-HBs antibodies at a titer of ≥10 mIU/ml (Hadler *et al.*, 1986). In such individuals (vaccine responders), subsequent infection has occasionally occurred as shown by the appearance of anti-HBc antibodies and an increase in anti-HBs antibody levels, but all such infections have been asymptomatic. These observations are based on long-term, follow-up studies of homosexual men vaccinated with three doses of plasma-derived HBsAg vaccine. After 5 years, the antibody titers usually declined below the protective level, but the need for a booster injection remains questionable (Resti *et al.*, 1997). The efficacy of HBsAg vaccination and simultaneous administration of hepatitis B immunoglobulin (HBIG) within 24 hours of birth has also been shown to reduce the carrier rate from 95% to 5% in perinatal transmission (Beasley *et al.*, 1983; Wong *et al.*, 1984). HBsAg vaccination alone reduced the carrier state to only 21%, implying that HBIG provided immediate protection until the infants could mount their own anti-HBs reponse. This assumption was based on the fact that seroconversion to a protective level of >10 mIU/ml in most of

vaccinated infants was only achieved after the second injection (Wong *et al.*, 1984; André & Zuckerman, 1994).

Vaccination with HBsAg vaccines results in production of anti-HBs antibodies directed predominantly against the group-specific determinant, *a*, as is the case in convalescent patients after resolution of HBV infection (Waters *et al.,* 1987a; Waters *et al.,* 1987b). Since the *a* determinant is common to all HBV subtypes, vaccination with HBsAg confers immunity to all HBV subtypes.

Despite its high efficacy, approximately 5-10% of people vaccinated with the current HBsAg vaccine fail to respond. This has led to the development of newer HBV vaccines containing pre-S1 and pre-S2 antigens in addition to HBsAg. Inclusion of both pre-S antigens has been reported to induce more rapid and higher anti-HBs responses in mice than with HBsAg vaccine alone and to circumvent non-responsiveness (Shouval *et al.,*1994). Similar observations have been reported in humans (Yap *et al.*, 1995). Nonetheless, it remains to be seen whether the enhanced seroconversion rate and the anti-HBs titer induced by this combined vaccine will significantly increase its protective efficacy and duration of immunity when compared to vaccination with HBsAg alone.

1.8.2. DNA vaccines.

Although the current HBsAg vaccines have been shown to be safe and efficacious, other vaccine alternatives which can provide long-lasting immunity and alleviate the need for booster vaccinations are still more desirable. In addition to higher efficacy, other features for newer vaccines such as simplicity of manufacture, the stability without the need of

cold chain, and lowered cost, would provide further advantages. An alternative approach which can theoretically meet the above demands has recently been developed, namely DNA-based vaccines (Whalen *et al.,* 1995).

DNA-based vaccination or DNA vaccination refers to a method of inducing immune response to protein expressed *in vivo* from a gene introduced directly in the form of pure (naked) plasmid DNA (Ulmer *et al.*, 1996). This method offers an alternative not only for classical vaccination against various intracellular pathogens, but also for anti-tumor therapy (Conry *et al.,* 1996) and autoimmune disease (Ulmer *et al.,* 1996). The benefits of DNA vaccination include: (i) the possibility of prolonged gene expression, resulting in sustained antigen presentation to the immune system which could obviate the need for booster injections; (ii) the synthesis of antigen *in vivo* which allows the presentation of antigen on the cell surface in association with MHC class I molecules via the cytosolic antigen presentation pathway, similar to that observed during natural viral infection or in response to live-attenuated vaccines resulting in induction of specific CTL responses; (iii) that DNA vaccines are highly stable, more simple to produce and to purify than recombinant vaccines, and (iv) the ability to design expression vectors with multiple inserted genes thus creating multivalent vaccines. The initial demonstration of efficiency of this approach was shown by a DNA vaccine encoding both the influenza virus nucleoprotein (NP) and surface hemagglutinin (HA). This cocktail vaccine has been shown to effectively induce CTL responses to NP protein and neutralizing antibodies to HA protein in ferrets, which are considered as the model of choice for immunological studies with influenza virus (Donelly *et al.,* 1995).

1.8.2.1. Characteristics of vectors for DNA vaccination.

For safety reasons, the vectors used for DNA vaccines are constructed without an origin of replication (*ori*) that is functional in eukaryotic cells. Hence such plasmids neither replicate in the mammalian host nor integrate within its chromosomal DNA (Donelly *et al.,* 1997). They do, however, share the basic characteristics of vectors developed for *in vitro* expression of genes in transfected cell lines such as: (i) an *ori* suitable for producing high yields of plasmid in *E. coli*; (ii) an antibiotic resistance gene to confer antibiotic-selected growth in *E. coli*, most commonly the ampicillin resistance (amp^r) gene, which was found to have an additional benefit due to the presence of specific CpG motifs in its sequence (see below); (iii) a strong enhancer/promoter and an mRNA transcript termination/polyadenylation sequence for directing expression in mammalian cells. The most frequently used are the human cytomegalovirus (CMV) immediate/early promoter, the Rous sarcoma virus (RSV) LTR, and the SV40 early promoter, in conjunction with the SV40 or bovine growth hormone 3'-untranslated region (BGH 3'-UTR) transcription termination and polyadenylation sequences (Donelly *et al.,* 1997). Other features such as introns, have also been included in a number of vectors, as expression of many mammalian genes may be dependent on, or may be increased by the presence of introns (Barry and Johnston, 1997; Donelly *et al.,* 1997).

1.8.2.2. Administration routes of DNA vaccines.

Skeletal muscle is the most commonly used site for administration of DNA vaccines, although other routes of inoculation such as intradermal, intravenous, intraperitoneal, intranasal, and subcutaneous, have also been explored (Fynan *et al.*, 1993). The mode of

entry of plasmid DNA into the muscle cells following intramuscular (i.m.) injection is suggested to be endocytosis, and the efficiency of DNA uptake is greatly enhanced in regenerating muscle. Normal mature muscle is permanently post-mitotic in the absence of muscle injury, but muscle regeneration can be induced with necrotizing agents such as snake toxins (Davis *et al.*, 1993b) or local anaesthetics such as bupivacaine HCl (Wells, 1993).

DNA delivery can also be improved by the use of a gene gun, in which plasmid DNA is coated onto colloidal gold particles and administered by particle bombardment through the skin, hence increasing the DNA uptake by professional APCs such as cutaneous dendritic cells (Condon *et al.*, 1996). Alternatively, plasmid DNA can be encapsulated in micron-sized particles composed of the biodegradable polymer polylactide-co-glycolide (PLGA) which have previously been used as drug delivery vehicles (Hedley *et al.*, 1998). Due to their size, PLGA particles have been shown to be engulfed by mononuclear phagocytic cells in the liver, lung, spleen, and bone marrow, which can all function as professional APC.

1.8.2.3. Immune responses elicited by DNA vaccines.

Numerous studies have demonstrated that mice vaccinated with DNA encoding HBsAg were able to elicit strong humoral and CMI responses as early as 1-2 weeks after DNA injection (Davis *et al.*, 1993a; Davis *et al.*, 1996a; Whalen *et al.*, 1995). The longevity of antibody responses elicited by these vaccines varied, although a single intramuscular injection of 100 μg DNA in young mice (6-8 weeks old) has been reported to elicit anti-HBs antibody titers of 1,000 mIU/ml which were sustained for 74 weeks (Davis *et al.*,

1996a). The long-term immunity elicited by DNA vaccines could be due to the prolonged survival of episomal DNA in non-dividing muscle cells. The kinetics of antibody responses induced by DNA vaccines show a classic shift from IgM to IgG isotypes, indicating that specific Th cells are involved (Michel *et al.,* 1995). The activation of Th cells requires antigen presentation by MHC class II molecules, which are not normally expressed by muscle cells with the exception of myoblasts (Hohlfeld and Engel, 1994). Therefore, professional APCs such as interstitial dendritic cells in the muscle tissue, or proliferating myoblasts that are present during muscle regeneration, induced by either local anaesthetic or snake toxin, might also present the antigen in the context of MHC class II molecules to the immune system.

The precise mechanism(s) by which DNA vaccines induce immune responses remains unclear. Since most DNA vaccines have been delivered intramuscularly, and to a lesser extent, intradermally, it was initially suggested that transfected muscle cells, or keratinocytes, might be presenting antigen to the immune system. However, muscle cells only express low levels of MHC class I antigens and do not express MHC class II or co-stimulatory molecules (Hohlfeld & Engel, 1994). Hence, it is more likely that resident professional APC such as macrophages or dendritic cells capture the antigen released by myocytes and present it in the context of MHC class I and II molecules.

A hypothetical mechanism was proposed (Fig. 1.7) in which bone marrow-derived cells but not the skeletal muscle cells, might be the important APC in this setting (Robinson, 1997). Professional APC (dendritic cells or Langerhans cells in the skin) may also be directly transfected by plasmid DNA, and the antigen would thereby be presented in the context of appropriate co-stimulation for T cell activation. Indeed, DNA vaccination by

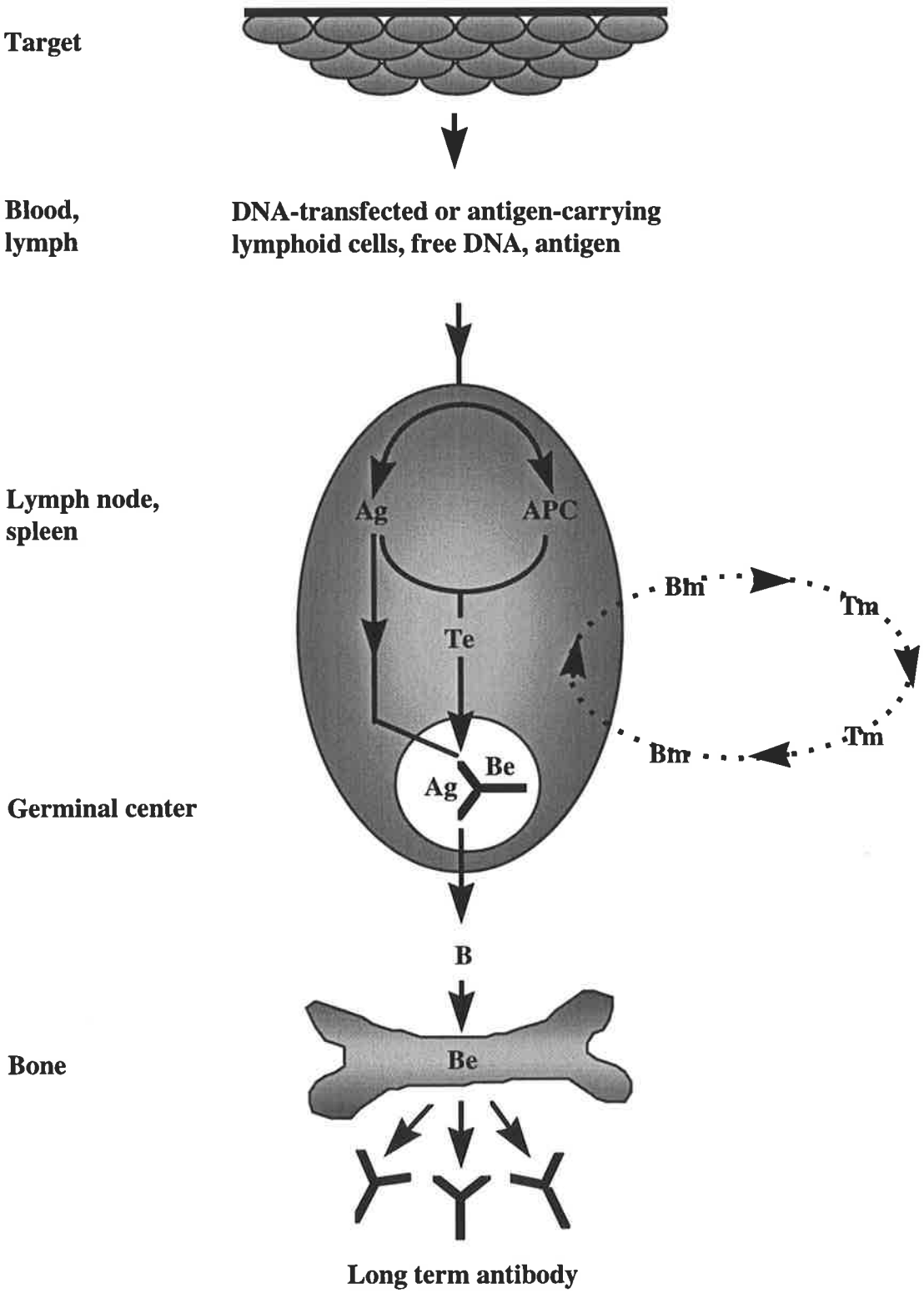


Figure 1.7. Hypothetical mechanism for the induction and maintenance of immune responses by DNA vaccines (as described in text). Skin is used as an example of the target site. *Ag,* antigen; *APC,* antigen presenting cell; *Te,* effector T lymphocytes; *Be,* effector B lymphocytes (plasma cells); *Bm,* memory B lymphocytes; *Tm,* memory T lymphocytes. Adapted from Robinson, H.I. (1997).

the gene gun technique has been demonstrated to directly transfect cutaneous dendritic cells, which then migrate to the draining lymph nodes (Condon *et al.,* 1996). The following course of events would be proposed. The transfected cells and the antigen they expressed would be carried via the bloodstream and lymph to the lymph nodes and spleen where antigen presentation would occur, and effector T and B lymphocytes would be generated from naive T and B lymphocytes. As the antibody responses are generated, germinal centers would form to support the deposition of antigen-antibody complexes on the follicular dendritic cells. The slow release of antigen from these complexes would maintain the B lymphocytes both as recirculating memory cells, and as effector lymphocytes localizing in the bone marrow.

1.8.2.4. Enhancement of the immunogenicity of DNA vaccines.

(i) Adjuvant property of CpG motifs.

Several other parameters are known to modulate the humoral and cellular immune responses to DNA vaccines, such as the adjuvant property of plasmid DNA, and co-expression of cytokines and/or co-stimulatory immune molecules which affect antigen processing by the transfected cells. Although plasmid DNA is commonly regarded as being immunologically inert, enhancement of immune responses by the CpG sequence motifs contained in most plasmid DNA has been reported (Klinman *et al.*, 1996; Roman *et al.,* 1997; Sato *et al.,* 1996). The unmethylated CpG motif (5'-Pur Pur CG Pyr Pyr-3'), termed as immunostimulatory sequence (ISS), present in the ampicillin resistance (*amp*^{r}) gene and the CMV promoter sequence of plasmid DNA, has been shown to exert Th1-type adjuvant effect. This property has been attributed to its unmethylated status (Krieg *et al.,* 1995; Pisetsky, 1997).

CpG motifs occur 20 times more commonly in bacterial than in mammalian DNA for at least two reasons: (i) the CpG suppression phenomenon, where C and G bases are present in tandem much less commonly in mammalian DNA than predicted by base composition; (ii) cytosine in mammalian DNA is commonly methylated at the C5 position (Pisetsky, 1997). Due to its ISS content, bacterial DNA has shown the ability to induce the production of cytokines (IL-12, TNF-α, IL-6, IFN-α/β/γ, and IL-10), polyclonal B cell activation, and stimulation of specific antibody responses. The adjuvant activity of ISS has also been demonstrated when it is co-administered with β-galactosidase (β-gal) protein. This approach markedly augments the production of anti-β-gal antibodies possibly by promoting the differentiation of naive Th cells to Th_1 lymphocytes (Roman *et al.,* 1997).

The immunostimulatory effects of ISS were initially discovered in the mycobacterial genome as DNA sequences that selectively enhanced NK cell activity (Kimura *et al.,*1994). For the same reason, the strong immunostimulatory effect of complete Freund's adjuvant which contains mycobacterial extract as its major constituent, probably relates to the ISS-enriched mycobacterial DNA (Roman *et al.,* 1997). In light of the concept of 'danger signals' as a trigger to induce host immune responses against invading pathogens (Matzinger, 1994), the conserved patterns of the ISS made by microbial pathogens, but not by vertebrate cells, may function as 'danger signals' that elicit immune responses in higher organisms (Pisetsky, 1997).

The adjuvant properties of bacterial DNA could theoretically be exploited for vaccination purposes. For example, vectors with more potent ISS would be used to develop more effective DNA vaccines. Likewise, the addition of ISS oligonucleotides to conventional protein/subunit vaccines may enhance their potency. On a cautionary note, however, the broad range of activities elicited by ISS may also stimulate non-specific immune responses and predispose to autoimmunity (Ulmer *et al.,* 1996).

(ii) Co-expression of DNA vaccines with cytokines or co-stimulatory molecules.

Injection of DNA vaccines co-expressing interleukin-2 (IL-2) and HBsAg have resulted in stronger humoral and CMI responses when compared to those expressing HBsAg alone, with preferential induction of Th_1 subsets in mice (Chow *et al.,* 1997). Other co-stimulatory immune molecules such as B7-1 molecules have also been demonstrated to enhance anti-tumor effects when co-expressed with human carcinoembryogenic antigen (CEA) compared to those generated by plasmid DNA encoding CEA alone (Conry *et al.,* 1996). The enhancement effect of both cytokines and co-stimulatory immune molecules was only achieved if these molecules and the desired antigen were delivered on the same plasmid (Chow *et al.,* 1997; Conry *et al.,* 1996), suggesting that the upregulation of immune response occurs if the antigen and the cytokines are expressed in the same cell.

1.8.2.5. Therapeutic potential of DNA vaccines for chronic HBV infection.

DNA vaccines encoding HBsAg have also been considered as therapeutic alternatives to overcome non-responsiveness to current HBsAg vaccines, and to circumvent immune tolerance in the chronic carrier state. Both applications were indicated by murine model

studies, in which certain mice strains shown to be unresponsive to recombinant HBsAg at the CTL level became responsive after DNA vaccination (Schirmbeck *et al.,* 1995).

The strong induction of Th_1 and CTL immune responses by DNA vaccines is particularly important in infection of newborns and young infants with intracellular pathogens where it is likely that the immaturity of cellular immune responses impairs their ability to clear the infection. In contrast to conventional vaccines that lead to Th_2 polarization in neonates (Barrios *et al.,* 1996), DNA vaccines have been shown to raise Th_1 and CTL responses in young mice, even immediately after birth (Bot *et al.,* 1996; Martinez *et al.,* 1997). The ability of DNA vaccines to induce Th_1 responses in neonates suggests that the immune tolerance observed in infants born to HBV carrier mothers can be circumvented by these vaccines.

In regard to its therapeutic value in patients with chronic HBV infection, DNA vaccines have been demonstrated to downregulate HBsAg expression, and subsequently, to induce anti-HBs antibody responses in HBsAg-expressing transgenic mice without any evidence of immune-mediated hepatic damage (Mancini *et al.,* 1996; Davis *et al.,* 1997a). Given the fact that at present there are ~350 million chronic HBV carriers worldwide without any effective treatment (Kane, 1996), effective therapeutic use of DNA vaccines would indeed be welcome. Nevertheless, in spite of their potent immunogenic properties, DNA vaccines have also been reported to induce tolerance, rather than stimulation of the immune system in neonates (Mor *et al.,* 1996).

1.9. DUCK HEPATITIS B VIRUS INFECTION AS AN ANIMAL MODEL FOR HBV INFECTION.

Suitable animal models are needed to determine the protective and therapeutic efficacy of new DNA vaccines against HBV infection, as the ultimate goals of vaccination are protection against viral challenge, or resolution or clearance of chronic infection. The inability of HBV to infect cultured cells and the narrow host range of this virus have limited experimental studies to higher primates. However, similarities between HBV and DHBV, have allowed the use of DHBV infection of Pekin ducks to study various aspects of hepadnavirus infection.

DHBV is closely related to HBV in regard to genomic organization, hepatotropism and mode of replication (Mason *et al.*, 1980). Likewise, the outcome of DHBV infection also shows age-related effects with acute infection following inoculation of adult ducks, whilst congenitally- and neonatally-infected ducklings will invariably become chronically-infected (Jilbert *et al.,* 1998). In addition, DHBV and its natural host, the domestic Pekin duck (*Anas domesticus)* permit the study of viral neutralization mechanisms both *in vitro* (in primary hepatocyte cultures) and *in vivo* (in neonatal ducklings and adult ducks).

1.9.1. GENOMIC ORGANIZATION AND STRUCTURE OF DHBV.

1.9.1.1. Genomic organization of DHBV.

Detailed information regarding the genomic organization of the DHBV is provided in Chapter 3. In brief, the DHBV genome (3021-3027 bp in different strains) shares only

40-55% nucleotide sequence homology to the HBV genome (Mandart *et al.,* 1984). The DHBV genome contains only 3 ORFs (the P, S, and pre-C/C genes), and lacks the fourth ORF (X gene) present in the HBV genome (Fig. 1.8). As in its HBV counterpart, the DHBV genome produces two classes of RNAs, the subgenomic and a greater than genomic-length transcripts. The 2.1 and 1.8 kb subgenomic transcripts are the mRNAs for the pre-S/S and S proteins, respectively. The greater than genomic-length 3.3 kb transcript serves as the mRNA for the polymerase and core proteins, and also acts as the RNA pregenome (Büscher *et al.,* 1985; Hirsch *et al.,* 1990).

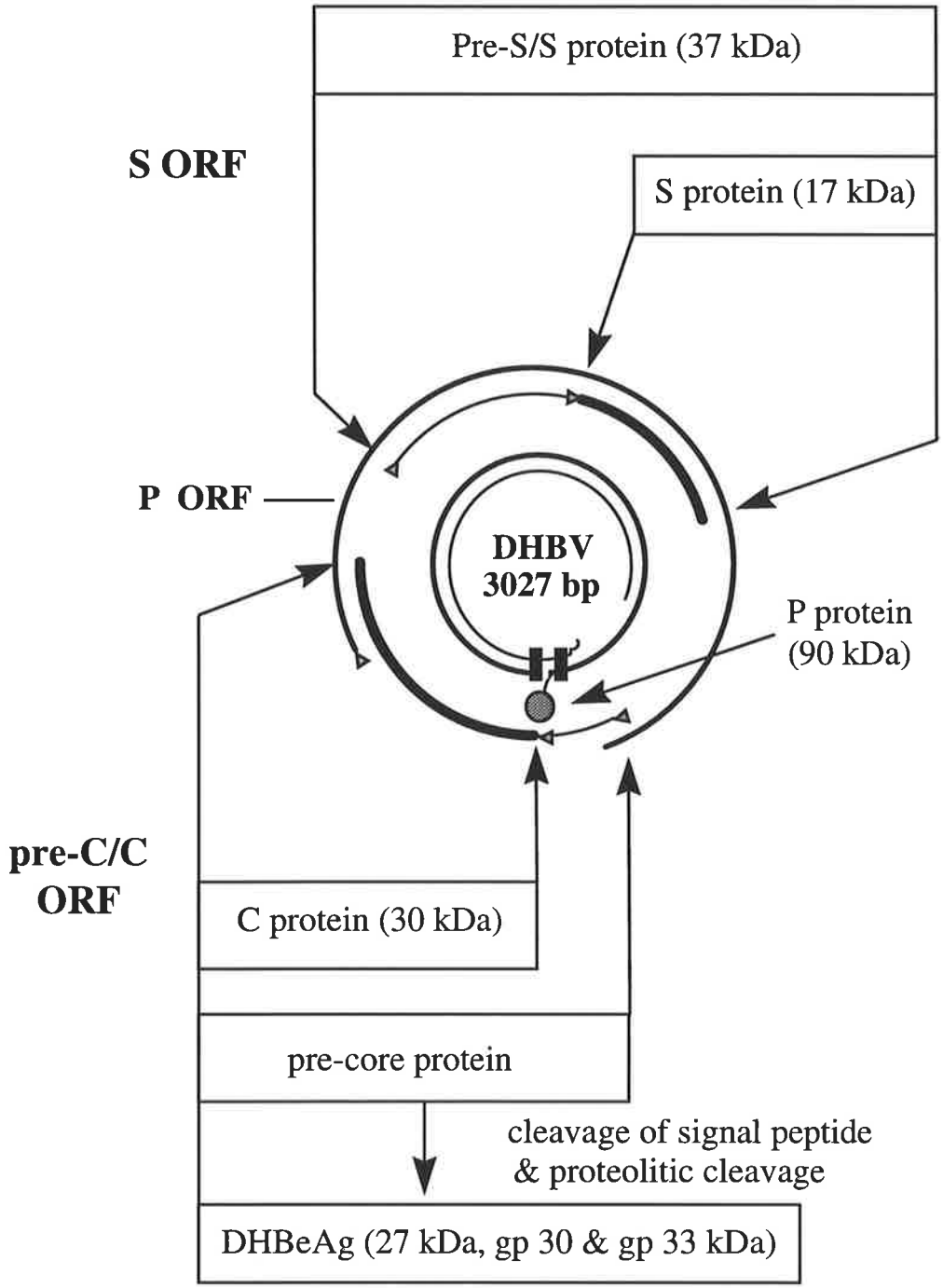
1.9.1.2. Structure of DHBV particles.

The virion of DHBV is pleomorphic with spherical particles of approximately 30-60 nm in diameter (Schödel *et al.*, 1989). DHBV lacks the filamentous forms of DHBsAg found with HBV. The subviral DHBsAg particles are almost indistinguishable from the virions due to their similarity in size (Klingmüller & Schaller, 1993).

1.9.2. OUTCOMES OF DHBV INFECTION.

DHBV has been found in domestic ducks and wild mallard ducks (*Anas domesticus platyrhyncos)* throughout the world; the incidence of chronic infection in individual flocks can range from 0-100% (Mason & Marion, 1994). Infections are primarily acquired *in ovo* from viremic ducks. The virus replicates in the yolk sac tissue of developing duck embryos and is then transferred to the embryonic hepatic tissue where replication is found by day 12 of incubation (O'Connell *et al.,* 1983). The outcome of this vertical transmission resembles that found in infants born to HBV-carrier mothers, where

Figure 1.8. Genomic structure of DHBV. *Inner circles* represent the partially double-stranded DNA genome found in the virion. *Black boxes* represent DR1 and DR2 sequences, the hatched grey circle is the P protein which is covalently attached to the 5' end of negative strand DNA. *Wavy line* is the RNA primer at the 5' end of the positive DNA strand. *Outer lines* represent the open reading frames (ORFs), with their 5' to 3' direction indicated by arrowheads. The P ORF covers ~80% of the entire genome and overlaps with the S and the pre-C/C ORFs. The S ORF has two start codons in the pre-S and the S region (thin and thick lines, respectively). Likewise, the pre-C/C ORF has two start codons, the pre-C and C, represented by the thin and the thick lines, respectively. DHBeAg is produced as both glycosylated (*gp*), and non-glycosylated forms.


Pre-S/S protein (37 kDa)
S ORF
S protein (17 kDa)
P ORF
DHBV
3027 bp
P protein
(90 kDa)
pre-C/C
ORF
C protein (30 kDa)
pre-core protein
cleavage of signal peptide
& proteolitic cleavage
DHBeAg (27 kDa, gp 30 & gp 33 kDa)

ducklings from DHBV-positive ducks invariably become chronically-infected, probably for their lifetime. The majority of hepatocytes are infected (>95%) and produce infectious virus which is released into the bloodstream causing high level persistent viremia (Jilbert *et al.,* 1992). Natural horizontal transmission has not been proved so far (Schödel *et al.*, 1989), although it may occur through open wounds, and would be expected to resolve in older ducks as a transient infection with subsequent immunity. DHBV is not known to be infectious to mammals.

DHBV can be experimentally transmitted by intravenous or intraperitoneal infection of DHBV-infected serum (Jilbert *et al.,* 1988), or by intrahepatic or intravenous injection of cloned viral DNA into 1-day-old ducklings (Sprengel *et al.*, 1984; Tagawa *et al.,* 1996). The susceptibility to DHBV infection in neonatal ducklings might also be related to the active liver cell division found at this young age, suggesting cell-to-cell spread of infection (Jilbert *et al.,* 1988). This notion was based on observations made following a partial hepatectomy, a condition which mimics the active cell division phase, where DHBV replication was markedly increased (Qiao *et al.,* 1992). The outcome of DHBV infection is also dose-dependent: inoculation of 14-day-old ducks with various doses resulted in transient or persistent infection, depending on the size of inoculum given (Jilbert *et al.,* 1998) (see also Chapter 6).

It is thought that congenitally DHBV-infected ducks fail to clear viral infection due to immunological tolerance as a consequence of early exposure to the viral antigens (Ridge *et al.,* 1996), a response which is also believed to occur in infants born to HBV-carrier mothers. The virus accumulates in the yolk sac, liver, and pancreas of the developing embryo, and there is no evidence of an immune-mediated liver disease in these ducks

(Marion *et al.,* 1984). The lack of significant cytopathology has also been observed in one-day-old ducks experimentally-infected with DHBV, despite persistent infection (Jilbert *et al.,* 1988). In adolescent ducks, DHBV infection of up to 80% of their hepatocytes was only accompanied by a mild hepatitis, and was followed by complete resolution of infection without evidence of massive cell death (Jilbert *et al.,* 1992; Jilbert *et al.,* 1998).

1.10. AIMS OF THESIS.

The project aimed to compare the protective efficacy of DNA vaccines encoding the DHBV surface proteins, that of with yeast-derived DHBsAg vaccines, against DHBV infection in both adult and neonatal ducks. DNA vaccines were also tested for their potential as therapeutic vaccines for chronic hepadnavirus infection. To achieve the above aims, a fully-characterized DHBV strain was required, which led to the isolation and cloning of the genome of an Australian DHBV strain (AusDHBV).

Detailed aims included:

(i) To isolate and clone the genome of AusDHBV (from a pool of congenitally-DHBV infected duck serum) into a suitable vector, to allow characterization of its nucleotides and amino acid sequence, and its comparison to other published DHBV isolates.

(ii) To subclone the pre-S/S and S genes of AusDHBV into a suitable yeast expression vector, and subsequently, to produce and to purify the yeast-derived DHBV pre-S/S and S proteins for vaccination and serological assays.

(iii) To determine the immune responses elicited by yeast-derived DHBsAg and to assess their protective efficacy against virus challenge.

(iv) To subclone the pre-S/S and S genes of AusDHBV into a suitable eukaryotic expression vector for use as DNA vaccines.

(v) To determine the protective efficacy of DNA vaccines against viral challenge.

(vi) To further analyze whether the antiserum from DNA-vaccinated ducks could confer protection against virus infection in both *in vivo* and *in vitro* systems.

(vii) To study whether DNA vaccines were able to: (i) protect newly hatched ducklings from becoming chronically-infected with DHBV, (ii) modulate the immune response to clear virus infection in chronically-infected ducklings, or (iii) break the immune tolerance state in congenitally DHBV-infected ducklings.

Chapter 2

Materials and Methods

2.1. TRANSFORMATION OF *Escherichia coli.*

2.1.1. Bacterial strains and preparation of competent cells.

Two bacterial strains were used in this study, *Escherichia coli* (*E. coli*) DH5α (Sambrook *et al.,* 1989) and *E. coli* TOP10F' (Invitrogen, San Diego, Calif.). Competent cells for bacterial transformation were prepared using a $CaCl_2$ method (Sambrook *et al.*, 1989). A single colony of *E. coli* DH5α or *E. coli* TOP10F' from a Luria-Bertani (LB) agar plate (Appendix) containing the appropriate antibiotics for each strain (100 μg/ml Ampicillin, or 60 μg/ml Ampicillin and 15 μg/ml Tetracycline, respectively), was inoculated into 100 ml of LB broth and grown at 37°C for 4 hour (hr) with agitation. Bacterial culture was transferred to a 50 ml polypropylene tube and cooled on ice for 10 minutes (min). Cells were harvested by centrifugation at 3,500 rpm (Beckman GPR) for 10 min at 4°C, the supernatant was decanted and the tubes were inverted for 1 min to drain any remaining supernatant. The cell pellet was resuspended in 10 ml of ice-cold 100 mM $CaCl_2$ and stored on ice for 20 min, followed by centrifugation at 3,500 rpm (Beckman GPR) for 10 min at 4°C, and the supernatant was decanted as above. Bacterial cells were resuspended in 2 ml of ice-cold 100 mM $CaCl_2$ for each 50 ml of the original culture, then stored at 4°C for 12-24 hr to increase the transformation efficiency.

Frozen stocks of competent cells were prepared as follows: 140 μl of DMSO was added per 4 ml of resuspended cells, mixed gently by swirling and stored on ice for 15 min. An additional 140 μl of DMSO was added to the cell suspension, mixed gently and held on

ice. Aliquots of 200 µl of cells were dispensed quickly into sterile, chilled Eppendorf tubes that were snap-frozen by immersing the tubes in dry ice and then stored at -70°C.

2.1.2. Transformation of competent cells.

(i) Heat-shock method.

100-500 ng of recombinant plasmid DNA, in a volume of <20 µl, was mixed gently with 200 µl of competent *E. coli* DH5α, or TOP 10F' cells and incubated on ice for 20 min. The cells were heat-shocked for 90 seconds (sec) at 42°C and immediately chilled on ice. Four hundred microlitres of SOC medium (Appendix) was then added and cells were incubated at 37°C with agitation for 20 min. The cells were spread on LB agar plates containing the appropriate antibiotics and incubated in an inverted position at 37°C for 24 hr.

(ii) Electroporation method.

This method (O'Callaghan, 1990) was performed on a BioRad Gene Pulser apparatus, and was the preferred method for transformation of *E. coli* DH5α with yeast plasmid following plasmid DNA extraction from yeast cells. Cells were grown in 100 ml LB broth (Appendix) to A_{600} of 0.6, then chilled on ice and harvested by centrifugation at 1,000 x g for 15 min at 4°C. The cell pellet was washed twice with 100 ml ice-cold distilled water (DW), once with 20 ml of ice-cold 10% glycerol in DW, and finally resuspended in 200 µl of 10% glycerol. Plasmid DNA was desalted on a 0.025 µm membrane (Millipore) floating on the surface of DW for 1 hr. A 40 µl aliquot of cells

was mixed with 1-2 µl of DNA in a chilled Eppendorf tube and transferred to a chilled cuvette (0.2 cm electrode gap). A single pulse of 12.5 kV/cm (2.5 kV, 200 Ω, 25 µF) was applied to the cuvette, and 1 ml of pre-warmed SOC medium was immediately added. The bacteria were transferred to an appropriate tube and grown for 1 hr at 37°C before plating onto LB agar containing 100 µg/ml Ampicillin.

2.1.3. Mini preparation (small-scale) of plasmid DNA.

Small-scale preparation of plasmid DNA was carried out using the alkaline lysis method (Sambrook *et al*, 1989). A single bacterial colony was grown overnight in 2 ml of LB broth containing the appropriate antibiotics at 37°C, with agitation. One and half ml of overnight culture was then transferred to a sterile Eppendorf tube and pelleted at 12,000 rpm for 2 min at room temperature (RT). The supernatant was aspirated, and the cell pellet was resuspended in 100 µl (ice-cold) Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) by vigorous vortex. Cells were then lysed by the addition of 200 µl freshly-prepared Solution II (0.2 N NaOH, 1% SDS) and invertion of the tubes several times. 150 µl (ice-cold) of Solution III (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml DW) was added and the mixture was kept on ice for 5 min. Cell debris and chromosomal DNA was removed by centrifugation at 12,000 rpm for 5 min at RT, and plasmid DNA in the supernatant was phenol:chloroform extracted and ethanol precipitated. DNA was redissolved in 50 µl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE) buffer containing 20 µg/ml DNase-free RNase A (Boehringer Mannheim) and stored at -20°C. Five microlitres of each DNA sample was used for restriction enzyme analysis to identify the correct clones.

2.2. STANDARD PROTOCOLS FOR DNA HANDLING.

2.2.1. Phenol:chloroform extraction and ethanol precipitation.

Crystallized phenol (BDH) was melted at 65°C then 8-hydroxyquinoline (BDH) was added to a final concentration of 0.1%. Phenol was equilibrated with an equal volume of 0.5 M Tris base buffer followed by repeated washes in 50 mM Tris-HCl pH 8.0 until the pH of the aqueous phase was 8.0. The equilibrated phenol was stored in 50 mM Tris-HCl pH 8.0 buffer. Chloroform:isoamyl alcohol was prepared by mixing 24 volumes of chloroform (BDH) and 1 volume of isoamyl alcohol (BDH). Phenol:chloroform:isoamyl alcohol (25:24:1) was a mixture of an equal volume of equilibrated phenol and chloroform:isoamyl alcohol. All preparations were stored at 4°C.

DNA solutions were extracted with an equal volume of phenol:chloroform:isoamyl alcohol. After centrifugation at 12,000 rpm for 5 min at RT, the upper aqueous phase was transferred to a sterile tube and extracted with an equal volume of chloroform:isoamyl alcohol and centrifuged at 12,000 rpm for 5 min at RT. The upper phase containing DNA was removed and the DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol at -20°C, overnight. DNA was pelleted by centrifugation at 12,000 rpm at 4°C for 15 min, washed three times with chilled (-20°C) 70% ethanol, dried in a Speed Vac (Savant) for 10-15 min, and finally redissolved in the required volume of DW or TE buffer.

2.2.2. DNA modifying enzymes.

(i) Restriction enzymes.

In a 20 µl reaction, 0.2-1 µg of DNA was digested with 2-4 units of the appropriate restriction enzymes (RE) (Pharmacia) in 1 x One-Phor-All Buffer PLUS [10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate (Pharmacia)] at 37°C, for ≥2 hr. The samples were analyzed by electrophoresis on a 1.2% agarose gel (Section 2.2.3) using *Pst* I-digested λ DNA (Appendix) as the MW marker to identify the restriction patterns of the DNA samples.

(ii) Alkaline phosphatase.

Prior to the cloning of a specific gene into plasmid vectors, 5'-phosphate groups were removed from linearized plasmid DNA to prevent self ligation. Plasmid DNA was digested with the appropriate RE, phenol:chloroform extracted, ethanol precipitated, and dissolved in 90 µl of 10 mM Tris-HCl pH 8.0. Dephosphorylation was then performed by addition of 10 µl of 1 x One-Phor-All-Buffer PLUS, 1 unit of alkaline phosphatase (calf intestinal mucosa; Pharmacia), and incubation at 37°C for 30 min. At the end of the reaction, a final concentration of 100 µg/ml proteinase K (Boehringer Mannheim), 5 mM EDTA and 0.5% SDS were added, followed by incubation at 56°C for 30 min. DNA was then phenol:chloroform extracted and ethanol precipitated with the addition of 0.1 volume of 3 M sodium acetate pH 7.0 (to avoid co-precipitation of 5 mM EDTA at pH 5.2) (Sambrook *et al.,* 1989) and 2.5 volumes of 100% ethanol.

(iii) DNA ligase.

Vector and insert DNA were ligated at 12°C for >16 hr in a 15 µl reaction mixture containing 1 unit of bacteriophage T4 DNA ligase (Boehringer Mannheim), 1 x ligase buffer (20 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 50 µg/ml BSA), and 5 mM ATP. A total of 100-500 ng DNA, with vector:insert molar ratio of 1:5 or 1:10, was added to the reaction. The ligation reaction was used to transform competent *E. coli* DH5α [for the full-length genome of an Australian DHBV (AusDHBV) isolate cloned in pBluescript IIKS+], or *E. coli* TOP10F' (for the pre-S/S and S genes of AusDHBV cloned in pcDNA I/Amp).

2.2.3. Agarose gel electrophoresis.

1.2% agarose gels were prepared by dissolving 1.2 g of agarose powder (ICN) in 100 ml of 1 x TAE buffer (40 mM Tris-HCl pH 8.0, 40 mM acetic acid, 1 mM EDTA) by heating in a microwave oven. The mixture was cooled to ~50°C, poured into the gel tray, and a 1-2 mm comb was inserted. The gel was allowed to set at RT then submerged in an electrophoresis tank containing 1 x TAE buffer and 0.5 µg/ml ethidium bromide (Sigma). The comb was then carefully removed from the gel.

Loading buffer (Appendix) was added to each DNA sample (0.1-1 µg) and samples with a final volume of 10-20 µl were loaded into the wells. Gel electrophoresis was performed at 100-120 V until the bromophenol blue dye marker migrated to ~2 cm from the bottom of the gel. DNA was visualized by UV illumination and photographed on polaroid type

667 film. DNA sizes were determined by comparison with the bands generated from *Pst* I-digested λ DNA (Appendix).

2.3. NUCLEIC ACID HYBRIDIZATION.

2.3.1. Preparation of ^{32}P-labeled DNA probes by random priming.

The random-priming method for labeling DNA probes was performed using the Megaprime DNA labeling kit (Amersham). The DNA template was the full-length DHBV genome released from pBL4.8 (pBluescript IIKS+ clone containing the full length genome of AusDHBV) by digestion with *Eco*R I and *Pvu* I. The digested DNA was run on a 1% agarose gel, and the 3027 bp of DHBV DNA fragment was excised from the gel with a sterile scalpel blade. DNA was recovered from the gel slice using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) as specified by the manufacturers. The concentration of DHBV DNA was determined using agarose gel electrophoresis by comparison with known amounts *Pst* I-digested λ DNA. The DNA was dissolved in DW at a concentration of 50 ng/μl.

For each DNA probe, 50 ng of DHBV DNA was labeled in a 40 μl reaction mixture containing 5 μl of random primer, 5 μl of each dATP, dGTP, and dTTP, 5 μl of reaction buffer, 5 μl of [α-^{32}P] dCTP (3000Ci/mmol; Bresatec, Adelaide), and 2 units of Klenow DNA polymerase I enzyme. The reaction was incubated at 37°C for 30 min, then stopped by the addition of 2 μl of 0.5 M EDTA. The efficiency of the radiolabeling reaction was monitored by trichloroacetic acid (TCA) precipitation. Briefly, a 1 μl sample was spotted

each onto two pieces of Whatman 542 filter paper. One filter was washed for 10 min in ice-cold 10% TCA, then dehydrated by successive washes in 100% ethanol, 50% ethanol/ether and 100% ether. The second filter was allowed to air dry for 10 min. Each filter was counted in 1-ml of scintillation liquid in a Beta scintillation counter (Beckman LS 6000TA). The percentage incorporation of ^{32}P was determined by the ratio of counts in the TCA-washed filter to the air-dried filter. The percentage of ^{32}P incorporation by this method is usually 75%.

Each DNA probe was then ethanol precipitated to remove unincorporated nucleotides, washed three times with chilled (-20°C) 70% ethanol, vacuum dried and dissolved in 100 µl DW containing 0.5% SDS. One microliter of the redissolved probe, was counted as above to determine the specific activity (cpm/ml) of each DNA probe obtained. The probe was denatured by boiling for 3 min and quenched on ice prior to use in hybridization reactions.

2.3.2. Southern blot hybridization.

Southern blot hybridization was performed as previously described (Sambrook *et al.*, 1989) with minor modifications. After electrophoresis on a 1.2% agarose gel, DNA samples were denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl buffer for 3 x 15 min. The gel was then neutralized in 1.5 M NaCl, 1.0 M Tris-HCl pH 7.4 buffer for 4 x 15 min. All denaturation and neutralization steps were carried out at RT with gentle agitation. For Southern blot analysis of cccDNA from liver tissue samples, ethidium bromide was omitted from the agarose gel and the gel buffer.

A piece of Hybond-C extra membrane (Amersham, Buckinghamshire, UK) was cut to the same size as the gel, then pre-wet in 10 x SSC buffer (Appendix) for 10-20 min. DNA was transferred onto the membrane by capillary action in a 20 x SSC buffer for 16-20 hr at RT. The membrane was rinsed for 5 min with 6 x SSC buffer and baked in a vacuum oven at 80°C for 2 hr. The membrane was then placed between 2 layers of printing silk and transferred to a siliconized glass hybridization tube (RATEK Instruments, Australia). Pre-hybridization and hybridization of the membrane was performed in 5-10 ml of solution (Appendix) at 42°C for 4 and 18 hr, respectively, in a hybridization oven (RATEK Instruments, Australia) with gentle rotation. The hybridization solution contained 5-10 x 10^6 cpm/ml of [α-^{32}P] dCTP-labeled DHBV DNA probe. Following hybridization, the membrane was washed as follows: First wash, 2 x SSC, 0.1% SDS for 2 x 15 min at 42°C; second wash, 2 x SSC, 0.1% SDS for 2 x 30 min at 55°C; and final wash, 0.1 x SSC, 0.1% SDS for 2 x 15 min at 55°C. The membrane was wrapped in plastic film, placed in a cassette with intensifying screens, and exposed to X-ray film (X-Omat LS; Kodak) at -70°C, and developed after exposure in an X-ray processor (ILFORD).

2.3.3. Spot blot hybridization.

Serum samples (5 µl) were spotted in duplicate onto an untreated Hybond-C extra (Amersham) membrane. The membrane was air-dried, soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 min, then in neutralization solution (1 M Tris-HCl pH 7.4, 1.5 M NaCl) for 15 min. Both steps were performed at RT with gentle agitation. The

membrane was air-dried, and baked in a vacuum oven at 80°C for 2 hr. The subsequent steps were performed exactly as above for Southern blot hybridization.

2.4. CLONING AND SEQUENCING OF AN AUSTRALIAN DHBV ISOLATE.

2.4.1. Source of the virus.

The AusDHBV strain used throughout this study was isolated from a pool of serum of congenitally DHBV-infected Pekin ducks *(Anas domesticus platyrhyncos)* (Jilbert *et al.*, 1996). The DHBV-infected ducks were purchased at one day of age from a commercial farm in Victoria, Australia. Blood samples were held at RT for 6 hr and then at 4°C overnight, to allow clot formation prior to centrifugation at 2,000 rpm at 4°C for 10 min. Serum samples were collected, filter sterilized, aliquoted, and stored at -70°C. A typical yield of 520 ml of pooled serum was obtained from 30 ducks sacrificed at 17 days post-hatch and bled by cardiac puncture. Serum was thawed immediately prior to use (Jilbert *et al.*, 1996).

2.4.2. Vector.

To facilitate characterization of the AusDHBV isolate, a full-length genome of AusDHBV DNA was cloned into a pBluescript IIKS+ (Stratagene). This plasmid contains a multiple cloning site flanked by T3- and T7- RNA polymerase promoters, allowing the use of T3 and T7 primers for sequencing of the inserted viral DNA. The *lacZ* gene and an ampicillin resistance gene within the plasmid provide α-complementation for blue/white

color selection of recombinant plasmids on media containing ampicillin at a concentration of 100 μg/ml (Sambrook *et al.*, 1989).

2.4.3. Pelleting of virus particles from serum.

Twenty ml of pooled serum from congenitally DHBV-infected ducks (containing ~800 ng DHBV DNA) was overlaid onto 4 ml of 20% sucrose (w/v) in TNE buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA), and centrifuged at 230,000 x g (Beckman 80 Ti rotor) for 4 hr at 4°C (Qiao, 1993).

2.4.4. Completion of the partially double-stranded viral DNA using the endogenous viral DNA polymerase.

The virus pellet was redissolved in 0.5 ml of buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% (v/v) NP40, 0.1 mM of dTTP, dATP, dCTP, and dGTP], and incubated at 37°C for 2 hr to repair the single-stranded region of DHBV DNA by the endogenous viral DNA polymerase (Uchida *et al.*, 1989). The viral DNA was then isolated after digestion with 100 μg/ml proteinase K in 10 mM Tris-HCl pH 7.8, 5 mM EDTA, 0.5% SDS, at 37°C for 1 hr, followed by phenol:chloroform extraction and ethanol precipitation. The DNA polymerase assay was monitored by incubating a 1/10 aliquot of the pelleted virus exactly as above, except with the addition of 1 μl each of ^{32}P-labeled dATP and dCTP (3000Ci/mmol). Two microliter samples were taken at 0, 30, 60, 90 min, 2, and 4 hr, spotted onto Whatman 524 filters and TCA precipitated (Section

2.3.1) to determine the incorporation of radiolabeled dNTP at each time point. A sample of normal duck serum (NDS) was similarly treated to provide a negative control.

2.4.5. End-filling reaction by T4 DNA polymerase.

To repair the nick at the 5'-end of the long negative strand of the DHBV DNA genome, a T4 DNA polymerase reaction was performed in 1 x Phor All buffer (Pharmacia), 5 mM $MgCl_2$, 0.1 mM dNTPs and 1 unit T4 DNA polymerase (Amersham) for 15 min at 12°C. The reaction was heated at 75°C for 10 min to inactivate the enzyme, followed by phenol:chloroform extraction. The DNA was then ethanol precipitated, washed three times with chilled 70% ethanol and redissolved in 25 µl of sterile DW.

2.4.6. Cloning of the full-length AusDHBV genome into pBluescript IIKS+.

Two hundred nanograms of fully double-stranded DHBV DNA was digested with *Eco*R I, then phenol:chloroform extracted and ethanol precipitated, and finally redissolved in 5 µl of DW. Three hundred fifty nanograms of pBluescript IIKS+ DNA was digested with *Eco*R I, phenol:chloroform extracted and ethanol precipitated, then dephosphorylated with alkaline phosphatase, and subsequently, treated with Proteinase K as described in Section 2.2.2. The dephosphorylated plasmid was phenol:chloroform extracted and ethanol precipitated, and finally redissolved in 5 µl of DW. DHBV DNA was ligated into the *Eco*R I site of pBluescript IIKS+ with vector:insert molar ratio of 1:5, and transformed into *E. coli* DH5α. Transformants carrying the recombinant plasmid (white colonies) were screened on LB plates [containing 100 µg/ml Ampicillin, 40 µl of X-gal (20 mg/ml

in dimethylformamide), and 4 µl of isopropylthio-β-D-galactoside (IPTG, 200 mg/ml)]. The orientation of the DHBV genome in the recombinant plasmids was identified by restriction enzyme analysis (with *Pvu* I and *Bgl* II enzymes) followed by Southern blot hybridization. The recombinant plasmid containing the DHBV genome (pBL4.8) was then amplified by a large-scale (maxi-prep) method (Section 2.6.3), redissolved at a concentration of 1 mg/ml in TE buffer and stored at -20°C.

2.4.7. Restriction mapping of the AusDHBV isolate.

The restriction pattern of the AusDHBV isolate (pBL4.8) was compared with that of the USA DHBV isolate (HBDG/Mandart *et al.*, 1984). Restriction enzymes used for comparison were: *Eco*R I, *Bam*H I, *Hpa* I, *Sma* I, and *Xba* I. DNA templates were pBL4.8 and pSP.DHBV 5.1 {USA DHBV isolate (DHBV16) cloned in pSP65, a gift from Dr. John Pugh, Fox Chase Center, USA}. Two hundred nanograms of each plasmid was digested with *Eco*R I alone, or *Eco*R I combined with *Bam*H I, *Hpa* I, *Sma* I, or *Xba* I, respectively (4 units of enzyme/reaction). Reactions were performed at 37°C for >2 hr, then DNA samples were electrophoresed in a 1.2% agarose gel. The restriction pattern of each DHBV DNA isolate was analyzed by UV illumination, and confirmed by Southern blot hybridization using an [α-^{32}P]dCTP-labeled full-length DHBV DNA probe.

2.4.8. Sequencing of the AusDHBV isolate.

(i) Sequencing strategy.

The sequencing of the DHBV DNA in pBL4.8 was carried out by the 'primer walking' method from both strands, starting with T3 and T7 primers located in the flanking region of the inserted viral DNA. DHBV primers (20 mer) (see Table 3.2) were designed based on the Primer Designer program version 2.0 (Scientific & Educational Software, 1991) with criteria as follows: GC content between 40-60%, melting temperature (Tm) between 55-70°C, no possibility of hairpin formation or of dimers at the 3' ends. Primers were synthesized by Genemed Biotechnologies, Inc (CA, USA) and were provided in a lyophilized form. After reconstitution in 1.5 ml of sterile DW (220 ng/µl), primer solutions were stored at -20°C until use. For each DNA sequence obtained with one primer (350-550 bp), the subsequent primer was designed to cover 50-80 bp at the 3' end of the previous sequence. DHBV-S31, the complete DHBV sequence from a Chinese isolate (Uchida *et al.*, 1989) was used as the DNA source for designing sequencing primers as well as for nucleotide sequence homology, as its restriction pattern was similar to the AusDHBV strain (see Chapter 3).

(ii) Sequencing protocols.

Sequencing reactions were performed using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). This method relies on four dye-labeled dideoxy nucleotides: G, A, T and C DyeDeoxy terminators that will be incorporated into the DNA chain during the sequencing reaction. The terminator premix included dITP (to minimize band compression) and the AmpliTaq DNA Polymerase (to

allow the reactions to be performed at high temperatures to minimize problems due to secondary structure). Five hundred nanograms of template DNA was mixed with 50-200 ng of primer and 8 µl of terminator premix [1.58 µM A-DyeDeoxy, 94.74 µM T-DyeDeoxy, 0.4 µM G-DyeDeoxy, 47.37 µM C-DyeDeoxy, 78.95 µM dITP, 15.79 µM dATP, 15.79 µM dCTP, 15.79 µM dTTP, 168.42 mM Tris-HCl pH 9.0, 4.21 mM $(NH_4)_2SO_4$, 42.1 mM $MgCl_2$, and 0.42 units/ul AmpliTaq DNA Polymerase] in a 20 µl reaction mixture overlaid with mineral oil. Sequencing reactions were carried out in a Perkin Elmer DNA Thermo Cycler preheated to 96°C, for 25 cycles: a denaturation step at 96°C for 30 sec, an annealing step at 50°C for 15 sec and an extension step at 60°C for 4 min, per cycle. At the completion of the reaction, DNA was precipitated with 2 µl of 3 M sodium acetate pH 5.2 and 50 µl of 100% ethanol on ice for 10 min. DNA was recovered by centrifugation at 18,000 rpm (Beckman JA-20 rotor) for 30 min at 4°C, and the DNA pellet was washed with 250 µl of chilled 70% ethanol and centrifuged at 18,000 rpm for 5 min at 4°C, then dried in a SpeedVac for 10 min. The nucleotide sequence of the DNA samples was read on a 373 DNA sequencer (Applied Biosystems) in the Microbial Pathogenesis Unit, Department of Microbiology & Immunology, University of Adelaide.

(iii) Nucleotide and amino acid sequence analysis.

DNA sequence and amino acid data were analyzed, respectively, by DNASIS and PROSIS programs version 7.00 (Hitachi, 1991). The phylogenetic tree of avian hepadnaviruses was determined using version 1.02 of *MEGA* (Kumar *et al.,* 1994). The predicted secondary structure of RNA sequence was determined using an RNAdraw software program (Windows). The hydrophobicity score of the pre-S/S and S proteins

was determined by a PROSIS software program based on the method of Hopp and Woods (1981).

2.5. SUBCLONING OF THE AusDHBV pre-S/S AND S GENES INTO A YEAST EXPRESSION PLASMID.

2.5.1. Amplification of the AusDHBV pre-S/S and S DNA.

To facilitate the cloning of the AusDHBV pre-S/S and S genes into the unique *Bam*H I cloning site of a yeast plasmid, pYCpG2 (Richardson *et al.,* 1989) (see Fig. 4.1), *Bgl* II sites were introduced to both ends of the genes by polymerase chain reaction (PCR). Primers (23 mer) were designed based on the DHBV-S31 (Uchida *et al.,* 1989) sequence as follows: 5'-GGC-*Bgl* II site-DHBV-S31 sequence-3' (see Section 4.3.1). The presence of the GGC overhang at the 5' end was to ensure that complete *Bgl* II digestion of the amplified PCR products could be accomplished. A 1062 bp PCR product containing the pre-S/S gene (nt 792-1854) and a 723 bp PCR product containing the S gene (nt 1131-1854) were amplified with a combination of either 31CG.792s (sense), or 31CG.1131s, and 31CG.1854as (anti-sense) primers, respectively (see Section 4.3.1).

All PCR reagents were redissolved in sterile DW and all procedures were carried out carefully to minimize contamination. A laminar flow hood was used to prepare the PCR master mix [10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 200 µM of each dNTP, 100 pM of each oligonucleotide primer, 1.25 units of Ampli Taq Polymerase (Pharmacia)], and an aliquot of 40 µl was dispensed into each PCR tube. On a separate

bench, 10 µl of the DNA sample (1-10 ng of pBL4.8), or 10 µl sterile DW (as negative control) was added to the reaction mix in each tube and overlaid with one drop of mineral oil (Sigma). Amplification of DHBV DNA fragments was carried out in on a Perkin-Elmer DNA Thermo Cycler as follows: The first cycle included a denaturation step at 94°C for 2 min 30 sec, an annealing step at 55°C for 1 min and an extension step at 72°C for 1 min. DNA was then amplified for 33 cycles (94°C for 50 sec, 55°C for 30 sec, 72°C for 40 sec) followed by the last extension step at 72°C for 5 min. A 2 µl aliquot of each PCR product was electrophoresed on a 1.2% agarose gel to analyze the size of the amplified DNA fragments. The amplified DNA fragments containing the pre-S/S and S genes were purified by QIAquick Spin PCR Purification Kit (QIAGEN) as specified by the manufacturer and dissolved in 5 µl of sterile DW.

2.5.2. Yeast expression plasmid and yeast strain.

The episomal pYCpG2 plasmid (11 kb) (Richardson *et al.,* 1989) (see Fig. 4.1) is a shuttle vector that can replicate both in *E. coli* and in *Saccharomyces cerevisiae (S. cerevisiae).* pYCpG2 was derived from plasmid pYCpG1[*CDC28*] (Mendenhall *et al.,* 1988) by removal of the *CDC28* coding region but retaining the 3' flanking sequences. The plasmid contains an inducible *GAL1* promoter, *CEN4* (centromere) and *ARS1* (*a*utonomously *r*eplicating *s*equences), *ori* (*ori*gin of replication), *amp*^{r} gene, *LEU2* and *URA3* yeast-selection markers. The presence of the *CEN4* and *ARS1* sequences ensure plasmid stability and the ability to be autonomously replicated in yeast, respectively. *LEU2* and *URA3* sequences complement the yeast cells so that survival and growth of the transformants is dependent on the presence of the plasmid, while *ori* and *amp*^{r} sequences

allow the plasmid to be selected for in *E. coli*. This plasmid has an unique *Bam*H I cloning site downstream of the *GAL1* promoter into which the AusDHBV pre-S/S or S gene was inserted, hence the expression of recombinant DHBV pre-S/S and S proteins will be induced by the addition of galactose to the culture medium. The yeast strain used was *S. cerevisiae* 15Dα (*ura3-, leu2-, his2-, ade1-*) (Richardson *et al.*, 1989).

2.5.3. Subcloning of the AusDHBV pre-S/S and S genes into pYCpG2 and transformation into *E. coli*.

Following PCR amplification of the DHBV DNA fragments containing the pre-S/S and S genes, they were digested with *Bgl* II, phenol:chloroform extracted and ethanol precipitated. *Bam*H I-digested pYCpG2 plasmid was also phenol:chloroform extracted and ethanol precipitated, followed by a dephosphorylation step to prevent self-ligation (Section 2.2.2). After proteinase K digestion of the dephosphorylated plasmid, DNA was recovered by phenol:chloroform extraction and ethanol precipitation. *Bgl* II-digested DHBV pre-S/S or S DNA was cloned into the *Bam*H I-site of pYCpG2 with vector:insert molar ratio of 1:5, prior to transformation into *E. coli* DH5α. Transformants were selected on LB plates (containing 100 μg/ml Ampicillin), and clones with the correct orientation were screened by restriction enzyme analysis followed by Southern blot hybridization. Restriction enzymes used for screening were *Eco*R I and *Hpa* I for the pYCpG2-pre-S/S clones, or *Eco*R I and *Xho* I for the pYCpG2-S clones.

2.5.4. Transformation of the pYCpG2-pre-S/S and pYCpG2-S plasmids into *S. cerevisiae.*

Both constructs, pYCpG2-pre-S/S and pYCpG2-S, were used to transform the *S. cerevisiae* strain 15Dα (*ura3-, leu2-, his2-, ade1-)* by the lithium acetate method (Becker & Fikes, 1993) with minor modifications. Cells were grown from a single colony in 50 ml of YPAD medium (Appendix) at 30°C to 5-10 x 10^7 cells/ml (an OD_{600} of 1 is equivalent to ~3 x 10^7 cells/ml). The cells were then harvested by centrifugation at 4,000-6,000 x g for 5 min at 4°C. After washing the cells in 10 ml sterile of DW with centrifugation as above, the cells were resuspended in 500 µl of freshly prepared 1 x TE/LiAc (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM lithium acetate pH 7.5, 25 mM DTT). The cells were then incubated at 30°C with shaking (180 rpm) for 30 min. For each transformation, 100 µg of high molecular weight (MW) single-stranded salmon sperm DNA (Sigma) (repeatedly boiled and chilled for 2 x 3 min) was mixed with 5 µg of plasmid DNA (pYCpG2-pre-S/S or -S) then added to 150 µl of the yeast cells. The positive and negative controls used in the transformation reaction were 5 µg of pYCpG2 alone, or 100 µg of high MW single-stranded salmon sperm DNA alone. Cells were grown for 30 min at 30°C with shaking (200 rpm), then 0.7 ml of LiPEG (0.1 M LiAc, 40% PEG 4000, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA) was added and the cells were allowed to grow for another 30 min. Cells were then heat shocked at 42°C for 10 min, inoculated into 5 ml of YPAD medium and allowed to regenerate for 3 hr. After pelleting the cells at 4,000 rpm for 2 min at RT, they were resuspended in 1 ml TE buffer and plated on SC-*ura* medium (Appendix), and grown at 30°C until transformants appeared.

2.5.5. Expression and purification of the AusDHBV pre-S/S & S proteins in *S. cerevisiae.*

S. cerevisiae carrying the desired plasmids were grown on SC-*ura* medium (Appendix) at 30°C. Ten ml of SC-*ura* medium was inoculated with a single colony from a SC-*ura* plate and grown for 24 hr, then subcultured into 200 ml of fresh SC-*ura* medium for another 24 hr until late-logarithmic phase. To induce protein expression, yeast cells were pelleted by centrifugation at 3,000 rpm (Beckman GPR) for 5 min, at RT, and resuspended in 400 ml of fresh SC-*ura* medium containing 2% galactose and grown for 24 hr. Cells were harvested by centrifugation at 3,500 rpm (Beckman GPR) for 15 min at 4°C, and resuspended in 4 volumes of lysis buffer [25 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT, 0.1% Triton X-100 and a mixture of protease inhibitors (Appendix)]. Three volumes of sterile, acid-washed, glass beads (425-600 microns; Sigma) were then added to the suspension, and cells were lysed by vortexing for 3 x 1 min at 4°C with a 1 min interval on ice. The cell extracts were first clarified at 10,000 x g (Beckman JA-20 rotor) for 30 min at 4°C, then at 100,000 x g (Beckman SW 41 rotor) for 40 min, at 4°C. The clarified supernatant (~15 ml obtained from 400 ml of culture) was aliquoted and stored at -70°C.

The DHBV pre-S/S or S proteins in the supernatant were purified by successive ultracentrifugation (Klingmüller & Schaller, 1993) with some modifications as follows. First, 1 ml of the clarified supernatant was diluted to 11 ml with lysis buffer, layered onto 1 ml of 70% (w/v) sucrose in a 14 x 89 mm ultracentrifuge tube (Nalgene, USA), and centrifuged in an SW 41 rotor at 20,000 x g for 14 hr. Five hundred microliter fractions

were collected from the bottom of the tube, and the fractions containing the recombinant proteins were pooled and dialysed thoroughly against PBSE (PBS containing 1 mM EDTA, 0.02% sodium azide), and concentrated with PEG 6000 powder. Protein samples were then overlaid onto 20-50% (w/v) sucrose gradients (2.6 ml of each 20, 30, 40, and 50% sucrose) and centrifuged in an SW 41 rotor (Beckman) at 190,000 x g, 18 hr. Fractions (0.5 ml) were collected from the bottom of the tube, and the fractions of interest were then pooled, dialyzed against PBSE, and concentrated with PEG 6000.

The final purification step was rate-zonal centrifugation in a 5-30% continuous sucrose gradient at 33,000 rpm in an SW 41 rotor (Beckman) for 6 hr. Fractions (0.5 ml) were collected from the bottom of the tube, dialyzed against PBSE buffer then concentrated with PEG 6000. All purification steps were carried out at 4°C, and the purity of each purification step was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5.6. Detection of the AusDHBV pre-S/S and S proteins expressed in *S. cerevisiae.*

(i) SDS-PAGE.

For protein analysis, aliquots from each purification step were subjected to SDS-PAGE and detected with Coomassie brilliant blue staining (Appendix). Whenever necessary, protein samples of each fraction after centrifugation were precipitated with 100% TCA (final concentration 10%) for 30 min on ice (Schlicht *et al.*, 1987a). The precipitated proteins were recovered by centrifugation at 12,000 rpm for 10 min, at 4°C, and the pellet was washed once with 1% TCA in PBSE. After removal of residual acid, proteins were

dissolved in 20 µl of sample buffer (3% SDS, 2% β-mercaptoethanol, 10% sucrose, 0.1% bromophenol blue, 5 mM EDTA, 200 mM Tris-HCl pH 8.8), boiled for 5-10 min, then applied onto a 12% acrylamide SDS-PAGE (Appendix). The gel was run in 25 mM Tris base, 192 mM glycine, 0.1% SDS buffer on a Hoefer SE 400 slab gel electrophoresis apparatus (Pharmacia) at 30 mA until the bromophenol blue dye reached the bottom of the gel. Low molecular weight (LMW; Pharmacia) markers were used to determine the size of the protein bands.

(ii) Western Blot.

After protein separation on SDS-PAGE, the gel was equilibrated in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3) for 15 min at RT. Membranes {Immobilon-P, 0.45 µm; (Millipore), or Hybond-C extra (Amersham)} were equilibrated in transfer buffer for 15 min at RT. Immobilon-P membrane was pre-wet in 100% methanol prior to equilibration in transfer buffer. Proteins were transferred to the membrane in a Mini Trans-Blot apparatus (Bio-Rad Laboratories) using pre-cooled (4°C) transfer buffer at 100 V for 1 hr. Following transfer, the membrane was fixed in 20% (v/v) methanol for 5 min, rinsed with DW, and stained with 0.1% Ponceau S solution (Appendix) for ~2 min to determine the completion of protein transfer. Ponceau S staining was removed by several washes with DW, and unoccupied sites were blocked by incubating the membrane in blocking buffer {5% (w/v) skim milk (Carnation) in TBS-T buffer (10 mM Tris-HCl pH 7.4, 0.9% NaCl, 0.05% Tween 20)} at 4°C overnight with gentle shaking. The membrane was washed for 5 min in TBS-T buffer and incubated with appropriate antibodies diluted in 5% skim milk/TBS-T buffer. All incubation steps

below were performed at RT with gentle shaking; and the membrane was washed for 3 x 5 min in TBS-T buffer between each step.

Pre-S/S protein was detected with 1H.1, an anti-pre-S specific monoclonal antibody (Pugh, *et al.,* 1995), at a dilution of 1/5,000 for 2 hr, followed by biotinylated, sheep-anti-mouse Ig $F(ab')_2$ (Amersham) at a dilution of 1/400 for 2 hr, and finally, with horseradish peroxidase (HRP)-conjugated streptavidin-biotin (Amersham) at a dilution of 1/400 for 1 hr. Bound antibody was detected by the addition of a freshly-prepared HRP substrate {0.5 mg/ml diaminobenzidine (DAB; Sigma), 12 µl H_2O_2 in 9 ml PBS} and incubation in the dark at RT. The membrane was washed thoroughly in DW after color development had occurred.

S protein was detected with an anti-DHBV antibody positive duck serum (B40R86) (Jilbert *et al.,* 1998) at a dilution of 1/4000 for 2 hr, followed by rabbit-anti-duck IgY (Bertram, 1997) at a dilution of 1/10,000 for 1 hr, and finally with HRP-conjugated goat-anti-rabbit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at a dilution of 1/5,000 for 1 hr. S protein was visualized with enhanced chemiluminescence (ECL) (Boehringer Mannheim) protocol as specified by the manufacturer, using X-OMAT LS film (KODAK) after 5 min exposure.

The positive controls for Western blot analysis were: (i) purified AusDHBV from congenitally DHBV-infected duck serum, and (ii) Aus-DHBV infected liver tissue. AusDHBV-infected serum was purified by sucrose gradient centrifugation as follows. Five ml of serum was layered onto 20-70% sucrose (5 ml and 1 ml of each, respectively) in a 14 x 89 mm ultracentrifuge tube (Nalgene, USA), and centrifuged in an SW 41 rotor

at 33,000 rpm for 4 hr at 4°C. Five hundred microliter fractions were collected from the bottom of the tube, fractions 2, 3 and 4 were pooled, dialyzed against TN (20 mM Tris-HCl pH 7.4, 150 mM NaCl), and concentrated with PEG 6000. A 100 µl aliquot of the pooled fractions was then applied onto a 15-35% sucrose gradient (1.8 ml each of 15, 20, 25, 30 and 35% sucrose) underlaid with 1 ml of 70% sucrose, and centrifuged in an SW 41 rotor at 33,000 rpm for 3 hr at 4°C. Five hundred microliter fractions were collected from the bottom of the tube, fractions 14, 15, and 16 were pooled, aliquoted and stored at -70°C (Jilbert, personal communication). For each SDS-PAGE and Western blot analysis, 50 µl of this preparation was used as a positive control.

Protein from AusDHBV-infected liver tissue was extracted as described (Wu *et al.*, 1991). In a petri dish, ~200 mg of frozen liver tissue was finely chopped with a sterile scalpel, added with 2 ml of lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP40, 1% aprotinin) and the mixture was held on ice for 10 min. The liver suspension was then transferred into a chilled glass homogenizer on ice, and homogenized for 25-30 strokes. The cell debris was removed by centrifugation at 10,000 rpm in a JA-20 rotor (Beckman) for 5 min at 4°C. The supernatant was collected, added with 1/10 vol of 1 M Tris-HCl pH 8.0 and precipitated with 5 volumes of chilled 100% ethanol at -20°C overnight. Protein samples were pelleted by centrifugation at 10,000 rpm for 5 min at 4°C, and finally resuspended in 600 µl of SDS-PAGE loading buffer (Appendix). A 10 µl of this preparation (~3 µg total protein) was used for SDS-PAGE and Western blot analysis.

2.6. SUBCLONING OF THE AusDHBV pre-S/S AND S GENES INTO A MAMMALIAN EXPRESSION VECTOR.

2.6.1. Mammalian expression vector pcDNA I/Amp.

The plasmid pcDNA I/Amp (Invitrogen, CA, USA) was chosen as the vector to express DHBV pre-S/S and S proteins *in vivo* following DNA vaccination. This plasmid (4.8 kb) is a multifunctional vector designed for the expression of cDNA in eukaryotic systems and cDNA analysis in prokaryotes. It contains an early CMV promoter/enhancer, splice segment and polyadenylation signal, Sp6 and T7 RNA promoters for the production of sense and anti-sense RNA transcripts and an ampicillin resistance gene for selection in any *E. coli* strain. The competent host used was *E. coli* TOP10F' harboring P3, a low-copy 60 kb episomal plasmid that encodes the kanamycin resistance gene as well as amber mutants of the tetracycline and ampicillin resistance genes. After transformation with pcDNA I/Amp, bacterial cells were resistant to both tetracycline and ampicillin.

2.6.2. Subcloning of the AusDHBV pre-S/S & S genes into pcDNA I/Amp.

The AusDHBV pre-S/S and S DNA fragments derived from pBL 4.8, were subcloned into the pcDNA I/Amp plasmid using the method described in Section 2.5.3. Briefly, PCR-amplified DHBV pre-S/S and S DNA fragments with *Bgl* II sites at both ends were digested with *Bgl* II, and ligated into the *Bam*H I site of dephosphorylated pcDNA I/Amp with a vector:insert molar ratio of 1:10. The ligation mixtures were used to transform competent *E. coli* TOP10F' and cells were plated on LB agar containing 60 μg/ml

Ampicillin and 15 μg/ml Tetracycline. Transformants carrying the recombinant plasmids were screened by restriction enzyme analysis (*Hpa* I for pcDNA I-pre-S/S clones; *Pvu* I and *Kpn* I for pcDNA I-S clones) followed by Southern blot hybridization. To assess the fidelity of Taq DNA polymerase during PCR, each DHBV insert in both clones was sequenced with T7 (sense) and SP6 (reverse) primers, and compared with the DNA sequence in the parental clone (pBL4.8).

2.6.3. Large-scale (maxi) preparation of plasmid DNA.

Transformants carrying pcDNA I-pre-S/S and pcDNA I-S clones were grown in 5 ml of LB broth containing the appropriate antibiotics for 8 hr at 37°C, then subcultured into 500 ml of LB broth for another 20 hr. Cells were harvested by centrifugation at 6,000 rpm (Beckman JA-10 rotor) for 15 min at 4°C. Recombinant plasmid DNA was purified by anion-exchange chromatography, with a QIAfilter Plasmid Maxi Prep kit (QIAGEN, Hilden, Germany) as specified by the manufacturer with minor modifications. Cell pellets were resuspended in 20 ml of P1 buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A) for each 500 ml of original culture. Cells were lysed by the addition of 20 ml of P2 buffer (200 mM NaOH, 1% SDS), mixed gently by inverting the tubes several times and left at RT for 5 min. Twenty ml of chilled P3 buffer (3 M potassium acetate, pH 5.5) was then added, and cellular debris was removed by centrifugation at 18,000 rpm (Beckman JA-20 rotor) for 30 min at 4°C. The anion-exchange resin, QIAGEN-tip 500, was equilibrated with 10 ml of QBT buffer (750 mM NaCl, 50 mM MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100), then the supernatant was filtered through a Qiafilter cartridge and loaded onto a pre-equilibrated QIAGEN-tip 500 by

gravity flow. To ensure maximum DNA binding to the resin, the flow through from the column was reapplied twice. The QIAGEN-tip 500 was then washed with 30 ml of QC buffer (1 M NaCl, 50 mM MOPS pH 7.0, 15% ethanol), and DNA was subsequently eluted with 15 ml of QF buffer (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% ethanol). DNA was precipitated by the addition of 10.5 ml (0.7 volume) of analytical grade (AR) isopropanol followed by centrifugation at 18,000 rpm (Beckman JA-20 rotor) for 45 min at 4°C. The DNA pellet was washed once with 5 ml of chilled 70% ethanol, air-dried, and redissolved in sterile PBS at a concentration of 1 mg/ml.

2.6.4. Transient transfection of the COS7 cell line.

To confirm the ability of recombinant plasmids to express the DHBV pre-S/S and S proteins, a SV40-transformed African green monkey kidney cell line (COS7) was transiently transfected with both recombinant plasmids. Cells were grown in a 24-well plate (Falcon; Beckton Dickinson) in DMEM (Dulbecco's Modified Eagle Medium; GIBCO) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 12 ng/ml Penicillin and 160 ng/ml Gentamycin, at 37°C in a 5% CO_2 incubator until the cell density reached 50-60% confluence. Cells were washed once with PBS, and 250 µl of DMEM (supplemented as above, except with 1% FBS) was added. Twenty microlitres of transfection mixture was prepared in a polystyrene, round-bottom tube (Falcon 2054; Beckton Dickinson) containing 1 µg (1 µg/ul) of DNA, 5 µg of N-[1-(2,3,-Dioleoyloxy) propyl]-N,N,N-trimethyl-ammoniummethylsulfate (DOTAP; Boehringer Mannheim), and 14 µl of Hepes Buffered Saline (150 mM NaCl, 20 mM Hepes); the mixture was held at RT for 15 min. The transfection mixture was added to the cells and the plate was swirled

gently. The cells were incubated at 37°C in 5% CO_2, overnight, and the medium was replaced on the following day with 1 ml of DMEM supplemented with 5% FBS.

In some experiments an alternative transfection reagent, FuGENE™ 6 (Boehringer Mannheim), was used. FuGENE™ 6 resulted in a higher transfection efficiency and less cytotoxicity than DOTAP. In these experiments, COS7 cells were grown in 6-well plates until the cell density reached 50-60% confluency. The cells were washed once with PBS, and 250 µl of DMEM (containing 5% FBS) was added. For each well, the transfection mixture was prepared as follows. In a sterile Eppendorf tube containing 97 µl of serum-free DMEM media, 3 µl of FuGENE 6 reagent was added directly into the medium, and the mixture was held at RT for 5 min. In a separate tube, 1 µg DNA (1 µg/µl) was slowly diluted with 100 µl of the FuGENE 6 mixture. The tube was tapped gently to mix the contents and was incubated at RT for 15 min. The mixture was then added slowly to the cells, and the plates were swirled to ensure even dispersal. The cells were incubated at 37°C in 5% CO_2 overnight, and the medium was replaced on the following day with 1 ml of DMEM supplemented with 5% FBS.

Expression of DHBV pre-S/S and S proteins was detected on day 2-3 after transfection by indirect immunofluorescence (IMF) as described (Kok *et al.,* 1993) with minor modifications. Briefly, cells were washed twice with cold PBS, air-dried in a 37°C incubator for 5 min, then fixed with chilled (-20°C) methanol for 10 min at RT. After removal of methanol and air-drying, 200 µl of the corresponding primary antibody was added: anti-pre-S monoclonal antibody (ascites fluid) (1H.1; Pugh *et al.,* 1995), diluted at 1/1,000 in PBS, to detect pre-S protein, or, rabbit anti-DHBs IgG (R4; Qiao *et al.,* 1990),

diluted at 1/50 in PBS, to detect S protein. The reaction was incubated for 30 min at 37°C, the cells were washed three times with PBS, and 200 µl of the corresponding secondary antibody {FITC conjugated sheep-anti-mouse (Silenius) or FITC conjugated anti-rabbit $F(ab')_2$ fragment (Silenius)} at a dilution of 1/50 in PBS was added. After further incubation at 37°C for 1 hr, the cells were washed as above, mounted in 90% glycerol, 50 mM Tris-HCl pH 8.6 in PBS, and examined under an inverted, fluorescent microscope (Olympus) with FITC filters.

2.7. VACCINATION AND VIRUS CHALLENGE PROTOCOLS.

2.7.1. Animals.

DHBV-negative ducks were purchased from a commercial farm (Inghams Tahmoor Hatchery, NSW, Australia). Congenitally DHBV-infected ducks were purchased at one day of age from a different commercial farm in Victoria, Australia. DHBV-negative and DHBV-infected ducks were kept separately in the Animal House facilities of the Institute of Medical and Veterinary of Science (IMVS), Adelaide. All animal handling procedures were approved by the IMVS and University of Adelaide Animal Ethics Committees, and followed the National Health and Medical Research Council (NH&MRC) guidelines.

2.7.2. Vaccination with yeast-derived pre-S/S and S antigens.

Two groups of 3-week-old DHBV-negative ducks (3 animals/group) were injected subcutaneously (s.c.) with 40 µg of either pre-S/S or S protein emulsified in Freund's

complete adjuvant (FCA). All ducks received 2 further s.c. doses of antigen emulsified in Freund's incomplete adjuvant 1 and 2 months after the first vaccination. Blood samples were collected every two weeks.

2.7.3. Protective vaccination of ducks with pcDNA I-pre-S/S & pcDNA I-S plasmids.

(i) DNA vaccination.

Two groups of 6-month-old DHBV-negative ducks consisting of 3 ducks/group were injected intramuscularly (i.m.) into the quadriceps anterior (QA) muscle with 750 μg of either pcDNA I-pre-S/S or -S plasmid DNA (1 mg DNA/ml in PBS). Another two groups of 3-week-old ducks were also injected i.m. with 250 μg of plasmid DNA. Five days prior to DNA vaccination, the vaccination sites were injected with 750 μl (6-month-old) or 250 μl (3-month-old) of 5 mg/ml bupivacaine hydrochloride 0.5% {2-piperidine carboxamide, 1-butyl-N-(2,6-dimethylphenyl)-monohydrochloride, monohydrate (Marcain; Astra Pharmaceuticals, Australia)} to induce muscle necrosis and, subsequently, muscle regeneration. Fifteen min before vaccination, the muscle sites were injected with 750 μl (6-month-old) or 250 μl (3-week-old) of 25% sucrose (w/v) in PBS. All injections were carried out using 1-ml syringe fitted with 26-gauge needle. DNA vaccination was repeated 3 and 6 weeks after the first vaccination. Blood samples were collected weekly for the first month then every two weeks.

(ii) Challenge of vaccinated ducks with DHBV.

Vaccinated and non-vaccinated ducks were challenged intravenously (i.v.) with a high-dose viral inoculum (1.9×10^{11} DHBV DNA genomes) via cannulation of the jugular vein

and inoculation of 20 ml of pooled serum containing 9.5 x 10^9 DHBV genomes/ml (Jilbert *et al.,* 1996). Blood samples were collected from the cannula before challenge (pre-bleed), and at 1, 5, 15, 30, 45, 60, 90 min, and 2 hr post-challenge (p.c.). Blood was allowed to clot by incubating the samples at 37°C for 1 hr, then kept at 4°C overnight. Serum was collected by centrifugation at 2,000 rpm for 10 min at 4°C and stored at -20°C.

2.7.4. Therapeutic vaccination of ducks with pcDNA I-pre-S/S & pcDNA I-S plasmids.

In the therapeutic vaccination study (Chapter 6), four groups of newly hatched ducks (groups A-D) were examined. DNA vaccines (1 mg DNA/ml in PBS) were injected i.m. into the QA muscle. Prior to inoculation, viral stocks were diluted in 100 µl of NDS and were injected i.v. through jugular vein. All injections were carried out using 1-ml syringes fitted with 26-gauge needles. Blood samples from each duck were collected weekly and assayed for DHBsAg, anti-DHBs antibodies and anti-DHBc antibodies by ELISA (Section 2.10), and for serum viral DNA by spot-blot hybridization (Section 2.3.3).

(A) Pre-infection.

This group consisted of 9 ducks that were vaccinated at 1 and 14 days of age with 100 µg of either pcDNA I-pre-S/S, -S, or 50 µg of each plasmid DNA (3 ducks for each type of vaccine). Muscle sites were not pre-treated with bupivacaine hydrochloride and sucrose prior to vaccination. All ducks were challenged at 14 days of age with 1 x 10^7 DHBV

genomes. Three non-vaccinated control ducks were inoculated at 14 days of age with an identical virus inoculum. Liver biopsies were performed in all ducks at 4 days p.c. Ducks were sacrificed at 6 weeks of age (4 weeks p.c.).

All ducks in groups (B-D) below received 5 doses of DNA vaccine. Each dose contained 50 μg of pcDNA I-pre-S/S and 50 μg of pcDNA I-S plasmid DNA. For the 4^{th} and the 5^{th} vaccine doses only, muscle sites were pre-treated with 100 μl of bupivacaine HCl and 100 μl of 25% (w/v) sucrose in PBS at 5 day and 15 min prior to DNA injection, respectively. In each group, 1-2 non-vaccinated ducks were included as controls. All ducks were sacrificed at 24 weeks of age.

(B) Simultaneous infection and vaccination.

This group consisted of 4 ducks that were simultaneously inoculated with 1 x 10^4 DHBV genomes and vaccinated at 1 day of age. Vaccination was repeated two times at 2 week intervals, and then at 11 and 13 weeks of age. Liver biopsies were performed in all ducks at 3 weeks of age (3 weeks p.c.).

(C) Delayed vaccination.

This group consisted of 5 ducks that were inoculated with 1 x 10^4 DHBV genomes at 1 day of age, then were given the first vaccination at 7 days of age. Vaccination was repeated two times at 2 week intervals, and then at 11 and 13 weeks of age. Liver biopsies were performed in all ducks at 2 weeks of age (1 week p.c.).

(D) Congenital infection.

This group consisted of 8 congenitally DHBV-infected ducks and were given the first vaccination at 1 day of age. Vaccination was repeated two times at 2 week intervals, and then at 11 and 13 weeks of age. Liver biopsies were performed at 1 week of age.

2.8. DETECTION OF VIRUS INFECTION IN SERUM AND LIVER.

2.8.1. Quantification of the rate of virus removal from the bloodstream.

Duck serum (100 µl) collected at each time point p.c. was overlaid onto 10-20% sucrose (1 ml each) in TN buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl). Virus was pelleted at 369,000 x g (55,000 rpm in a Beckman TLS-55 rotor) for 3 hr at 4°C (Jilbert *et al.,* 1996). Viral DNA was isolated by digesting the pellet with 50 µl of TN buffer containing 2 mg/ml pronase, 20 ng/µl salmon sperm DNA, 0.1% SDS, 10 mM EDTA, and incubation at 37°C for 1 hr. Pronase was inactivated by the addition of 20 mM EDTA, and samples were stored at -20°C. To assess the extent of DNA loss during sample processing, 100 µl of the pooled serum inoculum containing a known amount of DHBV DNA (26 ng/ml) (Jilbert *et al.,* 1996) was pelleted separately and pronase/SDS digested in the same manner. Twenty-five µl of each extracted serum sample (equivalent to 50 µl of original serum) and 5 µl of extracted inoculum (equivalent to 10 µl of inoculum and containing 260 pg of DHBV DNA) were analyzed by agarose gel electrophoresis followed by Southern blot hybridization.

The relative amounts of viral DNA remaining in the bloodstream at each time point p.c. were quantitated by a Molecular Phosphor Imager System using 5 µl of extracted inoculum (260 pg) and 50 pg of DHBV DNA (gel-purified from pBL4.8) as standards. The rate of virus removal from the bloodstream was calculated based on the DHBV DNA concentration at each time point p.c. compared to the 100% value. The 100% value was defined as the DHBV DNA concentration of the inoculum corrected for a 10 fold dilution in the bloodstream occurring immediately after inoculation, i.e. 20 ml inoculum diluted in a total blood volume of 200 ml (estimated as 7% of body weight).

2.8.2. Liver biopsy.

In the yeast-derived vaccination study (Section 2.7.2) and the protective vaccination study (Section 2.7.3), liver biopsies were performed at 4 days p.c., and in some cases, 2 weeks before challenge. In the therapeutic vaccination study (Section 2.7.4), liver biopsy were performed at various times after infection or challenge. Anaesthesia was induced with a combination of N_2O/O_2/5 vol% isoflourane (Forthane; Abbott), by placing the animal in a closed chamber for 5-10 min. After intubation with a 2.5 mm endotracheal tube (Contour™; Mallinckrodt Medical, Ireland), anaesthesia was then maintained with N_2O/O_2/3 vol% isoflourane. Ducks were placed in a recumbent position on an electric heating pad, and feathers were cut from the ventral and right lateral abdomen. The surgical site was aseptically cleansed with 0.5% chlorhexidine in 70% alcohol, then a sterile laparotomy drape was placed to retract the peripheral feathers and to isolate the surgical site. A 2-3 cm incision was made on the right abdomen, 1-2 cm caudal and parallel to the last rib. Dissection proceeded through the subcutaneous fat layer and the abdominal muscle layer until the abdominal wall (peritoneum) was visible. To protect the

underlying viscera, the peritoneum was lifted with forceps then was carefully cut to expose the liver. An abdominal clamp was inserted underneath the edge of the right lobe that localized a ~0.5x1 cm^3 section of the lobe, which was then resected with a scalpel. The clamp was left for a few minutes to control haemorrhage from the biopsy site, and if necessary, a piece of adsorbent gelatin sponge (Gelfoam-7mm; Upjohn) was placed adjacent to the biopsy site. A continuous 4-0 Vicryl suture was used to suture the abdominal wall and the subcutaneous fat layer. The skin edges were closed with interrupted subcuticular sutures, then Flexible Collodion B.P. solution (Incorporating David Craig Pharmaceuticals, QLD, Australia) (Appendix) was applied to the wound surface to aid the healing process.

Following surgery, ducks were kept in cage warmed with a red light for 30 min to stabilize body temperature and to encourage a quiet recovery. The amount of tissue obtained ranged between 250-500 mg. Liver tissues were cut immediately into three pieces of up to 0.5 cm^3, a portion of which was put into an Eppendorf tube and snap-frozen in liquid N_2 for extraction of total and covalently closed circular (ccc) DNA (Section 2.8.3). The other tissue samples were fixed in: (i) ethanol:acetic acid (EAA) (3:1) for 30 min at RT, then in chilled 70% ethanol at 4°C overnight, and in (ii) 10% formalin in PBS, then embedded in paraffin wax and sectioned onto gelatin-coated glass slides by the Tissue Pathology Department, IMVS.

2.8.3. Extraction of total and cccDNA from liver tissue.

(i) Tissue homogenization.

In a petri dish, ~100 mg of frozen liver tissue was finely chopped with a sterile scalpel blade and resuspended in 3 ml of 10 mM Tris-HCl pH 7.4, 10 mM EDTA. The chopped liver suspension was then transferred to a sterile chilled glass homogenizer, and the tissue was homogenized for 25-30 strokes. The liver homogenate was divided into 2 x 1.5 ml aliquots for extraction of total and cccDNA. All steps were carried out on ice.

(ii) Extraction of total DNA.

One aliquot (1.5 ml) of liver homogenate was diluted to 4 ml with 10 mM Tris-HCl pH 7.4, 10 mM EDTA. Four ml of pronase/SDS solution (final concentration: 4 mg/ml pronase, 0.1% SDS, 0.15 M NaCl, 10 mM Tris-HCl pH 7.4, 10 mM EDTA) was then added. The mixture was vortexed thoroughly, and incubated at 37°C for 2 hr with intermittent vortex until no lumps were visible. The digested liver sample was then extracted once with phenol:chloroform:isoamyl alcohol followed by chloroform:isoamyl alcohol. DNA in the upper (aqueous) phase was precipitated with a 1/10 volume of 3 M sodium acetate pH 5.2 and 2 volumes of 100% ethanol at -20°C overnight. DNA was recovered by pelleting at 10,000 rpm (Beckman JA-20.1 rotor) for 15 min at 4°C, washed twice with chilled 70% ethanol, dried, and redissolved in 400 µl TE buffer containing 100 µg/ml RNase A.

(iii) Extraction of cccDNA.

The remaining 1.5 ml of liver homogenate was diluted in 6 ml of 10 mM Tris-HCl pH 7.4, 10 mM EDTA, and mixed with 400 µl of 10% SDS (final concentration: 0.5% SDS) to lyse the cells. Finally, 2 ml of 2.5 M KCl (final concentration: 0.5 M KCl) was added. The mixture was mixed thoroughly, and incubated at RT for 30 min to precipitate protein-bound DNA. Protein-bound DNA was then removed by centrifugation at 10,000 rpm (Beckman JA-20.1 rotor) for 20 min at 4°C. cccDNA in the supernatant was extracted once with phenol:chloroform:isoamyl alcohol, then with chloroform:isoamyl alcohol. After precipitation with 2 volumes of 100% ethanol at RT for ≥30 min, cccDNA was recovered as above (ii) and redissolved in 200 µl of TE buffer (without RNase A). Samples equivalent to 10 µg of DNA for each extract were analyzed by agarose gel electrophoresis and Southern blot hybridization.

2.8.4. Detection of DHBsAg in tissue sections by immunoperoxidase staining.

The presence of viral surface antigen in liver tissue was detected by immunoperoxidase staining as previously described (Jilbert *et al.,* 1996). EAA-fixed wax-embedded sections were dried at 37°C overnight, dewaxed in xylene for 2 x 10 min (in a fume hood), and successively re-hydrated in absolute (100%), 90%, and 70% ethanol, each for 2 min. Sections were washed in PBS for 2 x 10 min, treated with 0.5% H_2O_2 in PBS at RT for 15 min to inactivate endogenous peroxidase, then washed in PBS for 2 x 5 min. To block non-specific binding, sections were incubated with 100 µl of normal sheep serum (NSS) at a dilution of 1/30 in PBS, for 30 min at RT. Primary antibody was then added after removal of the NSS but without washing: 100 µl of an anti-pre-S/S monoclonal antibody

(1H.1; Pugh *et al.,* 1995) at a dilution of 1/200 in 10% normal duck serum (NDS) in PBS for 1 hr at 37°C, then at 4°C, overnight. After washing in PBS for 2 x 5 min, sections were incubated with HRP-conjugated sheep-anti-mouse IgG (Amersham) at a dilution of 1/40 in 10% NDS in PBS for 1 hr at RT, then washed in PBS for 2 x 5 min. To prevent drying of the sections, all incubations were performed with glass coverslips in a humid box.

Bound conjugate was visualized by incubation with 0.5 mg/ml diaminobenzidine (DAB; Sigma), 0.03% H_2O_2 in PBS, at RT for 9 min. Sections were washed in PBS for 5 min, counterstained with haematoxylin for 30 sec, washed in PBS for 3 x 1 min, and successively re-hydrated in 70%, 90%, and 100% ethanol, each for 2 min. Sections were treated in Histoclear (National Diagnostics) for 2 x 5 min and mounted in Histomount (National Diagnostics) under glass coverslips. The specificity of viral antigen staining was confirmed by the absence of staining in uninfected tissues.

2.9. NEUTRALIZATION ASSAYS.

2.9.1. *In vivo* neutralization.

Ten microlitres of viral inoculum containing 1 x 10^6 DHBV DNA genomes, equal to 1 x 10^6 ID_{50} diluted in NDS (Jilbert *et al.,* 1996), was incubated alone, or with serum collected from the pre-S/S (R76) or S (R81) DNA-vaccinated ducks (anti-pre-S/S or anti-S serum, respectively) at 37°C, for 1 hr. Different volumes of neat anti-pre-S/S serum (20, 40, and 80 µl), or anti-S serum (5, 10, and 20 µl) were used, based on the results of

the rate of virus removal from the bloodstream of vaccinated ducks. After 1 hr incubation (with or without antiserum), the total volume of each inoculum was adjusted to 100 µl with NDS, and inoculated i.v. into groups of one-day-old ducklings (3 animals/volume of serum tested). Serum samples were collected each week and were analyzed for DHBsAg and DHBV DNA.

2.9.2. *In vitro* neutralization.

Primary duck hepatocytes (PDH) were obtained from 2-3-week old ducklings by collagenase perfusion of the liver as previously described (Tuttleman *et al.,* 1986). PDH were seeded at 1.5 x 10^6 cells per well in 6-well plates (Falcon, Beckton Dickinson Labware, NJ, USA) in 3 ml of Leibovitz's L-15 medium (Gibco BRL) supplemented with 5% FBS, 2 mM L-glutamine, 12 ng/ml Penicillin, 160 ng/ml Gentamycin, 1 µg/ml insulin (Sigma), 10 units/ml nystatin (Sigma), and 10^{-5} M hydrocortisone hemisuccinate (Sigma). The plates were incubated at 37°C without CO_2. Following overnight incubation the medium was replaced with L-15 supplemented as above but without FBS. The medium was then changed everyday and *in vitro* neutralization assays were performed one day post-plating. The virus inoculum (sucrose gradient purified-AusDHBV from serum) contained 7 x 10^7 DHBV DNA genomes, equivalent to 5.4 x 10^4 $TCID_{50}$ (Qiao, unpublished). Samples of inoculum were pre-incubated with serum from DNA vaccinated ducks for 1 hr at 37°C. The serum samples were 35 or 70 µl of either anti-S, anti-pre-S/S, or both antisera (35 or 70 µl of each serum). After 1 hr incubation, the volumes of the virus/antiserum mixtures were adjusted to 1 ml with L-15 medium then inoculated into individual PDH cultures. The inoculum was incubated with the cells for

12 hr at 37°C, then another 2 ml of fresh L-15 medium was added without removing the original inoculum. As a positive control, the viral inoculum was pre-incubated with NDS. Cells were incubated at 37°C and harvested at 7 days post inoculation (p.i.). Total intracellular DNA was extracted as described (Qiao, unpublished), and the DHBV DNA content in each sample was analyzed by Southern blot hybridization.

2.10. SEROLOGICAL ASSAYS.

2.10.1. DHBsAg.

The presence of DHBsAg in duck serum was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Jilbert *et al.,* 1996). A 96-well microdilution plate (Disposable Products Pty. Ltd) was coated with 100 µl of rabbit anti-DHBs IgG (Qiao *et al.*, 1990) at a dilution of 1/500 in 0.1 M $NaHCO_3$ pH 9.6 for 1 hr at 37°C, then at 4°C overnight. Samples of duck serum (100 µl) were serially diluted at five-fold dilutions (starting at 1/100 in PBS) and were added to the plate, and incubated at 37°C for 1 hr. Non-specific sites were blocked by the addition of 100 µl of 5% skim milk (Carnation) in PBS containing 0.5% Tween 20 (PBS-T) and incubation at 37°C, for 1 hr. The plates were then subsequently incubated with 100 µl of an anti-pre-S MAb 1H.1 (Pugh *et al.,* 1995) at a dilution of 1/10,000, followed by 100 µl of horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Amersham) at a dilution of 1/4,000. The presence of DHBsAg was visualized by addition of 100 µl of HRP substrate {40 mg of *o*-phenylendiamine (Sigma), 0.012% H_2O_2 in 100 ml of 0.1 M citrate-phosphate buffer (Appendix)} and incubation in the dark for 15 min; the reaction was stopped by adding 50

µl of 2.5 M H_2SO_4. The optical density at 490 nm (OD_{490}) was read on an automatic ELISA reader (Dynatech MR5000). The amount of DHBsAg in the samples was calculated by comparison with a pool of congenitally DHBV-infected duck serum that contained 50 µg/ml DHBsAg (Jilbert *et al.,* 1996).

In both the DHBsAg assay (above) and the total anti-DHBs antibody assay (see Section 2.10.2), all incubation steps were carried out at 37°C for 1 hr. Plates were washed three times with PBS-T between each step, except prior to addition of the HRP substrate, when they were washed three times with PBS. All dilutions were made in PBS-T containing 5% skim milk.

2.10.2. Total anti-DHBs antibodies.

Total anti-DHBs antibodies were measured by antibody capture-ELISA as described previously (Jilbert *et al.,* 1998). One hundred microliter samples of anti-pre-S MAb 1H.1 (ascites fluid at a dilution of 1/10,000 in 0.1 M $NaHCO_3$ pH 9.6) was used to coat the ELISA plate at 37°C for 1 hr, then at 4°C overnight. Non-specific sites were blocked with 150 µl of 5% skim milk in PBS-T, and then 100 µl of DHBsAg {1 ng/µl; purified on sucrose gradients from serum of congenitally DHBV-infected ducks (see Section 2.5.6)} was added to each well. The plates were then incubated with five-fold dilutions of individual serum samples (starting at a dilution of 1/25) and then with 100 µl of rabbit anti-duck IgY (purified from duck egg yolk; Bertram, 1997) at a dilution of 1/5,000. Finally, the plates were incubated with 100 µl of HRP-conjugated goat anti-rabbit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at a dilution of 1/5,000.

Bound antibody was visualized by the addition of HRP substrate as described above. The antibody titer in each serum sample was defined as the highest serum dilution that resulted in an OD 490nm of 0.5. In some cases, relative levels of antibody are expressed as OD 490nm readings.

2.10.3. Anti-S antibodies.

Yeast-derived (recombinant) DHBV S protein was used to coat ELISA plates to detect anti-S specific antibodies. One hundred microliters of purified yeast-derived S protein (1 ng/µl) in 0.1 M $NaHCO_3$ (pH 9.6) was added to each well and incubated at 37°C overnight. Non-specific sites were blocked with 100 µl of 5% skim milk in PBS-T, and the plates were incubated with fivefold dilutions of serum (starting at a dilution of 1/25). Subsequent steps were performed exactly as for the total anti-DHBs assay.

2.10.4. Anti-DHBc antibodies.

Anti-core antibodies were detected using an antibody-capture ELISA as described (Jilbert *et al.*, 1998). Plates were coated with 100 µl of recombinant DHBcAg (Jilbert *et al.*, 1992) at a concentration of 1 µg/ml in PBS, and incubated at 37°C for 1 hr, then at 4°C overnight. Non-specific sites were blocked with 100 µl of 5% skim milk in PBS-T at 37°C for 1 hr, then 100 µl of each serum sample (starting at a dilution of 1/100 in 5% BSA-PBS-T) was added, and diluted in five-fold steps across the plate. Plates were then incubated with 100 µl of rabbit anti-duck IgY (Bertram, 1997) at a dilution of 1/5,000 for 1 hr, followed by 100 µl of HRP-conjugated goat anti-rabbit (Kirkegaard & Perry

Laboratories, Inc., Gaithersburg, Md) at a dilution of 1/5,000. Visualization of bound antibodies was performed as above, and the antibody titer in each serum sample was defined as the highest serum dilution that resulted in an OD 490nm of 0.5. In some cases, relative levels of antibody are expressed as OD 490nm readings.

2.11. TECHNICAL APPENDIX.

2.11.1. Media.

Luria Bertani (LB) medium:

10 g of Bacto tryptone (Difco), 5 g of Bacto yeast extract (Difco), and 10 g of NaCl per liter medium. Adjust the pH to 7.0. To make solid media, 2% Bacto agar (Difco) was added. Sterilize by autoclaving.

Protease inhibitor cocktails (100 x solution):

200 µg/ml aprotinin (Sigma), 10 mM sodium metabisulfite, 50 µg/ml leupeptin trifluoroacetate salt (Sigma), 100 µg/ml pepstatin A (Sigma), 10 mM benzamidine HCl.

SOB medium:

2% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$. Mix tryptone, yeast extract, NaCl and KCl in the required volume of DW, and autoclave. Make a 2 M stock of Mg^{2+}, consisting of 1 M $MgCl_2$ and 1 M $MgSO_4$, and sterilize by filtration through a 0.22 µm membrane.

Similarly prepare a 2 M stock of glucose and store at -20°C. Just prior to use, combine the medium with Mg^{2+} (and glucose for SOC) and sterilize by filtration.

SOC medium:

Identical to SOB medium with the addition of 20 mM glucose.

SC-*ura* medium:

0.67% yeast nitrogen base without amino acids (Difco), 2% sucrose (BDH). To induce DHBV pre-S/S and S expression in yeast, sucrose was substituted with 2% D(+) galactose (Sigma). To make solid media, 2% Bacto-agar (Difco) was added. Sterilize by autoclaving, cool to 50°C then add 1/10 volume of a 10 x solution of *ura* dropout powder.

YPAD medium:

1% (w/v) Bacto yeast extract (Difco), 2% (w/v) Bacto peptone (Difco), 40 mg/l adenine (Sigma), 2% (w/v) D-glucose (BDH). Sterilize by autoclaving.

***Ura* dropout powder (10 x solution):**

Prepare a premix of *ura* dropout powder containing:

Amino acids (Sigma)	Amount in dropout powder (g)	Final conc. in prepared media (µg/100 ml)
adenine (hemisulfate salt)	2.5 g	40
L-arginine (HCl)	1.2 g	20
L-aspartic acid	6.0 g	100
L-glutamic acid	6.0 g	100
L-histidine	1.2 g	20
L-leucine	3.6 g	60
L-lysine	1.8 g	30
L-methionine	1.2 g	20
L-phenylalanine	3.0 g	50
L-serine	22.5 g	375
L-theonine	12.0 g	200
L-tryptophan	2.4 g	40
L-tyrosine	1.8 g	30
L-valine	9.0 g	150

To make a 10 x solution of *ura* dropout powder: dissolve 13 g of *ura* dropout powder in 100 ml distilled water. Sterilize by filtration and store at 4°C.

2.11.2. Antibiotics and antiseptics.

Ampicillin 100 mg/ml:

A stock solution of 100 mg/ml ampicillin (Promega) was prepared in DW, and stored in 0.5 ml aliquots at -20°C. Ampicillin was added into the culture media at a concentration of 60 μg/ml (for *E. coli* TOP 10F'), or 100 μg/ml (for *E. coli* DH5α).

Flexible Collodion B.P. solution:

Pyroxylin 1.6% (w/v), ethanol 21.6% (v/v), and diethyl ether 70% (v/v). The solution was purchased from Biotech Pharmaceuticals.

Tetracyclin 5 mg/ml:

A stock solution of tetracyclin (Promega) was prepared at 5 mg/ml in 97% ethanol and was stored in 5 ml aliquots at -20°C. Tetracyclin was added into the culture media at a concentration of 15 μg/ml.

2.11.3. Dyes.

Coomassie brilliant blue:

0.025% (w/v) Coomassie brilliant blue R-250 (Bio-Rad), 25% (v/v) methanol, 10% (v/v) acetic acid. Filter though Whatman filter paper.

DNA loading buffer (10 x):

60% sucrose, 1% sarkosyl, 1 x TAE buffer, 0.1% bromophenol blue (Sigma), 0.1% xylene cyanol (Ajax Chemicals). Dispense into 500 µl aliquots and store at 4°C.

Ponceau S:

0.1% (w/v) Ponceau S powder (Sigma), dissolve in 5% acetic acid (v/v).

Store in the dark at RT.

2.11.4. Buffers.

1 x PBS (0.13 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4):

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per 1 liter medium. Adjust pH to 7.4. Sterilize by autoclaving.

20 x SSC (2.99 M NaCl, 0.29 M sodium citrate):

175.3 g of NaCl and 88.2 g of sodium citrate per liter. Adjust the pH to 7.0 with 10 N NaOH. Sterilize by autoclaving.

50 x TAE buffer:

484 g Tris base, 114.2 ml glacial acetic acid, 37.2 g EDTA per 2 liter solution.

2.11.5. ELISA reagents.

Coating buffer:

0.1 M $NaHCO_3$ pH 9.6. Adjust the pH with 5 N NaOH. Sterilize by autoclaving.

0.1 M citrate-phosphate buffer pH 5.0:

Mix 12.125 ml of 0.1 M citric acid and 12.875 ml of 0.2 M phosphate (Na_2HPO_4) buffer.

HRP substrate:

Mix 40 mg of *o*-phenylendiamine (Sigma) and 40 µl H_2O_2 per 100 ml citrate-phosphate buffer pH 5.0. Make the solution prior to use in an appropriate tube wrapped with aluminium foil.

2.11.6. SDS-PAGE reagents.

Separating gel (12% acrylamide):

7.5 ml of 40% acrylamide/bis solution (19:1) (Bio-Rad), 11 ml of 0.75 M Tris-HCl pH 8.8, 3 ml DW. The solution was de-aerated for 5 min, then mixed with 470 µl of 10% (w/v) SDS, 95 µl of 10% (w/v) ammonium persulfate (APS) (Sigma), and 16 µl of N, N, N', N'-tetramethylethylenediamine (TEMED) (Sigma). The solution was poured into the gel apparatus and overlaid with DW. The separating gel was allowed to polymerize for ~1 hr before pouring the stacking gel.

Stacking gel (6% acrylamide):

1.25 ml of 40% acrylamide/bis solution (19:1) (Sigma), 3.75 ml of 0.25 M Tris-HCl pH 6.8, and 2.4 ml DW. The solution was de-aerated for 5 min, then mixed with 75 µl of 10% SDS, 18 µl of 10% APS, and 15 µl of TEMED. The stacking gel was poured on top of the separating gel after the overlaid DW was decanted, and finally the comb was inserted.

SDS-PAGE running buffer:

25 mM Tris base, 192 mM glycine, 0.1% SDS.

SDS-PAGE loading buffer (10 x):

3% SDS, 2% β-mercaptoethanol, 10% sucrose, 0.1% bromophenol blue, 5 mM EDTA, 200 mM Tris-HCl pH 8.8.

2.11.7. Southern blot hybridization reagents.

Pre-hybridization solution:

50% deionized formamide, 500 µg/ml denatured salmon sperm DNA, 0.1% (w/v) polyvinyl pyrolodone (PVP), 0.1% (w/v) Ficoll 400, 1% (w/v) glycine, 50 mM Na_2HPO_4, NaH_2PO_4 buffer pH 6.5, 1 mg/ml BSA, 5 x SSC. Store at -20°C.

Hybridization solution:

50% deionized formamide, 100 μg/ml denatured salmon sperm DNA, 0.02% (w/v) PVP, 0.02% Ficoll 400, 25 mM Na_2HPO_4, NaH_2PO_4 buffer pH 6.5, 0.2 mg/ml BSA, 5 x SSC, 10% (w/v) dextran sulphate (Sigma). Store at -20°C.

Lambda-*Pst* I DNA standard

[Lambda DNA (48,502 bp) digested with *Pst* I].

Fragment size (bp)	ng DNA/Fragment/Total DNA loaded			
	125	250	375	500
11502	30	60	90	120
5077	13	26	39	53
4749	12.5	25	37.5	50
4507	11.3	22.5	33.8	45
2838	7.4	14.8	22.1	29.5
2556 }				
2459 }	19.3	38.5	57.8	77
2443 }				
2140	5.5	11	16.5	22
1959	5	10	15	20
1700	4.4	8.8	13.1	17.5
1159	3	6	9	12
1093	2.9	5.6	8.6	11.5
805	2.1	4.3	6.4	8.5
514	1.4	2.8	4.1	5.5
468 }				
448 }	2.4	4.8	7.1	9.5
339	0.9	1.8	2.6	3.5
264	0.6	1.3	1.9	2.5
247	0.6	1.3	1.9	2.5
216 }				
211 }	1.5	3.0	4.5	6.0
200 }				
164	0.4	0.8	1.1	1.5
150	0.4	0.8	1.1	1.5
99	0.3	0.5	0.8	1.0
94	0.3	0.5	0.8	1.0
87	0.3	0.5	0.8	1.0
15	0.04	0.08	0.11	0.15

Table 2.2. Physical specifications of Beckman preparative ultracentrifuge rotors.

Rotor	Maximum Speed (rpm)	Maximum Force (*g*)	*k* Factor	*k* Factor for Half-Filled Tubes	Number of Tubes x Nominal Tube Volume (ml)	Nominal Rotor Capacity (ml)	Rotor Radius (mm)	
							r_{min}	r_{max}
Fixed Angle								
Type 80 Ti	80,000	602,000	28	12	8 x 13.5	108	41.0	84.0
Type 75 Ti	75,000	502,000	35	14	8 x 13.5	108	36.9	79.7
Type 70 Ti	70,000	505,000	44	18	8 x 38.5	308	39.0	92.0
Type 70.1 Ti	70,000	450,000	37	15	12 x 13.5	162	40.0	82.0
Type 65	65,000	368,000	45	19	8 x 13.5	108	36.8	77.8
Type 60 Ti	60,000	363,000	63	25	8 x 38.5	308	37.0	90.0
Type 55.2 Ti	55,000	340,000	64	26	10 x 38.5	385	46.8	100.3
Type 50.2 Ti	50,000	302,000	72	29	12 x 38.5	462	53.0	108.0
Type 50 Ti	50,000	226,000	78	32	12 x 13.5	162	37.4	80.8
Type 50.3 Ti	50,000	224,000	52	23	18 x 6.5	117	48.0	80.0
Type 50	50,000	199,000	66	28	10 x 10	100	37.0	71.0
Type 45 Ti	45,000	236,000	129	48	6 x 94	564	37.0	104.0
Type 42.2 Ti	42,000	223,000	9	—	72 x 230 µl	16.5	104.0	113.0
Type 42.1	42,000	196,000	134	52	8 x 38.5	308	39.0	99.0
Type 40	40,000	145,000	120	50	12 x 13.5	162	38.0	81.0
Type 40.3	40,000	143,000	81	35	18 x 6.5	117	48.0	80.0
Type 40.2	40,000	143,000	131	54	12 x 6.5	78	35.0	80.0
Type 35	35,000	143,000	225	82	6 x 94	564	35.0	104.0
Type 30	30,000	106,000	209	84	12 x 38.5	462	50.0	105.0
Type 30.2	30,000	94,800	113	49	20 x 10.5	210	63.0	94.0
Type 25	25,000	92,500	62	29	100 x 1	100	113.4	132.1
Vertical Tube								
VTi 80	80,000	510,000	8	—	8 x 5.1	40.8	57.9	71.1
VTi 65	65,000	404,000	10	—	8 x 5.1	40.8	72.1	85.4
VTi 50	50,000	242,000	36	—	8 x 39	312	60.8	86.6
VAI 26	26,000	70,300	122	—	8 x 39	312	67.1	92.9
Swinging Bucket								
SW 65 Ti	65,000	421,000	46	20	3 x 5.0	15	41.2	89.0
SW 60 Ti	60,000	485,000	45	19	6 x 4.4	26.4	63.1	120.3
SW 55 Ti	55,000	369,000	49	20	6 x 5.0	30	61.0	109.0
SW 50.1	50,000	300,000	59	25	6 x 5.0	30	59.7	107.3
SW 41 Ti	41,000	286,000	125	50	6 x 13.2	79.2	67.0	152.0
SW 40 Ti	40,000	285,000	137	54	6 x 14	84	66.7	158.8
SW 28.1	28,000	150,000	276	109	6 x 17	102	72.9	171.3
SW 28	28,000	141,000	245	99	6 x 38.5	231	75.3	161.0
SW 25.2	25,000	107,000	335	133	3 x 60	180	66.7	152.3
SW 25.1	25,000	90,400	338	134	3 x 34	102	56.2	129.2
Continuous Flow					Flow Rate			
CF-32 Ti	32,000	102,000	42	—	to 9 l/h	430	80.0	89.0
Zonal					Sample Volume (ml)			
Z-60	60,000	256,000	101	33*	10-15	330	15.5	63.5
Ti-14	48,000	172,000	173	55*	20-50	665	26.7	66.7
Al-14	35,000	91,000	328	105*	20-50	665	26.7	66.7
Ti-15	32,000	102,000	459	135*	50-200	1675	29.1	88.9
Al-15	22,000	48,000	968	286*	50-200	1675	29.1	88.9

**These rotors must be run full. If half the normal gradient/sample volume is used, add buffer solution to fill the rotor.*

Table 2.3. Values of time integral for sucrose gradient centrifugation.

Temperature 5.0°C Particle Density 1.30

WT. PCT. SUCROSE	Z0=5	Z0=0	Z0=-5	Z0=-10	Z0=-15	Z0=-20	Z0=-25	Z0=-30	Z0=-40	Z0=-60	Z0=-100
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0000	0.0000	0.2648	0.1437	0.0987	0.0752	0.0607	0.0509	0.0385	0.0259	0.0156
2	0.0000	0.0000	0.5296	0.2873	0.1973	0.1503	0.1213	0.1018	0.0769	0.0517	0.0312
3	0.0000	0.5930	0.7450	0.4195	0.2927	0.2249	0.1827	0.1538	0.1169	0.0790	0.0479
4	0.0000	1.1860	0.9603	0.5516	0.3881	0.2995	0.2440	0.2058	0.1568	0.1062	0.0646
5	0.0000	1.5655	1.1484	0.6768	0.4820	0.3746	0.3066	0.2594	0.1985	0.1351	0.0825
6	0.0000	1.9449	1.3364	0.8020	0.5758	0.4497	0.3691	0.3130	0.2402	0.1640	0.1003
7	1.1347	2.2408	1.5084	0.9233	0.6695	0.5261	0.4335	0.3688	0.2841	0.1948	0.1196
8	2.2693	2.5367	1.6804	1.0446	0.7632	0.6024	0.4979	0.4245	0.3279	0.2255	0.1389
9	2.8487	2.7903	1.8431	1.1645	0.8581	0.6809	0.5649	0.4829	0.3744	0.2585	0.1598
10	3.4280	3.0439	2.0058	1.2843	0.9530	0.7594	0.6318	0.5412	0.4208	0.2915	0.1806
11	3.8521	3.2740	2.1639	1.4047	1.0502	0.8410	0.7021	0.6029	0.4704	0.3271	0.2034
12	4.2762	3.5041	2.3219	1.5251	1.1474	0.9225	0.7723	0.6645	0.5200	0.3627	0.2262
13	4.6302	3.7214	2.4788	1.6478	1.2482	1.0081	0.8466	0.7302	0.5733	0.4014	0.2512
14	4.9841	3.9387	2.6356	1.7705	1.3490	1.0936	0.9208	0.7958	0.6265	0.4400	0.2761
15	5.3017	4.1502	2.7942	1.8974	1.4547	1.1842	1.0001	0.8663	0.6842	0.4823	0.3037
16	5.6193	4.3617	2.9528	2.0242	1.5604	1.2748	1.0794	0.9367	0.7418	0.5246	0.3313
17	5.9184	4.5727	3.1158	2.1571	1.6725	1.3717	1.1648	1.0130	0.8047	0.5712	0.3620
18	6.2175	4.7837	3.2788	2.2899	1.7846	1.4686	1.2501	1.0893	0.8676	0.6177	0.3926
19	6.5093	4.9986	3.4490	2.4307	1.9047	1.5733	1.3430	1.1727	0.9369	0.6694	0.4269
20	6.8011	5.2135	3.6191	2.5715	2.0247	1.6780	1.4358	1.2560	1.0061	0.7211	0.4612
21	7.0940	5.4366	3.7993	2.7226	2.1549	1.7923	1.5377	1.3479	1.0829	0.7790	0.5000
22	7.3869	5.6597	3.9794	2.8737	2.2850	1.9065	1.6395	1.4398	1.1597	0.8368	0.5387
23	7.6879	5.8953	4.1729	3.0379	2.4276	2.0326	1.7524	1.5420	1.2457	0.9021	0.5828
24	7.9889	6.1309	4.3664	3.2021	2.5702	2.1586	1.8652	1.6442	1.3317	0.9674	0.6268
25	8.3049	6.3837	4.5771	3.3827	2.7282	2.2990	1.9916	1.7591	1.4290	1.0418	0.6774
26	8.6209	6.6364	4.7877	3.5632	2.8862	2.4394	2.1180	1.8740	1.5262	1.1161	0.7280
27	8.9590	6.9119	5.0202	3.7642	3.0633	2.5977	2.2611	2.0045	1.6372	1.2017	0.7866
28	9.2970	7.1873	5.2526	3.9652	3.2403	2.7559	2.4041	2.1350	1.7482	1.2872	0.8451
29	9.6654	7.4922	5.5127	4.1920	3.4414	2.9364	2.5679	2.2850	1.8764	1.3866	0.9137
30	10.0338	7.7971	5.7728	4.4188	3.6424	3.1169	2.7317	2.4349	2.0046	1.4859	0.9823
31	10.4427	8.1401	6.0681	4.6781	3.8735	3.3254	2.9216	2.6092	2.1544	1.6028	1.0635
32	10.8515	8.4830	6.3634	4.9374	4.1046	3.5339	3.1114	2.7835	2.3041	1.7197	1.1447
33	11.3135	8.8750	6.7038	5.2382	4.3741	3.7780	3.3345	2.9889	2.4814	1.8588	1.2420
34	11.7754	9.2669	7.0442	5.5390	4.6436	4.0221	3.5575	3.1942	2.6586	1.9979	1.3392
35	12.3077	9.7232	7.4435	5.8939	4.9631	4.3125	3.8238	3.4400	2.8716	2.1661	1.4576
36	12.8399	10.1794	7.8427	6.2488	5.2825	4.6029	4.0900	3.6857	3.0846	2.3343	1.5759
37	13.4655	10.7205	8.3194	6.6749	5.6676	4.9542	4.4130	3.9846	3.3447	2.5408	1.7222
38	14.0910	11.2615	8.7961	7.1009	6.0526	5.3055	4.7360	4.2835	3.6048	2.7472	1.8684
39	14.8429	11.9171	9.3772	7.6227	6.5262	5.7390	5.1356	4.6542	3.9286	3.0056	2.0524
40	15.5947	12.5726	9.9582	8.1445	6.9997	6.1724	5.5351	5.0248	4.2523	3.2639	2.2364
41	16.5216	13.3865	10.6837	8.7989	7.5957	6.7196	6.0409	5.4950	4.6644	3.5945	2.4732
42	17.4484	14.2003	11.4091	9.4533	8.1917	7.2667	6.5466	5.9651	5.0765	3.9251	2.7100
43	18.6219	15.2375	12.3383	10.2949	8.9608	7.9748	7.2027	6.5763	5.6141	4.3583	3.0221
44	19.7953	16.2746	13.2675	11.1365	9.7299	8.6829	7.8587	7.1874	6.1516	4.7915	3.3342
45	21.3234	17.6330	14.4902	12.2481	10.7489	9.6236	8.7323	8.0027	6.8711	5.3740	3.7561
46	22.8514	18.9914	15.7128	13.3596	11.7679	10.5642	9.6058	8.8180	7.5906	5.9565	4.1779
47	24.9082	20.8296	17.3744	14.8756	13.1617	11.8541	10.8062	9.9405	8.5841	6.7644	4.7661
48	26.9649	22.6677	19.0360	16.3916	14.5555	13.1440	12.0065	11.0630	9.5776	7.5723	5.3542
49	29.8450	25.2542	21.3833	18.5401	16.5364	14.9814	13.7199	12.6680	11.0024	8.7358	6.2055
50	32.7251	27.8407	23.7305	20.6886	18.5172	16.8188	15.4332	14.2729	12.4272	9.8993	7.0568
51	36.9604	31.6613	27.2104	23.8835	21.4703	19.5642	17.9981	16.6796	14.5696	11.6559	8.3483
52	41.1957	35.4819	30.6902	27.0784	24.4234	22.3095	20.5630	19.0863	16.7120	13.4125	9.6398
53	47.8271	41.4886	36.1797	32.1328	29.1066	26.6722	24.6464	22.9239	20.1373	16.2320	11.7226
54	54.4584	47.4952	41.6692	37.1872	33.7897	31.0349	28.7297	26.7615	23.5626	19.0515	13.8054
55	65.8345	57.8388	51.1522	45.9418	41.9199	38.6238	35.8448	33.4586	29.5555	24.0031	17.4799
56	77.2105	68.1824	60.6352	54.6964	50.0500	46.2126	42.9599	40.1557	35.5483	28.9546	21.1544
57	99.8914	88.8782	79.6655	72.3092	66.4419	61.5420	57.3560	53.7257	47.7213	39.0491	28.6793
58	122.5723	109.5740	98.6957	89.9220	82.8338	76.8713	71.7521	67.2957	59.8942	49.1435	36.2041
59	188.1689	169.6339	154.0808	141.3076	130.7586	121.7720	113.9878	107.1650	95.7462	78.9823	58.5478
60	253.7655	229.6937	209.4658	192.6931	178.6833	166.6727	156.2235	147.0343	131.5981	108.8210	80.8914

Chapter 3

Cloning and sequencing of an Australian DHBV strain

3.1. INTRODUCTION AND AIMS.

As members of the hepadnavirus family, DHBV and HBV share a common genomic structures, although the DHBV genome is ~200 bp shorter (3021-3027 bp in different strains; Sprengel *et al.,* 1991) and contains only 3 ORFs (the P, S, and pre-C/C genes; Sprengel *et al.,* 1985). The P gene of DHBV encodes the DNA polymerase (P protein) which has a molecular weight of approximately 90 kDa (Hu & Seeger, 1996; Uchida *et al.,* 1989). As in the HBV, the P protein of DHBV exhibits reverse transcriptase (RT) and RNase H activities, and can be divided into four functional domains: the N-terminal domain, the spacer, the central reverse transcriptase domain, and the C-terminal RNase H domain (Hu and Seeger, 1996).

Similar to HBV, the pre-C/C gene of DHBV contains two in-frame start codons with a common carboxy terminus. Translation from the first ATG produces e antigen (DHBeAg) that is found as 33, 30, and 27 kDa proteins. The 33- and 30-kDa protein are the doubly and singly glycosylated forms of the 27 kDa non-glycosylated DHBeAg. In contrast, HBeAg is a non-glycosylated protein (Schneider *et al.,* 1991). As with HBeAg, DHBeAg is not essential for core particle formation, viral DNA replication or virion formation (Chang *et al.,* 1987; Schlicht *et al.,* 1987b). It has been shown that DHBeAg is detected rather late in DHBV infection, which may be due to the low-level of putative pre-C mRNA that is produced which, at best, is only 5% of the amount of C mRNA (Schneider *et al.,* 1991). On the other hand, HBeAg is regarded as an early marker of active viral replication and can be detected around the same time as the pre-S protein and HBV DNA (Lok *et al.,* 1985).

Translation from the second ATG in the pre-C/C gene of DHBV produces core (nucleocapsid) protein with a molecular weight of approximately 30 kDa. Despite the presence of two well-conserved glycosylation signals (Asn-Ala-Ser and Asn-Val-Thr) at AA positions 4 and 160 of the C region, the core protein of DHBV is not glycosylated. This presumably is due to the fact that during its assembly, the core protein does not enter the secretory pathway where the glycosylation process takes place (Schlicht *et al.,* 1987b).

By analogy to HBV, the S gene of DHBV is divided into the pre-S and the S regions. This gene encodes two surface proteins, the pre-S/S (large) and S (small) proteins, respectively. There are several start codons within the pre-S region of this gene, but it is likely that the major pre-S/S protein is translated from the second ATG (nt 801). This prediction resulted in a protein product of 330 AA that consistent with the size of pre-S/S protein which has molecular weight of approximately 37 kDa (Summers *et al.,* 1990). Results from a previous study have also shown that the 2.1 kb mRNA transcript that serves as a template for translation of the DHBV pre-S/S protein corresponds to the second AUG of the pre-S/S sequence (Büscher *et al.,* 1985). The internal ATGs within the pre-S region at nt positions 825, 882 and 957, were previously shown to encode the minor pre-S/S proteins of 35, 33 and 30 kDa, respectively, which were occasionally detected in the liver cell extracts of DHBV-infected ducks (Fernholz *et al.,* 1993). The existence of a DHBV pre-S2 protein similar to that of HBV remains uncertain.

The S region of the S gene encodes the S protein that consists of 167 AA with a molecular weight of 17 kDa. In contrast to HBV, the S protein of DHBV is not glycosylated

(Schlicht *et al.,* 1987a), although the glycosylation signal (Asn-X-Ser/Thr) is conserved within the S domain of all DHBV isolates (Sprengel *et al.,* 1985; Uchida *et al.,* 1989).

At the beginning of this study, 11 published DHBV and HHBV sequences from different geographical isolates were already available (Mandart *et al.*, 1984; Sprengel *et al.*, 1985, Uchida *et al.*, 1989; Sprengel *et al.*, 1991), although none of them had been isolated from DHBV-infected Pekin ducks in Australia. Based on the percentage of DNA sequence divergence between those 11 isolates, an evolutionary tree of avian hepadnaviruses had been proposed which consisted of three major branches, namely Chinese DHBV, Western country DHBV, and HHBV (Sprengel *et* al., 1991). This tree, however, did not include the new DHBV isolate from India, DHBVCG (Munshi *et al.,* 1993), and DHBV isolate from Ross goose, HPUGENM (Shi *et al.,* 1993), that were reported afterwards.

The DHBV strain (AusDHBV) used throughout this study, was purified from serum of congenitally DHBV-infected ducks obtained from a single commercial duck flock in Australia (Jilbert *et al.,* 1996). Although the ID_{50} and $TCID_{50}$ of this virus pool had previously been determined (Jilbert *et al.,* 1996; Qiao, unpublished), the heterogeneity of the pool had not been assessed and the genome of AusDHBV had not been cloned.

The aims of this study were: (i) to clone and sequence the AusDHBV genome, subsequently, allowing the design of primers for PCR and the choice of cloning sites, (ii) to locate the AusDHBV strain within the phylogenetic tree of the avian hepadnaviruses based on a comparison of the sequence divergence with other isolates.

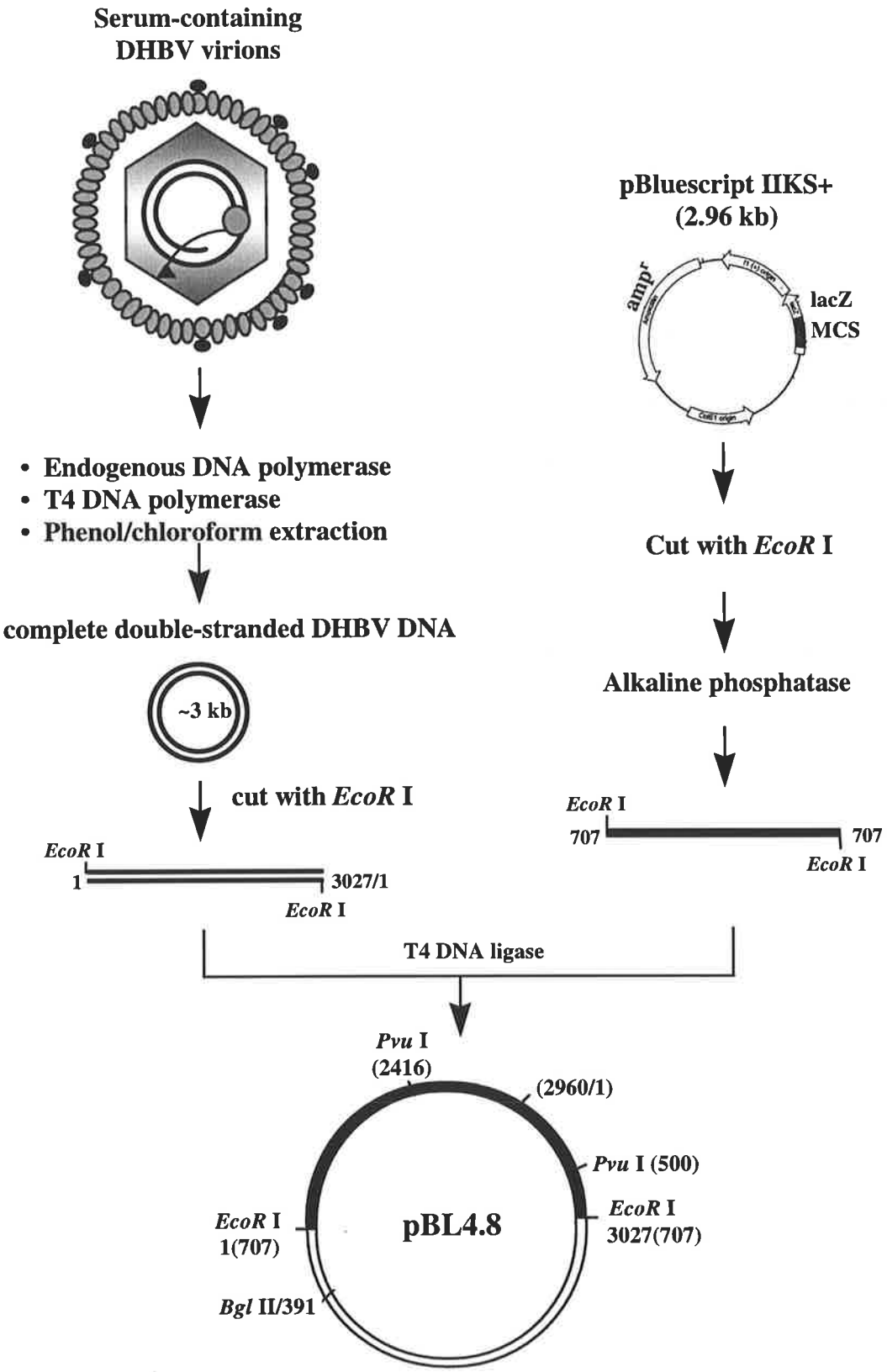
3.2. EXPERIMENTAL DESIGN.

Virus particles were isolated from 20 ml of congenitally DHBV-infected duck serum by pelleting through 20% sucrose and viral DNA was purified from virions following two polymerase reactions to form a complete double-stranded DNA. First, the short (positive) strand of viral DNA was repaired by endogenous DNA polymerase. Second, the nick at the 5' end on the long (negative) strand was repaired by the addition of T4 DNA polymerase (Section 2.4).

The full length genome of double-stranded viral DNA was cloned into the *Eco*R I site of pBluescript IIKS+. An overview of the cloning strategy is shown in Fig. 3.1. Following transformation of *E. coli* DH5α, transformants were identified on LB plates using a screening method based on α-complementation of β-galactosidase activity (Section 2.1). To confirm correct size and orientation of the recombinant plasmids, they were screened by restriction enzyme (RE) analysis and Southern blot hybridization using a [α-^{32}P]dCTP-labeled pSP.DHBV5.1 DNA probe (see below).

The restriction pattern of the AusDHBV clone was compared to the USA DHBV clone (pSP.DHBV5.1, a full-length genome of DHBV16 cloned in the pSP65 vector) (Tuttleman *et al.,* 1986) and to a published Chinese DHBV isolate, DHBV-S31 (Uchida *et al.,* 1989). Five restriction enzymes were chosen which could distinguish between the two major branches of the avian hepadnaviruses, namely the Chinese and the Western country DHBV isolates. This restriction pattern analysis did not include the HHBV that has the most distant relationship with other DHBV isolates (Sprengel *et al.,* 1991).

Figure 3.1. Schematic of the cloning of a full-length AusDHBV genome into pBluescript IIKS+. Virus particles were isolated from congenitally DHBV-infected duck serum, and the partially double-stranded viral DNA was repaired *in situ* by two DNA polymerase reactions. The *Eco*R I-digested DHBV DNA was cloned into the *Eco*R I site of pBluescript IIKS+ and the recombinant plasmid (pBL4.8) containing the full-length DHBV DNA in the same orientation as the lacZ promoter, was obtained. The restriction enzyme sites used for screening of transformants are shown (numbers in parentheses are the restriction sites located within pBluescript IIKS+). The nucleotide positions in AusDHBV genome have been numbered with *Eco*R I at position 1/3027. *amp*^{r}, ampicillin resistance gene; lacZ, lacZ promoter that provides α-complementation for blue/white color selection of recombinant plasmids; MCS, multi cloning sites.


Serum-containing
DHBV virions
• Endogenous DNA polymerase
• T4 DNA polymerase
• Phenol/chloroform extraction
complete double-stranded DHBV DNA
~3 kb
cut with EcoR I
EcoR I
1
3027/1
EcoR I
pBluescript IIKS+
(2.96 kb)
ampr
lacZ
MCS
Cut with EcoR I
Alkaline phosphatase
EcoR I
707
707
EcoR I
T4 DNA ligase
Pvu I
(2416)
(2960/1)
Pvu I (500)
EcoR I
3027(707)
pBL4.8
EcoR I
1(707)
Bgl II/391

Nucleotide sequencing of the AusDHBV clone was carried out by 'primer walking' from both strands, starting with the T3 and T7 primers located in the regions flanking the inserted viral DNA. The position of AusDHBV in the phylogenetic tree of avian hepadnaviruses was determined using version 1.02 of *MEGA* (Kumar *et al.*, 1994). The hydrophobicity profile of the pre-S/S protein was analyzed using the PROSIS software program based on the method of Hopp and Woods (1981). The predicted secondary structure of RNA sequences was determined using the RNAdraw software program (Windows).

3.3. RESULTS.

3.3.1. Endogenous viral DNA polymerase assay.

Virus particles were pelleted from 20 ml of DHBV-positive duck serum through 20% sucrose at 230,000 x g for 4 hr (Section 2.4), and an endogenous DNA polymerase assay was performed on a 1/10 portion of the pellet. As can be seen in Fig. 3.2, the maximum incorporation of ^{32}P-labeled dATP and dCTP into viral DNA was achieved within 90 min.

3.3.2. Cloning of the full-length DNA genome of AusDHBV into pBluescript IIKS+.

The recombinant plasmid (pBL4.8) containing the full-length AusDHBV genome in the same orientation as the lacZ promoter (Fig. 3.1) was obtained after screening plasmids harboured by transformants (white colonies) with (i) *Bgl* II and *Pvu* I, and (ii) *Bgl* II and

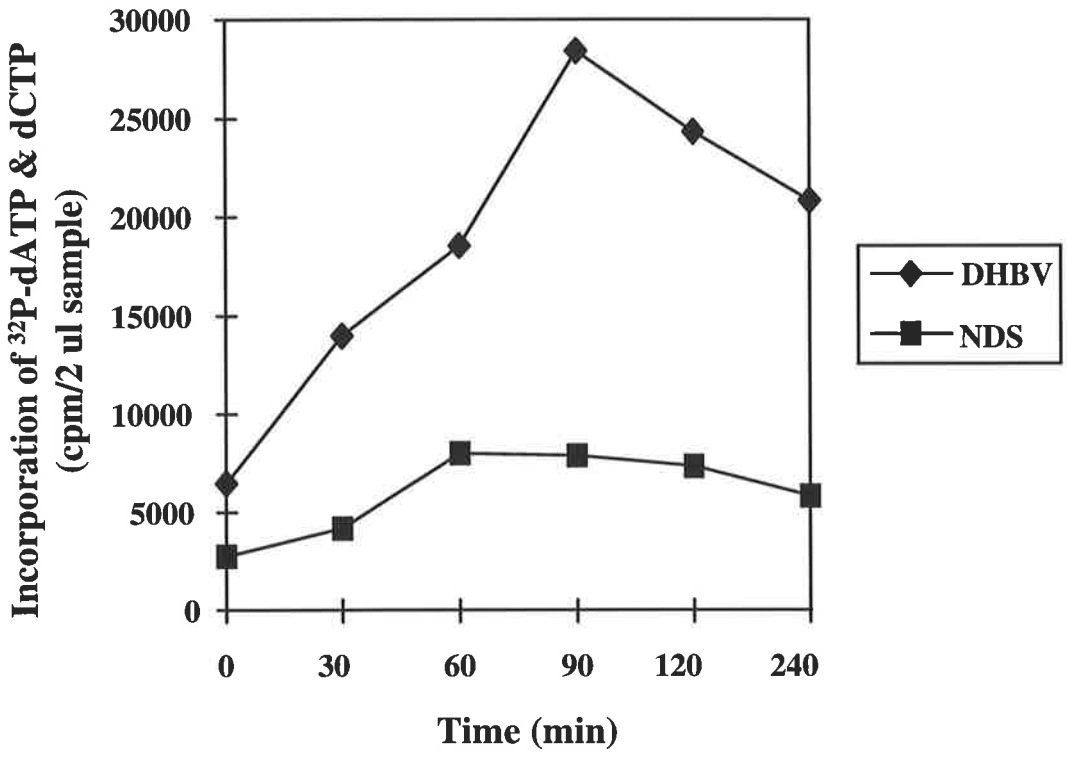


Figure 3.2. The endogenous viral DNA polymerase assay. A 50 microliter aliquot (equal to 2 ml of original serum) of congenitally DHBV-infected duck serum was incubated with ^{32}P-labeled dATP and dCTP (3000Ci/mmol) in an endogenous DNA polymerase assay (Section 2.4.4). Two microliter samples were taken at each time point indicated and were assayed for the incorporation of radiolabeled dNTPs, indicating elongation of the short (positive) strand of viral DNA. NDS (normal duck serum) treated in the same manner was used as a negative control. cpm= counts per minute.

*Eco*R I enzymes. The predicted restriction sites within the AusDHBV genome using these sets of RE as described below were based on the observed similarity of its restriction pattern to that of DHBV-S31, a complete genome of the Chinese DHBV isolate (Uchida *et al.,* 1989; see Section 3.3.3). Both *Bgl* II and *Eco*R I have one restriction site in DHBV-S31, at nt 391 and nt 1/3027, respectively, whereas *Pvu* I has two restriction sites in pBluescript IIKS+ DNA (at nt 500 and nt 2416), but none in DHBV-S31 (Fig. 3.1). Following digestion with (i) and (ii), the pBL4.8 clone showed the expected bands (2843, 2100 and 1044 bp) and (2960, 2636 and 391 bp), respectively, after electrophoresis in a 1.2% agarose gel (Fig. 3.3A). The bands containing DHBV DNA were confirmed by Southern blot hybridization using a ^{32}P-labeled DHBV probe (Fig. 3.3B). The AusDHBV clone (pBL4.8) is most likely to represent the predominant AusDHBV strain found in the pool of congenitally DHBV-infected duck serum used in this study, since 24 transformants (white colonies) screened with both sets of enzymes showed identical RE profiles (data not shown).

3.3.3. Restriction mapping of the AusDHBV clone.

The restriction patterns of the AusDHBV clone and the USA DHBV clone were compared using RE digestion followed by Southern blot hybridization. The results are presented in Table 3.1, which shows that the restriction pattern of the AusDHBV clone was more similar to that of the Chinese isolate/DHBV-S31 (Uchida *et al.,* 1989) than the USA isolate/DHBV16 (Mandart *et al.,* 1984). Therefore, primers for sequencing of the AusDHBV genome (Table 3.2) were designed, based on the DHBV-S31 sequence.

Figure 3.3. (A) Restriction enzyme analysis of the AusDHBV clone. The AusDHBV clone (pBL*4*.8) showed the expected bands after digestion with *Bgl* II and *Pvu* I: 2843, 2100, and 1044 bp (lane 1), and with *Bgl* II and *Eco*R I: 2960, 2636, and 391 bp (lane 2). The digested-DNA samples were electrophoresed on a 1.2% agarose gel. M: DNA marker (*Pst* I-digested λ DNA). **(B) Southern blot analysis of the pBL4.8 clone.** Following restriction enzyme analysis as above, the gel was transferred to a Hybond C-extra membrane and hybridized with a ^{32}P[dCTP]-labeled pSP.DHBV 5.1 DNA probe. Bands containing the DHBV DNA insert (lane 1: 2843 and 2100 bp; lane 2: 2636 and 391 bp) were specifically reactive with the probe. Several low intensity bands (marked as *) were also detected on the Southern blot, possibly due to star activity of the *Eco*R I, or hybridization between the pBluescript IIKS+ and pSp65 (the parental vector of pSP.DHBV 5.1 which was used as probe) sequences.

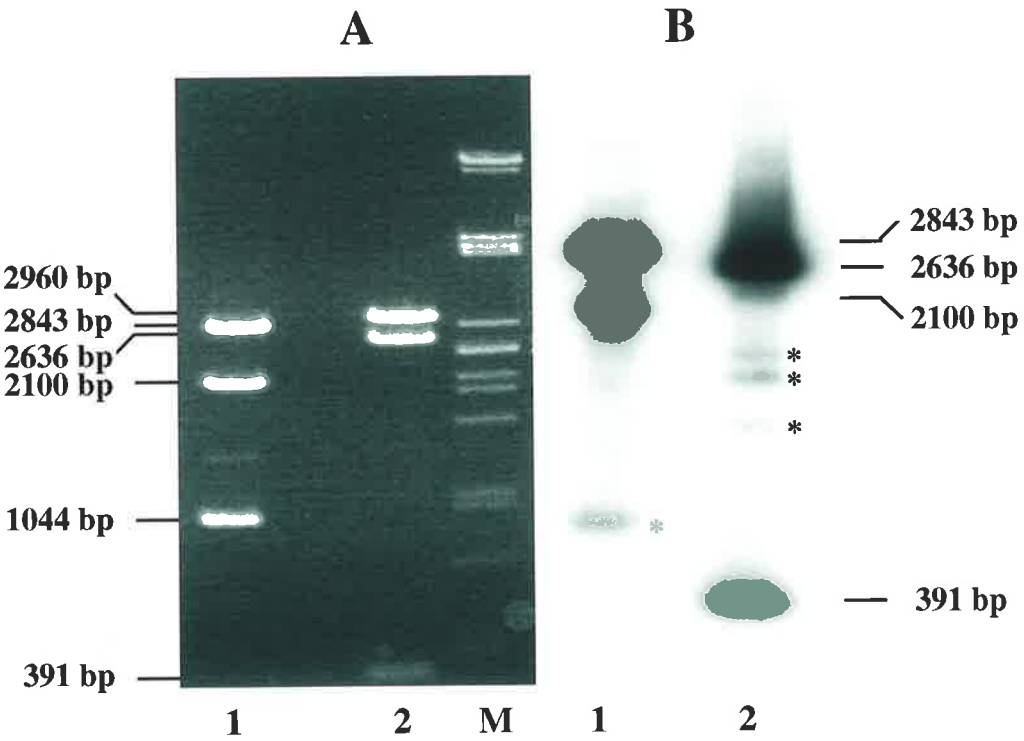

A
B
2960 bp
2843 bp
2636 bp
2100 bp
1044 bp
391 bp
2843 bp
2636 bp
2100 bp
391 bp
1
2
M
1
2

Table 3.1. Comparison of the RE digestion patterns of AusDHBV with the Chinese and the USA DHBV clones.

Restriction enzymes	Chinese DHBV (DHBV-S31)	AusDHBV	USA DHBV (DHBV16)
***Eco*R I**	nt 1/3027	nt 1/3027	nt 1/3021
***Bam*H I**	none	none	nt 1658
***Hpa* I**	nt 1022, 2057	nt 1022, 2057	none
***Sma* I**	none	nt 323	nt 320, 1176
***Xba* I**	nt 2668	nt 2668	nt 1358, 2662

Table 3.2. Primers for sequencing of the AusDHBV clone.

Name of primer (site)	5'-3' sequences	Sense/ complementary
pBL/T3 (MCS of pBS)	ATT AAC CCT CAC TAA AG	**sense**
pBL300 (nt 300-319)	TGG GAG AAG ACG TTC AAA GT	**sense**
pBL699 (nt 699-718)	CAG GAA TCC TTT ATA AGC GG	**sense**
pBL944 (nt 944-963)	TCA TGT GCA AAC AAT GGA GG	**sense**
pBL1561 (nt 1561-1577)	CAG GCT TGC TGT ATC TG	**sense**
pBL1759 (nt 1759-1778)	TAG CCA CGC TGT CTG CAC TT	**sense**
pBL/T7 (MCS of pBS)	AAT ACG ACT CAC TAT AG	**complementary**
pBL2750c (nt 2750-2731)	GCG TCT TTA GCA TCC CTT AC	**complementary**
pBL2379c (nt 2379-2360)	GCC AAC ATA GCA AAA TGC CA	**complementary**
pBL1978c (nt 1978-1959)	CCC GAC AAA TCT TTG AAT AC	**complementary**
pBL1445c (nt 1445-1426)	GTC CGT CAG ATA CAG CAA GC	**complementary**

MCS = multiple cloning sites **pBS = pBluescript 11KS+**

3.3.4. The nucleotide sequence of the cloned AusDHBV genome.

The sequencing strategy used is illustrated in Fig. 3.4 and the complete nucleotide sequence of the negative strand of the AusDHBV clone is presented in Fig. 3.5. The AusDHBV genome is 3027 bp in length and has three overlapping open reading frames (ORFs), termed P, S, and pre-C/C that consist of 2368, 1056 and 918 bp, respectively. The AusDHBV genome, like other avian hepadnaviruses, lacks the X gene present in mammalian hepadnavirus genomes. The AusDHBV clone is the same length as DHBV-S31 and is 6 bp longer than known Western country DHBV genomes (Sprengel *et al.*, 1991). The additional 6 bp were found in the overlapping region of the pre-S and P genes, as occurs in DHBV-S31. As predicted from restriction mapping analysis, the AusDHBV clone showed higher homology to DHBV-S31 than to DHBV16 (95.8% and 89.7% homology, respectively).

The P gene of AusDHBV (nt 170-2536) covers nearly the whole genome and overlaps the S and C genes. At its 5'end, the P gene overlaps the 3' end of the C gene by 243 nt, and in the middle overlaps the entire S gene. At its 3'end, the P gene overlaps the first 12 nt of the pre-C region. The P gene of AusDHBV shows 96.4% and 89.7% homology to that of DHBV-S31 and DHBV16, respectively. Sequence variation in the P gene was mainly found in the region that overlapped the pre-S region, which codes for the spacer domain of the P protein. This domain is not highly conserved among different isolates (Sprengel *et al.*, 1991) and does not seem to be required for the known enzymatic functions of this protein (Hu & Seeger, 1996).

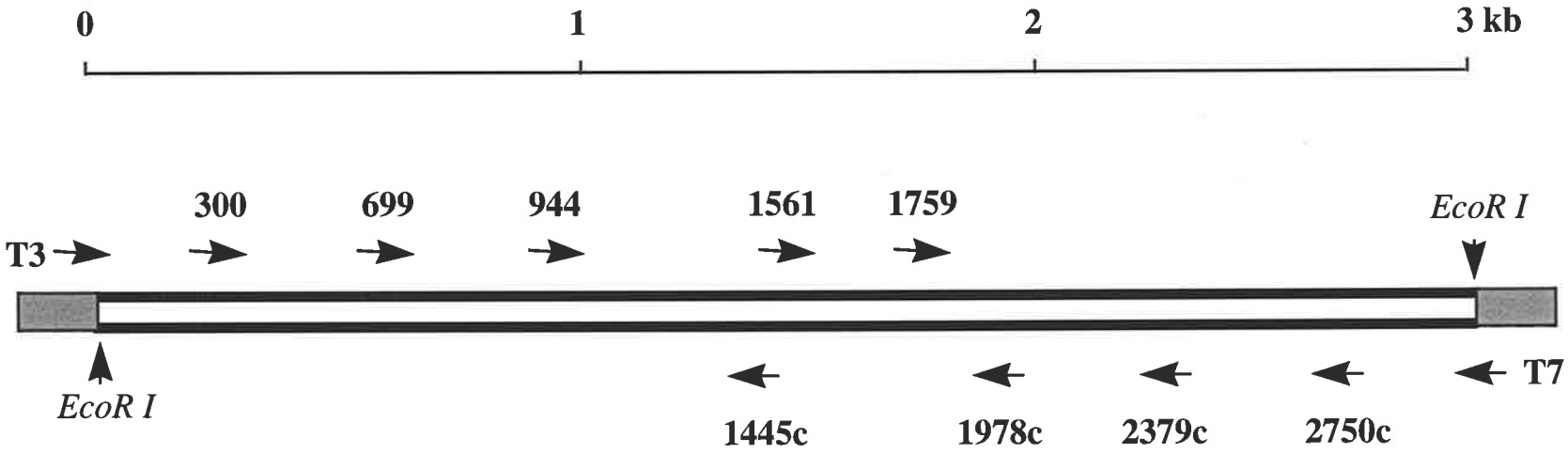


Figure 3.4. The sequencing strategy for the AusDHBV clone. The AusDHBV clone (pBL4.8) was sequenced by 'primer walking' methods, and the numbers shown are the nucleotide positions at 5'end of the primers used as listed in Table 3.2. Arrows show the direction of sequencing of the AusDHBV genome from both strands, starting at the flanking regions of DHBV DNA in the pBluescript IIKS+ using T3 and T7 primers.

As stated above, the pre-S/S gene of DHBV is divided into two parts, the pre-S and S regions. The S region of the S gene (nt 1290-1793) is highly conserved and showed 99.6% and 95.4% homology to DHBV-S31 and DHBV16, respectively. The pre-S region (nt 693-1289) was less conserved, with only 94.3% homology to DHBV-S31 and 81.8% homology to DHBV16. There were 5 in-frame initiation codons within the pre-S region at nt positions 693, 801, 825, 882 and 957 (Fig. 3.5). Sequence variability within the pre-S region has also been observed in the 13 other published avian hepadnaviruses (see below), which may be due to one of its proposed functions as a host range determinant. Evidence for this possibility has been provided by the demonstration that replacement of 69 AA residues of the pre-S domain of the HHBV L protein by the DHBV counterpart (encoded by nt 864-1071) allowed HHBV to infect primary duck hepatocyte cultures (Ishikawa & Ganem, 1995). In contrast, phenotypic mixing of an HBV *env*$^-$ mutant with the L protein of DHBV did not result in formation of virus particles in COS7 cell line, perhaps due to the absence of homology in the pre-S region of the two viruses (Gerhardt & Bruss, 1995).

The C gene of AusDHBV (nt 2653-414) is highly conserved and showed 98.4% homology to DHBV-S31 and 91.4% homology to DHBV16. The pre-C region, starting 129 nt upstream of the major C ATG at nt 2524, represents the signal sequence necessary for secretion of the pre-core protein (DHBeAg). The region encoding this signal sequence has been mapped between nt 2629 and 2652 just upstream of the C gene ATG (Schlicht *et al.,* 1987b). This region is well conserved between the AusDHBV, DHBV-S31, and DHBV16 genomes.

Figure 3.5. The complete nucleotide sequence of the AusDHBV clone. The positions of the start and termination codons for the S, C, and P genes are indicated above the sequence by < and >, respectively. Four other in-frame start codons within the pre-S region (nt 693, 825, 882, and 957) are indicated as <<<. A previous report has shown that the ATG at nt 801, and not nt 693, is the start codon for the pre-S/S protein (Summers *et al.*, 1991). Compared to DHBV16, the AusDHBV clone contains two 3-bp inserts located at nt 1236-1238 and 1284-1286 within the overlapping region of pre-S and P genes, and depicted as the lines above the sequence. Two copies of direct repeat sequences (D.R.) at nt 2483-2494 and nt 2541-2552, are shown. Likewise, the epsilon (ε) sequence at nt 2566-2622 (indicated as *), and the poly(A) addition signal [p(A)] at nt 2778-2783 are shown.

```
                                                                    60
  1 CATGCACACC TAAAAGCATA TGCAAAGATT AATGAGGAAT CATTGGATCG GGCTAGGAGA
                                                                   120
 61 TTGCTTTGGT GGCATTACAA TTGTTTACTG TGGGGAGAAG CTAACGTTAC TAATTATATT
                                                     <P start      180
121 TCTCGGCTCC GTACTTGGTT GTCAACACCT GAGAAGTACC GAGGCCGTGA TGCCCCAACC
                                                                   240
181 ATTGAAGCAA TCACTAGACC AATCCAAGTG GCTCAGGGAG GCAGAAAAAC ATCTTCGGGA
                                                                   300
241 ACTAGAAAAC CTCGTGGACT CGAACCTAGA AGAAGAAAAG TTAAAACCAC AGTTGTCTAT
                                                                   360
301 GGGAGAAGAC GTTCAAAGTC CCGGGAAAGG AGAGCCCCTA CACCCCAACG TGCGGGCTCC
                                                     C stop>       420
361 CCTCTCCCAC GTAGTTCGAG CAGCCACCAC AGATCTCCCT CGCCTAGGAA ATAAATTACC
                                                                   480
421 TGCTAAGCAT CACTTGGGGA AATTGTCAGG TTTATATCAA ATGAAGGGTT GCACCTTTAA
                                                                   540
481 CCCTGAATGG AAAGTACCTG ATATTTCGGA TACTCATTTT GATTTACAAA TAACTAATGA
                                                                   600
541 GTGCCCTTCC CGAAATTGGA AATATTTGAC TCCAGCCAAA TTTTGGCCCA AGAGCATTTC
                                                                   660
601 CTACTTTCCT GTACATGCAG GGGTTAAACC AAAATATCCT GACAATGTGA TGCAGCATGA
                                          <<<                      720
661 GGCAATAGTA GGTAAATATT TAAACAGGCT CTATGAAGCA GGAATCCTTT ATAAGCGGAT
                                                                   780
721 ATCTAAACAT TTGGTTGCAT TCAAAGGCAA GCCTTATCAT TGGGAACTTC AATACCTTGT
                          <pre-S start          <<<                840
781 CAAGCAACAT CAAGTTCCTG ATGGGACAAC AACCTGCAAA ATCAATGGAC GTGCGGAGAA
                                                 <<<                 900
841 TCGAAGGAGG AGAACTCCTG CTAAATCAAT TAGCAGGCCG CATGATACCA AAAGGGACTG
                                                                  <<<
901 TCACATGGTC GGGCAAATTT CCAACAATAG ATCACCTATT AGATCATGTG CAAACAATGG
                                                                  1020
961 AGGAGGTAAA TACTCTTCAG CAACAAGGCG CATGGCCTGC TGGGGCAGGA AGACGTTTGG
                                                                  1080
1021 GGTTAACCAA TCCGGCACCC CAAGAACCTC CTCAGCCCCA GTGGACTCCC GAAGAAGATC
                                                                  1140
1081 AGAAAGCACG GGAAGCCTTT CGTCGTTATC AAGAAGAGAG ACCACCGGAA ACCACCACAA
                                                                  1200
1141 TTCCACCAAC GTCACCAACT CCGTGGAAAC TACAACCAGG GGACGATCCC CTACTCGAGA
                                                                  1260
1201 ACAAATCTCT GCTCGAGACT CATCCTCTTT ACCAGAATCC GGAGCCGGCC GTGCCTGTGA
                                  <S start                        1320
1261 TAAAGACTCC TCCCCTCAAG AAGAAGAAAA TGGCTGGTAC CTTCGGGGGA ATACTAGCTG
                                                                  1380
1321 GCCTAATCGG ATTACTGGTA GGCTTTTTCT TGTTGATAAA AATTCTCGAA ATACTACGGA
                                                                  1440
1381 GGCTAGATTG GTGGTGGATT TCTCTCAGTT CTCCAAAGGG AAAAATGCAA TGCGCTTTCC
                                                                  1500
1441 AAGATACTGG AGCCCAAATC TCTCCACACT ACGCAGGATT CTGCCCGTGG GGATGCCCAG
                                                                  1560
1501 GATTTCTCTG GACCTATCTC AGGCTTTTTA TCATCTTCCT CTTAATCCTG CTAGTAACAG
                                                                  1620
1561 CAGGCTTGCT GTATCTGACG GACAACATGT CTATTATTTT AGGAAAGCTC CAATGGGAGT
                                                                  1680
1621 CGGTCTCAGC CCTTTTCTCC TCCATCTCTT CACTACTGCC CTCGGATCAG AAATCGCTCG
                                                                  1740
1681 TCGCTTTAAT GTTTGGACTT TTACTTATAT GGATGACTTC CTCCTCTGCC ACCCAAACGC
                                                     S stop>      1800
1741 TCGTCACCTT AACTCAATTA GCCACGCTGT CTGCACTTTT CTACAAGAAT TAGGAGTGCG
                                                                  1860
1801 CATAAACTTT GACAAAACAA CGCCATCTCC AGTAAATGAC ATACGATTCC TCGGATACCA
                                                                  1920
1861 GATTGATCAG AAATTCATGA AGATCGAAGA AAGCAGATGG AAAGAATTAA GGACTGTTAT
                                                                  1980
1921 TAAGAAAATA AAAATTGGCG CGTGGTATGA TTGGAAATGT ATTCAAAGAT TTGTCGGGCA
                                                                  2040
1981 TTTAAATTTC GTGTTACCTT TCACTAAGGG TAATATTGAA ATGTTAAAAC CAATGTATGC
                                                                  2100
2041 TGCTATTACT AACAAAGTTA ACTTTAGCTT CTCTTCTGCA TATAGGACTT TATTGTATAA
```

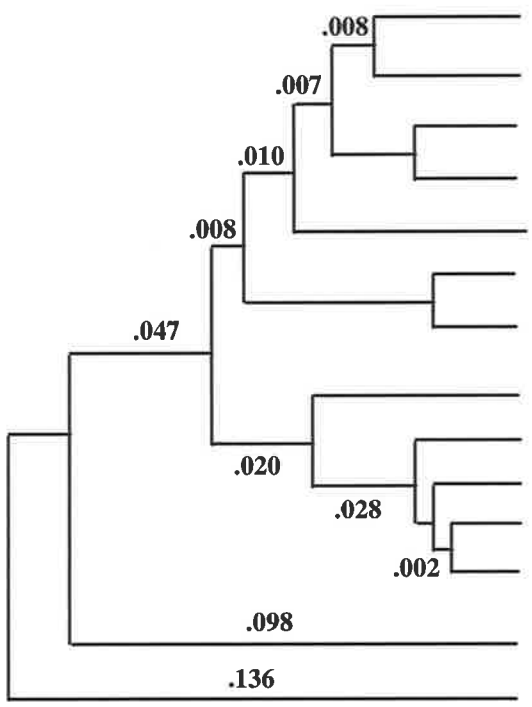
```
                                                                    2160
2101 ACTAACCATG GGTGTTTGTA AATTAGCCAT TAGACCAAAG TCCTCTGTAC CTTTGCCACG
                                                                    2220
2161 TGTAGCCACA GATGCTACTC CAACACATGG CGCAATATCC CATATCACCG GCGGGAGCGC
                                                                    2280
2221 AGTGTTTGCT TTTTCAAAGG TCAGAGATAT ACATATACAG GAATTGCTGA TGGTATGTTT
                                                                    2340
2281 AGCTAGGATA ATGATTAAAC CCAGATGTAT ACTTTCCGAT TCTACCTTTG TTTGTCACAA
                                                                    2400
2341 ACGTTATCAG ACGTTACCAT GGCATTTTGC TATGTTGGCC AAACAATTGT TATCTCCTAT
                                                                    2460
2401 ACAATTGTAC TTTGTTCCGA GTAAATATAA TCCTGCTGAC GGCCCATCCA GGCACAAACC
                                  _____D.R.____          pre-C start 2520
2461 GCCTGATTGG ACGGCTCTTA CATACACCCC TCTCTCGAAA GCAATATATA TTCCACATAG
        <       P stop>     _____D.R.____                           2580
2521 GCTATGTGGA ACTTAAGAAT TACACCCCTC TCCTTCGGAG CTGCCTGCCA AGGTATTTTT
                                                                    2640
2581 ACGTCTACAT TGCTGTTGTC AGCCTTGACT GTACCTTTGG TATGTACCAT TGTTTATGAT
                     <C start                                       2700
2641 TCTTGCTTAT ATATGGATAT CAATGCTTCT AGAGCCTTAG CAAATATATA TGATCTGCCT
                                                                    2760
2701 GATGATTTCT TTCCTAAAAT AGATGATCTT GTAAGGGATG CTAAAGACGC TTTAGAACCT
                           __p(A)_                                  2820
2761 TATTGGAAAT CTGATTCAAT AAAGAAACAT GTTTTAATTG CAACTCACTT TGTGGATCTT
                                                                    2880
2821 ATTGAAGATT TCTGGCAGAC CACTCAGGGT ATGCATGAAA TTGCCGAAGC ACTAAGAGCA
                                                                    2940
2881 GTAATACCAC CTACCACTGC TCCGGTACCT ACAGGGTATC TCATTCAGCA CGAAGAAGCA
                                                                    3000
2941 GAAGAGATAC CCTTAGGGGA TTTATTTAAA CATCAAGAGG AAAGAATAGT AAGTTTCCAG
                                  3027
3001 CCTGACTATC CGATTACGGC AAGAATT
//
```

A characteristic feature of the genome of hepadnaviruses is their circular, partially double-stranded DNA; the circular conformation of the genome is maintained by a cohesive overlap between the 5'ends of the two strands (Sattler & Robinson, 1979). In the DHBV16 genome, it is known that the cohesive overlap region is 69 ± 4 bp in length and contains a pair of 12-bp direct repeat (D.R.) sequences (Molnar-Kimber *et al.,* 1984). The 12-bp D.R. sequences (nt 2483-2494 and nt 2541-2552) are completely conserved in the AusDHBV genome. Another important feature in this region is the RNA encapsidation signal sequence, also known as epsilon (ε), which forms a stem-loop structure with a bulge and a loop (Wang & Seeger, 1993). The ε sequence (nt 2566-2622) of the AusDHBV genome shows some nucleotide differences from most of other avian hepadnaviruses described so far (see below). Nevertheless, the TTAC motifs, located at nt 2540 in D.R.1 and nt 2579 in the bulge region of the ε sequence, are conserved. These motifs correspond to the UUAC motif in the RNA pregenome and are used as the initiation site for synthesis of negative-strand DNA (Wang & Seeger, 1993). The poly(A) addition signal sequence (nt 2778-2783) is the same as that found in DHBV-S31.

3.3.5. The position of AusDHBV in the phylogenetic tree of avian hepadnaviruses.

As predicted from the nucleotide sequence homology, AusDHBV is closely positioned to the Chinese DHBV isolate (DHBV-S31) in the phylogenetic tree of avian hepadnaviruses (Fig. 3.6). AusDHBV is also closely related to the other published Shanghai/Chinese DHBV isolates including DHBVQCA34, DHBV-S5, and DHBVS18-B, and to a lesser extent, DHBV22 and DHBV26. The five members of the Western country DHBV branch showed less homology to AusDHBV. The third branch of avian hepadnaviruses, HHBV,

Figure 3.6. The phylogenetic tree of the avian hepadnaviruses. The phylogenetic tree was determined using version 1.02 of *MEGA (Molecular Evolutionary Genetics Analysis)* software. The numbers shown are the average number of nucleotide substitutions per site between two DHBV sequences. The evolutionary distance was plotted based on the method of Tamura and Nei (1993). C/PRCH, Chi-tung County/ People's Republic of China; S/PRCH, Shanghai/People's Republic of China; E/FRG, Ermke/Federal Republic of Germany; USA, United States of America.



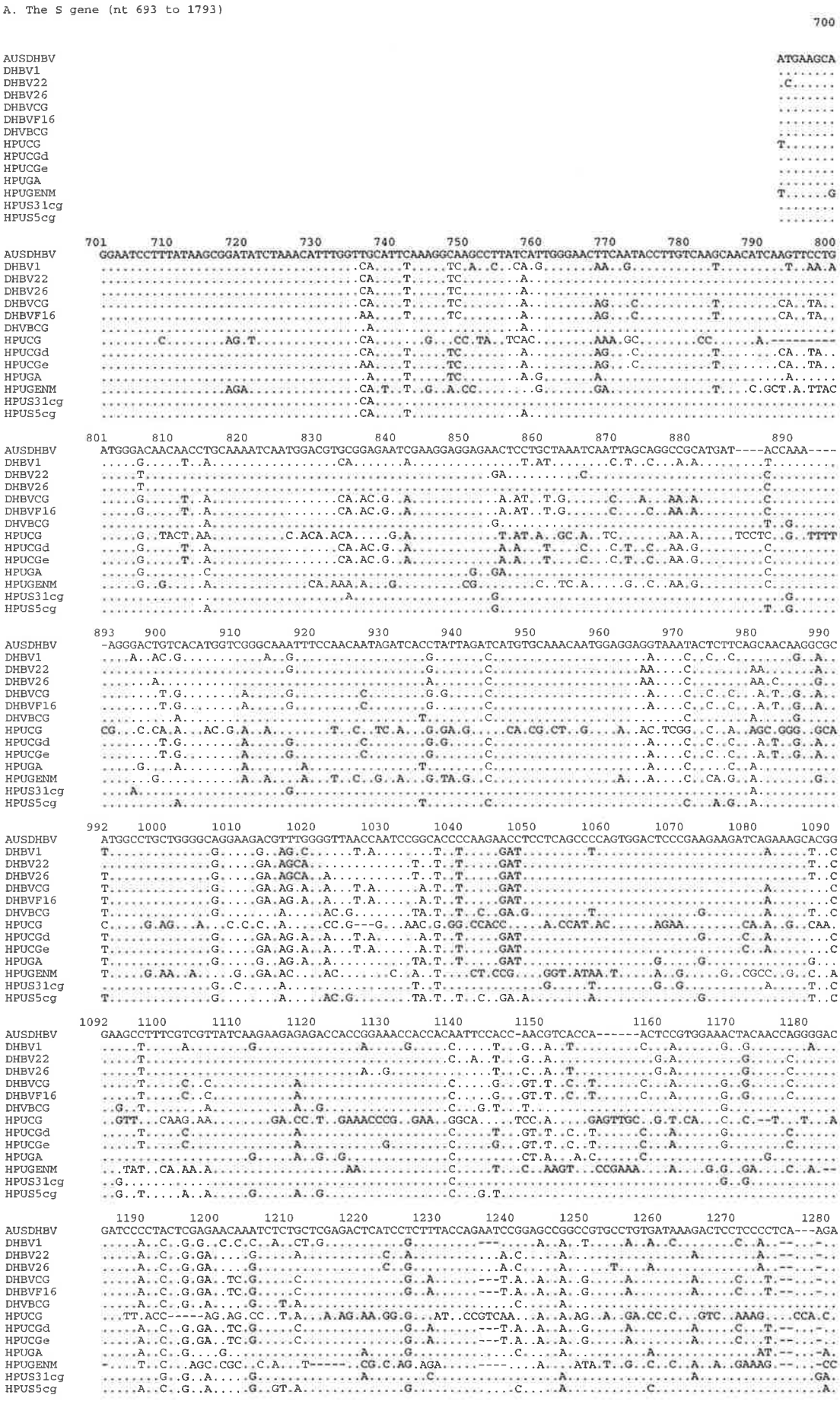
Genbank name	Isolate	Genome length (bp)	Origin		Reference
			country	animal	
AusDHBV	AusDHBV	3027	Australia	Pekin duck	Triyatni (1998b)
HPUS31.CG	DHBV-S31	3027	S/PRCH	White duck	Uchida (1989)
DHVBCG	DHBVQCA34	3027	S/PRCH	Domestic duck	Tong (1991)
HPUS5CG	DHBV-S5	3027	S/PRCH	Brown duck	Uchida (1989)
HPUGA	DHBVS18-B	3024	S/PRCH	Domestic duck	Tong (1990)
DHBV22	DHBV22	3024	C/PRCH	Domestic duck	Sprengel (1991)
DHBV26	DHBV26	3024	S/PRCH	Domestic duck	Sprengel (1991)
DHBV1	DHBV1	3021	E/FRG	Domestic goose	Sprengel (1991)
HPUCGE	HPUCGE	3021	USA	Pekin duck	Thomas (1991)
HPUCGD	DHBV16	3021	USA	Pekin duck	Mandart (1984)
DHBVCG	DHBVCG	3021	India		Munshi (1993)
DHBVF16	DHBVF1-6	3021	E/FRG	Pekin duck	Mattes (1990)
HPUGENM	HPUGENM	3018	USA	Ross goose	Shi (1993)
HPUCG	HPUCG	3027	FRG	Grey heron	Sprengel (1988)

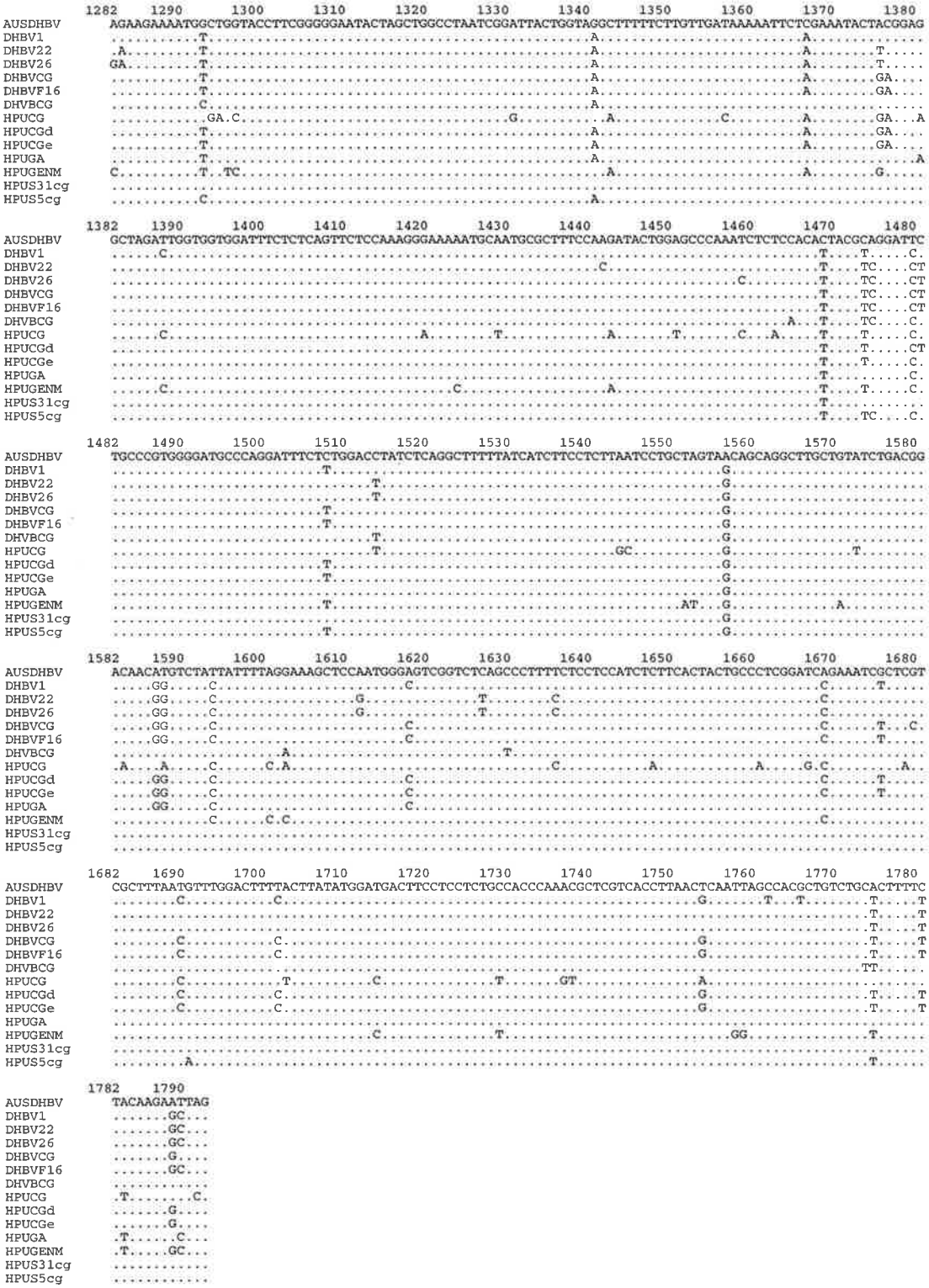
which consists of 2 isolates (HPUGENM and HPUCG), has the most distant relationship to AusDHBV. Accordingly, the AusDHBV genome can be classified as a new member of the Chinese DHBV branch of avian hepadnaviruses, which now consists of seven DHBV isolates.

Despite overall nucleotide differences which separated the avian hepadnaviruses sequenced so far into the three branches, three regions in the genome were highly conserved (Fig. 3.7). The first is the S region of the S gene (nt 1290-1793; Fig. 3.7A). The second is the cohesive overlap region which contains two D.R. sequences (nt 2483-2494 and nt 2541-2552). The third is the pre-C region encoding the signal sequence for secretion of DHBeAg (nt 2629-2652; Fig. 3.7C). The pre-S region (nt 693-1289) was the most variable region found among all of the 14 isolates (Fig. 3.7A). The ε sequence (nt 2566-2622) of the AusDHBV genome, was similar to only two other Chinese DHBV isolates, DHBVQCA34 and DHBV-S5 (Fig. 3.7B). A predicted secondary structure of the RNA deduced from the ε sequence of the AusDHBV genome (Fig. 3.7D) indicated the stem-loop structure of ε is slightly different from the structure proposed for DHBV16 (Wang & Seeger, 1993). The bulge region of AusDHBV ε that separates the lower and upper stems is identical to ε structure of DHBV16 (Wang & Seeger, 1993) but the upper stem is shorter (3 bp in AusDHBV versus 9 bp in DHBV16), and is followed by a big apical loop similar to that of HHBV (Beck & Nassal, 1997).

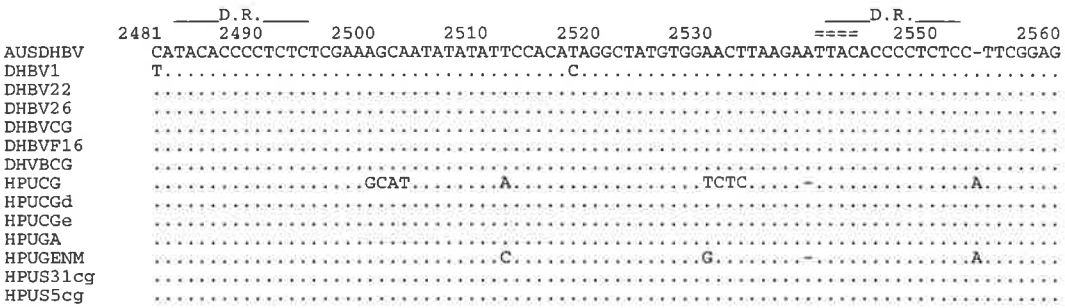
Figure 3.7. Sequence homology within the genomes of the avian hepadnaviruses. Comparison of the nucleotide sequences was performed using version 1.02 of *MEGA*. AusDHBV has been used as the reference isolate and the dots represent the nucleotide homology with other strains/isolates. **(A) The S gene (nt 693 to 1793).** The pre-S region (nt 693 to 1289) which has the most variable sequences among hepadnaviruses is shown for comparison with the S gene (nt 1290 to 1793) which is highly conserved. **(B) The cohesive overlap region (nt 2483 to 2622).** This region contains two copies of D.R. sequences (nt 2483-2494 and nt 2541-2552) and the ε sequence (nt 2566-2622). The ε sequence of AusDHBV genome shows similarities to only two other DHBV Chinese isolates, DHBVQCA34 and DHBV-S5. The positions of the TTAC motifs (=) which correspond to the UUAC motifs on RNA pregenome and are important for initiation of negative DNA strand synthesis, are shown at nt 2540 in D.R.1 and nt 2579 in ε sequences, respectively. **(C) The pre-C region encoding the signal sequence for secretion of DHBeAg (nt 2629-2652). (D) The predicted secondary structure of the ε region in the RNA pregenome.** The numbers shown (1-57) correspond to nt 2566-2622. The UUAC motifs, located in the bulge region (13-16), are similar to other DHBV sequences. However, the ε sequence of the AusDHBV RNA pregenome has a bigger apical loop that similar to that of HHBV (Beck & Nassal, 1997).

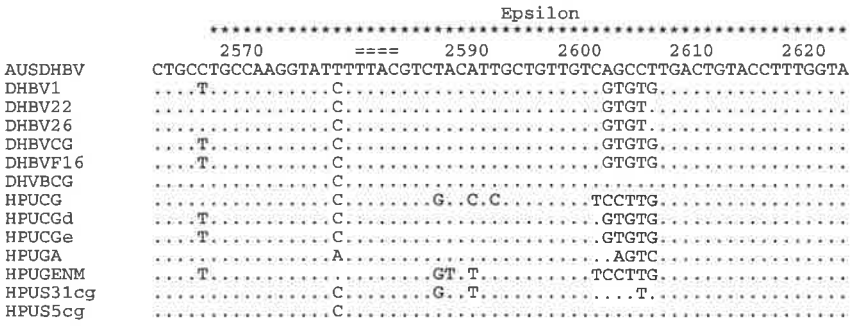
A. The S gene (nt 693 to 1793)



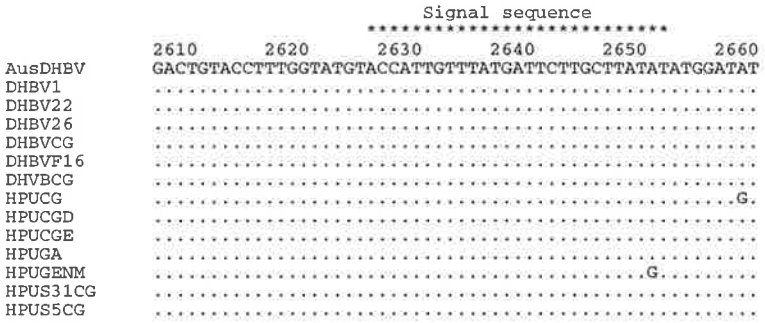

1282 1290 1300 1310 1320 1330 1340 1350 1360 1370 1380
AUSDHBV AGAAGAAAATGGCTGGTACCTTCGGGGGAATACTAGCTGGCCTAATCGGATTACTGGTAGGCTTTTTCTTGTTGATAAAAATTCTCGAAATACTACGGAG
DHBV1 ...........T.....A.....A.....
DHBV22 .A.....T.....A.....A.....T.....
DHBV26 GA.....T.....A.....A.....T.....
DHBVCG ...........T.....A.....A.....GA....
DHBVF16 ...........T.....A.....A.....GA....
DHVBCG ...........C.....A.....
HPUCG ...........GA.C.....G.....A.....C.....A.....GA...A
HPUCGd ...........T.....A.....A.....GA....
HPUCGe ...........T.....A.....A.....GA....
HPUGA ...........T.....A.....A
HPUGENM C.....T..TC.....A.....A.....G.....
HPUS31cg .....
HPUS5cg ...........C.....A.....
1382 1390 1400 1410 1420 1430 1440 1450 1460 1470 1480
AUSDHBV GCTAGATTGGTGGTGGATTTCTCTCAGTTCTCCAAAGGGAAAAATGCAATGCGCTTTCCAAGATACTGGAGCCCAAATCTCTCCACACTACGCAGGATTC
DHBV1 ......C.....T....T.....C.
DHBV22 .....C.....T....TC....CT
DHBV26 .....C.....T....TC....CT
DHBVCG .....T....TC....CT
DHBVF16 .....T....TC....CT
DHVBCG .....A..T....TC....C.
HPUCG ......C.....A.....T.....A.....T.....C..A....T....T.....C.
HPUCGd .....T....T.....CT
HPUCGe .....T....T.....C.
HPUGA .....T.....C.
HPUGENM ......C.....C.....A.....T....T.....C.
HPUS31cg .....T.....
HPUS5cg .....T....TC....C.
1482 1490 1500 1510 1520 1530 1540 1550 1560 1570 1580
AUSDHBV TGCCCGTGGGGATGCCCAGGATTTCTCTGGACCTATCTCAGGCTTTTTATCATCTTCCTCTTAATCCTGCTAGTAACAGCAGGCTTGCTGTATCTGACGG
DHBV1 .....T.....G.....
DHBV22 .....T.....G.....
DHBV26 .....T.....G.....
DHBVCG .....T.....G.....
DHBVF16 .....T.....G.....
DHVBCG .....T.....G.....
HPUCG .....T.....GC.....G.....T.....
HPUCGd .....T.....G.....
HPUCGe .....T.....G.....
HPUGA .....G.....
HPUGENM .....T.....AT..G.....A.....
HPUS31cg .....G.....
HPUS5cg .....T.....G.....
1582 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
AUSDHBV ACAACATGTCTATTATTTTAGGAAAGCTCCAATGGGAGTCGGTCTCAGCCCTTTTCTCCTCCATCTCTTCACTACTGCCCTCGGATCAGAAATCGCTCGT
DHBV1 .....GG.....C.....C.....C.....T.....
DHBV22 .....GG.....C.....G.....T.....C.....C.....
DHBV26 .....GG.....C.....G.....T.....C.....C.....
DHBVCG .....GG.....C.....C.....C.....T...C.
DHBVF16 .....GG.....C.....C.....C.....T.....
DHVBCG .....A.....T.....
HPUCG .A....A.....C.....C.A.....C.....A.....A.....G.C.....A..
HPUCGd .....GG.....C.....C.....C.....T.....
HPUCGe .....GG.....C.....C.....C.....T.....
HPUGA .....GG.....C.....C.....
HPUGENM .....C.....C.C.....C.....
HPUS31cg .....
HPUS5cg .....
1682 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780
AUSDHBV CGCTTTAATGTTTGGACTTTTACTTATATGGATGACTTCCTCCTCTGCCACCCAAACGCTCGTCACCTTAACTCAATTAGCCACGCTGTCTGCACTTTTC
DHBV1 .....C.....C.....G.....T...T.....T.....T
DHBV22 .....T.....T
DHBV26 .....T.....T
DHBVCG .....C.....C.....G.....T.....T
DHBVF16 .....C.....C.....G.....T.....T
DHVBCG .....TT.....
HPUCG .....C.....T.....C.....T.....GT.....A.....
HPUCGd .....C.....C.....G.....T.....T
HPUCGe .....C.....C.....G.....T.....T
HPUGA .....
HPUGENM .....C.....T.....GG.....T.....
HPUS31cg .....
HPUS5cg .....A.....T.....
1782 1790
AUSDHBV TACAAGAATTAG
DHBV1 .....GC...
DHBV22 .....GC...
DHBV26 .....GC...
DHBVCG .....G....
DHBVF16 .....GC...
DHVBCG .....
HPUCG .T.....C.
HPUCGd .....G....
HPUCGe .....G....
HPUGA .T.....C...
HPUGENM .T.....GC...
HPUS31cg .....
HPUS5cg .....

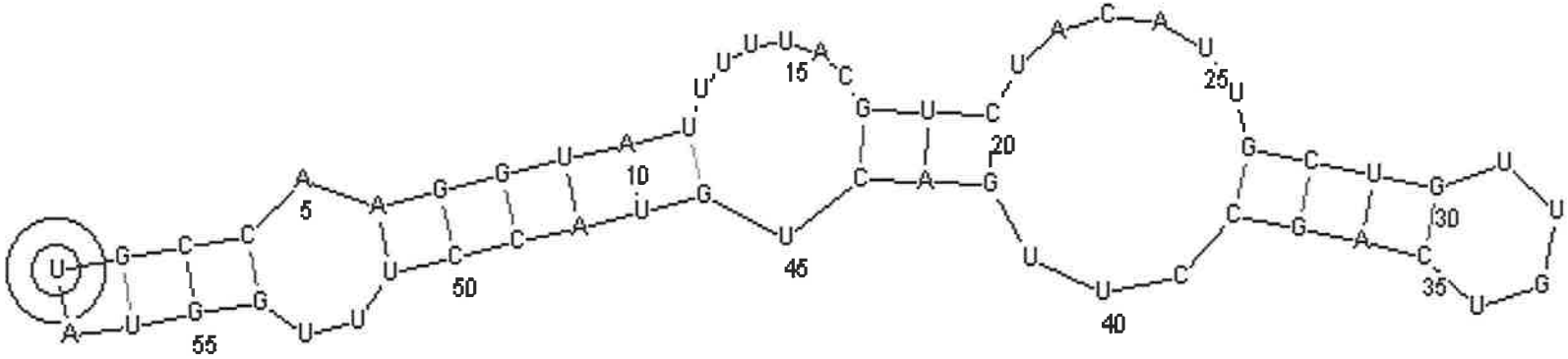
B. The cohesive overlap region of the avian hepadnavirus genomes.





C. The pre-C region of the avian hepadnavirus genomes




5
10
15
20
25
30
35
40
45
50
55

3.3.6. Amino acid sequences of the cloned AusDHBV genome.

The viral proteins produced from the AusDHBV genome were not examined in this study, however, the deduced amino acid sequences from the cloned AusDHBV genome were considered. The polymerase protein of DHBV, similar to HBV, is responsible for initiating the synthesis of negative-strand DNA by binding to the ε region of the RNA pregenome through a tyrosine residue at AA position 96 within its N-terminal domain (Hu & Seeger, 1996; see also Section 1.3). This Tyr-96 residue is conserved in the AusDHBV genome.

As in its HBV counterpart, the surface protein of DHBV contains some cysteine residues within the S domain. However, the S protein of DHBV contains only 3 cysteine residues, in comparison to the 14 found in HBV (Mangold & Streeck, 1993). In both HBV and DHBV, the three cysteine residues found at positions 48, 65 and 69, together with histidine at position 60, have been demonstrated to be indispensable for secretion of subviral particles of both mammalian and avian hepadnaviruses (Mangold & Streeck, 1993). Not surprisingly, in the S protein of AusDHBV, cysteine (C) residues at positions of 48, 65, and 69, and histidine (H) at position 60, are well conserved (Fig. 3.8).

The glycosylation signal (Asn-X-Ser/Thr) has been conserved within the S domain of all DHBV isolates reported so far (Sprengel *et al.,* 1985; Uchida *et al.,* 1989). This also holds true for AusDHBV; the sequence Asn-Met-Ser was conserved at AA position 99 of the S protein (Fig. 3.8). As stated earlier, despite the presence of this sequence, the S protein of DHBV is unglycosylated, presumably due to poor accessibility of the

Figure 3.8. The amino acid sequences of the S protein. The cysteine (C) residues at positions 48, 65, and 69 (marked as *), and histidine (H) at position 60 (marked as #), were conserved among all members of the avian hepadnaviruses. The glycosylation signal (Asn-X-Ser) at position 99 (marked as line above the sequence) was also conserved, although in contrast to the mammalian hepadnaviruses, the S proteins of the avian hepadnaviruses are not glycosylated. The two hydrophobic transmembrane domains (AA 8-39 and AA 73-96) are shown (marked as ^). The position of the recognition site of 7C.12, an anti-S monoclonal antibody raised against the USA DHBV isolate (Pugh *et al.,* 1995), has been mapped at AA 106-111 (marked as =). The alanine (A) residue at AA 110 is critical for antigen-antibody binding (Schaller & Grgacic, personal communication), hence this antibody does not recognize AusDHBV that has a glutamic acid (E) at AA 110.

```
                1      ^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^          *           #
AusDHBV         MAGTFGGILAGLIGLLVGFF LLIKILEILRRLDWWWISLS SPKGKMQCAFQDTGAQISPH
DHBV1           .S..............S.. .................... ....................
DHBV22          .S..............S.. .................... ..........H.........
DHBV26          .S..............S.. .................... ................T...
DHBVCG          .S..............S.. .................... ....................
DHBVF16         .S..............S.. .................... ....................
DHVBCG          .P..............S.. .................... ..................Q.
HPUCG           .GA.............. ..T.......K......... ...E..L....N....T...
HPUCGD          .S..............S.. .................... ....................
HPUCGE          .S..............S.. .................... ....................
HPUGA           .S..............S.. ..........K......... ....................
HPUGENM         .SS............... .................... ....N......N........
HPUS31CG        ................... .................... ....................
HPUS5CG         .P..............S.. .................... ....................

                61   *   *  ^^^^^^^^ ^^^^^^^^^^^^^^^^^  __ _     ======      120
AusDHBV         YAGFCPWGCPGFLWTYLRLF IIFLLILLVTAGLLYLTDNM SIILGKLQWESVSALFSSIS
DHBV1           .V.S.............. .........A.........G .T........A..........
DHBV22          .V.S.............. .........A.........G .T.....R....L..S....
DHBV26          .V.S.............. .........A.........G .T.....R....L..S....
DHBVCG          .V.S.............. .........A.........G .T........A..........
DHBVF16         .V.S.............. .........A.........G .T........A..........
DHVBCG          .V.S.............. .........A.......... ....E.........V......
HPUCG           .V.S.............. .....L...A....F..E.K .T.FE..........S...Y
HPUCGD          .V.S.............. .........A.........G .T........A..........
HPUCGE          .V.S.............. .........A.........G .T........A..........
HPUGA           ...S.............. .........A.........G .T........A..........
HPUGENM         .V.S.............. .......H.A.......... .T.FA...............
HPUS31CG        ................... .........A.......... ....................
HPUS5CG         .V.S.............. .........A.......... ....................

              121                 140                 160
AusDHBV         SLLPSDQKSLVALMFGLLLI WMTSSSATQTLVTLTQLATL SALFYKN*
DHBV1           ......P......T...S.. ..................V.. ......S.
DHBV22          ......P.............. ..................... ......S.
DHBV26          ......P.............. ..................... ......S.
DHBVCG          ......P...L..T...S.. ..................... ......S.
DHBVF16         ......P......T...S.. ..................... ......S.
DHVBCG          ..................... ..................... .V......
HPUCG           .....EP......T...F.. .T....V..V.......... ....F..S
HPUCGD          ......P......T...S.. ..................... ......S.
HPUCGE          ......P......T...S.. ..................... ......S.
HPUGA           ..................... ..................... ....F...
HPUGENM         ......P.............. .T....V.........G... ....F.S.
HPUS31CG        ..................... ..................... ........
HPUS5CG         .............I...... ..................... ........
```

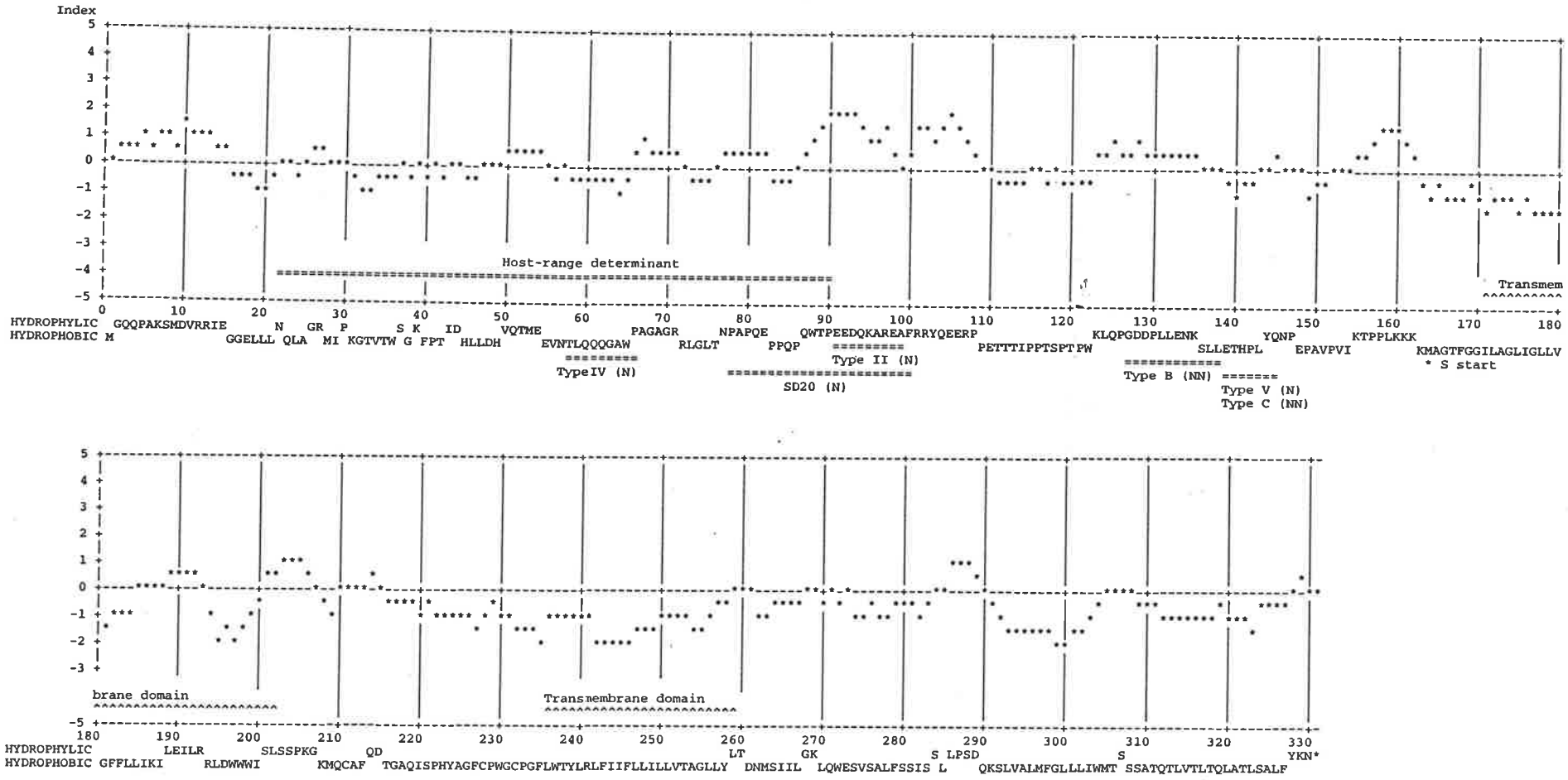
glycosylation signal which is located close to the second translocation signal sequences (AA 73-96; Fig. 3.9). In comparison, the glycosylation signal of the HBV S protein (Asn 146) is located in the second hydrophilic region, and is therefore exposed on the outside of the membrane (Berting *et al.,* 1995).

3.3.7. Hydrophobicity analysis of the AusDHBV surface protein.

The hydrophobicity analysis of the pre-S/S protein of AusDHBV reveals that the pre-S domain is rather hydrophilic, whereas the S domain contains two hydrophobic peaks (Fig. 3.9). In comparison, the primary structure of the S protein of HBV (HBsAg) is divided into three hydrophobic and two hydrophilic regions. Two of the hydrophobic regions of HBsAg contain type I (AA 11-29) and type II (AA 80-98) translocation signal sequences which mediate translocation of the S protein into the ER membrane twice with a cytosolic loop between the two translocation signals (Eble *et al.,* 1990). The hydrophilic region of S HBV (AA 120-160) contains the subtype- and group-specific HBV antigenic determinants with the dominant epitopes, the *a* determinant, found between AA 121-147 (Ashton-Rickardt *et al.,* 1989).

By analogy with HBV, the hydrophobic regions of DHBV S protein also contains a type I (AA 8-39) and a type II (AA 73-96) translocation signal (Guo & Pugh, 1997). The well-conserved cysteine residues at positions 48, 65, and 69, are located in the cytosolic loop between these two transmembrane sequences. So far, epitope(s) within the hydrophilic region of the S domain of DHBV that can elicit neutralizing antibodies *in vivo* have not been identified. Nevertheless, the presence of such epitope(s) in the S domain DHBV can

Figure 3.9. Hydrophilicity profile of the AusDHBV pre-S/S protein. The hydrophilicity score of pre-S/S protein was determined by the PROSIS software program based on the method of Hopp & Woods (1981). The pre-S domain contains 163 AA residues. AA 1 denotes the translation product from nt 801 within the pre-S region of the S gene. The 69 AA residues (AA 22-90) in the pre-S domain proposed to be implicated as the host-range determinant (Ishikawa & Ganem, 1995) are shown. Several epitopes in the pre-S domain have been mapped earlier by others (Lambert *et al.,* 1990; Yuasa *et al.,* 1991) using anti-preS monocolonal antibodies type IV (AA 58-65), type II (AA 91-99), SD20 (AA 78-100), type B (AA 127-138), type V and C (AA 139-145). N and NN denote neutralizing and non-neutralizing, respectively. The S protein starts at AA residue 164 (marked as *). The two hydrophobic transmembrane domains in S protein are shown (marked as ^).


Index
Host-range determinant
Transmem
HYDROPHYLIC GQQPAKSMDVRRIE N GR P S K ID VQTME PAGAGR NPAPQE QWTPEEDQKAREAFRRYQEERP KLQPGDDPLLENK YQNP KTPPLKKK
HYDROPHOBIC M GGELLL QLA MI KGTVTW G FPT HLLDH EVNTLQQQGAW RLGLT PPQP Type II (N) PETTTIPPTSPTPW SLLETHPL EPAVPVI KMAGTFGGILAGLIGLLV
TypeIV (N)
SD20 (N)
Type B (NN)
Type V (N)
Type C (NN)
* S start

brane domain
Transmembrane domain
HYDROPHYLIC LEILR SLSSPKG QD LT GK S LPSD S YKN*
HYDROPHOBIC GFFLLIKI RLDWWWI KMQCAF TGAQISPHYAGFCPWGCPGFLWTYLRLFIIFLLILLVTAGLLY DNMSIIL LQWESVSALFSSIS L QKSLVALMFGLLLIWMT SSATQTLVTLTQLATLSALF

not be ruled out, although the S gene of DHBV lacks 54 codons that correspond to the HBV sequence which encodes the subtype- and group-specific determinants of HBsAg (Sprengel *et al.,* 1985). Studies using DNA vaccines have shown that ducks vaccinated with S DNA were protected against virus challenge, and serum from these vaccinated ducks was able to neutralize DHBV infectivity in both *in vivo* and *in vitro* systems (Triyatni *et al.,* 1998a; see also Chapter 5).

The hydrophilic regions of pre-S protein from the Western country DHBV isolates have been shown to contain several antigenic sites, mapped between AA residues 58 to 145 (Fig. 3.9), with the dominant epitopes that can elicit neutralizing antibody responses to be located between AA 78 and 100 (Lambert *et al.,* 1990; Yuasa *et al.,* 1991). It is interesting to note that the location of AA 22-90 residues proposed to be part of a host-range determinant (Ishikawa & Ganem, 1995) overlaps with two epitopes (AA 58-65 and AA 78-100) which induced neutralizing antibodies (type IV and SD20 MAbs, respectively; Lambert *et al.,* 1990; Yuasa *et al.,* 1991).

As is the case with the pre-S/S protein of HBV, it has been shown that the pre-S/S protein of DHBV displays a dual topology, with the N-terminal pre-S domain located both on the inside and the outside of virus particles (Prange & Streeck, 1995; Swameye & Schaller, 1997). This unique feature was probably related to the functions of pre-S domain that required its presence on both sides of particles: (i) internally, to form a complete virion (envelopment of the budding virion), and to downregulate the intracellular amplification of cccDNA (Lenhoff & Summers, 1994; Summers *et al.,* 1990), and (ii) externally, as a ligand for receptor binding and viral infectivity. A topological model of the DHBV pre-

S/S protein has been proposed (Swameye & Schaller, 1997). According to this model, half of the pre-S/S molecules expose their pre-S domain on the particle surface; while the others have an internal pre-S domain because the translocation signal type I is not inserted into the membrane (Fig. 3.10).

3.4. DISCUSSION

The time (~90 min) required to complete the endogenous polymerase reaction was presumably due to the fact that >80% of DHBV virions isolated from serum already contain complete genomes, and the endogenous polymerase reaction only increases the proportion of virions with complete genomes to >95% (Molnar-Kimber *et al.*, 1984). It is therefore not surprising that the pooled serum used in this study (estimated to contain 9.5 x 10^9 DHBV genomes/ml) has been shown earlier to have an ID_{50} of 1.5 x 10^{10}/ml, indicating that one virus DNA genome is infectious in newly hatched ducks (Jilbert *et al.*, 1996).

The AusDHBV clone contains a full-length double-stranded viral genome which is 3027 bp in length; a similar length to most of the Chinese DHBV isolates, and 6 bp longer than the Western country DHBV isolates. As stated above, three important features associated with virus replication are well conserved in the AusDHBV clone. The first is the cohesive overlap region which maintains the circular conformation of hepadnavirus genomes (Sattler & Robinson, 1979). The second is the poly(A) addition signal sequence which is necessary for termination of viral mRNA transcription. The third is the tyrosine

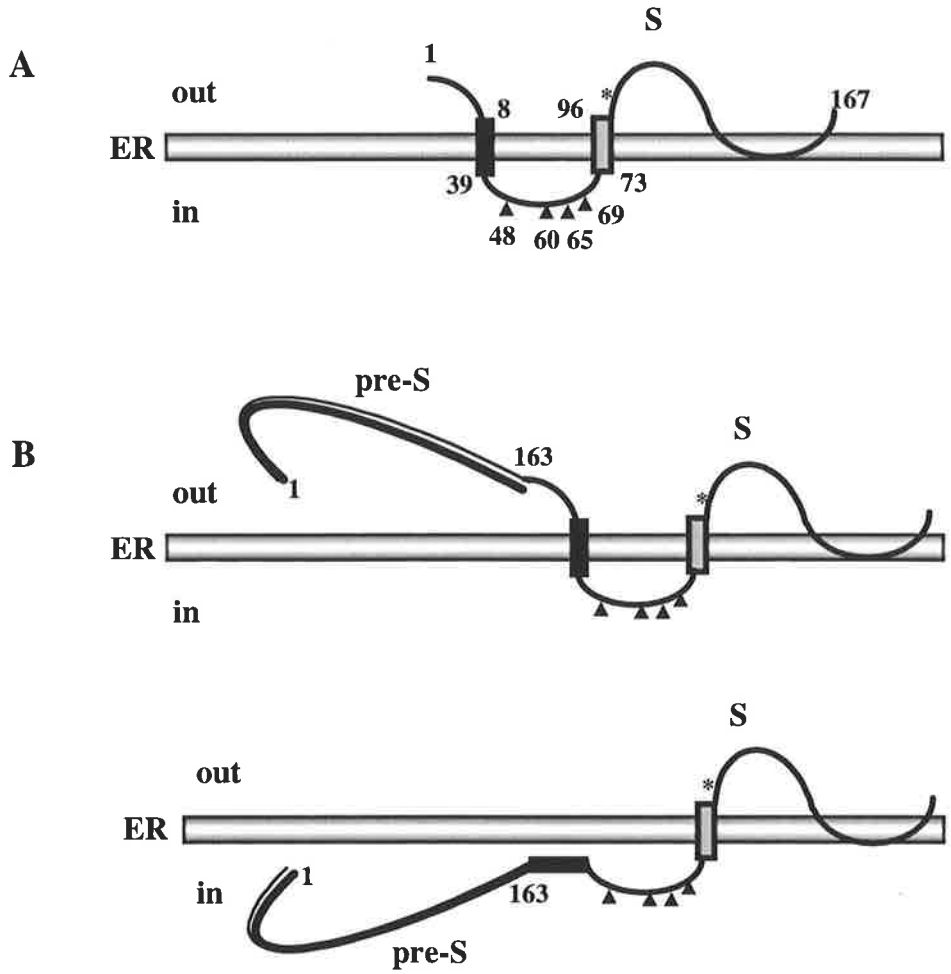


Figure 3.10. Proposed transmembrane topology of the DHBV surface proteins. The numbers in **(A)** and **(B)** indicate the AA within the S and the pre-S domains, respectively. **(A)** The S protein traverses the ER membrane twice with both transmembrane domains I (AA 8-39) and II (AA 73-96) (black and grey box, respectively) inserted into the membrane, in analogy to HBV. The cysteine (Cys) at positions 48, 65, and 69, and histidine (H) at position 60 (black triangles), known to be critical for secretion of subviral particles of hepadnaviruses, are located in the cytosolic loop between two domains (Mangold & Streeck, 1993). The glycosylation signal (*) located at AA 99 is shown. **(B)** During DHBV morphogenesis, the pre-S domain is initially located in the cytoplasmic space of the ER, and is then post translationally translocated to the outer membrane to expose the pre-S domain on the particle surface. The pre-S/S protein, however, displays a dual topology, with half of the molecules still remaining inside the particle because the transmembrane domain I (black box) is not inserted into the ER membrane. Adapted from Swameye & Schaller (1997).

residue at position AA 96 within the N-terminal domain of P protein which serves as the binding site to the ε sequence in the pregenomic RNA for negative strand DNA synthesis.

The similarity in restriction pattern and nucleotide sequence (95.8% homology), between the AusDHBV clone and DHBV-S31 suggested that both isolates may have a common ancestor. Pekin ducks introduced to Australia may have been imported from China, hence DHBV harboured by these animals and used in this study could have originated from the Chinese DHBV isolates. A phylogenetic tree of avian hepadnaviruses presented in this study also supports this hypothesis since the position of AusDHBV is closely located to DHBV-S31 and five other Chinese DHBV isolates.

The high variability found in the pre-S region of the S gene and consequently, in the pre-S domain of L protein, is well characterized in different hepadnaviruses (Lauder *et al.,* 1993; Sprengel *et al.,* 1985). The proposed function of the pre-S domain as a host-range determinant (Ishikawa & Ganem, 1995) might account for the high variability of this region. The presence of a so-called 28 kDa pre-S2 protein in a Chinese DHBV isolate (Yokosuka *et al.,* 1988) and the close association between AusDHBV and the Chinese DHBV isolates, raised the possibility that the 28 kDa pre-S2 protein may also be present in the AusDHBV. Evidence for this hypothesis was provided from Western blot analysis of the yeast-derived AusDHBV pre-S/S protein, and also from AusDHBV-infected serum and liver samples (see Section 4.3.4). Furthermore, others have shown by Western blot analysis that an anti-pre-S monoclonal antibody, SD20, identified the 28 kDa pre-S protein found in some DHBV isolates from French Pekin duck, French wild mallard, two Chinese brown ducks, and a German domestic Pekin duck (Lambert *et al.,* 1990). It is

conceivable that this protein could be translated from any internal AUG, although it may also represent a proteolytic processing product from the major pre-S/S protein (see Chapter 4).

In contrast to the pre-S domain, the S domain is the most conserved gene product of hepadnaviruses. The highly conserved S gene is indeed well characterized for HBV, perhaps related to the role of S protein (HBsAg) which carries the *a* determinant that elicits neutralizing antibodies, in establishing virus infection. The vast numbers of subviral particles that mostly consist of the S protein, were produced during HBV infection, and it was postulated that these empty particles act as decoy for the host neutralizing antibodies. In accordance to this hypothesis, three cysteine residues and a histidine residue within the S protein that responsible for secretion of subviral particles are well conserved among the mammalian and avian hepadnaviruses reported so far (Mangold & Streeck, 1993). These cysteine residues that are found at positions 48, 65, and 69, and histidine at position 60, are also conserved in the S protein of AusDHBV.

In summary, data presented in this chapter show that the AusDHBV strain can be classified as a new Chinese DHBV isolate, as defined by phylogenetic tree of avian hepadnaviruses. Although the infectivity of the AusDHBV clone was not determined in this study, it contains the full-length viral genome, and hence would be useful as a DNA template for various purposes such as for cloning of other gene(s), mapping of epitopes of the pre-S/S and the pre-C/C gene products, and designing specific probes that can be used to distinguish AusDHBV from other DHBV isolates for use in co- and super-infection experiments.

Chapter 4

The production & efficacy of yeast-derived DHBV pre-S/S & S proteins as vaccines

4.1. INTRODUCTION AND AIMS.

The restricted availability of HBsAg-positive human plasma and the stringent purification processes required to produce plasma-derived HBsAg vaccines have led to the development of recombinant HBV vaccines. By using various promoters in yeast expression systems, recombinant HBsAg vaccines have been produced in *S. cerevisiae* (Valenzuela *et al.,* 1982; McAleer *et al.*, 1984). Although yeast-derived HBsAg is not a secreted protein, sedimentation analysis and electron microscopy showed that HBsAg synthesized in yeast cells formed particles or aggregates (Valenzuela *et al.,* 1982) that were detected in the ER lumen (Biemans *et al.,* 1992). Further transport along the secretion pathway seemed to be blocked and, unlike native HBsAg, yeast-derived HBsAg was not glycosylated.

The yeast-derived HBsAg vaccines proved to be antigenically indistinguishable from plasma-derived HBsAg vaccines and to be of a similar immunogenicity. The vaccines elicited antibody responses to the *a* determinant of HBsAg, similar to those produced by plasma-derived vaccines (Waters *et al.,* 1987a; Waters *et al.,* 1987b). Long-term studies showed that yeast-derived HBsAg vaccines and plasma-derived vaccines were equally effective (McMahon & Wainwright, 1993). Nevertheless, 5-10% of individuals remained non-responsive after three doses of either type of vaccine. In addition, the emergence of vaccine-escape mutants of HBV has been reported (Carman, *et al.,*1990; Okamoto *et al.,*1992), emphasizing the need for more effective vaccines. Several attempts to design such vaccines have been made, these include the addition of pre-S1 and pre-S2 proteins into the current recombinant HBsAg vaccines (Yap *et al.,* 1995) and, more recently, the use of DNA vaccines (Davies *et al.,* 1993a).

Using DHBV infection of Pekin ducks as a model, the protective efficacy of yeast-derived and DNA vaccines can be compared. The aims of this chapter were: (i) to produce DHBsAg vaccines containing AusDHBV pre-S/S and S proteins expressed in yeast, (ii) to determine the protective efficacy of yeast-derived pre-S/S and S vaccines against DHBV infection in ducks, and (iii) to use the yeast-derived S protein as a source of antigen for detection of anti-DHBs antibody responses in vaccinated ducks by ELISA.

4.2. EXPERIMENTAL DESIGN.

To allow cloning of the pre-S/S and S genes of the AusDHBV clone (pBL4.8) into the unique *Bam*H I site of a yeast plasmid pYCpG2 (Fig. 4.1), *Bgl* II sites were introduced to both ends of the genes by PCR (Section 2.5.1 and 2.5.3). This was done because *Bgl* II has a cohesive terminus compatible with *Bam*H I (Sambrook *et al.,* 1989).

The PCR-amplified AusDHBV pre-S/S and S genes were then cloned into the *Bam*H I site of pYCpG2 downstream of a *GAL* promoter, and used to transform *E. coli* DH5α. The presence of recombinant plasmids were detected using restriction enzyme (RE) analysis and confirmed by Southern blot hybridization with a ^{32}P[dATP]-labeled AusDHBV DNA probe (Section 2.5.3).

Both clones were used to transform *S. cerevisiae* 15Dα (*ura3-, leu2-, his2-, ade1-),* and transformants were selected on SC-*ura* medium (Section 2.5.4). Expression of DHBV pre-S/S or S protein by the respective plasmids in yeast cells was driven by the *GAL* promoter following addition of 2% galactose into the medium, and the recombinant

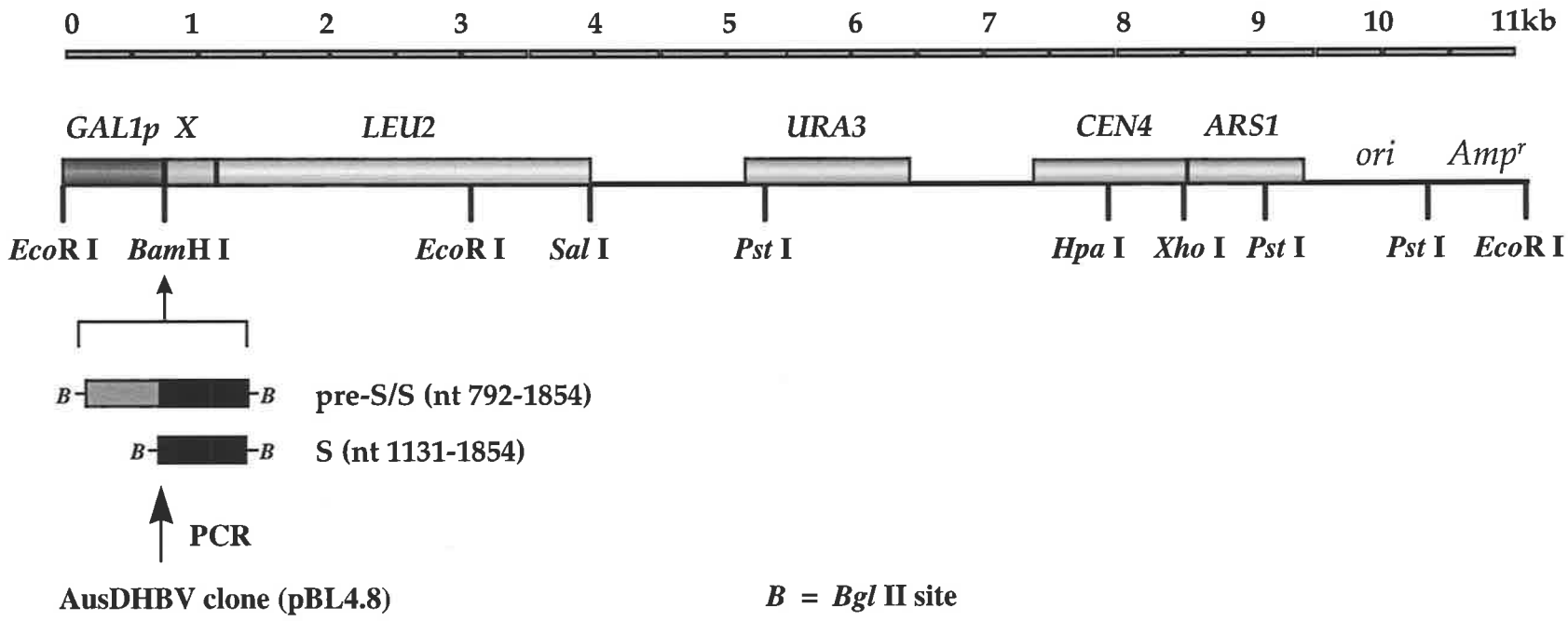


Figure 4.1. Map of pYCpG2 and the strategy for insertion of the AusDHBV pre-S/S and S genes.

The pre-S/S and S genes were amplified by PCR with *Bgl* II sites at both ends to allow cloning into the unique *Bam*H I site of the yeast plasmid, pYCpG2, downstream of the *GAL1* promoter (Richardson *et al.,* 1989; Section 2.5.3). This plasmid is derived from pYCpG1[*CDC28*] (Mendenhall *et al.,*1988) by removing the *CDC28* coding region but retaining the 3' flanking sequences (*X*). *LEU2* and *URA3* are yeast-selectable markers. *CEN4* is a centromeric sequence that stabilizes the plasmid in single copy number, and *ARS1* (*a*utonomously *r*eplicating *s*equence) allows the plasmid to replicate autonomously in *S. cerevisiae. ori* is origin of replication, and *Amp*^{r} encodes beta-lactamase; both properties permit the plasmid to be grown and selected for in *E. coli*.

proteins were purified by successive sucrose gradient centrifugation (Section 2.5.5). The properties of the yeast-derived proteins were analyzed by: (i) SDS-PAGE and Western blot (Section 2.5.6), (ii) their S value and buoyant density (in $CsCl_2$), and (iii) the ability of yeast-derived S protein to detect anti-S specific antibodies in the serum of vaccinated ducks by ELISA.

Three-week-old ducks were vaccinated by injecting them three times at 4 week intervals, with 40 μg of yeast-derived pre-S/S or S protein. Both proteins were emulsified in complete Freund's adjuvant (CFA) in the first injection, and in incomplete Freund's adjuvant (IFA) in the second and the third injections (Section 2.7.2). Blood samples were taken serially every two weeks and were measured for total anti-DHBs antibody responses by ELISA (Section 2.10.2). Four weeks after the third injection, ducks were challenged intravenously with a high dose of DHBV (Section 2.7.4), and the protective efficacy of the vaccines was assessed by monitoring: (i) the rate of virus removal from the bloodstream within 2 hr p.c., and (ii) the extent of virus replication in the liver at 4 days p.c. (Section 2.8).

4.3. RESULTS.

4.3.1. Amplification of the AusDHBV pre-S/S and S genes by PCR and cloning of the amplified DNA into pYCpG2.

The pre-S/S and S genes were amplified using two sets of primers, 31CG.792s/31CG.1854as and 31CG.1131s/31CG.1854as, respectively. Each primer contained the *Bgl* II site, AGA TCT. The sequences of primers were: 31CG.792s (5'-

GGC AGA TCT AAG TTC CTG ATG GG-3'), 31CG.1131s (5'-GGC AGA TCT ACC ACC ACC ATT CC-3'), and 31CG.1854as (5'-GGC AGA TCT CCG AGG AAT CGT AT-3'). The PCR products showed the expected sizes, i.e. 1062 bp for pre-S/S, and 723 bp for S gene (Fig. 4.2). Both DNA fragments were purified and subsequently digested with *Bgl* II (Section 2.5.1); the pre-S/S or S DNA was then cloned into the *Bam*H I site of pYCpG2 and the recombinant plasmids were used to transform *E. coli* DH5α (Section 2.5.3).

4.3.2. Screening for the pYCpG2-pre-S/S and pYCpG2-S plasmids.

Transformants grown on LB plates (containing 100 μg/ml Ampicillin) were screened by RE analysis for recombinant plasmids with the correct orientation. Two sets of RE, namely (i) *Eco*R I and *Hpa* I, and (ii) *Eco*R I and *Xho* I, were used to screen for the pYCpG2-pre-S/S and pYCpG2-S plasmids, respectively. Following digestion with the appropriate sets of respective RE as shown in Fig. 4.3A&B, two pre-S/S clones were identified, designated as pYCpG2-pre-S/S4 and pYCpG2-pre-S/S1D; the latter contains a head-to-tail dimer of the pre-S/S DNA insert. Digestion of pYCpG2-pre-S/S4 produced bands of 4350, 3830, 1947, 1000 and 915 bp, while digestion of pYCpG2-pre-S/S1D produced an additional 1062 bp band that hybridized with a ^{32}P-labeled AusDHBV DNA probe (Fig. 4.3C). Likewise, digestion of the S clone pYCpG2-S41 produced bands of the expected sizes (4895, 3250, 2592 and 766 bp). These results confirmed that the pYCpG2-pre-S/S4, pYCpG2-pre-S/S1D, and pYCpG2-S41 plasmids contained DHBV gene sequences in the correct orientation.

Figure 4.2. PCR amplification of the AusDHBV pre-S/S and the S genes. The pre-S/S and S genes of the AusDHBV clone (pBL4.8) were amplified with two sets of primers, 31CG.792s/31CG.1854as, and 31CG.1131s/31CG.1854as, respectively (Section 2.5.1). The PCR products were analyzed on a 1.2% agarose gel, and the amplified DNA bands were of the expected size, 1062 bp for pre-S/S (lane 1) and 723 bp for S (lane 2). M is a molecular weight marker (*Pst* I digested-λ DNA).

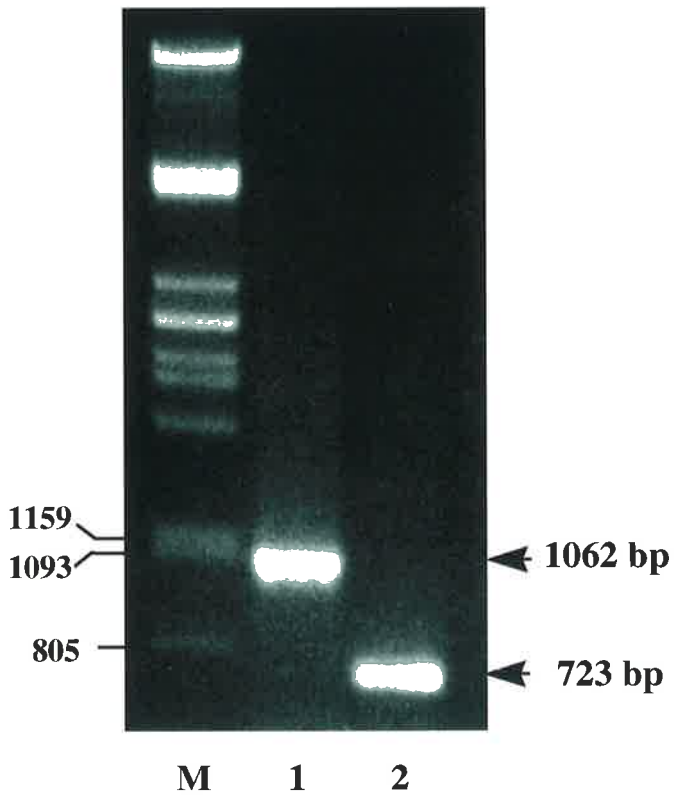
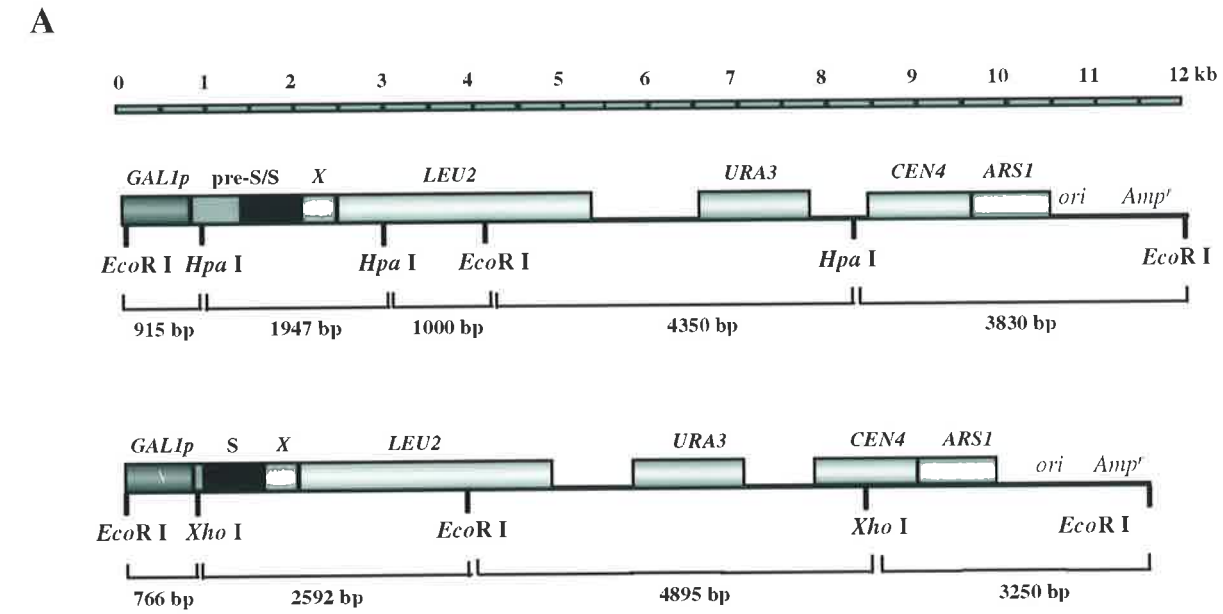
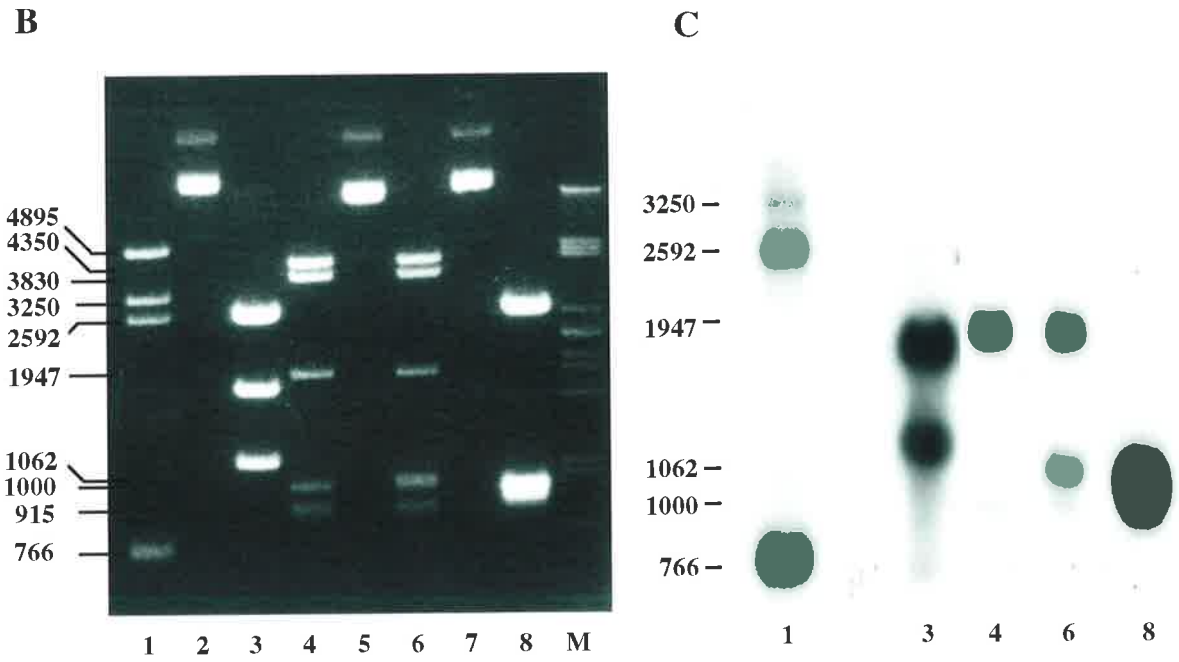

1159
1093
805
1062 bp
723 bp
M
1
2

Figure 4.3. Restriction enzyme (A) and Southern blot (B) analysis of the pYCpG2-pre-S/S and pYCpG2-S plasmids. **(A)** Recombinant yeast plasmids, pYCpG2-pre-S/S and pYCpG2-S, were screened with the following RE: *Eco*R I and *Hpa* I (for two pre-S/S clones), and *Eco*R I and *Xho* I (for the S clone). The predicted sizes of the plasmid DNA bands after digestion are shown.

(B) Following RE digestion, the recombinant plasmid DNA was analyzed on a 1.2% agarose gel. Lane *1*, digestion of pYCpG2-S41 produced bands of 4895, 3250, 2592, and 766 bp; lane *2*, undigested pYCpG2-S41; lane *3*, digestion products of the parental AusDHBV clone (pBL4.8) with *Eco*R I and *Xho* I (2961, 1815 and 1212 bp); lane *4*, digestion of pYCpG2-pre-S/S4 produced bands of 4350, 3830, 1947, 1000 and 915 bp; lane *5*, undigested pYCpG2-pre-S/S4; lane *6*, digestion of pYCpG2-pre-S/S1D which contains a head-to-tail dimer of the pre-S/S insert, produced an additional 1062 bp band; lane *7*, undigested pYCpG2-pre-S/S1D; lane *8*, digestion products of pBL4.8 with *Eco*R I and *Hpa* I (2961, 1035, 1022 and 970 bp). M, MW marker (*Pst* I-digested λ DNA)

(C) Southern blot hybridization using a ^{32}P[dCTP]-labeled AusDHBV DNA probe was performed following restriction enzyme analysis as shown in **(B)**. The undigested plasmids were not included. As a dimer, the pYCpG2-pre-S/S1D clone shows an extra band (1062 bp; lane 6) that not present in the monomer, pYCpG2-pre-S/S4 clone (lane 4).


A
0 1 2 3 4 5 6 7 8 9 10 11 12 kb
GAL1p pre-S/S X LEU2 URA3 CEN4 ARS1 ori Ampr
EcoR I Hpa I Hpa I EcoR I Hpa I EcoR I
915 bp 1947 bp 1000 bp 4350 bp 3830 bp
GAL1p S X LEU2 URA3 CEN4 ARS1 ori Ampr
EcoR I Xho I EcoR I Xho I EcoR I
766 bp 2592 bp 4895 bp 3250 bp


B
4895
4350
3830
3250
2592
1947
1062
1000
915
766
1 2 3 4 5 6 7 8 M
C
3250
2592
1947
1062
1000
766
1 3 4 6 8

4.3.3. Expression and purification of the recombinant DHBV pre-S/S and S proteins in *S. cerevisiae*.

(i) Selection of *URA3* transformants and expression of the recombinant proteins.

Yeast cells carrying the recombinant plasmids (*URA3* transformants) were obtained following transformation of *S. cerevisiae* 15Dα (*ura3-, leu2-, his2-, ade1-)* with the pYCpG2-pre-S/S4, pYCpG2-pre-S/S1D, or pYCpG2-S41 plasmids, and selection of transformants on *SC-ura* medium, a selective medium lacking the amino acid uracil (Section 2.5.4). Substitution of 2% sucrose with 2% galactose in the *SC-ura* medium induced the expression of recombinant DHBV pre-S/S and S proteins that were detected 12 hr after induction. Recombinant proteins were harvested by lysis of the cells with glass beads and removal of cell debris by centrifugation (Section 2.5.5). The procedures described below were used for purification of both yeast-derived DHBV pre-S/S and S proteins, although data for the S protein only are shown.

(ii) Purification of the yeast-derived pre-S/S and S proteins.

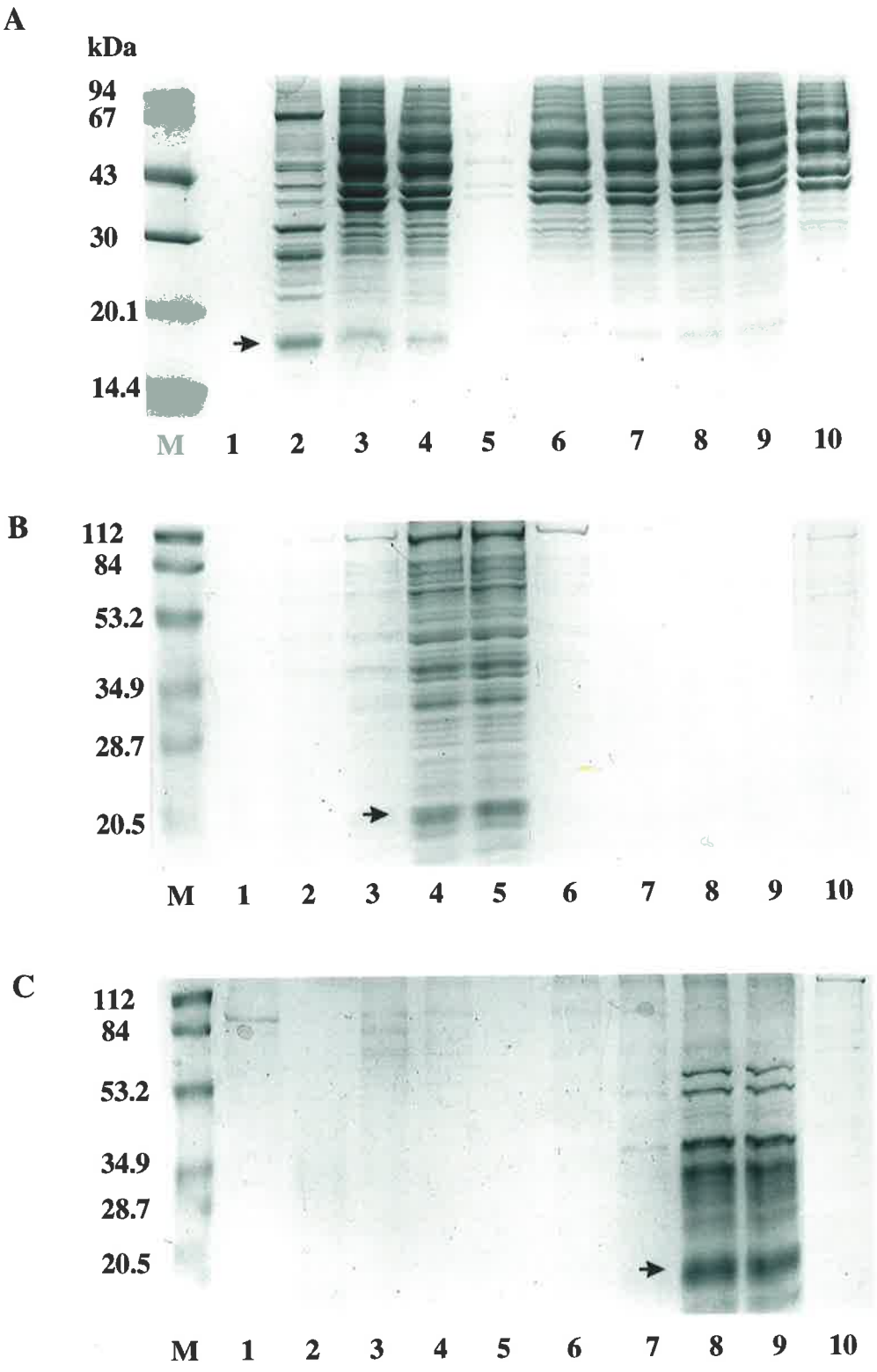
All ultracentrifugation steps were performed in 14 x 89 mm ultracentrifuge tubes in an SW41 rotor (Beckman) at 4°C. Following centrifugation, 500 µl fractions (usually a total of 22) were collected from the bottom of each tube. At each step, only the first 10 fractions are shown (Fig. 4.4A-C) as other fractions contained only high molecular weight (MW) bands of yeast protein. Fractions containing the recombinant DHBV surface proteins were pooled, dialyzed thoroughly against PBSE (containing 0.02% sodium azide) and concentrated with PEG 6000 prior to the next centrifugation step.

Figure 4.4. Purification of yeast-derived AusDHBV S protein. Following lysis of the cell and removal of the cell debris, the clarified supernatant containing the S protein was subjected to successive sucrose gradient ultracentrifugation performed in an SW41 rotor (Beckman) at 4°C as described (Section 2.5.5). In all steps (A-C), 500 µl fractions were collected from the bottom of the tube, and a 90 µl sample of each fraction was TCA precipitated (Schlicht *et al.,* 1987), run on a SDS-PAGE and stained with Coomassie brilliant blue. The numbers represent the fraction numbers from the bottom of the tube. M is the low molecular weight (LMW) marker (Pharmacia).

(A) After pelleting onto a 70% sucrose cushion. Protein samples were applied onto 1 ml of 70% sucrose and centrifuged at 12,500 rpm for 12 hr. The S protein (arrow) was detected mostly in fraction 2, with a small proportion in fractions 3 and 4.

(B) After centrifugation on a 20-50% sucrose equilibrium gradient. A pool of fraction 2 from (A) was dialyzed thoroughly against PBSE (containing 0.02% sodium azide), concentrated with PEG 6000, and centrifuged through a 20-50% continuous sucrose gradient at 39,000 rpm for 16 hr. The S protein (arrow) was banded in fractions 4 and 5.

(C) After centrifugation on a 5-30% sucrose rate-zonal gradient. A pool of fractions 4 and 5 from (B) was dialyzed thoroughly against PBSE (containing 0.02% sodium azide), concentrated with PEG 6000, and subjected to a rate-zonal centrifugation on a 5-30% continuous sucrose gradient at 33,000 rpm for 6 hr. The S protein (arrow) was located in fractions 8 and 9, which also contained high MW bands.


A
kDa
94
67
43
30
20.1
14.4
M 1 2 3 4 5 6 7 8 9 10
B
112
84
53.2
34.9
28.7
20.5
M 1 2 3 4 5 6 7 8 9 10
C
112
84
53.2
34.9
28.7
20.5
M 1 2 3 4 5 6 7 8 9 10

In the first step, the clarified supernatant containing the yeast-derived DHBV surface proteins was overlaid onto 1 ml of 70% (w/v) sucrose and centrifuged at 20,000 x g (12,500 rpm) for 14 hr (Fig. 4.4A). A 17 kDa band, the expected size of the DHBV S protein, was found above the cushion mostly in fraction 2, with less in fractions 3 and 4. Protein samples from fraction 2 were then overlaid onto a 20-50% equilibrium sucrose gradient and centrifuged at 190,000 x g (39,000 rpm) for 12 hr. The 17 kDa S protein was located in fractions 4 and 5 (Fig. 4.4B), and had been separated from most of the yeast proteins that were found later in fractions 16-22 (data not shown). However, several protein bands of high MW were also found in these two fractions containing the S protein. To purify the S protein from these high MW bands, protein samples from fractions 4 and 5 of the 20-50% gradient were then overlaid onto a 5-30% equilibrium sucrose gradient and subjected to rate-zonal centrifugation at 33,000 rpm for 6 hr (Fig. 4.4C). The 17 kDa S protein was detected in two fractions that had a density of 22% sucrose, namely fractions 8 and 9. As was observed following centrifugation on the 20-50% sucrose gradient above, high MW yeast protein bands were also present in these two fractions containing the S protein. This result suggested that the recombinant DHBV proteins were associated with these cellular proteins which presumably are yeast membrane proteins, that co-assembled during particle formation. Dissociation of other cellular proteins from the S protein was attempted by incubating 10 µl of the S protein with an equal volume of detergents (Triton X-100 and Triton X-114 at final concentrations of 0.25-2%, and Sarkosyl at 0.83%) and high salt (at final concentration of 0.5-2 M NaCl) for 15 min at 4°C (Bordier, 1981). After centrifugation at 20,000 rpm in JA-20 rotor (Beckman) for 15 min, and resuspension of the pellet in 10 µl of 25 mM Tris-HCl pH 7.4, 150 mM NaCl, both pellet and supernatant phases were analyzed on SDS-PAGE. The S protein was

found in the pellet phase and still associated with other yeast proteins (data not shown), suggesting that the yeast-derived DHBV pre-S/S and S proteins were likely to be present as integral membrane proteins, presumably due to the hydrophobic character of the S domain (see Section 3.3.7). Due to this association, the yeast-derived pre-S/S and S proteins after the rate zonal centrifugation still contain ~30% of other yeast proteins.

(iii) Yield of the recombinant pre-S/S and S proteins.

The yield of yeast-derived DHBV pre-S/S and S proteins were 0.62 and 1.5 mg/l of culture, respectively. The low expression of yeast-derived pre-S/S protein was shown by both pre-S/S constructs (pYCpG2-pre-S/S4 and pYCpG2-pre-S/S1D) (data not shown). Higher expression of S than the pre-S/S protein in yeast cells have also been reported earlier for DHBV (Klingmüller & Schaller, 1993) and for HBV (Imamura *et al.,* 1987).

4.3.4. Characteristics of the yeast-derived DHBV pre-S/S and S proteins.

(i) The Svedberg (S) value.

The S value of yeast-derived DHBV pre-S/S and S proteins was estimated according to Griffith (1979) following a rate zonal centrifugation on 5-30% sucrose gradient in an SW41 rotor (see above), where pre-S/S and S proteins were detected in two fractions at a density of 22% sucrose.

First, the Z_0 for rotor and gradient was calculated from formula:

Z_o for rotor and gradient:

$$\frac{Z_1 r_2 - Z_2 r_1}{r_2 - r_1}$$

Z_1 = minimum % w/w of sucrose gradient (5%).

Z_2 = maximum % w/w of sucrose gradient (30%).

r_1 = meniscus of gradient/minimum radial distance from centrifugal axis (6.7 cm)*.

r_2 = bottom of tube/maximum radial distance from centrifugal axis (15.2 cm)*.

Z_o = solute concentration corresponding to extrapolation of a linear gradient distribution to zero radius.

$$Z_o = \frac{(5 \times 15.2) - (30 \times 6.7)}{15.2 - 6.7} = \frac{76 - 201}{8.5} = \frac{-125}{8.5} = -14.7 = -15$$

* The values for SW41 rotor according to the physical specifications of Beckman preparative ultracentrifuge rotors (see Table 2.2).

Second, the Time Integral (I) values of sucrose at the meniscus of the gradient and at the separated zone for particle was determined (see Table 2.3). In this study, a particle density of 1.30 (generally used for most proteins and some plants and bacterial viruses) measured at 5°C was used.

ΔI = Time integral value (at the meniscus of the gradient and at the separated zone for the particles).

I (5% at the meniscus) = 0.4820^

I (22% at the separated zone) = 2.2850^

ΔI = 1.803

^ The Time Integral values of sucrose for particle density of 1.30 measured at 5°C (see Table 2.3).

Finally, the S value is calculated from formula:

$$S = \Delta I / w^2 t$$

where $w^2 = (0.10472 \times rpm)^2$

$= (0.10472 \times 33{,}000)^2$

$= (3455.76)^2$

$$= 1.194 \times 10^7 \text{ sec}$$

$$t = 6 \text{ hr} = 21{,}600 \text{ sec}$$

$$S = \frac{1.803}{1.194 \times 10^7 \times 21{,}600} = \frac{1.803}{25.79 \times 10^{10}}$$

$$= 0.0699 \times 10^{-10} = 699 \times 10^{-13}$$

$$= 70S$$

The S value above (70S) is in agreement with purified serum-derived DHBsAg particles which have an S value of ~77S (Tsiquaye *et al.,* 1985). For comparison, the S value of serum- and yeast-derived HBsAg particles is 55-60S (Burrell, 1975; Valenzuela *et al.,* 1982), while plant-derived HBsAg has the S value of ~60S (Mason *et al.,* 1992). The yeast-derived DHBV pre-S/S and S particles had a buoyant density in $CsCl_2$ of 1.2-1.21 g/cm^3 (data not shown). This buoyant density was slightly higher than that shown for serum-derived DHBsAg particles (1.16 g/cm^3; Schlicht *et al.,* 1987a; Tsiquaye *et al.,* 1985).

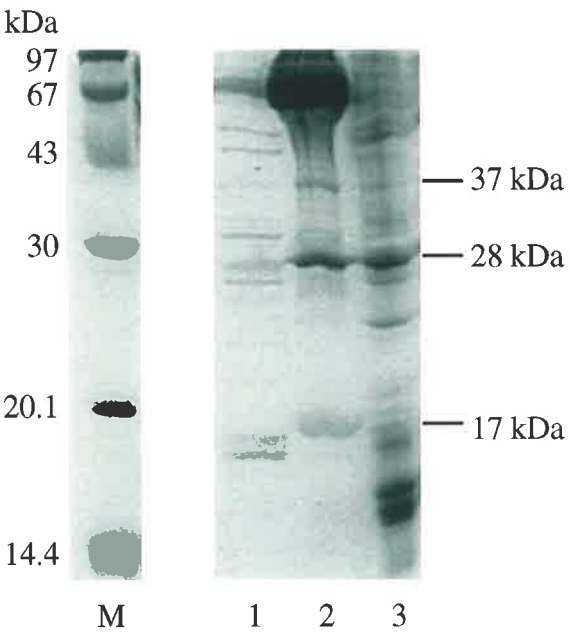
The similarities in the S value and buoyant density of both yeast-derived DHBV pre-S/S and S particles would not be surprising if both proteins were assembled as particles. A similar result has been reported earlier for the M and S proteins of HBV expressed in *S. cerevisiae* that showed a buoyant density in $CsCl_2$ at 1.2 g/ml (Imamura *et al.,* 1987).

(ii) Immunogenicity of the pre-S/S and S proteins.

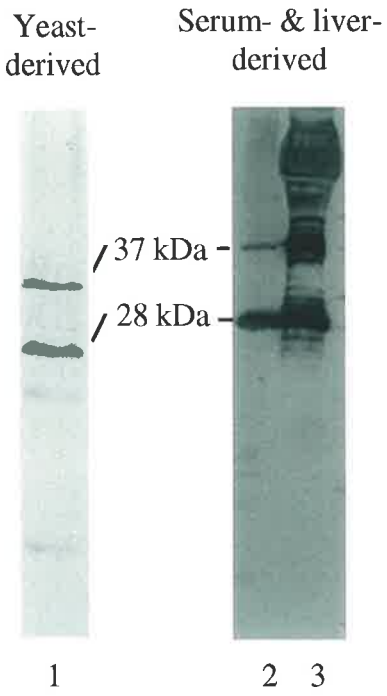
Western blot analysis of the yeast-derived pre-S/S protein using 1H.1, an anti-pre-S specific monoclonal antibody (Pugh *et al.,* 1995) revealed the presence of two bands (37 and 28 kDa). Both the 37 and the 28 kDa pre-S/S bands were also present in the AusDHBV-infected serum and liver samples (Fig. 4.5). Of note, in a recent study, the

Figure 4.5. SDS-PAGE and Western blot analysis of the yeast-derived AusDHBV pre-S/S protein. **(A)** Following centrifugation on a 5-30% continuous sucrose gradient, a 90 µl sample of fraction 9 of the yeast-derived pre-S/S protein was run on a SDS-PAGE, and stained with Coomassie brilliant blue. Lane: *1*, fraction 9; *2*, sucrose gradient-AusDHBV purified from infected serum; *3*, AusDHBV infected-liver tissue; M, LMW (Pharmacia). Methods to obtain samples 2 and 3 were described in Section 2.5.6.
(B) An identical gel was transferred to a PVDF membrane (Section 2.5.6). After blocking in 5% skim milk in TBS-T buffer (10 mM Tris-HCl 7.5, 0.9% NaCl, 0.05% Tween 20) overnight at 4°C, the pre-S/S protein was detected with the following antibodies (prepared in 5% skim milk-TBS-T buffer). First, 1H.1, an anti-pre-S specific monoclonal antibody, at a dilution of 1/5000 for 2 hr. Second, sheep-anti-mouse Ig, biotinylated $F(ab')_2$ fragment (Amersham) at a dilution of 1/400. Finally, streptavidin-biotinylated HRP complex (Amersham) at a dilution of 1/400. The pre-S/S protein was then visualized after addition of HRP substrate (0.5 mg/ml DAB, 12 µl H_2O_2 in 9 ml PBS). All incubation steps were performed at RT with gentle shaking; unbound antibodies were removed by 2 x 5 min washing in TBS-T between each step. Two bands (37 and 28 kDa) were detected in the yeast-derived, and also in the serum derived AusDHBV and infected liver tissue samples.

(A) SDS-PAGE



(B) Western blot



^{125}I-labeled virus purified from the same pool of AusDHBV-infected serum did not show the presence of the 28 kDa pre-S/S protein following autoradiography (A. Jilbert, personal communication). The possible explanations for this difference are discussed below.

Western blot analysis was also used to characterize the yeast-derived S protein. Using an anti-DHBV positive serum from duck (B40R86) that had resolved DHBV infection (Jilbert *et al.,* 1998), the yeast-derived S protein showed the expected 17 kDa band (Fig. 4.6B lane 1-3) similar to that of purified AusDHBV-infected serum run as positive control (Fig. 4.6B). Interestingly, although the AusDHBV-infected serum sample contains both pre-S/S and S proteins, the anti-DHBV positive duck serum only identified the 17 kDa S protein band, but not the 37 kDa or 28 kDa pre-S/S bands (Fig. 4.6B).

(iii) Development of anti-S specific antibody ELISA.

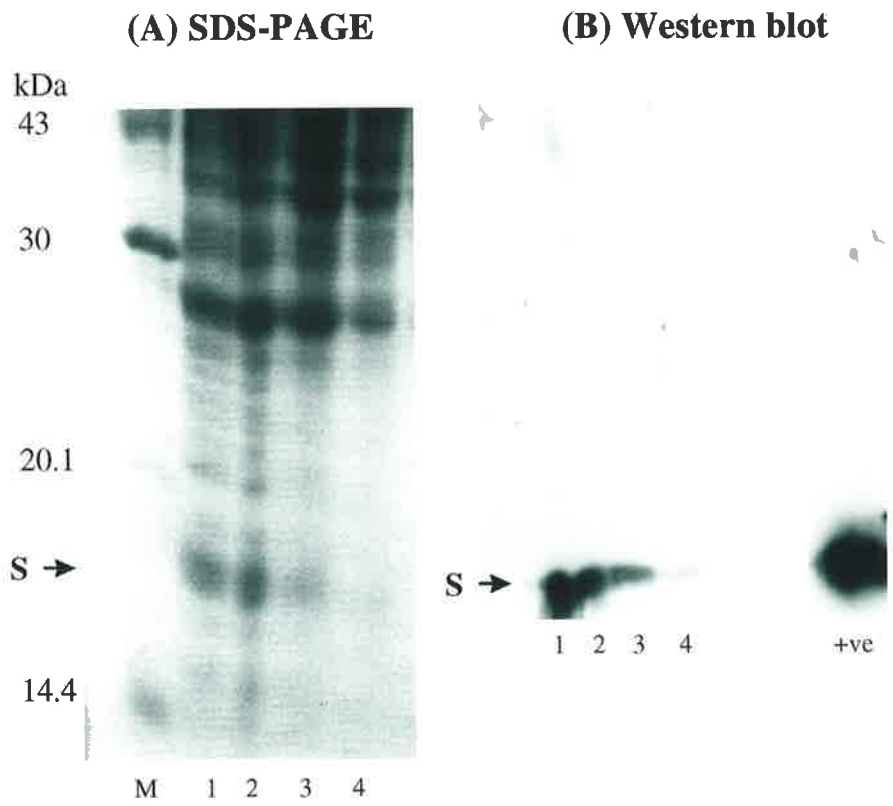
The yeast-derived S protein has been used to develop an anti-S specific antibody ELISA (Section 2.10.3). This assay has been used to detect the presence of anti-S specific antibody responses in the serum of ducks vaccinated with pre-S/S and S DNA (see Section 5.3.2; Triyatni *et al.,* 1998a). However, a detailed study on the sensitivity of this assay was not performed.

4.3.5. Anti-DHBs antibody responses following vaccination with yeast-derived DHBV pre-S/S and S proteins.

As stated earlier, following injection of 3-week-old ducks with three doses of 40 μg of the yeast-derived pre-S/S or S protein in Freund's adjuvant, serum samples were collected every two weeks and were measured for anti-DHBs antibody responses by ELISA

Figure 4.6. SDS-PAGE and Western blot analysis of the yeast-derived AusDHBV S protein. **(A)** Fractions 2-5 of the yeast-derived DHBV S protein collected following centrifugation on 1 ml of 70% sucrose cushion at 12,500 rpm for 14 hr, at 4°C, were run on a SDS-PAGE. Lanes: *1*, fraction 2; *2*, fraction 3; *3*, fraction 4; *4*, fraction 5; M, LMW marker (Pharmacia).

(B) An identical gel was transferred to Hybond-C extra membrane (Amersham) (Section 2.5.6). After blocking in 5% skim milk-TBST buffer, overnight at 4°C, the S protein was detected with the following antibodies (prepared in 5% skim milk-TBS-T buffer). First, anti-DHBV positive duck serum (B40R86) at a dilution of 1/4000 in for 2 hr. Then, rabbit-anti-duck IgY (Bertram, 1997) at a dilution of 1/10,000 for 1 hr. Finally, HRP-conjugated goat anti-rabbit IgG at a dilution of 1/5000 for 1 hr. All incubation steps were performed at RT with gentle shaking; unbound antibodies were removed by 2 x 5 min washing in TBS-T buffer between each step. The S protein was visualized using the enhanced chemiluminescence (ECL; Boehringer Mannheim) procedure. Exposure time: 5 min. Lanes: *1*, fraction 2; *2*, fraction 3; *3*, fraction 4; *4*, fraction 5; *+ve*, sucrose gradient-AusDHBV purified from infected serum (Section 2.5.6).


(A) SDS-PAGE
(B) Western blot
kDa
43
30
20.1
S
14.4
M 1 2 3 4
S
1 2 3 4
+ve

(Section 2.10.2). By 2 weeks after the first injection, anti-DHBs antibody responses were detected in all ducks (OD 490nm of 0.1-0.4 at a serum dilution of 1/25). The antibody levels slowly increased following subsequent injections, and reached an OD 490nm of 0.7-1.0 (at a serum dilution of 1/25) at 4 weeks after the third vaccination (Fig. 4.7).

4.3.6. The rate of virus removal from the bloodstream.

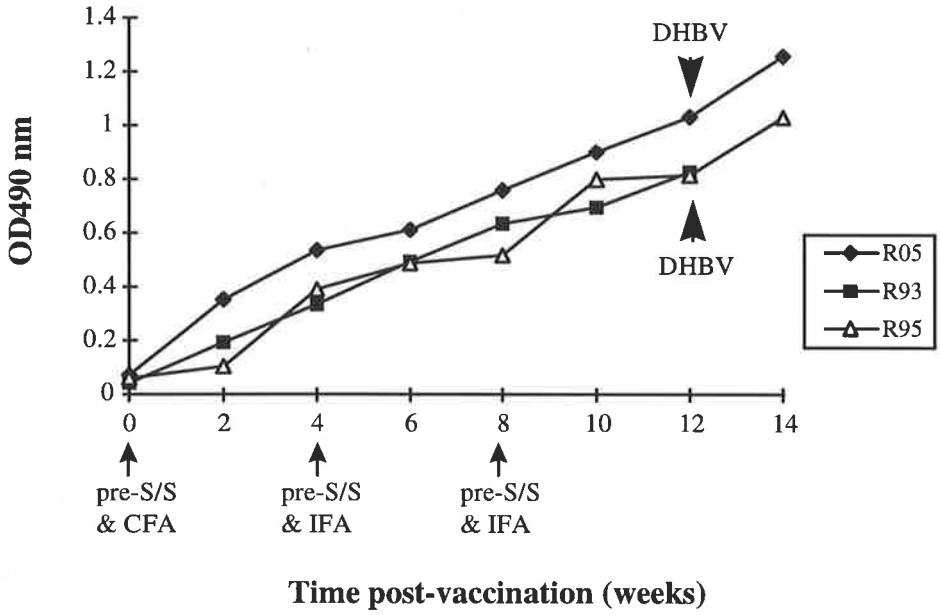
One of the parameters used in the laboratory to analyze the kinetics of DHBV infection following inoculation of a high dose of virus into non-vaccinated ducks is to measure the rate of virus removal from the bloodstream during the first 2 hr p.c. To assess whether the vaccinated ducks could remove the virus inoculum faster than non-vaccinated ducks, 4 ducks were challenged i.v. with high dose of inoculum ($1.9x10^{11}$ DHBV genomes in a total volume of 20 ml) four weeks after the third injection. The rate of virus removal from the bloodstream within 2 hr p.c. was measured (Section 2.7.4) and compared with that of a non-vaccinated control duck (R46) inoculated with an identical viral dose (Fig. 4.8). Both pre-S/S vaccinated ducks (R05 and R95) removed 90% of the inoculum within 60 and 110 min that was not different from the rate observed in the control duck (R46, 90% removal in 70 min). The S vaccinated ducks (R01 and R19) removed 90% of the inoculum slightly faster, between 30 and 45 min p.c.

4.3.7. Virus replication in the liver at 4 days p.c.

Immunohistological staining of liver sections taken from liver biopsy at 4 days p.c. showed that both vaccinated groups had restricted virus infection which affected ~40% of hepatocytes (Fig. 4.9). By comparison, the non-vaccinated duck (R46) showed

Figure 4.7. Total anti-DHBs responses following vaccination with yeast-derived pre-S/S and S proteins. A group of three-old-week ducks (R05, R93, R95) were vaccinated with yeast-derived pre-S/S protein (**A**); another group (R01, R04, R19) with yeast-derived S protein (**B**). Each dose contained 40 μg of either protein emulsified in CFA (first dose), or IFA (second and third doses). The presence of anti-DHBs antibody responses were measured by ELISA (Section 2.10.2). Four ducks (R05 and R95 vaccinated with pre-S/S; R19 and R23 vaccinated with S) were challenged i.v. with $1.9x10^{11}$ DHBV genomes (marked as DHBV) after the third vaccination. Serum samples were diluted at 1/25 in 5% skim milk-PBS-T buffer. CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

A


OD490 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DHBV
DHBV
R05
R93
R95
0
2
4
6
8
10
12
14
pre-S/S & CFA
pre-S/S & IFA
pre-S/S & IFA
Time post-vaccination (weeks)

B

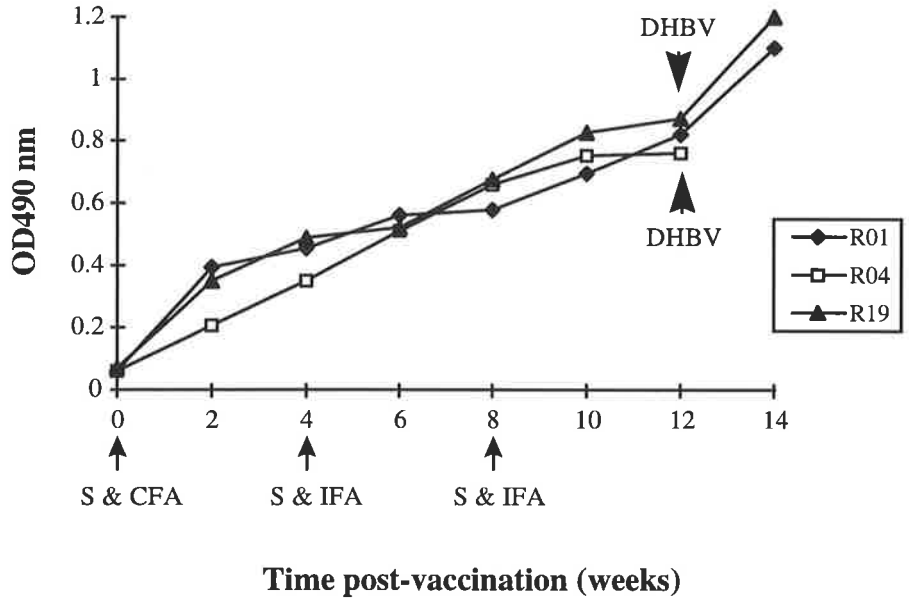
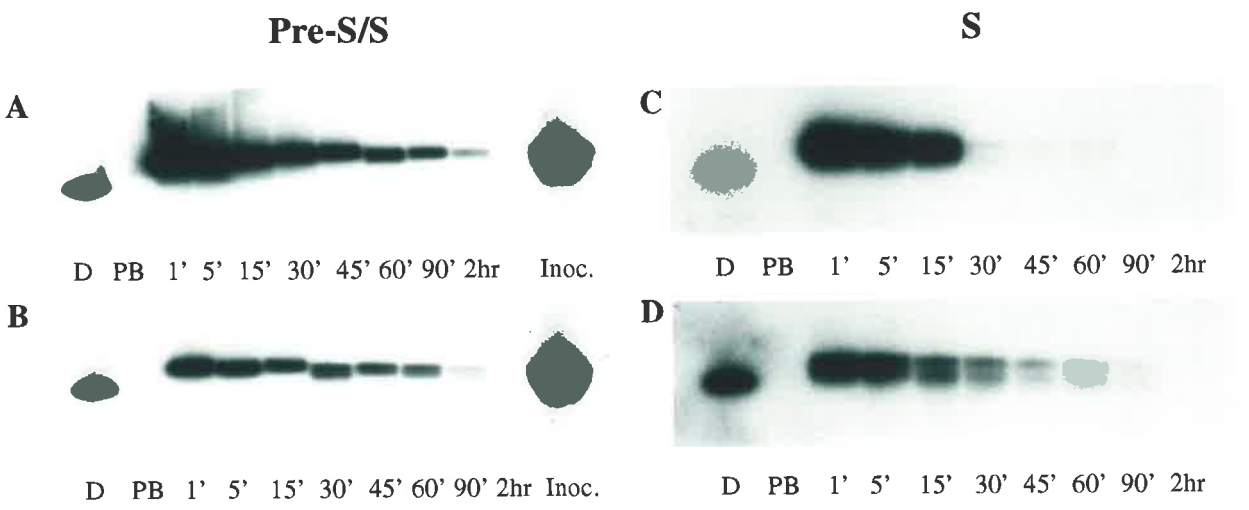
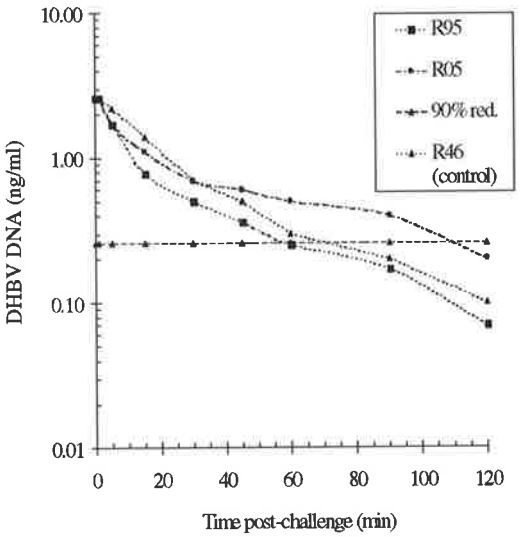

OD490 nm
1.2
1
0.8
0.6
0.4
0.2
0
DHBV
DHBV
R01
R04
R19
0
2
4
6
8
10
12
14
S & CFA
S & IFA
S & IFA
Time post-vaccination (weeks)

Figure 4.8. Southern blot analysis of virus removal from the bloodstream of pre-S/S and S vaccinated ducks. **(A-D)** Two ducks of each group which received yeast-derived vaccines: pre-S/S, R05 **(A)** and R95 **(B)**; and S, R19 **(C)** and R23 **(D),** were challenged i.v. with a high dose viral inoculum ($1.9x10^{11}$ DHBV genomes/20 ml pooled serum). Serum samples were taken at the indicated times p.c. and were extracted for total DNA (section 2.8.1). Samples equal to 50 µl of the original serum were electrophoresed on a 1.2% agarose gel followed by Southern blot hybridization using a ^{32}P[dCTP]-labeled AusDHBV DNA probe. Lanes: D, 50 pg of DHBV DNA/pBL4.8; PB, pre-bleed before challenge; Inoc., 5 µl of extracted inoculum, equivalent to 260 pg DHBV DNA.

(E & F) The profiles of the rate of virus removal from the bloodstream of pre-S/S and S (yeast-derived) vaccinated ducks. The profiles of the rate of virus removal from all pre-S/S **(E)** and S **(F)** vaccinated ducks are shown. The rate of removal of an identical dose of virus from a non-vaccinated control duck (R46) is also shown as comparison. The concentration in the inoculum (0 min) was calculated after correction for 10 x dilution effect (20 ml/200 ml total blood volume) occurring immediately after inoculation, i.e. 2.6 ng DHBV DNA/ml. The *y* axis shows the relative amount of viral DNA remaining in the bloodstream at each indicated time p.c. The DHBV DNA concentration remaining in the bloodstream after 90% removal of the inoculum is indicated as a horizontal line (90% red.).


Pre-S/S
S
A
C
D PB 1' 5' 15' 30' 45' 60' 90' 2hr Inoc.
D PB 1' 5' 15' 30' 45' 60' 90' 2hr
B
D
D PB 1' 5' 15' 30' 45' 60' 90' 2hr Inoc.
D PB 1' 5' 15' 30' 45' 60' 90' 2hr


10.00
1.00
0.10
0.01
DHBV DNA (ng/ml)
0
20
40
60
80
100
120
Time post-challenge (min)
R95
R05
90% red.
R46 (control)

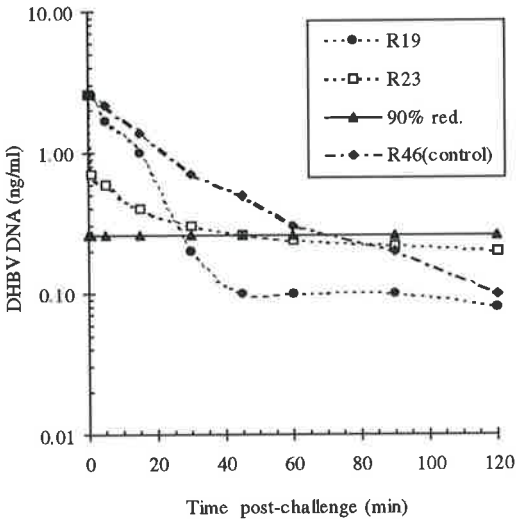
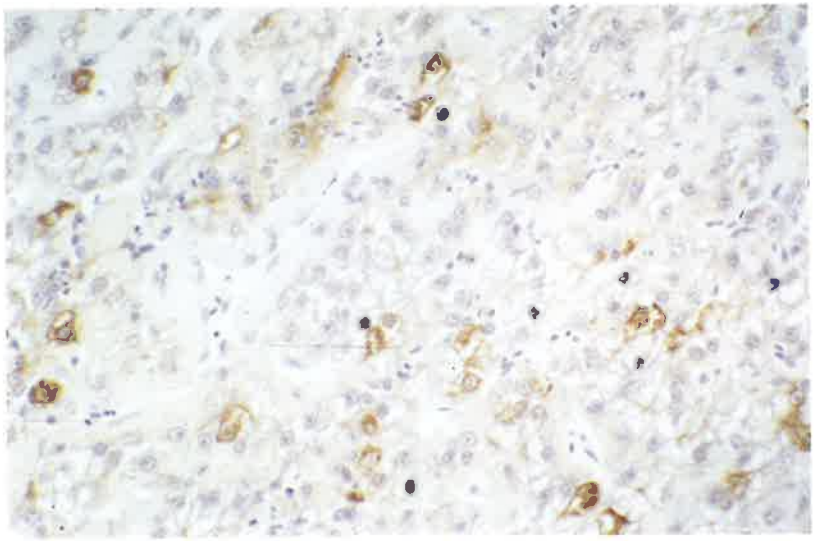
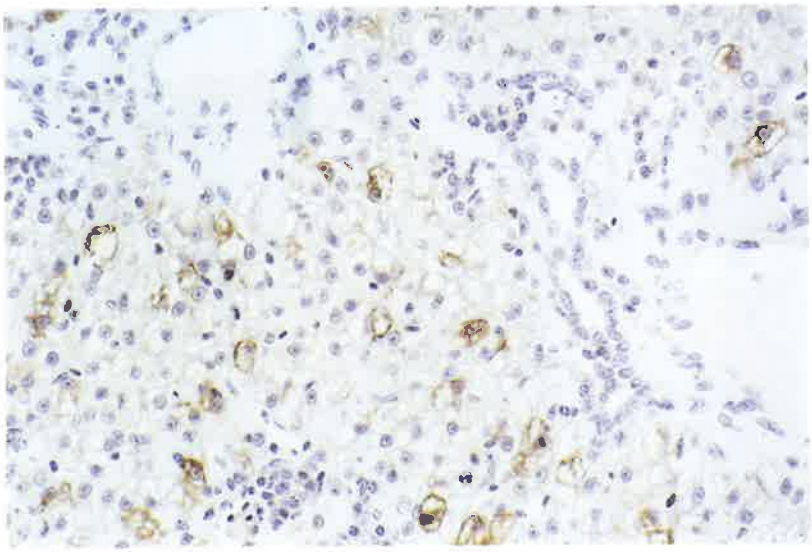

10.00
1.00
0.10
0.01
DHBV DNA (ng/ml)
0
20
40
60
80
100
120
Time post-challenge (min)
R19
R23
90% red.
R46(control)

Figure 4.9. Detection of DHBsAg in the liver at 4 days p.c. by immunohistological staining. Ducks vaccinated with yeast-derived pre-S/S, R05 (**A**), and S, R19 (**B**) were biopsied at 4 days p.c., and the presence of DHBsAg in the liver was detected by immunoperoxidase staining (Section 2.8.5). An anti-pre-S specific monoclonal antibody, 1H.1 (Pugh *et al.,* 1995) was used. Magnification: *40x*.

A



B



widespread virus replication that affected 90-95% of hepatocytes (see Fig. 5.6C). The extent of virus infection in the liver of the pre-S/S and S vaccinated ducks was approximately equal and, consistent with the earlier observation that both groups showed no significant differences in terms of virus removal rate from the bloodstream. Unfortunately, the monitoring of viremia to evaluate the long-term protective efficacy of yeast-derived vaccines could not be performed; all animals had developed secondary amyloidosis shortly or not long after the virus challenge. The factors responsible for secondary amyloidosis remain unknown, but possibly include housing problems, side effects of the Freund's adjuvant, or a combination of both.

4.4. DISCUSSION.

This study showed that transformed yeast cells expressed the AusDHBV pre-S/S and S proteins in the form of particles, confirming the earlier reports for yeast-derived HBsAg (Valenzuela *et al.,* 1982), and DHBsAg (Klingmüller & Schaller, 1993). However, the yeast-derived AusDHBV pre-S/S and S proteins had slightly higher buoyant density in $CsCl_2$ (1.2-1.21 g/cm^3) than that of serum-derived DHBsAg (1.16 g/cm^3; Schlicht *et al.,* 1987a; Tsiquaye *et al.,*1985), presumably due to their association with yeast membrane proteins. This association, together with the hydrophobic character of the DHBV S domain, could result in the formation of aggregates or micelles. This analysis was supported by a previous study which showed that the buoyant density of micelles of yeast-derived HBsAg in $CsCl_2$ was found to be 1.25 g/ml (Skelly *et al.,* 1981), in comparison to 1.19 g/ml for intact yeast-derived HBsAg particles (Valenzuela *et al.,* 1982).

The poor yield of yeast-derived DHBV pre-S/S protein compared to the S protein has been reported earlier by others (Klingmüller & Schaller, 1993), and is consistent with the observations made with yeast-derived HBV pre-S2/S protein (Imamura *et al.,* 1987). It was suggested that problems related to the low production of HBV pre-S2/S protein in yeast cells were due to hyperglycosylation, protease sensitivity of the pre-S2 sequence and incorrect folding of the assembled polyproteins (Schödel, 1998). Likewise, over-expression of the L (pre-S1/pre-S2/S) protein of HBV in yeast cells did not lead to the assembly of native-like particles; secretion of the L particles in the mammalian expression system requires the co-synthesis of L, M and S proteins in a ratio approximately similar to that found in virions (Schödel, 1998).

Two findings from Western blot analysis of the recombinant pre-S/S and S proteins are worthy of discussion. First, the presence of the 28 kDa pre-S/S protein in AusDHBV-infected serum and liver deserved comment. Currently, the 28 kDa pre-S/S protein of DHBV is commonly believed to represent the proteolytic product of the major 37 kDa product (Fernholz *et al.,* 1993). However, its presence could also be strain-related since it was detected in some DHBV isolates such as from the French Pekin duck, the French wild mallard, two Chinese brown ducks, and a German domestic Pekin duck (Lambert *et al.,* 1990; Yokosuka *et al.,* 1988). In a recent study, ^{125}I-labeled AusDHBV virus from DHBV-infected serum, did not show the presence of a 28 kDa pre-S/S protein (A. Jilbert, personal communication). There are few possibilities for the detection of 28 kDa protein in this study. Firstly, the 28 kDa protein could be a proteolytic product of the 37 kDa pre-S/S protein as has been reported earlier (Fernholz *et al.,* 1993). Alternatively, the 28 kDa protein could represent a component of duck serum protein that associates with the viral pre-S protein, and cross-reacts with the anti-pre-S monoclonal antibody used. Finally,

that the 28 kDa protein is indeed a second pre-S/S protein that present in some DHBV isolates (Lambert *et al.,* 1990; Yokosuka *et al.,* 1988). So far, based on the close relationship between the AusDHBV and the Chinese DHBV isolates, the specificity of MAb used, and the presence of this protein band in the AusDHBV-infected serum and liver samples, it can not be ruled out that the AusDHBV isolate indeed expresses the 28 kDa pre-S/S protein.

The second finding from Western blot analysis was that the observation that an anti-DHBV positive duck serum (B40R86) reacted to the 17 kDa protein, but not to the 37 kDa band, of the pre-S/S protein. It is conceivable that the S domain of AusDHBV surface protein is more immunogenic than the pre-S domain, hence antibodies produced following DHBV infection and challenge were predominantly raised against the S, and not the pre-S, domain.

Both yeast-derived DHBV pre-S/S and S proteins were able to elicit anti-DHBs antibody responses in three-week-old ducks following three doses of vaccine. The antibody titers elicited by yeast-derived DHBV surface proteins were, however, much lower than those produced following DNA vaccination. As comparison, the matched age ducks injected with pre-S/S and S DNA vaccines, developed anti-DHBs antibody titers at least >3 log higher than seen in this study (see Chapter 5). At least three possibilities could account for the lower immunogenicity of yeast-derived DHBV surface proteins. Firstly, the presence of ~30% of yeast proteins in the preparation given as vaccines. These yeast proteins may interfere with DHBV surface proteins in inducing immune responses in ducks by antigen competition. Secondly, the recombinant proteins may have undergone structural alteration that occurred during the purification process until the time of

injection (see below). Finally, the amount of pre-S/S and S proteins given (40 μg) at each injection may not have been sufficient to elicit strong antibody responses, due to the presence of other yeast proteins.

The Freund's complete adjuvant given with the first injection in this study is also known as a strong adjuvant. Its potency has been postulated to be due to the immunostimulatory sequences (ISS)-enriched mycobacterial DNA as one of its constituent, together with the cell-wall ingredients such as lipids and protein (Roman *et al.,* 1997). However, Freund's adjuvant can also cause severe inflammatory and toxic side effects attributed to the paraffin oil and the mycobacterial cell-wall products (Munoz, 1964). Nevertheless, the unfortunate development of secondary amyloidosis in ducks in this study was unlikely due to the side effect of Freund's adjuvant since the same problems were also found in 3 non-vaccinated ducks used as controls (data not shown).

The observation of restricted virus infection in the liver (~40% of hepatocytes) at 4 days p.c. demonstrated that both yeast-derived pre-S/S and S vaccines could provide some protection, albeit only partial, against DHBV infection. The inclusion of the pre-S protein did not result in enhanced immunogenicity and protection, when compared to that induced by the S vaccine. It should be pointed out, however, that due to the limited number of ducks examined, the single vaccine dose tested (40 μg per injection), and the secondary amyloidosis problems which might have affected the immune system of animals studied, differences may have been observed if various doses of vaccines were tested on a large number of healthy animals. Of note, although the inclusion of pre-S1 or pre-S2 domain has been shown to overcome genetic non-responsiveness to HBsAg in the murine model

(Milich *et al.,* 1991) and in humans (Zuckerman *et al.,* 1996), other clinical trials in non-responders have yielded conflicting results (Rutgers *et al.,* 1993).

Technical problems were encountered in this study and are worth mentioning as they have hampered the use of this method for production of suitable vaccines. Firstly, the yield of yeast-derived DHBV pre-S/S and S proteins was generally poor. Despite the commonly held view that yeast cells provide suitable eukaryotic expression system, several requirements are needed to optimize their utilization. Good aeration is an absolute requirement for their optimal growth (Vasavada, 1995) that is normally provided by fermentation techniques, especially when they are grown in selective media that theoretically slows the rate of cell growth. The use of selective medium (SC-*ura*) in a shake flask resulted in poor growth of yeast cells, and subsequently, in less reproducible yields of the recombinant proteins. For comparison, 20 mg/l of HBsAg has been produced in a fed-batch fermentation system where HBsAg expression is driven by a constitutive promoter (Vasavada, 1995).

Second, the lack of proper equipment for cell lysis (homogenizer) lengthened the protein purification process and increased the risk of proteolytic degradation of the recombinant proteins even in the presence of protease inhibitors. Third, ~30% of the yeast proteins could not be separated from the fractions containing yeast-derived DHBV surface proteins after rate-zonal centrifugation on a 5-30% continuous sucrose gradient. The recombinant DHBV surface proteins were therefore, still associated with these cellular proteins that were most likely yeast membrane proteins. Other purification methods that have successfully been applied to obtain pure recombinant HBsAg vaccines such as immunoaffinity chromatography could not be performed in this study due to the lack of

anti-S specific monoclonal antibodies. The low yield of the yeast-derived DHBV S protein has hampered its use for routine immunoassays such as ELISA, and for other studies where large amounts of relatively pure S protein are needed.

Taken together, this study has shown that yeast-derived DHBV surface proteins can be used to vaccinate ducks against DHBV infection, and to provide a source of antigen for ELISA. However, to optimize the utilization of yeast-derived DHBV surface proteins for the above purposes, further improvements should be made such as: (i) Designing a vector with a secretion signal upstream of the DHBV pre-S/S and S genes to allow secretion of the recombinant proteins. This would minimize proteolytic degradation of the recombinant proteins and simplify the purification process. (ii) Optimizing the culture methods by the use of fermentation culture technique to achieve higher protein yields.

Chapter 5

Protective efficacy of DNA vaccines against DHBV infection in adult and young ducks

5.1. INTRODUCTION AND AIMS.

HBV vaccines which contain the small (S) envelope protein of the virus induce anti-HBs antibodies >10mIU/ml in 80-95% of recipients who are then protected against subsequent HBV infection (Hadler *et al.,* 1986). However, an improvement in HBV vaccine efficacy is still desirable to induce protective responses in those who are non-responsive to the current vaccine, and to eliminate the need for booster injections. Inclusion of the pre-S peptide in the current vaccines has shown some benefit, as it resulted in production of anti-HBs antibodies in a limited number of non-responders (Yap *et al.,* 1995; Zuckerman *et al.,* 1996). An alternative approach is to use DNA vaccines (Davis *et al,* 1993a). They induce synthesis of foreign protein(s) *in vivo* from injected plasmid DNA and thereby should induce long-lasting humoral and cellular immune responses. For example, it has been shown that a DNA vaccine encoding HBsAg elicited anti-HBs antibody responses in mice (Davis *et al.,* 1993a) and chimpanzees (Davis *et al.,* 1996b).

The ultimate goal of vaccination is protection against natural infection. This means that the protective efficacy of novel HBV vaccines can only be tested by vaccination of higher primates such as chimpanzees followed by experimental HBV challenge (Prince *et al.,* 1997). Nevertheless, information about the protective efficacy of DNA vaccines against hepadnavirus infection can be obtained in other animal models including ducks experimentally infected with DHBV as described in this chapter (see also Triyatni *et al.,* 1998a).

DHBV is closely related to HBV in regard to genomic organization, hepatotropism, and mode of replication (Mason *et al.,* 1980). Furthermore, DHBV infection in its natural

host, the domestic Pekin duck, permits the study of virus neutralization mechanisms *in vitro* and protective immune responses *in vivo*. As with HBV, the large (pre-S/S) and small (S) envelope proteins of DHBV have been shown to carry epitopes which elicit neutralizing antibodies. Using an *in vitro* neutralization assay, several neutralizing epitopes have been mapped within the DHBV envelope proteins, four within the pre-S domain, and one in the S domain (Cheung *et al.*, 1989; Yuasa *et al.,* 1991). Others have reported that monoclonal antibodies against specific antigenic sites within the pre-S domain (AA 77-100) were able to reduce DHBV infectivity *in vivo* (Lambert *et al.,* 1990; Chassot *et al.,* 1993; Sunyach *et al.,* 1997). The ability of DHBV S protein alone to induce antibodies that protect against infection *in vivo* remains uncertain, although it has been demonstrated that monoclonal antibodies specific for S protein were able to neutralize DHBV infectivity *in vitro* (Cheung *et al.,* 1989; Pugh *et al.,* 1995).

This study was designed to examine the protective efficacy of DNA vaccines against DHBV infection in ducks. Three factors were measured: (i) the ability of DNA vaccines encoding the DHBV pre-S/S and S proteins to elicit anti-DHBs antibody responses in ducks; (ii) the efficacy of immune responses elicited by DNA vaccines to protect the animals against virus challenge, as determined by the rate of virus removal from the bloodstream, the presence of early virus replication in the liver, and the development of viremia; and (iii) the ability of serum from vaccinated ducks to neutralize virus infectivity in both *in vivo* and *in vitro* systems.

5.2. EXPERIMENTAL DESIGN.

The AusDHBV pre-S/S and S genes were cloned into the *BamH* I site of an eukaryotic expression system (pcDNA I/Amp) downstream of a CMV promoter and used as the DHBV pre-S/S and S DNA vaccines, respectively (Fig. 5.1). The pcDNA I-pre-S/S and pcDNA I-S constructs obtained were verified by restriction enzyme analysis followed by Southern blot hybridization, and nucleotide sequencing (Section 2.6.2).

The ability of both constructs to express DHBV pre-S/S and S proteins was confirmed by transient transfection of COS7 cells followed by indirect IMF using the appropriate antibodies at 2 days post-transfection (Section 2.6.3).

Six-month- and 3-week-old ducks (hereafter are called adult and young ducks, respectively) were injected i.m., three times, with 750 μg (adult) or 250 μg (young) of pcDNA I-pre-S/S or pcDNA I-S plasmid. All injections were performed into the quadriceps anterior (QA) muscles pre-treated with 750 μl (adult) and 250 μl (young) of 5 mg/ml bupivacaine hydrochloride 0.5% (Marcain; Astra Pharmaceuticals, Australia) at 5 days, and 25% (w/v) sucrose at 15 min, prior to DNA injection, respectively (Section 2.7.3).

Following vaccination, sequential blood samples were collected to monitor serological responses by ELISA. All vaccinated young ducks and one non-vaccinated duck (of a similar age) were then challenged with a high dose viral inoculum containing 1.9×10^{11} DHBV genomes (Jilbert *et al.*, 1996). Several parameters were measured: (i) the rate of

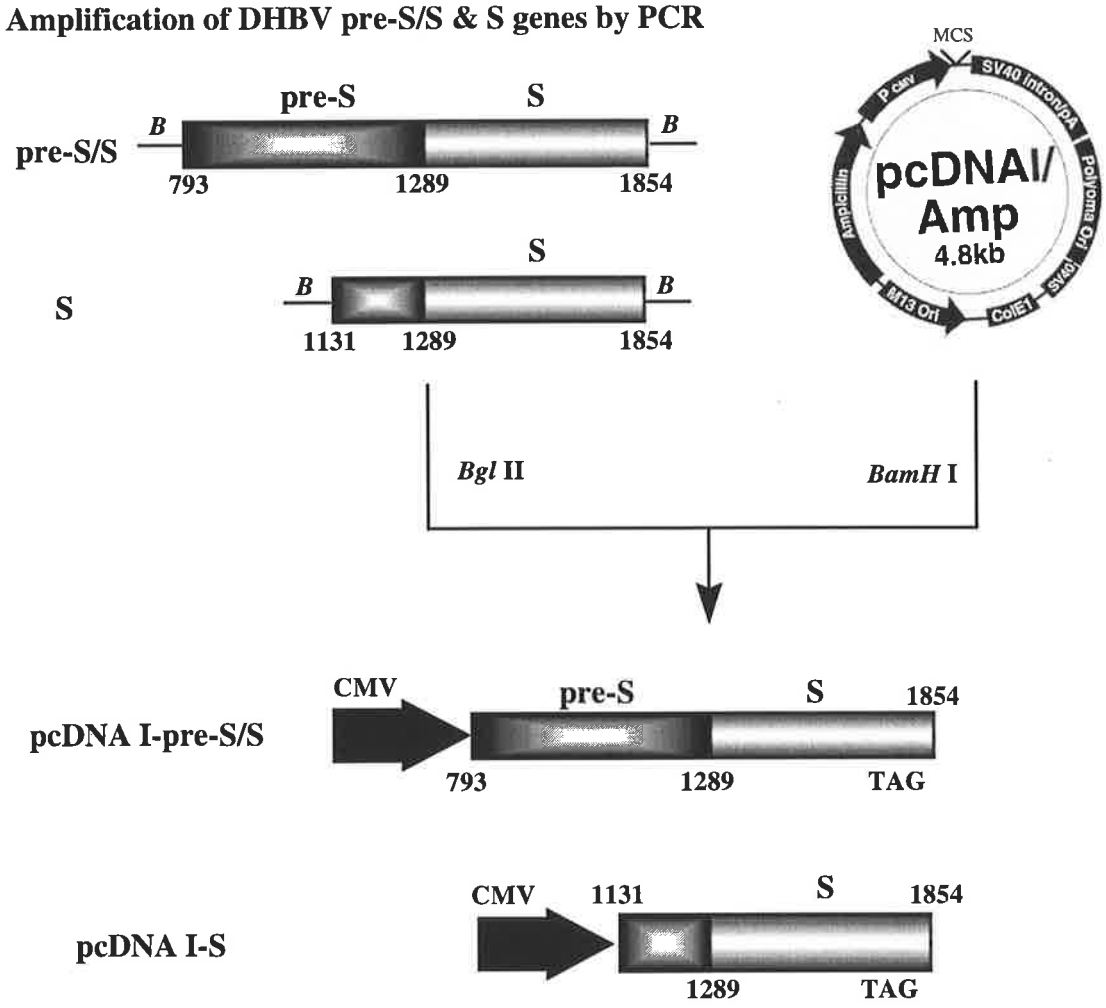


Figure 5.1. Schematic diagram of the cloning of the pre-S/S and S genes of AusDHBV into pcDNA I/Amp. The pre-S/S and S genes of the AusDHBV clone (pBL4.8) were introduced with *Bgl* II sites at both their 5' ends by PCR, and the PCR products were cloned into the *BamH* I site of pcDNA I/Amp plasmid downstream of a CMV promoter. The numbers shown are the nucleotide (nt) positions in the pre-S/S gene, according to DHBV-S31 (Uchida *et al.,* 1989). The start codon for the pre-S/S gene in the pcDNA I-pre-S/S construct is positioned at nt 801. The pcDNA I-S construct contains an extra 158 nucleotides of pre-S sequence (nt 1131-1289) upstream of the S start codon (nt 1290) due to the primer site chosen for PCR. An ATG is not present within this extra pre-S sequence nor within the CMV promoter, hence protein will be translated from the S start codon. TAG is the stop codon for the pre-S/S and S genes, positioned at nt 1793; *B, Bgl* II sites; MCS, multiple cloning sites; CMV, cytomegalovirus.

virus removal from the bloodstream, (ii) the extent of virus replication and mononuclear cell infiltration in liver tissue at 4 days p.c. (in some cases, liver tissues were also collected 2 weeks before challenge, to exclude any pre-existing non-specific histological changes), and (iii) the development of viremia (Section 2.8). The grading of histological changes in the liver tissues was based on the extent of (a) periportal necrosis, (b) intralobular degeneration and focal necrosis, (c) portal inflammation, and (d) fibrosis (Knodell *et al.,* 1981).

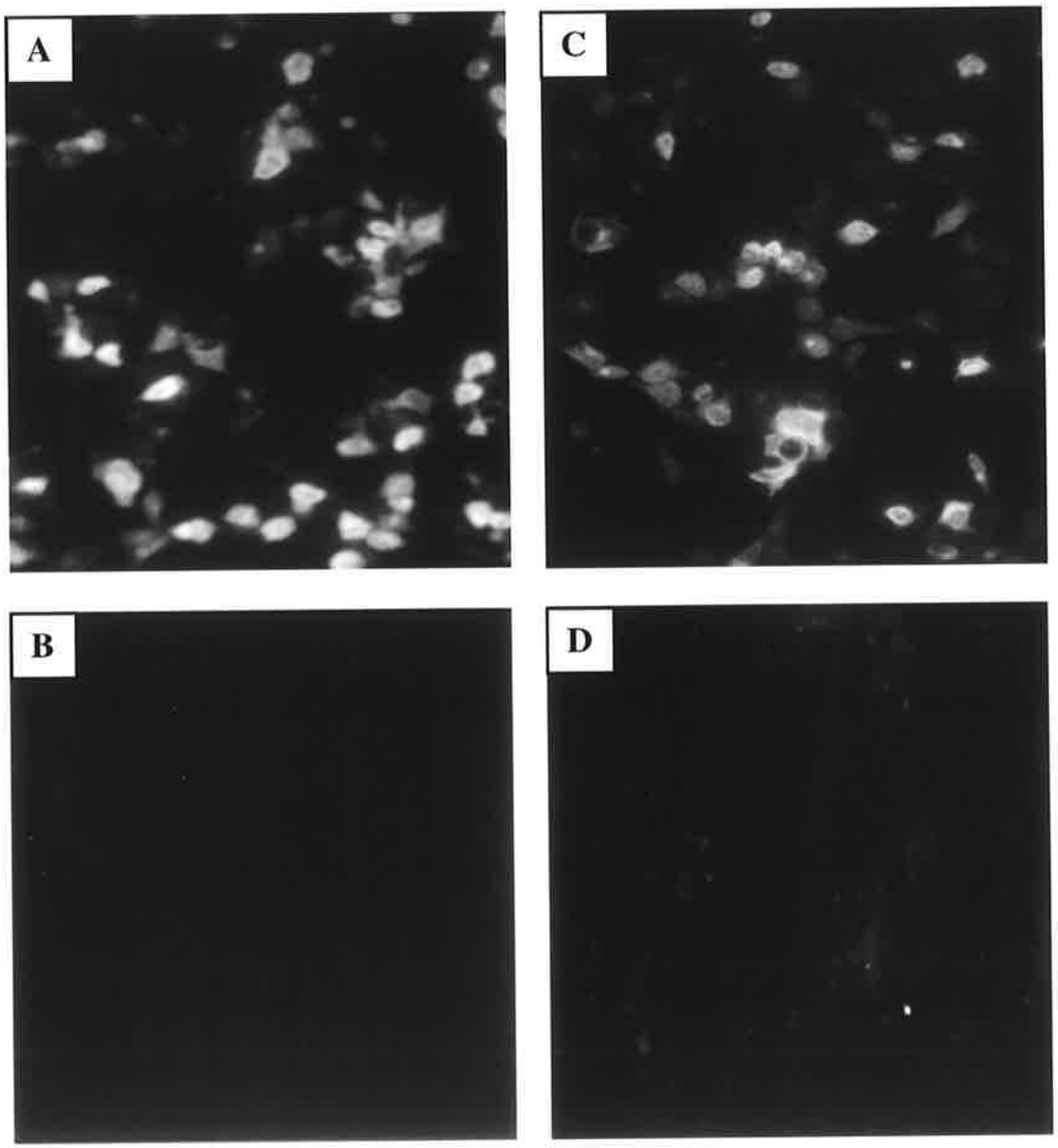
In vitro and *in vivo* neutralization assays were performed to assess whether antibodies elicited by DNA vaccines could neutralize viral infectivity prior to inoculation of primary duck hepatocyte cultures, and inoculation into 1-day-old ducks, respectively (Section 2.9).

5.3. RESULTS.

5.3.1. Expression of the pre-S/S & S proteins *in vitro* by pcDNA I-pre-S/S & pcDNA I-S plasmids.

COS7 cells were transiently transfected with either pcDNA I-pre-S/S or pcDNA I-S plasmid DNA, with the parental plasmid (pcDNA I/Amp) serving as a negative control. Examination of these cells at 2 days post-transfection by indirect IMF (Section 2.6.4) showed that pre-S/S and S proteins were expressed at approximately equivalent intensity from both constructs (Fig. 5.2A & C). On the other hand, cells transfected with the pcDNA I/Amp, did not react with the antibodies used (Fig. 5.2B & D). Western blot analysis was also performed using anti-pre-S 1H.1 (Pugh *et al.,* 1995) and anti-S 1B.10 (Cheung *et al.,* 1990) monoclonal antibodies (kindly donated by Dr. J. Pugh and Dr. P.

Figure 5.2. Transient transfection of COS7 cells with pcDNA-pre-S/S and pcDNA-S plasmid DNA. DHBV protein expression in COS7 cells was detected by indirect IMF 2 days post-transfection (Section 2.6.4). An anti-pre-S 1H.1 monoclonal antibody was used to detect pre-S protein **(A)** expressed by pcDNA I-pre-S/S plasmid. Rabbit-anti-DHBs antibody was used to detect S protein **(C)** expressed by pcDNA I-S plasmid. Cells transfected with the parental plasmid, pcDNA I/Amp, did not stain when treated with the anti-pre-S **(B)**, or anti-DHBs antibodies **(D).**

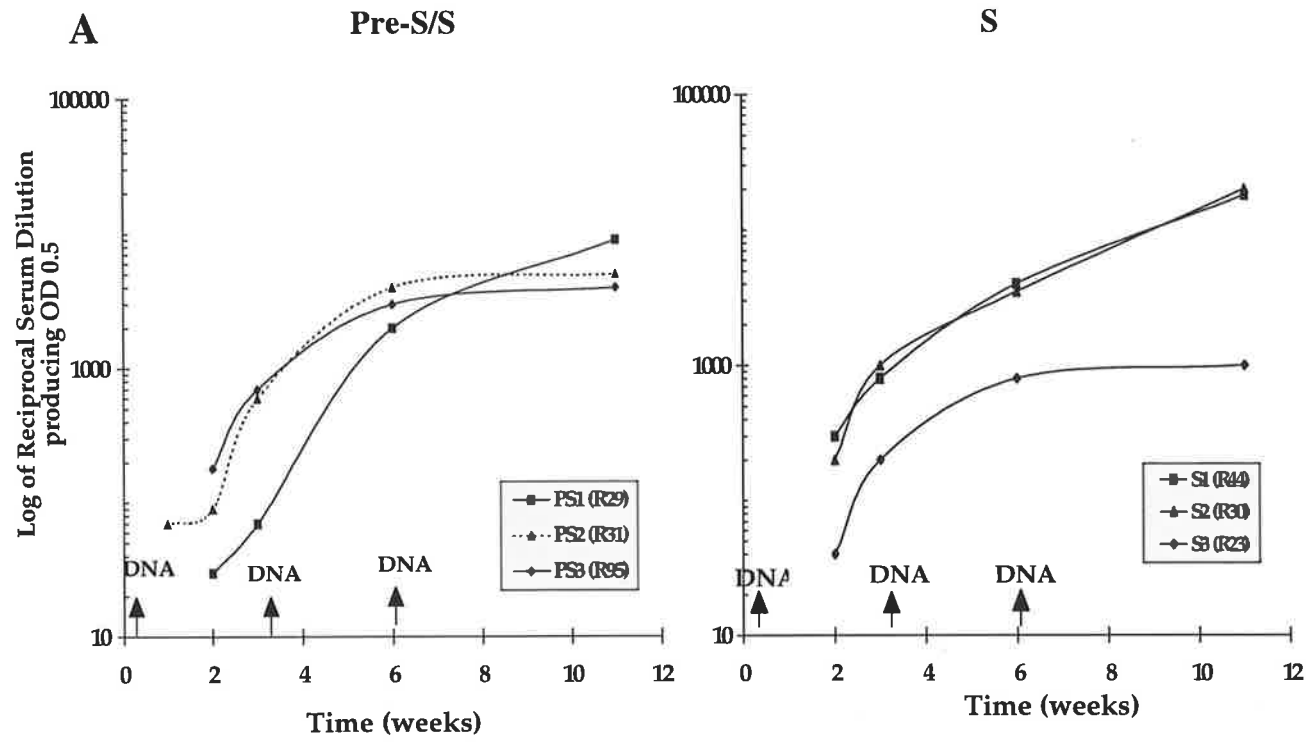

A
C
B
D

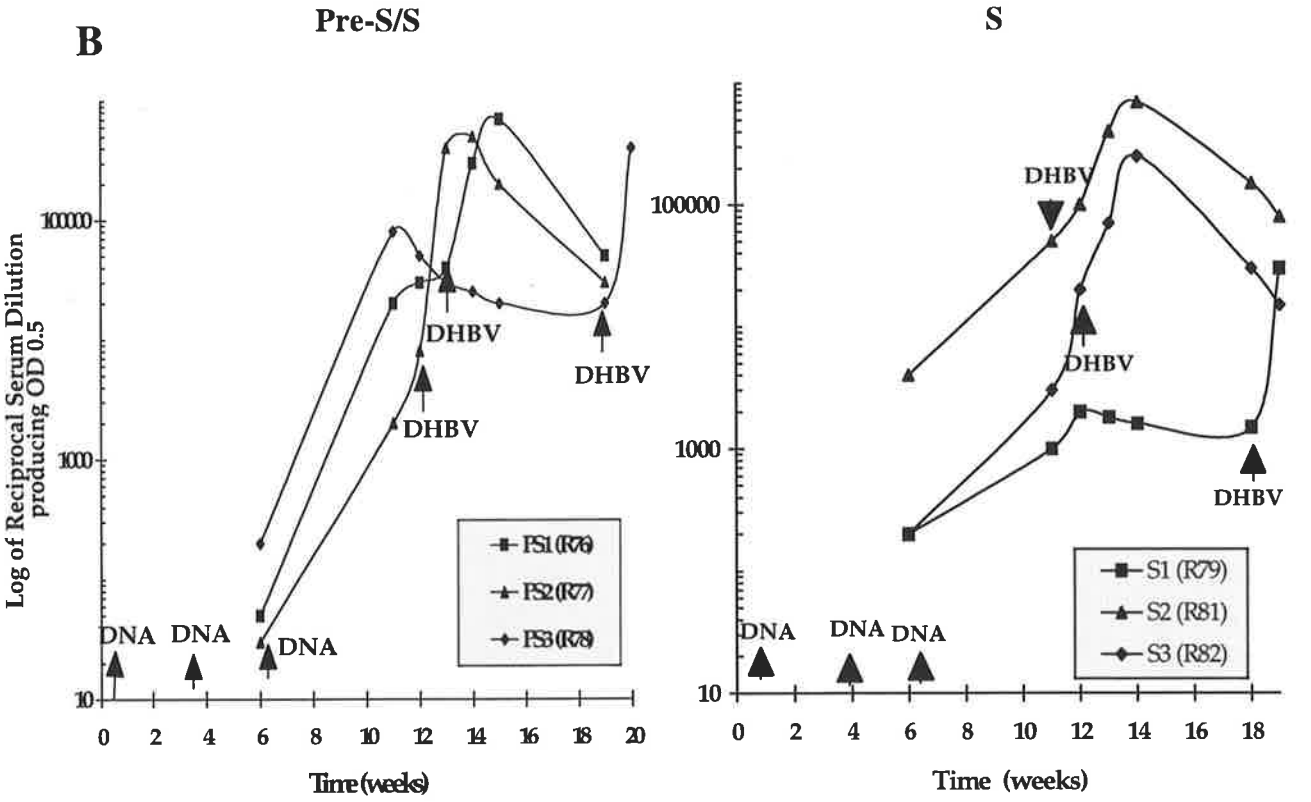
Marion, respectively), to characterize further the protein species expressed by both constructs in COS7 cells. Pre-S/S protein (MW 37 kDa) and a very small amount of S protein (17 kDa) were detected in cell lysates, but not in the culture medium, of cells transfected with the pcDNA I-pre-S/S plasmid. In contrast, the pcDNA I-S construct expressed S protein (MW 17 kDa) which was detected in both cell lysates and culture medium, suggesting that the S protein was secreted by the cells transfected with pcDNA I-S (data not shown).

5.3.2. Anti-DHBs antibody responses following DNA vaccination.

Both pre-S/S and S DNA-vaccinated adult ducks developed anti-DHBs antibody responses following vaccination. Anti-DHBs antibodies was detected two weeks after the first DNA injection, and the titers increased with subsequent vaccination to reach between 3,500-8,000 by 6 weeks after the third injection (Fig. 5.3A). Since purified DHBsAg from congenitally DHBV-infected serum was used as the antigen source in the ELISA, it could be inferred that antibodies raised by DNA vaccination recognized the native form of serum-derived DHBsAg. One duck (R23) that gave a poor antibody response was diagnosed subsequently by Congo Red staining of liver tissue (Elghetany & Saleem, 1988) to be suffering from secondary amyloidosis. DNA vaccination in young ducks also induced high titers of anti-DHBs antibodies. Although the antibody response were delayed until after the third injection, the antibody titers in young ducks were higher than those produced by the adult ducks. By six weeks after the third injection, the antibody titers of pre-S/S & S DNA-vaccinated young ducks reached between 10,000-40,000 and 20,000-50,000, respectively (Fig. 5.3B).

Figure 5.3. Anti-DHBs antibody responses following DNA vaccination in adult and young ducks. Two groups each of 6-month-old **(A)** and 3-week-old **(B)** ducks were vaccinated with either pre-S/S or S DNA vaccines, three times, at 3 weekly intervals. Total anti-DHBs antibody levels were measured by an antibody-capture ELISA with DHBsAg purified from serum of congenitally DHBV-infected ducks as the source of antigen (Section 2.10.2). The antibody titer was defined as the highest serum dilution that gave an OD 490nm of 0.5. In 3-week-old ducks **(B)**, anti-DHBs antibody responses were also measured following virus challenge (indicated by DHBV →) which was performed at different times after the third vaccination.


A
Pre-S/S
S
Log of Reciprocal Serum Dilution producing OD 0.5
100000
1000
10
DNA
DNA
DNA
PS1 (R29)
PS2 (R31)
PS3 (R95)
S1 (R44)
S2 (R30)
S3 (R23)
0 2 4 6 8 10 12
Time (weeks)


B
Pre-S/S
S
Log of Reciprocal Serum Dilution producing OD 0.5
100000
1000
10
DNA
DNA
DNA
DHBV
DHBV
DHBV
DHBV
DHBV
DHBV
PS1 (R76)
PS2 (R77)
PS3 (R78)
S1 (R79)
S2 (R81)
S3 (R82)
0 2 4 6 8 10 12 14 16 18 20
Time (weeks)

Because the above assay was design to detect antibodies to both pre-S and S antigens, the specific anti-S antibody responses in pre-S/S and S DNA-vaccinated young ducks was then determined by ELISA with yeast-derived S protein as the source of antigen (Section 2.5.5). The specific anti-S titers in both groups were approximately equivalent, ranging between 2,500-4,000 by six weeks after the third vaccination (Fig. 5.4).

5.3.3. The rate of virus removal from the bloodstream of vaccinated ducks.

As described in Chapter 4, one of the parameters used to assess the kinetics of DHBV infection following high dose virus inoculation in vaccinated ducks was to assess the rate of virus removal from the bloodstream within 2 hr p.c. Intravenous viral challenge was performed 3-8 weeks after the third vaccination in all young ducks (i.e. aged 10-12 weeks of age at the time of challenge). The different times chosen for viral challenge were due to technical constraints, since not all ducks could be challenged at the same time. The rate of virus removal from the bloodstream was analyzed by determining the DHBV DNA content of serum prepared from blood samples collected at various time points (pre-bleed, 1, 5, 15, 30, 45, 60, 90 min, and 2 hr p.c.). Examples of virus removal profiles following i.v. challenge of both groups of DNA vaccinated ducks are depicted in Fig. 5.

Pre-S/S DNA-vaccinated ducks removed 90% of the inoculum in 60-100 min (Fig. 5.5A & 5.5B). This is similar to the rate of virus removal measured in non-vaccinated ducks inoculated with an identical dose of virus (e.g., 70 min for R46; Fig. 5.5B). In contrast, two of four S DNA-vaccinated ducks (R81 and R82) removed 90% of the inoculum in less than 5 min p.c., while two other S DNA-vaccinated ducks (W17 and R79) removed

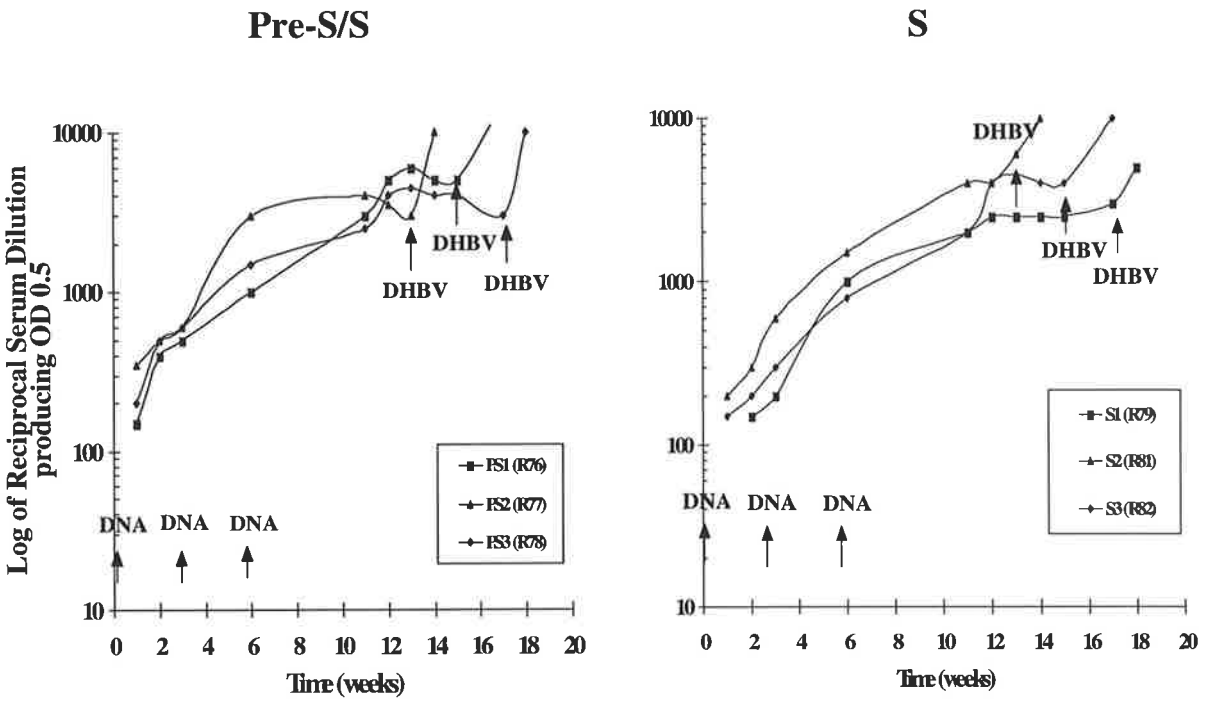
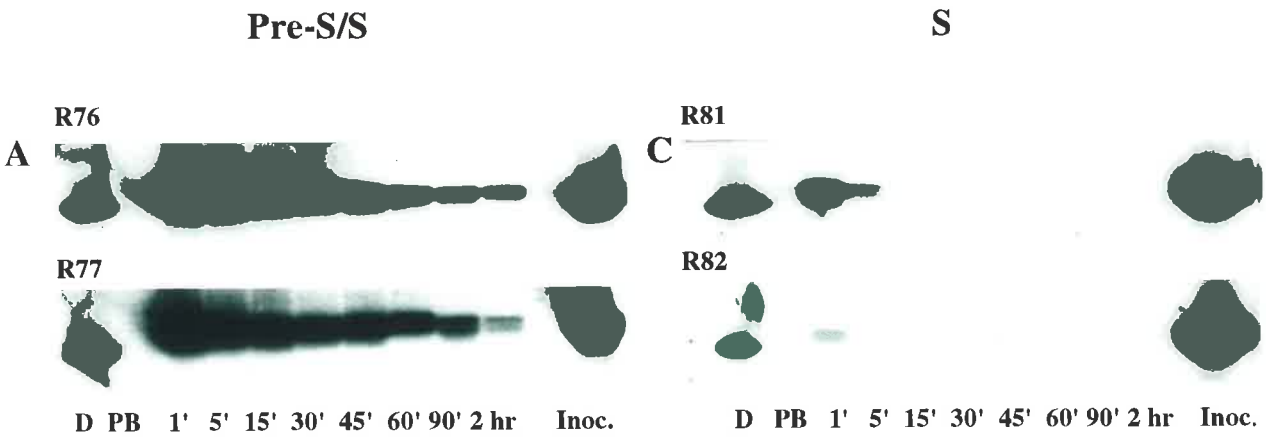
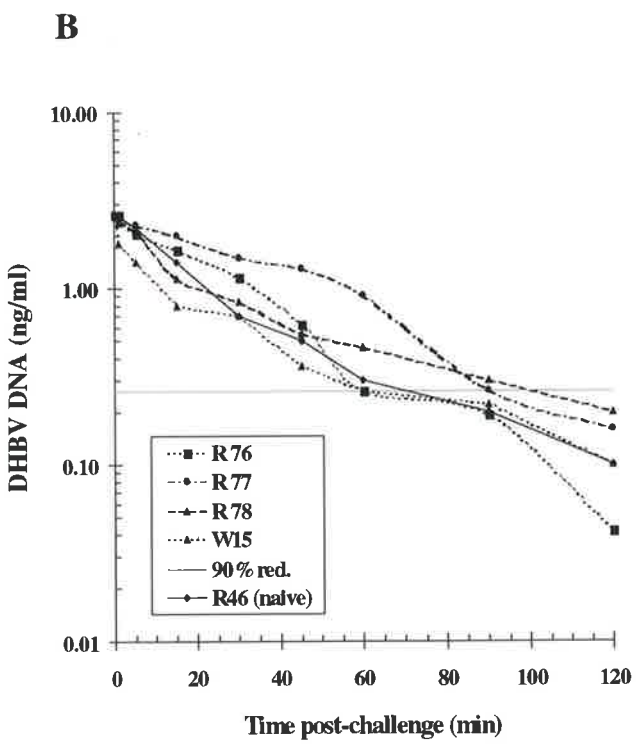


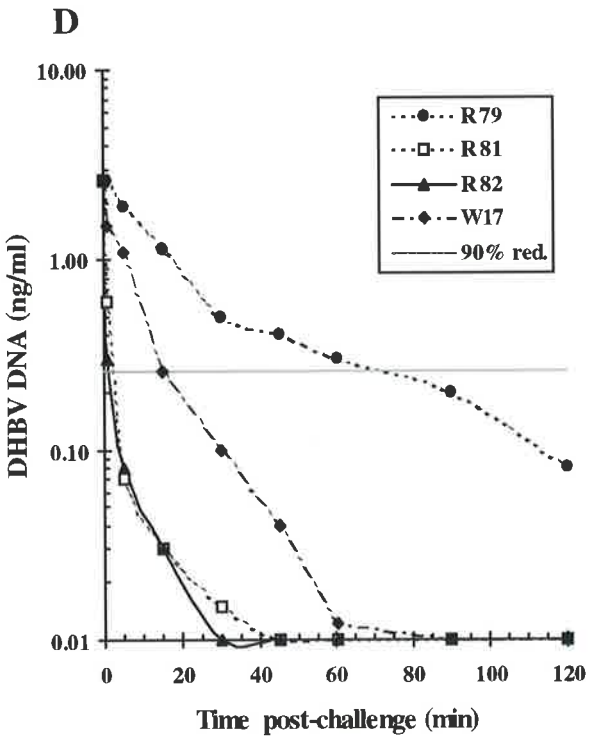
Figure 5.4. Anti-S specific responses in the pre-S/S & S DNA-vaccinated young ducks. Sera from the same ducks described in Fig. 5.3B were also assayed for the presence of anti-S specific antibodies by ELISA with yeast-derived DHBV S protein as the source of antigen (Section 2.5.5). The antibody titers were defined as the highest serum dilution that gave an OD 490nm of 0.5. Virus challenge (indicated by DHBV ➔) was performed at different times after the third vaccination.

Figure 5.5. Removal of DHBV from the bloodstream of vaccinated ducks following virus challenge. (A & C) Southern blot analysis of virus removal from the bloodstream of pre-S/S and S DNA-vaccinated ducks, respectively. The results from two ducks of each group (R76 and R77 for pre-S; and R81 and R82 for S) are shown. Serum samples were taken serially at the indicated times p.c. and were extracted for DHBV DNA (Section 2.8.1). Lanes: D, 50 pg of DHBV DNA/pBL4.8; PB, pre-bleed before challenge; Inoc, 5 µl of extracted inoculum, equivalent to 260 pg DHBV DNA.

(B & D) The rate of virus removal from the bloodstream of all pre-S/S and S DNA-vaccinated ducks (four ducks per group). The rate of virus removal of an identical dose of virus from a non-vaccinated duck (R46) is also shown **(B)**. A theoretical concentration of 2.6 ng DHBV DNA/ml in the circulation at 0 min was calculated by correcting for a 10 x dilution (20 ml inoculum/200 ml of total blood volume) occurring immediately after inoculation. The *y* axis shows the relative amount of viral DNA remaining in the bloodstream at each indicate time p.c. The DHBV DNA concentration remaining after removal of 90% of the inoculum is shown (90% red.).


Pre-S/S
S
A
R76
R77
C
R81
R82
D PB 1' 5' 15' 30' 45' 60' 90' 2 hr Inoc.
D PB 1' 5' 15' 30' 45' 60' 90' 2 hr Inoc.


B
DHBV DNA (ng/ml)
10.00
1.00
0.10
0.01
0
20
40
60
80
100
120
Time post-challenge (min)
R76
R77
R78
W15
90% red.
R46 (naive)


D
DHBV DNA (ng/ml)
10.00
1.00
0.10
0.01
0
20
40
60
80
100
120
Time post-challenge (min)
R79
R81
R82
W17
90% red.

the inoculum in 15 and 70 min, respectively (Fig. 5.5C & 5.5D). The duck showing the slowest removal of inoculum (R79) had also produced the lowest level of anti-DHBs antibodies (Fig. 5.3B).

5.3.4. Detection of viral replication in liver tissue at 4 days p.c.

(i) Pre-S/S DNA-vaccinated ducks.

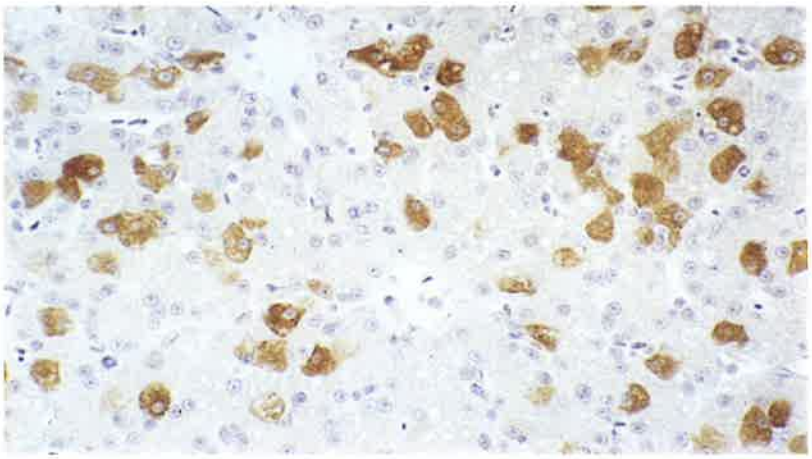
In the pre-S/S DNA-vaccinated ducks, DHBsAg was detected in 10-40% of hepatocytes in the liver by 4 days p.c. (Fig. 5.6A), and significant levels of viral DNA were also detected in liver by Southern blot analysis (Fig. 5.7, lane 3, 5, and 7). Nonetheless, virus infection in the liver was restricted because viremia (determined by analysis of serum for DHBsAg and DHBV DNA) was not detected during 8 weeks of monitoring. In contrast, when non-vaccinated ducks of a similar age (4-month-old) were inoculated with an identical dose of DHBV, widespread virus infection affecting >95% of hepatocytes (Fig. 5.6C) was observed and transient viremia was normally seen (Jilbert *et al.,* 1998).

(ii) S DNA-vaccinated ducks.

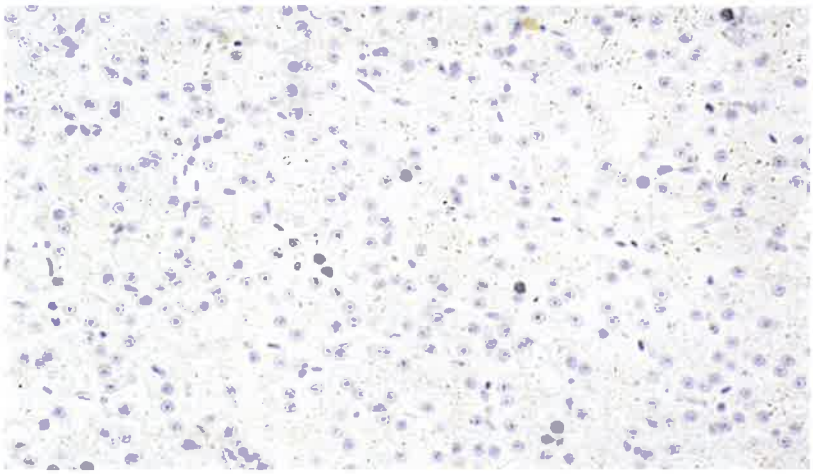
DHBsAg-positive hepatocytes were not detected in three of four S DNA-vaccinated ducks (Fig. 5.6B) and very few positive hepatocytes (~2%) were found in the fourth duck (R79) (data not shown). This duck (R79) also showed a slower rate of initial antibody response to vaccination and slower removal of the challenge inoculum from the bloodstream (90% removal in 70 min) compared to the other ducks in this group. Results of Southern blot analysis of viral DNA extracted from liver biopsies of these ducks were consistent with the above findings, with no evidence of virus replication in those S DNA-vaccinated

Figure 5.6. Detection of DHBsAg by immunoperoxidase staining of liver tissues of vaccinated and non-vaccinated ducks at 4 days p.c. Liver biopsy samples taken at 4 days p.c. were examined for the presence of DHBV infection by the immunoperoxidase staining method (Section 2.8.4) using 1H.1, an anti-pre-S specific monoclonal antibody (Pugh *et al.,* 1995). DHBsAg-positive cells were found in 10-40% hepatocytes of pre-S/S **(A)**, but not S **(B)** DNA-vaccinated ducks. In contrast, a non-vaccinated duck showed more widespread infection where >95% hepatocytes were DHBsAg-positive **(C).** (Magnification: *40x*).

A



B



C

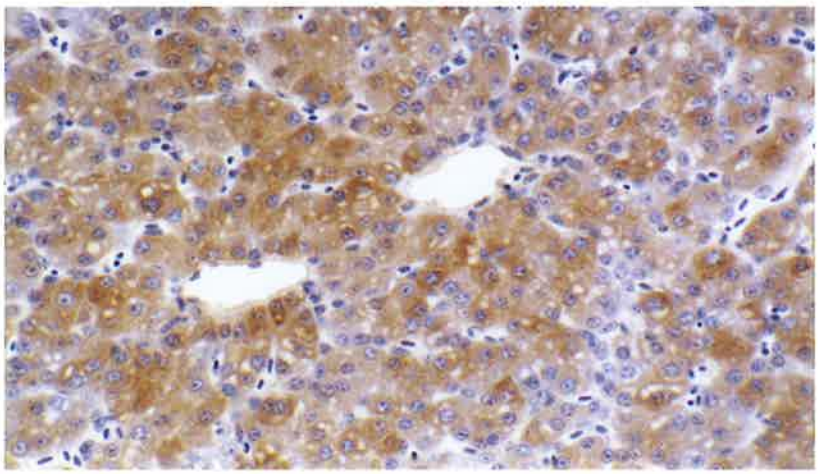
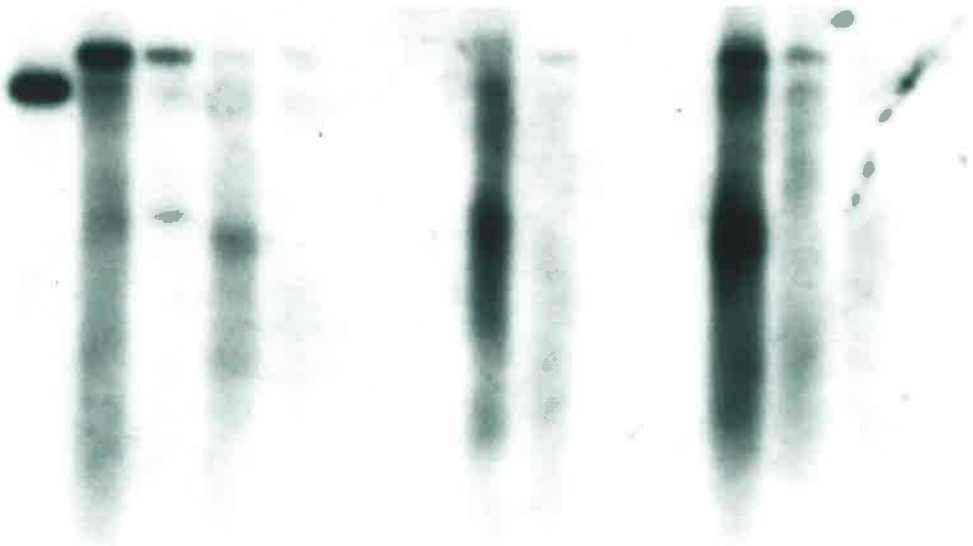


Figure 5.7. Detection of viral replication (total DNA and cccDNA) in the liver of vaccinated-ducks 4 days p.c. Total DNA was extracted from liver tissues as described (Section 2.8.3), and samples containing 10 μg of total DNA were analyzed by Southern blot hybridization. Lanes: 1, 50 pg of linear DHBV DNA (pBL4.8); 2, total DNA (a) & cccDNA (b) of the positive control (extracted from the liver of a congenitally DHBV-infected duck); 3, 5, and 7, total DNA (a) & cccDNA (b) of three pre-S/S DNA-vaccinated ducks (R76, R77, and R78, respectively); 4, 6, and 8, total DNA (a) & cccDNA (b) of three S DNA-vaccinated ducks (R81, R82, and R79, respectively). Exposure time: 18 hr.


a b a b a b a b a b a b a b
1 2 3 4 5 6 7 8

ducks which removed virus rapidly from the bloodstream, and only low levels of virus DNA in duck R79 (Fig. 5.7, lanes 4, 6, and 8).

5.3.5. Histological changes in liver tissue at 4 days p.c.

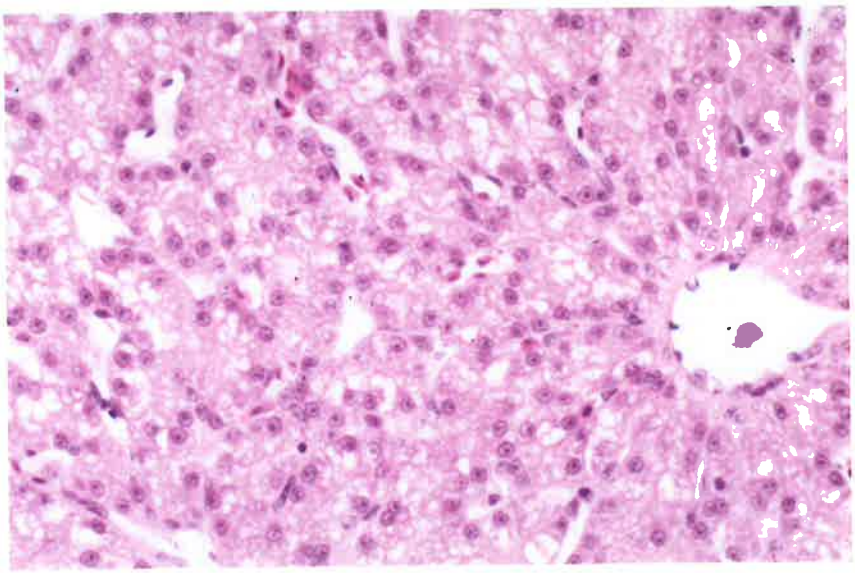
In both pre-S/S and S DNA-vaccinated ducks, but not in non-vaccinated ducks, a range of mild to moderate mononuclear cell inflammation (Knodell *et al.,* 1981) was present around the portal areas of the liver at 4 days p.c., but not prior to challenge (Fig. 5.8A and B). It is therefore possible that both CMI and humoral responses may have played a role in preventing more widespread viral replication in these vaccinated ducks.

5.3.6. *In vivo* neutralization.

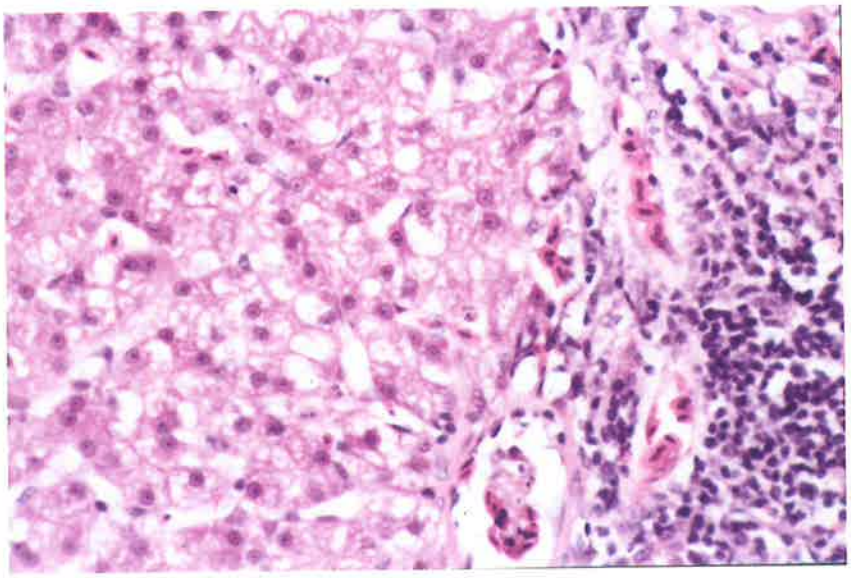
To determine whether the protection against virus challenge seen in vaccinated ducks was due to humoral immunity, an inoculum containing 1 x 10^6 DHBV genomes (equivalent to 1 x 10^6 ID_{50}) was pre-incubated with serum from both pre-S/S and S DNA-vaccinated ducks (R76 & R81, respectively) taken one week after the third DNA vaccination. Based on the results of the DHBV challenge of S DNA-vaccinated ducks (see above), it was estimated that the inoculum of 1.9 x 10^{11} DHBV genomes would have come into contact *in vivo* with circulating antibody present in a total blood volume of 200 ml. Hence, theoretically 1 x 10^6 DHBV genomes could be neutralized by approximately 1 µl of serum from S DNA vaccinated ducks. Pre-incubation of the virus inoculum with 5, 10, or 20 µl of anti-S duck serum at 37°C for 1 hr prior to i.v. inoculation into one-day-old ducklings (3 animals/group) prevented the development of viremia (monitored weekly by DHBsAg

Figure 5.8. Detection of mononuclear cell infiltrates in liver tissue of S DNA-vaccinated ducks following viral challenge. Liver tissue (represented by W17) collected 2 weeks pre-challenge **(A)** showed normal liver histology. In contrast, mild to moderate mononuclear cell infiltrates were detected at 4 days p.c. **(B).** Sections stained by H & E. (Magnification: *40x*).

A



B



assay and viral DNA by spot blot hybridization) during a 4 week observation period in all ducks. In contrast, viremia developed in all ducks receiving virus that had been pre-incubated with 20 or 40 µl anti-pre-S/S duck serum, and in two out of three ducks that received inoculum which had been pre-incubated with 80 µl of anti-pre-S/S duck serum. In the control group, all ducklings (3/3) inoculated with virus developed viremia that was detected until the end of the study at 4 weeks p.i. (Table 5.1). Thus, 5 µl anti-S antiserum neutralized virus infectivity under the *in vivo* conditions used, while with anti-pre-S/S antiserum only partial neutralization was seen with the largest volume (80 µl) used.

5.3.7. *In vitro* neutralization assay.

The neutralizing ability of serum from a vaccinated duck was also tested in primary duck hepatocyte (PDH) cultures inoculated with 7×10^7 DHBV genomes (equivalent to 5.4×10^4 $TCID_{50}$), which were examined for replicative viral DNA at 7 days p.i. by Southern blot hybridization. Pre-incubation of virus with either 35 or 70 µl of anti-S serum at 37°C for 1 hr reduced the final level of intracellular DHBV DNA by 90-95% compared to the positive control (Fig. 5.9, lane 2, 3 and 4). These results confirmed the above calculations which indicated 1×10^6 DHBV genomes might have been neutralized by approximately 0.5-1 µl of anti-S serum *in vivo*. In contrast, virus infectivity was not affected by pre-incubation with 35 µl of anti-pre-S/S serum, and was only reduced 50% after pre-incubation with 70 µl of anti-pre-S/S serum (Fig. 5.9, lane 5 and 6).

The next step was to test whether the reduced neutralizing ability of the pre-S/S antiserum was due to a reduced neutralizing ability of its S antibody component, or inhibition of

Table 5.1. ***In vivo*** **neutralization assay.**

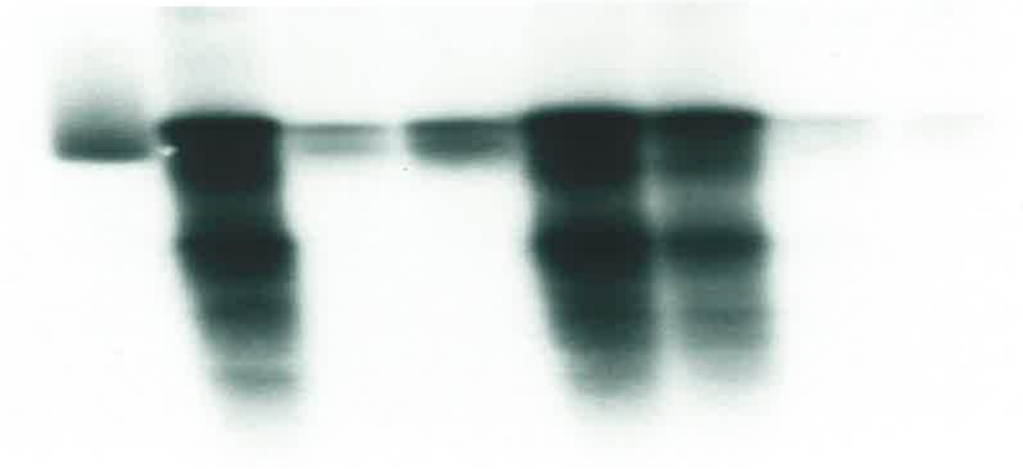
Antibodies	Viral inoculum[a]	Neutralization[b]
Anti-S		
5 µl	1 x 10^6 vge	100%
10 µl	1 x 10^6 vge	100%
20 µl	1 x 10^6 vge	100%
Anti-pre-S/S		
20 µl	1 x 10^6 vge	0%
40 µl	1 x 10^6 vge	0%
80 µl	1 x 10^6 vge	33%
None [c]	1 x 10^6 vge	0%

[a] Viral inoculum was pre-incubated with anti-S or anti-pre-S/S serum at each volume tested, at 37°C for 1 hr. The volume was then adjusted to 100 µl with NDS and inoculated i.v. to six groups of one-day-old ducks (3 ducks/group).

[b] Neutralization was monitored by the absence of viremia (by DHBsAg assay and spot blot hybridization) until the end of the study at 4 weeks p.i.

[c] In the control group, the viral inoculum was pre-incubated alone at 37°C for 1 hr prior to inoculation.

Figure 5.9. *In vitro* neutralization assay. PDH cultures were inoculated with 7 x 10^7 DHBV genomes (equivalent to 5.4 x 10^4 $TCID_{50}$), which had been pre-incubated for 1 hr at 37°C with 35 or 70 µl of either anti-S (lane 3 and 4), anti-pre-S/S (lane 5 and 6), or mixture of equal volumes of both antisera (35 or 70 µl of each antiserum; lane 7 and 8). Cells were harvested at 7 days p.i. Total cellular DNA was extracted and analyzed for viral DNA by Southern blot hybridization. Each lane represents the total DNA extracted from individual wells of a 6-well plate. Lanes: 1 (D), 50 pg DHBV DNA; 2 (V), virus alone (5.4 x 10^4 $TCID_{50}$).



1	2	3 4	5 6	7 8
D	V	+ anti-S	+Anti-pre-S/S	+Anti-S&-pre-S/S

neutralization by co-existing pre-S antibody. When equal volumes of anti-S and anti-pre-S/S sera were combined (35 or 70 µl of each serum), the extent of neutralization was slightly enhanced compared to that observed with anti-S serum alone, as viral DNA was reduced by 96% (Fig. 5.9, lane 7 and 8). This result demonstrated that the reduced neutralizing capacity of the anti-pre-S/S antiserum seen consistently, was likely to be due to ineffective neutralizing capacity of the S antibody component of this antiserum (despite equivalent ELISA titers), and not due to an inhibitory effect of anti-pre-S antibody counteracting the effect of potent anti-S antibody. A summary of the findings in this study is presented in Table 5.2.

5.4. DISCUSSION.

This study demonstrated the ability of DNA vaccines to elicit humoral immune responses against DHBV surface proteins in ducks, as has been reported earlier for HBV surface proteins in mice and chimpanzees (Chow *et al.,* 1997; Davis *et al.,* 1993a; Davis *et al.,* 1996b; Prince *et al.,* 1997). Ducks vaccinated i.m. with pcDNA I/Amp encoding either the DHBV pre-S/S or S protein produced very high titers of anti-DHBs antibodies. The strong antibody responses may have been facilitated by the use of local anesthetic (bupivacaine hydrochloride) to induce muscle regeneration (Davis *et al.,* 1993b; Wells, 1993), and injection of 25% (w/v) sucrose to aid even distribution of DNA uptake by muscle cells (Davis *et al.,* 1993b). In addition, the use of pcDNA I/Amp which contains several repeats of unmethylated CpG motifs in its *ampR* gene sequence and the CMV promoter region (Roman *et al.,* 1997; Sato *et al.,* 1996), may also have contributed to the strong humoral immune responses seen in this study. Previous studies in mice have demonstrated that DNA vaccination with a vector containing CpG motifs induced

Table 5.2. Summary of pre-S/S- and S-DNA vaccination in young (3-week-old) ducks.

	Pre-challenge			Post-challenge		
DNA vaccine	Total anti-DHBs titer[a]	Neutralization *in vivo*[b]	Neutralization *in vitro*[c]	Virus removal rate[d]	Viral replication[e]	Histological changes[f]
S-DNA	2-5x10^4 (4/4)	3/3 neutralized by >5 ul of anti-S	90-95% reduction in viral DNA by 35&70 ul of anti-S	<5-15 min (3/4) 70 min (1/4)	not detected (3/4) ~2% hepatocytes (1/4)	mild (3/4) marked (1/4)
Pre-S/S-DNA	1-4x10^4 (4/4)	1/3 neutralized by 80 ul of anti-pre-S/S, no neutralization by 20 & 40 ul of serum.	50% reduction in viral DNA by 70 ul of anti-pre-S/S, no reduction by 35 ul of serum.	60-100 min (4/4)	10-40% hepatocytes (4/4)	mild-moderate (4/4)

[a] The range of total anti-DHBs antibody titers as measured by ELISA one week after the third vaccination from 4 ducks/group.

[b] Neutralizing ability of serum from vaccinated-ducks when pre-incubated with 1x10^6 DHBV genomes (1x10^6 ID_{50}) prior to inoculation into one-day-old ducklings (3 animals/group). Different volumes of neat anti-S (5, 10, & 20 ul) or anti-pre-S/S (20, 40, & 80 ul) sera from ducks R81 & R76, respectively, were used.

[c] Neutralizing ability of serum when pre-incubated with 7x10^7 DHBV DNA genomes prior to inoculation of PDH cultures.

[d] The time taken for removal of 90% of virus inoculum from the bloodstream p.c. as determined by extracting viral DNA from serum and subjected to Southern blot hybridization.

[e] Viral replication in the liver at 4 days p.c., determined by the presence of total and cccDNA, as well as by the presence of viral antigen (DHBsAg)-positive hepatocytes.

[f] Histological changes seen in the liver tissue samples taken at 4 days p.c., in relation to the presence of (i) periportal necrosis, (ii) intralobular degeneration and focal necrosis, (iii) portal inflammation, and (iv) fibrosis, according to Knodell *et al.,* 1981.

significantly higher antibody and CMI responses against the expressed protein than was seen with a vector lacking this motif (Sato *et al.,* 1996; Klinman *et al.,* 1996; Roman *et al.,* 1997).

Although ducks from both groups (6-month- and 3-week-old) mounted anti-DHBs responses, the titers found in young ducks were higher (10,000-50,000) than in older ducks (3,500-8,000). An influence of age on the expression of plasmid DNA injected i.m. has been reported previously: young mice (4-week-old) expressed significantly higher levels of the reporter gene product chloramphenicol acetyltransferase (Wells and Goldspink, 1992), or luciferase (Barry and Johnston, 1997), than animals older than 10 weeks. It was proposed that this could be due to a difference in DNA uptake related to the growth rate of the animal, since mice show rapid growth from 3 to 10 weeks of age. A similar mechanism might operate with ducklings that show rapid growth until ~4 months of age and responded better than older ducks to DNA vaccines. Alternatively, it is also known that MHC class I molecules are expressed at higher levels on the surface of immature muscle fibers (Hohlfeld and Engel, 1994). This phenomenon might increase the presentation of DHBV envelope proteins on the surface of transfected muscle cells in the context of MHC class I. It was notable that the anti-DHBs titers found in vaccinated 6-month-old ducks were still at least three times higher than those obtained following primary DHBV infection (Jilbert *et al.,* 1998). The significance of this findings, however, remains unknown, since only small numbers of ducks were examined in this study.

Vaccination of ducks with either pre-S/S- or S-DNA plasmid prevented the development of viremia following virus challenge. However, significant differences were found in the rate of virus removal from the bloodstream p.c., and in the presence/absence of early virus

replication in the liver. With the exception of one duck, S DNA-vaccinated ducks rapidly removed the inoculum from the bloodstream and showed no detectable DHBsAg nor viral replication in their hepatocytes at 4 days p.c. These findings were similar to those seen after challenge of ducks that had resolved primary infection and developed anti-DHBs (Jilbert *et al.,* 1998). Marked mononuclear cell infiltrates around the portal areas of the liver at day 4 p.c. were also observed, which could be virus antigen specific T lymphocytes induced by DNA vaccination that subsequently accumulated at sites of antigen localization within the liver. This phenomenon could be seen as the potent priming of CTL responses by DNA vaccination, as has been demonstrated previously in mice injected i.m. with an HBsAg-expressing DNA vaccine (Schirmbeck *et al.,* 1995).

The protection conferred by the S DNA vaccine seen in this study may have been due to a combined effect of humoral and cell-mediated immunity induced by the vaccine. However, humoral antibodies alone abolished virus infectivity *in vivo* and in *in vitro* neutralization assays. The precise mechanism(s) of virus neutralization in the S DNA-vaccinated ducks are yet to be determined, although several possibilities exist. First, anti-S antibodies might inhibit attachment of virus to its specific receptor, either by direct binding to the ligand or sterically, although this mechanism alone is generally considered as an inefficient process for virus neutralization (Dimmock, 1993). Alternatively, formation of virus-antibody complexes might lead to: (i) enhanced phagocytosis by macrophages and other phagocytic cells via Fc receptors, or (ii) the formation of aggregates by cross-linking virus particles, thus reducing their infectivity (Dimmock, 1993). The above mechanisms of virus neutralization might also require the cooperation of cellular immune responses in the *in vivo* system. For example, a possible role for T lymphocytes in clearing virus infection from hepatocytes and preventing cell-to-cell

spread of DHBV could be inferred from the marked mononuclear cell infiltrates seen in the liver 4 days p.c.

The surprising finding from this study was that in contrast to S DNA-vaccinated ducks, all pre-S/S DNA-vaccinated ducks removed virus inoculum from the bloodstream at similar rates to naive ducks. However, this vaccine still provided protection since by 4 days p.c., virus infection of the liver only affected 10-40% of hepatocytes and none of these ducks developed viremia (the limit of the assay sensitivity is ~0.5 pg DNA by spot blot hybridization) during 8 weeks of monitoring. The abortive virus infection observed in these ducks was presumably due to the combined actions of humoral and CMI responses, as judged by the moderate mononuclear cell infiltrates present in the liver at 4 days p.c.

The *in vivo* and *in vitro* neutralization assays using serum from vaccinated ducks also revealed that the marked difference between the protective efficacy of both vaccines could be ascribed in part at least to the difference in the humoral component. The reason for the reduced ability of anti-pre-S/S antibodies to neutralize virus infectivity is unknown. Both anti-S and anti-pre-S/S sera contained equivalent levels of anti-S antibody in an S-antigen specific ELISA, but the antiserum mixing experiment demonstrated that reduced efficiency of the pre-S/S antiserum was likely to be due to an uneffective function of its anti-S component, rather than inhibition by the anti-pre-S component. One possibility could be related to the nature of the intracellular DHBV pre-S/S protein expressed in the transfected muscle cells. It was found that, unlike the S protein, pre-S/S protein was detected only intracellularly when expressed in COS7 cells transfected with pcDNA I-pre-S/S plasmid (data not shown). This phenomenon was similar to earlier reports with co-expression of the L and S proteins of HBV in a number of mammalian expression

systems. In these systems, the large (pre-S1/pre-S2/S) protein was not secreted, and in fact inhibited the expression and the secretion of S protein (Chisari *et al.,* 1986; Chow *et al.,* 1997; Persing *et al.,* 1986). If impaired secretion is also a feature of pre-S/S protein expression in muscle *in vivo*, this may have affected the correct conformation of the antigens produced, and subsequently, the biological function of the anti-S antibodies induced. The immunogenicity of DHBV S protein has been reported earlier to be conformation dependent (Yokosuka *et al.,* 1988). Hence, the specific anti-S antibodies raised in the pre-S/S DNA-vaccinated ducks might be conformationally different from those produced following primary DHBV infection or in S DNA-vaccinated ducks. Thus, although both pre-S/S and S antisera reacted equally with yeast-derived S protein in ELISA, some differences in biological function between the anti-S specific antibodies produced in the pre-S/S and S DNA-vaccinated ducks might have occurred.

In summary, the results described in this chapter demonstrate the importance of anti-S antibodies alone in preventing DHBV infection in both *in vitro* and *in vivo* systems, consistent with the well established role for anti-HBs antibodies in HBV infection in man (Hadler *et al.,* 1986; Waters *et al.,* 1987a; Waters *et al.,* 1987b). The markedly poorer protection conferred by pre-S/S vaccination despite apparently comparable levels of anti-S antibodies by ELISA needs further clarification, particularly in the context of the development of human HBV vaccines containing pre-S proteins where serological responses can be readily monitored but protective efficacy data are difficult to obtain.

Chapter 6

Protective & therapeutic efficacy of DNA vaccines against DHBV infection in newly hatched ducks

6.1. INTRODUCTION AND AIMS.

HBV infection occurring during the perinatal period or in early childhood from HBV-carrier mothers will result in 90% of the newborns becoming chronic carriers (Beasley *et al.*, 1983). Once the infection becomes chronic, HBsAg carriage persists in most individuals, with an overall incidence of HBsAg clearance in only 0.6% of carrier children per year (Chen, 1993). The inverse relationship between HBV chronicity rates and the age of host at the time of infection is believed to reflect the reduced immunological competence of neonates and the very young. Inability to clear virus infection has been linked to the induction of immunological tolerance in neonates (Lok, 1992; Milich *et al.*, 1993). This form of immune response has been shown to operate when initial exposure to a persisting antigen occurs prior to immunological maturation, i.e. *in utero* or during the neonatal period, resulting in the host 'accepting' the foreign antigen as 'self' and therefore, not mounting an immune response which is effective in eliminating that antigen (Billingham *et al.,* 1953; Sarzotti, 1997). Accordingly, it has been suggested that *in utero* passage of HBV antigens from the mother to the fetus may induce the unresponsiveness to HBsAg vaccines observed in newborns born to HBV-carrier mothers (Lazizi *et al.,* 1997).

Immaturity of the immune system of the newborn may also explain the finding that, although >90% of vaccinated infants born to HBV-carrier mothers developed anti-HBs antibodies after a course of three injections, the protective anti-HBs antibody level of >10 mIU/ml was achieved in most infants only after the third injection (given between 3 and 6 months of age; Wong *et al.,* 1984).

Recent studies, however, have demonstrated that neonates can indeed be immunized, or be made specifically unresponsive, and that their immune response can also be modulated to mount mainly Th_1 or Th_2 responses depending on the dose of antigen, the adjuvant used, and the type of antigen-presenting cells involved in the response (Ridge *et al.,* 1996; Sarzotti *et al.,* 1996; Forsthuber *et al.,* 1996). An effect of antigen dose on the ability of newborns to clear infection is suggested for perinatal transmission of HBV, where the outcome of infection can be directly related to the quantity of viral DNA to which the infants are exposed (Burk *et al.,* 1994; Ip *et al.,* 1989). These two studies demonstrated that chronic infection developed in 79% or 97.2% of infants born to HBV-carrier mothers with serum HBV DNA levels greater than 5 pg/ml or 1.4 ng/ml, respectively.

In contrast to conventional vaccines that lead to humoral (Th_2) responses in neonates, DNA vaccines have been shown to generate CTL or Th_1, cell-mediated responses in young mice, even when injected immediately after birth (Bot *et al.,* 1996; Hassett *et al.,* 1997; Martinez *et al.*, 1997). The ability of DNA vaccines to induce Th_1 responses in neonates has raised the possibility that the ineffective immune responses normally mounted by infants born to HBV carrier mothers can be circumvented by these vaccines. For similar reasons, others have also considered the use of DNA vaccines to reduce the number of non-responders to the current HBsAg vaccines (Schirmbeck *et al.,* 1995). In regard to their potential as therapeutic agents for chronic infection, DNA vaccines have been shown to downregulate HBsAg expression and, subsequently, to induce anti-HBs antibody responses in HBsAg-expressing transgenic mice without any signs of immune-mediated hepatic damage (Davis *et al.,* 1997a; Mancini *et al.,* 1996). Given that at present there are ~350 million chronic HBV carriers worldwide (Kane, 1996), the

potential for therapeutic use of DNA vaccines is substantial. However, the report by Mor *et al* (1996) that DNA vaccination in neonates could induce immunological tolerance rather than stimulation, indicates that further studies to assess the therapeutic value of DNA vaccines are needed.

At face value, ducks infected with DHBV should provide an excellent model for such studies. Similar to HBV, the outcome of DHBV infection also shows age- and dose-related patterns. Persistent infection in newly hatched ducks is invariably seen following *in ovo* transmissions, and in 1- or 14-day-old ducks after inoculation with 1 x 10^0 or 1 x 10^6 DHBV genomes, respectively. On the other hand, persistent infection in 4-month-old ducks could be induced in a proportion of ducks when an inoculum of 2 x 10^{11} DHBV genomes was used (Jilbert *et al.,* 1998).

Hence, the ultimate aim of this study was to determine whether the pre-S/S and S DNA vaccines were able to alter the course of infection in congenitally DHBV-infected ducks. However, before commencing the study in congenitally DHBV-infected ducks, three other experimental settings were also designed to assess the efficacy of DNA vaccines in more favourable situations than the congenital infection, i.e. when it was given prior to infection, simultaneously, or 7 days post-infection.

6.2. EXPERIMENTAL DESIGN.

Four different experimental settings were examined:

A. **Pre-infection.** Three groups of ducks (3 ducks/group) were vaccinated at 2 days of age and were challenged with 1 x 10^7 DHBV genomes at 14 days of age, when they were also given another (final) dose of vaccine.

B. **Simultaneous infection and vaccination.** Six ducks were inoculated with 1 x 10^4 DHBV genomes at one day of age and were simultaneously given the first dose of vaccine. Another 4 doses of vaccine were administered subsequently.

C. **Delayed vaccination.** Six ducks were inoculated with 1 x 10^4 DHBV genomes at one day of age, and the first dose of vaccine was given at 7 days of age. Another 4 doses of vaccine were administered subsequently.

D. **Congenital infection.** Eight congenitally DHBV-infected ducks were given the first dose of vaccine at one day of age, followed by another 4 doses subsequently.

Each group included an additional 2-3 non-vaccinated control ducks which were inoculated with an identical dose of virus.

In Group **A** above **(Pre-infection)**, the efficacy of three different DNA vaccines was examined. Three groups (3 ducks/group) were injected with 100 µg of either pcDNA I-pre-S/S, or pcDNA I-S, or a mixture containing 50 µg of each plasmid DNA (Section 2.7.4). All vaccinated ducks in Groups **B**, **C** and **D** were injected with a mixture containing 50 µg of pcDNA I-pre-S/S and 50 µg of pcDNA I-S plasmids. Each injection was performed i.m. into the QA muscle using a 1-ml syringe fitted with a 29-gauge needle. All ducks were challenged with 100 µl of virus inoculum given i.v. into the jugular vein. The source of inoculum was a pool of serum containing 9.5 x 10^9 DHBV genomes/ml (Jilbert *et al.*, 1996) diluted in 100 µl of NDS to give the appropriate dose

(Section 2.7.4). The 50% infective dose (ID_{50}) of the inoculum has previously been determined to be equivalent to 1 viral genome (Jilbert *et al.*, 1996). The schedule of vaccinations, virus challenges, and liver biopsies for all ducks is shown in Fig. 6.1.

Serum samples were obtained weekly and analyzed for: (i) serological markers (DHBsAg, total anti-DHBs antibodies, and anti-DHBc antibodies by ELISA; Section 2.10), and (ii) viral DNA by spot blot hybridization (Section 2.3.3).

Liver tissue samples (~100 mg) obtained from biopsy of each duck were used for: (i) determining total and cccDNA by Southern blot hybridization, (ii) histological analysis, and (iii) immunohistological analysis by immunoperoxidase staining (Section 2.8). Group **A** ducks were sacrificed at 6 weeks of age, while all ducks in Groups **B**, **C**, and **D** were sacrificed at 24 weeks of age. Liver samples taken at autopsy were also analyzed as described above.

6.3. RESULTS.

6.3.1. GROUP A (PRE-INFECTION).

6.3.1.1. Serological responses.

Following vaccination at 2 days of age, all ducks developed anti-DHBs antibody responses (Fig. 6.2A). The antibody titers were subsequently increased after viral challenge, presumably due to the priming effect of the first vaccination, rather than due to the exposure to the virus, since non-vaccinated ducks did not produce an anti-DHBs response (Fig. 6.2A). There was no significant difference in antibody titers between

Figure 6.1. The schedule of DNA vaccination, viral challenge, and liver biopsy in newly hatched ducks. **(A) Pre-infection.** Three groups (3 ducks/group) were vaccinated with 100 μg of pcDNA I-pre-S/S, pcDNA I-S, or a mixture of 50 μg of each plasmid DNA, at 2 & 14 days of age. Ducks were challenged at 14 days of age with 1 x 10^7 DHBV genomes. Liver biopsies were performed at 4 days p.c. with autopsy at 4 weeks p.c. **(B) Simultaneous infection & vaccination.** Six one-day-old ducks were vaccinated with 100 μg of a mixture of pcDNA I-pre-S/S & pcDNA I-S (50 μg of each). Ducks were simultaneously inoculated i.v. with 1 x 10^4 DHBV genomes. Vaccinations were repeated 2 times at 2 week intervals (at 2 and 4 weeks of age). Liver biopsy was performed at 3 weeks of age. **(C) Delayed vaccination.** Six one-day-old ducks were inoculated i.v. with 1 x 10^4 DHBV genomes. The first DNA vaccination was given at 7 days of age as described in group B, and then was repeated twice at 2 week intervals (at 3 and 5 weeks of age). Liver biopsy was performed at 2 weeks of age. **(D) Congenital infection.** Eight congenitally DHBV-infected ducks were first vaccinated at one day of age as described in group B. Liver biopsy was performed at 1 week of age. In groups **B**, **C** and **D**, further doses of vaccine were given at 11 and 13 weeks of age. Before administrating these final doses of DNA vaccine, the muscle sites were pre-treated with 100 μl of bupivacaine HCl and 100 μl of 25% (w/v) sucrose at 5 days and 15 min prior to injection, respectively. All ducks were sacrificed at 24 weeks of age.

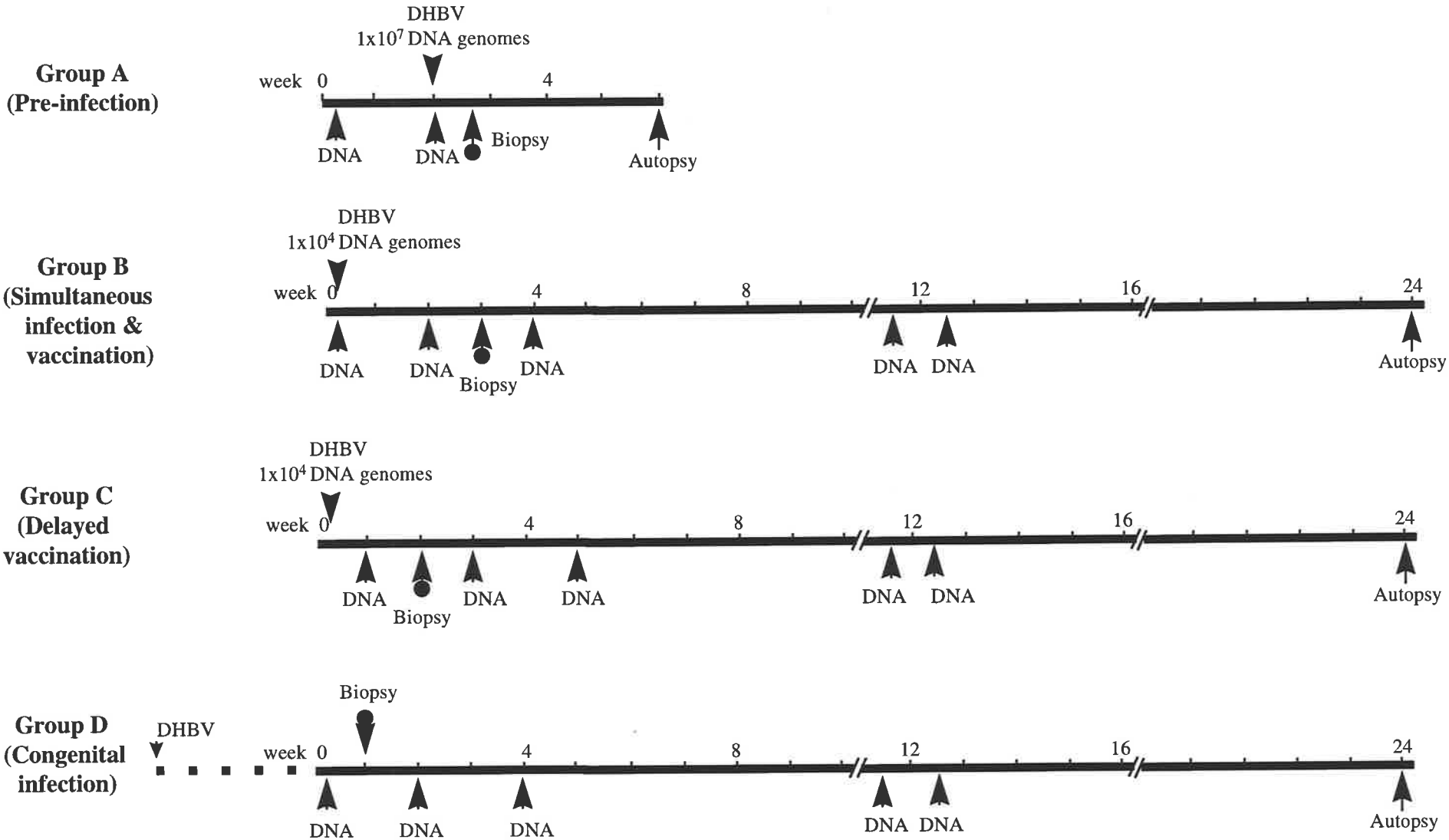
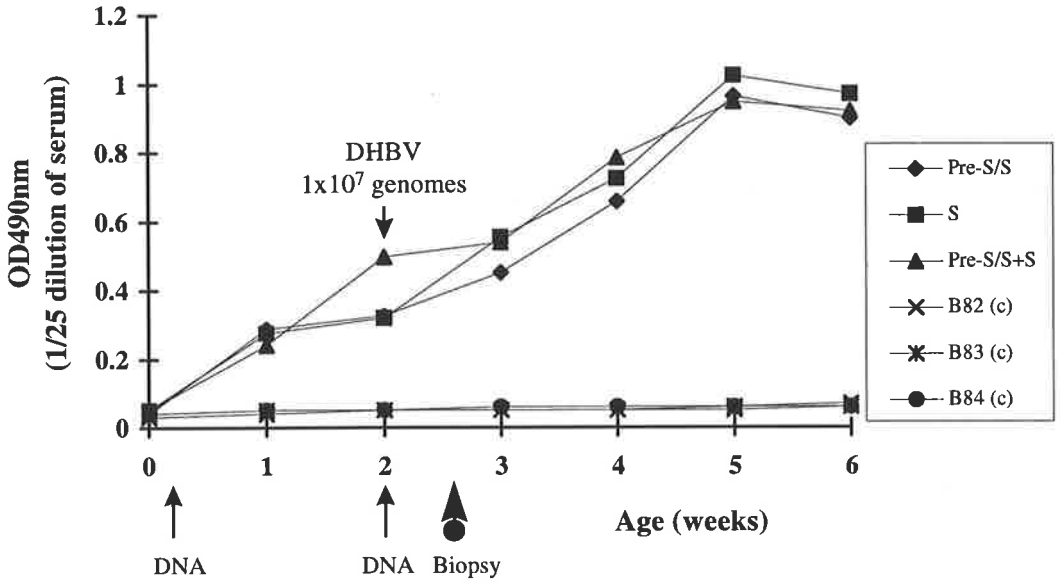

Group A
(Pre-infection)
DHBV
1x10^7 DNA genomes
week 0
4
DNA
DNA
Biopsy
Autopsy
Group B
(Simultaneous
infection &
vaccination)
DHBV
1x10^4 DNA genomes
week 0
4
8
12
16
24
DNA
DNA
Biopsy
DNA
DNA
DNA
Autopsy
Group C
(Delayed
vaccination)
DHBV
1x10^4 DNA genomes
week 0
4
8
12
16
24
DNA
Biopsy
DNA
DNA
DNA
DNA
Autopsy
Group D
(Congenital
infection)
DHBV
Biopsy
week 0
4
8
12
16
24
DNA
DNA
DNA
DNA
DNA
Autopsy

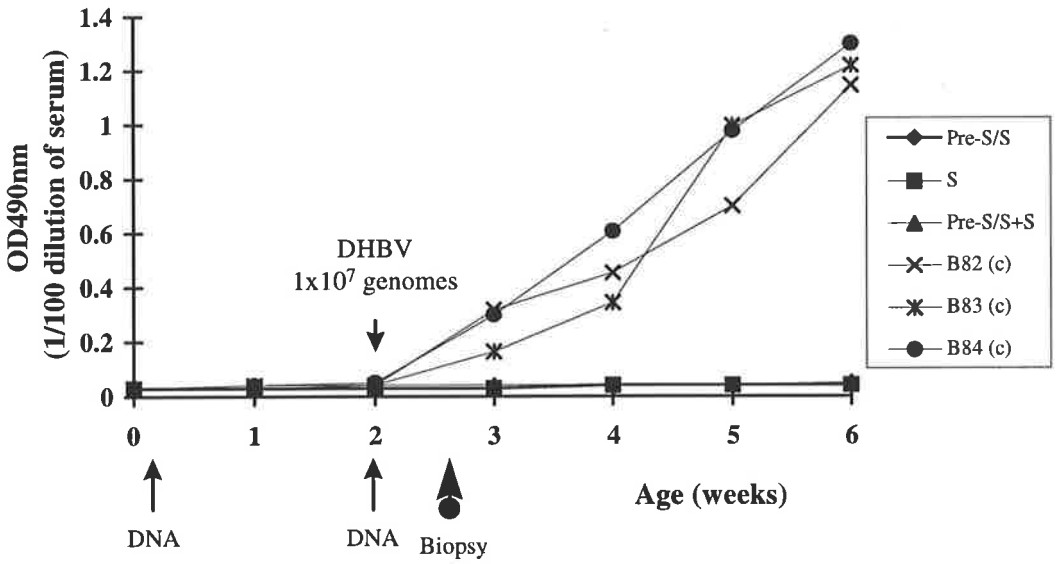
Figure 6.2. (A) Anti-DHBs antibody responses in Group A ducks (Pre-infection). Following vaccination at 2 days of age with pcDNA I-pre-S/S, pcDNA I-S, or a mixture of both, serum samples were obtained every week and analyzed for anti-DHBs antibody responses by ELISA (Section 2.10.2). All serum samples were assayed at a dilution of 1/25 and each line represents the average OD 490nm from the 3 ducks in each vaccination group (pre-S/S, S, and pre-S/S+S). Serum samples from three non-vaccinated control ducks (B82, B83, and B84) were also assayed for anti-DHBs antibodies and the OD 490nm for each duck is shown. At 2 weeks of age, all ducks received a second dose of DNA vaccine by the i.m. route and were simultaneously challenged i.v. with 1 x 10^7 DHBV genomes. Liver biopsy was performed at 4 days p.c. Non-vaccinated control ducks were also infected with 1 x 10^7 DHBV genomes at 2 weeks of age, and liver biopsies were performed at 4 days p.c.

(B) DHBsAg in serum of Group A ducks (Pre-infection). Serum samples from the same ducks as in (A) were assayed for the presence of DHBsAg by ELISA (Section 2.10.1). Serum samples were assayed at a dilution of 1/100, and the three lines designated as pre-S/S, S, and pre-S/S+S, represent the average OD 490nm from the 3 ducks in each vaccinated group. Serum samples from three non-vaccinated control ducks (B82, B83, and B84) were also assayed for DHBsAg and the OD 490nm for each duck is shown. All control ducks developed viremia that was first detected on day 7 post-infection i.e., at 3 weeks of age.

A. Anti-DHBs antibody.



B. DHBsAg.



ducks vaccinated with either pre-S/S, S, or a combination of both DNA plasmids. However, the anti-DHBs antibody titers in these young ducks were much lower than those produced by the 3-month-old ducks vaccinated with the same DNA vaccines (see Chapter 5). Serum DHBsAg and viral DNA were not detected in any vaccinated ducks during the 4 weeks of monitoring following viral challenge. In contrast, all of the non-vaccinated ducks (3/3) developed persistent viremia that was first detected on day 7 p.c. (Fig. 6.2B).

6.3.1.2. Detection of virus infection in the liver.

Liver biopsy tissues collected from all ducks on day 4 p.c. were analyzed for DHBsAg by immunoperoxidase staining of hepatocytes. In all vaccinated and non-vaccinated ducks, DHBsAg was detected in only ~0.16% of hepatocytes (Fig. 6.3A&B). Southern blot analysis of the total DNA extracted from the liver tissues collected on day 4 p.c. was negative for DHBV DNA (Fig. 6.4), presumably because levels of virus replication were too low to be detected by this assay (the limit of sensitivity was 0.5 pg DNA). Vaccination did, however, result in a significant change in the course of infection. Examination of autopsy liver tissue for DHBsAg at 4 weeks p.c. indicated that none of the vaccinated ducks had developed widespread infection of the liver regardless of the type of DNA vaccine given (Fig. 6.3C). In contrast, all of the non-vaccinated ducks had widespread DHBsAg expression in >95% hepatocytes in the liver (Fig. 6.3D). Virus infection was also detected in the kidney, spleen, and pancreas of the non-vaccinated ducks (data not shown). Southern blot analysis of the liver tissues at 4 weeks p.c. showed high levels of DHBV DNA in non-vaccinated ducks, while DHBV DNA was not detected in autopsy liver tissue from any of the vaccinated ducks (Fig. 6.4).

Figure 6.3. Detection of DHBV infection in liver tissues at 4 days & 4 weeks p.c. in Group A ducks (Pre-infection). Viral infection of the liver was detected by immunoperoxidase staining for DHBsAg (Section 2.8.5) using the anti-pre-S specific MAb 1H.1 (Pugh *et al.,* 1995). Figures A & C show representative examples of liver tissues from vaccinated ducks; figures B & D represent liver tissue samples from non-vaccinated ducks. **(A) Vaccinated duck at 4 days p.c.** In all vaccinated ducks (3 groups of 3 ducks) very few hepatocytes (~0.16%) were DHBsAg-positive (arrow) at 4 days p.c. with no significant difference in the number of infected cells between each group. **(B) Non-vaccinated duck at 4 days p.c.** All non-vaccinated ducks (3/3), also had ~0.16% of DHBsAg-positive hepatocytes (arrow) in the liver at 4 days p.c. **(C) Vaccinated duck at 4 weeks p.c.** Autopsy liver tissue taken from the same duck as shown in **(A).** Virus infection was no longer detected in the liver. **(D) Non-vaccinated duck at 4 weeks p.c.** All non-vaccinated ducks (3/3) developed widespread infection where >95% of hepatocytes were DHBsAg-positive. Magnification of **(A, B, & D)**: *40x.* Magnification of **(C)**: *20x.*

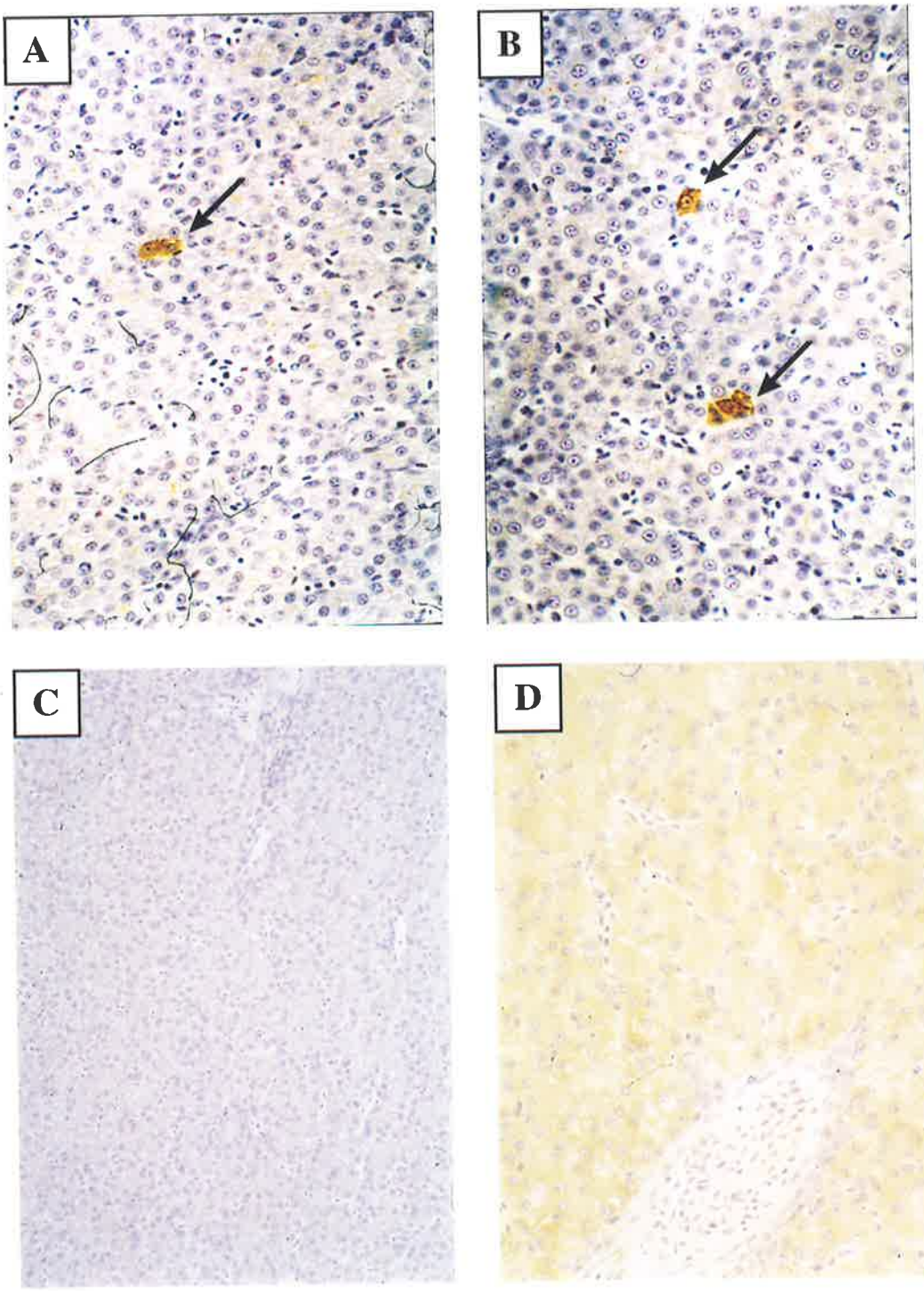

A
B
C
D

Figure 6.4. Southern blot analysis of total DNA extracted from the liver tissues of Group A ducks at 4 days and 4 weeks p.c. Total DNA was extracted from liver tissues (Section 2.8.3), and samples containing 10 μg of total DNA were analyzed by Southern blot hybridization using a ^{32}P-labeled [dATP] AusDHBV DNA probe. Four pairs of samples (at 4 days and 4 weeks p.c.) obtained from ducks vaccinated either with pre-S/S, S, or a mixture of both, and a non-vaccinated duck, are shown. D is a 50 pg sample of linear DHBV DNA. The limit of sensitivity of the Southern blot was 0.5 pg DNA. Autoradiography exposure time: 18 hr. Viral DNA in the liver samples of vaccinated ducks was not detected even after a 48 hr exposure (data not shown).

D 4d 4w 4d 4w 4d 4w 4d 4w

Pre-S/S S Pre-S/S + S Non-vaccinated

Results obtained from Group **A** above indicated that there was no difference in terms of the efficacy of DNA pre-S/S, S, or combination of both. Therefore, to conduct the three following experiments with a manageable number of ducks, it was decided that all ducks in the other groups (**B**, **C** and **D**) received only a combination of both DNA plasmids (50 µg of each). Pre-treatment of the muscle sites with bupivacaine hydrochloride and sucrose prior to DNA injection was only performed for the 4th and the 5th DNA injections in Group **B**, **C** and **D** ducks. These pre-treatments have been shown to result in the development of strong antibody responses (Davis *et al.,* 1993b; Wells, 1993; see also Chapter 5).

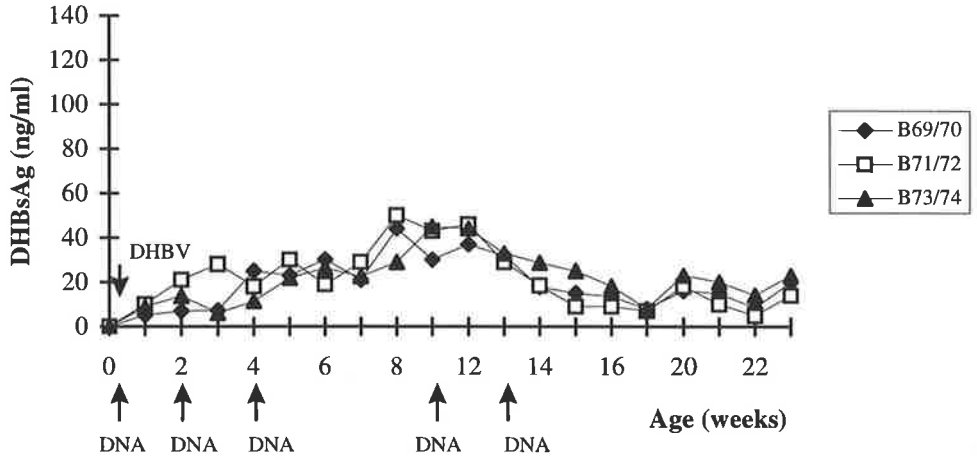
6.3.2. GROUP B (SIMULTANEOUS INFECTION & VACCINATION).

6.3.2.1. Detection of viremia.

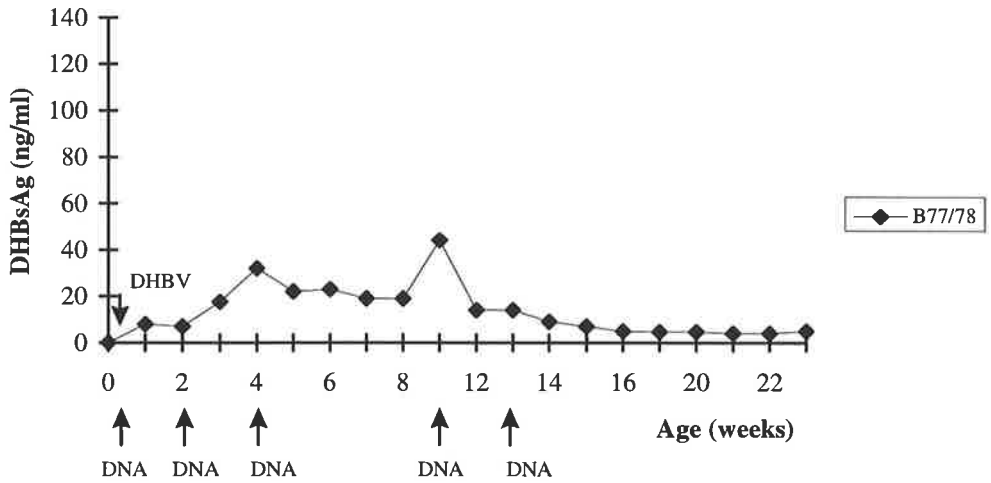
Following simultaneous infection and DNA vaccination at one day of age and 4 subsequent doses of vaccine, serum DHBsAg was detected in all animals from 1-24 weeks of age (Fig. 6.5A), except in one duck (B77/78) which showed a marked reduction in DHBsAg (5 ng/ml) notably after the 4th and 5th doses of DNA vaccine (Fig. 6.5B). At the time of autopsy (24 weeks of age), all vaccinated ducks had lower level of DHBsAg (5-24 ng/ml) compared to non-vaccinated ducks (62-74 ng/ml) (Fig. 6.5C). Viremia was also demonstrated in all ducks by the persistence of circulating viral DNA until 24 weeks of age. Levels of DHBV DNA ranged between 0.05-2.3 x 10^9 vge/ml (Fig. 6.6A&B), or in the case of non-vaccinated ducks, between 5.3-5.5 x 10^9 vge/ml (Fig. 6.6C).

Figure 6.5. Detection of DHBsAg in the bloodstream in Group B ducks (Simultaneous infection and vaccination). **(A)** Levels of serum DHBsAg in three ducks inoculated i.v. with 1 x 10^4 DHBV genomes and simultaneously vaccinated at one day of age. Vaccination was repeated at the time points indicated. **(B)** One vaccinated duck (B77/78), showed a marked reduction in DHBsAg after the second course of vaccination. **(C)** Two non-vaccinated control ducks (B79/80 and B81/82) were inoculated with an identical dose of virus. The presence of DHBsAg in the serum samples was measured by ELISA at a serum dilution of 1/500 (Section 2.10.1). The total amount of DHBsAg per ml of serum was calculated using a positive serum control containing a known amount of DHBsAg.

A


DHBsAg (ng/ml)
140
120
100
80
60
40
20
0
DHBV
0 2 4 6 8 12 14 16 20 22
Age (weeks)
DNA DNA DNA DNA DNA
B69/70
B71/72
B73/74

B


DHBsAg (ng/ml)
140
120
100
80
60
40
20
0
DHBV
0 2 4 6 8 12 14 16 20 22
Age (weeks)
DNA DNA DNA DNA DNA
B77/78

C

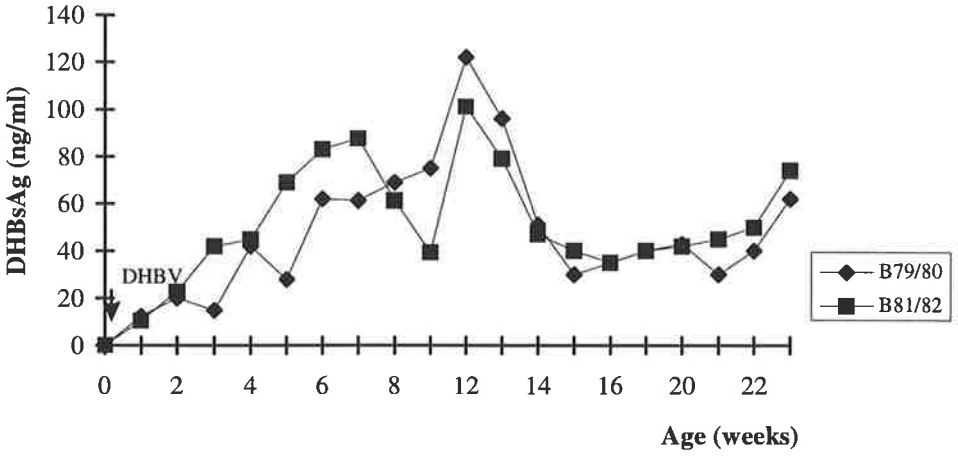
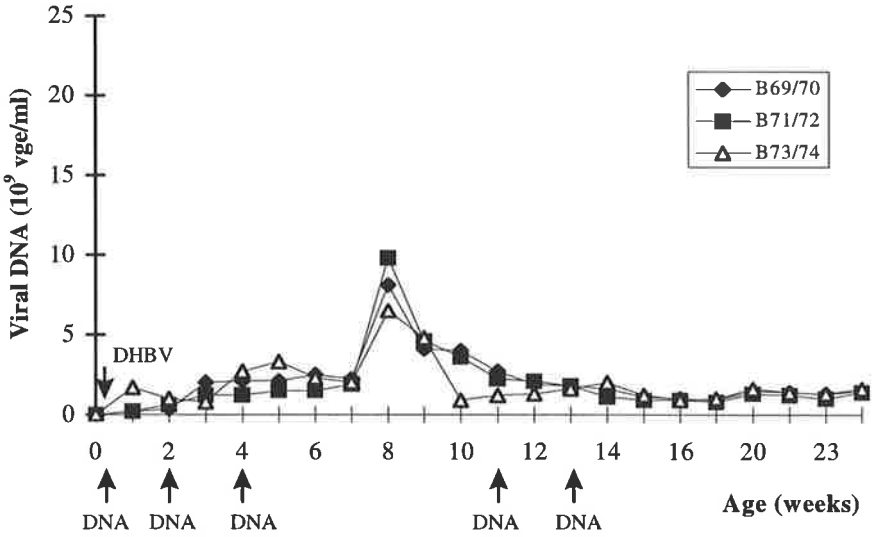
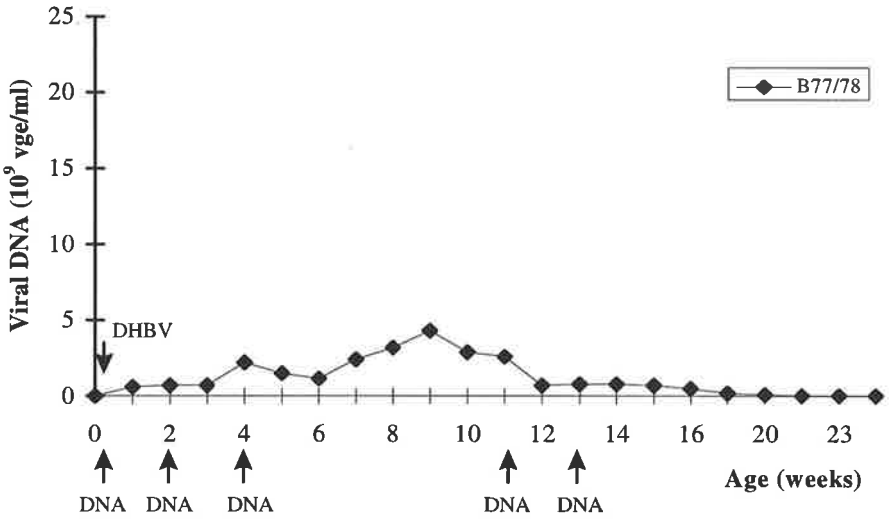

DHBsAg (ng/ml)
140
120
100
80
60
40
20
0
DHBV
0 2 4 6 8 12 14 16 20 22
Age (weeks)
B79/80
B81/82

Figure 6.6. Detection of viral DNA in the bloodstream in Group B ducks (Simultaneous infection and vaccination). Viral DNA in the bloodstream of ducks following simultaneous infection and vaccination (**A and B**), or infection alone (**C**), was detected by spot blot hybridization using ^{32}P[dATP]-labeled DHBV DNA probe (Section 2.3.3). The amount of viral DNA in each sample (5 µl serum) was measured using a Phosphor Imager System. Congenitally DHBV-infected duck serum containing a known amount of viral DNA (4.75×10^7 vge/5 µl serum) was used as a standard.

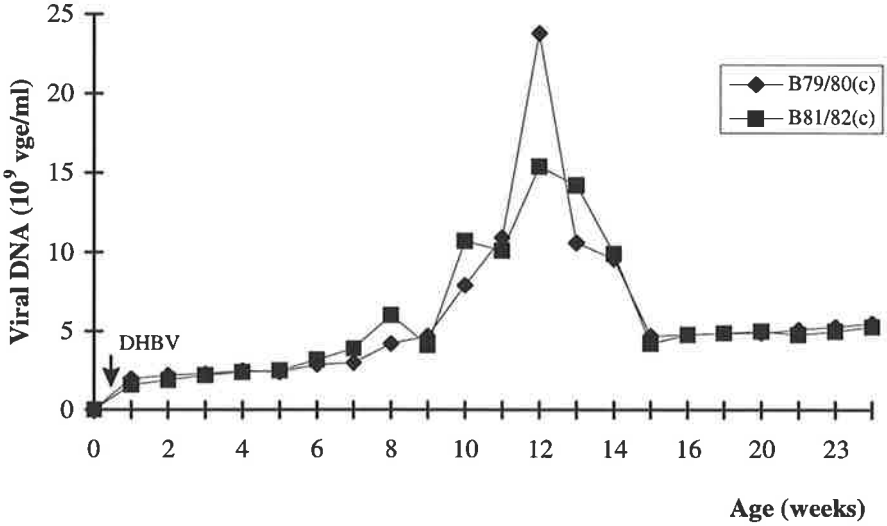
A


Viral DNA (10^9 vge/ml)
25
20
15
10
5
0
B69/70
B71/72
B73/74
DHBV
0 2 4 6 8 10 12 14 16 20 23
Age (weeks)
DNA DNA DNA DNA DNA

B


Viral DNA (10^9 vge/ml)
25
20
15
10
5
0
B77/78
DHBV
0 2 4 6 8 10 12 14 16 20 23
Age (weeks)
DNA DNA DNA DNA DNA

C


Viral DNA (10^9 vge/ml)
25
20
15
10
5
0
B79/80(c)
B81/82(c)
DHBV
0 2 4 6 8 10 12 14 16 20 23
Age (weeks)

6.3.2.2. Anti-DHBc antibody responses.

All vaccinated and non-vaccinated ducks mounted anti-DHBc antibody responses. The relative levels of antibodies were estimated by testing all serum samples by ELISA at a dilution of 1/500 (Section 2.10.4). Accurate titers of anti-DHBc antibodies in each sample were not defined in this study. The time taken to reach an antibody level which gave an OD 490nm reading of 0.5 was used arbitrarily as a measure of the rate of development of anti-DHBc antibody responses. Two vaccinated ducks (B71/72 and B77/78) showed a higher level of anti-DHBc antibodies (OD 490nm >0.5) starting at 3-6 weeks of age than the other two (B69/70 and B73/74) which reached antibody levels of >0.5 at OD 490nm only at 21 weeks of age (Fig. 6.7A). Both non-vaccinated ducks showed strong anti-DHBc antibody responses, with OD 490nm >0.5 after 8 weeks (B79/80) or 17 weeks (B81/82), of age (Fig. 6.7B).

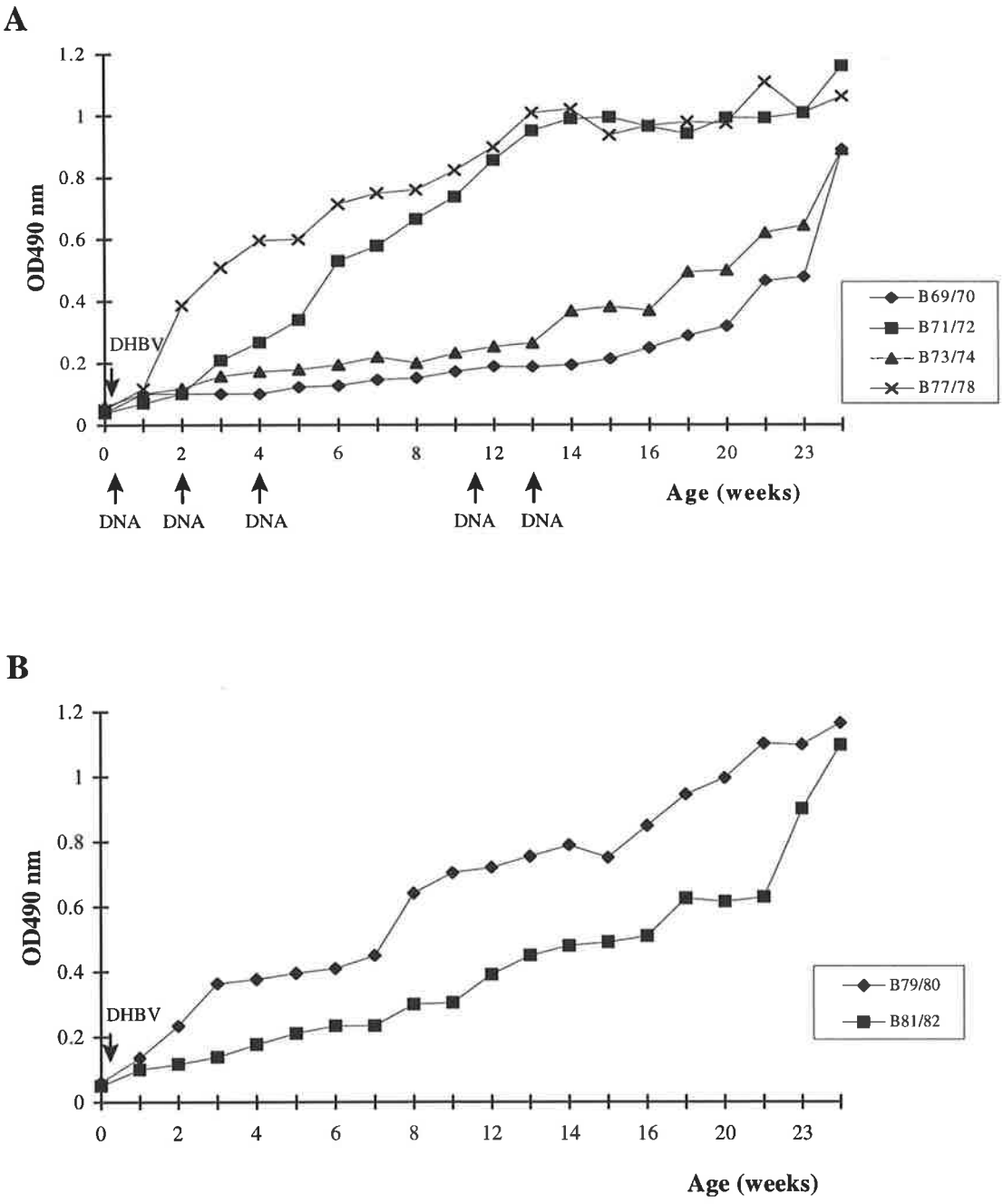


Figure 6.7. Anti-DHBc antibody responses in Group B ducks (Simultaneous infection and vaccination). **(A)** Serum samples from four vaccinated ducks were assayed for anti-DHBc antibody responses by ELISA (Section 2.10.4). **(B)** Anti-DHBc antibody responses in two non-vaccinated control ducks, B79/80 & B81/82. Serum samples were diluted at 1/500 in 5% BSA/PBS-T for this assay and results are expressed as an OD reading at 490nm.

6.3.3. GROUP C (DELAYED VACCINATION).

6.3.3.1. Detection of viremia.

Delaying the first vaccination until 7 days of age, following DHBV inoculation at 1 day of age, did not prevent the development of viremia or establishment of persistent infection in all ducks examined. All vaccinated ducks showed persistent viremia as indicated by serum levels of DHBsAg ranging in different ducks between 5.8-20.5 ng/ml (Fig. 6.8A), and 0.8-2 x 10^9 vge/ml of viral DNA (Fig. 6.9A) at 24 weeks of age. In comparison, at 24 weeks of age all non-vaccinated control ducks (B55/56 and B65/66) had higher levels of DHBsAg and viral DNA that ranged between 83-92 ng/ml and 12-14 x 10^9 vge/ml, respectively (Fig. 6.8B and 6.9B). The high levels of viremia in both control ducks were especially detected after 20 weeks of age, and are likely to be due to increased levels of viral replication in the liver.

6.3.3.2. Anti-DHBc antibody responses.

All vaccinated ducks developed strong anti-DHBc antibody responses, with a 1/500 dilution of serum giving an OD 490nm reading of >0.5 in this assay as early as 3 weeks of age in one duck (B53/54), and between 5-12 weeks of age in the others (Fig. 6.10A). Likewise, both non-vaccinated control ducks developed an anti-DHBc antibody responses, albeit at slower rates, reaching an OD 490nm >0.5 after 18-20 weeks of age (Fig. 6.10B).

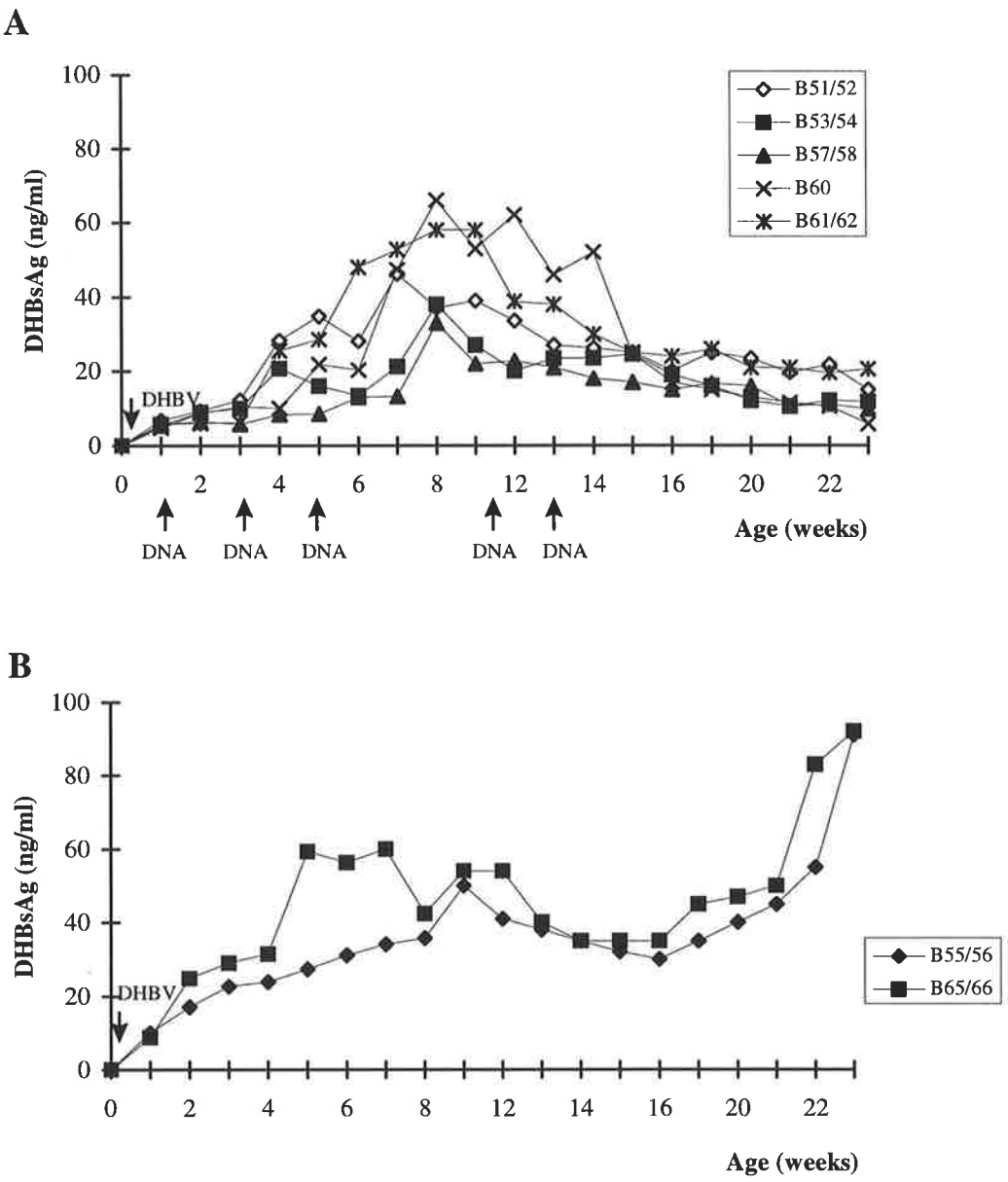


Figure 6.8. Detection of DHBsAg in the bloodstream of Group C ducks (Delayed vaccination). **(A)** Five ducks were inoculated i.v. with 1 x 10^4 DHBV genomes at one day of age, and the first vaccination was was given at 7 days of age. Vaccination was repeated at the time points indicated. **(B)** Two non-vaccinated control ducks (B55/56 and B65/66) were inoculated with an identical dose of virus. Serum DHBsAg was measured by ELISA at a serum dilution of 1/500 (Section 2.10.1). The total amount of DHBsAg in the serum was calculated using a positive serum control containing a known amount of DHBsAg.

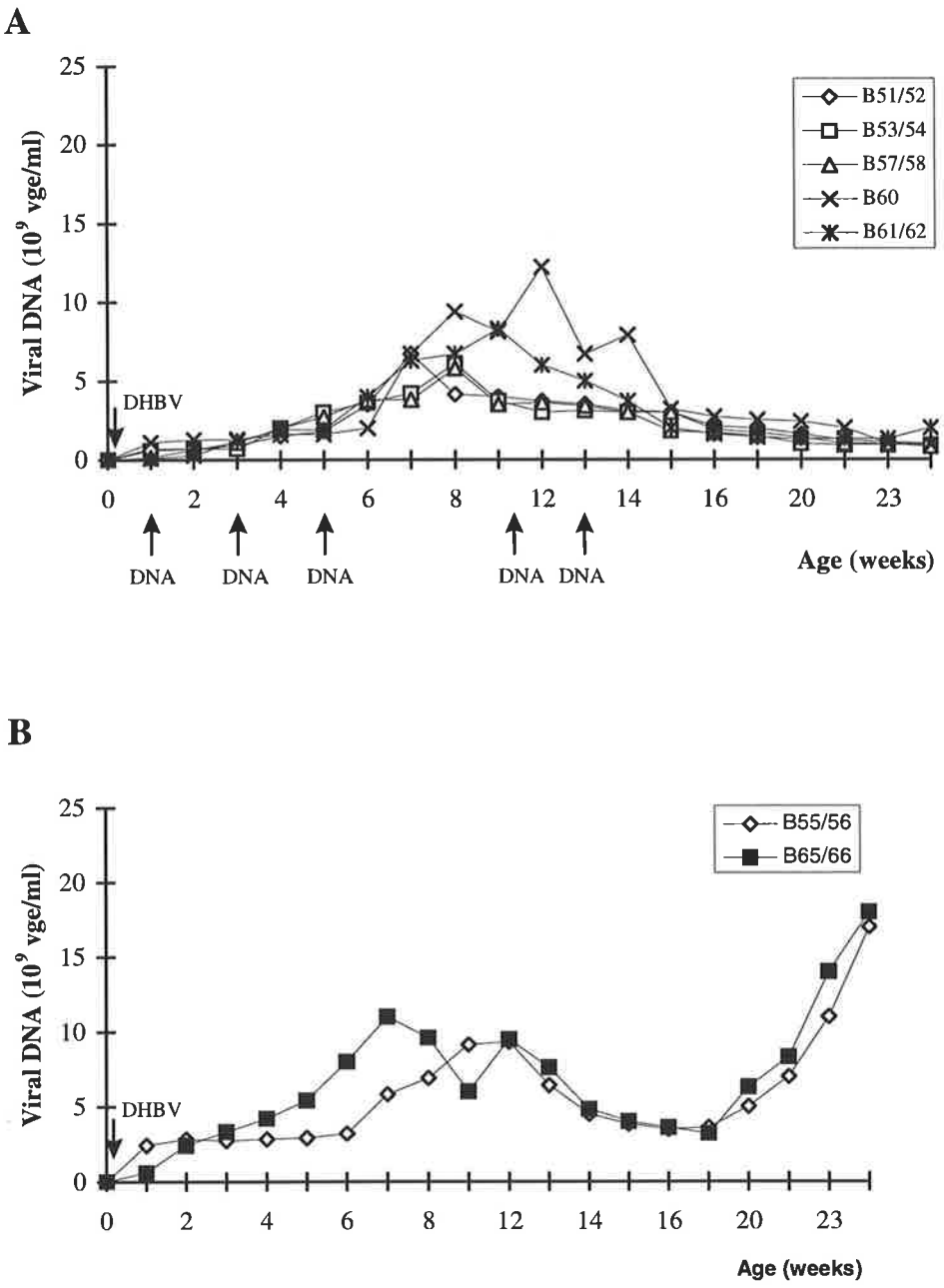


Figure 6.9. Detection of viral DNA in the bloodstream of Group C ducks (delayed vaccination). Viral DNA in the bloodstream of ducks following delayed vaccination **(A)**, or infection alone **(B)**, was detected by spot blot hybridization using a ^{32}P[dATP]-labeled DHBV DNA probe (Section 2.3.3). The amount of viral DNA on each sample (5 ul serum) was determined using a Phosphor Imager System. Congenitally DHBV-infected duck serum containing a known amount of viral DNA (4.75 x 10^7 vge/5 ul serum) was used as a standard.

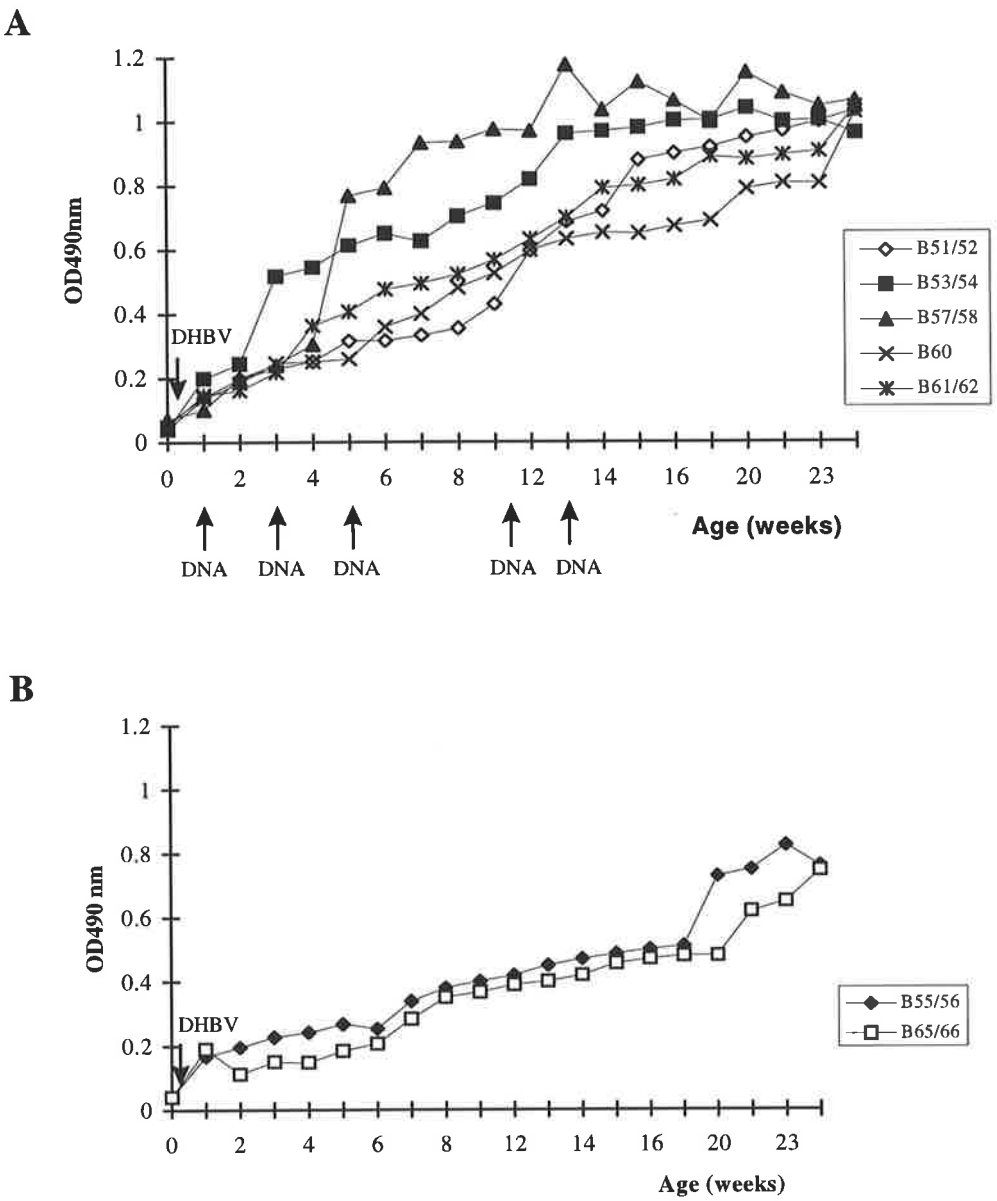


Figure 6.10. Anti-DHBc antibody responses in Goup C ducks (Delayed vaccination). **(A)** Serum samples from five ducks following i.v. inoculation with 1 x 10^4 DHBV genomes at one day of age, and DNA vaccination commencing at 7 days of age, were measured for anti-DHBc antibody responses by ELISA (Section 2.10.4). **(B)** The anti-DHBc antibody responses from two noon-vaccinated control ducks, B55/56 and B65/66 are also shown. Serum samples were assayed at a dilution of 1/500 in 5% BSA/PBS-T and results are expressed as an OD reading at 490nm.

6.3.4. GROUP D (CONGENITAL INFECTION).

6.3.4.1. Detection of viremia.

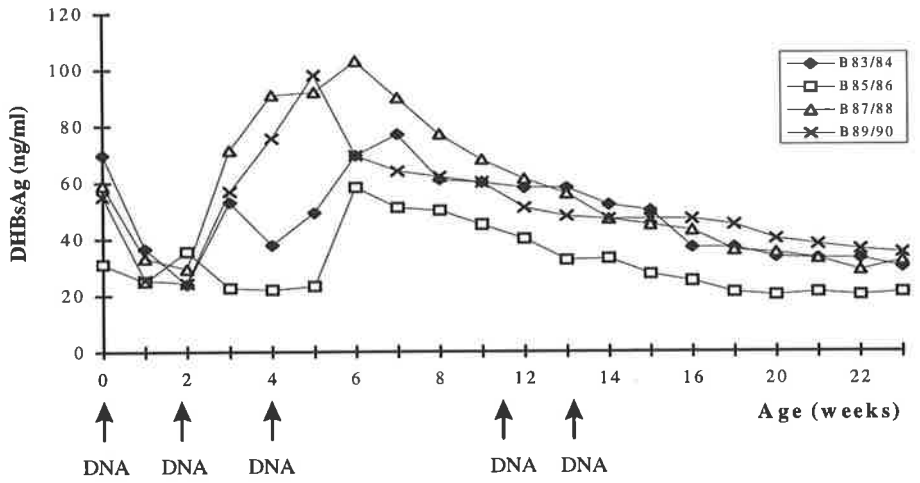
As shown in Fig. 6.11 and Fig. 6.12, all congenitally DHBV-infected ducks at 1 day of age had serum DHBsAg levels between 29.5-97.7 ng/ml and viral DNA levels between 10.2-47.5 x 10^9 vge/ml. These high levels of viremia have been reported to be due to virus replication during development of the embryo following *in ovo* transmission (O'Connell *et al.,* 1983). DNA vaccination did not alter the course of persistent infection in this group, although the level of viremia decreased over time, mainly after the second course of vaccination. By the time of autopsy performed at 24 weeks of age, the DHBsAg and viral DNA levels were found to be between 21-35 ng/ml and 3-6.7x10^9 vge/ml, respectively, in all vaccinated ducks (Fig. 6.11 and Fig. 6.12). The levels of these viral markers were, however, lower than those in the non-vaccinated duck (B39) at 24 weeks of age, which had a DHBsAg level of 91 ng/ml (Fig. 6.11C) and a viral DNA level of 14 x 10^9 vge/ml (Fig. 6.12C).

6.3.4.2. Anti-DHBc antibody responses.

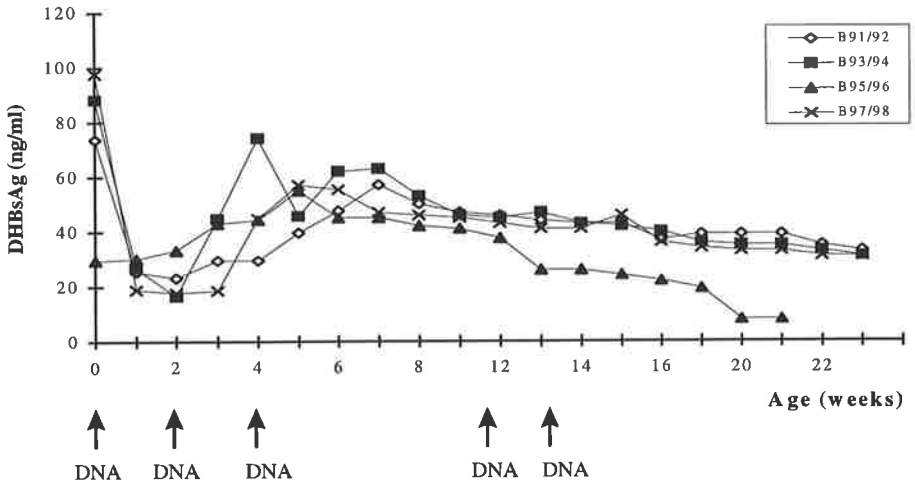
Anti-DHBc antibody responses were first detected in congenitally DHBV-infected ducks at 17 weeks of age. Most of the vaccinated ducks developed antibody responses with an OD 490nm >0.5 (at 1/500 dilution of serum) after 20 weeks of age, except in duck B93/94 which had low antibody response up to 24 weeks of age when all ducks were sacrificed (Fig. 6.13). The non-vaccinated duck, B39, also developed an anti-DHBc antibody response, albeit a lower level than the vaccinated ducks (Fig. 6.13C).

Figure 6.11. Detection of DHBsAg in the bloodstream of Group D ducks (Congenital infection). (A & B) Eight congenitally DHBV-infected ducks were given the first dose of DNA vaccine (containing 50 μg each of pre-S/S and S DNA) at one day of age. Vaccinations were repeated at the time points indicated. One control duck (B39) did not receive DNA vaccines **(C)**. The presence of DHBsAg in the serum samples was measured by ELISA at a serum dilution of 1/500 (Section 2.10.1). The total amount of DHBsAg in serum was calculated using a positive serum control containing a known amount of DHBsAg.

A


DHBsAg (ng/ml)
120
100
80
60
40
20
0
0
2
4
6
8
12
14
16
20
22
Age (weeks)
B 83/84
B 85/86
B 87/88
B 89/90
DNA
DNA
DNA
DNA
DNA

B


DHBsAg (ng/ml)
120
100
80
60
40
20
0
0
2
4
6
8
12
14
16
20
22
Age (weeks)
B 91/92
B 93/94
B 95/96
B 97/98
DNA
DNA
DNA
DNA
DNA

C

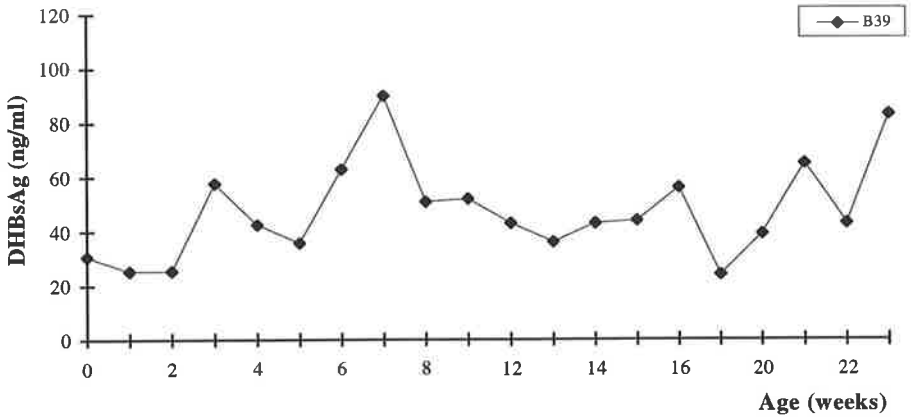
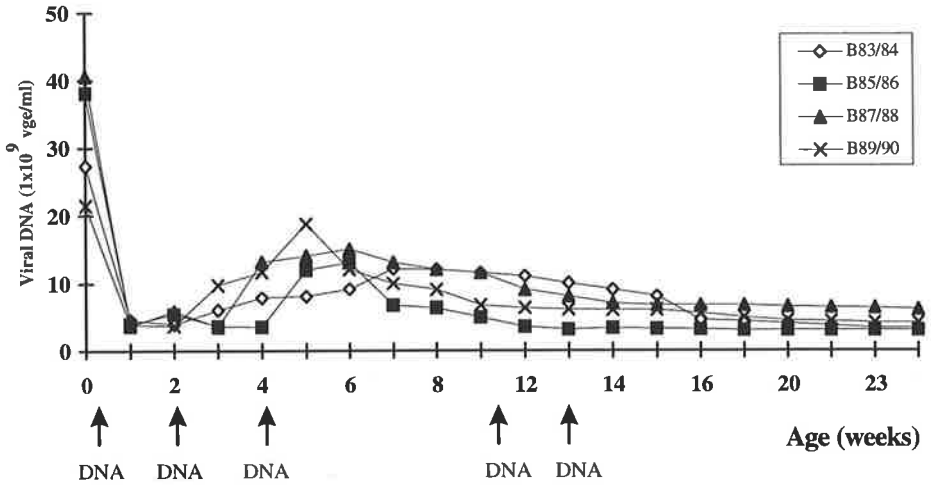
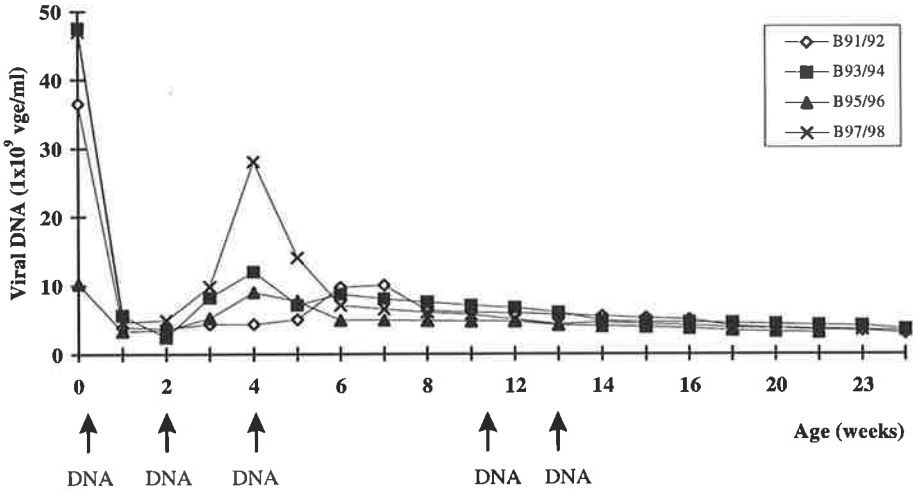

DHBsAg (ng/ml)
120
100
80
60
40
20
0
0
2
4
6
8
12
14
16
20
22
Age (weeks)
B39

Figure 6.12. Detection of viral DNA in the bloodstream of Group D ducks (Congenital infection). Viral DNA in the bloodstream of congenitally DHBV-infected ducks following initial vaccination at 1 day of age **(A & B)**, was detected by spot blot hybridization using ^{32}P[dATP]-labeled DHBV DNA probe (Section 2.3.3). Vaccination was repeated at the time points indicated. The level of viral DNA in a non-vaccinated duck, B39 **(C)**, is shown for comparison. The amount of viral DNA in each sample (5 µl serum) was determined using a Phosphor Imager System. Congenitally DHBV-infected duck serum containing a known amount of viral DNA (4.75 x 10^7 vge/5 µl serum) was used as a standard.

A


Viral DNA (1x10 9 vge/ml)
50
40
30
20
10
0
0
2
4
6
8
12
14
16
20
23
Age (weeks)
DNA
DNA
DNA
DNA
DNA
B83/84
B85/86
B87/88
B89/90

B


Viral DNA (1x10 9 vge/ml)
50
40
30
20
10
0
0
2
4
6
8
12
14
16
20
23
Age (weeks)
DNA
DNA
DNA
DNA
DNA
B91/92
B93/94
B95/96
B97/98

C

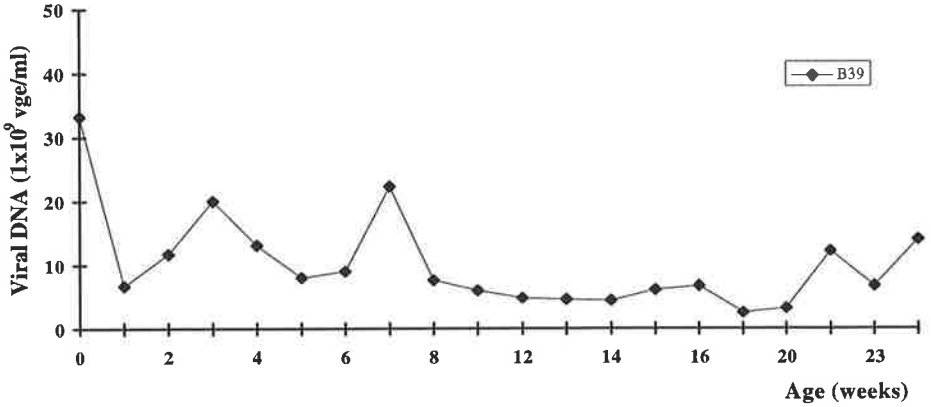
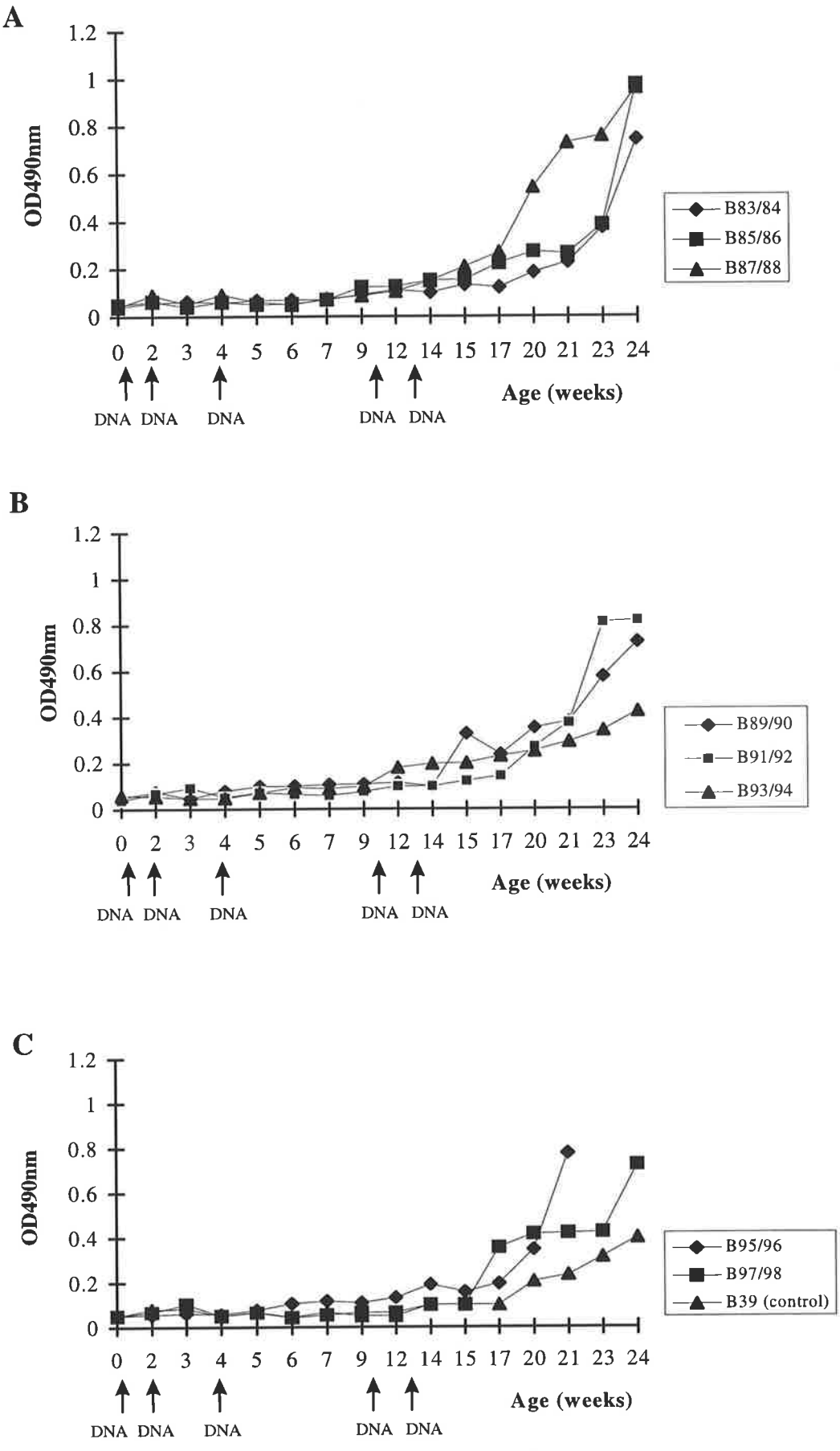

Viral DNA (1x10 9 vge/ml)
50
40
30
20
10
0
0
2
4
6
8
12
14
16
20
23
Age (weeks)
B39

Figure 6.13. Anti-DHBc antibody responses following DNA vaccination of Group D ducks (Congenital infection). Eight congenitally DHBV-infected ducks were given DNA vaccines as shown (**A, B & C**), and their serum samples were used to measure anti-DHBc antibody responses by ELISA (Section 2.10.4). Serum samples from a non-vaccinated control duck B39 (**C**) were also measured for anti-DHBc antibody responses. Serum samples were diluted at 1/500 in 5% BSA/PBS-T for this assay and results are expressed as an OD reading at 490nm.


A
OD490nm
1.2
1
0.8
0.6
0.4
0.2
0
0 2 3 4 5 6 7 9 12 14 15 17 20 21 23 24
Age (weeks)
DNA DNA DNA DNA DNA
B83/84
B85/86
B87/88
B
OD490nm
1.2
1
0.8
0.6
0.4
0.2
0
0 2 3 4 5 6 7 9 12 14 15 17 20 21 23 24
Age (weeks)
DNA DNA DNA DNA DNA
B89/90
B91/92
B93/94
C
OD490nm
1.2
1
0.8
0.6
0.4
0.2
0
0 2 3 4 5 6 7 9 12 14 15 17 20 21 23 24
Age (weeks)
DNA DNA DNA DNA DNA
B95/96
B97/98
B39 (control)

6.3.5. VIRUS INFECTION IN THE LIVER OF DUCKS FROM GROUPS B, C, & D.

Immunohistochemical staining was used to analyze liver biopsy tissue samples taken at different times p.i. All liver samples from ducks in the simultaneous (Group B), delayed (Group C), and congenital infection (Group D) biopsied at 3, 2, and 1 weeks of age, respectively, showed that >95% of cells were DHBsAg-positive (data not shown). Similarly, liver tissues collected during autopsy at 24 weeks of age also showed DHBV infection in >95% of hepatocytes. It is noteworthy, however, that the DHBsAg-positive hepatocytes in all vaccinated ducks had lower levels of viral antigen than the non-vaccinated ducks (Fig. 6.14), consistent with the observations of the reduced levels of viremia in all vaccinated ducks. In addition, mild to moderate levels of mononuclear cell infiltrates were also observed around the portal areas of the livers of vaccinated ducks, but absence in those non-vaccinated ones (data not shown). This could reflect a cell-mediated type of immune responses which, although not effective in clearing virus infected-cells, may have downregulated viral antigen production by these cells.

A summary of the viral markers examined from serum and liver tissue samples of Group **B**, **C** and **D** ducks taken from autopsy at 24 weeks of age is presented in Table 6.1.

Figure 6.14. Detection of virus infection in the liver of ducks from Groups B, C & D at 24 weeks p.i. Virus infection of the liver at the time of autopsy (24 weeks of age) was detected by immunoperoxidase staining (Section 2.8.5) using 1H.1, an anti-pre-S specific monoclonal antibody (Pugh *et al.*, 1995). All liver tissue samples had DHBsAg staining in >95% of hepatocytes, although the liver of vaccinated ducks showed a lower level of antigen staining. **(A & B) Group B ducks (Simultaneous infection and vaccination).** Duck B77/78 **(A)** had low level virus infection of the liver; the strongly DHBsAg-positive cells were mostly detected around the portal areas and are likely to represent bile duct cells. This duck showed marked reduction of viremia from 20 weeks p.i (see Fig. 6.5B). For comparison, virus infection of the liver in the non-vaccinated duck B79/80 **(B)** is shown. **(C & D) Group C ducks (Delayed vaccination).** More than 95% of hepatocytes of duck B53/54 remained DHBsAg-positive **(C)**. Mononuclear cell infiltrates were also present around the portal areas. Liver tissue staining of a non-vaccinated duck B65/66 **(D)** is shown as comparison. **(E & F) Group D ducks (Congenital infection).** Duck B85/86 **(E)** showed low level virus infection of the liver, the DHBsAg-positive cells were mostly present around the portal area. The liver tissue staining of a non-vaccinated duck B39 **(F)**, is shown for comparison.

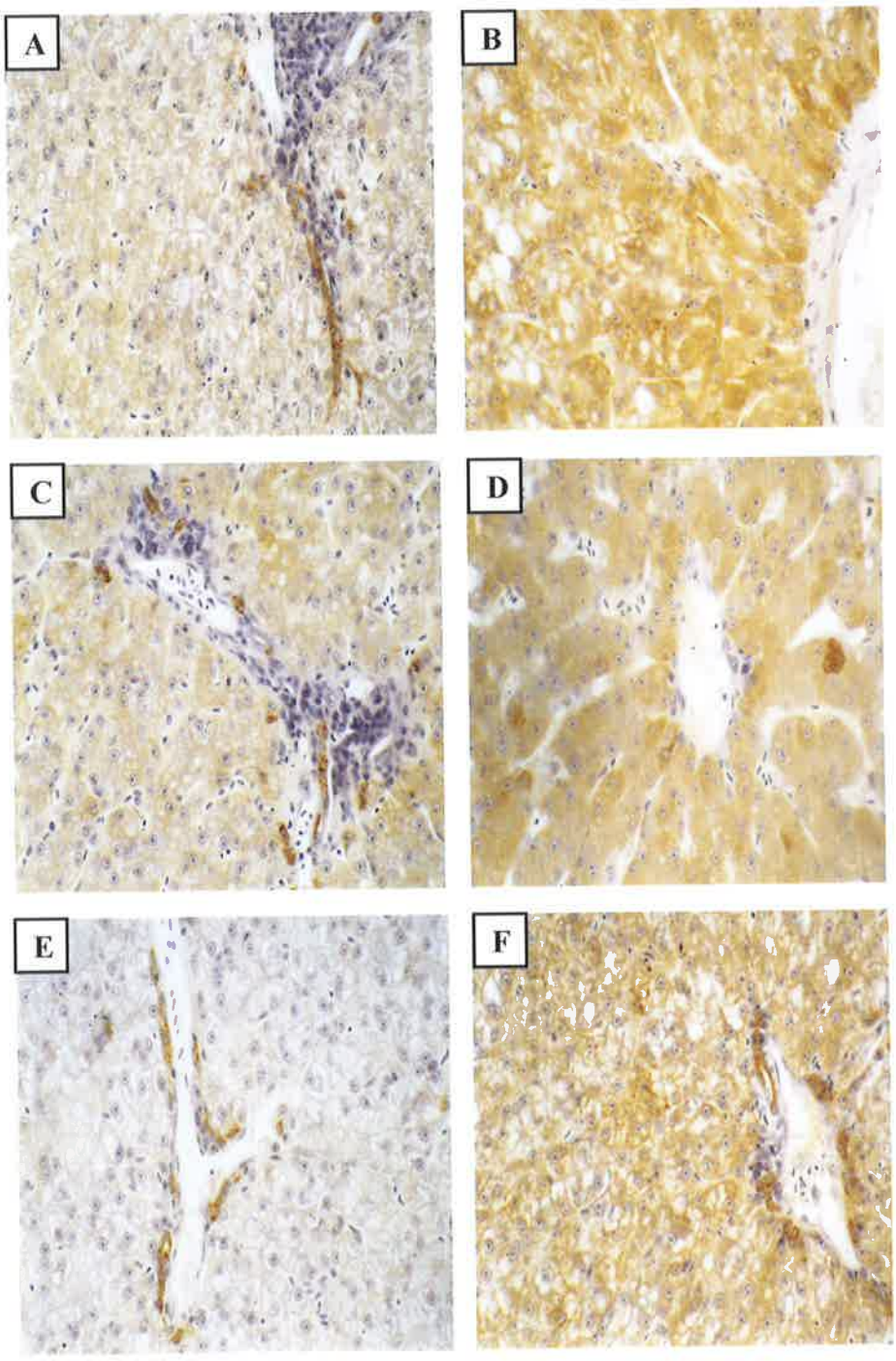

A
B
C
D
E
F

Table 6.1. Markers of DHBV infection at 24 weeks of age

		DHBsAg (ng/ml)	viral DNA (1×10^9 vge/ml)	Anti-DHBc (OD 490nm at 1/500 dil)	Liver (DHBsAg +ve cells)
Group B	vaccinated	5 - 24	0.05 - 2.3	0.89 - 1.16	>95%[a]
	non-vaccinated	62 - 74	5.3 - 5.5	1.01 - 1.16	>95%
Group C	vaccinated	5.8 - 20.5	0.8 - 2	0.96 - 1.06	>95%[a]
	non-vaccinated	83 - 93	12 - 14	0.75 - 0.76	>95%
Group D	vaccinated	21 - 35	3 - 6.7	0.42 - 0.97	>95%[a]
	non-vaccinated	91	14	0.4	>95%

[a] Although the livers of all vaccinated ducks showed >95% DHBsAg-positive hepatocytes by immunoperoxidase staining, the levels of antigen staining in the livers of the vaccinated ducks were lower than those expressed by non-vaccinated ducks.

6.4. DISCUSSION.

The notion that immaturity of the host immune system and the size of the virus load determine the outcome of HBV infection in newborns has been suggested in clinical studies (Burk *et al.,* 1994; Ip *et al.,* 1989). By analogy with HBV, the outcome of DHBV infection is also affected by both the dose of virus inoculated and the age of the duck at the time of inoculation (Jilbert *et al.,* 1998). Chronic infection in infants born to HBV-carrier mothers is observed following perinatal transmission or during childhood, and a similar outcome of infection is consistently found in congenitally DHBV-infected ducks following *in ovo* transmission. Chronic infection is unlikely to be resolved in both hosts, presumably due to the early exposure to antigen prior to immunological maturation as stated earlier (Section 6.1).

The ultimate goal in this study was to assess the efficacy of DNA vaccines in congenitally DHBV-infected ducks as a model for treatment of HBV chronic carriers in human. This idea was proposed based on the observation that DNA vaccines could elicit anti-HBs antibody and HBsAg-specific CTL responses in HBV transgenic mice (Mancini *et al.,* 1996). It is known that HBV transgenic mice have high levels of virus in serum (between 10^7 to 10^8 HBV genomes/ml) and in liver (100 to 200 copies of HBV replicative intermediate/hepatocyte) without any evidence of cytopathic effects in the liver (Guidotti *et al.,* 1995). The amount of circulating virus in these animals are comparable to that found in the sera of chronically infected humans (Ranki *et al.,* 1995). A similar observation is also found in the congenitally DHBV-infected ducks. Eight ducks examined in this study had 10.2-47.5 x 10^9 DHBV genomes/ml in their serum at one day of age (Section 6.3.4.1). Previously, others have also showed that liver of the

congenitally DHBV-infected ducks contain between 100-1,000 DHBV genomes in each hepatocyte (O'Connell *et al.,* 1983).

The efficacy of DNA vaccines in three other more favourable settings (pre-infection, simultaneous infection & vaccination, and delayed vaccination) than congenital infection were also evaluated. In the Group **A** (Pre-infection) ducks, DNA vaccines given at 2 and 14 days of age were able to induce protective immunity when they were challenged at 14 days of age with 1 x 10^7 DHBV genomes, ten times more than needed to ensure persistent infection in non-vaccinated ducks of the same age (Jilbert *et al.,* 1998). However, it is noteworthy that both vaccinated and non-vaccinated ducks showed similar percentages of DHBsAg-positive hepatocytes in liver biopsy tissue taken on day 4 p.c., and the differences in the levels of viral markers in serum and liver of the vaccinated ducks developed beyond this point of time. It is likely that vaccinated ducks developed vaccine-induced immunity up to ≥14 days of age, and henceforth the host immune response was able to ensure abortion of virus infection following challenge at 14 days of age. The presence of vaccine-induced antibodies by the time of virus challenge may have restricted virus infection in the liver and, thus tipped the balance in favour of virus elimination rather than persistence.

To some extent, this observation resembles the simultaneous administration of HBIG and HBsAg vaccine to infants born to HBV-carrier mothers when given at birth. Long-term studies have demonstrated that HBIG significantly increases the protective efficacy of HBsAg vaccines (André & Zuckerman, 1994) since it provides immediate (passive) protection until neonates are able to mount their own anti-HBs antibody response. The

results observed in Group **A** ducks are in accordance with a previous report showing that other avians such as chickens, were able to produce moderate level of antibody in response to antigenic stimulation at around four weeks of age (Gordon & Jordan, 1982).

DNA vaccines could not prevent the development of viremia or alter the course of virus infection in the other groups of ducks (**B**, **C** or **D**) examined in this study. As can be seen in Table 6.1, all Groups **B**-**D** ducks showed persistent viremia up to 24 weeks of age. Nonetheless, the levels of DHBsAg and viral DNA in vaccinated ducks were lower than those of non-vaccinated ones in each group, notably after the 4^{th} and the 5^{th} doses of vaccine were given. It must be noted, however, that variation in the levels of DHBsAg and viral DNA were also observed within non-vaccinated ducks over the 24 weeks of the study. The reduced levels of viremia at the later stages (>14 weeks of age) could be due to the enhanced DNA uptake induced by muscle regeneration following bupivacaine hydrochloride treatment with the 4^{th} and 5^{th} doses of vaccine. Therefore, the pre-S/S and S antigens might have been expressed more efficiently, or in different way, to the immune system. Alternatively, ducks have also reached full immunocompetence at around this age, and thus were able to respond to the vaccine. These immune responses might include cell-mediated immunity, as suggested from the presence of mononuclear cell infiltrates around the portal areas of the livers of the vaccinated ducks at the time of autopsy.

Groups **B** and **C** ducks were inoculated with 1×10^4 DHBV genomes (equivalent to 0.03 pg of DHBV DNA) which is 10,000-fold higher than the dose required to produce persistent infection in one-day-old ducks. This large viral dose may have contributed to the failure of DNA vaccines to alter the course of infection when given simultaneously or

7 days after the viral inoculum. In an attempt to relate these data to HBV infection in human, the viral dose required for HBV to produce chronic infection in 97.2% of infants born to HBV-carrier mothers was >1.4 ng/ml of maternal HBV DNA (Burk *et al.,* 1994). However, it must also be considered that due to the nature of virus transmission in this setting (blood contamination during labour, and rarely, *in utero* passage), the virus load received by infants can not accurately be quantitated.

The inability of DNA vaccines to alter the course of persistent infection in congenitally DHBV-infected ducks in this study was disappointing, but especially with hindsight, not entirely surprising. Despite two reports that showed HBsAg clearance following DNA vaccination in transgenic mice that constitutively express HBsAg (Davis *et al.,* 1997a; Mancini *et al.,* 1996), others have failed to demonstrate similar results (Schödel, 1998).

The observation that congenitally DHBV-infected ducks were able to elicit anti-DHBc antibody responses at around 17 weeks of age has not been previously reported. From transgenic mice studies, it is known that HBcAg/HBeAg-specific T cell tolerance is reversible following the regression of the thymus, and only persists for 12-16 weeks, after which the host can develop antibody responses against HBcAg, but not HBeAg (Milich & McLachlan, 1986). The exact time for the regression of thymus in avian is unknown, although it has regressed completely by the time bird reaches sexual maturity at 24 weeks of age (Gordon & Jordan, 1982). Hence, it is possible that the regression of thymus in ducks started at around 17 weeks age, and the development of anti-DHBc antibody responses in congenitally DHBV-infected ducks at around this time may have been due to a similar mechanism to that seen in transgenic mice.

Taken together, this study showed that the potential use of DNA vaccines as therapeutic vaccines requires further evaluation. Under the conditions and within the time span studied, once persistent infection has been established in the newborns, DNA vaccines were unable to eliminate infection. As stated above, the outcome of DHBV infection in ducks is related to the size of viral load and the age of the host. In the experimental settings examined in this study, it likely that the relative timing of development of vaccine-induced immunity versus extent of virus replication dictated the outcome of virus infection. Reduction of viral dose inoculum to somewhere between 1×10^0 to 1×10^3 DHBV genomes might help to discriminate whether the inability of DNA vaccines to induce protective immunity was due to the viral load, the immaturity of the immune system, or both. However, the consistent reduction in virus and antigen load observed in the vaccinated ducks in Groups **B-D**, although small in biological terms, suggested that vaccination played some role in accelerating virus removal from the bloodstream or in reducing virus production, and provides strong encouragement for further studies of this effect. Furthermore, it is possible that a combination of DNA vaccine and antiviral drugs that reduce viral load may be a more effective way to induce humoral and cellular immune responses in congenitally DHBV-infected ducks, and thereby, to alter the course of persistent infection.

Chapter 7

Concluding remarks

7.1. INTRODUCTION.

At the commencement of this study, concerns existed regarding vaccination against HBV infection, despite the widespread successful use of currently licensed plasma-derived and recombinant HBV vaccines. One of these concerns stemmed from the failure of 5-10% of vaccine recipients to develop protective levels of anti-HBs antibodies following the standard vaccination protocols. Furthermore, the necessity for and the timing of booster injections were open to debate (Resti *et al.,* 1997; Zuckerman & Zuckerman, 1998). Finally, vaccine-escape mutants had been reported from a wide range of countries (Carman *et al.,* 1990; Harrison *et al.,* 1991; Okamoto *et al.,* 1992; Karthigesu *et al.,* 1994; Zuckerman *et al.,* 1994; Oon *et al.,* 1995).

The newer generation of recombinant HBV vaccines include either pre-S2 or pre-S1 proteins in addition to the S protein. Observations from limited numbers of individuals vaccinated with the pre-S2 and pre-S1 containing HBV vaccines showed that the antibody responses against both pre-S proteins appeared earlier than to the S protein, thereby enhancing the rate of seroconversion to anti-HBs antibodies when compared with the standard HBV vaccine containing only S protein (Shouval *et al.,* 1994; Yap *et al.,* 1995). In addition, the pre-S1 containing HBV vaccine was able to elicit anti-HBs antibody responses in 70% health care workers who had failed to mount antibody responses to the standard HBsAg vaccine (Zuckerman *et al.,* 1996). However, it has not been established whether use of pre-S containing HBV vaccines reduces the need for booster injections.

The observation that injection of DNA vaccines encoding HBsAg elicited anti-HBs antibody responses in mice (Davis *et al.,* 1993a), has highlighted the potential of DNA vaccines as third generation HBV vaccines. In the current study, DHBV infection in ducks was used as a non-primate model for HBV infection in humans, to evaluate the efficacy of the pre-S/S and S DNA vaccines as new candidates for use in vaccination against HBV. The major findings obtained from this study are discussed below.

7.2. CHARACTERIZATION OF THE GENOME OF AusDHBV.

As an initial step in designing DHBV vaccines, a full-length genome of an AusDHBV strain was cloned from a pool of serum from congenitally DHBV-infected ducks. Although the infectivity of the AusDHBV clone was not examined, it was shown that several important sequences necessary for viral replication were conserved (Section 3.3.5). Comparison of the nucleotide sequence of this clone with the 13 other published DHBV isolates (Sprengel *et al.,* 1991; Munshi & Panda, 1993; Shi *et al.,* 1993) indicated that the AusDHBV isolate is a new member of the Chinese DHBV branch in the phylogenetic tree of the avian hepadnaviruses. This result was not entirely surprising since both strains may have a common ancestor (discussed in Section 3.4). The close relationship between the AusDHBV and Chinese DHBV is consistent with the finding in this study of the presence of a 28 kDa pre-S protein in AusDHBV-infected serum and liver tissue samples, and also in the yeast-derived pre-S/S protein preparations (Section 4.3.4); the same observation has been reported earlier with a

Chinese DHBV isolate (Yokosuka *et al.,* 1988) and also with a few other DHBV isolates (Lambert *et al.,* 1990). Although several internal ATGs are present within the pre-S region (nt 825, 882 and 957) of DHBV genome (Mandart *et al.,* 1984; Triyatni *et al.,* 1998b; Uchida *et al.,* 1989), the deduced translation products are 35, 33 and 30 kDa protein, respectively. Therefore, it is more likely that the 28 kDa protein is the proteolytic product of the 37 kDa or other pre-S/S proteins; the precise reason why this should occur with AusDHBV and certain DHBV strains but not with others was not examined, but it would be interesting to define.

7.3. THE USE OF YEAST-DERIVED AusDHBV pre-S/S & S PROTEINS AS VACCINES.

Recombinant yeast plasmids encoding the pre-S/S and S proteins of AusDHBV were used to produce yeast-derived DHBV pre-S/S and S protein vaccines. Analysis of the sedimentation coefficients (S values) and the buoyant density (in CsCl) of both proteins indicated that they were assembled as intracellular particles or aggregates. Three injections of 40 μg of yeast-derived protein elicited only moderate levels of antibody in ducks compared to those produced by age matched ducks injected three times with 250 μg of pre-S/S or S DNA vaccines (> 3 log lower). Under the conditions used in this study, the yeast-derived DHBV pre-S/S vaccine did not show enhanced immunogenicity and better protective efficacy against DHBV infection compared to the vaccine containing DHBV S protein. Although the amount of pre-S/S and S proteins produced *in vivo* following DNA vaccination could not be determined, it is possible that

the difference in response observed in these two studies was related to the amount and sustained presentation of protein to stimulate immune responses. The mode of presentation of antigen may also have influenced the response obtained. In any case, this study does indicate that DNA vaccines are likely to be much more effective than protein vaccines, especially since CFA was added to the first dose of protein.

As discussed in Chapter 4, the adjuvant properties of CFA were likely to be in part due to the presence of mycobacterial DNA which contains specific CpG motifs acting as ISS (Roman *et al.,* 1997), similar to that seen in plasmid DNA vaccines (see Chapter 5). However, due to the presence of paraffin oil and other bacterial cell wall extracts, CFA can also produce detrimental side effects such as inflammatory responses at the site of injection (Munoz, 1964), and therefore is not available for use in humans. A more effective approach that could be assessed for its ability to enhance the immunogenicity of recombinant HBsAg vaccines is to combine the antigens with synthetic ISS-oligonucleotides (ISS-ODN) that act as an adjuvant. Such combinations (protein/ISS-ODN) have been shown to elicit eightfold higher antibody titers than those produced by protein alone (Roman *et al.,* 1997).

7.4. DNA VACCINES ENCODING DHBV pre-S/S & S PROTEINS AS POTENTIAL THIRD GENERATION HBV VACCINES.

This study demonstrated for the first time that DNA vaccines encoding the AusDHBV pre-S/S and S proteins induced strong antibody responses in 6-month- and 3-week-old

ducks (Chapter 5; see also Triyatni *et al.,* 1998a). An interesting observation (and somewhat unexpected) was that the protective efficacy of the S DNA vaccine proved far greater than that of the pre-S/S DNA vaccine (Chapter 5). The rapid removal of DHBV injected into the bloodstream of S DNA vaccinated ducks (90% of 2 x 10^{11} DHBV genomes removed within 5-15 min) was comparable to the rate of HBsAg removal by chimpanzees following challenge with HBV-infected blood. When HBV-vaccinated chimpanzees (body weight 18 kg) were challenged with 180 ml of HBV infected-blood containing 10^8 chimpanzee infectious doses (CID_{50}) and HBsAg titer of 2^{11} (by reverse passive hemagglutination, RPHA), the HBsAg titer was reduced to 2^4 5 min after transfusion, although complete removal of HBsAg occurred only 5 hr after transfusion. These chimpanzees had been injected twice with 20 μg of plasma-derived HBsAg vaccine and at the time of challenge, had an anti-HBs titer of 2^9 as determined by PHA. They showed no biochemical and histological signs of hepatitis during the 126 weeks of the study (Karasawa *et al.,* 1983). Unfortunately, the rate of removal of HBV DNA was not determined in this study. However, an estimation of this rate is possible given that subviral HBsAg particles in serum can present up to 1,000-10,000 fold higher than virions (Nassal, 1996), it is likely that 10^8 CID_{50} of HBV DNA were removed from the bloodstream of chimpanzees ≥5 min after transfusion; this calculation was inferred from the observation that reduction of HBsAg titer from 2^{11} to 2^4 occurred 5 min after transfusion.

The lower efficacy demonstrated with the pre-S/S DNA vaccine in protecting against DHBV challenge, despite similar anti-DHBs titers with both vaccines, was unexpected.

This may be related to the finding that DHBV pre-S/S protein was not secreted following transient transfection of COS7 cells with this construct, in contrast to the S protein expressed by the S DNA construct (Section 5.3.1; Triyatni *et al.,* 1998a). Recently, others have also reported a similar finding for DHBV pre-S/S protein expressed in an avian hepatoma cell line, LMH (Rollier *et al.,* 1998). Both these results confirmed the earlier observations that in contrast to the HBV S protein, HBV pre-S/S protein was not secreted in mammalian expression systems (Chisari *et al.,* 1986; Standring *et al.,* 1986; Chow *et al.,* 1997). It follows that the two different proteins may have been presented to the immune system in a different way i.e. denatured form of pre-S/S and native form of S protein. Alternatively, folding of conformational epitopes in S protein may differ according to whether or not covalently linked pre-S sequences were also present. The immunogenicity of DHBV S protein has been reported earlier to be conformation dependent (Yokosuka *et al.,* 1988). As a consequence, the specificity of the anti-pre-S (and anti-S) antibodies produced by the pre-S/S DNA vaccinated ducks might be directed to conformationally different determinant(s) from those produced following primary DHBV infection (or by S DNA-vaccinated ducks).

So far, epitopes that elicited neutralizing antibodies against DHBV infection were predominantly mapped within the pre-S domain (Cheung *et al.,* 1989; Cheung *et al.,* 1990; Lambert *et al.,* 1990; Yuasa *et al.,* 1991), and only one within the S domain (Cheung *et al.,* 1989; Pugh *et al.,* 1995). The *in vivo* role of the neutralizing epitope(s) of the DHBV S protein has not extensively been studied, whereas it is well known that the S protein of HBV carries the 'a' determinant as major neutralizing epitope, to which

the neutralizing anti-HBs antibodies are raised. Although the AA sequences corresponding to the 'a' determinant in HBV are absent in DHBV (Mandart *et al.,* 1984; Sprengel *et al.,* 1985; Triyatni *et al.,* 1998b), it is conceivable that the S protein of DHBV contains epitope(s) that elicit neutralizing antibodies *in vivo*. In any case, this study emphasized the role of anti-S antibodies in preventing DHBV infection, consistent with the well established role for anti-HBs antibodies in HBV infection in human (Hadler *et al.,*1986; Waters *et al.,*1987a; Waters *et al.,* 1987b).

7.5. THERAPEUTIC DNA VACCINES FOR CHRONIC HEPADNAVIRUS INFECTION.

Chronic HBV infection reflects sustained viral replication without the ability of the host immune system to eliminate the virus. The wide array of histopathological changes seen in chronic hepatitis (Scheuer *et al.,* 1996) and the fact that HBV is non cytopathic, led to the view that HBV clearance is mediated principally by destruction of infected cells by HBV-specific CTL. More recently, it has been shown that viral clearance might also occur by a non-cytolytic mechanism mediated by cytokines secreted from CTL (Guidotti *et al.,* 1996). Circumstantial evidence exists that cellular immune responses, notably HBcAg/HBeAg-specific T helper cells, are involved in liver injury and viral clearance; they were predominantly seen in acute hepatitis and CAH, but were absent in chronic infection (Bertoletti *et al.,* 1991; Jung *et al.,* 1995). Furthermore, Maruyama *et al* (1993) have already shown that, based on the IgG subclass of anti-HBc and anti-HBe antibodies produced, asymptomatic chronic HBV carriers showed Th_2-

type immune responses. In contrast, acute hepatitis and CAH patients preferentially showed Th_1-type or combined Th_1 and Th_2 immune responses. Data from HBsAg-expressing transgenic mouse studies also supported the view that CMI plays a major role in down-regulating HBV gene expression. Vaccination with DNA encoding HBsAg, adoptive transfer of HBV-specific CTL, or infusion of TNF-α and IFN-γ all resulted in the reduced expression of HBV genes (Guidotti *et al.,* 1996; Mancini *et al.,* 1996). Therefore, DNA vaccines have also been seen to have potential as therapeutic agents that could drive the immune response of chronically infected individuals to mediate viral clearance.

The therapeutic efficacy of DNA vaccines in congenitally DHBV-infected ducks was compared with 3 other more favourable vaccination settings (vaccination prior to infection, at the time of challenge, and 7 days post-infection). These three settings were chosen because they mimic the situation in infants born to HBV-carrier mothers where infection mostly occurs during labour or during early childhood (Chen, 1993), while vaccination can be given as early as one day of age, or 1 month later (Beasley *et al.,* 1983; Wong *et al.,* 1984). Congenitally DHBV-infected ducks, on the other hand, could reflect the situation of chronic adult carriers in terms of comparable high levels of viremia present in the blood and liver of the respective hosts (Section 6.4). DNA vaccines were effective in aborting DHBV infection only in the pre-infection situation.

The failure of DNA vaccines in the other settings was most likely due to the high viral challenge used (10,000 fold higher than is needed to produce chronic infection of one-

day-old ducks) which tipped the balance against the ability of the host's immune system to eliminate virus infection. However, the observation that vaccinated ducks from Groups B, C and D (Pre-infection, Simultaneous infection & vaccination and Delayed vaccination, respectively; see Chapter 6) showed reduced levels of viremia after the second course of vaccination (at 11 and 13 weeks) and lower levels of DHBsAg staining of the liver autopsy tissue sections (at 24 weeks of age) compared to the non-vaccinated control ducks, suggested that DNA vaccines could suppress, albeit partially, DHBV replication in the livers of chronically infected ducks. It is possible that the relatively short duration of this study (24 weeks) may have not been long enough to observe the therapeutic effect of DNA vaccines (see below). Alternatively, DNA vaccines may be more effective against challenge with a lower dose of virus, which may more closely reflect HBV transmission in typical human situations. In this case, administration of antiviral drugs to reduce the viral load prior to DNA vaccination may be a more effective approach for the treatment of chronic hepadnavirus infection.

During the period of this study, another research group have conducted similar experiments with promising results (Rollier *et al.,* 1998). Following chronic infection induced by inoculation of 2 x 10^8 DHBV genomes into 3 day-old ducks, 100 μg of pre-S/S & S DNA vaccine were administered starting at 4 weeks of age (including bupivacaine pre-treatment 5 days earlier) for a total 4 doses of vaccine. Two out of 6 ducks cleared the virus infection completely after 32 weeks, although the other 4 ducks remained infected until 42 weeks of age. The difference in the outcome between these two studies could be due to: (i) the ID_{50} of the inoculum used by Rollier *et al* (1998)

may be less than that used in the present study (the ID_{50} of AusDHBV is equivalent to 1 viral genome; Jilbert *et al.,* 1996), (ii) the duration of observation. In the present study, ducks were sacrificed at 24 weeks of age, while in the other study ducks were monitored for 42 weeks and the absence of viremia in two ducks was first noted from 32 weeks of age.

7.6. FUTURE DIRECTIONS.

This study has demonstrated that i.m. injection of DNA vaccines encoding DHBV pre-S/S and S proteins elicited strong antibody responses (and possibly CMI) in ducks, although the mechanism whereby such immune responses were induced is not completely understood. It has been suggested that bone marrow-derived APC, recruited as a result of inflammation induced by injection, are responsible for triggering the immune response (Robinson, 1997). Given the scarcity of professional APC in muscle tissue (Hohlfeld & Engel, 1994), i.m. injection of DNA may be effective only when potent antigens are used or large doses of antigen administered. In this regard, DNA vaccines have the advantage of flexibility in allowing construction of 'cocktail' vaccines to enhance the host immune responses against weak or small doses of antigen. These 'cocktail' vaccines could include a mixture of several antigens as shown for influenza virus (Donelly *et al.,* 1995), a combination of antigen and cytokine such as HBsAg and IL-2 (Chow *et al.,* 1997), or by coupling DNA with a specific ligand that targets the complex to APC or to lymphoid tissue (Boyle *et al.,* 1998). The latter combination would result in prolonged presentation of antigen to the immune system,

that not only should enhance the immune responses but may also eliminate the need for booster injection.

To improve the immunogenicity of future HBV vaccines, a combination of HBsAg with another viral antigen such as nucleocapsid (HBcAg), or with cytokines such as IFN-α, IFN-γ, and IL-2 are worth assessing. HBcAg is highly immunogenic even in the absence of adjuvant and is able to induce both strong antibody and CMI responses (Milich *et al.,* 1997). HBcAg is both a T cell dependent and T cell independent antigen (Milich & McLachlan, 1986). However, the use of HBcAg alone as a HBV vaccine may not be effective since anti-HBc antibodies persist for many years in the sera of chronic HBV infected patients (Hollinger, 1990), suggesting that antibodies against HBcAg alone can not eliminate infection. This observation was supported by an earlier study which showed that monoclonal anti-HBc antibodies were not able to protect chimpanzees against HBV challenge (Pignatelli *et al.,*1987). In contrast, others have shown conflicting results in an experimental model system; vaccination of woodchucks with the WHV nucleocapsid antigen protected the animals from developing viremia following WHV challenge (Schödel *et al.,* 1993). The protection seen may have been due to the fact that HBcAg-specific helper T cells can act as helper T cells for B cells specific for HBsAg (Milich *et al.,* 1987). Alternatively, HBcAg-specific CTL may have mediated the rapid clearance of virus from the small number of cells initially infected with HBV. Experiments are in progress in our laboratory to evaluate DHBcAg and DHBeAg as protective and therapeutic vaccines.

The potential of 'combination' HBV vaccines containing HBsAg and cytokines such as IFN-α, IFN-γ, or IL-2 has been demonstrated recently in mice (Chow *et al.,* 1997). So far, IFN-α has been used as a therapeutic agent for chronic HBV infection although rebound virus replication normally occurs following cessation of treatment (Haria & Benfield, 1995). This combination therapy approach should be explored further in the duck model to evaluate various combinations of antigens and cytokines for their effectiveness in the treatment of chronic hepadnavirus infection. Cloning of duck cytokines genes has been commenced in our laboratory with some promising results, and will be used for such studies.

Despite the results reported from numerous studies so far on the potency of DNA vaccines, safety issues need to be addressed. These include the possibility of integration of the DNA plasmids with the host chromosome, either randomly or as result of homologous recombination, which may limit their application as commercial vaccines. Several lines of evidence have shown that this possibility is very unlikely. Firstly, vectors used for DNA vaccines are usually designed so that they are unable to replicate inside mammalian cells. It has been shown that the optimal conditions for integration as a result of homologous recombination to be concurrent replication of the host and plasmid DNA and the presence of large (>600 bp) closely spaced regions of homology between the host and the plasmid (Donelly *et al.*, 1997). In the case of i.m DNA vaccination, the transfected cells (myocyte or resident macrophage/dendritic cells) are known to be non-dividing cells (Hohlfeld & Engel, 1994). Additionally, direct

studies of random integration by PCR methods (able to detect 1 copy/150,000 nuclei) in mice injected with plasmid DNA have not detected integration of the injected plasmid (Nichols *et al.,* 1995). The absence of integration can also be inferred from studies with the DNA plasmid encoding the luciferase reporter gene that was still detected in the cytoplasm, and not in the nucleus, of mouse muscle cells 19 months after injection (Wolff *et al.,* 1992). In addition, others have also demonstrated that transfected mouse muscle fibers expressing HBsAg were destroyed 10 days after injection of DNA, although muscle fibers transfected with luciferase-expressing DNA plasmid maintained the antigen expression for at least 60 days (Davis *et al.,* 1997b).

Altogether, these results suggest that i.m. injection of DNA vaccines, at least for those encoding HBV pre-S/S and S DNA, is unlikely to result in the integration of plasmid DNA into the host chromosome. Nonetheless, long-term studies to eliminate this possibility are still needed, preferably in higher primates. Provided that all the safety concerns can be overcome, the flexibility of designing 'cocktail' vaccines offered by DNA vaccines could be very beneficial, not only for the prevention, but also for the treatment of chronic HBV infection by inducing both effective humoral and CMI responses.

REFERENCES

André, F.E., and Zuckerman, A.J. (1994). Review: protective efficacy of hepatitis B vaccines in neonates. *J. Med. Virol.* **44:**144-151.

Araki, K., Shiosaki, K., Araki, M., Chisaka, O., and Matsubara, K. (1990). The essential region for assembly and particle formation in hepatitis B virus surface antigen produced in yeast cells. *Gene* **89:**195-201.

Ashton-Rickardt, P.G., and Murray, K. (1989). Mutants of the hepatitis B virus surface antigen that define some antigenically essential residues in the immunodominant *a* region. *J. Med. Virol.* **29:**196-203.

Bancroft, W.H., Mundon, F.K., and Russel, P.K. (1972). Detection of additional antigenic determinants of hepatitis B antigen. *J. Immunol.* **109:**842-848.

Barrios, C., Brawand, P., Berney, M., Brandt, C., Lambert, P.-H., and Siegrist, C.-A. (1996). Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th_2-biased pattern which persists after adult boosting. *Eur. J. Immunol.* **26:**1489-1496.

Barry, M.A., and Johnston, S.A. (1997). Biological features of genetic immunization. *Vaccine* **15:**788-791.

Beasley, R.P., Hwang, L.Y., Lee, G.C., *et al.* (1983). Prevention of perinatally transmitted hepatitis B virus infections with hepatitis B immune globulin and hepatitis B vaccine. *Lancet* **ii:**1099-1102.

Beck, J., and Nassal, M. (1997). Sequence- and structure-specific determinants in the interaction between the RNA encapsidation signal and reverse transcriptase of avian hepatitis B viruses. *J. Virol.* **71:**4971-4980.

Becker, D. M. and Fikes, J. D. (1993). Cloning and functional analysis of heterologous eukaryotic transcription factors in yeast. In: Hames, B.D. and Higgins, S.J. (eds), *Gene transcription, a practical approach,* Oxford University Press, New York, 295-318.

Berting, A., Hahnen, J., Kroger, M., and Gerlich, W.H. (1995). Computer-aided studies on the spatial structure of the small hepatitis B surface protein. *Intervirol.* **38:**8-15.

Bertoletti, A., Ferrari, C., Fiaccadori, F., Penna, A., *et al.* (1991). HLA class-I restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. *Proc. Natl. Acad. Sci. USA* **88:**10445-10449.

Bertram, E.M. (1997). Characterisation of duck lymphoid cell populations and their role in immunity to duck hepatitis B virus. Ph.D. Thesis, University of Adelaide, Australia.

Biemans, R., Thines, D., Petre-Parent, B., Wilde, M., Rutgers, T., and Cabezon, T. (1992). Immunoelectron microscopic detection of the hepatitis B virus major surface protein in dilated perinuclear membranes of yeast cells. *DNA Cell Biol.* **11:**621-626.

Billingham, R.E., Brent, L., and Medawar, P.B. (1953). Actively required tolerance of foreign cells. *Nature* **172:**603-606.

Bot, A., Bot, S., Garcia-Sastre, A., Bona, C. (1996). DNA immunization of newborn mice with a plasmid-expressing nucleoprotein of influenza virus. *Viral Immunol.* **9:**207-210.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256:**1604-1607.

Böttcher, B., Wynne, S.A., and Crowther, R.A. (1997). Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. *Nature* **386:**88-91.

Boyle, J.S., Brady, J.L., and Lew, A.M. (1998). Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction. *Nature* **392:**408-411.

Brown, S.E., Howard, C.R., Zuckerman, A.J., and Steward, M.W. (1984). Affinity of antibody responses in man to hepatitis B vaccine determined with synthetic peptides. *Lancet* **ii:**184-187.

Bruss, V., and Ganem, D. (1991). The role of envelope proteins in hepatitis B virus assembly. *Proc. Natl. Acad. Sci. USA* **88:**1059-1063.

Bruss, V., and Gerlich, W.H. (1988). Formation of transmembraneous hepatitis B e antigen by cotranslational *in vitro* processing of the viral precore protein. *Virology* **163:**268-275.

Bruss, V., and Vieluf, K. (1995). Functions of the internal pre-S domain of the large surface protein in hepatitis B virus particle morphogenesis. *J. Virol.* **69:**6652-6657.

Burrell, C.J. (1975). Host components in hepatitis B antigen. *J. Gen. Virol.* **27:**117-126.

Burk, R.D., Hwang, L.-Y., Ho, G.Y.F., Shafritz, D.A., and Beasley, R.P. (1994). Outcome of perinatal hepatitis B virus exposure is dependent on maternal virus load. *J. Inf. Dis.* **170:**1418-1423.

Büscher, M., Reiser, W., Will, H., and Schaller, H. (1985). Transcripts and the putative RNA pregenome of duck hepatitis B virus: implications for reverse transcription. *Cell* **40:**717-724.

Carman, W.F., Zanetti, A.R., Karayiannis, P. *et al.* (1990). Vaccine induced escape mutant of hepatitis B virus. *Lancet* **336:**325-329.

Carman, W.F., Fagan, E.A., Hadziyannis, S. *et al.* (1991). Association of a precore variant of hepatitis B virus with fulminant hepatitis. *Hepatology* **14:**219-222.

Carman, W.F. (1993). Viral genetic variation: hepatitis B as a clinical example. *Lancet* **341:**349-353.

Celis, E., Zurawski, V.R., and Chang, T.W. (1984). Regulation of T-cell function by antibodies: enhancement of the response of human T-cell clones to hepatitis B surface antigen by antigen-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* **81:**6846-6850.

Centers for Disease Control. (1991). Hepatitis B: A comprehensive strategy for eliminating transmission in the United States through universal childhood vaccination: Recommendation of the Immunization Practices Advisory Committee (ACIP). *Morbidity and Mortality Weekly Report* **40:**1-25.

Chang, C., Enders, G., Sprengel, R., Peters, N., Varmus, E., and Ganem, D. (1987). Expression of the precore region of an avian hepatitis B virus is not required for viral replication. *J. Virol.* **61:**3322-3325.

Chang, M.-H., Chen, C.-J., Lai, M.-S., Hsu, H.-M., Wu, T.-C., *et al.* (1997). Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. *N. Engl. J. Med.* **336:**1855-1859.

Chassot, S., Lambert, V., Kay, A., Godinot, C., Roux, B., Trepo, C., and Cova, L. (1993). Fine mapping of neutralization epitopes on duck hepatitis B virus (DHBV) pre-S protein using monoclonal antibodies and overlapping peptides. *Virology* **192:**217-223.

Chen, D.S., Hoyer, B.H., Nelson, J., Purcell, R.H., and Gerin, J.L. (1982). Detection and properties of hepatitis B viral DNA in liver tissue from patients with hepatocellular carcinoma. *Hepatology* **2:**S42-46.

Chen, D.-S. (1993). Natural history of chronic hepatitis B infection: New light on an old story. *J. Gastroenterol. Hepatol.* **8:**470-475.

Cheung, R.C., Robinson, W.S., Marion, P.L., and Greenberg, H.B. (1989). Epitope mapping of neutralizing monoclonal antibodies against duck hepatitis B virus. *J. Virol.* **63:**2445-2451.

Cheung, R.C., Trujillo, D.E., Robinson, W.S., Greenberg, H.B., and Marion, P.L. (1990). Epitope-specific antibody response to the surface antigen of duck hepatitis B virus in infected ducks. *Virology* **176:**546-552.

Chirillo, P., Pagano, S., Natoli, G., Puri, P.L., Burgio, V.L., Balsano, C., and Levrero, M. (1997). The hepatitis B virus X gene induces p53-mediated programmed cell death. *Proc. Natl. Acad. Sci. USA* **94:**8162-8167.

Chisari, F.V., Filippi, P., McLachlan, A., Milich, D.R., Riggs, M., Lee, S., Palmiter, R.D., Pinkert, C.A., and Brinster, R.L. (1986). Expression of hepatitis B virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. *J. Virol.* **60:**880-887.

Chow, Y.-H., Huang, W.-L., Chi, W.-K., Chu, Y.-D., and Tao, M.-H. (1997). Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. *J. Virol.* **71:**169-178.

Condon, C., Watkins, S.C., Celluzzi, C.M., Thompson, K., and Falo, Jr, L.D. (1996). DNA-based immunization by *in vivo* transfection of dendritic cells. *Nat. Med.* **2:**1122-1128.

Conry, R.M., Widera, G., LoBuglio, A.F., Fuller, J.T., Moore, S.E., Barlow, D.L., turner, J., Yang, N.-S., and Curiel, D.T. (1996). Selected strategies to augment polynucleotide immunization. *Gene Ther.* **3:**67-74.

Conway, J.F., Cheng, N., Zlotnick, A., Wingfield, P.T., Stahl., S.J., and Steven, A.C. (1997). Visualization of a 4-helix bundle in the hepatitis B virus capsid by cryo-electron microscopy. *Nature* **386:**91-94.

Davis, H.L., Michel, M.L., and Whalen, R.G. (1993a). DNA based immunization for hepatitis B induces continuous secretion of antigen and high levels of circulating antibody. *Hum. Molec. Genet.* **2:**1847-1851.

Davis, H.L., Whalen, R.G., and Demeneix, B.A. (1993b). Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression. *Hum. Gene Ther.* **4:**151-159.

Davis, H.L., Mancini, M., Michel, M.-L., and Whalen, R. G. (1996a). DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. *Vaccine* **14:**910-915.

Davis, H.L. McCluskie, M.J., Gerin, J.L., and Purcell, R.H. (1996b). DNA vaccine for hepatitis B: immunogenicity in chimpanzees and comparison with other vaccines. *Proc. Natl. Acad. Sci. USA* **93:**7213-7218.

Davis, H.L., Millan, C.L.B., Mancini, M., McCluskie, M.J., Hadchouel, M., Comanita, L., Tiollais, P., Whalen, R.G., and Michel, M.-L. (1997a). DNA-based immunization against hepatitis B surface antigen (HBsAg) in normal and HBsAg-transgenic mice. *Vaccine* **15:**849-852.

Davis, H.L., Millan, C.L.B., and Watkins, S.C. (1997b). Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. *Gene Ther.* **4:**181-188.

Dimmock, N.J. (1993). Neutralization of animal viruses. *Curr. Top. Microbiol. Immunol.* **183:**3-31.

Donelly, J.J., Friedman, A., Martinez, D., Montgomery, D.L., Shiver, J.W., Motzel, S., Ulmer, J.B., Liu, M.A. (1995). Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. *Nat. Med.* **1:**583-587.

Donelly, J.J., Ulmer, J.B., Shiver, J.W., and Liu, M. (1997). DNA vaccines. *Annu. Rev. Immunol.* **15:**617-648.

Eble, B.E., Lingappa, V.R., and Ganem, D. (1990). The N-terminal (pre-S2) domain of a hepatitis B virus surface glycoprotein is translocated across membranes by downstream signal sequences. *J. Virol.* **64:**1414-1419.

Elghetany, M.T., and Saleem, A. (1988). Methods for staining amyloid in tissues: a review. *Stain. Technol.* **63:**201-212.

Fernholz, D., Wildner, G., and Will, H. (1993). Minor envelope proteins of duck hepatitis B virus are initiated at internal pre-S AUG codons but are not essential for infectivity. *Virology* **197:**64-73.

Forsthuber, T., Yip, H.C., and Lehmann, P.V. (1996). Induction of Th_1 and Th_2 immunity in neonatal mice. *Science* **271:**1728-1730.

Fynan, E.F., Webster, R.G., Fuller, D.H., and Haynes, J.R. (1993). DNA vaccines: protective immunizations by parenteral, mucosal and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* **90:**11478-11482.

Galibert, F., Mandart, F., Fitoussi, F., Tiollais, P., and Charnay, P. (1979). Nucleotide sequence of the hepatitis B virus genome (subtype *ayw*) cloned in *E. coli*. *Nature* **281:**646-650.

Ganem, D., and Varmus, H.E. (1987). The molecular biology of the hepatitis B viruses. *Annu. Rev. Biochem.* **56:**651-693.

Gerhardt, E., and Bruss, V. (1995). Phenotypic mixing of rodent but not avian hepadnavirus surface proteins into human hepatitis B virus particles. *J. Virol.* **69:**1201-1208.

Girones, R. and Miller, R. (1989). Mutation rate of the hepadnavirus genome. *Virology* **170:**595-597.

Gordon, R.F., and Jordan, F.T.W. (1982). The avian immune system. In: Poultry Diseases, 2nd ed, Bailliere Tindall, London, 328-340.

Griffith, O.M. (1979). Techniques of preparative, zonal, and continuous flow ultracentrifugation. *Applications Research Department, Spinco Division, Beckman Instruments, Inc,* 22-23.

Guidotti, L.G., Matzke, B., Schaller, H., and Chisari, F.V. (1995). High-level hepatitis B virus replication in transgenic mice. *J. Virol.* **69:**6158-6169.

Guidotti, L.G., Ishikawa, T., Hobbs, M.V., Matzke, B., Schreiber, R., and Chisari, F.V. (1996). Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity* **4:**25-36.

Guo, J.-T., and Pugh, J.C. (1997). Topology of the large envelope protein of duck hepatitis B virus suggests a mechanism for membrane translocation during particle morphogenesis. *J. Virol.* **71:**1107-1114.

Hadler, S.C., Francis, D.P., Maynard, J.E., Thompson, S.E., Judson, F.N. *et al.* (1986). Long-term immunogenicity and efficacy of hepatitis B vaccine in homosexual men. *N. Engl. J. Med.* **315:**209-214.

Haria, M., and Benfield, P. (1995). Interferon-alpha-2a. A review of its pharmacological properties and therapeutic use in the management of viral hepatitis. *Drugs* **50:**873-896.

Harrison, T.J., Hopes, E.A., Oon, C.J., Zanetta, A.R., and Zuckerman, A.J. (1991). Independent emergence of a vaccine-induced escape mutant of hepatitis B virus. *J. Hepatol.* **13:**105-107.

Hassett, D.E., Zhang, J., and Whitton, J.L. (1997). Neonatal DNA immunization with a plasmid encoding an internal viral protein is effective in the presence of maternal antibodies and protects against subsequent viral challenge. *J. Virol.* **71:**7881-7888.

Hedley, M.L., Curley, J., and Urban, R. (1998). Microspheres containing plasmid-encoded antigens elicit cytotoxic T-cell responses. *Nat. Med.* **4:**365-368.

Heermann, K.H., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H., and Gerlich, W.H. (1984). Large surface proteins of hepatitis B virus containing the preS sequence. *J. Virol.* **52:**396-402.

Heermann, K.H., and Gerlich, W.H. (1991). Surface proteins of hepatitis B viruses. In: McLachlan, A. (ed), *Molecular biology of the hepatitis B virus*, CRC Press, Boca Raton, 145-169.

Hirsch, R.C., Lavine, J.E., Chang, L.-J., Varmus, H.E., and Ganem, D. (1990). Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. *Nature* **344:**552-555.

Hohlfeld, R, and Engel, A.G. (1994). The immunobiology of muscle. *Immunol. Today* **15:**269-274.

Hollinger, F.B. (1990). Hepatitis B virus. In: Fields, B.N. (ed), *Virology*, 2nd ed, Raven Press, Ltd, 2171-2220.

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

Hopp, T.P., and Woods, K.R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78:**3824-3828.

Hu, J., and Seeger, C. (1996). Expression and characterization of hepadnavirus reverse transcriptases. *Methods Enzymol.* **275:**195-209.

Imamura, T., Araki, M., Miyanohara, A., Nakao, J., Yonemura, H., Ohtomo, N., and Matsubara, K. (1987). Expression of hepatitis B virus middle and large surface antigen genes in *Saccharomyces cerevisiae. J. Virol.* **61:**3543-3549.

Ip, H.M.H., Lelie, P.N., Wong, V.C.W., Kuhns, M.C., and Reesink, H.W. (1989). Prevention of hepatitis B virus carrier state in infants according to maternal serum levels of HBV DNA. *Lancet* **i:**406-410.

Ishikawa, T., and Ganem, D. (1995). The pre-S domain of the large viral envelope protein determines host range in avian hepatitis B viruses. *Proc. Natl. Acad. Sci. USA* **92:**6259-6263.

Iwarson, S., Tabor, E., Thomas, H.C., Goodall, A., Waters, J., Snoy, P., Shih, J.W., and Gerety, R.J. (1985). Neutralization of hepatitis B virus infectivity by a murine monoclonal antibody: an experimental study in the chimpanzee. *J. Med. Virol.* **16:**89-95.

Jilbert, A.R., Freiman, J.S., Burrell, C.J., Holmes, M., Gowans, E.J., Rowland, R., Hall, P., and Cossart, Y.E. (1988). Virus-liver cell interactions in duck hepatitis B virus infection. A study of virus dissemination within the liver. *Gastroenterol.* **95:**1375-1382.

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

Jilbert, A.R., Miller, D.S., Scougall, C.A., Turnbull, H., and Burell, C.J. (1996). Kinetics of duck hepatitis B virus infection following low dose virus inoculation: one virus DNA genome is infectious in neonatal ducks. *Virology* **226:**338-345.

Jilbert, A.R., Botten, J.A., Miller, D.S., Bertram, E.M., Hall, P., Kotlarski, I., and Burrell, C.J. (1998). Characterization of age- and dose-related outcomes of duck hepatitis B virus infection. *Virology* **243:**273-282.

Junker-Niepman, M., Bartenschlager, R., and Schaller, H. (1990). A short *cis*-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. *EMBO J.* **9:**3389-3396.

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

Kane, M.A. (1996). Global status of hepatitis B immunization. *Lancet* **348:**696.

Karasawa, T., Shikata, T., Abe, K., Horiuchi, R., Takahashi, T., Yoshihara, N., Mayumi, M., Suzuki, H., and Oda, T. (1983). Efficacy of hepatitis B vaccine in chimpanzees given transfusions of highly infective blood. *J. Inf. Dis.* **147:**327-335.

Karthigesu, V.D., Allison, L.M.C., Fortuin, M., Mendy, M., Whittle, H.C., and Howard, C.R. (1994). A novel hepatitis B virus variant in the sera of immunized children. *J. Gen. Virol.* **75:**443-448.

Kimura, Y., Sonehara, K., Kuramoto, E., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T., and Tokunaga, T. (1994). Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. *J. Biochem.* **116:**991-994.

Klinman, D.M., Yi, A.-K., Beaucage, S.L., Conover, J., and Krieg, A.M. (1996). CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ. *Proc. Natl. Acad. Sci. USA* **93:**2879-2883.

Klingmüller, U., and Schaller, H. (1993). Hepadnavirus infection requires interaction between the viral preS domain and a specific hepatocellular receptor. *J. Virol.* **67:**7414-7422.

Knodell, R.G., Ishak, K.G., Black, W.C., Chen, T.S., Craig, R., Kaplowitz, N., Kiernan, T.W., and Wollman, J. (1981). Formulation and application of a numeric scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* **1:**431-435.

Kok, T.W., Payne, L.E., Bailey, S.E., and Waddell, R.G. (1993). Urine and the laboratory diagnosis of *Chlamydia trachomatis* in males. *Genitourin. Med.* **69:**51-53.

Krieg, A.M., Yi, A.-K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A., Klinman, D.M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374:**546-549.

Kumar, S., Tamura, K., and Nei, M. (1994). MEGA: molecular evolutionary genetics analysis software for microcomputers. *Comput. Appl. Biosci.* **10:**189-195.

Lambert, V., Fernholz, D., Sprengel, R., Fourel, I., Deléage, G., Wildner, G., Peyret, C., Trépo, C., Cova, L., and Will, H. (1990). Virus-neutralizing monoclonal antibody to a conserved epitope on the duck hepatitis B virus pre-S protein. *J. Virol.* **64:**1290-1297.

Lanford, R.E., Chavez, D., Brasky, K.M., Burns III, R.B., and Rico-Hesse, R. (1998). Isolation of a hepadnavirus from the woolly monkey, a New World primate. *Proc. Natl. Acad. Sci. USA* **95:**5757-5761.

Lauder, I. J., Lin, H.-J., Lau, J.Y.N., Siu, T.-S., and Lai, C.-L. (1993). The variability of the hepatitis B virus genome: statistical analysis and biological implications. *Mol. Biol. Evol.* **10:**457-470.

Lazizi, Y., Badur, S., Perk, Y., Ilter, O., and Pillot, J. (1997). Selective unresponsiveness to HBsAg vaccine in newborns related with an *in utero* passage of HBV DNA. *Vaccine* **15:**1194-1199.

Le Bouvier, G.L. (1971). The heterogeneity of the Australia antigen. *J. Infect. Dis.* **123:**671-675.

Lenhoff, R.J., and Summers, J. (1994). Coordinate regulation of replication and virus assembly by the large envelope protein of an avian hepadnavirus. *J. Virol.* **68:**4565-4571.

Liang, T., Hasegawa, K., Rimon, N., Wands, J.R., and Ben-Porath, E. (1991). A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N. Engl. J. Med.* **324:**1705-1709.

Liang, T.J., Hasegawa, K., Munoz, S.J., Shapiro, C.N., Yoffe, B., *et al.* (1994). Hepatitis B virus precore mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation. *J. Clin. Invest.* **93:**550-555.

Lok, A.S.F. (1992). Natural history and control of perinatally acquired hepatitis B virus infection. *Dig. Dis.* **10:**46-52.

Mancini, M., Hadchouel, M., Davis, H.L., Whalen, **R.G., Tiollais, P., and Michel, M.-L. (1996).** DNA-mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state. *Proc. Natl. Acad. Sci. USA* **93:**12496-12501.

Mandart, E., Kay, A., and Galibert, F. (1984). Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. *J. Virol.* **49:**782-792.

Mangold, C.M.T., and Streeck, R.E. (1993). Mutational analysis of the cysteine residues in the hepatitis B virus small envelope protein. *J. Virol.* **67:**4588-4597.

Marion, P.L., Oshiro, L.S., Regnery, D.C., Scullard, G.H., and Robinson, W.S. (1980). A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. *Proc. Natl. Acad. Sci. USA* **77:**2941-2945.

Marion, P.L., Knight, S.S., Ho, B.-K., Guo, Y.-Y., Robinson, W.S., and Popper, H. (1984). Liver disease associated with duck hepatitis B virus infection of domestic ducks. *Proc. Natl. Acad. Sci. USA* **81:**898-902.

Martinez, X., Brandt, C., Saddallah, F., Tougne, C., Barrios, C., Wild, F., Dougan, G., Lambert, P.-H., and Siegrist, C.-A. (1997). DNA immunization circumvents deficient induction of T helper type 1 and cytotoxic T lymphocyte responses in neonates and during early life. *Proc. Natl. Acad. Sci. USA* **94:**8726-8731.

Maruyama, T., McLachlan, A., Iino, S., Koike, K., Kurokawa, K., and Milich, D.R. (1993). The serology of chronic hepatitis B infection revisited. *J. Clin. Invest.* **91:**2586-2595.

Mason, H.S., Lam, D.M.-K., and Arntzen, C.J. (1992). Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl. Acad. Sci. USA* **89:**11745-11749.

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

Mason, W.S., Taylor, J.M., Seal, G., and Summers, J. (1981). An HBV-like virus of domestic ducks. In: Szmuness, W., Alter, H.J., and Maynard, J.E. (eds), *Viral Hepatitis 1981 International Symposium,* 107-116.

Mason, W.S., Halpern, M., England, J., Seal, G., Egan, J., Coates, L., Aldrich, C., and Summers, J. (1983). Experimental transmission of duck hepatitis B virus. *Virology* **131:**375-384.

Mason, W.S., and Marion, P.L. (1994). Avian hepatititis B viruses. In: Webster, R.G. and Granoff, A. (eds), *Encyclopedia of Virology,* volume 2, Academic Press, 564-568.

Mattes, F., Tong, S., Teubner, K., and Blum, H.E. (1990). Complete nucleotide sequence of a German duck hepatitis B virus. *Nucl. Acids. Res.* **18:**6140. *GenBank X12798.*

Matzinger, P. (1994). Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12:**991-1045.

McAleer, W.J., Buynak, E.B., Maigetter, R.Z., Wampler, D.E., Miller, W.J., and Hilleman, M.R. (1984). Human hepatitis B vaccine from recombinant yeast. *Nature* **307:**178-180.

McMahon, G., Ehrlich, P.H., Moustafa, Z.A. *et al.* (1992). Genetic alterations in the gene encoding the major HBsAg: DNA and immunological analysis of recurrent HBsAg derived from monoclonal antibody-treated liver transplant patients. *Hepatology* **15:**757-766.

McMahon, B., and Wainwright, R. (1993). In: Ellis, R (ed), *Hepatitis B vaccines in clinical practice,* New York, Marcel Dekker, Inc., 243-261.

Mendenhall, M.D., Richardson, H.E., and Reed, S.I. (1988). Dominant negative protein kinase mutations that confer a G_1 arrest phenotype. *Proc. Natl. Acad. Sci. USA* **85:**4426-4430.

Michel, M.-L., Davis, H.L., Schleef, M., Mancini, M., Tiollais, P., and Whalen, R.G. (1995). DNA-mediated immunization to the hepatitis B surface antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. *Proc. Natl. Acad. Sci. USA* **92:**5307-5311.

Milich, D.R., and McLachlan, A. (1986). The nucleocapsid of hepatitis B virus is both a T-cell independent and a T-cell-dependent antigen. *Science* **234:**1398-1401.

Milich, D.R., Jones, J.E., Hughes, J.L., Price, J., Raney, A.K., and McLachlan, A. (1990). Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance *in utero*? *Proc. Natl. Acad. Sci. USA* **87:**6599-6603.

Milich, D.R. (1991). Immune response to hepatitis B virus proteins: relevance of the murine model. *Semin. Liver Dis.* **11:**93-112.

Milich, D.R., Jones, J., Hughes, J., and Maruyama, T. (1993). Role of T-cell tolerance in the persistence of hepatitis B virus infection. *J. Immunother.* **14:**226-233.

Milich, D.R., Schödel, F., Hughes, J.L., Jones, J.E., and Peterson, D.L. (1997). The hepatitis B virus core and e antigens elicit different Th cell subsets: antigen structure can affect Th cell phenotype. *J. Virol.* **71:**2192-2201.

Molnar-Kimber, K.L., Summers, J.W., and Mason, W.S. (1984). Mapping of the cohesive overlap of duck hepatitis B virus DNA and of the site of initiation of reverse transcription. *J. Virol.* **51:**181-191.

Molnar-Kimber, K.L., Jarocki-Witek, V., Dheer, S.K., Vernon, S.K., Conley, A.J., Davis, A.R., and Hung, P.P. (1988). Distinctive properties of the hepatitis B virus envelope proteins. *J. Virol.* **62:**407-416.

Mor, G., Yamshchikov, G., Sedegah, M., Takeno, M., Wang, R., Houghten, R.A., Hoffman, S., and Klinman, D.S. (1996). Induction of neonatal tolerance by plasmid DNA vaccination of mice. *J. Clin. Invest.* **98:**2700-2705.

Munoz, J. (1964). Effect of bacteria and bacterial products on antibody response. *Adv. Immunol.* **4:**397-440.

Munshi, A., and Panda, S.K. (1993). Cloning, sequencing and sequence comparison of the Indian isolate of duck hepatitis B virus. *GenBank X74623.*

Nassal, M., and Schaller, H. (1993). Hepatitis B virus replication. *Trends Microbiol.* **1:**221-228.

Nassal, M. (1996). Hepatitis B virus morphogenesis. *Curr. Top. Microbiol. Immunol.* **214:**297-337.

Nassal, M., and Schaller, H. (1996). Hepatitis B virus replication - an update. *J. Vir. Hep.* **3:**217-226.

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

Nichols, W.W., Ledwith, B.J., Manam, S.V., and Troilo, P.J. (1995). Potential DNA vaccine integration into the host cell genome. *N. Y. Acad. Sci.* **772:**30-39.

O'Callaghan, and Charbit, A. (1990). High efficiency transformation of *Salmonella typhimurium* and *Salmonella typhi* by electroporation. *Mol. Gen. Genet.* **223:**156-158.

O'Connell, A.P., Urban, M.K., and London, W.T. (1983). Naturally occurring infection of Pekin duck embryos by duck hepatitis B virus. *Proc. Natl. Acad. Sci. USA* **80:**1703-1706.

Okamoto, H., Yotsumoto, S., Akahane, Y., Yamanaka, T., Miyazaki, Y., Sugai, Y., Tsuda, F., Tanaka, T., Miyakawa, Y., and Mayumi, M. (1990). Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J. Virol.* **64:**1298-1303.

Okamoto, H., Yano, K., Nozaki, Y., *et al.* (1992). Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. *Pediatr. Res.* **32:**264-268.

Omata, M., Ehata, Y., Yokosuka, O., Hosoda, K., Ohto, M. (1991). Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N. Engl. J. Med.* **324:**1699-1704.

Oon, C.-J., Lim, G.-K., Ye, Z., *et al.* (1995). Molecular epidemiology of hepatitis B virus variants in Singapore. *Vaccine* **13:**699-702.

Orito, E., Mizokami, M., Ina, Y., Moriyama, E.N., Kameshima, N., Yamamoto, M., and Gojobori, T. (1989). Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. *Proc. Natl. Acad. Sci. USA* **86:**7059-7062.

Ou, J.H., Laub, O., and Rutter, W.J. (1986). Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. *Proc. Natl. Acad. Sci. USA* **83:**1578-1582.

Persing, D.H., Varmus, H.E., and Ganem, D. (1986). Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science* **234:**1388-1391.

Pignatelli, M., Waters, J., Lever, A., Iwarson, S., Gerety, R., Thomas, H.C. (1987). Cytotoxic T cell responses to nucleocapsid protein of HBV in chronic hepatitis: evidence that antibody modulation may cause protracted infection. *J. Hepatol.* **4:**15-21.

Pisetsky, D.S. (1997). Immunostimulatory DNA: A clear and present danger? *Nat. Med.* **3:**829-831.

Prange, R., and Streeck, R.E. (1995). Novel transmembrane topology of the hepatitis B virus envelope proteins. *EMBO J.* **14:**247-256.

Prince, A.M., Whalen, R., Taylor, P.E., and Brotman, B. (1997). Protective efficacy of DNA-based immunization against HBV newborn chimpanzees. In: Brown, F. *et al.*, (eds), *Vaccines 97,* Cold Spring Harbor Laboratory, 141-144.

Pugh, J.C., Sninsky, J.J., Summers, J.W., and Schaeffer, E. (1987). Characterization of a pre-S polypeptide on the surfaces of infectious avian hepadnavirus particles. *J. Virol.* **61:**1384-1390.

Pugh, J. C., Di, Q., Mason, W. S., and Simmons, H. (1995). Susceptibility to duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles. *J. Virol.* **69:**4814-4822.

Qiao, M., Gowans, E. J., Bailey, S. E., Jilbert, A. R., and Burrell, C. J. (1990). Serological analysis of duck hepatitis B virus infection. *Virus Res.* **17:**3-14.

Qiao, M., Gowans, E.J., and Burrell, C.J. (1992). Intracellular factors, but not virus receptor levels, influence the age-related outcome of DHBV infection of ducks. *Virology* **186:**517-523.

Qiao, M. (1993). Studies on the pathogenesis and the early events of hepadnavirus replication. Ph.D. Thesis, University of Adelaide, Australia.

Ranki, M., Schätzl, H.M., Zachoval, M., Uusi-Oukari, M., and Lehtovaara, P. (1995). Quantification of hepatitis B virus DNA over a wide range from serum for studying viral replicative activity in response to treatment and in recurrent infection. *Hepatology* **21:**1492-1499.

Resti, M., Azzari C., Mannelli, F., Rossi, M.E., Lionetti, P., and Vierucci, A. (1997). Ten-year follow-up study of neonatal hepatitis B immunization: are booster injections indicated? *Vaccine* **15:**1338-1340.

Richardson, H. E., Wittenberg, C., Cross, F., and Reed, S. I. (1989). An essential G1 function for cyclin-like proteins in yeast. *Cell* **59:**1127-1133.

Ridge, J.P., Fuchs, E.J., and Matzinger, P. (1996). Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* **271:**1723-1726.

Robinson, H.L. (1997). Nucleic acid vaccines: an overview. *Vaccine* **15:**785-787.

Rollier, C., Sunyach, C., Barraud, L., Madani, N., Jamard, C., Trepo, C., and Cova, L. (1998). Protective and therapeutic effect of DNA-based immunization against hepadnavirus large envelope protein. *Gastroenterol. (in press).*

Roman, M., Martin-Orozco, E., Goodman, J.S., Nguyen M.-D, Sato, Y., Ronaghy, A., Kornbluth, R.S., Richman, D.D., Carson, D.A., and Raz, E. (1997). Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nature Med.* **3:**849-854.

Rutgers, T., *et al.* (1993). In: Ellis, R (ed), *Hepatitis B vaccines in clinical practices,* Marcel Dekker, New York, 383-407.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed, Cold Spring Harbor Laboratory Press.

Sarzotti, M., Robbins D.S., and Hoffman, P.M. (1996). Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* **271:**1726-1728.

Sarzotti, M. (1997). Immunologic tolerance. *Curr. Opin. Hematol.* **4:**48-52.

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.-D., Silverman, G.J., Lotz, M., Carson, D.A., and Raz, E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273:**352-354.

Sattler, F., and Robinson, W.S. (1979). Hepatitis B viral DNA molecules have cohesive ends. *J. Virol.* **32:**226-231.

Scheuer, P.J., Davies, S.E., and Dhillon, A.P. (1996). Histopathological aspects of viral hepatitis. *J. Vir. Hep.* **3:**277-281.

Schirmbeck, R., Bohm, W., Ando, K., Chisari, F.V., and Reiman, J. (1995). Nucleic acid vaccination primes hepatitis B virus surface antigen-specific cytotoxic T lymphocytes in non responder mice. *J. Virol.* **69:**5929-5934.

Schlicht, H. J., Kuhn, C., Guh, B., Mattaliano, R. J., and Schaller, H. (1987a). Biochemical and immunological characterization of the duck hepatitis B virus envelope protein. *J. Virol.* **61:**2280-2285.

Schlicht, H.-J., Salfeld. J., and Schaller, H. (1987b). The duck hepatitis B virus pre-C region encodes a signal sequence which is essential for synthesis and secretion of processed core proteins but not for virus formation. *J. Virol.* **61:**3701-3709.

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

Schneider, R., Fernholz, D., Wildner, G., and Will, H. (1991). Mechanism, kinetics, and role of duck hepatitis B virus e-antigen expression *in vivo*. *Virology* **182:**503-512.

Schödel, F., Sprengel, R., Weimer, T., Fernholz, D., Schneider, R., and Will, H. (1989). Animal hepatitis B viruses. In: Klein, G.(ed), *Advances in Viral Oncology* **8:**73-100.

Schödel, F., Neckerman, G., Peterson, D., Fuchs, K., Fuller, S., Will, H., and Roggendorf, M. (1993). Immunization with recombinant woodchuck hepatitis virus nucleocapsid antigen or hepatitis B virus nucleocapsid antigen protects woodchucks from woodchuck hepatitis virus infection. *Vaccine* **11:**624-628.

Schödel, F. (1998). Hepatitis B virus vaccines. In: Koshy, R., & Caselman, W.H. (eds), *Hepatitis B virus, molecular mechanisms in disease and novel strategies for therapy,* Imperial College Press, 219-250.

Shi, H., Cullen, J.M., and Newbold, J.E. (1993). A novel isolate of duck hepatitis B virus. *GenBank M95589.*

Shiels, M.T., Taswell, H.F., Czaja, A.J., Nelson, C., and Swenke, P. (1987). Frequency and significance of concurrent hepatitis B surface antigen and antibody in acute and chronic hepatitis B. *Gastroenterol.* **93:**675-680.

Shouval, D., Ilan, Y., Adler, R., Deepen, R., Panet, A., Even-Chen, Z., Gorecki, M., and Gerlich, W.H. (1994). Improved immunogenicity in mice of a mammalian cell-derived

recombinant hepatitis B vaccine containing pre-S1 and pre-S2 antigens as compared with conventional yeast-derived vaccines. *Vaccine* **12:**1453-1459.

Skelly, J., Howard, C.R., and Zuckerman, A.J. (1981). Hepatitis B polypeptide vaccine preparation in micelle form. *Nature* **290:**51-54.

Sprengel, R., Kuhn, C., Manso, C., and Will, H. (1984). Cloned duck hepatitis B virus DNA is infectious in Pekin ducks. *J. Virol.* **52:**932-937.

Sprengel, R., Kuhn, C., Will, H., and Schaller, H. (1985). Comparative sequence analysis of duck and human hepatitis B virus genomes. *J. Med. Virol.* **15:**323-333.

Sprengel, R., Kaleta, E.F., and Will, H. (1988). Isolation and characterization of a HBV endemic in herons. *J. Virol.* **62:**3832-3839.

Sprengel, R., Schneider, R., Marion, P.L., Fernholz, D., Wildner, G., and Will, H. (1991). Comparative sequence analysis of defective and infectious avian hepadnaviruses. *Nucl. Acids Res.* **19:**4289.

Standring, D.N., Ou, J.-H., and Rutter, W.J. (1986). Assembly of viral particles in *Xenopus* oocytes: pre-surface antigens regulate secretion of the hepatitis B viral surface envelope particle. *Proc. Natl. Acad. Sci. USA* **83:**9338-9342.

Stibbe, W., and Gerlich, W.H. (1983). Structural relationships between minor and major proteins of hepatitis B surface antigen. *J. Virol.* **46:**626-628.

Stirk, H.J., Thornton, J.M., and Howard, C.R. (1992). A topological model for hepatitis B surface antigen. *Intervirology* **33:**148-158.

Su, Q., Schroder, C.H., Hofmann, W.J., Otto, G., Pichlmayr, R., and Bannasch, P. (1998). Expression of hepatitis B virus X protein in HBV-infected human livers and hepatocellular carcinomas. *Hepatology* **27:**1109-1120.

Summers, J., Smolec, J.M., and Snyder, R. (1978). A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc. Natl. Acad. Sci. USA* **75:**4533-4537.

Summers, J., Smith, P.M., and Horwich, A.L. (1990). Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J. Virol.* **64:**2819-2824.

Sunyach, C., Chassot, S., Jamard, C., Kay, A., Trepo, C., and Cova, L. (1997). *In vivo* selection of duck hepatitis B virus pre-S variants which escape from neutralization. *Virology* **234:**291-299.

Swameye, I., and Schaller, H. (1997). Dual topology of the large envelope protein of duck hepatitis B virus: determinants preventing pre-S translocation and glycosylation. *J. Virol.* **71:**9434-9441.

Tagawa, M., Yokosuka, O., Imazeki, F., Ohto, M., and Omata, M. (1996). Gene expression and active virus replication in the liver after injection of duck hepatitis B virus DNA into the peripheral vein of ducklings. *J. Hepatol.* **24:**328-334.

Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10:**512-526.

Terazawa, S., Kojima, M., Yamanaka, T., Yotsumoto, S., Okamoto, H., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1991). Hepatitis B virus mutants with precore region defects in two babies with fulminant hepatitis and their mothers positive for antibody to hepatitis B e antigen. *Pediatr. Res.* **29:**5-9.

Testut, P., Renard, C.-A., Terradillos, O., Vivitski-Trepo, V., Tekaia, F., Degott, C., Blake, J., Boyer, B., and Buendia, M.A. (1996). A new hepadnavirus endemic in arctic ground squirrels in Alaska. *J. Virol.* **70:**4210-4219.

Thanavala, Y., Yang, Y.F., Lyons, P., Mason, H.S., and Arntzen, C. (1995). Immunogenicity of transgenic plant-derived hepatitis B surface antigen. *Proc. Natl. Acad. Sci. USA* **92:**3358-3361.

Thomas, R.F., and Newbold, J.E. (1991). Nucleotide sequence analysis of the duck hepatitis B virus clone P2-3 from Maple Leaf farms. *GenBank M60677.*

Tong, S., Mattes, F., Teubner, K., and Blum, H.E. (1990). Complete nucleotide sequence of a Chinese duck hepatitis B virus. *Nucl. Acids. Res.* **18:**6139. *GenBank M21953.*

Tong, S., Mattes, F., Blum, H.E., Fernholz, D., Schneider, R., and Will, H. (1991). Complete nucleotide sequence of a Chinese duck hepatitis B virus. *GenBank X60213.*

Triyatni, M., Jilbert, A.R., Qiao, M., Miller, D.S., and Burrell, C.J. (1998a). Protective efficacy of DNA vaccines against duck hepatitis B virus infection. *J. Virol.* **72:**84-94.

Triyatni, M., Qiao, M., Ey, P., Burrell, C.J., and Jilbert, A.R. (1998b). Cloning and sequencing of an Australian duck hepatitis B virus. *EMBL AJ006350.*

Tsiquaye, K.N., Rapicetta, M., McCaul, T.F., and Zuckerman, A.J. (1985). Experimental *in ovo* transmission of duck hepatitis B virus. *J. Virol. Methods* **11:**49-57.

Tuttleman, J., Pugh, J., and Summers, J. (1986). *In vitro* experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. *J. Virol.* **58:**17-25.

Twu, J.S., Lai, M.Y., Chen, D.S., and Robinson, W.S. (1993). Activation of protooncogene c-jun by the X protein of HBV. *Virology* **192:**3-3350.

Uchida, M., Esumi, M., and Shikata, T. (1989). Molecular cloning and sequence analysis of duck hepatitis B virus genomes of a new variant isolated from Shanghai ducks. *Virology* **173:**600-606.

Ulmer, J.B., Sadoff, J.C., and Liu, M.A. (1996). DNA vaccines. *Curr. Opin. Immunol.* **8:**531-536.

Valenzuela, P., Medina, A., Rutter, W.J., Ammerer, G., and Hall, B.D. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* **298:**347-350.

Vasavada, A. (1995). Improving productivity of heterologous proteins in recombinant *Saccharomyces cerevisiae* fermentations. *Adv. Appl. Microb.* **41:**25-54.

Wang, G.-H., and Seeger, C. (1993). Novel mechanism for reverse transcription in hepatitis B viruses. *J. Virol.* **67:**6507-6512.

Wang, Y., Xiang, Z., Pasquini, S., and Ertl, H.C.J. (1997). Immune response to neonatal genetic immunization. *Virology* **228:**278-284.

Waters, J.A., Pignatelli, M., Brown, D., O'Rourke, S., Lever, A., and Thomas, H.C. (1987a). The immune response to hepatitis B virus. In: André, F.E. *et al.* (eds), *Postgrad. Med. J.* **63** (Suppl. 2)**:**51-56.

Waters, J.A., O'Rourke, S.M., Richardson, S.C., Papaevangelou, G., and Thomas, H.C. (1987b). Qualitative analysis of the humoral immune response to the *'a'* determinant of HBs antigen after inoculation with plasma-derived or recombinant vaccine. *J. Med. Virol.* **21:**155-160.

Wells, D.J., and Goldspink, G. (1992). Age and sex influence expression of plasmid DNA directly injected into mouse skeletal muscle. *FEBS Lett.* **306:**203-205.

Wells, D.J. (1993). Improved gene transfer by direct plasmid injection associated with regeneration in mouse skeletal muscle. *FEBS Lett.* **332:**179-182.

Whalen, R.G., Leclerc, C., Deriaud, E., Schirmbeck, R., Reimann, J., Davis, H.L. (1995). DNA-mediated immunization to the hepatitis B surface antigen: activation and entrainment of the immune response. *Ann. N Y Acad. Sci.* **95:**64-76.

Wolff, J.A., Ludtke, J.J., Ascadi, G., Williams, P., and Jani, A. (1992). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.* **1:**363-369.

Wong, V.C.W., HMH, I.P., Reesinck, H.W., *et al.* (1984). Prevention of the HBsAg carrier state in newborn infants of mothers who are chronic carriers of HBsAg and HBeAg by

administration of hepatitis B vaccine and hepatitis B immune globulin: Double-blind randomized placebo-controlled study. *Lancet* **i:**921-926.

Wu, T.-T., Condreay, L.D., Coates, L., Aldrich, C., and Mason, W. (1991). Evidence that less-than-full-length *pol* gene products are functional in hepadnavirus DNA synthesis. *J. Virol.* **65:**2155-2163.

Yamamoto, K., Horikita, M., Tsuda, F. *et al.* (1994). Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. *J. Virol.* **68:**2671-2676.

Yap, I., Guan, R., and Chan, S.H. (1995). Study on the comparative immunogenicity of a recombinant DNA hepatitis B vaccine containing pre-S components of the HBV coat protein with non pre-S containing vaccines. *J. Gastroenterol. Hepatol.* **10:**51-55.

Yokosuka, O., Omata, M., and Ito, Y. (1988). Expression of pre-S1, pre-S2, and C proteins in duck hepatitis B virus infection. *Virology* **167:**82-86.

Yuasa, S., Cheung, R.C., Pham, Q., Robinson, W.S., and Marion, P.L. (1991). Peptide mapping of neutralizing and non-neutralizing epitopes of duck hepatitis B virus pre-S polypeptide. *Virology* **181:**14-21.

Zuckerman, A.J., Harrison, T.J., and Oon, C.-J. (1994). Mutations in the S region of hepatitis B virus. *Lancet* **343:**737-738.

Zuckerman, J.N., Sabin, C., Craig, F.M., *et al.* (1996). Immune response to a new hepatitis B vaccine in health care workers who had not responded to hepatitis B vaccination. *Br. Med. J.* **314:**329-333.

Zuckerman, J.N., and Zuckerman, A.J. (1998). Is there a need for boosters of hepatitis B vaccines? *Viral Hep. Rev.* **4:**43-46.

JOURNAL OF VIROLOGY, Jan. 1998, p. 84–94
0022-538X/98/$04.00+0

Vol. 72, No. 1

Protective Efficacy of DNA Vaccines against Duck Hepatitis B Virus Infection

M. TRIYATNI,[1]* A. R. JILBERT,[1,2] M. QIAO,[2] D. S. MILLER,[1] AND C. J. BURRELL[1,2]

Department of Microbiology and Immunology, University of Adelaide, Adelaide, South Australia 5005,[1] and Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Adelaide, South Australia 5000,[2] Australia

Received 12 May 1997/Accepted 18 September 1997

The efficacy of DNA vaccines encoding the duck hepatitis B virus (DHBV) pre-S/S and S proteins were tested in Pekin ducks. Plasmid pcDNA I/Amp DNA containing the DHBV pre-S/S or S genes was injected intramuscularly three times, at 3-week intervals. All pre-S/S and S-vaccinated ducks developed total anti-DHBs and specific anti-S antibodies with similar titers reaching 1/10,000 to 1/50,000 and 1/2,500 to 1/4,000, respectively, after the third vaccination. However, following virus challenge, significant differences in the rate of virus removal from the bloodstream and the presence of virus replication in the liver were found between the groups. In three of four S-vaccinated ducks, 90% of the inoculum was removed between <5 and 15 min postchallenge (p.c.) and no virus replication was detected in the liver at 4 days p.c. In contrast, in all four pre-S/S-vaccinated ducks, 90% of the inoculum was removed between 60 and 90 min p.c. and DHBsAg was detected in 10 to 40% of hepatocytes. Anti-S serum abolished virus infectivity when preincubated with DHBV before inoculation into 1-day-old ducklings and primary duck hepatocyte cultures, while anti-pre-S/S serum showed very limited capacity to neutralize virus infectivity in these two systems. Thus, although both DNA vaccines induced high titers of anti-DHBs antibodies, anti-S antibodies induced by the S-DNA construct were highly effective in neutralizing virus infectivity while similar levels of anti-S induced by the pre-S/S-DNA construct conferred only very limited protection. This phenomenon requires further clarification, particularly in light of the development of newer HBV vaccines containing pre-S proteins and a possible discrepancy between anti-HBs titers and protective efficacy.

Hepatitis B virus (HBV) vaccines which contain the small envelope protein (S-HBs) of the virus provide significant protection against HBV infection. Global HBV vaccination programs as recommended by the World Health Organization may eventually reduce the number of HBV carriers, at present estimated to be 350 million people worldwide (21). In natural HBV infection and in HBV vaccine recipients, the presence of antibodies directed to the surface antigen of the viral envelope protein (anti-HBs antibodies) is a marker of immunity. The surface gene of HBV contains a single open reading frame with three in-frame translation start codons that identify the pre-S1, pre-S2, and S genes, which code for the large (L-HBs), middle (M-HBs), and small (S-HBs) proteins, respectively. All three envelope proteins have the same carboxyl terminus but differ in length at their amino terminus. The S-HBs protein, also termed the major surface antigen (HBsAg), carries a group-specific determinant, *a*, which is common to all subtypes. The *a* determinant of HBsAg is an immunodominant epitope to which anti-HBs responses following natural infection and vaccination are predominantly directed (37). The antigenicity of the *a* determinant depends on its conformational structure maintained by disulfide bridges between amino acids 124 and 137 and 139 and 147 (1, 3). Injection of a monoclonal antibody raised against the *a* determinant of HBsAg (anti-*a*) into chimpanzees conferred protection against HBV infection (16). Likewise, HBV subunit vaccines (yeast derived) that contain only S-HBs protein confer protection against HBV infection in vaccinees who develop an anti-HBs titer of >10 mIU/ml (15).

Despite the effectiveness of the current HBV vaccine, several problems concerning this vaccine still exist, e.g., nonresponsiveness in 5 to 10% of vaccinees and the emergence of vaccine-escape mutants (3). Therefore, further development of the current HBV vaccines to improve the efficacy of vaccination would be desirable. The benefit of inclusion of the pre-S protein into the current HBV vaccine has not been established, although a preliminary study has demonstrated that incorporation of pre-S protein into the HBV vaccine resulted in seroconversion in a small number of nonresponders to the conventional vaccine (40). Another possible approach is DNA-based vaccination (10, 12), which allows synthesis of a foreign protein(s) in vivo from the injected plasmid DNA. An important feature of this method is that the viral protein(s) enters the major histocompatibility complex (MHC) class I pathway of the cell, leading to the induction of cytotoxic T-lymphocyte responses. Theoretically, the presence of plasmid DNA within the transfected cells will allow sustained viral antigen expression in vivo, with prolonged induction of both humoral and cell-mediated immune responses. This type of immune response induced by DNA vaccines mimics that of live attenuated viral vaccines yet avoids some of the possible problems associated with live vaccines. It has been shown that intramuscular (i.m.) injection of plasmid DNA encoding HBsAg in mice (10) and chimpanzees (12) led to the production of anti-HBs antibodies in vivo. However, the protective efficacy of DNA vaccines against HBV infection could be tested only in primates, and hence other animal models such as ducks experi-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Adelaide, Adelaide, South Australia 5005, Australia. Phone: 61-8-8303 5399. Fax: 61-8-8303 4362. E-mail: mtryatni@microb.adelaide.edu.au.

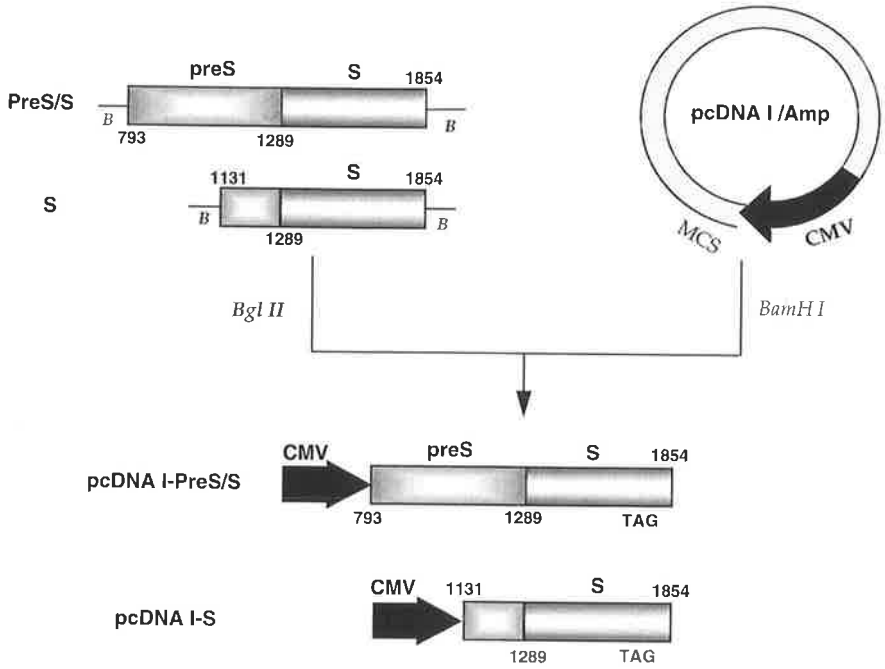


FIG. 1. Schematic diagram of the cloning of the pre-S/S and S genes of AusDHBV into pcDNA I/Amp. The pre-S/S and S genes of AusDHBV were amplified, *Bgl*II sites were introduced at both 5′ ends by PCR, and the products were subcloned into the *Bam*HI site of pcDNA I/Amp downstream of a CMV promoter. The numbers shown are the nucleotide positions in the pre-S/S and S genes, according to HBDS31.CG (36). The start codon for the pre-S/S gene in the pcDNA I–pre-S/S DNA construct is positioned at nucleotide 801. The pcDNA I-S construct contains an extra 158 nucleotides of pre-S sequence (nucleotides 1131 to 1288) upstream of the S start codon (nucleotide 1289) due to the primer site chosen for PCR. TAG is the stop codon for the pre-S/S and S genes, positioned at nucleotide 1793. *B*, *Bgl*II restriction site; MCS, multiple-cloning site; CMV, cytomegalovirus.

mentally infected with duck HBV (DHBV) have been explored.

DHBV is closely related to HBV with regard to genomic organization, hepatotropism, and mode of replication (26). In addition, DHBV in its natural host, the domestic Pekin duck, permits the study of virus neutralization mechanisms both in vitro and in vivo. The envelope proteins of DHBV, the large (pre-S/S) and small (S) surface proteins, have been shown to be involved in viral infectivity and to carry neutralization epitopes. Previous studies have demonstrated several neutralizing epitopes in DHBV envelope proteins, four within the pre-S domain, and one in the S domain (6). Others have also reported that monoclonal antibodies against specific antigenic sites within the pre-S domain (amino acids 77 to 100) were able to reduce DHBV infectivity in vivo (4, 5). The role of DHBV S protein alone in inducing neutralizing antibodies remains uncertain in vivo, although it has been shown that monoclonal antibodies specific for S protein were able to neutralize DHBV infectivity in vitro (6, 28).

Using DHBV as a model for HBV, we report the protective efficacy of DNA vaccines against DHBV infection in ducks. We have used DNA vaccines coding for the DHBV pre-S/S and S proteins to test the hypothesis that current HBV vaccines might be improved by the inclusion of pre-S protein.

MATERIALS AND METHODS

Source of the virus and plasmid. The Australian strain of DHBV (AusDHBV) used throughout this study was isolated from a pool of congenitally DHBV-infected Pekin duck (*Anas domesticus platyrhyncos*) serum (34). A full-length clone of the AusDHBV genome, pBL 4.8 (34), was obtained by insertion of DHBV genome at the *Eco*RI site of pBluescript 11KS+ (Stratagene). pBL 4.8 was used as the DNA template for subcloning by PCR (see below) and as a DNA probe for Southern blot hybridization.

Subcloning of DHBV pre-S/S and S genes into the eukaryotic expression vector. pcDNA I/Amp (Invitrogen, San Diego, Calif.) containing the cytomegalovirus early promoter/enhancer sequence and the polyadenylation signal from simian virus 40 was chosen as the vector to express DHBV pre-S/S and S proteins in vivo following DNA vaccination. Cloning of the pre-S/S and S genes into the *Bam*HI site of the plasmid was facilitated by introducing *Bgl*II sites (AGATCT) into both ends of the DHBV DNA fragments by PCR (Fig. 1). Two pairs of primers (31CG.792/31CG.1854c and 31CG.1131/31CG.1854c, numbered according to the HBDS31.CG sequence, a full-length genomic sequence of a Chinese DHBV isolate [36]) were used to amplify the pre-S/S and S genes, respectively. The sequences of the primers are as follows: 31CG.792, 5′-GGC-AGATCTAA GTTCCTGATGGG-3′; 31CG.1131, 5′-GGC-AGATCT-ACCACCACCATTC C-3′; and 31CG.1854c, 5′-GGC-AGATCT-CCGAGGAATCGTAT-3′. A 10-ng portion of pBL 4.8 DNA was amplified in a 50 μl of PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM $MgCl_2$, 200 μM [each] deoxynucleoside triphosphates, 100 mM each primer, 1.25 U of AmpliTaq polymerase [Pharmacia]). The first cycle was performed at 94°C for 2 min 30 s, 55°C for 1 min, and 72°C for 1 min. DNA was then amplified for 33 cycles (94°C for 50 s, 55°C for 30 s, and 72°C for 40 s) followed by a final extension step at 72°C for 5 min. The PCR products were purified with a QIAquick Spin PCR purification kit (Qiagen) as specified by the manufacturer and redissolved in distilled water. The amplified pre-S/S or S genes were digested with *Bgl*II and cloned into the *Bam*HI site of pcDNA I/Amp before transformation into *Escherichia coli* TOP10F′ (Invitrogen). The pcDNA I–pre-S/S and pcDNA I-S plasmids were confirmed by restriction enzyme analysis and Southern blot hybridization with an α-^{32}P-labeled full-length DHBV probe. The nucleotide sequences of the amplified pre-S/S and S genes in both constructs were verified by sequencing and compared with the sequence of the parental AusDHBV clone. Plasmid DNA was purified by anion-exchange chromatography, with a QIAfilter Plasmid Maxi Prep kit (Qiagen) as specified by the manufacturer, and DNA was dissolved in sterile phosphate-buffered saline (PBS) at 1 mg/ml.

Transient transfection of the COS7 cell line. COS7 cells were grown at 37°C in 5% CO_2 in 24-well plates (Falcon, Becton Dickinson Labware) containing Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 12 ng of penicillin per ml, and 160 ng of gentamicin per ml until the cells reached 50 to 60% confluency. The cells were washed once with PBS, and 300 μl of DMEM (supplemented as above, except with 1% FBS) was added. Then 20 μl of transfection mixture [1 μg of plasmid DNA, 5 μg of *N*-[1-(2,3,-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammoniummethylsulfate (DOTAP; Boehringer), 14 μl of HEPES-buffered saline (150 mM NaCl, 20 mM

Hepes)] was held at room temperature (RT) for 15 min and added to the cells, which were then incubated at 37°C in 5% CO_2 overnight. The medium was replaced on the following day with 1 ml of DMEM supplemented with 5% FBS, and the cells were grown for 2 days. Expression of the DHBV pre-S/S and S proteins was detected by indirect immunofluorescence (IMF) as described previously (24) with minor modifications. Briefly, the cells were washed twice with cold PBS, air dried, and fixed with chilled (−20°C) methanol for 10 min at RT. After removal of the methanol and air drying, 200 μl of the corresponding primary antibody was added: a 1/1,000-dilution of anti-pre-S monoclonal antibody 1H.1 (ascites fluid) (28), or a 1/50 dilution of rabbit anti-DHBs (29). The reaction mixture was incubated for 30 min at 37°C, the cells were washed three times with PBS, and 200 μl of the corresponding secondary antibody [fluorescein isothiocyanate-conjugated sheep anti-mouse (Silenius) or fluorescein isothiocyanate-conjugated anti-rabbit $F(ab')_2$ fragment (Silenius)] at a 1/50 dilution in PBS was added. After a further incubation at 37°C for 1 h, the cells were washed as above, mounted in 90% glycerol–50 mM Tris-HCl (pH 8.6) in PBS, and examined under an inverted fluorescence microscope.

Vaccination protocols. Ducks (6 months old and 3 weeks old) were vaccinated i.m. in the quadriceps anterior muscle with 750 or 250 μg, respectively, of pcDNA I/Amp containing either the pre-S/S or the S gene. At 5 days before DNA vaccination, the injection sites were treated with 750 μl (6-month-old ducks) or 250 μl (3-week-old ducks) of bupivacaine HCl 0.5% (Marcain; Astra) to induce muscle necrosis and subsequent muscle regeneration (11, 39). At 15 min before vaccination, the muscle sites were injected with 750 μl (6-month-old ducks) or 250 μl (3-week-old ducks) of 25% (wt/vol) sucrose in PBS. All injections were carried out with 1-ml syringes fitted with 26-gauge needles. The DNA vaccination was repeated 3 and 6 weeks later by the same procedure.

Serological assays. (i) Detection of total anti-DHBs antibodies by antibody capture ELISA. Serum samples were collected weekly after vaccination and analyzed for the presence of anti-DHBs antibodies by antibody capture enzyme-linked immunosorbent assay (ELISA) (20). A 100-μl sample of anti-pre-S monoclonal antibody 1H.1 (1/10,000 dilution of ascites fluid in 0.1 M $NaHCO_3$ [pH 9.6]) (28) was used to coat 96-well microdilution plates (Disposable Products Pty. Ltd.) at 37°C for 1 h and then at 4°C overnight. Nonspecific sites were blocked with 150 μl of 5% skim milk (Carnation) in PBS–0.05% Tween 20 (PBS-T), and then 100 μl of DHBsAg (1 ng/μl; purified on sucrose gradients from the serum of congenitally DHBV-infected ducks [30]) was added to each well. The plates were incubated with fivefold dilutions of serum samples (starting at a dilution of 1/25) and then with 100 μl of rabbit anti-duck immunoglobulin Y (purified from duck egg yolks) (2) at a 1/5,000 dilution. Finally, the plates were incubated with 100 μl of horseradish peroxidase (HRP)-conjugated goat-anti-rabbit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) at a dilution of 1/5,000. Bound antibodies were visualized by the addition of 100 μl of HRP substrate (40 mg of *o*-phenylendiamine [Sigma] plus 0.012% H_2O_2 in 100 ml of 0.1 M citrate-phosphate buffer [pH 5.0]) and incubation in the dark for 15 min; the reaction was stopped by adding 50 μl of 2.5 M H_2SO_4. The optical density at 490 nm (OD_{490}) was read on an automatic ELISA reader (Dynatech MR5000). The antibody titer in each serum sample was defined as the highest serum dilution that resulted in an OD_{490} of 0.5. Each incubation step was carried out for 1 h at 37°C, and the plates were washed three times with PBS-T between each step, except prior to addition of the HRP substrate, when they were washed three times with PBS. All dilutions were made in PBS-T containing 5% skim milk.

(ii) Detection of anti-S antibodies by ELISA. Yeast-derived (recombinant) DHBV S protein was used to coat plates in a specific assay for anti-S antibodies. For this purpose, the S gene of AusDHBV was cloned downstream of a GAL promoter in a yeast plasmid, pYCpG2 (31). The S protein was expressed in *Saccharomyces cerevisiae* after induction with 2% galactose for 8 to 12 h. Yeast-derived DHBV S protein was recovered after lysis of the yeast cells with glass beads (diameter, 425 to 600 μm [Sigma]) and purification by sequential ultracentrifugation in an SW 41 rotor, first onto a 1-ml 70% sucrose cushion (12,500 rpm for 14 h at 4°C) and then onto 20 to 50% continuous sucrose gradients (39,000 rpm for 14 h at 4°C) (22). To detect anti-S antibodies, the plates were coated with 100 μl (1 ng/μl) of purified yeast-derived DHBV S protein in 0.1 M $NaHCO_3$ (pH 9.6) at 37°C overnight. Nonspecific sites were blocked with 150 μl of 5% skim milk in PBS-T, and the plates were incubated with fivefold dilutions of serum samples (starting at a dilution of 1/25). Subsequent steps were performed exactly as for the total anti-DHBs assay.

Virus challenge. All vaccinated young ducks and one nonvaccinated duck were challenged with a high-titer dose of DHBV (1.9×10^{11} DHBV DNA genomes). The ducks were cannulated via the jugular vein, and 20 ml of pooled serum containing ~9.5×10^9 DHBV genomes/ml (18) was injected through the cannula. Blood samples were collected before virus challenge (prebleed) and at 1, 5, 15, 30, 45, 60, and 90 min and 2 h postchallenge (p.c.). For viral DNA extraction from serum after the virus challenge, 100 μl of duck serum collected at each time point p.c. was centrifuged through 1 ml each of 10 and 20% sucrose in TN buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl) at 55,000 rpm in a Beckman TLS-55 rotor for 3 h at 4°C (19). The pellet containing viral DNA was digested for 1 h at 37°C in 50 μl of TN buffer containing 2 mg of pronase per ml, 20 ng of salmon sperm DNA per ml, 0.1% sodium dodecyl sulfate (SDS), and 10 mM EDTA. The reaction was terminated by adding 20 mM EDTA, and samples were stored at −20°C. To assess the extent of DNA loss during sample processing, 100 μl of pooled serum inoculum containing a known amount of DNA (26 ng of DHBV DNA/ml) (18) was pelleted separately and digested with pronase and SDS in the same manner. Extracted samples equivalent to 50 μl of original serum or 10 μl of pooled serum inoculum (containing 260 pg of DNA) were analyzed by agarose gel electrophoresis and Southern blot hybridization. The relative amounts of viral DNA remaining in the bloodstream at each time point p.c. were quantitated by a Molecular Dynamics PhosphorImager system with 260 pg of extracted inoculum and 50 pg of DHBV DNA (gel purified from pBL 4.8 DNA after digestion with *Eco*RI and *Pvu*I to release a full-length DHBV genome) as standards. The percent virus removal was calculated based on the DHBV DNA concentration at each time point p.c. compared to the 100% value defined as the DHBV DNA concentration of the inoculum corrected for a 10× dilution effect (20 ml of inoculum/200 ml of total blood volume, calculated as 7% of body weight) occurring immediately after inoculation.

Liver biopsy. Liver biopsies were performed at 4 days p.c. and in some cases 2 weeks before challenge. Liver tissue samples (~200 mg) were dissected from the right lobe and divided into three pieces: (i) snap-frozen in liquid N_2 for total DNA and covalently closed circular DHBV DNA (cccDNA) extraction, (ii) fixed in formalin for histological analysis, and (iii) fixed in ethanol-acetic acid (EAA) (3:1) for viral antigen detection. EAA fixation was performed at room temperature for 30 min and was followed by treatment in chilled (−20°C) 70% ethanol overnight; then the blocks were processed into paraffin wax and sectioned onto gelatin-coated slides.

(i) Total and cccDNA extraction from liver tissue. Viral DNA (total and cccDNA) was extracted from liver tissues as described previously (17). A 100-mg sample of frozen liver tissue was homogenized in 3 ml of 10 mM Tris-HCl (pH 7.4)–10 mM EDTA buffer on ice. Total DNA was extracted from 1.5 ml of liver homogenate, initially diluted to 4 ml with 10 mM Tris-HCl (pH 7.4)–10 mM EDTA, and digested with an equal volume of pronase-SDS (final concentrations, 4 mg of pronase per ml, 0.1% SDS, 0.15 M NaCl, 10 mM Tris-HCl [pH 7.4], and 10 mM EDTA) at 37°C for 2 h. The total DNA was phenol-chloroform extracted, ethanol precipitated at −20°C overnight, washed three times with 70% ethanol, and redissolved in 400 μl of TE (10 mM Tris HCl [pH 7.4], 1 mM EDTA) buffer containing 100 μg of RNase A per ml. The cccDNA was extracted from the remaining 1.5 ml of liver homogenate by incubation with 10 mM Tris-HCl (pH 7.4)–10 mM EDTA–0.5% SDS–0.5 M KCl buffer at RT for 30 min followed by centrifugation at 10,000 rpm (Beckman JA-20.1 rotor) for 20 min at 4°C. The cccDNA in the supernatant was then phenol-chloroform extracted, ethanol precipitated at RT for >30 min, washed three times with 70% ethanol, and redissolved in 200 μl of TE buffer. Samples (25 μl each) of total and cccDNA were subjected to agarose gel electrophoresis followed by Southern blot hybridization.

(ii) Viral antigen detection in tissue sections. Viral pre-S antigen was detected in EAA-fixed liver tissues by standard immunoperoxidase techniques (18) with anti-pre-S monoclonal antibody 1H.1 (28), followed by HRP-conjugated sheep anti-mouse antibody (Amersham). Bound conjugate was visualized with diaminobenzidine (Sigma), counterstained with hematoxylin, mounted with DPX (Koch-light Laboratories) under glass coverslips, and examined by light microscopy.

In vivo neutralization assay. A sample of virus inoculum diluted to 10 μl in normal duck serum (NDS) containing 10^6 DHBV DNA genomes (equivalent to 10^6 50% infective doses [ID_{50}] [18]), was preincubated at 37°C for 1 h alone or with serum collected from the pre-S/S (R76) and S (R81) DNA-vaccinated ducks (anti-pre-S/S and anti-S serum, respectively). Based on the results of virus removal from the bloodstream of vaccinated ducks (see Results), different volumes of neat anti-pre-S/S serum (20, 40, and 80 μl) or anti-S serum (5, 10, and 20 μl) were used. The total anti-DHBs antibody titers of both antisera were similar as determined by ELISA. After 1 h of incubation, the volume of the mixture was adjusted to 100 μl with NDS, and the mixture (100 μl) was inoculated intravenously (i.v.) into groups of 1-day-old ducklings (three animals/volume of serum tested). The ducks were bled weekly, and viremia was detected by ELISA for DHBsAg (18) and by spot blot hybridization for viral DNA (30). The detection limit of spot blot hybridization was 0.5 pg of DNA.

In vitro neutralization assay. Primary duck hepatocytes (PDH) were obtained from 2- to 3-week-old ducklings by collagenase perfusion of the liver as described previously (30, 35). The cells were seeded at 1.5×10^6 cells per well in six-well plates (Falcon, Beckton Dickinson) in Leibovitz's L-15 medium (Gibco-BRL) supplemented with 5% FBS, 2 mM L-glutamine, 12 ng of penicillin per ml, 160 ng of gentamicin per ml, 1 μg of insulin per ml, 10 U of nystatin per ml, and 10^{-5} M hydrocortisone hemisuccinate and were incubated at 37°C without CO_2. The maintenance medium (L-15 medium without FBS) was changed every day, and the in vitro neutralization assay was performed 1 day postplating. A sample of 30 μl of virus inoculum (sucrose gradient-purified DHBV from serum containing 7×10^7 DHBV DNA genomes, equivalent to 5.4×10^4 50% tissue culture infective doses [$TCID_{50}$] [30]) was preincubated with 35 or 70 μl of either anti-S, anti-pre-S/S, or a mixture of equal volumes (35 or 70 μl) of each antiserum for 1 h at 37°C, and the volume was adjusted to 1 ml with L-15 medium prior to inoculation into each well of PDH. The cells were incubated with 1 ml of virus-antibody mixture for 12 h at 37°C, and then a further 2 ml of fresh L-15 medium was added without removing the inoculum. As a positive control of infection, the same concentration of virus preincubated with NDS was used. The cells were incubated at 37°C and harvested at 7 days postinoculation (p.i.) for detection of DHBV replication. The total intracellular DNA was extracted as

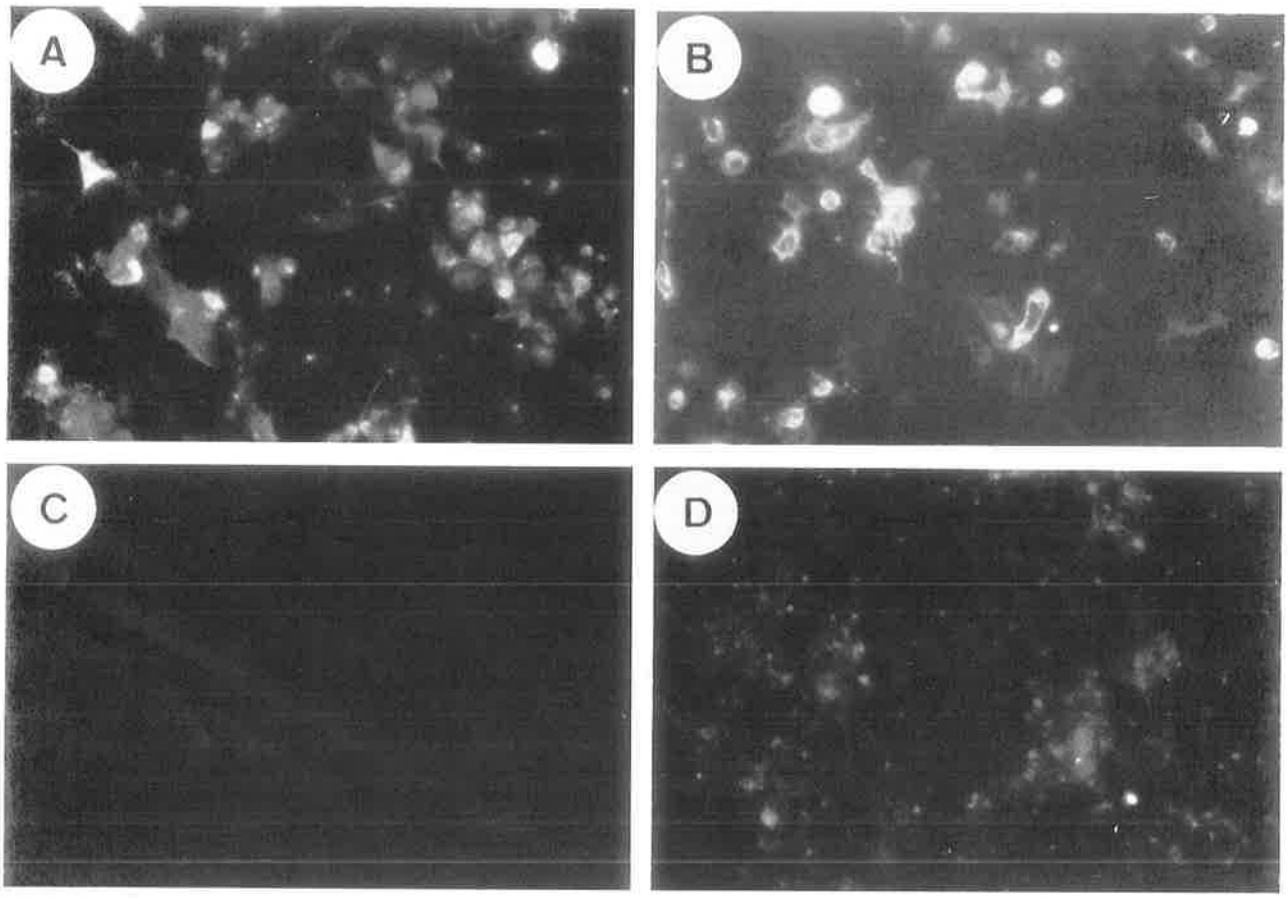


FIG. 2. Transient transfection of COS7 cells with pcDNA I–pre-S/S and pcDNA I-S plasmids. DHBV protein expression in COS7 cells was detected by indirect IMF 2 days after transfection with the respective plasmids (see Materials and Methods). Anti-pre-S 1H.1 monoclonal antibodies were used to detect pre-S protein (A) expressed by pcDNA I–pre-S/S; and rabbit-anti-DHBs antibodies were used to detect S protein (B) expressed by pcDNA I-S. Cells transfected with the parental plasmid, pcDNA I/Amp, showed negative results when reacted with anti-pre-S (C) or anti-DHBs (D) antibodies.

described previously (30), and the DHBV DNA content in each sample was analyzed by Southern blot hybridization.

RESULTS

Expression of DHBV pre-S/S and S proteins in vitro by pcDNA I–pre-S/S and pcDNA I-S plasmids. The ability of recombinant pcDNA I/Amp plasmids to express DHBV pre-S/S and S proteins was confirmed by transient transfection of COS7 cells, with the parental plasmid, pcDNA I/Amp, serving as a negative control. As shown in Fig. 2A and B, the expression of DHBsAg proteins was detected at 2 days posttransfection by indirect IMF at approximately equivalent intensity with both pcDNA I–pre-S/S and pcDNA I-S constructs, while cells transfected with the parental plasmid, pcDNA I/Amp, did not react with either antiserum (Fig. 2C and D). Western blot analysis was also performed to determine the protein species expressed by pcDNA I/Amp constructs in COS7 cells with anti-pre-S 1H.1 (28) and anti-S 1B.10 (7) monoclonal antibodies (kindly donated by J. Pugh and P. Marion, respectively). The pre-S/S protein (37 kDa) and a very small amount of S protein (17 kDa) were detected in cell lysates, but not in the culture medium, of the cells transfected with the pcDNA I–pre-S/S plasmid. In contrast, the pcDNA I-S construct expressed the S protein (17 kDa), which could be detected in both cell lysates and the culture medium, suggesting that the S protein was secreted by the cells transfected with pcDNA I-S (data not shown).

Anti-DHBs responses following DNA vaccination. Both pre-S/S and S DNA-vaccinated ducks (6 months old) elicited high titers of anti-DHBs antibodies following vaccination (Fig. 3A). The use of purified DHBsAg from serum as the antigen source in ELISA indicated that antibodies raised by DNA vaccination recognized the native form of serum-derived DHBsAg. Anti-DHBs could be detected 2 weeks after the first DNA injection, and the titers increased with subsequent vaccinations. Total anti-DHBs titers after the third vaccination ranged between 3,500 and 8,000. One duck (R23) that gave a poor antibody response was diagnosed subsequently by Congo red staining on liver tissue as suffering from secondary amyloidosis. Young (3-week-old) ducks also developed high titers of anti-DHBs antibodies following DNA vaccination (Fig. 3B). After the third vaccination, the antibody titers in pre-S/S and S DNA-vaccinated ducks ranged between 10,000 and 40,000 and between 20,000 and 50,000, respectively.

Since the above assay would detect antibodies to both pre-S and S antigens, we then determined the specific anti-S antibody responses in pre-S/S and S DNA-vaccinated young ducks by ELISA with yeast-derived S protein as the source of antigen. The specific anti-S titers in the two groups were equivalent, ranging between 2,500 and 4,000 after the third vaccination (Fig. 4).

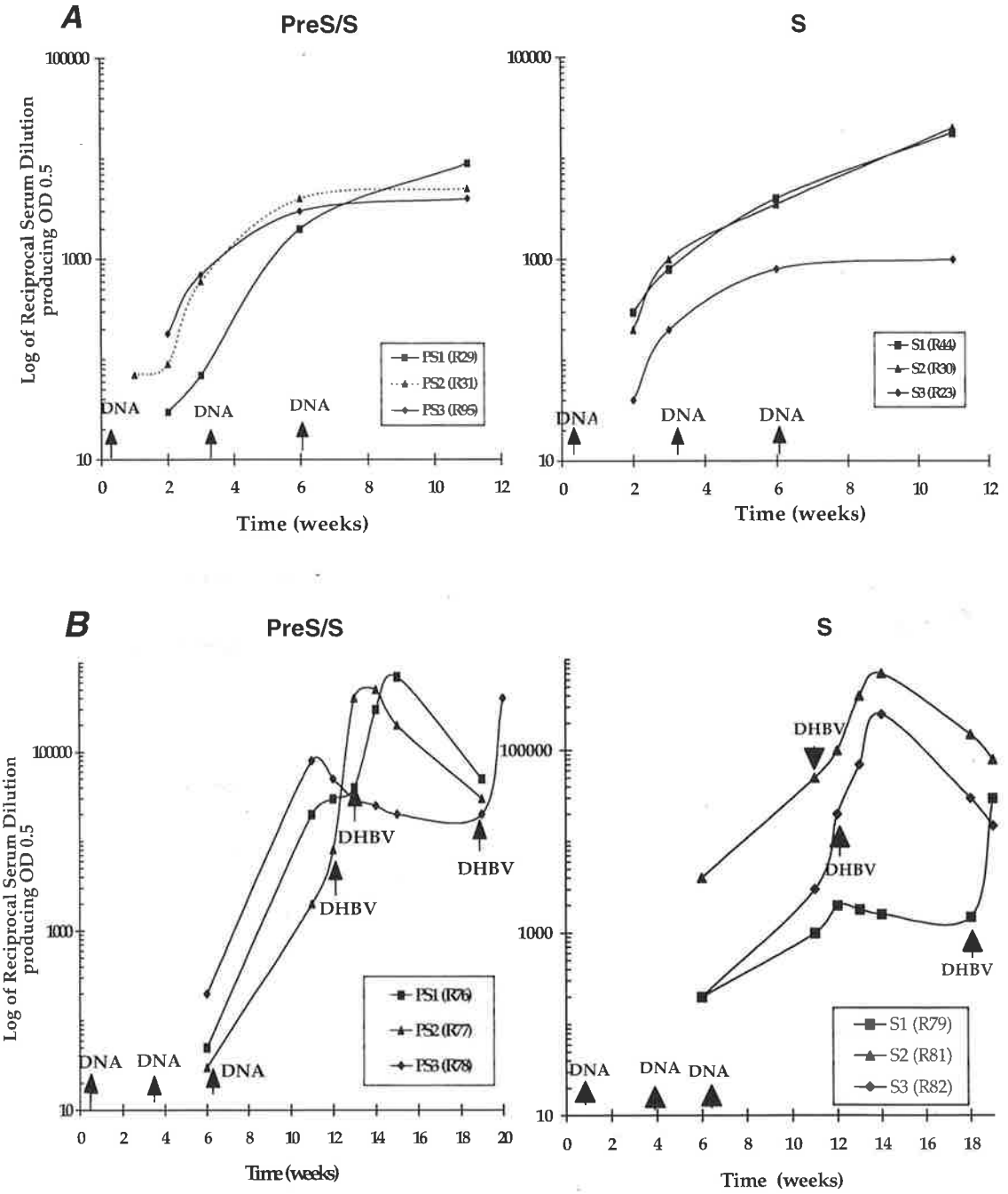


FIG. 3. Anti-DHBs antibody responses following DNA vaccination in ducks. Two groups each of 6-month-old (A) and 3-week-old (B) ducks were vaccinated with either pre-S/S or S DNA vaccines three times at 3-week intervals. Total anti-DHBs antibody levels were measured by an antibody capture ELISA with DHBsAg purified from the serum of congenitally DHBV-infected ducks as the source of antigen (see Materials and Methods). The antibody titer was defined as the highest serum dilution that gave an OD_{490} of 0.5. In 3-week-old ducks, anti-DHBs antibody responses were also measured following virus challenge (indicated by DHBV →), which was performed at various times after the third vaccination.

Removal of DHBV from the bloodstream of DNA-vaccinated ducks following virus challenge. An i.v. virus challenge was performed 3 to 8 weeks after the third vaccination in all young ducks (10 to 18 weeks old at the time of challenge). The rate of virus removal from the bloodstream was analyzed by determining the DHBV DNA content of serum samples collected from 1 min to 2 h p.c. Examples of virus removal profiles following i.v. inoculation in ducks vaccinated either with pre-S/S or S DNA are shown in Fig. 5A and B and Fig. 5C and D, respectively. The pre-S/S DNA-vaccinated ducks (R76 and R77) showed 90% removal of the inoculum after 60 and 90 min (Fig. 5A). Two other pre-S/S DNA-vaccinated ducks (R78 and W15) showed similar results, with 90% removal of the virus inoculum in 90 and 60 min (Fig. 5B). This is similar to the rate of virus removal measured in nonvaccinated ducks inoculated with an identical dose of virus (e.g., 70 min for R46), as shown in Fig. 5B. In contrast, the S DNA-vaccinated ducks (R81 and R82) showed 90% removal of the inoculum in less than 5 min

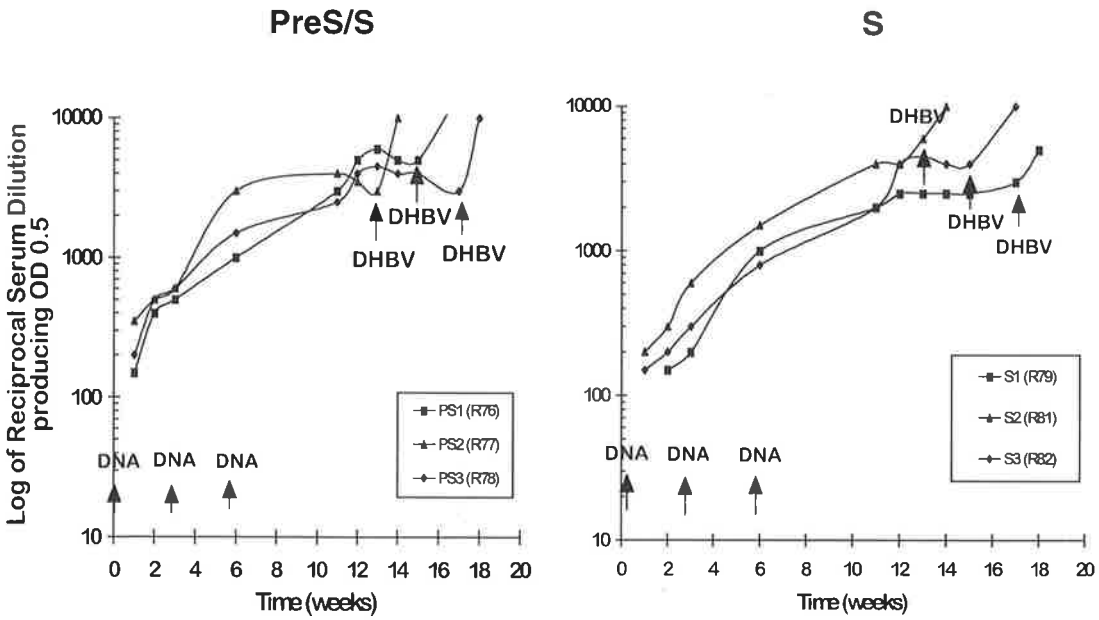


FIG. 4. Anti-S-specific responses in the pre-S/S and S DNA-vaccinated 3-week-old ducks. The same ducks as those used for the experiment in Fig. 3B were also measured for the presence of anti-S-specific antibodies in serum by ELISA with recombinant DHBV S protein (yeast derived) as the source of antigen (see Materials and Methods). The antibody titers were defined as the highest serum dilution that gave an OD_{490} of 0.5. Virus challenge (indicated by DHBV →) was performed at various times after the third vaccination.

p.c. (Fig. 5C). Two other S DNA-vaccinated ducks (W17 and R79) showed 90% removal of the inoculum in 15 and 60 min, respectively (Fig. 5D).

Detection of viral replication in liver tissue after challenge. Liver biopsies were performed 4 days p.c. to assess the extent of virus replication. DHBsAg was found in 10 to 40% of hepatocytes in the liver of pre-S/S DNA-vaccinated ducks (Fig. 6A), and significant levels of viral DNA were detected in Southern blot analysis (Fig. 7, lanes 3, 5, and 7). Nonetheless, virus infection in the liver was likely to be restricted, because viremia (determined by analysis of serum for DHBsAg and DHBV DNA) was not detected during 8 weeks of monitoring. In both pre-S/S and S DNA-vaccinated ducks, a range of mild to moderate mononuclear cell inflammation was present around the portal areas of the liver at 4 days p.c. but not prior to challenge (Fig. 6C and D). It is therefore possible that both cell-mediated and humoral immune responses played a role in preventing more widespread viral replication in these vaccinated ducks. In contrast, when nonvaccinated ducks of a similar age (4 months old) were inoculated with a similar high dose of DHBV, widespread viral infection affecting more than 95% of hepatocytes (Fig. 6E) and transient viremia were normally seen (20).

On the other hand, DHBsAg-positive hepatocytes were not detected in three of four S DNA-vaccinated ducks (Fig. 6B), while very few positive hepatocytes (~2%) were found in the fourth duck (R79) (data not shown). Duck R79 also showed a slower initial antibody response to vaccination and slower removal of challenge inoculum (45 min) than did the other ducks in this group. Southern blot analysis of viral DNA extracted from the above biopsy specimens was consistent with the above findings, with no evidence of virus replication in those S DNA-vaccinated ducks that showed rapid removal of virus from the bloodstream and low levels of virus DNA in duck R79 (Fig. 7, lanes 4, 6, and 8). The different outcome in this duck might be related to the lower antibody titer at the time of virus challenge compared to the others (Fig. 3B).

In vivo neutralization. To determine whether the protection against virus challenge seen in the vaccinated ducks was due to humoral antibody, serum from both pre-S/S and S DNA-vaccinated ducks (R76 and R81, respectively), obtained 1 week after the third DNA vaccination, was preincubated with 10^6 DHBV DNA genomes (equivalent to 10^6 ID_{50}). Based on the results of DHBV challenge of S DNA-vaccinated ducks (see above), we estimated that a total inoculum of 1.9×10^{11} DHBV DNA genomes had been neutralized in vivo in the presence of circulating antibody equivalent to 200 ml of total blood volume, i.e., 7% of body weight. Therefore, theoretically 10^6 DHBV DNA genomes might be neutralized by approximately 1 μl of serum. Preincubation of the virus inoculum with 5, 10, or, 20 μl of anti-S serum at 37°C for 1 h prior to i.v. inoculation into 1-day-old ducklings (three animals/group) completely prevented the development of viremia during a 4-week observation period in all the ducks in all the groups. In contrast, viremia developed in all the ducks receiving virus that had been preincubated with 20 or 40 μl of anti-pre-S/S serum, and in two of three ducks that received inoculum which had been preincubated with 80 μl of anti-pre-S/S serum. In the control group, all three ducklings inoculated with virus developed persistent viremia, which was monitored until 4 weeks p.i. Thus, 5 μl of anti-S antiserum neutralized virus infectivity completely under the in vivo conditions used, while with anti-pre-S/S antiserum, only partial neutralization was seen with the largest volume (80 μl) used (data not shown).

In vitro neutralization assay. PDH cultures were inoculated 1 day postplating with 30 μl of virus inoculum containing 7×10^7 DHBV DNA genomes (equivalent to 5.4×10^4 $TCID_{50}$), and the cells were examined for replicative DHBV DNA at 7 days p.i. by Southern blot hybridization. Preincubation of virus at 37°C for 1 h with either 35 or 70 μl of anti-S serum reduced

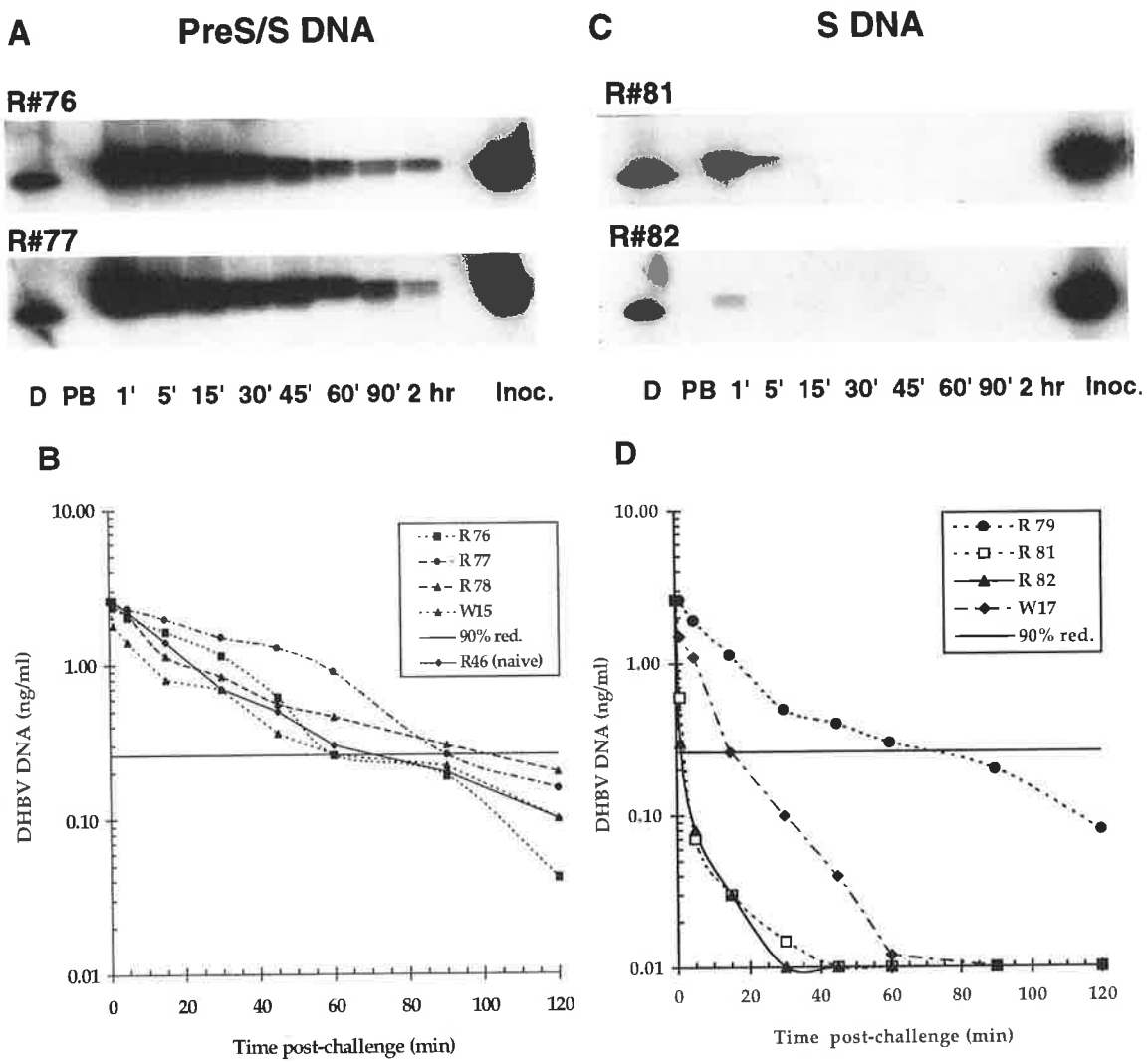


FIG. 5. Removal of DHBV from the bloodstream of DNA-vaccinated ducks following virus challenge. (A and C) Southern blot analysis of virus removal from the bloodstream of pre-S/S and S DNA-vaccinated ducks. The results for two ducks of each group (R76 and R77 for pre-S/S, and R81 and R82 for S) are shown. Serum samples were taken serially at the indicated times p.c. and were extracted for DHBV DNA as described in Materials and Methods. Lanes: D, 50 pg of DHBV DNA/pBL4.8; PB, prebleed before challenge; Inoc., 5 μl of extracted inoculum, equivalent to 260 pg of DHBV DNA. (B and D) Rate of virus removal from the bloodstream of all pre-S/S and S DNA-vaccinated ducks (four ducks per group). The rate of removal of an identical dose of virus from a nonvaccinated duck (R46) is also shown (B). A concentration of 2.6 ng of DHBV DNA/ml in the inoculum at 0 min was calculated by correction for the 10× dilution effect (20 ml/200 ml of total blood volume) occurring immediately after inoculation. The *y* axis shows the relative amount of viral DNA remaining in the bloodstream at each indicated time p.c. The DHBV DNA concentration remaining after removal of 90% of the inoculum is shown (90% red.).

the final level of intracellular DHBV DNA by 90 to 95% compared to that in the positive control (Fig. 8, lanes 2 to 4), consistent with the above calculations that 10^6 DHBV DNA genomes might have been neutralized by approximately 0.5 to 1 μl of anti-S serum in vivo. In contrast, virus infectivity was not affected by preincubation with 35 μl of anti-pre-S/S serum and was reduced only 50% after preincubation with 70 μl of anti-pre-S/S serum (lanes 5 and 6). We next wished to test whether the reduced neutralizing ability of the pre-S/S antiserum was due to a reduced neutralizing ability of the S antibody component, or inhibition of neutralization by pre-S antibody. When equal volumes of anti-S and anti-pre-S/S sera were combined (35 or 70 μl of each serum), the extent of neutralization was enhanced slightly, since the amount of DHBV DNA was reduced by 96% (lanes 7 and 8). This result demonstrated that the reduced neutralizing capacity of the anti-pre-S/S antiserum, seen consistently above, was likely to be due to an impaired neutralizing capacity of the S antibody component of this antiserum (despite equivalent ELISA titers), not to an inhibitory effect of anti-pre-S antibody in the presence of potent anti-S antibody. A summary of the findings in this study is presented in Table 1.

DISCUSSION

This study demonstrated the ability of DNA vaccines to elicit humoral immune responses against DHBV surface proteins in ducks, as has been reported previously for HBV surface proteins in mice and chimpanzees (9, 10, 12). Ducks vaccinated i.m. with pcDNA I/Amp containing either the DHBV pre-S/S or S genes produced very high titers of anti-DHBs antibodies. The strong antibody responses may have been facilitated by the use of a local anesthetic (bupivacaine HCl) to induce muscle regeneration (11, 39) and injection of 25% (wt/vol) sucrose to

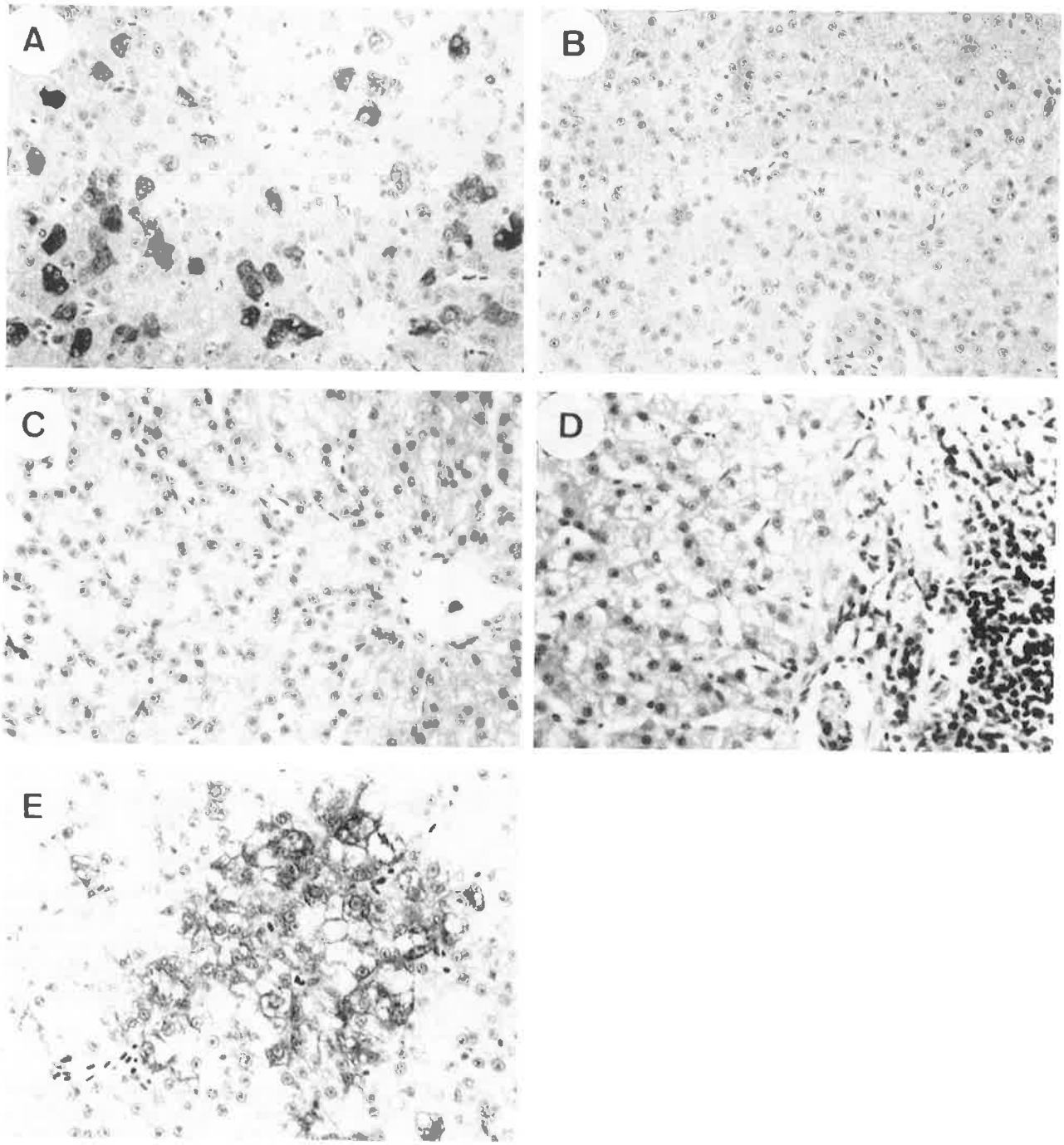


FIG. 6. (A, B, and E) Detection of DHBsAg by immunoperoxidase staining in liver tissues of pre-S/S and S DNA-vaccinated and nonvaccinated ducks 4 days p.c. DHBsAg was detected in 10 to 40% of the hepatocytes of pre-S/S (A), but not S (B) DNA-vaccinated ducks. In contrast, a nonvaccinated duck showed more widespread virus infection, with more than 95% of hepatocytes being DHBsAg positive (E). (C and D) Detection of mononuclear cell infiltrates in liver tissue of S DNA-vaccinated ducks before and after challenge. Significant mononuclear cell infiltrates were not detected in the liver tissue (represented by W17) taken 2 weeks before challenge (C) but were present in the sample taken 4 days p.c. (D). Sections were stained with hematoxylin-eosin. Magnification, ×40.

aid the even distribution of DNA uptake by muscle cells (11). In addition, the use of pcDNA I/Amp, which contains two repeats of unmethylated CpG motifs in its *ampR* gene sequence, could contribute to the strong humoral (and cellular) immune responses seen in this study. A previous study in mice has demonstrated that DNA vaccination with a vector containing CpG motifs induced significantly higher antibody and cellular immune responses against the expressed protein β-galactosidase than was seen with a vector lacking this motif (32). The CpG motif (5′-Pur Pur CG Pyr Pyr-3′) present in bacterial DNA has been shown to preferentially activate B cells that simultaneously encounter their specific antigen, and this adjuvant property has been attributed to its unmethylated status (25).

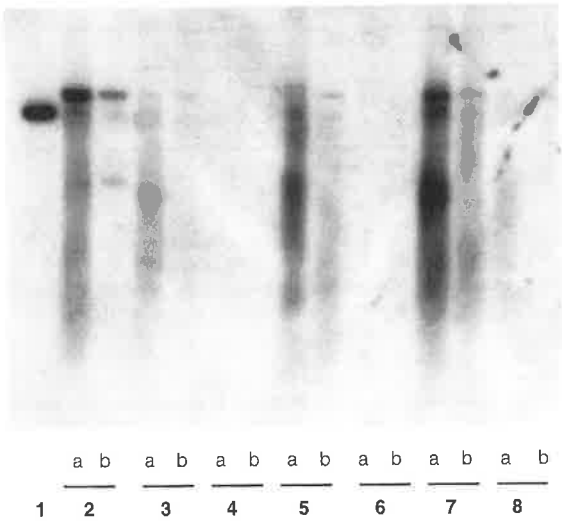


FIG. 7. Detection of viral replication (total and cccDNA) in the liver of DNA vaccinated-ducks 4 days p.c. Viral DNA was extracted from liver tissues as described in Materials and Methods and analyzed by Southern blot hybridization. Lanes: 1, 50 pg of linear DHBV DNA (pBL4.8); 2, total DNA and cccDNA of the positive control (extracted from the liver of a congenitally DHBV-infected duck); 3, 5, and 7, total DNA and cccDNA of three pre-S/S DNA-vaccinated ducks (R76, R77, and R78); 4, 6, and 8, total DNA and cccDNA of three S DNA-vaccinated ducks (R81, R82, and R79) (lanes a contain total DNA; lanes b contain cccDNA).

Although ducks from both groups (6 month old and 3 weeks old) elicited anti-DHBs responses, the titers found in young ducks were much higher (10,000 to 50,000) than those found in older ducks (3,500 to 8,000). The influence of age on the expression of plasmid DNA injected i.m. has been reported previously (38): young mice (4 to 6 weeks old) expressed significantly higher levels of the reporter gene product chloramphenicol acetyltransferase than did mice older than 10 weeks. It was proposed that this could be due to a difference in DNA uptake related to the growth rate of the animal, since mice show rapid growth from 3 to 10 weeks of age. A similar mechanism might operate with ducklings, which show rapid growth until ~4 months of age and responded better than older ducks to DNA vaccines. Alternatively, it is known that MHC class I molecules are expressed at higher levels on the surface of immature muscle fibers (14). This phenomenon might increase the presentation of DHBV envelope proteins on the surface of transfected muscle cells in the context of MHC class I. It was notable that the anti-DHBs titers found in vaccinated 6-month-old ducks were still at least three times higher than those obtained following primary DHBV infection (20).

Vaccination of ducks with either pre-S/S or S DNA vaccines prevented the development of viremia following virus challenge. However, despite the presence of approximately equal titers of anti-DHBs antibody in the two groups of vaccinated ducks at the time of challenge, significant differences were found in the rate of virus removal from the bloodstream p.c. and in the presence or absence of early virus replication in the liver. With the exception of one duck, S DNA-vaccinated ducks showed rapid removal of the inoculum from the bloodstream and showed no detectable DHBsAg or viral replication in their hepatocytes at 4 days p.c. These findings were similar to those seen after challenge of ducks that had resolved their primary infection and developed anti-DHBs (19). We also observed marked mononuclear cell infiltrates around the portal areas of the liver on day 4 p.c., which could represent virus antigen-specific T lymphocytes induced by DNA vaccination that subsequently accumulated at sites of passive uptake of challenge antigen within the liver. Potent priming of CTL responses by DNA vaccination has been demonstrated previously in mice injected i.m. with an HBsAg-expressing DNA vaccine (33).

The protection conferred by the S DNA vaccine in this study may have been due to a combined effect of humoral and cell-mediated immunity induced by the vaccine. However, humoral antibodies alone abolished virus infectivity in in vitro and in vivo neutralization assays. The precise mechanism(s) of virus neutralization in the S DNA-vaccinated ducks has yet to be determined, although several possibilities exist. First, anti-S antibodies might inhibit the attachment of virus to its specific receptor, either by direct binding to the ligand or sterically, although this mechanism alone is generally considered an inefficient process for virus neutralization (13). Alternatively, the formation of virus-antibody complexes might lead to (i) enhanced phagocytosis by macrophages and other phagocytic cells in vivo via Fc receptors or (ii) formation of aggregates by cross-linking virus particles, thus reducing their infectivity (13). The above mechanisms of virus neutralization might also require cooperative specific cellular immune responses in the in vivo system. For example, a possible role for T lymphocytes in clearing virus infection from hepatocytes and preventing cell-to-cell spread of DHBV could be inferred from the marked mononuclear cell infiltrates seen in the liver 4 days p.c.

The surprising finding from this study was that in contrast to S DNA-vaccinated ducks, all pre-S/S DNA-vaccinated ducks showed removal of the virus inoculum from the bloodstream at similar rates to nonvaccinated ducks and that virus replication was detected in 10 to 40% of hepatocytes in the liver at 4 days p.c. and was accompanied by mild to moderate inflammatory changes in the liver. However, in contrast to the nonvaccinated ducks, the vaccinated ducks did not develop transient viremia (the limit of our assay sensitivity is ~0.5 pg of DNA by spot blot hybridization). Virus replication in the vaccinated ducks was presumably restricted due to the action of cellular and/or humoral immune responses and therefore has little clinical significance. The in vivo and in vitro neutralization assays with serum from vaccinated ducks also revealed that the marked difference between the protective efficacy of the two vaccines could be ascribed in part at least to the difference in the humoral component. The reason for the reduced ability of

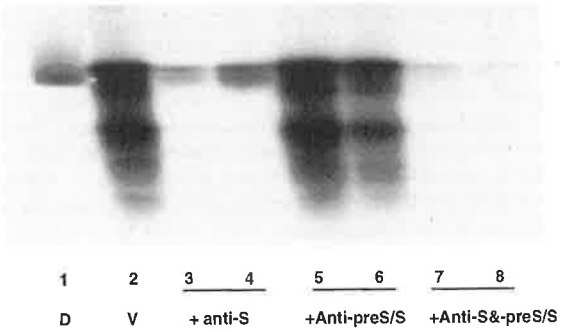


FIG. 8. In vitro neutralization assay. PDH cultures were inoculated with a virus inoculum containing 7×10^7 DHBV DNA genomes (equivalent to 5.4×10^4 $TCID_{50}$), which had been preincubated for 1 h at 37°C with 35 or 70 μl of either anti-S (lanes 3 and 4), anti-pre-S/S (lane 5 and 6), or a mixture of equal volumes of the two antisera (35 or 70 μl of each antiserum) (lanes 7 and 8). The cells were harvested 7 days p.i. and analyzed for viral replication by Southern blot hybridization. Each lane represents the total DHBV DNA extracted from individual wells of a six-well plate. Lane 1 (D) contains 50 pg of DHBV DNA; lane 2 (V) contains virus alone (5.4×10^4 $TCID_{50}$).

TABLE 1. Summary of pre-S/S- and S-DNA vaccination in 3-week-old ducks

DNA vaccine	Prechallenge			Postchallenge		
	Total anti-DHBs titer[a]	Neutralization		Virus removal rate (min)[d]	Viral replication[e]	Histological changes[f]
		In vivo[b]	In vitro[c]			
S-DNA	(2–5) × 10^4 (4/4)	3/3 neutralized by >5 μl of anti-S	90–95% reduction in viral DNA by 35 and 70 μl of anti-S	<5–15 (3/4)	Not detected (3/4)	Mild (3/4)
				45 (1/4)	~2% hepatocytes (1/4)	Marked (1/4)
Pre-S/S-DNA	(1–4) × 10^4 (4/4)	1/3 neutralized by 80 μl of anti-preS/S, no neutralization by 20 and 40 μl of serum	50% reduction in viral DNA by 70 μl of anti-pre-S/S; no reduction by 35 μl	45–90 (4/4)	10–40% hepatocytes (4/4)	Mild to moderate (4/4)

[a] The range of total anti-DHBs antibody titers as measured by ELISA after the third vaccination from four ducks/group. Numbers in parentheses show the number of ducks.

[b] Neutralizing ability of serum from vaccinated ducks when preincubated with 10^6 DHBV DNA genomes (10^6 ID_{50}) prior to inoculation into 1-day-old ducklings (three animals/group). Different volumes of neat anti-S (5, 10, and 20 μl) or anti-pre-S/S (20, 40, and 80 μl) sera from ducks R81 & R76, respectively, were used.

[c] Neutralizing ability of serum when preincubated with 7×10^7 DHBV DNA genomes prior to inoculation of PDH cultures.

[d] The time taken for removal of 90% of virus inoculum from the bloodstream PC as determined by Southern blot hybridization. Numbers in parentheses show the number of ducks.

[e] Viral replication in the liver 4 days p.c., determined by the presence of total DNA and cccDNA, as well as by the presence of viral antigen (DHBsAg)-positive hepatocytes. Numbers in parentheses show the number of ducks.

[f] Histological changes seen in the liver tissue samples taken 4 days p.c., in relation to the presence of periportal necrosis, intralobular degeneration and focal necrosis, portal inflammation, and fibrosis (23). Numbers in parentheses show the number of ducks.

anti-pre-S/S antibodies to neutralize virus infectivity is unknown. Anti-S and anti-pre-S/S sera contained equivalent levels of anti-S antibody in an S-antigen-specific ELISA, but the antiserum mixing experiment demonstrated that reduced efficiency of the pre-S/S antiserum was likely to be due to an impaired function of its anti-S component rather than to inhibition by the anti-pre-S component. One possibility could be related to the nature of the intracellular DHBV pre-S/S protein expressed in the transfected muscle cells. We found that unlike the S protein, pre-S/S protein was detected only intracellularly when expressed in COS7 cells transfected with pcDNA I–pre-S/S plasmid (data not shown). This was similar to earlier reports with HBV that, unlike the major (S) envelope protein, the middle (pre-S2/S) protein was not secreted in a number of expression systems and, in fact, inhibited the expression and/or secretion of S protein (8, 9, 27). If impaired secretion is also a feature of pre-S/S protein expression in muscle in vivo, this may have affected the correct conformation of the antigens produced and subsequently the biological function of the anti-S antibodies induced. The immunogenicity of DHBV S protein has been reported previously to be conformation dependent (41). Hence, the specific anti-S antibodies raised in the pre-S/S DNA-vaccinated ducks might be conformationally different from those produced following primary DHBV infection or in S DNA-vaccinated ducks. Thus, although both pre-S/S and S antisera reacted equally with yeast-derived S protein in ELISA, some differences in biological function between the anti-S-specific antibodies produced in the pre-S/S and S DNA-vaccinated ducks might have occurred.

In summary, the results described here demonstrate the importance of anti-S antibodies alone in preventing DHBV infection in both in vitro and in vivo systems, consistent with the well-established role for anti-HBs in HBV infection in humans (15, 16, 37). The markedly poorer protection conferred by pre-S/S vaccination, despite the presence of apparently comparable levels of anti-S antibodies by ELISA, needs further clarification, particularly in the context of the development of human HBV vaccines containing pre-S proteins where serological responses can be readily monitored but protective efficacy data are difficult to obtain. Finally, this study demonstrated the advantage of DNA vaccines as a tool to explore the role of immune responses against hepadnavirus infection, thus permitting further studies on the detailed mechanism(s) of hepadnavirus neutralization in vivo and characterization of alternative vaccine strategies.

ACKNOWLEDGMENTS

We thank E. Bertram for providing rabbit anti-duck IgY and for assisting in the challenge experiments, C. Scougall for preparing PDH cultures, G. Mayrhofer for helpful discussions, and P. Marion for the gift of 1B.10 monoclonal antibodies. We also thank the staff of the Veterinary Services Branch, IMVS, for animal care; the Division of Tissue Pathology, IMVS, for section preparation; and Photography Services, IMVS, for assistance in preparation of the figures. We are particularly indebted to J. Pugh for the gift of the 1H.1 monoclonal antibodies.

This research was supported by a project grant from the National Health and Medical Research Council of Australia and a postgraduate fellowship (to M.T.) from the Australian Government (AusAID).

REFERENCES

1. **Ashton-Rickardt, P. G., and K. Murray.** 1989. Mutants of the hepatitis B virus surface antigen that define some antigenically essential residues in the immunodominant *a* region. J. Med. Virol. **29:**196–203.
2. **Bertram, E. M.** 1997. Characterisation of duck lymphoid cell populations and their role in immunity to duck hepatitis B virus. Ph.D. thesis. University of Adelaide, Adelaide, Australia.
3. **Carman, W. F., A. R. Zanetti, P. Karayiannis, J. Waters, G. Manzillo, E. Tanzi, A. J. Zuckerman, and H. C. Thomas.** 1990. Vaccine-induced escape mutant of hepatitis B virus. Lancet **336:**325–329.
4. **Chassot, S., V. Lambert, A. Kay, C. Godinot, B. Roux, C. Trepo, and L. Cova.** 1993. Fine mapping of neutralization epitopes on duck hepatitis B virus (DHBV) Pre-S protein using monoclonal antibodies and overlapping peptides. Virology **192:**217–223.
5. **Chassot, S., V. Lambert, A. Kay, C. Godinot, C. Trepo, and L. Cova.** 1994. Identification of major antigenic domains of duck hepatitis B virus Pre-S protein by peptide scanning. Virology **200:**72–78.
6. **Cheung, R. C., W. S. Robinson, P. L. Marion, and H. B. Greenberg.** 1989. Epitope mapping of neutralizing monoclonal antibodies against duck hepatitis B virus. J. Virol. **63:**2445–2451.
7. **Cheung, R. C., D. E. Trujillo, W. S. Robinson, H. B. Greenberg, and P. L. Marion.** 1990. Epitope-specific antibody response to the surface antigen of duck hepatitis B virus in infected ducks. Virology **176:**546–552.
8. **Chisari, F. V., P. Filippi, A. McLachlan, D. R. Milich, M. Riggs, S. Lee, R. D. Palmiter, C. A. Pinkert, and R. L. Brinster.** 1986. Expression of hepatitis B

virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. J. Virol. **60**:880–887.
9. **Chow, Y.-H., W.-L. Huang, W.-K. Chi, Y.-D. Chu, and M.-H. Tao.** 1997. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. J. Virol. **71**:169–178.
10. **Davis, H. L., M. L. Michel, and R. G. Whalen.** 1993. DNA-based immunization for hepatitis B induces continuous secretion of antigen and high levels of circulating antibody. Hum. Mol. Genet. **2**:1847–1851.
11. **Davis, H. L., R. G. Whalen, and B. A. Demeneix.** 1993. Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression. Hum. Gene Ther. **4**:151–159.
12. **Davis, H. L., M. J. McCluskie, J. L. Gerin, and R. H. Purcell.** 1996. DNA vaccine for hepatitis B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. Proc. Natl. Acad. Sci. USA **93**:7213–7218.
13. **Dimmock, N. J.** 1993. Neutralization of animal viruses. Curr. Top. Microbiol. Immunol. **183**:3–31.
14. **Engel, A. G., and R. Hohlfeld.** 1994. Immunobiology of muscle tissue. Immunol. Today **15**:269–274.
15. **Hadler, S. C., D. P. Francis, J. E. Maynard, S. E. Thompson, F. N. Judson, et al.** 1986. Long-term immunogenicity and efficacy of hepatitis B vaccine in homosexual men. N. Engl. J. Med. **315**:209–214.
16. **Iwarson, S., E. Tabor, H. C. Thomas, A. Goodall, J. Waters, P. Snoy, J. W. Shih, and R. J. Gerety.** 1985. Neutralization of hepatitis B virus infectivity by a murine monoclonal antibody: an experimental study in the chimpanzee. J. Med. Virol. **16**:89–95.
17. **Jilbert, A. R., T.-T. Wu, J. M. England, P. Hall, N. D. Carp, A. P. O'Connell, and W. S. Mason.** 1992. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvement. J. Virol. **66**:1377–1388.
18. **Jilbert, A. R., D. S. Miller, C. A. Scougall, H. Turnbull, and C. J. Burrell.** 1996. Kinetics of duck hepatitis B virus infection following low dose virus inoculation: one virus DNA genome is infectious in neonatal ducks. Virology **226**:338–345.
19. **Jilbert, A. R., D. S. Miller, E. M. Bertram, L. Mickan, I. Kotlarski, P. Hall, and C. J. Burrell.** Unpublished data.
20. **Jilbert, A. R., D. S. Miller, J. D. Botten, E. M. Bertram, P. Hall, I. Kotlarski, and C. J. Burrell.** Characterisation of age and dose-related outcomes of duck hepatitis B virus infection. Submitted for publication.
21. **Kane, M. A.** 1996. Global status of hepatitis B immunisation. Lancet **348**:696.
22. **Klingmüller, U., and H. Schaller.** 1993. Hepadnavirus infection requires interaction between the viral pre-S domain and a specific hepatocellular receptor. J. Virol. 67:7414–7422.
23. **Knodell, R. G., K. G. Ishak, W. C. Black, T. S. Chen, R. Craig, N. Kaplowitz, T. W. Kiernan, and J. Wollman.** 1981. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology **1**:431–435.
24. **Kok, T. W., L. E. Payne, S. E. Bailey, and R. G. Waddell.** 1993. Urine and the laboratory diagnosis of *Chlamydia trachomatis* in males. Genitourin. Med. **69**:51–53.
25. **Krieg, A. M., A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman.** 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature **374**:546–549.
26. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. **36**:829–836.
27. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1986. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science **234**:1388–1391.
28. **Pugh, J. C., Q. Di, W. S. Mason, and H. Simmons.** 1995. Susceptibility to duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles. J. Virol. **69**:4814–4822.
29. **Qiao, M., E. J. Gowans, S. E. Bailey, A. R. Jilbert, and C. J. Burrell.** 1990. Serological analysis of duck hepatitis B virus infection. Virus Res. **17**:3–14.
30. **Qiao, M., C. A. Scougall, A. Duszynski, and C. J. Burrell.** Unpublished data.
31. **Richardson, H. E., C. Wittenberg, F. Cross, and S. I. Reed.** 1989. An essential G1 function for cyclin-like proteins in yeast. Cell **59**:1127–1133.
32. **Sato, Y., M. Roman, H. Tighe, D. Lee, M. Corr, M.-D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz.** 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science **273**:352–354.
33. **Schirmbeck, R., W. Böhm, K. Ando, F. V. Chisari, and J. Reimann.** 1995. Nucleic acid vaccination primes hepatitis B surface antigen-specific cytotoxic T lymphocytes in nonresponder mice. J. Virol. **69**:5929–5934.
34. **Triyatni, M., A. R. Jilbert, M. Qiao, and C. J. Burrell.** Unpublished data.
35. **Tuttleman, J. S., J. C. Pugh, and J. W. Summers.** 1986. In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. J. Virol. **58**:17–25.
36. **Uchida, M., M. Esumi, and T. Shikata.** 1989. Molecular cloning and sequence analysis of duck hepatitis B virus genomes of a new variant isolated from Shanghai ducks. Virology **173**:600–606.
37. **Waters, J. A., S. M. O'Rourke, S. C. Richardson, G. Papaevangelou, and H. C. Thomas.** 1987. Qualitative analysis of the humoral immune response to the '*a*' determinant of HBs antigen after inoculation with plasma-derived or recombinant vaccine. J. Med. Virol. **21**:155–160.
38. **Wells, D. J., and G. Goldspink.** 1992. Age and sex influence expression of plasmid DNA directly injected into mouse skeletal muscle. FEBS Lett. **306**:203–205.
39. **Wells, D. J.** 1993. Improved gene transfer by direct plasmid injection associated with regeneration in mouse skeletal muscle. FEBS Lett. **332**:179–182.
40. **Yap, I., and S. H. Chan.** 1996. A new pre-S containing recombinant hepatitis B vaccine and its effect on non-responders: a preliminary observation. Ann. Acad. Med. Singapore **25**:120–122.
41. **Yokosuka, O., M. Omata, and Y. Ito.** 1988. Expression of pre-S1, pre-S2, and C proteins in duck hepatitis B virus infection. Virology **167**:82–86.