THE UNIVERSITY
OF ADELAIDE
AUSTRALIA

Detection and Analysis of Residual Duck Hepatitis B Virus DNA

Marc Le Mire
B.M., B.S.

School of Molecular and Biomedical Science
The University of Adelaide
South Australia

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

April 2003

Abstract

Hepatitis B virus (HBV) is estimated to have infected approximately 2 billion people worldwide, 350 million of these remain persistently infected, most of whom acquired infection in infancy or early childhood. Infection acquired in adulthood is usually cleared with the development of neutralising anti-surface antibodies and immunity to reinfection. Despite this apparent recovery from infection, residual HBV DNA can be detected by PCR in serum and liver, months or years later. Cytotoxic T-cell responses also persist and reactivation of infection can occur following immunosuppression or after liver transplantation.

This study of the persistence of hepadnaviral DNA used duck hepatitis B virus (DHBV) infection of Pekin ducks as a model for HBV infection of humans. The project aims were to determine whether DHBV DNA persists following transient infection, to study the effect of inoculum dose on levels of residual DNA and to identify the sites and possible mechanism of residual DHBV DNA persistence.

Polymerase chain reaction (PCR) assays were developed to detect and quantify traces of residual DHBV DNA. The first was a nested PCR targeting part of the DHBV core open reading frame (ORF) with a sensitivity of 1 copy of DHBV DNA/350000 cells). The second was a quantitative real time PCR targeting a region of the polymerase ORF with a sensitivity of 10 copies/80000 cells. This assay was found to have an intra-assay coefficient of variation of 10% and an inter-assay coefficient of variation of 21% when measuring a sample containing ~322 copies of template.

The third assay was a selective quantitative PCR for detection of DHBV covalently closed circular DNA (cccDNA), using primers spanning the "direct repeat" (DR) region in the DHBV genome. CccDNA is the transcriptional template of the virus that is formed in infected cells from the relaxed circular DNA (RC DNA) genome. The DR region of the genome is continuous in cccDNA, but contains a nick and gap in RC DNA, a difference that can be exploited to enable selective amplification of hepadnavirus cccDNA. The quantitative PCR assay enabled PCR detection of as little as 10 copies of cccDNA but required 10^4 copies of RC DNA to produce detectable product. This assay was found to have a sensitivity of 10 copies/80000 cells, an intra-assay coefficient of variation of 15.5% and an inter-assay coefficient of variation of 24.6% when measuring a sample containing ~1787 copies.

In each of these assays a head-to-tail dimer of DHBV DNA from an Australian strain of DHBV (AusDHBV) cloned into pBluescript IIKS+, was used to prepare DNA standards for the PCR reaction. This head-to-tail dimer was constructed using a monomeric clone of AusDHBV and was shown to be infectious when inoculated intravenously into 2-day-old ducklings (Triyatni *et al.* 2001).

Ducks with high-level congenital and experimental DHBV infection were studied to confirm the distribution of DHBV-infected cells and to compare the levels of DHBV DNA detected using the quantitative PCR assays with levels and forms of viral DNA detected by Southern blot hybridization. DHBV DNA was found in a greater range of tissues by PCR than by Southern Blot hybridization or immunohistochemical detection of viral antigens. This is consistent with the greater sensitivity of PCR assays. However, in widespread high-level infection quantitation of DHBV DNA by PCR showed similar levels to those measured by Southern Blot hybridization.

To set up transient infection, 3 groups (A, B, C) of five 6-week-old ducks were inoculated intravenously with 10^7, 10^8 or 10^{10} DHBV virions. Eleven of the 15 ducks had a transient infection and were monitored throughout the course of infection until autopsy approximately 8 months later. The percentage of DHBsAg-positive cells detected in liver at day 3-4 post-inoculation (p.i.) correlated with the inoculum dose. At day 31 p.i. DHBsAg was no longer detectable in liver and anti-surface antibodies were detectable in the serum. However, approximately 8 months later DHBV DNA was still detected by nested PCR in autopsy liver samples from 10 out of the 11 ducks. Using the quantitative PCR, DHBV DNA was found in autopsy liver samples from 1/4 Group A ducks, 3/4 Group B ducks and 3/3 Group C ducks, with highest levels in Group C (190-1260 copies per 8×10^4 cells).

DHBV DNA was also detected in 7 spleen, 2 kidney, 1 heart and 1 adrenal tissue samples from the total group of 11 ducks, with autopsy spleen samples from Group C ducks showing 20-70 copies of DHBV DNA per 8×10^4 cells. Peripheral blood mononuclear cells (PBMC) and skeletal muscle had undetectable levels of DHBV DNA.

Using the quantitative PCR for DHBV cccDNA, it was found that 42-89% of residual DHBV DNA detected in autopsy liver of Group C ducks was cccDNA. The relative depletion of RC DNA compared to acutely infected liver is consistent with suppression of DHBV replication, possibly by immune mechanisms. However, an attempt to confirm this by administration of immunosuppressive drugs to a similar group of recovered ducks did not lead to an increase in levels of residual viral DNA as measured by quantitative PCR.

Southern blot hybridization confirmed the presence of cccDNA and showed a band migrating as cccDNA in most of the liver samples tested. In addition, amplification of full-length DHBV genomes with primers designed to amplify cccDNA indicated that residual cccDNA was indistinguishable in size from wild-type. Sequencing of the DR region showed only 1 nucleotide substitution (without a predicted amino acid change) in the (503 bp) region sequenced.

Infectivity of the residual DHBV DNA could not be demonstrated despite trying a number of strategies including intraperitoneal (i.p.) and intravenous inoculation (i.v.) of homogenates of fresh and frozen liver samples and inoculation of cultured spleen cells and supernatant into newly hatched ducks. The demonstrated transmission of HBV infection following liver transplantation, and woodchuck hepatitis virus (WHV) infection by inoculation of cultured PBMC from recovered woodchucks, may reflect different conditions from those in this study. It is possible that removal of infected cells from a setting of immune control might allow increased replication and release of infectious virions.

To establish whether viral replication was required for maintenance of residual DHBV DNA, comparison was made between the observed rate of decline of levels of residual DHBV DNA within the liver and the predicted rate according to a computational model assuming an absence of viral replication and no new production of cccDNA. Liver cell turnover was measured by incorporation of bromodeoxyuridine (BrdU), and the model allowed for loss of cccDNA with cell death, but maintenance of cccDNA with cell division.

Daily cell turnover was measured by BrdU labelling as 14% in 6-week-old ducks, 3 days p.i., and 2% in 8-month-old ducks, 6 months p.i.. DHBV cccDNA was measured as 0.048+/-0.018 copies per liver cell by Southern blot hybridization at 3 days p.i.. Assuming 14% daily cell turnover, DHBV cccDNA was predicted to decline from 0.05 to 0.0007 copies per cell from day 3 to 31 p.i.. Assuming a daily turnover of 2% for the next 8 months, cccDNA was predicted to decline to 0.00001 copies per cell at 9 months p.i.. This is below the threshold for detection with the quantitative PCR (~0.000125 copies/liver cell). Samples of liver were collected at 3 days and 1, 3, 6 and 9 months p.i. and cccDNA was measured by Southern blot hybridization and/or quantitative PCR. The observed levels of cccDNA declined more slowly than predicted and were maintained at 0.004+/-0.006 copies per cell from 3-9 months p.i.. This finding is consistent with the hypothesis that virus replication is required for persistence of residual hepadnavirus, but does not exclude the possibility that DHBV DNA persists in a subset of long-lived liver cells. Further studies are needed to distinguish between these hypotheses.

Future studies will focus on the events around the time of recovery from transient DHBV infection to examine the mechanisms of clearance and the reason why clearance is incomplete. The state of residual hepadnavirus infection will also be studied, in particular, the change over time in residual DHBV DNA levels, evidence of replication and the cell type in which DHBV DNA persists. It is hoped that a better understanding of residual hepadnavirus infection may assist with the design of improved strategies for the treatment of HBV infection.

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 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 of Adelaide Library being made available for loan and photocopying.

3/4/2003

Marc Le Mire Date.....

Publications and presentations arising from the research presented in this thesis

Publications:

Triyatni M, Ey P, Tran T, Le Mire MF, Qiao M, Burrell CJ, Jilbert AR (2001). Sequence comparison of an Australian duck hepatitis B virus strain with other avian hepadnaviruses. *J Gen Virol* 82: 373-8.

Le Mire MF, Miller DS, Foster W, Burrell CJ, Litwin S, Jilbert AR (2001). Persistence of duck hepatitis B Virus DNA after transient infection. *J Gastroenterology Hepatology* 16 (Supplement): A78.

Le Mire MF, Miller DS, Foster W, Burrell CJ, Litwin S, Jilbert AR (2002) Analysis of residual duck hepatitis B virus DNA after transient infection. *Hepatology* 36 (4, Pt 2): 369a.

Oral presentations:

Le Mire MF, Miller DS, Jilbert AR, Burrell CJ. Persistence of duck hepatitis B virus DNA after transient infection. Australian Centre for Hepatitis Virology Inc Annual Scientific Meeting, Sydney, 31st March - 2nd April 2000.

Le Mire MF, Miller DS, Burrell CJ Jilbert AR. Persistence of duck hepatitis B virus DNA after transient infection. Australian Centre for Hepatitis Virology Inc Annual Scientific Meeting, Sydney, 30th Mar - 1st April 2001.

Le Mire MF, Miller DS, Burrell CJ Jilbert AR. Persistence of duck hepatitis B virus DNA after transient infection. International Meeting on the Molecular Biology of the Hepatitis B Viruses, Amherst, MA, USA, 29th July - 2nd August 2001.

Le Mire MF, Miller DS, Foster W, Burrell CJ, Litwin S, Jilbert AR. Persistence of duck hepatitis B virus DNA after transient infection. Australian Centre for Hepatitis Virology Inc Annual Scientific Meeting, Alfred Hospital Melbourne, 23rd March 2002.

Le Mire MF, Miller DS, Foster W, Burrell CJ, Litwin S, Jilbert AR. Residual duck hepatitis B virus (DHBV) infection. International Meeting on the Molecular Biology of the hepatitis B viruses, Asilomar, California, USA, 29th September - 3rd October 2002.

Poster presentations:

Le Mire MF, Miller DS, Burrell CJ Jilbert AR. Persistence of Duck Hepatitis B Virus DNA after Transient Infection. International Congress of Virology Meeting, Paris, 28th July – 3rd August 2002.

Le Mire MF, Miller DS, Foster W, Burrell CJ, Litwin S, Jilbert AR. Analysis of residual duck hepatitis B virus (DHBV) DNA after transient Infection. AASLD Meeting Boston, 1st – 5th November, 2002.

Manuscript in Preparation:

Le Mire MF, Miller DS, Foster W, Burrell CJ, Litwin S, Jilbert AR (2002) Residual duck hepatitis B virus DNA after transient infection. In preparation.

Acknowledgements

This research project was carried out in the Department of Microbiology and Immunology and subsequently in the Department of Molecular Biosciences at the University of Adelaide and the Institute of Medical and Veterinary Science (IMVS). I wish to express my gratitude to the staff and students of both institutions who helped with this work.

I wish to thank my supervisors Dr Allison Jilbert and Prof Christopher Burrell for their enthusiasm, guidance, support and friendship throughout the project. I thank the many other members of the Hepatitis Research Laboratory, including Darren Miller, Wendy Foster and Cathy Scougall for their help and companionship.

I also appreciate the help of Shaun McColl, Nick Coates and the members of the Chemokine Laboratory in providing PCR facilities, and the advice and company of Ieva Kotlarski, Renato Morona, Jamie Botten, Tony Richardson, John Mackrill, Ros Hammond, Gary Penney, Gail Bezuidenhoudt and Chris Cursaro amongst many in the Microbiology discipline.

Thanks are also due to staff of the IMVS photo and imaging service, tissue pathology, HIV and Infectious Diseases Laboratories and Veterinary Services. In particular, thanks to Dr Tuckweng Kok for help with quantitative PCR facilities. Elsewhere I received the generous help of Drs Scott Bowden and Margaret Littlejohn at VIDRL and Andreas Nitsche at Tib Molbiol with real time PCR.

I am grateful for a scholarship from the University of Adelaide (1999), an NHMRC medical post-graduate scholarship (2000-1) and for a travelling fellowship from the Medical School of the University of Adelaide.

Finally, I wish to thank Louise, my wife, for her patience and support for the duration of this project, Eliza, for making us both laugh and my parents for being there whenever help was needed.

List of Abbreviations

AA	amino acids
ALT	alanine transaminase
Anti-DHBc	anti-duck hepatitis B virus core
Anti-DHBs	anti-duck hepatitis B virus surface
Anti-HBc	anti-hepatitis B virus core
Anti-HBe	anti-hepatitis B virus e
Anti-HBs	anti-hepatitis B virus surface
APC	antigen presenting cells
AST	aspartate transaminase
AusDHBV	Australian strain of duck hepatitis B virus
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cccDNA	covalently closed circular deoxyribonucleic acid
cm	centimetre
CM	culture medium
CPM	counts per minute
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
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine-5'-triphosphate
DR	direct repeat
DS-DNA	double-stranded DNA
DSL-DNA	double-stranded linear DNA
DW	deionized water
EAA	ethanol acetic acid (3:1)

E. coli	*Escherichia coli*
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
H&E	haematoxylin and eosin
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBSS	Hanks balanced salt solution
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
hr	hour
HRP	horseradish peroxidase
HSV	herpes simplex virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
kb	kilo base (pair)
kD	kilodalton
kg	kilogram
LB	Luria Bertani
MHC	major histocompatibility complex
µCi	microcurie
µg	micrograms
µl	microlitres
µM	micromolar
mA	milliamps
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger RNA

NDS	normal duck serum
NDL	normal duck liver
NK	natural killer (cells)
nm	nanometres
NRS	normal rabbit serum
NSS	normal sheep serum
nt	nucleotide
OD	optical density
OPD	*o*-phenylenediamine
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-T	PBS with 0.05% Tween 20
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
p.i.	post-inoculation
polyA	polyadenylated
PVP	polyvinyl pyrolodone
rDHBcAg	recombinant DHBcAg
RI	replicative intermediates
RT	room temperature
RC DNA	relaxed circular DNA
RNA	ribonucleic acid
rpm	revolution per minute
SAP	shrimp alkaline phosphatase
SD	standard deviation
SDS	sodium dodecyl sulphate
SSC	0.15 M NaCl, 0.15 M Na_3Citrate pH 7.0
sec	second(s)
SS-DNA	single-stranded DNA
TAE	Tris acetate EDTA buffer
TE8	Tris-HCl EDTA buffer pH8
Th1	T helper 1
Th2	T helper 2
TNF-α	tumour necrosis factor alpha

Tris	3,3,5,5-tetramethylbenzidine
WHO	World Health Organisation
WHV	woodchuck hepatitis virus
v/v	volume per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Table of Contents

1. Introduction

1.1 Historical Background

"Serum hepatitis" following vaccination and administration of contaminated blood products has been recognised since the 1930s and 1940s. However, an earlier report of an epidemic of jaundice following smallpox vaccination in Bremen dates back to 1883 and is now considered the earliest known description of hepatitis virus infection (Robinson 1990).

The finding of an antigen in serum, "Australia antigen," now termed hepatitis B surface antigen (HBsAg) was the first step in identifying an agent responsible for serum hepatitis (Blumberg *et al.* 1967). Subsequently, in 1970, Dane described a particle now known to be the infectious virion (Dane *et al.* 1970). This agent is now named the human hepatitis B virus (HBV) and represents the prototype of the Hepadnavirus family, named so because of its **hepa**totropic nature and **DNA** genome. The structure, genome and unique replicative strategy have since been described and an effective vaccine developed. This is despite the lack of a convenient animal model or tissue culture method for studying human HBV infection.

1.2 Epidemiology

The problems posed by HBV infection remain considerable despite advances in our knowledge. Estimates of prevalence are that 350 million people are persistently infected and 2 billion have had transient HBV infection (WHO 2001). Morbidity and mortality are considerable, exemplified by the approximately 1 million cases of hepatocellular carcinoma (HCC) occurring annually, a significant proportion of which are thought to be HBV-related (WHO 2001).

Regional prevalence rates of persistent HBV infection vary enormously with 5-15% of the population in parts of China, South-East Asia and sub-Saharan Africa infected. This compares with 1-5% in Eastern Europe, the Mediterranean and the Middle East and up to 1% in areas of low prevalence such as North America, most of Western Europe and Australia. The prevalence rate of current HBV infection in the USA between 1988 and 1994 was reported to be 0.42% and the rate of either past or current infection over the same period 4.9% (McQuillan 1999). In Australia the rate of current HBV infection has been estimated at 1% with the highest rates being seen in Aborigines and Torres Strait Islanders (Gust 1992).

Transmission of HBV occurs through several mechanisms. Perinatal infection is acquired at or shortly after delivery in babies born to persistently HBV-infected mothers. *In utero* infection is not the usual means of transmission as shown by the lack of surface antigenaemia for 1-3 months post-partum (White and Fenner 1994). Perinatal transmission of HBV infection can therefore be prevented in a majority of cases by immunisation of neonates. Important modes of transmission during the early childhood years include intrafamilial spread by contaminated body fluids such as blood, oozing fluid from minor wounds and saliva. In adolescents and adults sexual transmission, injecting drug use and occupational exposure to body fluids are important routes of transmission (Hollinger 1990). In high prevalence regions perinatal infection is the primary means of transmission and horizontal transmission during childhood is less common. In low prevalence areas sexual transmission between adults is the most important route.

1.3 Family Hepadnaviridae

The hepadnavirus family is divided into two genera - orthohepadnaviruses, which infect some mammals, and avihepadnaviruses, which infect certain avian species (see Table 1-1). Following the discovery of HBV, several viruses with features similar to HBV were found in animals (Summers *et al.* 1978; Mason *et al.* 1980). The distinguishing features of hepadnavirus family include high levels of viral replication in liver and the presence of a double-stranded DNA genome. Hepadnaviruses contain a DNA polymerase (P) which demonstrates reverse transcriptase activity and they possess a lipid envelope within which is a nucleocapsid core containing the genome and polymerase. Another common feature is the secretion of greater numbers of non-infectious HBsAg-containing particles than virions (Dane *et al.* 1970; Summers *et al.* 1978; Mason *et al.* 1980).

Table 1-1: The Hepadnavirus Family

Orthohepadnaviruses	*Avihepadnaviruses*
Hepatitis B virus (HBV)	Duck hepatitis B virus (DHBV)
Woodchuck hepatitis virus (WHV)	Heron hepatitis B virus (HHBV)
Ground squirrel hepatitis virus (GSHV)	Ross goose hepatitis B virus (RGHBV)
Arctic squirrel hepatitis virus (ASHV)	Snow goose hepatitis B virus (SGHBV)
Wooly monkey hepatitis B virus (WMHBV)	

1.4 Hepatitis B Virus Structure and Genome Organisation

HBV virions are 42 nm in diameter. They have a 7 nm thick envelope consisting of a lipid membrane into which are embedded copies of the 3 HBV surface proteins (see below). Within is a 28 nm icosahedral nucleocapsid consisting of 180 or 240 subunits of core

protein (Wingfield *et al.* 1995; Bottcher *et al.* 1997; Conway *et al.* 1997). The HBV genome consists of a partially double-stranded DNA molecule of 3182-3221 bp. The genome has a short discontinuity in the minus strand and lacks 15-50% of the plus strand. It is held in relaxed circular (RC) conformation and packaged with viral polymerase in the nucleocapsid. In addition to virions, HBsAg particles are secreted as 22 nm spheres and filaments of 22 nm diameter and variable length. HBsAg particles are present in serum in numbers greatly exceeding that of virions (Dane *et al.* 1970).

The genome contains 4 open reading frames (ORFs) – the so called preS-S-ORF; preC-C-ORF; P-ORF; and the X-ORF. The 4 ORFs represent genes coding for surface (preS-S), core (preC-C), e antigen, polymerase (P) and X proteins. There is considerable overlap in the ORFs that significantly increases the coding capacity of the genome (Figure 1.1).

The HBV preS-S ORF is transcribed to produce 2 mRNA species (2.4 and 2.1 kb in length) with a common 3' end but differing 5'ends. The 2.4 kb mRNA codes for "L" protein, which contains $preS_1$, $preS_2$ and S domains and is 409 amino acids (AA) long. The 2.1 kb mRNA codes for both "M" protein, which contains $preS_2$ and S domains, and is 281 AA long; as well as "S" protein, which consists of the S domain and is 226 AA long (Monjardino 1998). HBV surface proteins exist in unglycosylated and glycosylated forms with molecular weights of 37 kD and 39 kD (unglycosylated and glycosylated) for L protein, 31 kD and 33 kD for M protein and 24 kD and 27 kD for S protein (Monjardino 1998).

The different subviral particles and virion of HBV contain varying quantities of each surface protein. Virions contain more L protein than do subviral particles. This may reflect the presence of a receptor recognition site in L protein (Klingmüller and Schaller

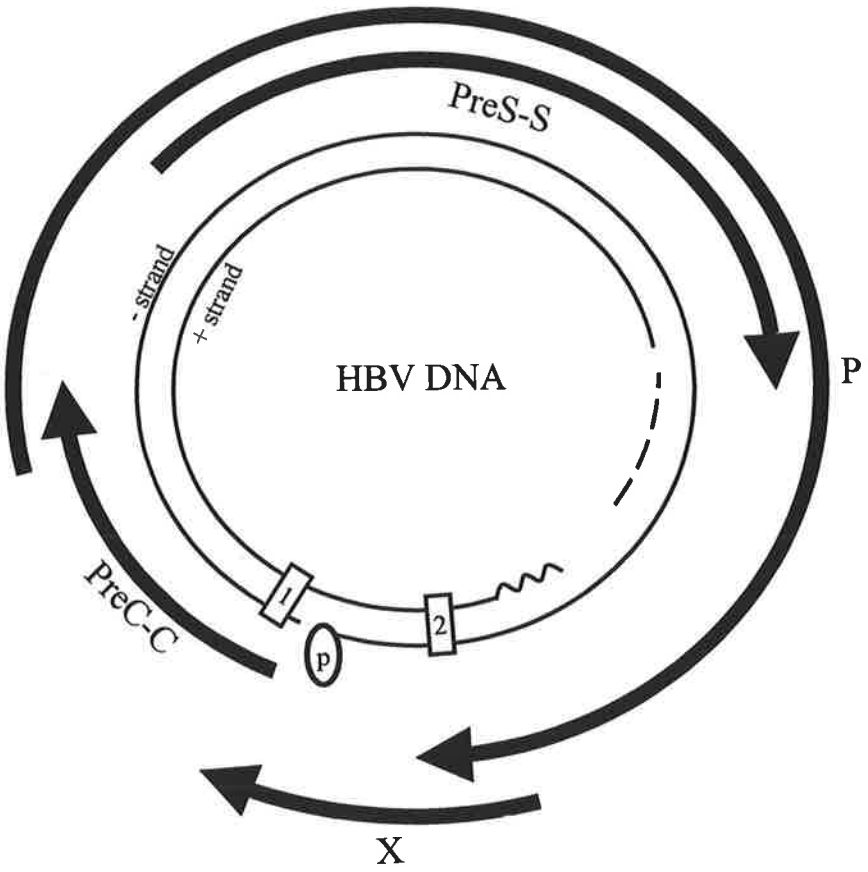


Figure 1.1: The HBV genome showing the locations of DR1 (1) and DR2 (2) and the 4 open reading frames (ORFs). The outer bold lines show the overlapping ORFs preS-S, preC-C, P and X. The viral polymerase (P) is attached to the 5'end of the minus strand DNA.

1993). Whilst S protein is the most abundant of the 3 proteins in all types of particle, i.e. viral and subviral, it is particularly concentrated in subviral particles. Subviral particles are collectively referred to as "HBsAg" particles.

The pre-C-C and P-ORFs are included in polyadenylated mRNA transcripts of ~3.4 kb in HBV and ~3.3 kb in DHBV. These longer than genome length RNAs, known as pre-genomic RNA, contain a terminal redundancy of 200 bp which results in their length exceeding that of the DNA from which they are derived. Pre-genomic RNA serves as the template for genome replication and for core and polymerase protein translation (Nassal and Schaller 1996). Translation of the pre-C+C regions produces a polypeptide, which is then cleaved in the endoplasmic reticulum to produce "e" antigen (HBeAg). HBeAg is secreted into the serum and does not form part of the virion. Translation of the C region produces the viral core protein (HBcAg) that forms the nucleocapsid. Core protein monomers appear, at least as expressed from recombinant *E.coli,* to self assemble into icosahedral core particles comprising 180 or 240 subunits with T3 or T4 symmetry (Monjardino 1998). Further examination of nucleocapsids by cryo-electron microscopy has shown that spikes formed by dimers of core protein protrude from the shell and channels penetrate through the shell (Bottcher *et al.* 1997; Conway *et al.* 1997).

P protein is translated by internal initiation from the same ~3.4 kb mRNA as the core protein, but in smaller quantities. It is closely associated with nucleocapsids consisting of core protein and pre-genomic RNA via interaction with the RNA encapsidation signal, ε, as discussed below. The X-ORF product is a transcriptional activator, the precise role of which is unclear. It is not found in virions.

1.4.1 Duck Hepatitis B Virus Structure and Genome Organisation

DHBV has a similar structure to other hepadnaviridae (Mason *et al.* 1980). It is an enveloped virus with a diameter of 45 nm (Schaefer *et al.* 1998). The genome of 3021-3027 bp (Mandart *et al.* 1984; Triyatni *et al.* 1998) is double-stranded DNA and in 80% of virions the plus strand is complete. The plus strand is incomplete in the remaining 20% of DHBV virions (Lien *et al.* 1987). The minus strand is full length but has a nick in the region of base pair 2536. The DNA genome and polymerase are contained within the 27 nm diameter viral nucleocapsid (Schaefer *et al.* 1998).

The DHBV envelope consists of a lipid membrane into which are embedded copies of the 2 viral surface proteins (in contrast to the 3 seen in the mammalian hepadnaviruses) (Schaefer *et al.* 1998). The 2 surface proteins, preS+S, are 330 and 167 AA long (Schaefer *et al.* 1998). As with HBV, viral surface protein particles with a diameter of 40-60 nm are secreted into the serum in excess of virions.

Avian hepadnavirus genomes contain 3 ORFs – preS-S, preC-C and P, unlike mammalian hepadnaviruses, which have a fourth, X-ORF. Recently, however it has been reported that an X-ORF is also present in DHBV, but commences with an atypical start codon (Chang *et al.* 2001).

1.5 ***Hepadnavirus Replication***

HBV virions attach to a cell surface receptor of unknown structure via the pre-S_1 protein (Klingmüller and Schaller 1993). After endocytosis the envelope is removed and the nucleocapsid is transported to the cell nucleus. The partially double stranded RC DNA genome is then converted to covalently closed circular DNA (cccDNA) from which mRNA transcripts are synthesized (Tuttleman *et al.* 1986; White and Fenner 1994). The

events are outlined in Figure 1.2. The mechanisms involved in conversion from RC DNA to cccDNA are unclear but the changes required include removal of the viral polymerase from the 5' end of the minus strand of DNA, removal of the poly-A RNA sequence from the 5' end of the plus strand, removal of one copy of terminally redundant sequence, completion of the plus strand, supercoiling of the circular molecule and ligation of both strands (Seeger and Mason 1998).

CccDNA exists in the nucleus in association with nucleosomes (Miller and Robinson 1984) and hence has been termed a "mini-chromosome," with 10 or 20 nucleosomes bound to each 3 kbp molecule (Newbold *et al.* 1995). It has been reported that up to 50 copies of cccDNA exist in each infected hepatocyte (Summers *et al.* 1990). CccDNA production continues during infection, hence maintaining the transcriptional template (Summers *et al.* 1990; Civitico and Locarnini 1994). Therapy with nucleoside analogues has been shown to reduce the quantity of RC DNA to a much greater extent than cccDNA, demonstrating the relative stability of cccDNA (Fourel *et al.* 1994).

RNA polymerase of cellular origin produces RNA transcripts using cccDNA as a template. The transcripts include the 3.4 kb pre-genome (3.3 kb in DHBV) used for genome replication and the subgenomic RNAs (Section 1.4) (Hollinger 1990). Pre-genomic RNA is capped with 7-methylguanosine at the 5' end, polyadenylated at the 3' end and transferred to the cytoplasm where core and P proteins are translated.

Pre-genomic RNA, core protein and polymerase are required for genome replication and nucleocapsid assembly. Within pre-genomic RNA, a specific encapsidation signal, epsilon (ε), is involved in nucleocapsid assembly (Calvert and Summers 1994). The encapsidation signal is a hairpin that contains a loop and bulge (Seeger and Mason 1998) (Figure 1.3).

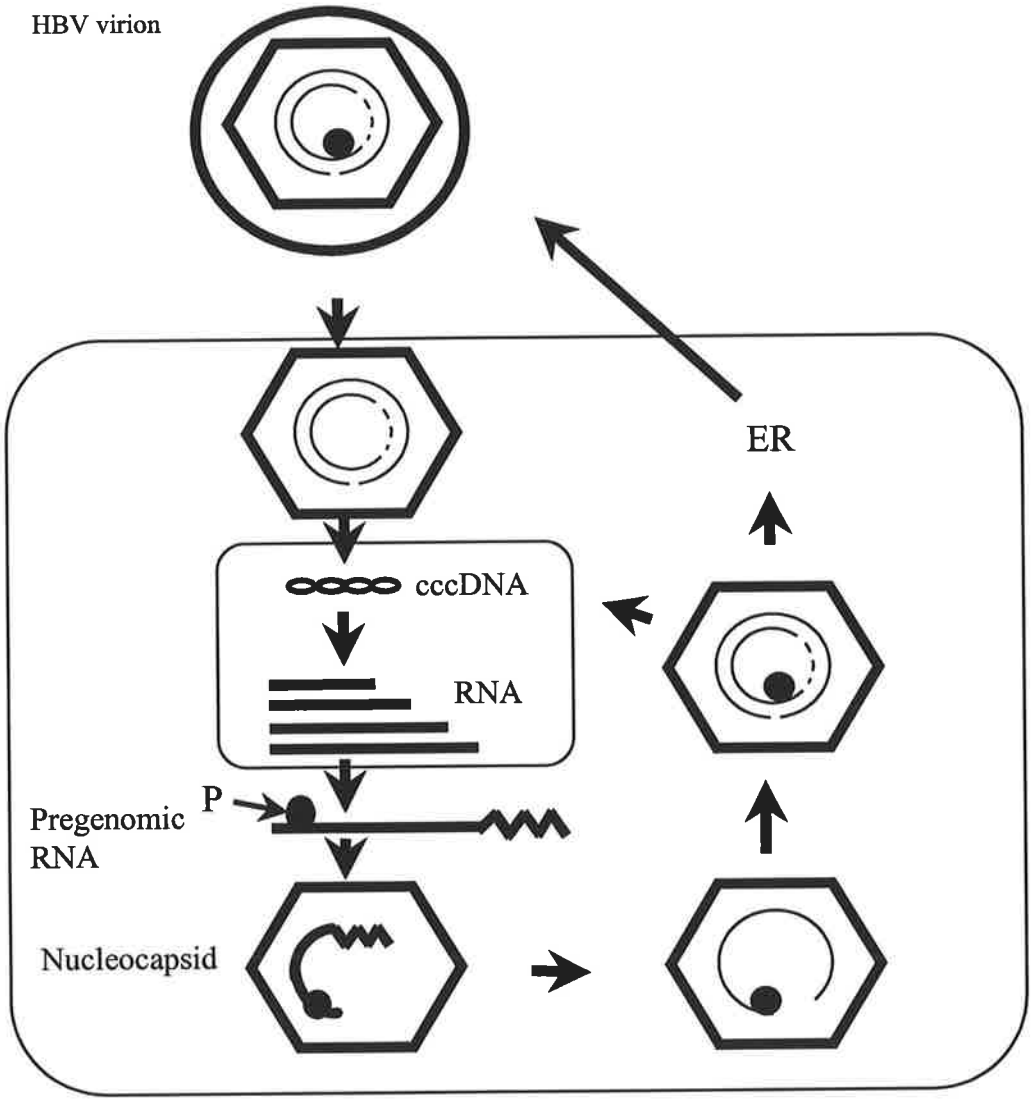


Figure 1.2: Hepadnavirus replication commences with binding of the preS1 proteind on the surface of the virion to an unknown receptor. Endocytosis of the virion is followed by uncoating and transfer of the nucleocapsid to the hepatocyte nucleus. In the nucleus RC DNA is repaired to produce cccDNA. This cccDNA is the template for RNA synthesis including the longer than genome length pre-genomic RNA and several shorter mRNAs. Pre-genomic RNA is translated to produce core and P proteins in the cytoplasm, allowing formation of a protein-RNA complex from which nucleocapsids are formed by the reverse transcription of DNA from pre-genomic RNA. Nucleocapsids containing RC DNA are enveloped in the endoplasmic reticulum (ER) and exported as virions or recycled to the nucleus.

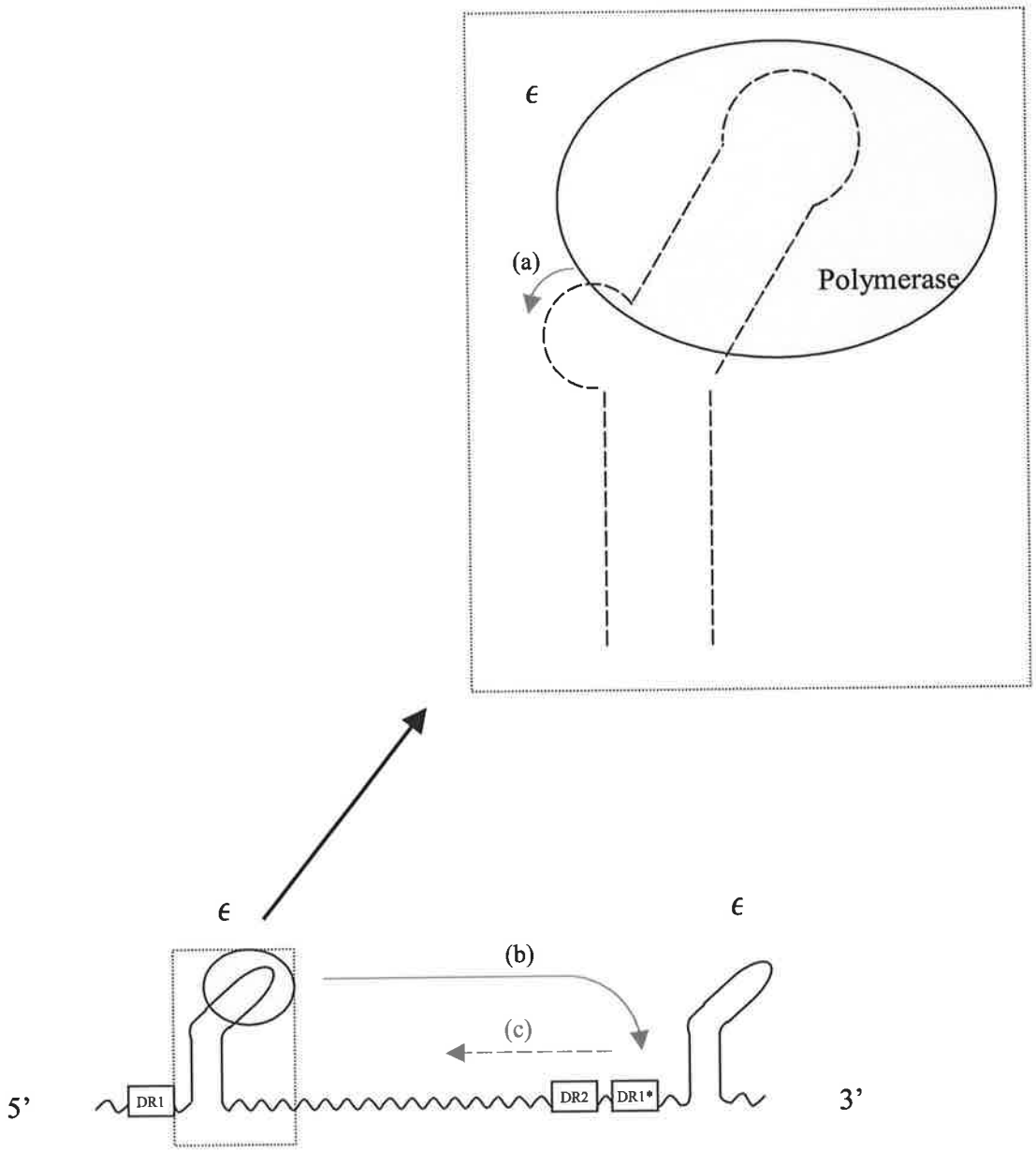


Figure 1.3: Pre-genomic RNA showing the positions terminal repeat sequence including DR1 and DR1* adjacent to the ϵ regions near the 3' and 5' ends of the RNA. The 5' ϵ acts as the encapsidation signal and binds to P protein as shown enlarged. Several nucleotides are transcribed as indicated by arrow (a) followed by transfer of the P protein to DR1* as shown by arrow (b) and elongation of the minus strand DNA indicated by (c).

Diagram adapted from D. Ganem and RJ Schneider, "Hepadnaviridae: the viruses and their replication", in Fields Virology, 4th Ed., 2001, (Philadelphia, Lippincott, Williams and Wilkins).

Two copies of ε are present, one within the 3' and one within the 5' region of pre-genomic RNA. Binding of polymerase to the 5' ε is essential for nucleocapsid assembly (Wang and Seeger 1993; Beck and Nassal 1997). Possibly as a result of the action of chaperone molecules, polymerase protein becomes linked to ε, an event which precedes the addition of core protein dimers to produce the viral nucleocapsid (Nassal and Schaller 1996).

Reverse transcription then commences at the 5' ε sequence with copying of several nucleotides to make a DNA primer which then translocates along with the covalently bound P to a region near the 3' end of the pre-genomic RNA called DR1*. Extension from the DR1* site results in production of minus strand DNA as shown in Figure 1.3 (Nassal and Schaller 1996). At the same time as minus strand DNA elongates pre-genomic RNA is degraded by an RNase H activity of the viral polymerase. A short 5' RNA sequence remains, which includes DR1 and forms the primer for plus strand synthesis (Lien *et al.* 1986).

Plus strand synthesis requires translocation of the RNA primer to the 5' end of the minus strand where it binds to a complementary sequence (DR2). Synthesis then commences, extending towards the 5' end of the minus strand. Another template shift is then required to complete the plus strand. This is produced by circularisation of the minus and partial plus strands enabled by the presence of the terminal repeat region as shown in Figure 1.4. Complementary segments of the plus strand and 3' minus strand terminal repeat sequence bind. After circularisation, plus strand synthesis continues for a variable length. In DHBV, as described above, most plus strands are completed (Lien *et al.* 1987), but in HBV plus strand synthesis stops at 50-85% of full length. The mechanism by which this occurs is not known but may relate to maturation of the nucleocapsid.

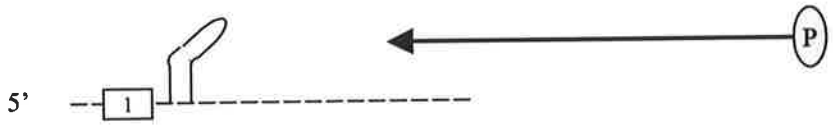
Figure 1.4: Hepadnavirus DNA Synthesis.

Diagram adapted from D. Ganem and R.J. Schneider, "Hepadnaviridae: the viruses and their replication", in Fields Virology, 4th Ed., 2001; (Philadelphia, Lippincott, Williams and Wilkins).

1. Minus strand DNA synthesis from the pre-genomic RNA template continuing after translocation of P from the 5' ϵ sequence to DR1* (translocation shown in Figure 1.3).
2. As the minus strand elongates, the RNase H activity of the viral polymerase degrades the RNA template.
3. A short segment of RNA containing DR1 remains undegraded at completion of minus strand synthesis.
4. This remaining RNA sequence becomes the primer for plus strand synthesis. It is translocated and anneals to DR2 from where a short segment of plus strand DNA synthesis reaches the 5' end of the minus strand.
5. Finally circularisation occurs as the short plus strand segment translocates to the 3' end of the minus strand, which contains a homologous sequence to that present at the 5' end. Elongation of the plus strand can then continue, although, in the case of HBV, it usually remains incomplete.

1.

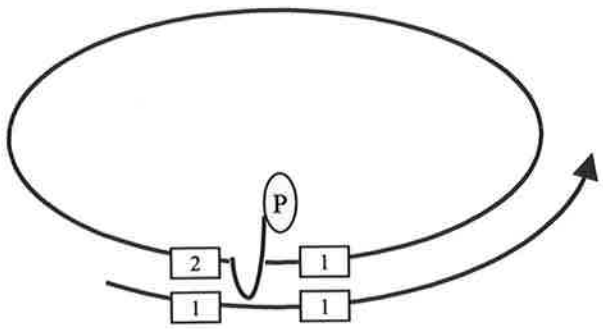
2.



3.

4.

5.



The DNA containing nucleocapsids are either recycled back to the nucleus to increase and maintain the copy number of cccDNA or are exported as virions which are formed in the endoplasmic reticulum by budding of the nucleocapsid through S and pre-S containing membranes (Bruss and Vieluf 1995; Monjardino 1998).

1.6 The Course and Outcome of Hepatitis B Virus Infection

HBV infection in adults usually causes an acute self-limited infection although in ≤1% fulminant hepatitis occurs. In 2-10% of adults infection persists at high level as chronic hepatitis B. The course of acute HBV infection and responses to infection are outlined below.

1.6.1 Transient HBV infection in adults

1.6.1.1 Serologic and Biochemical Events

Exposure to virus is followed by an incubation period of 45-120 days (Hollinger 1990). During this time virus replication occurs in liver and surface antigen initially becomes detectable in serum at 7-84 days, usually within 21-42 days (Robinson 1995). In around a third of adults infected with HBV, surface antigen is not detectable in routinely submitted clinical samples. In the remainder, following the appearance of surface antigen, HBeAg and virions appear in serum. A specific immune response is elicited and hepatocyte necrosis and apoptosis are thought to result from this, rather than a direct cytopathic effect of HBV. Hepatocyte necrosis is reflected in serum as transaminase elevation, due to leakage of enzymes from damaged hepatocytes (see Figure 1.5). Convalescence is marked by loss of HBeAg, appearance of anti-HBe antobodies and then disappearance of HBsAg followed 2-8 weeks later by appearance of anti-HBs antibodies (Hollinger 1990).

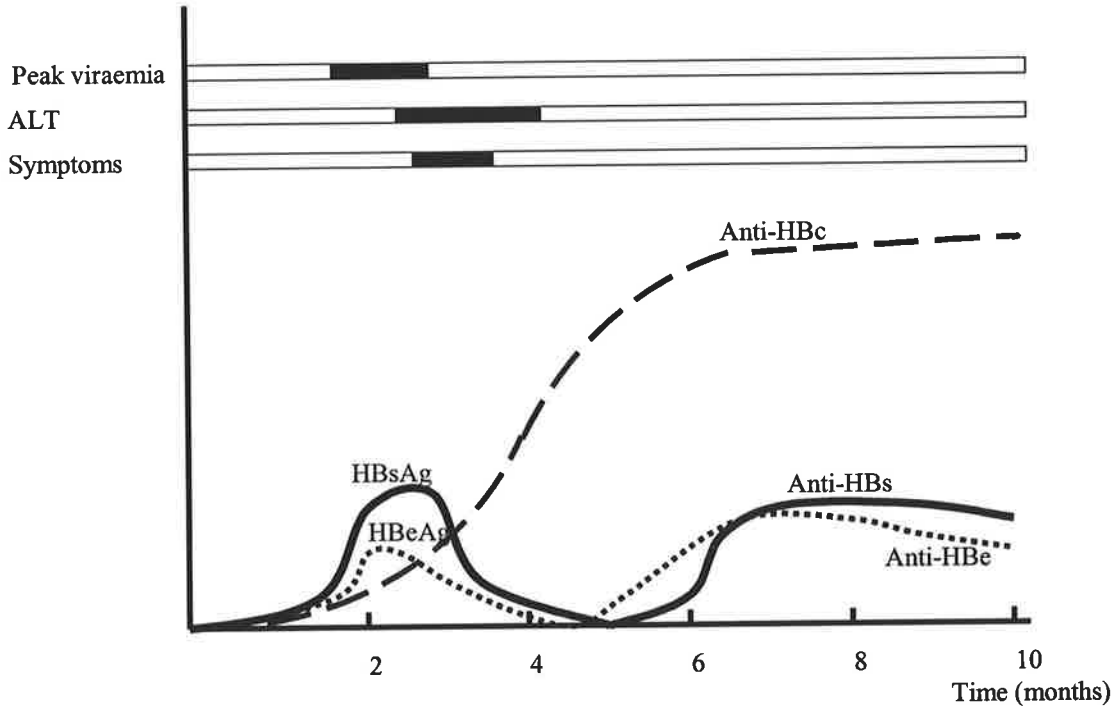


Figure 1.5: Serological and biochemical profile of acute hepatitis B.
The earliest serological marker of infection is HBsAg, followed by HBeAg and peak viraemia. The onset of effective cell mediated immune responses is reflected in hepatocyte injury and alanine transaminase (ALT) elevation, which is closely followed by symptoms if present. Anti-HBc antibodies are the first antibodies to be detectable and often persist many years after recovery. Recovery from infection is marked by appearance of anti-HBe and then anti-HBs antibodies. Anti-HBe antibodies are detectable 1-2 years after infection and anti-HBs antibodies may last many years.

1.6.1.2 Histologic Changes

Histologic changes seen in the liver with acute hepatitis B include damage to hepatocytes, inflammatory infiltrate and regeneration. Hepatocyte damage may lead to necrosis with enlargement of the nucleus and cytoplasmic ballooning or apoptosis with shrinkage of the nucleus and cytoplasm. Kupffer cell hypertrophy with phagocytosis of hepatocyte debris is also seen.

An inflammatory infiltrate, predominantly lymphocytic but also neutrophils and eosinophils is seen in portal tracts. Cytotoxic lymphocytes (CD8+) are present in greatest numbers. Some spillover of portal infiltrate into the lobule (interface hepatitis) may be evident, as may signs of cholestasis (Ferrell 2002)

In the recovery phase changes in liver include regenerative change with increased mitosis and a diminishing inflammatory infiltrate. At approximately 8-12 weeks post onset of infection, parenchymal tissue has generally been restored.

1.6.1.3 Symptoms

Symptoms occur in only a minority of infections (Hoofnagle *et al.* 1987). Prodromal symptoms of mild fever, fatigue, malaise, myalgia, anorexia and nausea occur for approximately one week. Later, symptoms of cholestasis - jaundice, dark urine and pale faeces accompanied by right upper quadrant discomfort and often-profound anorexia and nausea may occur.

1.7 The Immune Response and Transient HBV Infection

1.7.1 Innate Immune Mechanisms

Even before specific humoral or cell mediated immune mechanisms become effective, innate or non-specific immune processes are likely to be initiated during HBV infection, as has been shown with other viruses. A likely mechanism is the release of α-interferon (IFN-α) from leukocytes, which leads to inhibition of viral protein synthesis as well as having an immuno-modulatory role (White and Fenner 1994). This may, in part, explain some of the prodromal symptoms such as malaise and myalgia.

Natural killer (NK) cells are found in periportal hepatic infiltrates in HBV-infected liver (Hollinger 1990) and are known to be an important part of first line defence against virus infections. They are able to recognise and destroy virus-infected cells by recognition of foreign cell surface molecules.

As well as the presence of innate immune responses a range of specific mechanisms appear essential for an effective response to HBV infection. These include viral antigen specific humoral and cell mediated immune responses.

1.7.2 Humoral Immune Responses

The role of the humoral response is neutralisation and clearance of extracellular virus, hence preventing initiation or cell-to-cell spread of infection. In the case of HBV infection, anti-HBs antibodies are neutralising. Stimulation of antibody production occurs via binding of antigen to specific B cell immunoglobulin receptors and is assisted by the action of T cell mediated cytokine production including IL-4, IL-5 and IL-10 produced following major histocompatibility complex (MHC) class II restricted antigen presentation (see below).

Antibodies are found to a range of viral proteins including viral core, polymerase, x, e-antigen, pre-S and S protein. Important events include the production of anti-HBc antibodies, the first to appear in the serum. They usually persist, often for the lifetime of the patient and are non-neutralising, a fact demonstrated by their presence, often at high levels, in patients with chronic infection. Another important marker is anti-HBe antibody, which foreshadows recovery from acute infection or suppression of high level replication in most cases of chronic infection. Finally, production of neutralising, anti-HBs antibodies, mark either recovery from acute infection or an antibody response to vaccination (see Table 1-2).

Table 1-2: Typical Antibody responses to HBV

Serological Marker	Acute HBV	Past infection	Vaccine recipient	Chronic HBV high-level	Chronic HBV low-level
HBsAg	+	–	–	+	+
HBeAg	+	–	–	+	–
HBV DNA, (copies/ml)	+	–	–	$\geq 10^5$	$<10^5$
anti-HBc	IgM+	IgG+	–	+	+
anti-HBs	–	+	+	–	–
anti-HBe	–	+ (1-2 years)	–	– (except pre-core mutant)	+

1.7.3 The Cell Mediated Immune Response

The cell-mediated response to HBV consists of class I and II MHC restricted T cell antigen recognition leading to cytotoxic (CD8+) T cell activity and T helper (CD4+) cell activity. MHC class I molecules are found on most cell types and contain an antigen binding groove. Infected cells display viral peptides within the binding groove of the MHC molecule on the cell surface and these peptide-MHC complexes are recognised by viral antigen specific CD8+ T cells. Following recognition, killing of the infected cell occurs

(Peters *et al.* 1991; Rehermann and Chisari 1998), usually by apoptosis. A number of antigenic epitopes have been identified on viral envelope (9), polymerase (5) and core (1) proteins which elicit cytotoxic T lymphocyte (CTL) responses in patients with acute hepatitis B (Milich 1997).

T helper stimulation occurs via MHC class II restricted antigen. MHC class II molecules are found only on professional antigen presenting cells (APC) including macrophages and dendritic cells. Viral antigens are taken up by the APC and are processed before being expressed as peptides in a complex with MHC class II molecules. Specific CD4+ T helper cells recognise the complex and mount a proliferative and cytokine response (Peters *et al.* 1991). The roles of the CD4+ cell may include assisting with B cell activation and to a lesser extent with CD8+ activity (Rehermann and Chisari 1998), inhibition of virus replication via (non-cytocidal) action of some cytokines (IFN-α and TNF-α shown in a murine model) (Guidotti *et al.* 1994; Guidotti *et al.* 1996) and possibly direct cytotoxicity (Milich 1997). Non-cytocidal mechanisms of viral clearance may be major contributors to viral clearance as observed in mice, woodchucks, ducks and chimpanzees (Jilbert *et al.* 1992; Kajino *et al.* 1994; Guidotti *et al.* 1996; Guidotti *et al.* 1999).

Unlike CD8+ CTL responses, which are directed to a broad range of epitopes, CD4+ cell responses are strongest against core and e peptides in both acute and chronic infections. However, the response in chronically infected patients is relatively weak (Milich 1997).

1.8 The Immune Response and Persistent High-Level HBV infection

In more than 90% of neonates and 2-10% of adults who become infected, HBV persists as shown by high levels of replication in the liver, surface antigenaemia and viraemia of up to

10^{10} virions/ml. Persistence is associated with immunological tolerance with weaker CD4+ and CD8+ T-cell responses than in transient HBV infection.

1.8.1 Host Tolerance and Persistent HBV Infection

Tolerance is well demonstrated in neonates by the development of high levels of viral replication and antigen expression and the apparent absence of effective immune responses. Suppression or deletion of specific T cells may occur, although limited data are available. Postulated mechanisms specific to HBV include the suppression of cell mediated immunity by binding of transplacentally acquired maternal antibody to viral antigens thus limiting their exposure to host T cells (Hollinger 1990). HBeAg is soluble and may also cross the placenta and could promote tolerance by its presence during development of the immune system (Peters *et al.* 1991). Typically, a degree of tolerance lasts until the second to fourth decade of life when a more vigorous immune response leads to suppression of high level replication, loss of serum HBeAg and conversion to anti-HBe antibody positive status.

In adults, tolerance is usually incomplete and T-cell responses are seen, but are weaker than those seen in transient HBV infection. CD4+ activity is strongest against HBcAg and HBeAg. CD8+ responses are also diminished in comparison to transient infection. These features are associated with less necroinflammatory activity except during flares. Anti-HBc antibodies are maintained at high levels. It is possible although as yet unproven that this reflects a skewing of T helper cytokine profile to a Th2 profile with IL-4, IL-5 and IL-10 production associated with a stronger humoral and weaker cell mediated immune response (Milich 1997; Milich 1997). In human immunodeficiency virus (HIV) infection a predominant Th2 response is associated with disease progression, whereas a Th1 response, with IL-2, IFN-γ and TNF-α, correlates with containment of infection (Milich 1997).

1.8.2 Viral Factors and Persistent HBV Infection

In addition to host factors, viral factors may contribute to persistence. High levels of replication and antigen expression may promote tolerance by depleting viral antigen specific T cells. Conversely, increased T cell responsiveness has been associated with reduction in viral load by antiviral therapy (Boni *et al.* 1998). Another example is mutation in the pre-core region of HBV that leads to defective synthesis of HBeAg. Wild-type HBV may be suppressed leading to loss of HBeAg and development of anti-HBe antibodies, however the emergence of pre-core mutant virus allows ongoing high level replication in some infected subjects. This represents selection of viral variants with diminished antigen expression that permit evasion of the immune response.

1.8.3 Loss of Tolerance and Chronic HBV Infection

As tolerance is lost, flares of chronic hepatitis occur over a variable period usually resulting in seroconversion from HBeAg to anti-HBe antibodies and suppression of virus replication to low levels. This state of low virus replication, which is often referred to as the "healthy carrier" state, has a more favourable prognosis (Hoofnagle *et al.* 1987). Seroconversion from HBeAg to anti-HBe antibodies, flares in liver disease and increases in liver enzymes, may be associated with symptomatic hepatitis. In 15-20% of subjects seroconversion to anti-HBe antibodies does not occur before the development of cirrhosis.

1.9 Persistent Low-Level Hepadnavirus Infection Following Transient Infection

Resolution is manifest serologically as clearance of HBsAg and development of anti-HBs antibodies, and until recently was thought to reflect complete eradication of virus.

However, several observations and now considerable experimental data support the presence of hepadnavirus persistence after serological resolution of acute infection.

1.9.1 Serological evidence

Antibodies to HBcAg are an early serological marker of HBV infection (Robinson 1990). They also persist for many years following acute infection and apparent resolution of infection (Penna *et al.* 1996). High levels of anti-HBc antibodies have also been found in the serum of HBsAg-negative blood shown to be the cause of transfusion associated HBV infection (Hoofnagle *et al.* 1978). Such donors are assumed to represent "low-level" carriers in whom HbsAg levels in serum are below the detection limit. Persistent antibodies to viral core protein have also been noted in the serum of animals previously infected with the woodchuck hepatitis virus (WHV) and correlate with infectivity and detection of viral nucleic acid in the serum and liver of animals following recovery from acute WHV infection (Michalak *et al.* 1999). The persistence of anti-HBc antibodies are in contrast to the more limited duration of anti-HBs antibodies following vaccination with recombinant HBsAg produced in yeast. This raises the possibility that after serological resolution of acute infection, ongoing antigenic stimulation of the immune system occurs by the production of low amounts of viral antigens, which are not detectable by conventional serodiagnostic methods.

1.9.2 Detection of Cell Mediated Immune Responses

Rehermann *et al* (Rehermann *et al.* 1995) detected CTL responses to a range of HBV epitopes, which persisted for more than a year after clearance of infection. This could be explained by either ongoing antigenic stimulation or by immunological memory. Stronger evidence of ongoing antigenic stimulation is the presence of MHC class II restricted

induction of T cell proliferation 2.2 - 13 years after serological resolution of hepatitis B. Penna *et al* (Penna *et al.* 1996) showed that this *in vitro* proliferative response was abrogated by depletion of T cells with markers of recent activation (i.e. HLA-DR, CD-69 and CD-25). This suggests ongoing antigenic stimulation rather than T cell memory. However, it was postulated that antigen could be stored by dendritic cells and need not represent persistent, replicative virus.

1.9.3 PCR Amplification of Hepadnavirus DNA

A number of studies of patient sera months or years after resolution of acute HBV infection demonstrate persistent viral DNA (Blum *et al.* 1991; Mason *et al.* 1992; Michalak *et al.* 1994; Rehermann *et al.* 1995; Yotsuyanagi *et al.* 1998).

Yotsuyanagi *et al* found traces of HBV DNA in 10 of 11 patient sera up to 19 months after the onset of transient HBV infection (Yotsuyanagi *et al.* 1998). Michalak *et al* reported persistent HBV DNA in 4 of 5 patients' serum and PBMC up to 70 months after resolution of infection. In addition, using ultracentrifugation and PCR, they found that the viral DNA-positive serum fraction sedimented at the same rate as HBV core particles and virions. This suggests that viral DNA was contained in virions (Michalak *et al.* 1994). Further evidence for the existence of replicative virus was the finding of HBV cccDNA in liver almost 4 years after resolution of HBV infection (Mason *et al.* 1998). The mechanism of persistence was not defined in these studies, but could relate to infection of immunologically privileged sites or to viral mutation (Blum *et al.* 1991).

1.9.4 Evidence from Organ Transplantation

Several published series from transplant centres have documented the risk of transplanting livers from donors who were anti-HBc antibody-positive and HBsAg-negative in the serum (Wachs *et al.* 1995; Dickson and Everhart 1996; Delmonico and Snydman 1998). Many of these cases involved donors who also had anti-HBs antibodies indicating recovery from HBV infection. All of these studies demonstrated that transmission of hepatitis B with such donor livers was common and occurred in 19 of 23 transplants in a multicentre study (Dickson and Everhart 1996), 3 of 6 transplants in San Francisco (Wachs *et al.* 1995) and 4 of 16 transplants in New York (Delmonico and Snydman 1998). This provides strong evidence that traces of viable HBV are harboured in the liver after recovery from HBV infection. The presence of anti-HBs antibodies in the recipient does not protect against such transmission. However, transmission of HBV infection following heart and kidney transplantation in a study in San Francisco occurred following transplant of only 1 out of 42 kidneys and 0 out of 7 hearts (Wachs *et al.* 1995). No other explanation could be found which might have accounted for the transmission associated with the kidney graft. No cases of HBV transmission were reported in New York following heart or kidney transplantation from donors with anti-HBc antibodies (Delmonico and Snydman 1998).

1.9.5 Animal Studies of Hepadnavirus Persistence

Michalak *et al* studied woodchucks after recovery from acute WHV infection for periods ranging up to almost 6 years (Michalak *et al.* 1999). Using PCR amplification they found traces of WHV DNA persisting in a range of sites including liver, PBMC and serum. They were able to show infectivity by inoculating PBMC-derived virus into WHV naïve woodchucks, which then developed typical WHV infection. Sequelae of virus persistence included mild hepatitis in 8 of 9 and HCC in 2 of the 9 woodchucks studied. Offspring of

female woodchucks, born after maternal recovery from WHV infection, also showed traces of WHV DNA (Coffin and Michalak 1999).

1.10 General Concepts of Viral Persistence with Reference to HBV

Survival strategies of viruses include persistence within a single host for a long period or a shorter duration of infection with frequent transmission between hosts. Hepadnaviruses employ the former strategy, with lifelong infection with HBV being the usual outcome after vertical transmission. This source of persistent infection provides most of the estimated 350 million cases of HBV infection worldwide.

It has been proposed that the requirements for viral persistence within a host are:

1. Non-cytopathic infection of host cells
2. Maintenance of the viral genome
3. Avoidance of immune-mediated clearance (Ahmed *et al.* 1996).

Hepadnaviruses satisfy all of these criteria for establishment of persistent infection, being non-cytopathic, possessing stable forms of DNA (cccDNA and integrated DNA) and appearing to induce immunological tolerance in some hosts particularly after congenital or neonatal infection.

It is thought that the loss of immunological tolerance in individuals persistently infected with HBV may eventually produce a marked fall in viral load and in some cases clearance of viraemia with seroconversion from HBsAg-positive to anti-HBs antibodies. Avoidance of immune mediated clearance appears important in the maintenance of high-level hepadnavirus replication. In contrast, during acute HBV infection vigorous cellular immune responses occur as described above resulting in rapid reduction in viral load. The

fact that this clearance of virus is frequently incomplete raises the possibility that additional mechanisms of immune avoidance may operate.

Examples of other strategies of immune avoidance are seen in a range of viral infections and include the following:

1. Restriction expression of viral genome reducing the presentation of viral antigens: an example is herpes simplex virus (HSV) infection of sensory ganglia.
2. Antigenic variation as found in HIV infection, where mutation of the *env* gene enables evasion of neutralising antibodies (Luciv 1996).
3. Infection of particular sites where immune clearance does not occur, such as in HSV infection of neurons that provide a site with relative protection from effector cells of the immune system.

It is not known whether the persistence of traces of HBV after recovery from acute infection requires restricted expression, mutation or infection of a particular site or cell type. This project will examine some of these possibilities using DHBV as a model for HBV infection.

1.11 Animal Models of Hepadnavirus Infection:

HBV is highly species specific as are the other members of the hepadnavirus family. Few non-human hosts have been found to be infectable with HBV. Chimpanzees inoculated with HBV develop infection similar to that in humans and have been used in some studies. The tupaia, a Chinese tree shrew, has been reported more recently to be susceptible to HBV infection (Kock *et al.* 2001). Because of the species-specific nature of HBV, research has relied heavily on the availability of several hepadnaviruses that specifically

infect animal hosts. DHBV and WHV are the most widely used in studies of hepadnaviruses.

A comparison of HBV, WHV and DHBV is found in Table 1-3. The WHV genome shows more than 60% nucleic acid homology with HBV (Galibert *et al.* 1982). It appears to produce a similar disease pattern to HBV infection in humans. A notable difference is that virtually 100% of chronically infected woodchucks develop hepatocellular carcinoma, a phenomenon which has proved useful in studies of hepadnavirus carcinogenesis (Schaefer *et al.* 1998).

Table 1-3: Structural features of hepadnaviruses

Hepadnavirus	virion size (nm)	sAg particle (nm)	Genome ORF	Genome length (bp)
HBV	42	22nm spheres, filaments[a]	s, c, p, x	3182-3221
WHV	45	20-25nm spheres, filaments[b]	S, C, P, X	3308-3323
DHBV	45	40-60nm spheres only[c]	S, C, P, (?X)[e]	3021-3027[f]

a: (Dane *et al.* 1970)
b: (Summers *et al.* 1978)
c: (Mason *et al.* 1980)
e: (Chang *et al.* 2001)
f: (Mandart *et al.* 1984; Triyatni *et al.* 1998)

DHBV and the other avian hepadnaviruses have only 40% nucleic acid homology with HBV (Mandart *et al.* 1984). Transmission of natural DHBV infection occurs *in ovo* (Tagawa *et al.* 1985) unlike HBV and WHV, which are usually transmitted at birth. Infection is lifelong with high level viral replication in liver, but usually with mild or no

hepatitis and without apparent major morbidity. DHBV is geographically widespread (Marion *et al.* 1984; Triyatni *et al.* 2001) and has been found in both domestic and wild duck populations.

Pekin ducks (*anas domesticus*) have been bred in captivity for centuries (Schaefer *et al.* 1998) and are easy to handle making them a very useful model for studying hepadnavirus replication. Furthermore the effect of age and dose of inoculum in experimental DHBV infection parallels the outcomes of exposure to HBV (Section 1.12), making DHBV a suitable model to study outcomes of infection.

1.12 Outcomes of DHBV Infection

As noted above natural infection with DHBV is by the vertical route with replication in the yolk sac and liver of developing embryos leading to persistent viral infection. Ducks infected by the vertical route, so called congenitally DHBV-infected ducks, have high levels of circulating virus (~9.5×10^9 virions/ml) and surface antigenaemia (50 μg/ml) (Jilbert *et al.* 1996) and develop anti-core antibodies in the serum from around day 80 post-hatch (Jilbert and Kotlarski 2000). Horizontal transmission of DHBV appears to be rare or absent except in laboratory settings. Several studies have examined the outcome of infection after inoculating varying doses of DHBV into ducks of different ages (Qiao *et al.* 1990; Jilbert *et al.* 1996; Vickery and Cossart 1996; Jilbert *et al.* 1998).

Newly hatched ducklings inoculated with as little as a single DHBV genome also develop persistent infection with high levels of circulating virus and surface antigen particles (Jilbert, 1996). Interestingly it was also shown in newly hatched ducks that DHBV infection spreads exponentially from a low percentage of initially infected cells with a mean doubling time of 16 hr resulting in infection of virtually every hepatocyte by 10-14

days after infection (Jilbert et al., 1996). This rapid dissemination of DHBV infection suggests that there are no major delays in virus replication within the liver and that innate and adaptive immune responses are insufficient to restrict virus spread or to result in clearance of infection. Newly hatched ducks that develop persistent infection have mild or no inflammatory changes in the liver (Jilbert 1998) again reflecting the lack of effective immune responses to DHBV. Antibodies to DHBV core antigen do develop in the serum within 1-2 weeks after inoculation, but antibodies to surface antigen are generally not detectable expect in some cases at low levels in complexes with surface antigen.

In contrast to neonatal ducklings, mature ducks inoculated with high doses of virus usually develop transient infection with or without viraemia leading to production of anti-surface antibodies and immunity to reinfection. Inflammatory changes are seen more frequently in liver tissue than with persistent infection, although they are usually only mild to moderate. Jilbert *et al* (Jilbert *et al.* 1998) infected 4-month-old ducks by intravenous inoculation with 2×10^{11} virions. Two of 3 ducks developed transient infection with viraemia. The other became persistently infected. Lower doses of virus (up to 10^9 virions) inoculated into 4-month-old ducks led to transient infection without high levels of viraemia, but to development of anti-DHBs antibodies.

From the neonatal period to maturity increasing doses of DHBV are required to produce persistent infection. This change can be seen in the first 14 days. Whilst 1 and 7-day-old ducklings receiving $4x10^4$ virions became persistently infected, 14-day-old ducklings inoculated with the same dose developed transient infection (Jilbert *et al.* 1998). Increasing the dose to 10^6 virions led to 2 out of 4, 14-day-old ducklings becoming persistently infected.

In a study by Vickery (Vickery and Cossart 1996), 26-day-old ducks were inoculated intraperitoneally with DHBV. Two out of 2 ducks inoculated with 7×10^8 virions developed persistent infection. A smaller dose (of 7×10^7 virions) caused transient infection in 3 out of 3 ducks inoculated.

The findings of these studies of outcome of DHBV infection are summarised in Table 1-4 and show a progressive, age-related, increase in the dose of virus required to produce persistent infection with high-level replication. This may be explained by increasing immunological maturity. Knowledge of the age- and dose-related outcomes of DHBV infection has been used to manipulate the characteristics and outcome of infection in the experiments described later.

Table 1-4: Effects of age and dose on the outcome of DHBV outcome

Age of Duck	Dose (genomes)	Route	Rate of persistence	Reference
1 day	4×10^4	i.v.	4/4	(Jilbert *et al.* 1998)
7 days	4×10^4	i.v.	3/4	(Jilbert *et al.* 1998)
14 days	4×10^4	i.v.	0/4	(Jilbert *et al.* 1998)
	10^6	i.v.	2/2	(Jilbert *et al.* 1998)
26 days	7×10^7	i.p.	0/3	(Vickery and Cossart 1996)
	7×10^8	i.p.	2/2	(Vickery and Cossart 1996)
4 weeks	10^6	i.v.	0/2	(Jilbert *et al.* 1998)
	3×10^8	i.v.	3/5	(Qiao *et al.* 1990)
6 weeks	3×10^8	i.v.	2/5	(Qiao *et al.* 1990)
4 months	10^3, 10^6, 10^9	i.v.	0/9	(Jilbert *et al.* 1998)
	2×10^{11}	i.v.	1/3	(Jilbert *et al.* 1998)

1.13 Tissue Localisation of Hepadnavirus Infection

The tissue localisation of hepadnavirus infection is of potential importance in persistence of residual virus after transient infection and is therefore considered here. The liver is the main site of hepadnavirus infection, but some other tissues support replication. The extrahepatic localisation of hepadnavirus infection has been studied in the setting of high-level persistent infection of ducks and woodchucks. Few data are available for HBV. Extrahepatic replication does not appear to be associated with pathological consequences and the significance, if any, of extrahepatic involvement in maintenance of persistent infection is unknown. Extrahepatic infection has been proposed as a possible mechanism of evasion of immune clearance although there is no direct evidence to support this. Extrahepatic replication may be important in reinfection of allografts following liver transplantation for chronic hepatitis B (Omata 1990) although residual circulating virions are an alternative explanation. Circulating virus means that the detection of antigen or viral DNA in a particular tissue does not prove infection at that site.

In studies of DHBV, antigen has been detected in both hepatocytes and bile duct epithelial cells within the liver (Halpern *et al.* 1983; Walter *et al.* 1991). Extrahepatic antigen has been reported in pancreas (Halpern *et al.* 1983; Jilbert *et al.* 1987; Walter *et al.* 1991), spleen (Jilbert *et al.* 1987) and kidney (Jilbert *et al.* 1988) although Halpern *et al* note that antigen in kidney was detected extracellularly and suggested that this reflects the presence of immune complexes rather than extrahepatic replication (Halpern *et al.* 1983).

In situ hybridization has detected DHBV DNA in hepatocytes, bile duct epithelial cells, spleen, pancreas, kidney (Jilbert *et al.* 1987; Freiman *et al.* 1988; Jilbert *et al.* 1988; Hosoda *et al.* 1990; Walter *et al.* 1991) and, in one study, lung (Hosoda *et al.* 1990). Freiman *et al* (1988) found PBMC were positive by dot blot hybridization and Southern

blot hybridization has shown DHBV DNA in heart, brain and intestine at low levels (Hosoda *et al.* 1990).

Replicative intermediates of DHBV have been found by Southern blot hybridization in pancreas, (Jilbert *et al.* 1987), pancreas and spleen (Walter *et al.* 1991) and in brain, heart, kidney, pancreas, spleen and intestine (Hosoda *et al.* 1990) suggesting that virus replication may occur in these sites.

Extrahepatic involvement has also been studied with WHV. Korba *et al* reported the presence of replicative WHV was restricted to liver and spleen although WHV DNA and RNA was found in PBMC, spleen, thymus, pancreas, kidney and ovary (Korba *et al.* 1988). This suggests a narrower range of tissues support viral replication in the woodchuck than in the duck model although the woodchuck model has been less extensively studied.

Studies of extrahepatic HBV show evidence of viral replication in a number of sites. Yoffe *et al* found replicative HBV DNA intermediates by Southern hybridization in PBMC from 14 patients with chronic infection (Yoffe *et al.* 1986).

In another study, this time of autopsy tissue from 2 patients with fulminant and one with acute resolving hepatitis B (the latter patient died from an unrelated cause), Yoffe *et al* found viral DNA and/or RNA by hybridization in lymph node, spleen, gonads, thyroid, kidneys, pancreas and adrenal glands. Little trace of viral nucleic acid was found in liver or serum, which may reflect the effect of immune responses in acute resolving or fulminant hepatitis B (Yoffe *et al.* 1990).

Further evidence of significant extrahepatic HBV replication comes from the study of 2 patients with end-stage chronic HBV who received baboon liver xenograft transplants (Lanford *et al.* 1995). Since baboons cannot be infected with HBV (Lanford *et al.* 1995), persistence of virus after transplantation is suggestive of extrahepatic replication. The patients survived for 27 and 70 days and at autopsy HBV DNA was found in PBMC, spleen, kidney, bone marrow and pancreas.

Table 1-5: Extrahepatic DHBV localisation

Tissue	Antigen	DNA by *in situ*	DNA by Sthn blot	RI DNA	References
Spleen	+	+	+	+	(Jilbert *et al.* 1987; Freiman *et al.* 1988; Hosoda *et al.* 1990; Walter *et al.* 1991)
Pancreas	+	+	+	+	(Halpern *et al.* 1983; Jilbert *et al.* 1987; Freiman *et al.* 1988; Hosoda *et al.* 1990; Walter *et al.* 1991)
Kidney	+	+	+	+	(Halpern *et al.* 1983; Hosoda *et al.* 1990; Walter *et al.* 1991)
Bile duct	+	+	N/A	N/A	(Halpern *et al.* 1983; Walter *et al.* 1991)
Lung		+	+	+	(Hosoda *et al.* 1990)
Brain			+	+	(Hosoda *et al.* 1990)
Heart			+	+	(Hosoda *et al.* 1990)
Intestine			+	+	(Hosoda *et al.* 1990)

RI: DNA replicative intermediates

In the setting of previous hepadnavirus infection with recovery less is known about tissue localisation. In addition to liver, serum and peripheral blood mononuclear cells have tested positive by PCR for viral DNA, both with HBV (Mason *et al.* 1992; Michalak *et al.* 1994; Rehermann and Chisari 1998) and WHV (Michalak *et al.* 1999). As explained in Section 1.9.4 studies of organ transplant recipients of kidneys or hearts from donors positive for

anti-HBc antibodies reveal only a single case of HBV infection - associated with kidney transplantation (Wachs *et al.* 1995).

1.14 Thesis Aims and Outline

The aims of the work were to study residual hepadnavirus infection remaining after the apparent resolution of transient infection. Although the persistence of residual HBV is well documented, the sites, quantity, form and mechanism of persistence are not known. These aspects were studied using DHBV infection as a model for HBV. The duck model was chosen because of the extensive laboratory methods available for detection of DHBV and its serological markers, the relative convenience for serum and tissue sampling and the well-defined age- and dose-related outcomes of experimental DHBV infection.

The first requirement of the study was to develop and evaluate methods for the detection and quantitation of small quantities of viral DNA. PCR methods were developed and are described in Chapter 3, including the development of a quantitative and selective PCR method for detection of DHBV cccDNA.

Chapter 4 describes the sites and quantity of DHBV DNA found in congenital and early experimental infection. These experiments provide a baseline for later studies of residual DHBV DNA and allow comparison of PCR with Southern blot hybridization for quantitation of DHBV DNA.

Transient infection of ducks with DHBV is described in Chapter 5, in experiments in which ducks were followed for 8 months after apparent resolution of infection and a range of tissues were examined for residual DHBV DNA.

Work in Chapter 6 aimed to further characterize the form of residual viral DNA and to formulate hypotheses to explain the mechanism of persistence. In particular, alternative methods were used for demonstration of intact cccDNA, the presence of infectious virions was sought, the effect of immunosuppression was studied and cccDNA decay was predicted by computational modeling based on cell turnover and compared to observed levels.

Finally the results and conclusions are summarised in Chapter 7 and future research directions are outlined.

2. Materials and Methods

2.1 Animals:

Ducks were obtained as 1-day-old ducklings from commercial duck farms. DHBV-negative ducks were obtained from a farm in New South Wales and ducks congenitally infected with an Australian strain of DHBV, AusDHBV, (Triyatni *et al.* 2001) from a second farm in Victoria. The details of ducks described in experiments are described in the following table.

Table 2-1: Age and inoculum of ducks by group

Group	Inoculum (DHBV virions)	Age at inoculation (days)
A	1×10^{7}	39
B	1×10^{8}	40
C	1×10^{10}	39
D	Infectivity experiment*	2
E	Infectivity experiment*	2
F	1×10^{8}	41
G	1×10^{10}	41
H	Infectivity experiment*	2
I	Infectivity experiment*	2
J	1×10^{10}	40
K	Congenitally infected#	N/A
L	Infectivity experiment*	2
W	1×10^{6}	14

* Inocula were prepared from liver, spleen cell cultures and serum as described in Chapter 6
\# natural transmission by infection *in ovo*

Ducks were housed in separate facilities for infected and uninfected ducks at the Institute of Medical and Veterinary Science (IMVS), Adelaide, South Australia. They were housed

and handled in accordance with guidelines set by the National Health and Medical Research Council (NHMRC) of Australia. All research involving ducks was approved by the Animal Ethics Committees of the University of Adelaide and the IMVS.

2.2 Procedures involving ducks

2.2.1 Intravenous Inoculation of Ducks with DHBV

Ducks were inoculated with aliquots of a stock of pooled DHBV-positive serum from congenitally DHBV-infected ducks that contained 9.5×10^9 DHBV genomes/ml. The DNA content of the stock was previously measured by comparison to plasmid DHBV DNA in Southern and spot blot hybridization (Jilbert *et al.* 1996). DHBV-positive serum was serially diluted in normal duck serum (NDS) to produce inocula.

After disinfection of the overlying skin and feathers with 70% ethanol, ducks were inoculated by intravenous (i.v.) injection via a 23 G or 25 G needle (Terumo, USA) with 1 ml of inoculum into the right jugular vein.

2.2.2 Intraperitoneal Inoculation of Ducklings with Cell Suspensions

Cellular suspensions were inoculated intraperitoneally (i.p.) into 1-2-day-old ducklings. Tissue was weighed, usually 250 mg, chopped on a sterile Petri dish with a sterile scalpel and mixed with normal saline in a sterile eppendorf tube. Usually 100 mg of homogenised tissue (e.g. liver or spleen) was mixed with 150 µl of 0.85% saline and injected via a 23 G needle. The skin was cleansed with 70% ethanol and the peritoneal cavity was localised by pinching a fold of skin between thumb and index finger and directing the needle tip beneath the skin, but above the abdominal organs to find a location where cells could be injected without significant resistance.

2.2.3 Administration and Monitoring of Immunosuppressive Drugs

Immunosuppressive drugs were administered as liquid formulations using an oral gavage tube, which was passed via the beak into the upper oesophagus. Cyclosporin A (Neoral™, Novartis, 100 mg/ml) 50 mg/kg/day and dexamethasone (Women's and Children's Hospital Pharmacy, Adelaide, 1 mg/ml) 0.5 mg/kg/day were used. Absorption of cyclosporin A was confirmed by sampling blood immediately before a dose and measurement of the cyclosporin concentration in blood by routine diagnostic assay (IMVS, Adelaide). Ducks were weighed thrice weekly and monitored daily for changes in behaviour.

2.2.4 Venipuncture

Blood samples were obtained by venipuncture of the jugular or wing vein after cleansing the area with 70% ethanol. Blood samples were transferred into 10 ml tubes containing clot activator and separator gel, for serum, and either lithium heparin or EDTA for anti-coagulated blood (Sarstedt, South Australia). To obtain serum blood samples were incubated for 1 hr at 37°C, centrifuged at 4100 g for 10 min at room temperature and the serum fraction was removed and frozen at -20°C.

2.2.5 Liver biopsy

Liver biopsy was performed at laparotomy under general anaesthesia. Ducks were fasted for 4 hr from solids. Anaesthesia was induced with 5% isofluorane (Forthane, Abbott, USA) in oxygen in an induction box and maintained spontaneously by ventilating with 3-

4% isofluorane via an endotracheal tube (size 2.0, Contour™ Mallinckrodt Medical, Ireland).

Liver biopsy was performed using instruments sterilised by autoclaving after careful cleaning. Wherever possible sterile, disposable materials were used. Ducks with high titre viraemia were biopsied after or on separate days from ducks with resolved DHBV infection.

Ducks were placed supine on a heated pad and feathers overlying the right lower thorax and upper abdomen were trimmed. Povidone/Iodine was used to disinfect the surgical field. A 2 cm right subcostal incision was made and dissection was performed through muscle and peritoneum to expose the inferior margin of the liver. A clamp was placed to isolate a 600-800 mg portion of liver, which was excised with a scalpel. The clamp was removed and the incision closed with interrupted 4/0 Vicryl sutures (Ethicon, USA) in 2 layers. Ducks were maintained in a heated recovery room for 1-2 hr before being transferred back to the duck holding facility.

Liver specimens were divided into 3 portions: 1 snap frozen in liquid nitrogen and stored at -70^{0}C, another placed in 10% formalin and the remainder in ethanol/acetic acid as described below.

2.2.6 Tissue Fixation and Embedding

Formalin fixation was achieved by immersion in 10% formalin (equivalent to 4% formaldehyde) in PBS overnight at RT. For antigen detection fresh ethanol/acetic acid (EAA) 3:1 v/v was prepared and tissue was immersed for 30 min at RT. The EAA was then poured off and replaced with 70% ethanol at 4^{o}C and stored overnight. Tissue was

then transferred to embedding cassettes (Techno-Plas, Adelaide, Australia) and embedded into paraffin wax.

Cassettes were placed in an automatic tissue-embedding machine (Leica Microsystems, Germany) and soaked 4 times in 100% ethanol (i.e. 20, 10, 15 and 30 min at 45°C) followed by 3 washes in chloroform (i.e. 20, 10 and 20 min at 45°C) and 2 washes in wax (i.e. 30 and 20 min at 65°C) before being transferred to moulds and cooled on a chilled plate. Sections of 5-6 μm were then cut and placed onto aminoalkylsilane (Sigma, USA, Cat A-3648) coated slides and dried at 60°C for 20 min or at RT overnight.

2.2.7 Autopsy

Ducks were sacrificed by intravenous administration of 80 mg/kg pentobarbitone sodium (Nembutal, Rhone Merieux Australia, Pinkenba, Queensland). Autopsies were performed using sterile instruments as for liver biopsy and the collected tissues were handled separately to avoid cross contamination.

The abdominal and thoracic cavities were entered and blood was collected by cardiac puncture into EDTA and serum tubes. One femur was then removed for bone marrow harvest. Tissue was then collected in order from the adrenal, lymph node/aorta, kidney, pancreas, spleen, heart, skeletal muscle and finally liver. These tissues were divided into 3 specimens as described above.

2.3 Preparation of cells from blood, spleen or bone marrow

2.3.1 Separation of peripheral blood mononuclear cells (PBMC)

Blood (4.5 ml) was collected into EDTA tubes (Greiner labortechnik) containing an equal volume of Hank's balanced salt solution (HBSS). Then 3 ml aliquots were gently pipetted onto 3 ml of Ficoll-Paque® which had been pre-warmed to 37°C (Pharmacia Biotech, Uppsala, Sweden) in a 10 ml polycarbonate tube and centrifuged at 200 g for 20 min at RT. Following centrifugation, PBMC were visible as a band at the interface with Ficoll-Paque and were removed with a sterile pasteur pipette into a 50 ml centrifuge tube containing 30 ml of HBSS. The cells were washed 3 times in 30 ml of HBSS by gentle mixing and then centrifugation at 200 g for 6 min at RT, removing the supernatant and resuspending in HBSS. At the last resuspension a 25 µl aliquot of resuspended cells was mixed in an equal volume of Trypan blue for determining the cell number using a Neubauer chamber. For extraction of DNA the final cell pellet was resuspended at 5×10^6 cells per ml and 1 ml aliquots were placed in screw top eppendorf tubes. These were centrifuged at 18000 g, the supernatant removed and the pellets were stored at -80°C. For lymphocyte proliferation assays PBMC were resuspended in culture medium (CM) at a concentration of 8×10^7 cells/ml. CM consisted of RPMI 1640 (Gibco) supplemented with 1.2 µg/100 ml penicillin, 16 µg/100 ml gentamicin, 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1 µg/ml indomethacin and 5% NDS.

2.3.2 Lymphocyte Proliferation Assay

Antigen presenting cells (APC), erythrocytes and Nylon wool fractionated lymphocytes were first prepared with the help of Cathy Scougall as follows:

1. APC were added to selected wells of a 96-well tissue culture tray by diluting the Ficoll-separated PBMC 1:10 in CM and adding 100 µl/well (8×10^5 cells) before incubating at 37°C for 1 hr in 5% CO_2 to allow adherence. Non-adherent cells were then removed by shaking on a tray shaker (Titertek™, Flow Laboratories, Germany) and aspirating the medium.

2. Ficoll separated PBMC were also nylon wool fractionated to deplete the PBMC of adherent B cells and monocytes, leaving a T-cell-enriched population. Nylon wool columns were prepared by placing 0.32 g of nylon wool in 5 ml syringe barrels and autoclaving. Columns were supported vertically in a rack and saturated with CM (Section 2.3.1) that was added to the top of the column. 1.25 ml of PBMC suspension containing 5×10^7 cells was added to the top of the column and incubated at 37°C for 1 hr in 5% CO_2. Non-adherent cells were collected by flushing the columns with 6.5 ml CM and saving the flow through. The cells were centrifuged at 200 g for 20 min at RT, resuspended in CM, counted (Section 2.3.1) and resuspended at 1.6×10^7 cells/ml.

3. Erythrocytes were prepared by removing 1 drop from the pellet obtained by Ficoll gradient separation (Section 2.3.1), counting and resuspending at 8×10^6 cells/ml in CM.

Proliferation assays were performed by adding 8×10^5 nylon wool-fractionated lymphocytes and 4×10^5 erythrocytes to wells containing APC. Antigens or mitogens were also added: either 15 µl of DHBV-positive serum, 200 µg/ml recombinant DHBV core antigen (rDHBcAg) (Jilbert *et al.* 1992), or 0.5 or 1 µg/ml PHA in a final volume of 200 µl. Where possible, assays were performed in quadruplicate and control wells omitting antigen, erythrocytes or APC, as well as wells containing only APC were included.

The plates were incubated at 37°C in 5% CO_2 for 3-6 days (5 days in the experiment reported in Section 6.2), then 50 µl RPMI containing 1 µCi tritiated-thymidine was added to each well and incubated for another 4 hr. Cells were harvested onto glass fibre paper using a semi-automatic harvester (Skatron Instruments, Lier, Norway) and placed in 4 ml scintillation vials containing 1 ml of scintillation fluid (Wallac Optiphase HiSafe 2, Finland). The radioactive signal was then measured using a β-counter (Beckman LS 6000 TA). Results were expressed as counts/min +/- standard deviation (CPM +/-SD).

2.3.3 Separation of bone marrow cells

Using sterile instruments the femur was removed at autopsy and the proximal end transected. Cells were obtained by flushing the exposed marrow with HBSS and collecting into a 9 ml EDTA tube maintained on ice. Purified bone marrow cells were separated on Ficoll-Paque gradients as described in Section 2.3.1 for separation of PBMC, counted and stored as pellets at -80°C.

2.3.4 Separation of spleen cells

The spleen was removed at autopsy and approximately half was placed in a sterile petri dish on ice. The tissue was chopped coarsely with a sterile scalpel and 8-9 ml HBSS was added to the petri dish. The tissue and HBSS were then aspirated into a 10 ml syringe and passed through 2 layers of sterile gauze to make a largely single cell suspension. Some of the remaining cell clumps were flushed through the gauze with another 20 ml of HBSS. Cells were then washed and counted as described for PBMC in Section 2.3.1and frozen as pellets containing 5×10^6 cells or cultured as described below.

2.4 Culture of Spleen Cells

After separation of spleen cells, 5×10^7 cells were removed and transferred to another 50 ml centrifuge tube, spun at 200 g for 6 min at RT and resuspended in 60 ml CM containing 5 µg/ml PHA.

Cells were seeded at 4×10^6 cells/well in 5 ml of CM into each well of a 6-well plate and incubated at 37°C in 5% CO_2. Twelve wells were set up and the contents of 4 wells harvested on each of 3 time points (days 3, 5 and 10), by removal and centrifugation at 4100 g for 6 min at RT. The cell pellet and supernatant were stored frozen at -80°C.

2.5 Serologic Assays

ELISA assays for detection and quantification of DHBsAg and detection of anti-DHBs and anti-DHBc antibodies were developed in the laboratory by Mr Darren Miller (Jilbert *et al.* 1998). The methods are outlined below.

2.5.1 Qualitative Detection of DHBsAg by ELISA

Each well of a 96-well microtitre plates was coated with duplicate 100 µl samples of duck serum diluted to 1/100 in PBS. High titre DHBsAg-containing serum (50 µg/ml, Section 2.2.1) was also serially diluted from 1/500 to 1/64000 in PBS and duplicate samples were added to the plate as a positive control and used to create a standard curve. NDS was added as a negative control. Plates were incubated at 37°C overnight covered in plastic film. Plates were then washed 3 times with PBS containing Tween 0.05% (PBS-T); (Tween 20, Sigma, USA). Non-specific binding was blocked by the addition of 200

µl/well of 5% skim milk in PBS-T and the plates were incubated at 37°C for 1 hr. The plates were then washed 3 times with PBS-T before the addition of 100 µl/well of a 1/100,000 dilution of monoclonal mouse antibodies to Pre-S antigen (1H.1, a kind gift from John Pugh (Pugh *et al.* 1995)) in 5% skim milk in PBS-T. The plates were then incubated at 37°C for 1 hr.

Following 3 washes with PBS-T, 100 µl/well of a 1/4,000 dilution of sheep anti-mouse horseradish peroxidase (HRP) conjugate (Amersham NXA931) was added and the plates were incubated at 37°C for 1 hr before washing 3 times with PBS.

A suitable substrate for HRP was then added - in all cases 100 µl/well of 0.04% o-phenylenediamine (OPD) in citrate buffer pH 5.0 and 0.03% H_2O_2 was added to each well and incubated in the dark at RT for 15 min. The reaction was stopped by the addition of 50 µl/well of 2.5 M H_2SO_4. Optical density was read at 490 nm using an automated plate reader (Dynatech). A positive result was defined as an OD value 3 times that of NDS.

2.5.2 Quantitative Detection of DHBsAg by ELISA

96-well microtitre plates were coated with 100 µl/well of a 1/500 dilution of ammonium sulphate precipitated polyclonal rabbit anti-DHBs antibodies in freshly prepared 100 mM $NaHCO_3$ and incubated at 37°C for 1 hr and stored at 4°C until use. The plates were then washed 3 times with PBS-T before 200 µl of 5% skim milk in PBS-T was added to each well to block non-specific binding sites. The plates were incubated at 37°C for 1 hr before 3 washes with PBS-T.

Duck serum samples (100 µl) diluted 1/100 in 5% skim milk PBS-T, were then added to each well as duplicate samples or controls. A standard curve was constructed using high titre duck serum containing DHBsAg (50 µg/ml, Section 2.2.1). The serum was serially diluted from 1/500 to 1/64,000 in 5% skim milk PBS-T containing a 1/100 dilution of NDS. NDS diluted at 1/100 in 5% skim milk PBS-T was also used as a negative control. Samples and control specimens were incubated at 37°C for 1 hr before 3 washes with PBS-T.

Monoclonal anti-DHBV 1H.1 antibodies (100 µl) diluted 1/1,000 in 5% skim milk PBS-T was added and incubated at 37°C for 1 hr before washing 3 times with PBS-T.

Sheep anti-mouse HRP (Amersham NXA931; 100 µl/well), diluted 1/5,000 in 5% skim milk, 5% normal sheep serum, 5% normal rabbit serum in PBS-T was then added and the plates were incubated at 37°C for 1 hr before 3 washes with PBS.

OPD substrate was added as above and the plates were incubated in the dark at RT for 15 min before the reaction was stopped by addition of 2.5 M H_2SO_4. Optical density was read at 490 nm using an automated plate reader (Dynatech).

Results from high titre positive serum were used to generate a standard curve of concentration of DHBsAg vs OD at 490 nm (MS-Excel version 7.0a). From this the concentration of DHBsAg in the unknown samples was calculated.

2.5.3 Detection of anti-DHBc Antibodies by ELISA

Each well of a 96-well microtitre plate was coated with 100 µl of 1 µg/ml of recombinant DHBV core antigen (rDHBcAg) (Jilbert *et al.* 1992) in PBS and incubated at 37°C for 1 hr

and then at 4°C overnight. The plates were then washed 3 times with PBS-T and non-specific binding was blocked by the addition of 200 µl/well of 5% skim milk in PBS-T followed by incubation at 37°C for 1 hr.

After 3 washes with PBS-T duplicate 100 µl samples of duck serum were diluted from 1/1,000 dilution to 1/16,000 and added to the plate. Serum from a duck (B40) with high titre anti-DHBc antibodies was used as a positive control. This duck had previously recovered from experimental DHBV infection and rechallenge and had high levels of anti-DHBc antibodies. This serum was diluted from 1/1,000 to 1/512,000. NDS diluted to 1/1,000 was included as a negative control.

After 3 washes with PBS-T, 100 µl/well of a 1/15000 dilution of rabbit anti-duck IgY in 5% skim milk PBS-T was added and incubated at 37°C for 1 hr followed by 3 washes with PBS-T. Rabbit anti-duck IgY was prepared in the laboratory by Mr Edward Bertram by immunizing rabbits with duck IgY prepared from duck egg yolks (Bertram 1997).

Then 100 µl/well of a 1/5000 dilution of goat anti-rabbit HRP (Kirkegaard and Perry Laboratories, Inc, Maryland, USA) in 5% skim milk in PBS-T was then added and incubated at 37°C for 1 hr before 3 washes with PBS. OPD substrate was added to the plates and the OD value determined as described above. Positive results were defined as an OD value of 3 times that of NDS diluted to 1/1,000.

2.5.4 Detection of Anti-DHBs Antibodies by ELISA

Anti-DHBs antibodies were assayed by antibody capture ELISA. 96-well microtitre plates were coated with 100 µl/well of a 1/1,000 dilution of 1H.1 monoclonal anti-PreS1 antibodies (Pugh *et al.* 1995) in fresh 100 mM $NaHCO_3$. The plates were incubated at

37°C for 1 hr and then at 4°C overnight. After 3 washes with PBS-T, non-specific binding sites were blocked by addition of 200 µl/well of 5% skim milk in PBS-T and incubation at 37°C for 1 hr.

DHBsAg was purified by sucrose cushion centrifugation using pooled sera from congenitally DHBV-infected ducks (Section 2.2.1). Sucrose cushions were prepared in 13.2 ml tubes (to fit a Beckman SW41 rotor) with a 1 ml cushion of 70% sucrose in 150 mM NaCl, 20 mM Tris HCl pH 7.4 (TN), overlaid with 5 ml of 20% sucrose in TN. 6 ml of pooled sera was then added to the tube which was centrifuged at 140,000 g for 3 hr at 4°C in a Beckman 8-80 ultracentrifuge.

Following centrifugation, the tube was placed in a fraction collector and 0.5-0.6 ml fractions were collected via puncture of the inferior surface of the tube and drainage into 1.5 ml eppendorf tubes. The gradient fractions were assayed by quantitative ELISA (Section 2.6.2) for DHBsAg concentration and fractions containing 30-60 µg/ml and >100 µg/ml were pooled separately. For use in the anti-DHBs ELISA, DHBsAg was diluted to 10 ng/ml in 0.1% BSA in PBS and 100 µl was added to each well and incubated at 37°C for 1 hr. The purified DHBsAg was then collected for re-use and the plates were washed 3 times with PBS-T.

Duplicate serum samples diluted from 1/50 to 1/6,400 in 5% skim milk PBS-T were added to each well. High titre anti-DHBs-positive serum (B40) was also added to separate wells at 5-fold dilutions from 1/50 to 1/31,250 as a positive control and NDS was added at 1/50 as the negative control. After incubation at 37°C for 1 hr the plates were washed 3 times with PBS-T.

100 µl/well of a 1/15,000 dilution of rabbit anti-duck IgY in 5% skim milk PBS-T was then added to each well and incubated at 37°C for 1 hr before washing 3 times with PBS-T. 100 µl/well of a 1/5,000 dilution of goat anti-rabbit HRP conjugate in 5% skim milk PBS-T was then added and incubated at 37°C for 1 hr before washing 3 times with PBS and adding OPD substrate as described above. The reaction was stopped and plates read as above. Positive results were defined as an OD value of 3 times that of a 1/50 dilution of NDS.

2.6 Histology and Immunohistochemical Detection of DHBV and Cell Turnover

Biopsy or autopsy tissue was divided into 3-5 mm^3 pieces and separate pieces were fixed in 10% formalin and ethanol/acetic acid for histological analysis and antigen detection respectively as described above (Section 2.2.6).

2.6.1 Histology

Sections of formalin-fixed tissue were stained with haematoxylin and eosin (H&E) for assessment of tissue histology and congo red for detection of amyloid. EAA-fixed tissue sections were heated in citrate solution as described below for antigen retrieval prior to immunohistochemical detection of DHBsAg.

2.6.2 Antigen Retrieval

Slides were first dewaxed and rehydrated through ethanol to PBS by placing in racks and immersing in xylene (2 × 5 min), 100% ethanol (2 × 2 min), 90% ethanol (2 × 2 min), 70% ethanol (2 × 2 min) and finally PBS (2 × 5 min).

Racks were then placed in 10 mM sodium citrate buffer pH 6 and heated just to boiling point in a microwave oven and maintained at that temperature for 10 min before cooling to 50°C and then transferring to PBS at RT.

2.6.3 Immunohistochemical Detection of DHBsAg

Tissue peroxidases were inactivated by placing slides in 0.5% H_2O_2 (Sigma H1009) in PBS for 15 min at RT with stirring. Non-specific binding sites were then blocked by covering the tissue section with 200 µl of a 1/30 dilution of normal sheep serum (NSS) in PBS and incubating for 30 min at RT in a humid container. The NSS was then replaced without washing the slides with 100 µl of a 1/100 dilution of anti-DHBV 1.H.1 monoclonal antibodies (Pugh *et al.* 1995) diluted in 10% FCS in PBS. The slides were then covered with cover slips and incubated in a humid container at 37°C for 60 min and then at 4°C overnight.

After washing in PBS (2 × 5 min), 100 µl/slide of a 1/40 dilution of sheep anti-mouse antibody-HRP conjugate (Amersham NXA931) in 10% FCS in PBS was then added and the slides covered with coverslips. The slides were then incubated for 60 min at RT and washed again in PBS (2 × 5 min). 0.04% diaminobenzidine tetrahydrochloride (DAB, Sigma D09105), plus 0.5% H_2O_2 in PBS was used to flood each slide (1 ml/slide) which was then incubated in the dark for 9 min at RT. Slides were then washed in PBS (2 × 5 min) and counterstained with haematoxylin by immersion for 1 min followed by washing in PBS (3 × 1 min) and dehydrating in 70% ethanol (2 × 2 min,) 90% ethanol (2 × 2 min), 100% ethanol (2 × 2 min) and xylene (2 × 5 min). Coverslips were then mounted onto the slides using Depex cement (BDH Australia Ltd) and the slides were then dried in a fume hood.

2.6.4 Detection of Dividing Cells by BrdU Labeling

Eight hours before collection of tissues by biopsy or autopsy 25 mg/kg BrdU (Sigma Cat, No. B5002) was injected i.p. as a 10 mg/ml solution in order to label cells entering S phase. Tissues were then fixed in ethanol/acetic acid (3:1 v/v), embedded and sectioned onto silane-coated slides (Section 2.2.6).

After antigen retrieval (Section 2.6.2), slides were treated with 0.5 ml of 0.1% Trypsin and incubated in a pre-warmed, humid container for 5 min at 37°C. The slides were washed in PBS (2 × 5 min) and then the DNA was denatured by immersion in 2 N HCl for 60 min at RT. The acid was neutralized by immersion in borate buffer (Section 1.1.1) for 2 × 5 min. After washing in PBS (2 × 5 min), tissue peroxidases were inactivated by placing slides in 0.5% H_2O_2 for 15 min at RT.

Non-specific binding of antibody was blocked by adding 200 µl/slide of 1 mg/ml BSA for 5 min at RT. Mouse anti-BrdU monoclonal antibodies (Roche 1 170 376; 100 µl/slide, 6 µg/ml in 1 mg/ml BSA in PBS) were then added and the slides were incubated in a humid container, with a coverslip, for 1 hr at 37°C, then at 4°C overnight.

Slides were washed in PBS (2 × 5 min) and sheep anti-mouse HRP conjugate 1:40 in 10% FCS in PBS (100 µl/slide) was added and incubated for 60 min at 37°C. After another wash in PBS (2 × 5 min) DAB substrate was added, the slides were stained in haematoxylin, dehydrated and mounted as described above (Section 2.6.3).

Counting nuclei, which stained positive for BrdU in a grid and dividing by the total number of nuclei in the same grid gave a measurement of the fraction of cells undergoing division. The result was expressed as a percentage.

2.7 Methods for Handling Viral and Cellular DNA

2.7.1 DNA extraction by the Phenol-Chloroform method for Southern Hybridization

Tissues stored at -70°C were thawed on ice; 150-300 mg was then placed on a sterile plastic petri dish and chopped finely with a sterile scalpel. The chopped tissue was then suspended in 3 ml of TE pH 8.0 (10:10) buffer held on ice.

2.7.1.1 Preparation of cccDNA

Half of the suspended tissue homogenate (1.5 ml) was mixed with 5.5 ml of TE (10:10) in a 10 ml centrifuge tube. 187 µl of 20% SDS was then added and carefully mixed to make a final concentration of 0.5% SDS. The mixture was then held for 10 min at RT to allow complete lysis of cells. 1.87 ml of 2.5 M KCl was then added to a final concentration of 0.5 M and the solution was held for 30-60 min at RT before centrifugation at 12,900 g for 20 min at RT. The supernatant containing the non-protein bound cccDNA was removed and retained in a 50 ml centrifuge tube. The pellet which contained protein bound DNA was discarded.

The supernatant sample was then extracted with an equal volume of phenol (BDH) saturated with 50 mM Tris HCl pH 8 by vortexing and centrifugation at 4100 g for 10 min at RT. The upper layer (aqueous) was retained and the phenol extraction was repeated once.

The upper layer was then extracted with an equal volume of phenol/chloroform (50:50) (BDH) saturated with 0.5 M Tris HCl pH 8 by vortexing and centrifugation at 4100 g for 10 min at RT. Again the aqueous upper layer was retained and 2 volumes of 100% ethanol were added and the mixture was left for 30-60 min at RT before centrifugation at 4100 g for 10 min at RT and washing of the pellet with 70% ethanol. The pellet was then air dried at RT and dissolved in 150 µl TE (10:2) per 300 mg of tissue extracted.

2.7.1.2 Preparation of Total DNA

The remaining 1.5 ml of original tissue homogenate was diluted with 2.5 ml TE pH 8.0 (10:10) and 4 ml of SDS/pronase was added and the mixture was incubated from 4-24 hr at 37°C in a shaking water bath. The SDS/pronase mixture consisted of 8 mg/ml pronase (Boehringer Mannheim), 0.2% SDS, 0.3 M NaCl, 20 mM Tris HCl pH 7.5 and 20 mM EDTA.

After the SDS pronase digestion the mixture was extracted once with phenol and once with phenol/choroform as outlined above. A one tenth volume of 3 M NaOAc pH 4.8 was then added followed by 2 volumes 100% ethanol. The contents of the tube were then mixed and incubated overnight at -20°C. The precipitated DNA was collected by centrifugation at 4100 g for 10 min at RT. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 300 µl TE (10:2) + 100 µg/ml RNase A (Boehringer Mannheim) per 300 mg of tissue extracted.

2.7.2 DNA extraction for PCR using Qiagen Dneasy™ Tissue Kits

Extractions were performed according to the manufacturer's instructions. Both normal duck liver (NDL) and test samples of duck liver were extracted. In brief, each tissue sample was chopped using aseptic technique on a petri dish and added to 180 µl lysis buffer "ATL". 25 mg of each tissue was digested except for spleen where 10 mg was digested and cell preparations (eg. PBMC and cultured spleen cells) where 5×10^6 cells were used. 20 µl of proteinase K was added and the mixture was vortexed before incubation at 55°C overnight. 4 µl of 100 mg/ml RNase A was then added and the mixture was vortexed and incubated for 2 min at RT.

200 µl of Buffer "AL" was then added and the mixture was incubated for 10 min at 70°C. 200 µl 100% ethanol was added and the mixture was vortexed and pipetted onto a Qiagen mini-column and centrifuged at 6000 g for 1 min at RT. The flow-through was discarded, leaving DNA bound to the spin column. 500 µl wash buffer "AW1" was added and the column was centrifuged in a new collection tube at 6000 g for 1 min at RT. The column was again placed in a new collection tube and 500 µl wash buffer "AW2" added and centrifuged at 14000 g for 3 min at RT.

Finally the column was placed in a sterile eppendorf tube and the bound DNA was eluted with 200 µl elution buffer "AE" which was added to the column, incubated for 1 min at RT and centrifuged at 6000 g for 1 min at RT. The eluted DNA was then measured by spectrophotometry as described below and stored chilled (-4°C) or frozen (-20°C or -80°C).

2.7.3 Extraction of DNA from Serum

After proteinase digestion of serum, viral DNA was extracted by adsorption onto a silicon filter, washing and elution with water. The High Pure Viral Nucleic Acid Kit (Roche Cat. 1 858 874) was used according to the manufacturer's instructions. In brief, 200 µl serum was supplemented with 20 µg poly (A) carrier RNA in 200 µl of nucleic acid binding buffer containing guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100, pH 4.4 and 50 µl of proteinase K. The mixture was incubated at 72°C for 10 min.

Isopropanol, 100 µl, was added and the sample was then pipetted into a filter tube placed above a collection tube and centrifuged at 8000 g for 1 min at RT. The flow through was discarded and the collection tube replaced with a new one. Then 500 µl of inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris HCl pH 6.6) was added and the tube was centrifuged at 8000 g for 1 min at RT. Two washes with addition of 450 µl of wash buffer, centrifugation at 8000 g for 1 min and removal were followed by centrifugation at 13000 g for 10 sec at RT. The flow through was discarded and the filter tube placed in a sterile tube. 50 µl of water was added to the filter membrane to elute the bound DNA and the filter tube was centrifuged at 8000 g for 1 min at RT yielding viral nucleic acid for storage at –20°C or use.

2.7.4 Measurement of DNA

Spectrophotometry was used for routine measurement of DNA concentration by UV absorbance. After extraction using the Qiagen DNeasy™ method 10 µl of product was diluted in 90 µl water or 10 mM Tris HCl pH 8.5 and the absorbance at 260 and 280 nm was measured (with a Biorad Smartspec™ 3000) in a cuvette with a 1 cm detection path.

The absorbance at 260 nm (A_{260}) was used to calculate the concentration of DNA in µg/ml by the following formula:

Concentration of sample= $50 \times A_{260} \times$ dilution factor (Sambrook *et al.* 1989)

Fluorimetry was used to measure the concentration of DNA where greater accuracy was required or where a mixture of DNA and RNA was present. Standards were prepared containing 0, 10, 20, 30 and 40 ng/µl of lambda DNA in water. A dye solution consisting of 2 µl of Hoechst dye 33258 (1 mg/ml) and 200 ml of buffer (2 M NaCl, 50 mM Tris HCl pH 7.5) was prepared. 1 µl of each of the 5 lamda DNA standards was added to 2 ml of Hoechst dye solution and the fluorescence was measured using a Perkin-Elmer MPF-3L fluorimeter to define a standard curve. Unknown samples, 1-20 µl, were also mixed with 2 ml of Hoechst dye solution and the fluorescence was measured. The DNA concentration of the unknown samples was then determined using the standard curve.

2.7.5 Detection of DHBV DNA by Southern Blot Hybridization

A 1.5% horizontal agarose gel was prepared in TAE buffer and placed in an electrophoresis bath with TAE buffer up to the level of the top of the gel i.e. not covering the gel. DNA extracted from tissue or serum or a 50–100 pg standard of cloned DHBV DNA was added to each well. Usually 5-20 µl of each DNA sample was mixed with 2.5 µl of 10× loading buffer and made up to 25 µl with water before loading.

The gel was run at 20 mV until the samples had entered the gel. The gel was then flooded with TAE and run at 30mV for approximately 16 hr, then stained with 0.3 µg/ml ethidium bromide in TAE for 30 min and photographed under UV light.

The gel was then placed in a bath containing 0.05 M NaOAc pH 4.2 for 60 min at RT. The solution was replaced with fresh 0.05 M NaOAc and the gel was incubated at 55°C for 60 min. The gel was then placed in a bath of Blot I (1.5 M NaCl, 0.5 M NaOH) for 45-60 min at RTand finally in Blot II (1 M NH_4OAc, 0.02 M NaOH pH 8) for 60 min at RT.

The gel was then transferred by capillary action to nitrocellulose in Blot II as follows. Three sheets of Whatmann 3 MM, 2 of the same length and width as the gel and one long enough to hang over into Blot II, were placed on a flat platform in a bath containing Blot II. Onto this was placed the gel, face down, then 1 sheet of Schleicher and Schuell BA85 nitrocellulose cut to the same length and width as the gel and 3 sheets of Whatmann 3 MM presoaked in Blot II under a 7-8cm stack of absorbent paper towels. Finally a glass plate and a 200 g weight were added to the top of the structure, which was covered in plastic wrap and held overnight at RT.

After dismantling the structure the nitrocellulose was washed in 2 × SSC for 2 × 5 min at RT and baked between 2 sheets of Whatmann 3MM at 80°C in a vacuum oven for 2 hr. The nitrocellulose was then placed in a hybridization jar with 12-15 ml pre-hybridization mix (see) and incubated in a rotating hybridization oven for 4 hr at 42°C.

A^{32}P-labelled DHBV DNA probe was produced by random primer labelling of the cloned genome of AusDHBV using Megaprime DNA Labelling System (Amersham, United Kingdom). A 50 µl reaction containing 50 ng of cloned full-length DHBV DNA, 5 µl of random primer, 5 µl of (α^{32}P) dATP (3000 Ci/mmole, Geneworks, South Australia), 4 µl of dTTP, 4 µl of dCTP, 4 µl of dGTP and 2 units of Klenow enzyme was incubated for 30 min at 37°C. After incubation and dilution with 50 µl of water the reaction was stopped by

the addition of 20 µl of 0.5 M EDTA. To remove unincorporated nucleotides the labelled DNA was precipitated by addition of 3 M NaOAc pH 4.8 to a final concentration of 0.3 M and 2 volumes of 100% ethanol and incubation for 3 hr at -80°C. The tube was then centrifuged at 14,000 g for 10 min, the supernatant discarded and the pellet washed twice with 70% ethanol before being redissolved in 50 µl of water.

Measurement of radioactivity was performed by sampling 1 µl of the probe onto Whatmann 3MM paper in a vial containing 1 ml of scintillation fluid (Wallac Optiphase "Hi-Safe", Finland). The radioactive signal was then measured using a β-counter (Beckman LS 6000 TA) usually demonstrating counts of 1-2 $\times 10^6$. Before use the probe was boiled for 2 min and then chilled on ice for 5 min.

At completion of pre-hybridization the mixture was poured out of the hybridization jar and replaced with 10 ml hybridization mix containing 5-10 $\times 10^6$ cpm/ml of radiolabelled probe and incubated, rotating in a hybridization oven for >16 hr at 42°C.

The hybridization liquid containing the probe was then removed from the jar, stored for reuse and the nitrocellulose was washed in 2 × SSC for 2 × 5 min then in 2 × SSC, 0.1% SDS for 2 × 30 min at 55°C and finally in 0.1 x SSC, 0.1% SDS for 2 × 30 min at 55°C in a rotating hybridization oven.

The nitrocellulose membrane was then blotted dry, wrapped in plastic and placed in an x-ray cassette with film (Kodak X-Omat AR) for 3 - 24 hr at -70°C. Films were developed using an automatic x-ray developer (Ilford). Quantitation of signal present in the nitrocellulose membrane was performed with a phosphorimager (Molecular Dynamics,

ImageQuant). Comparison with a 50 or 100 pg plasmid control was used to quantitate amounts of RC DNA, cccDNA and RI in each sample.

2.7.6 Detection of DHBV DNA in Serum by Spot Blot Hybridization

Schleicher and Schuell BA85 nitrocellulose was cut to a size allowing 2 x 1 cm^2 per sample and 2 x 1 cm^2 for both positive and negative control sera. Then 1 cm^2 squares were marked in pencil on the nitrocellulose and 5 µl samples were spotted onto the membrane in duplicate. Positive control serum from congenitally DHBV-infected ducks was diluted in NDS and spotted in duplicate neat and in doubling dilutions from 1/2 to 1/1024. NDS was spotted in duplicate as a negative control.

The membrane was air-dried and then denatured by floating on a bath containing 0.1 M NaOH, 1 M NaCl for 20 min at RT. The membrane was then rinsed with 0.1 M Tris HCl pH 8, 1 M NaCl (2 × 2 min) then neutralised by placing in 2 × SSC for 20 min at RT.

The membrane was then baked in a vacuum oven between 2 sheets of Whatmann 3MM at 80°C for 2 hr. After baking, pre-hybridization, hybridization and autoradiography were carried out as for Southern blot hybridization as described above (Section 2.7.5).

2.8 PCR Protocols

2.8.1 Handling of PCR Materials and DNA

Most work was undertaken in the Hepatitis Research Laboratory in which DHBV DNA including viral, cloned and amplified PCR product were also handled. Therefore, in order to minimise the risk of contamination of PCR reactions with amplified DNA, cloned

DHBV DNA or extraneous viral DNA, strict contamination prevention measures were adhered to.

Primers, Taq polymerase, PCR buffer and magnesium (Geneworks, Adelaide, Australia) were stored and prepared in a separate laboratory free of DHBV, on a separate floor and in an area not used by other staff of the Hepatitis Research Laboratory. Master mixes were prepared in aliquots and frozen until use when they were thawed and Taq polymerase was added. Gowns and gloves were used and whenever possible "clean" work was done before entering laboratories containing DHBV DNA. No equipment used for handling DHBV DNA was used in the "clean" area and filter tip pipettes were exclusively employed. Decontamination of bench surfaces with 1.25% hypochlorite and of pipettes with DNA-Zap™ was performed.

DNA extraction was performed in a third laboratory removed from the Hepatitis Research Laboratory using the same precautions as for primers and other reagents. In order to monitor for contamination, DNA was extracted from samples of DHBV-negative duck liver in parallel with other samples and tested by PCR.

PCR reactions were assembled in the Hepatitis Research Laboratory on a specific bench using dedicated pipetting equipment. PCR was then performed using a Perkin-Elmer GeneAmp 2400 for nested PCR or a Roche Lightcycler for quantitative PCR.

2.8.2 PCR Primers

Table 2-2: PCR primers

Primers		Site*	Sequence 5' to 3'
Nested core round 1:	C1	2862-2881	TGCCGAAGCACTAAGAGCAG
	C2**	437-418	CCAAGTGATGCTTAGCAGGT
Nested core round 2:	C3	2926-2945	CAGCACGAAGAAGCAGAAGA
	C4**	267-248	AGGTTCGAGTCCACGAGGTT
Quantitative Total DNA	P3	1316-1335	AGCTGGCCTAATCGGATTAC
	P4**	1584-1565	TGTCCGTCAGATACAGCAAG
Quantitative cccDNA	CC2	2462-2481	CCTGATTGGACGGCTCTTAC
	R2**	52-32	CCCGATCCAATGATTCCTCAT
Full-Length PCR	FL1	1669-1688	AGAAATCGCTCGTCGCTTTA
	FL2**	1668-1649	GATCCGAGGGCAGTAGTGAA

* Nucleotide positions are located on the AusDHBV genome (Triyatni *et al.* 2001) using the *Eco* RI site to define nt 1.
** Compementary strand primer.

2.8.3 Nested PCR Amplification of DHBV DNA using primers specific for the core ORF

A 50 µl reaction containing 40 µl reaction mix and 10 µl template was used for the first round. Final concentrations were 0.4 µM of each of primers C1 and C2, 200 µM dNTPs, 2 mM $MgCl_2$, 5 µl/reaction buffer (670 mM Tris-HCl, pH 8.8, 166 mM $(NH_4)_2SO_4$ 2 mg/ml gelatin, 4.5% Triton X-100) and 1 unit/reaction Taq polymerase (Geneworks, Adelaide, Australia).

The PCR reaction was comprised of denaturation step of 4.5 min at 94°C; an annealing step of 30 sec at 60°C; an extension step of 30 sec at 72°C and a denaturation step of 30 sec at 94°C. Steps 2-4 were repeated for 35 cycles and then a final 7 min extension step was performed at 72°C.

For the second round, 1 µl of the first round reaction was added to a 50 µl reaction containing 1 µM of each primer C3 and C4 (Table 2-), 200 µM dNTPs, 2 mM $MgCl_2$, 5 µl/reaction buffer and 1 unit/reaction Taq polymerase. PCR cycles were as outlined above with 20 cycles of steps 2-4.

2.8.3.1 Detection of PCR Products: Nested PCR

Products of nested PCR were detected in ethidium bromide-stained agarose gels followed by Southern blot hybridization with a radiolabelled DHBV DNA probe. An aliquot of 18 µl of each PCR reaction was mixed with 2 µl 10 × TAE loading buffer and loaded onto a 1% agarose gel. After electrophoresis the gel was photographed and immersed in 0.25 M HCl 20-30 min or 10 min after the marker dyes had changed colour. Then, after rinsing in water, the gel was immersed in 0.4 M NaOH for 15-20 min or until the dye colours were restored. The transfer apparatus was then assembled as described above (Section 2.7.5) except that, in place of nitrocellulose membrane, a nylon membrane was used (Hybond™ - N+, Amersham, United Kingdom) and transfer was carried out in 0.4 M NaOH. After overnight transfer, the gel was immersed in 2 × SSC, placed between 2 sheets of Whatmann 3MM paper and baked for 2 hr in a vacuum oven at 80°C. Pre-hybridization and hydridization with a radiolabelled DHBV probe were then performed and the membrane was exposed to x-ray film (Kodak X-Omat AR) and developed as described above (Section 2.7.5).

2.8.4 Quantitative PCR to detect total DHBV DNA using primers specific for the POL ORF

Real time PCR was carried out using a Roche Lightcycler. The reaction contained 10 µl of reaction mix and 10 µl of template. Reaction mix included 2 µl of Roche FastStart Master SYBR Green 1 (Roche Molecular Biochemicals Cat. No. 2 239 26), 2.4 µl of $MgCl_2$ added to make a final concentration of 4 mM, 0.5 µl of 10 µM primers P3 and P4 (Table 2-, final concentration of 0.5 µM) and 5.1 µl of water. Template consisted of 120 or 200 ng of extracted DNA diluted with water to 10 µl or plasmid DNA standards.

Plasmid DNA standards contained 120 or 200 ng of extracted NDL DNA to which was added a known copy number of plasmid DHBV DNA genomes. Standards were set up as follows:

A working stock of plasmid DHBV DNA (DHBV 4.8 × 2; Section 3.2) was prepared by diluting plasmid DNA to a concentration of 90 ng/µl or 10^{10} genomes/µl as determined by measurement with a spectrophotometer (Section 2.7.4). A stock of 12 or 22 ng/µl of NDL DNA in water was also prepared. Both plasmid and NDL DNA were maintained on ice and a series of 10-fold dilutions of 2 µl of plasmid DNA in 18 µl of NDL DNA were carefully prepared. This resulted in standards containing 2×10^1, 2×10^2 2×10^3, 2×10^4 and 2×10^5 copies of the DHBV genome and these were used to produce a standard curve. Each PCR run included plasmid DNA standards, a NDL DNA negative control sample and a number of test samples.

The reaction mix and template were loaded into glass capillary tubes (Roche Molecular Biochemicals Cat. No. 1 909 339) and centrifuged briefly at 3000 rpm. They were loaded

into the sample carousel as per the manufacturer's instructions and the reaction commenced.

The protocol included an initial denaturation step of 10 min at 95°C to activate the FastStart Taq polymerase and then 40 cycles of amplification. These consisted of 5 sec at 95°C, 10 sec at 55°C and 15 sec at 72°C with ramping rate of 20°C/sec. After amplification a melting curve was generated with heating to 95°C, rapid cooling to 65°C and then slow heating to 95°C whilst fluorescent signal was measured. The acquisition phase was performed with a ramp rate of 0.1°C/sec. Finally the samples were cooled to 40°C.

Detection of product was by measurement of SYBR Green fluorescence at the end of the elongation segment of each amplification cycle. This permitted the construction of a curve of fluorescence vs cycle number for plasmid DNA and test samples. For each sample a "crossing point" was created, representing the cycle number when the fluorescent signal reached a defined level (Roche Lightcycler software version 3). The crossing point was calculated from data during the log-linear phase of amplification and allowed a curve of crossing point cycle number vs log concentration to be created from the plasmid DNA standards. The copy number of unknown samples was then determined by measurement of the crossing point cycle number and obtaining the corresponding log concentration using this curve.

As well as quantification of fluorescence, analysis of the melting temperature curves of samples was used as per the manufacturer's recommendations to determine the specificity of the PCR products. A specific product was defined as having a melting peak at the same temperature as that of the plasmid DNA standards. In cases of doubt product was run on a

1% agarose gel to determine its size and to distinguish specific product from non-specific products such as primer dimers.

2.8.5 Selective PCR Detection of Residual cccDNA using primers spanning the cohesive overlap region

The reaction mix was similar to that used for PCR detection of total DHBV DNA and contained 10 µl of reaction mix and 10 µl of template. The reaction mix also included 2 µl of Roche FastStart Master SYBR Green 1 (Roche Molecular Biochemicals Cat. No. 2 239 26), 2.4 µl of $MgCl_2$ to achieve a final concentration of 4 mM, 0.5 µl of 10 µM of each primer CC2 and R2 (Table 2-2) to achieve a final concentration of 0.5 µM and 5.1 µl of water. Template consisted of 200 ng of extracted DNA diluted with water to 10 µl.

Plasmid DNA standards were set up as for the total DHBV DNA PCR and tubes containing 10^1, 10^2, 10^3, 10^4 and 10^5 copies were used in each assay.

The protocol included an initial denaturation step of 10 min at 95°C to activate the FastStart Taq polymerase and then 40 cycles of amplification. These consisted of 5 sec at 95°C, 10 sec at 60°C and 24 sec at 72°C with ramping rate of 20°C/sec. After amplification a melting curve was generated and samples were cooled to 40°C. Analysis was as described above (Section 2.8.4).

2.8.6 Selective PCR Detection of cccDNA During Active Replication

In an attempt to develop a selective quantitation method for cccDNA during the active phase of DHBV infection, samples of extracted DNA were first depleted of linear DNA i.e. non ccc DHBV DNA and chromosomal DNA. PCR was then performed using a similar

method to that described in Section 2.8.5. This method was later discontinued as discussed later in Section 3.5.2.

The concentration of extracted DNA was measured by fluorimetry (Section 2.7.4) and 160 ng of DNA was denatured at 95^0C for 10 min. The DNA was then digested with Plasmid-Safe™ ATP-dependent DNase to deplete the sample of linear DNA, both single- and double-stranded, in a reaction containing 1.6 µl of reaction buffer, 0.6 µl of 20 mM ATP, 10 units of enzyme and water to make up to 16 µl total reaction volume.

The digestion was performed at 37°C for 2 hr and followed by inactivation of the Plasmid-Safe™ enzyme by incubation at 70°C for 30 min. 1 µl of the digested DNA and 9 µl water were added as template to a PCR reaction mix identical to that described in Section 2.8.5.

Quantitative PCR was performed (Section 2.8.5) using plasmid DNA standards containing 10^3, 10^4, 10^5, 10^6 and 10^7 copies of the target DNA, but without addition of extracted NDL DNA.

2.8.7 PCR amplification of full-length DHBV DNA

Long range PCR amplification similar to that described by Gunther *et* al (Gunther *et al.* 1998) and Netter e*t al* (Netter *et al.* 1997) was performed. The reaction contained 1 µg of extracted DNA template in 10 µl and 39 µl of reaction mix. The reaction mix consisted of 1 µl of a 10 mM solution of dNTP for a final concentration of 200 µM; 2 µl of a 10 µM solution of each of primers FL1 and FL2 (Table 2-) for a final concentration of 200 nM; 5 µl of Finnzymes™ EXT 10× reaction buffer (Finnzymes, Finland) for a final concentration

of 1.5 mM $MgCl_2$, 10 mM Tris HCl pH8.8, 50 mM of KCl and 0.1% Triton X-100 and 29 µl water.

The reaction tubes were heated to ≥80°C and 1 µl of 1 unit/µl Finnzymes™ EXT DNA polymerase (Finnzymes, Finland) was added to each tube before a denaturation step at 94 °C for 40 sec, then an annealing step 58°C for 90 sec, then an extension step at 68 °C for 180 sec (extension). After 10 cycles the extension step was increased from 180 to 300 sec for the remaining 30 cycles. After completion of the PCR reaction 18 µl of the PCR product was analysed by gel electrophoresis and Southern blot hybridization as described in Section 2.8.3.1. The presence of 3.0 kb product which hybridized to a radiolabelled genome-length DHBV DNA probe was considered to represent PCR detection of full-length DHBV DNA.

2.9 Sequencing of DNA

Sequencing of cloned DNA was carried out using BigDye Terminator Cycle Sequencing Kit (PE Biosystems). Sequencing reactions were set up with 400 ng template plasmid DNA or 4 µl PCR product, 3.2 pmol of primer (see relevant results section for primer description) and 4 µl of BigDye Terminator Mix made up to 10 µl with water. PCR product was first purified using the Qiaquick® PCR purification kit (Qiagen, Melbourne Australia) according to the manufacturer's instructions.

The reaction was performed on a Perkin-Elmer GeneAmp 2400 thermal cycler with 30 cycles of 10 sec at 96°C, 10 sec at 50°C and 4 min at 60°C. The product was purified by isopropanol precipitation by the addition of 80 µl of 75% isopropanol. The mixture was left for 20 min at RT then centrifuged at 13000 g for 20 min at RT before removal of the

supernatant. Two washes were performed using 250 µl of 75% isopropanol, followed by centrifugation at 13000 g for 1 min and removal of the supernatant. The pellet containing the sequencing product was then applied to a 5% polyacrylamide gel and electrophoresis and sequence analysis was performed in the Division of Molecular Pathology, IMVS, with an Applied Biosystems 377 DNA Sequencer.

2.10 Production of Cloned Dimeric DHBV DNA

2.10.1 Preparation of DNA

Plasmid and DHBV DNA was obtained from pBluescript™ IIKS+ containing a single copy of an Australian strain of DHBV, pBL4.8 (Triyatni *et al.* 2001) cloned at the *Eco* R1 site. *E. coli* DH5α transformed with pBL4.8 was cultured and a "maxi prep" of plasmid DNA was performed as described below.

The cloning strategy involved obtaining a partially *Eco* R1 digested linear form of pBL4.8 containing a monomer of DHBV DNA and inserting a second *Eco* R1 digested copy of DHBV DNA in the correct orientation followed by circularisation. In order to obtain linearised pBL4.8, aliquots of purified DNA (5 µg pBL4.8 in 1.5 µl) were digested with *Eco* R1 in a reaction containing 5 µg DNA, 1 µl (1 unit) of *Eco* R1 (Boehringer Mannheim) and 2 µl of 10 × One Phor All (OPA) buffer made up to 20 µl with water. In order to achieve partial *Eco* R1 digestion incubation the mixture was incubated at 37°C for 10 min, then at 65°C for 20 min to inactivate enzyme and placed on ice.

In a second reaction, DHBV DNA was completely excised from pBL4.8 in an identical reaction mix was incubated for 40 min at 37°C before enzyme inactivation at 65°C for 20

min. 12 units of *Sca* 1 (Boehringer Mannheim) were then added to cleave the pBluescript vector and the mixture was incubated at 37°C for 2 hr before inactivating at 85°C for 20 min.

The products of the digests were then separated by electrophoresis on a 1% agarose horizontal gel with a molecular weight marker (SPP1 phage digested with *Eco* R1; Appendix 2.12.15). The digested DNA was run in several lanes and the outer lanes were excised and stained with ethidium bromide to confirm the presence and location of the desired bands. Linearised pBL4.8 (6 kb), was then excised from the unstained portion of the gel from the first reaction and similarly linear genome-length DHBV DNA (3 kb) was excised from a second gel avoiding the additional smaller bands generated by *Sca* I digestion of the pBluescript IIKS+ vector.

DNA was extracted from the excised gel fragments using a QIAquick™ Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. The excised gel fragment was weighed and dissolved in a volume of buffer QG equal to 3 times the weight of the fragment by incubating at 50°C for 10 min with occasional vortex mixing. Then one gel volume of isopropanol was added and mixed before transferring to a Qiaquick spin column in a collection tube and centrifuging at 18000 g for 1 min at RT. The flow through was discarded and the column was placed in the collection tube.

To wash, 750 µl of Buffer PE was added and centrifuged at 18,000 g for 1 min at RT. The flow through was discarded and the centrifuge step was repeated to remove residual buffer PE. The column was then placed in a sterile 1.5 ml tube and the DNA was eluted by addition of 50 µl of buffer EB (10 mM Tris-HCl pH 8.5), with repeat centrifugation for 1 min.

The concentration of purified DNA was then estimated by comparison to a known quantity of SPP1 DNA when aliquots of each were run on a horizontal 1% agarose gel, which was stained with ethidium bromide and examined with UV transillumination.

2.10.2 Dephosphorylation of Plasmid DNA

Linearised pBL4.8 was treated with shrimp alkaline phosphatase (SAP, Boehringer Mannheim, Germany) to dephosphorylate the DNA, to reduce religation of monomeric forms of DNA and to promote insertion of the 3.0 kb DHBV DNA. The reaction consisted of 340 ng in 17 µl of linearised pBL4.8, 2 µl of 10 × SAP digestion buffer and 1 unit of SAP. The mixture was incubated at 37°C for 90 min, and then inactivated at 65°C for 20 min.

2.10.3 Ligation and Desalting of Cloned DNA

A ligation reaction was prepared containing a slight molar excess of insert (3.0 kb DHBV DNA) over vector (linearised 6.0 kb pBL4.8) in order to increase the probability of insertion compared with recircularisation of pBL4.8. The ligation reaction included 68 ng of pBL4.8 in 4 µl, 40 ng of genome-length DHBV DNA in 4 µl, 1 µl of T4 ligase (Boehringer Mannheim) and 1 µl of ligation buffer (10× T4 ligase buffer, Boehringer Mannheim).

The reaction was incubated at ~18°C overnight. The ligation reaction was desalted before being used in transformation of bacteria by placing 7.5 µl onto a 0.025 µm pore size filter membrane with a 13 mm diameter (Millipore) and floating the membrane on sterile

deionised water in a petri dish for 2-3 hr. For subsequent transformation 5 µl of desalted ligated DNA was used as described below.

2.10.4 Preparation of Competent *E. coli*

An overnight culture of *E. coli* DH5α in nutrient broth was set up. On the next day 2 ml of this culture was added to 500 ml of Luria Bertani (LB) broth and was cultured for 8 hr. The culture was then transferred into two 250 ml centrifuge tubes, chilled on ice and centrifuged with a Beckman JA-20 rotor at 4000 g for 20 min. The supernatant was removed and the cells were washed twice by resuspending in 250 ml cold water, centrifugation as above and removal of the supernatant. The cells were then resuspended in 2 × 250 ml of cold 10% glycerol in water and centrifuged at 4000 g for 20 min at 4°C. Finally the cells were resuspended in 1.5 ml of cold 10% glycerol and stored in 40 µl aliquots at -70°C.

2.10.5 Transformation of *E. coli*

An aliquot of 40 µl of competent *E. coli* DH5α was thawed on ice and pipetted into an electroporation cuvette with a 0.2 cm electrode gap. Approximately 50-100 ng desalted purified plasmid DNA consisting of pBluescript™ IIKS+ containing one or two copies of the Australian strain of DHBV (Triyatni *et al.* 2001) was added to the cuvette. Transformation was then performed using a Bio-Rad Gene Pulser™ set with capacitance 25 µF, resistance 200 Ω and pulsed at 2.5 kV giving a time constant of 4.3-4.6 msec. After pulsing, 1 ml of nutrient broth at 37°C was added to each cuvette and samples were transferred to nutrient agar containing 100 µg/ml ampicillin, 32 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-Galactopyranoside, Boehringer Mannheim) and 32 pg/ml isopropyl-β -D-thio-galactopyranoside (IPTG,Sigma) and cultured overnight.

Single white colonies (white colonies indicate the presence of vector with insert) were selected and grown in 10 ml Nutrient broth containing 100 µg/ml ampicillin for 18 hr. Plasmid DNA was extracted by mini plasmid prep kit (MO BIO Laboratories, Inc, California) according to the manufacturer's instructions as outlined below (Section 2.10.6).

2.10.6 Screening of Colonies by Restriction Enzyme Analysis

Purified plasmid DNA from individual colonies was linearised by cutting with *Sca* 1 (Boehringer Mannheim) in a reaction containing 1 µg plasmid DNA, 6 units *Sca* 1 and 1 µl 10 × OPA buffer made up to 10 µl with water. After incubation at 37°C for 1 hr and inactivation at 65°C for 20 min, the length of plasmid and inserted DNA was measured by electrophoresis in a horizontal 1% agarose gel stained with ethidium bromide. As a size marker, λ DNA digested with *Pst 1* was also run on the gel (Appendix 8.2.17).

DNA of the desired size (9 kb) was then digested in a separate reaction with *Bgl* II in a reaction containing 1 µg plasmid DNA, 1 µl (15 units) *Bgl* II and 1 µl 10 × OPA buffer made up to 10 µl with water. This enzyme cuts at a unique site in DHBV DNA allowing the orientation of the second copy of DHBV DNA to be determined by the size of the resulting restriction fragments (Section 3.2.3). The digested DNA was subjected to electrophoresis with a size marker as above. The desired cloned DNA was then sequenced in the region of the join using primers C1 and C4 (Table 2-) to confirm the orientation and integrity of the inserted DNA (Section 2.9).

2.10.7 Preparation of Cloned DHBV DNA

pBluescript™ IIKS+ containing 1 or 2 copies of DHBV DNA was transformed into *E. coli* DH5α and stored frozen in 10% glycerol until needed. The transformed *E. coli* was then thawed and plated onto Nutrient agar containing 100 μg/ml of ampicillin for overnight culture. A single colony was then transferred into 10 ml Nutrient broth containing 100 μg/ml of ampicillin and cultured at 37°C with agitation either overnight for small scale DNA preparation or for 4 hr for larger scale DNA preparation. For larger scale preparation the 4 hr culture was transferred into a larger volume of the same medium (eg. 500 ml) and cultured overnight.

Small-scale preparation of plasmid DNA was performed using Ultraclean Mini Plasmid Prep Kit (MO BIO Laboratories, Inc, California, USA) according to the manufacturer's instructions. First 2-5 ml of overnight culture was centrifuged at 13000 g for 1 min to pellet cells. The cells were then resuspended in 50 μl of Solution 1 (Tris, EDTA and RNase A) and subjected to alkaline lysis by addition of 100 μl Solution 2 (a lysis solution containing SDS and NaOH). Solution 3 (binding buffer, containing potassium acetate), was then added, the tube was inverted once and centrifuged at 10,000-13,000 g for 1 min. The supernatant was then transferred to a spin filter, centrifuged at 13000 g for 30 sec, the flow through was discarded and the spin filter was replaced in the collection tube. Next, 300 μl of Solution 4 (ethanol wash buffer, containing ethanol, Tris and NaCl) was added and centrifuged at 13000 g for 30 sec. Without contacting the flow through, the spin filter was placed in a new collection tube and 50 μl of Solution 5 (elution buffer: 10mM Tris HCl) was pippetted onto the middle of the filter membrane before centrifuging at 13000 g for 30 sec. As a final step, the spin filter was discarded and the eluted DNA was used or stored at –20°C.

For larger scale preparation of cloned DHBV DNA a BRESApure™ Maxi Kit (Geneworks, Australia) method was used with some modifications to the method. An overnight culture of *E. coli* DH5α, usually 500 ml LB broth, cultured on a shaker at 37°C, was decanted into 250 ml centrifuge buckets and centrifuged at 3840 g in a Beckman J2-21M centrifuge with a JA-14 rotor for 10 min at RT. The supernatant was discarded and the pellets were each resuspended in 10 ml of 50 mM Tris, 10 mM pH 8.0 EDTA with 100 μg/ml RNase A.

10 ml of lysis buffer containing 200 mM NaOH and 1% SDS was then added to the cell suspension and mixed gently for 5 min by inverting the buckets. Neutralisation buffer containing 3.2 M potassium acetate pH 5.5 was then added and mixed by gentle inversion for 5 min. The neutralised lysate was then transferred to a 50 ml polypropylene (Oakridge type) centrifuge tube, mixed briefly by gentle inversion and centrifuged at 39,200 g in a Beckman J2-21M centrifuge with a JA-20 rotor for 10 min at RT.

The supernatant was collected into a 50 ml disposable centrifuge tube (BLUE MAX™, Becton Dickinson) and solid debris was removed by pipetting and by repeat centrifugation in a 50 ml (Oakridge type) tube at 39,200 g for 10 min at RT followed by transfer of the supernatant into a clean 50 ml disposable centrifuge tube.

The supernatants were then loaded onto a pre-equilibrated BRESApure™ Maxi column (Geneworks, Australia). To equilibrate each column, 30 ml of equilibration buffer containing 0.6 M NaCl, 100 mM sodium acetate and 0.15% Triton X-100 pH 5.0, was added and allowed to flow through by gravity. After the supernatant stopped flowing through the column, 2 × 30 ml of wash buffer containing 0.8 M NaCl, 100 mM sodium

acetate pH 5 was added, then the DNA was eluted with 15 ml of elution buffer containing 1.25 M NaCl, 100 mM Tris HCl pH 8.5.

Each tube of plasmid DNA was then precipitated by addition and mixing with 10.5 ml isopropanol, and centrifugation in a 50 ml (Oakridge type) centrifuge tube at 39,200 g for 30 min at 4°C. The supernatant was removed without delay and the pelleted DNA was washed twice with 5 ml 70% ethanol with centrifugation at 39,200 g for 15 min at 4°C. Care was taken not to dislodge the pellet when removing the wash supernatant. After air-drying for 30 min at RT, 200 µl PBS was added to each tube, the contents were vortexed and left at 4°C overnight to dissolve.

3. Development of PCR Methods

3.1 Introduction and Aims

The detection of traces of residual hepadnavirus DNA requires the use of sensitive and specific PCR methods, the development of which is described in this chapter. To assist with interpretation of results, particularly those from quantitative PCR, sensitivity and accuracy were measured for each assay. The importance of accuracy was such that the development and assessment of each of the assays is discussed separately.

In addition to PCR detection of viral nucleic acids, persistence of DHBV was examined by testing for the presence of infectious virus, since a single virion, administered intravenously, has been shown to produce DHBV infection in 2-3-day-old ducklings (Jilbert *et al.* 1996). Studies of infectivity are described in a later chapter.

A nested PCR assay was developed to improve sensitivity. Since an increase in sensitivity leads to a higher risk of contamination of PCR reactions with traces of amplicon, virus or cloned viral DNA, the specificity of the assay was a major concern and careful measures were taken to reduce the chance of contamination and include appropriate negative control reactions. In order to maximise the sensitivity and specificity, Southern blot hybridization of PCR products and detection with a radiolabelled DHBV DNA probe were employed.

Other studies of residual hepadnavirus DNA following transient infection have relied on qualitative PCR (Blum *et al.* 1991; Mason *et al.* 1998; Yotsuyanagi *et al.* 1998; Michalak *et al.* 1999). The recent development of real-time fluorescence detection during PCR has allowed accurate quantitation of template without the need for labour intensive methods such as competitive PCR and without the need for handling product (Nitsche *et al.* 1999) with an increased risk of contamination. Quantitative PCR assays were developed to

compare different inocula, sites of persistence, changes with time and to quantify residual cccDNA.

Cloned DHBV DNA containing known numbers of DHBV genomes was used as a standard to determine the sensitivity of the PCR and to construct standard curves. Suitable cloned DHBV DNA of the Australian strain of DHBV was not available for use as an infectious clone (for work not described in this thesis) and therefore, a head-to-tail dimer of the DHBV genome was constructed for use as a standard. The clone was also tested to confirm that the AusDHBV genome (Triyatni *et al.* 2001) was infectious.

The aims of work in this chapter were to

1. Construct a dimeric clone of AusDHBV DNA in a plasmid vector and test its infectivity and use as a PCR control.
2. Develop a sensitive and specific nested PCR for detection of DHBV DNA.
3. Develop a quantitative PCR for detection of DHBV DNA and assess its accuracy.
4. Develop a quantitative PCR for selective detection of DHBV cccDNA and to assess its specificity and measurement accuracy.

3.2 Cloning of a head-to-tail dimer of DHBV DNA

3.2.1 Introduction

Some primers, which target the core and DR regions of the DHBV genome, flank the *Eco* R1 restriction site. Since the available monomeric cloned DHBV DNA was cut at this site and inserted into a plasmid it could not be used as a standard with such primers (Figure 3.1). In contrast, a head-to-tail orientated dimer of AusDHBV DNA joined at the *Eco* R 1

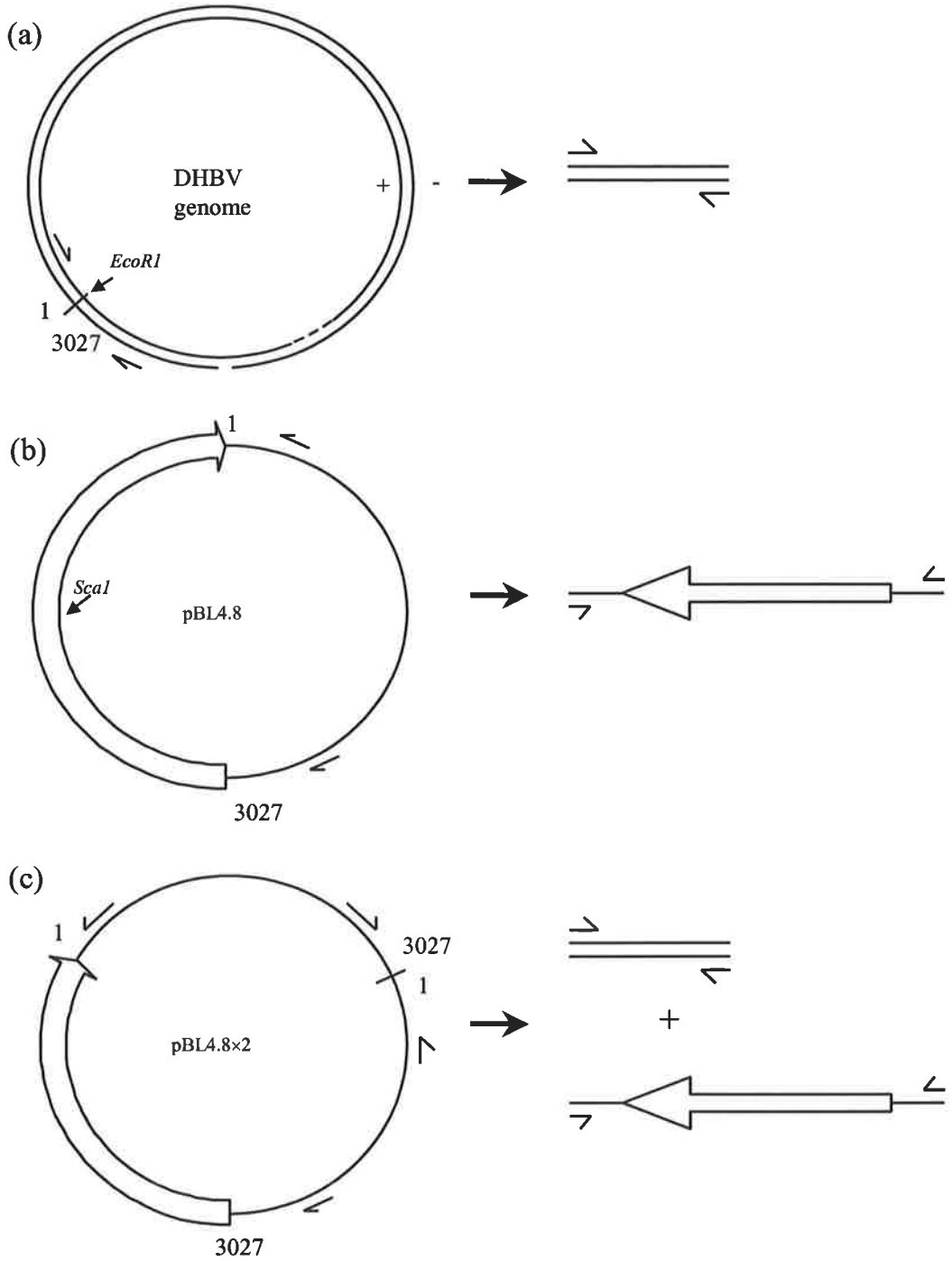


Figure 3.1: Plasmid Standards for PCR :
(a) DHBV RC DNA showing primers C3 and C4 spanning the *Eco* R1 site and amplicon of 268 bp. (b) Plasmid PBL4.8: a cloned monomeric DHBV DNA genome cut at the *Eco* R1 *site* in pBluescript IIKS+. PCR primers spanning the *Eco* R1 site are divergent and yield an amplicon of >3 kb length, which is not achievable with standard PCR methods. In order to allow for efficient amplification of plasmid, a dimer of DHBV DNA was cloned as in (c), to yield a combination of the 2 products only one of which (the shorter) is effectively amplified.

site could act as a template for primers targeting any region of the circular viral genome. It was for this reason that a clone of a head-to-tail dimer of DHBV DNA was constructed.

The second purpose for cloning dimeric DHBV DNA was to provide an infectious AusDHBV DNA plasmid. During genome replication the circular DHBV genome is transcribed to a longer than genome length pre-genomic RNA. Infectious cloned DHBV DNA must allow synthesis of a similar, longer than genome length RNA and, unless the template is circular as is the case with DHBV DNA, a second copy or part copy of the cloned DHBV genome is required as shown in Figure 3.2. Cloned dimers of DHBV DNA from Chinese and German strains have been used successfully to infect ducklings by i.v. or intrahepatic administration of purified plasmid DNA (Sprengel *et al.* 1984; Tagawa *et al.* 1996).

3.2.2 Cloning Strategy

A head-to-tail dimer of DHBV DNA was subcloned from the monomeric DNA genome of an Australian strain of DHBV, AusDHBV (Triyatni 1998). The monomer had been synthesised in the laboratory by Miriam Triyatni from purified viral DNA by linearisation and insertion into PBluescript IIKS+ (pBL4.8). This 6000 nt plasmid, pBL4.8, was linearised by digestion with *Eco* R 1 (Section 2.10.1) and a 3027 nt genome length DHBV DNA was isolated by gel purification and ligated in a head-to-tail orientation to make pBL4.8×2. This is outlined in Figure 3.3.

3.2.3 Results

E. coli DH5α transformed with pBL4.8 was cultured and plasmid DNA was purified using a BRESApure™ Maxi Kit (Geneworks, Australia) (Section 2.10.7). The concentration of

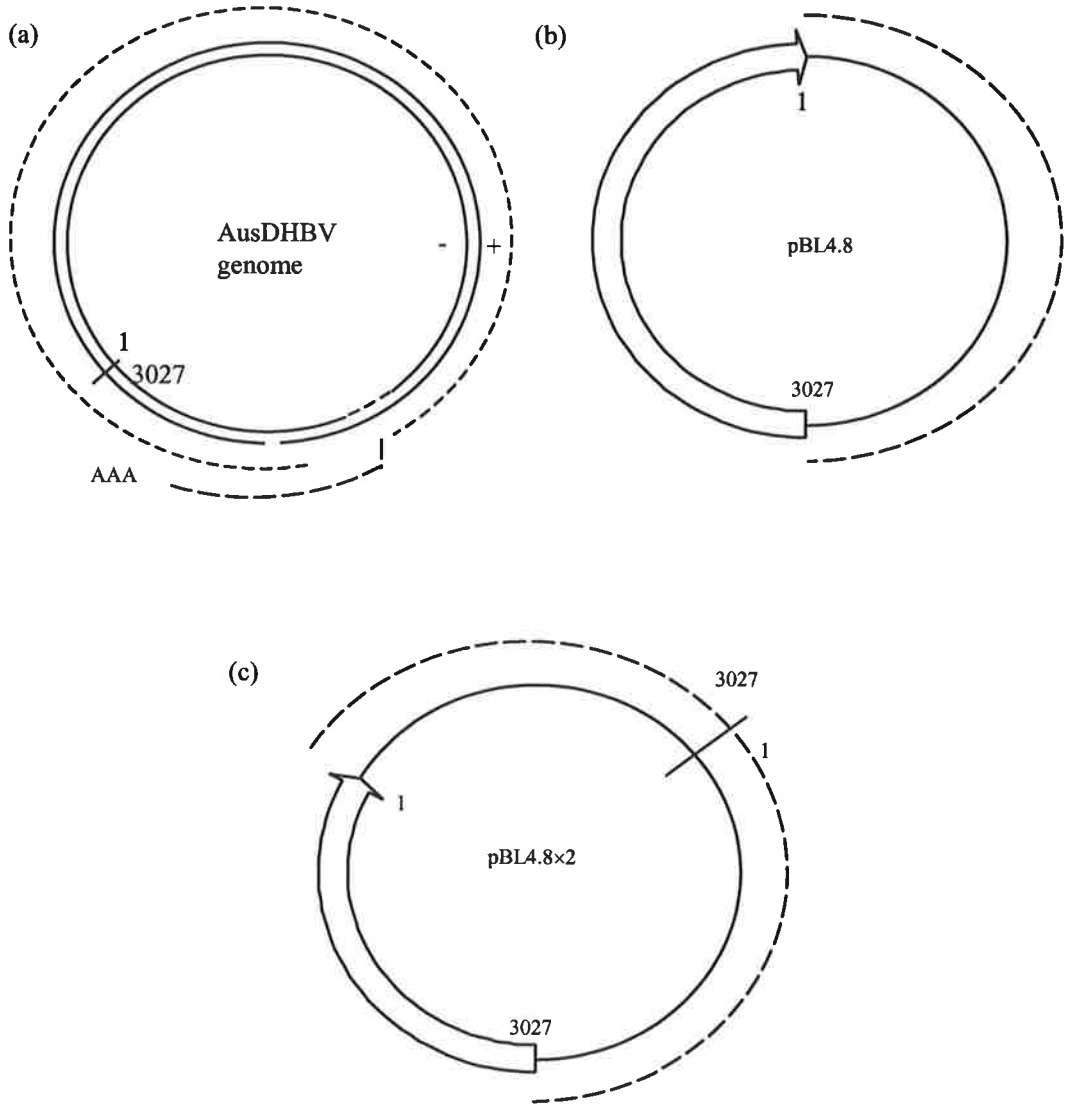


Figure 3.2: Infectious clone of DHBV DNA

DHBV DNA is transcribed to a longer than genome length "pre-genomic RNA" in order for genome replication to occur. (a) The 3.2 kb pre-genomic RNA is shown as a broken line surrounding the 3 kb DHBV DNA. (b) Transcription of a plasmid monomer of DHBV DNA yields RNA of the same length, 3 kb, insufficient for efficient genome replication, however addition of a second copy of DHBV DNA to produce a dimer, (c), enables a longer than genome length RNA to be transcribed as shown by the broken line. The pBluescript plasmid does not contain a promoter for expression of DHBV pre-genomic RNA. Transcription of the viral RNAs including pre-genomic RNA occur using the natural core and pre-S promoters and result in establishment of infection following i.v. or intrahepatic inoculation of plasmid DNA.

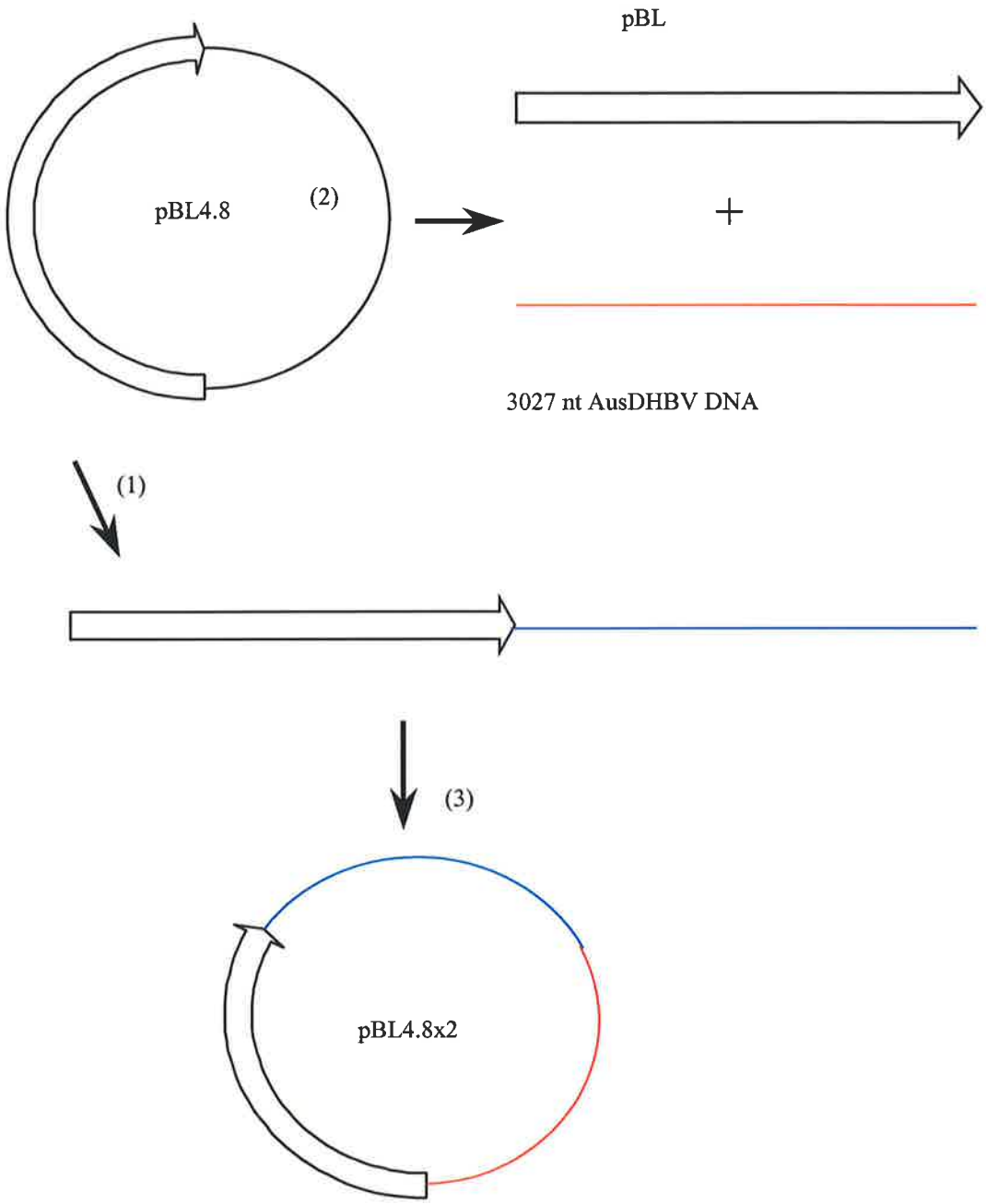


Figure 3.3: Cloning Strategy
(1) A partial digestion to linearise pBL4.8 at the *Eco* R1 restriction site was performed and, in a separate reaction, (2), a complete digestion to excise AusDHBV DNA from pBL4.8. After gel purification, the products were ligated as shown in (3) to produce pBL4.8×2, a dimeric plasmid AusDHBV DNA in a head-to-tail orientation.

the purified plasmid DNA, when measured by UV spectrophotometry was 3.5 μg/μl with a total yield of approximately 350 μg obtained from a 100 ml original culture volume.

Approximately 5 μg of purified pBL4.8 was cut with 1 unit *Eco* R I (Section 2.10.1), to linearise. The reaction was allowed to incubate for only 10 min so that most of the pBL4.8 was cut at 1 of the 2 *Eco* R I sites. In a second reaction, another 5 μg purified pBL4.8 was cut at both *Eco* R I sites by incubating with 25 units of *Eco* R I. After inactivating *Eco* R I, *Sac* 1 was added to cut pBluescript IIKS+ into 2 fragments of approximately 1.5 kb allowing the 3027 nt AusDHBV genome to be separated from the plasmid by gel electrophoresis in a 1% agarose gel.

The linearised pBL4.8 and DHBV genome were purified from 1% agarose gels after electrophoresis using a QIAquick™ Gel Extraction Kit (Section 2.10.1). Electrophoresis of a 0.5 μl aliquot of the DNA on a 1% agarose gel was performed in parallel with a known amount of SPP1 marker DNA. Comparison of the ethidium bromide staining intensity of the DNA bands was used to give an estimate of DNA quantity. The estimated concentration of AusDHBV4.8 was 10 ng/μl and linearised pBL4.8 was 20 ng/μl.

Linearised pBL4.8 was treated with alkaline phosphatase and a second copy of 3027 nt AusDHBV4.8 inserted in a ligation reaction with T4 ligase (2.10.2 and 2.10.3). After desalting, the ligation reaction mix was used for transformation of *E. coli* DH5α by electroporation and 10 white colonies were screened for the presence of plasmid containing 2 copies of full length DHBV DNA. This was done by extracting DNA using a plasmid "mini-prep" (Section 2.10.7) followed by gel electrophoresis and detection of a 9 kb band representing vector+insert as seen in Figure 3.4.

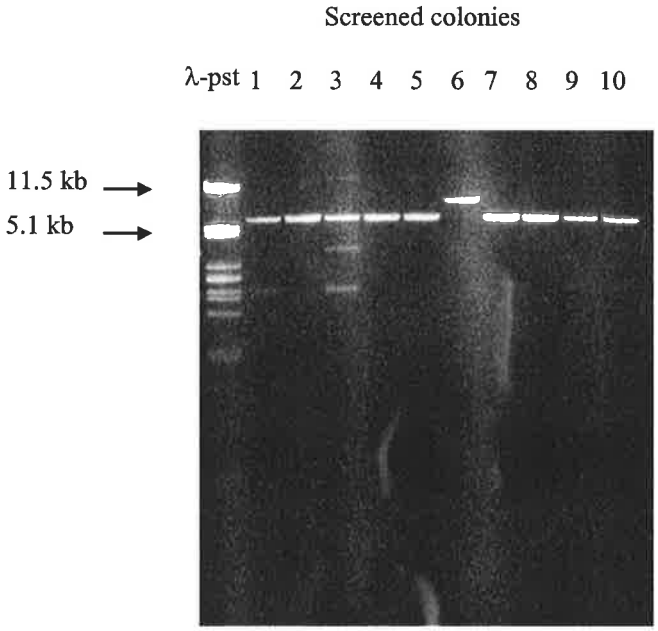


Figure 3.4: The product of the ligation reaction was used for bacterial transformation. After culture colonies were selected ,plasmid DNA was extracted and loaded onto an agarose gel. Electrophoresis of DNA including λ-pst as a molecular weight marker shows DNA prepared from Colony 6 migrating as a 9 kb band indicating insertion of a second copy of the DHBV genome. The remaining samples show plasmid DNA migrating as 6 kb bands, suggesting that religation of linear pBL4.8 has occurred.

The candidate clone was analysed by restriction digestion with *Bgl* II, which is shown and demonstrated the correct orientation of the insert in the candidate clone as shown in Figure 3.5. Sequencing across the region of the ligation between each copy of the AusDHBV genome in pBL4.8×2 confirmed that the sequence was correctly orientated and intact.

3.2.4 Infectivity of pBL4.8×2

PBL4.8×2 DNA was purified from a 500 ml culture of transformed *E. coli* DH5α yielding approximately 760 µg of DNA. Four aliquots of 50 µg of DNA, each equivalent to 5.5×10^{12} dimeric DHBV genomes, were diluted in sterile 0.85% saline to 200 µl. The diluted DNA was injected, half intrahepatically and half i.v. into 2-day-old ducklings from a duck flock free of DHBV.

The ducklings were housed in a pen separate from DHBV-infected ducks and samples of blood were collected for detection of serum DHBsAg by ELISA assay (Section 2.5.1). At day 7 all ducks were negative but on days 13, 20 and 27 sera from 2 of the 4 ducks were positive for surface antigen indicating that DHBV infection could be transmitted via pBL4.8×2.

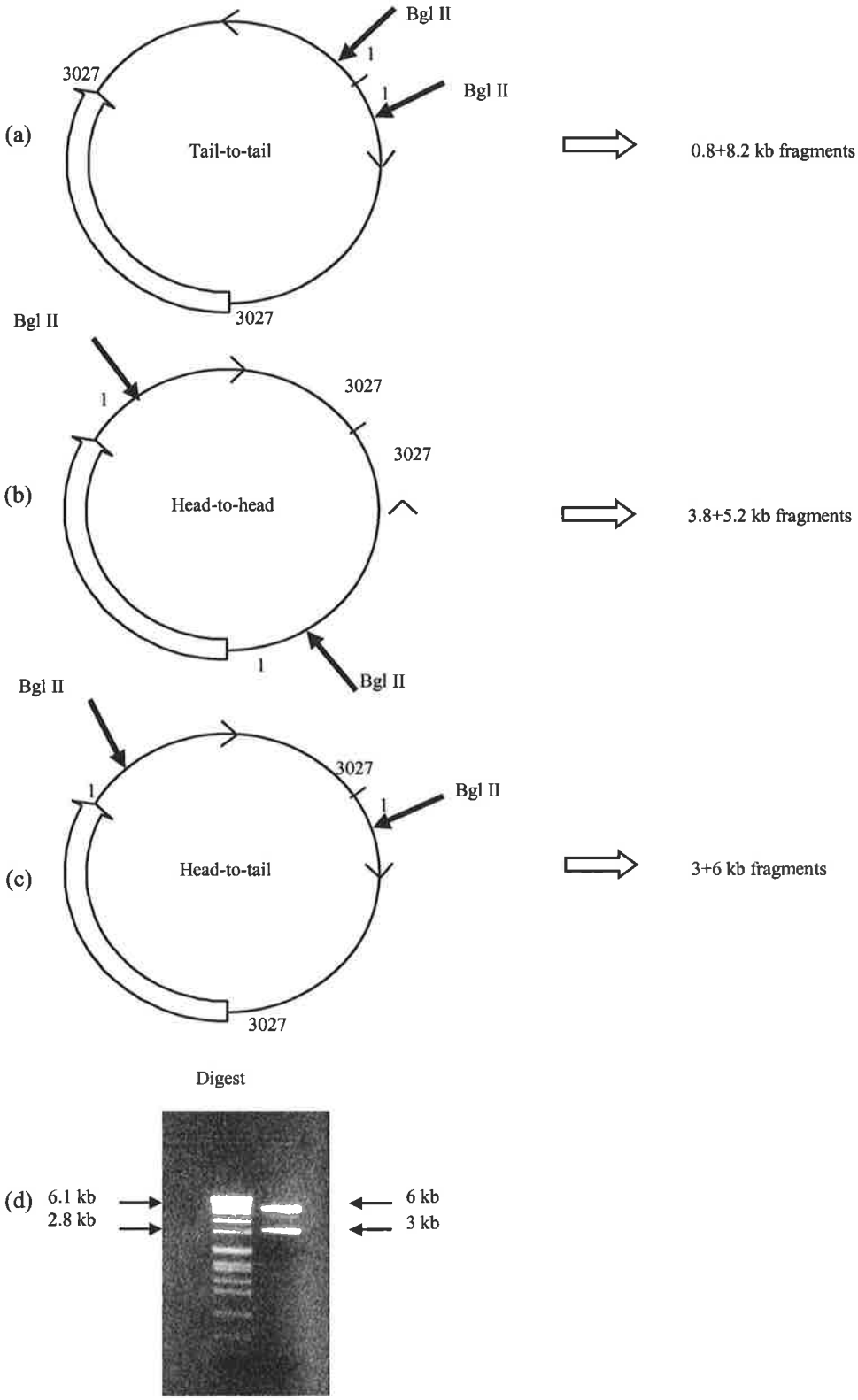
3.3 Nested PCR

3.3.1 Introduction

An outer primer set was chosen targeting a 603 nt segment from nt 2862-437 overlapping the precore-core open reading frame (ORF) which extends from nt 2524-414. Magnesium and primer concentrations were adjusted to 2 mM and 0.4 µM respectively to give maximal amplification efficiency and to minimise primer dimer formation (data not

Figure 3.5: Possible products of *Bgl* II digestion of DHBV DNA dimers in pBluescript II KS+

Plasmid DNA was cut with *Bgl* II, which has a unique restriction site within DHBV DNA but does not cut pBluescript. The digested DNA was analysed by gel electrophoresis. The size of the possible *Bgl* II digest products are shown for each of the 3 possible DHBV dimers. (a), (b) & (c) show possible fragment sizes and (d) actual digestion of plasmid DNA from colony 6, which matches the sizes predicted in (c). This confirms the head-to-tail orientation of DHBV DNA.


(a)
Bgl II
Bgl II
3027
Tail-to-tail
3027
0.8+8.2 kb fragments
(b)
Bgl II
3027
3027
Head-to-head
Bgl II
3.8+5.2 kb fragments
(c)
Bgl II
3027
Bgl II
Head-to-tail
3027
3+6 kb fragments
Digest
(d)
6.1 kb
2.8 kb
6 kb
3 kb

shown). The inner set of primers targeted a 369 bp segment from position 2926-267 within the precore-core ORF.

3.3.2 Results: Sensitivity

The sensitivity of the nested PCR was measured using serial ten-fold dilutions of cloned AusDHBV DNA containing from 10^{-1} to 10^{4} copies. First round PCR detected 10 copies and round 2 PCR detected 1 copy using ethidium bromide gel detection of product (Figure 3.6). When Southern blot hybridization was used to detect PCR products, both assays demonstrated a sensitivity of one copy of template, without contamination of the negative control (Figure 3.6).

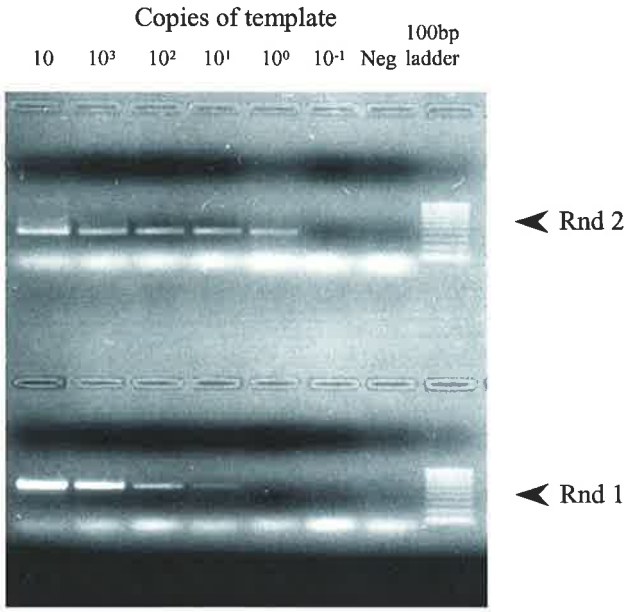
In order to assess the effect of adding non-specific chromosomal DNA, dilutions of AusDHBV plasmid DNA were subjected to PCR with or without the addition of 600 ng of DNA extracted from DHBV-uninfected normal duck liver (NDL). This did not alter the sensitivity of nested PCR detection of the plasmid DNA measured by ethidium bromide staining (not shown) or Southern blot hybridization as shown in Figure 3.7.

3.4 Quantitative PCR

3.4.1 Introduction and Aims

An assay using the Roche Lightcycler was designed to quantify low copy numbers of residual DHBV DNA, to allow comparisons to be made between different experimental situations. This enabled study of different inocula, time-points and comparison of liver and extrahepatic sites.

(a)



(b)

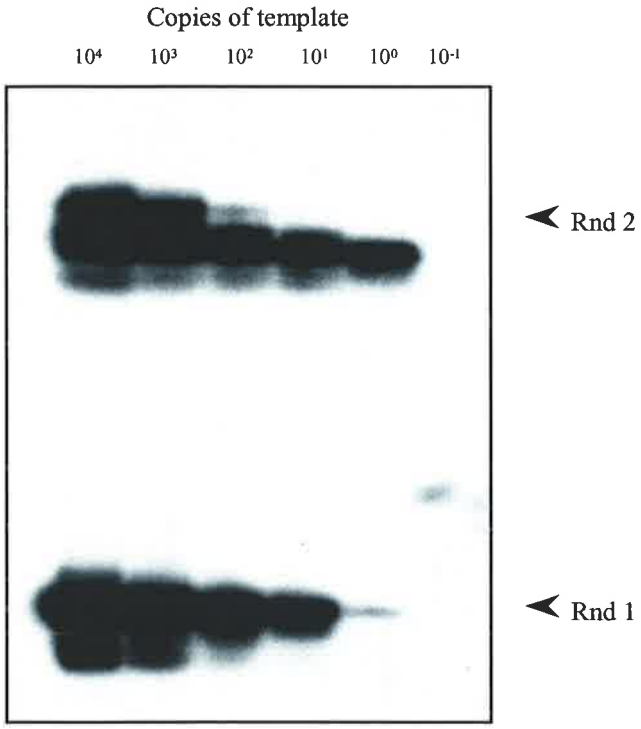


Figure 3. 6: Sensitivity of nested PCR
Nested PCR was performed with primers C1 & C2 for Round 1 and C3 & C4 for Round 2. Titrations of plasmid DHBV DNA were used to determine the sensitivity with detection of product by ethidium bromide and Southern hybridisation.
(a) Ethidium bromide stained gel showing detection of 1 copy DHBV DNA in Round 2.
(b) Southern blot showing detection of 1 copy DHBV DNA in both Round 1 and 2.

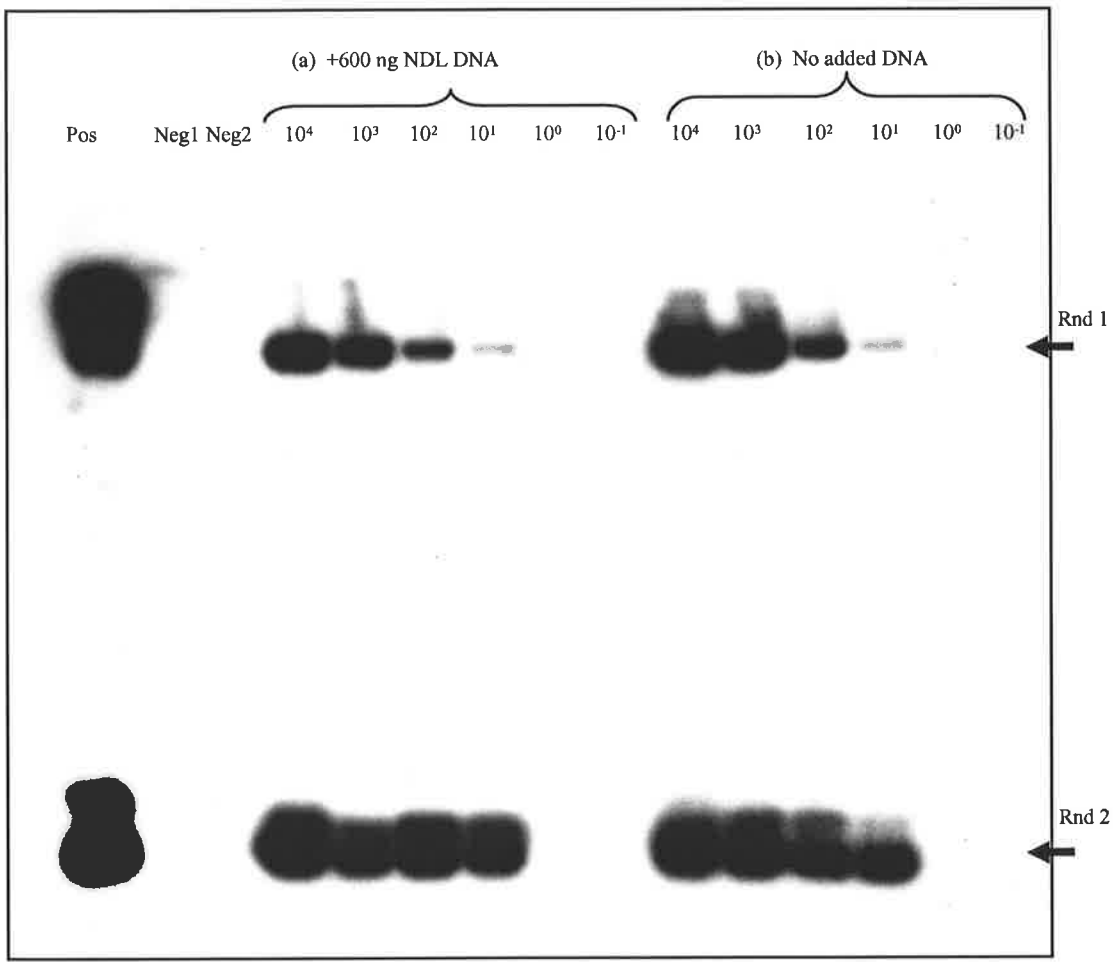


Figure 3.7: Sensitivity of nested PCR with the addition of 600 ng of extracted DNA.
This Southern blot shows the products of nested PCR using plasmid DNA as template at a range of concentrations. In both Round 1 and 2, 10 copies of template are detectable and no change in sensitivity is shown in reactions marked (a) when 600 ng of NDL DNA is added to the reaction. Positive (Pos) and negative controls (Neg1 and Neg2) were included.

Real time PCR is a recently developed method for continuous detection of PCR product throughout the amplification reaction. Conventional PCR does not allow accurate and convenient quantitation since the amount of product at the end of the reaction is not proportional to the quantity of starting template due to a variable reduction in reaction efficiency in the late rounds. This loss of efficiency occurs when PCR substrates are depleted and its timing varies according to the amount of starting template. Useful quantitative data are obtainable by measuring product during 4-5 cycles of log-linear amplification, the timing of which depends on the amount of starting template. An example of the log-linear phase is shown in Figure 3.8(a). Hence the capacity to measure product during every cycle enables quantification of starting template. The method generates a standard curve from fluorescence readings of standards of plasmid DNA of known concentration, plotting the cycle number to reach a "crossing point" (a point just above background), versus the log of the concentration in the standard. An example is shown in Figure 3.8(b).

Unknown samples are compared with controls usually consisting of known copy numbers of plasmid DNA diluted from a stock (previously measured by UV absorbance or dye fluorescence). It is important when seeking to measure absolute copy numbers, rather than performing comparisons, that the reaction conditions in the unknown samples are identical to those of the standards. For this reason NDL DNA was added to plasmid DNA to standardise the quantity of duck DNA in all samples.

In developing this assay several primer sets were tested, one targeting the polymerase region, showing efficient amplification and minimal primer-dimer production was chosen. The reaction conditions were optimised and the sensitivity and accuracy were determined. Primer dimer production can be measured by melting curve analysis as well as by gel

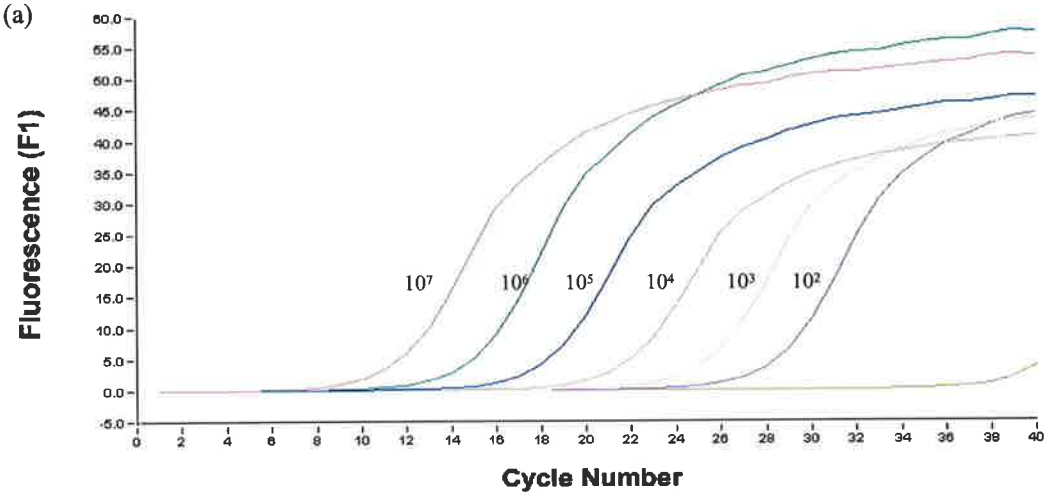
Figure 3.8: Quantitative Real Time PCR using Lightcycler.

(a) Fluorescence is proportional to the quantity of PCR product and increases log-linearly for several cycles during amplification. The cycle number when this occurs is determined by the template copy number as demonstrated in a series of 10 fold dilutions from 10^2-10^7 copies.

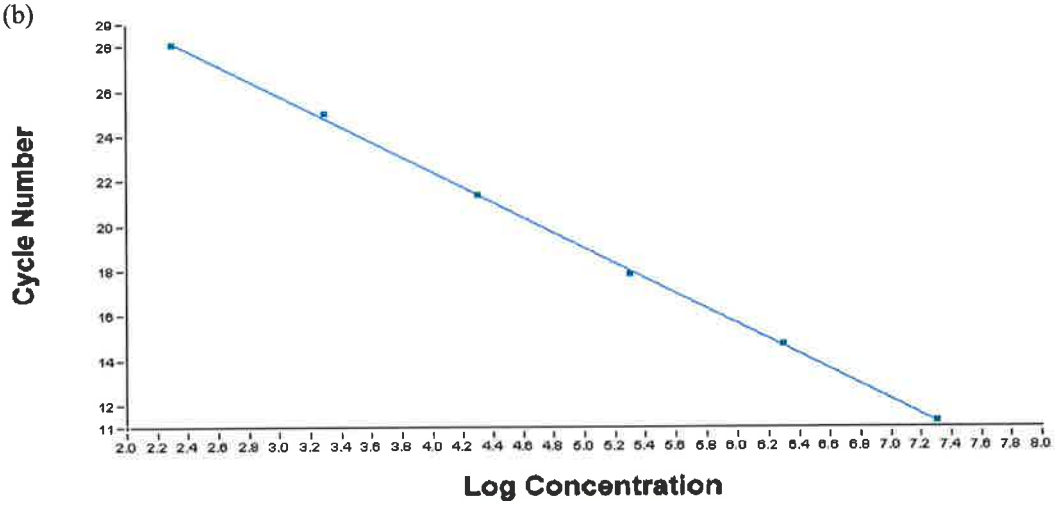
(b) A standard curve of log [concentration of template] vs cycle number to reach a threshold fluorescence level known as the crossing point. The crossing point is chosen arbitrarily, but must be above the background fluorescence level.

(c) Melting peak analysis of PCR products at the completion of cycling measures the melting temperature, which is used to differentiate primer dimer (lower temperature melting peak) from specific products (higher temperature melting peak). The graph shows the melting peaks for the products seen in (a) and (b), each represented as a coloured line.

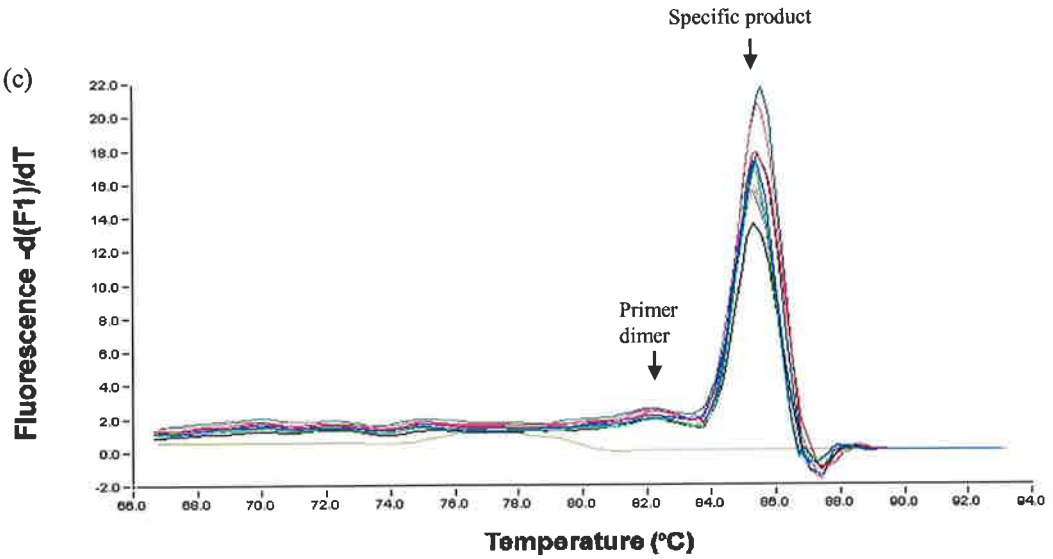
(a)


Fluorescence (F1)
Cycle Number
10⁷
10⁶
10⁵
10⁴
10³
10²

(b)


Cycle Number
Log Concentration

(c)


Specific product
Primer dimer
Fluorescence -d(F1)/dT
Temperature (°C)

electrophoresis. Primer dimers usually denature at a lower temperature than specific PCR product. The fall in fluorescence when the PCR product is heated slowly from 65°C to 95°C may be plotted to produce a melting curve. Temperatures where a rapid fall in fluorescence occurs correspond to denaturation of particular PCR products e.g. primer dimer or specific PCR product. If these data are expressed as rate of decline in fluorescence i.e. the derivative of fluorescence, the temperatures at which rapid decline in fluorescence occurs appear as peaks, i.e. melting peaks. Figure 3.8(c) shows an example of a PCR product melting peak using primers P3 and P4.

It has been reported that quantitative PCR has a reduced accuracy at lower copy numbers, therefore the accuracy and reproducibility of the optimised assay were assessed by measuring coefficients of variation using samples containing approximately 10^2 and 10^3 copies.

3.4.2 Results

3.4.2.1 Primers

Primer sets C1C2, C3C4 and P3P4 (Section 2.8.2) were tested by melting curve analysis (Figure 3.9). The former 2 sets produced significant primer dimers and accurate quantification was not achieved. In contrast, primers P3P4 showed efficient amplification with very little primer dimer production (Figure 3.9(c)).

3.4.2.2 Magnesium

Magnesium concentrations of 2, 3, 4 and 5 mM were examined using 10, 1000 and 100000 copies of plasmid DNA as template and primers P3 and P4. 4 mM was chosen because

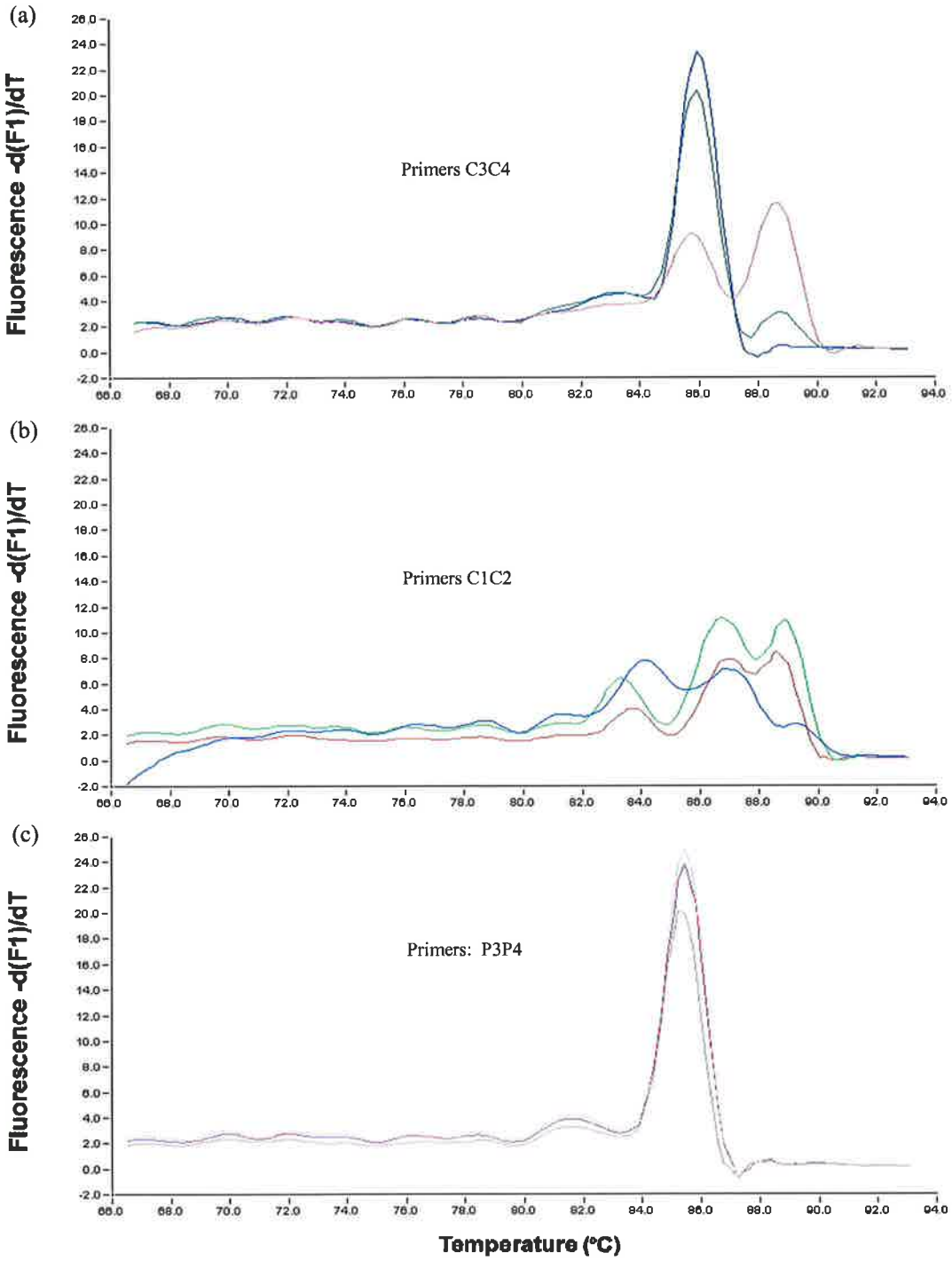


Figure 3.9: Melting peak analysis of PCR products using the 3 primer sets.
Each primer set (C3C4, C1C2 and P3P4) has been used in 3 reactions containing different concentrations of template. (a) and (b) show more than one melting peak – demonstrating non-specific product, e.g. primer dimer formation. (c) shows predominantly specific product as a single melting peak.

this concentration allowed assays to reach the crossing point, or defined threshold fluorescence, at the earliest cycle numbers (see bold figures in Table 3-1).

Table 3-1: Effect of magnesium concentration on PCR

Magnesium Concentration	Template Copy Number	Crossing Point
2	10	39.76
2	1000	29.47
2	100000	21.08
3	10	43.82
3	1000	25.64
3	100000	18.42
4	**10**	**31.03**
4	**1000**	**25.19**
4	**100000**	**17.80**
5	10	33.93
5	1000	25.20
5	100000	17.84

3.4.2.3 Sample DNA Quantity

Next, the quantity of total extracted DNA to be added to each reaction was titrated. Too little DNA would reduce the sensitivity of the assay and too much could inhibit the PCR reaction or increase background fluorescence. The efficiency of the reaction is measured as the slope of the standard curve. In a reaction with perfect efficiency 2 copies of product

are produced for each copy of template in a given round of the reaction. This can be expressed mathematically as the equation - copy number = starting copy number$\times E^n$ - where efficiency, E=2, for a reaction where 2 copies are generated in each round. In practice, a reaction efficiency between 1.5 and 2.2 is achievable; this corresponds to a slope of between –5.7 and –2.9, with a value of ~3.3 signifying E=2 (Anonymous).

Either 60 ng, 120 ng or 300 ng of NDL DNA was added to plasmid standards containing 10^2, 10^4 and 10^6 copies of template and assayed in parallel. The slopes of the standard curves ranged from –3.0 to –3.2, which represents excellent efficiency and suggest that up to 300 ng of extracted DNA can be assayed. 120 ng or 200 ng were used in later assays.

3.4.2.4 Coefficients of Variation

Intra- and inter-assay variation was measured using 2 samples, 1 containing approximately 1000 copies and the other approximately 300 copies of liver derived DHBV DNA. Samples were tested in quadruplicate and the coefficient of variation calculated as the standard deviation divided by the mean and expressed as a percentage.

Sample 1, with mean measured copy number 322 had an intra-assay coefficient of variation of 10% and inter-assay variation of 21%. Sample 2, with a mean copy number of 923 had an intra-assay coefficient of variation of 2.5% and inter-assay variation of 21%.

3.5 Selective and Quantitative PCR for DHBV covalently closed circular DNA (cccDNA)

3.5.1 Introduction and Aims

The presence of cccDNA defines infection in a particular tissue, since the viral genome is converted from RC DNA to cccDNA, only within infected cells. In contrast, detection of RC DNA in material from a particular site may be due to the presence of blood-borne virus rather than true infection of that site. In previous studies, tissue localisation of hepadnavirus infection has been measured by detecting cccDNA by Southern blot (Section 1.13). PCR detection increases sensitivity and may therefore define sites of residual hepadnavirus when only small quantities are present.

Kock and Schlicht in 1993 first described the selective PCR detection of hepadnavirus cccDNA using primers spanning the DR region of the genome (Kock and Schlicht 1993). Subsequently others have improved the selectivity by enzymatic digestion of RC and linear DNA before PCR (Mason *et al.* 1998). The effect of digestion is to deplete the sample of non-cccDNA forms before performing PCR.

The location of CC2 and R2 primers (Figure 3.10) demonstrates targeting the DR region of DHBV. RC DNA is not efficiently amplified because denaturation produces 2 linear strands that do not bind the divergent primers. This strategy is, however, not completely specific for cccDNA amplification as inefficient amplification of RC DNA occurs at high template concentrations. This may be due to repair of RC DNA by Taq polymerase or "priming" of one strand on the other.

Mung bean nuclease (New England Biolabs, USA) has been used to make the assay more selective. This enzyme cuts in single-stranded regions such as the gap in the plus strand of

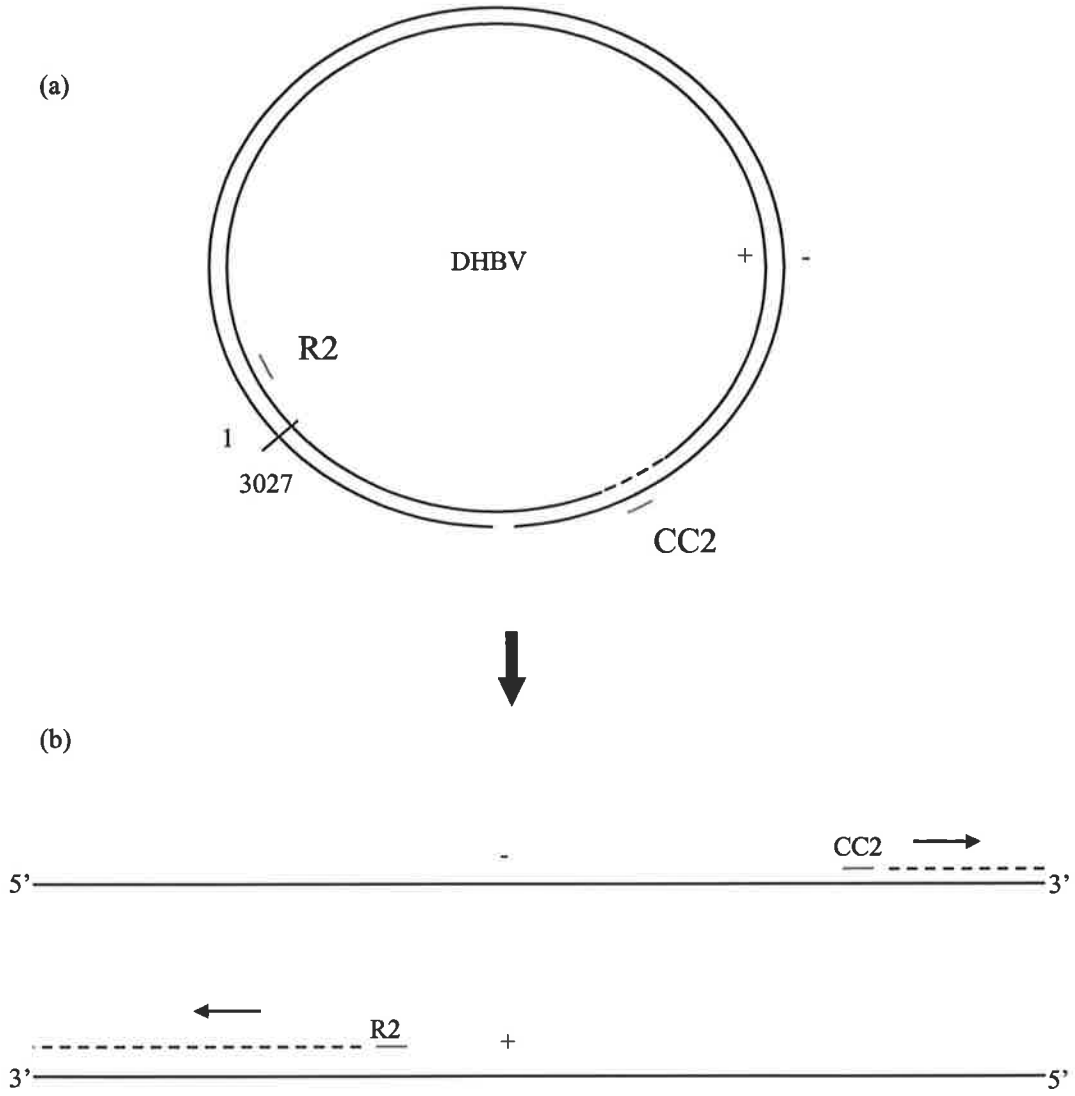


Figure 3.10: PCR primers for selective detection of cccDNA.
(a) Location of the plus and minus strand primers, CC2 and R2, which span discontinuities in the DR region of the RC DNA genome.
(b) PCR after denaturation: primers CC2 and R2 are divergent, each new strand is incomplete and does not provide a location for primer binding in the next round.

HBV. It does not cut at nicks and for this region may be less effective in digesting DHBV RC DNA since, unlike HBV, the plus strand of DHBV is complete in 85% of virions (Lien *et al.* 1987).

In order to increase the selectivity of the PCR for DHBV cccDNA an alternative enzyme, Plasmid-Safe™ ATP-dependent DNase (Epicentre Technologies, USA), was used to digest non-cccDNA. This enzyme cuts double-stranded linear DNA and is used to remove traces of chromosomal DNA from plasmid DNA preparations. In addition, the enzyme cuts single-stranded linear DNA less efficiently and has been used in selective PCR detection of HBV cccDNA since it cuts the single-stranded region caused by the incomplete plus strand of HBV (Bowden 2000).

A selective and quantitative PCR assay for DHBV cccDNA was developed using primers spanning the DR region of the genome and enzymatic digestion of non-cccDNA; this is described and evaluated in the following section.

3.5.2 Results

3.5.2.1 Primer selection and annealing temperature

Three sets of primers were evaluated in PCR reactions using plasmid and liver DNA as template. They included a common plus strand primer, CC2 and 3 different minus strand primers - R1, R2 and R3. The primers R1, R2 and R3 were designed with the assistance of Andreas Nitsche of Tib Molbiol (Berlin, Germany). As can be seen in Figure 3.11 (a), non-specific product was seen with all sets, but CC2 and R2 showed the most efficient amplification and were therefore selected for further development. Raising the annealing temperature from 52°C to 58°C led to loss of the non-specific bands (Figure 3.11 (b)).

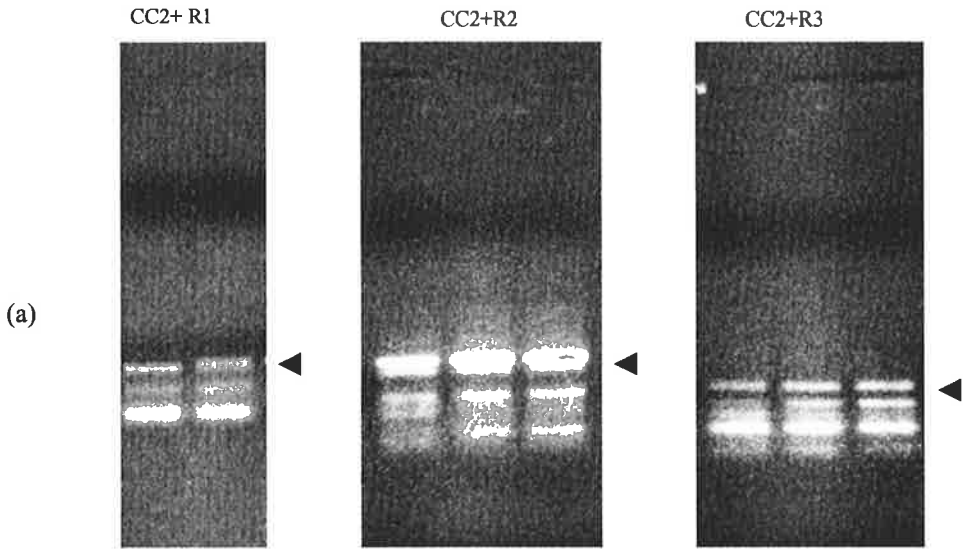


Figure 3.11: PCR primers for selective detection of cccDNA.

Comparison of primers showing non-specific amplification. In each case the specific product is shown by an arrow (a), CC2+R2 primers showed relatively efficient amplification compared with the 2 other primer combinations tested. (b) Increasing the annealing temperature to 58°C resulted in CC2+R2 showing loss of non-specific amplification products.

Use of these primers on the Lightcycler demonstrated satisfactory amplification of 10 copies of plasmid DNA, with less primer dimer formation at 58°C than at 52°C (Figure 3.12). The annealing temperature was subsequently increased further to 60°C.

3.5.2.2 Magnesium

Magnesium concentrations from 3-5 mM were tested and 4 mM was selected (data not shown).

3.5.2.3 Specificity of PCR for cccDNA

To compare amplification of cccDNA and RC DNA, plasmid DNA (pBL 4.8×2) was used in place of cccDNA. It was assumed that the double stranded circular plasmid DNA, which contains the same target sequence as DHBV cccDNA, would amplify with similar efficiency to cccDNA. Since cccDNA preparations contain small amounts of other forms of DHBV DNA, such DNA was unsuitable. RC DNA was obtained by extracting DHBV DNA from the serum of congenitally AusDHBV-infected ducks. Initially the sensitivity of PCR amplification of both forms of viral DNA was measured.

Firstly, the concentration of plasmid pBL4.8×2 DNA was measured by dye fluorescence and UV absorption. These gave similar results of approximately 2000 ng/µl. A single DHBV DNA genome has a mass of 3×10^{-6} pg, hence -

concentration in copies per µl = concentration in ng per µl / mass of 1 genome in ng

$= 2000/3\times10^{-9}$

$= 6.67\times10^{11}$ copies/µl

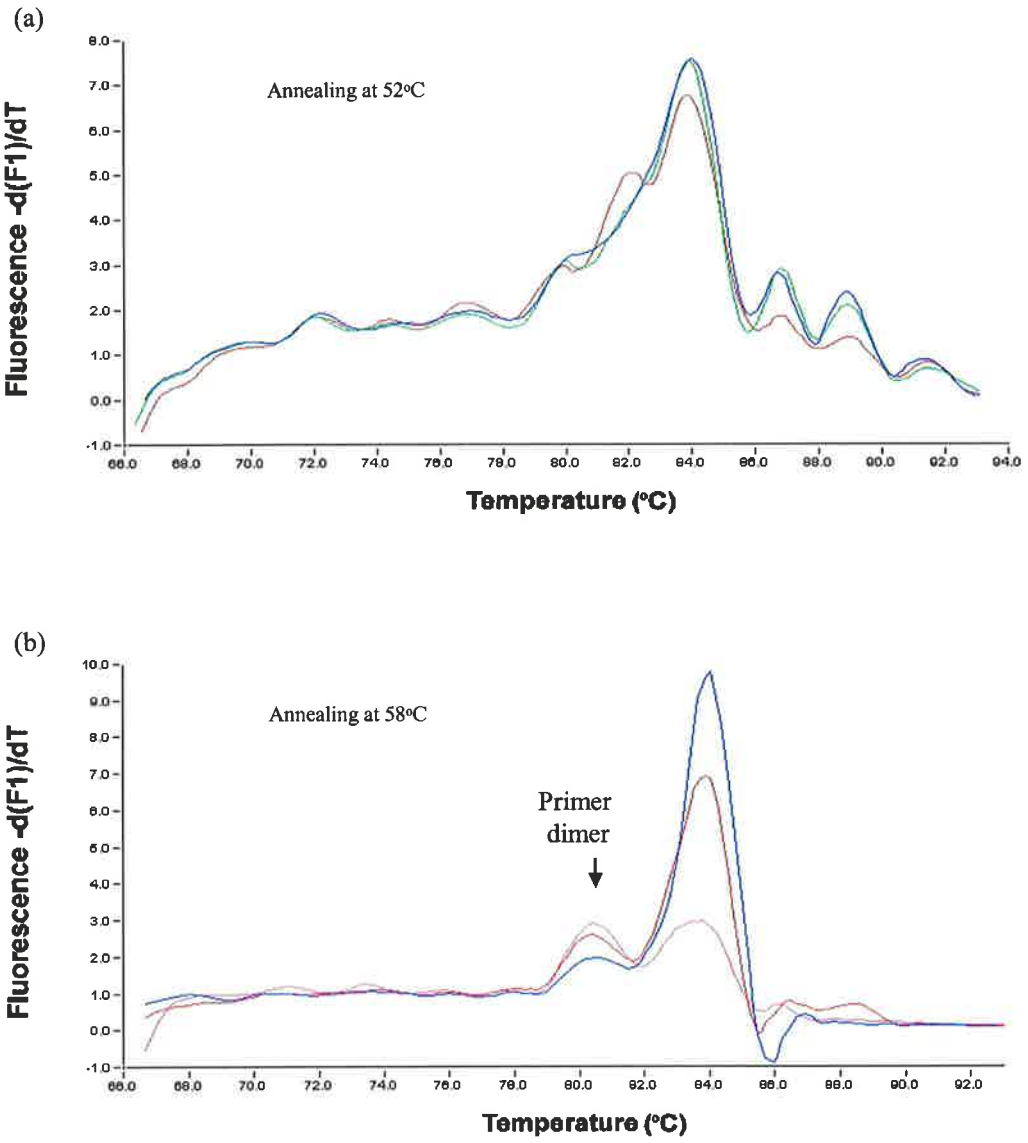


Figure 3.12: Comparison of PCR product melting peaks at 2 annealing temperatures- Different annealing temperatures were evaluated and one chosen with minimal amplification of non-specific product.
(a) Several melting peaks are seen when annealing at 52°C i.e. non-specific amplification
(a) Reduction of primer dimer and other non-specific products by increasing the annealing temperature to 58°C.

Then the sensitivity of the PCR assay for cccDNA was measured by assaying 10-fold dilutions of plasmid with cccDNA primers. Dimeric plasmid DNA contains only one copy of the target DNA sequence for the primer set CC2/R2 because the primers span the *Eco* RI restriction site as demonstrated in Figure 3.1. The results of one such assay performed with conventional PCR (Figure 3.13) demonstrated detection of 2 copies of plasmid DNA template.

In order to measure the sensitivity of detection of RC DNA a stock of RC DNA was prepared from serum using the Roche HighPure Kit method for extraction (Section 2.7.3). The concentration of RC DNA in the serum extract was then assayed by quantitative PCR using primers P3 and P4, which target the polymerase region and efficiently amplify RC DNA. The concentration was measured at 2×10^8 copies/µl of extract. 10-fold dilutions of this stock were then assayed using cccDNA primers. Product from this reaction was detectable in an ethidium bromide stained gel when 2×10^5 copies of template were used in conventional PCR format (Figure 3.13).

A similar comparison of sensitivities was made using cccDNA primers on the Lightcycler. The sensitivity of detection of plasmid DNA was ≤10 copies, but using 10-fold dilutions of RC DNA from serum, 4×10^4 copies of template were required to produce detectable PCR product (result not shown graphically). Thus, there was at least 10^3 greater efficiency in amplification of cccDNA compared with RC DNA.

When higher copy numbers of template were present, inefficient amplification of non-ccc DHBV DNA forms was also seen. To assess whether this would produce significant inaccuracy in measurement of cccDNA in samples with moderate to high copy numbers of DHBV DNA (i.e. $\geq10^4$ copies), samples containing known copy numbers of serum-derived

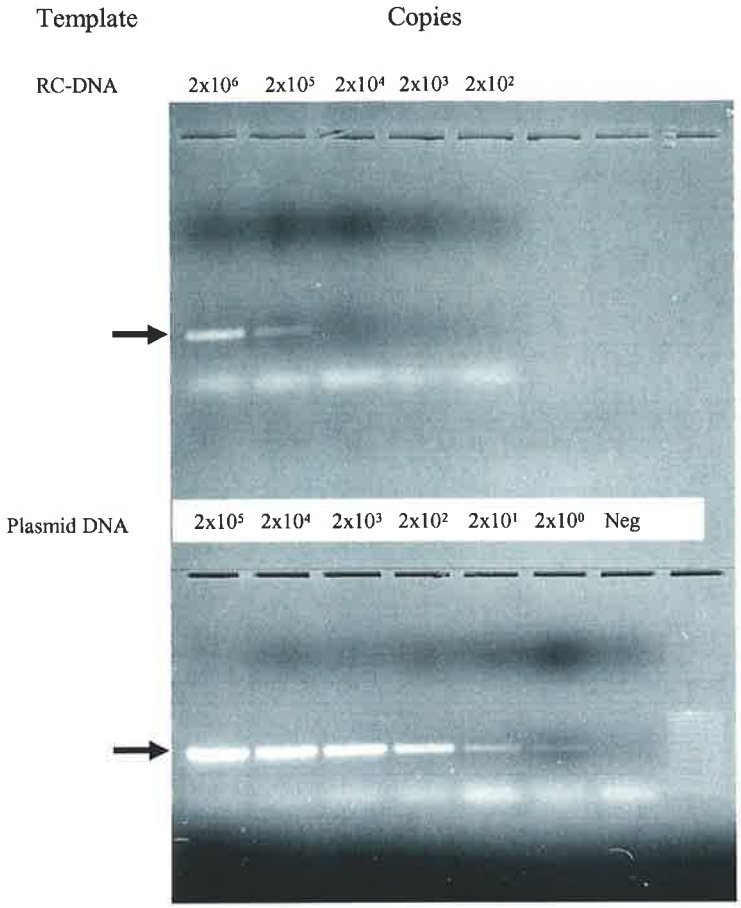


Figure 3.13: Specificity of selective PCR amplification of cccDNA.
The upper part of the gel shows inefficient amplification of serum-derived RC DNA with the PCR product indicated by an arrow. Using RC DNA as template $2x10^5$ copies are required for detection of product. The lower part of the gel shows efficient amplification of plasmid DHBV DNA used in place of cccDNA with 2 copies being detectable and indicated with an arrow.

RC DNA were assayed by cccDNA PCR and compared to the results for standards containing 10^1, 10^2, 10^3, $10^4$and 10^5 copies of target plasmid DNA. The results in Table 3.2 demonstrate an approximately 10^4-fold reduction in apparent copy number with RC DNA compared to cccDNA, while with cccDNA, the assay response was linear at template copy numbers up to 2×10^7. Hence the assay appears specific for cccDNA at or below 2×10^4 copies and highly selective for cccDNA at higher template copy numbers.

The absence of detectable product when 2×10^4 or fewer copies of RC DNA were used as template for cccDNA PCR suggests that the assay can be used for selective detection of cccDNA when low copy numbers of DHBV DNA template are present. This was confirmed by adding 10^3 copies of RC DNA template to a sample containing ~180 copies of plasmid DNA and performing quantitative PCR. No increase in the measured copy number was produced; showing that quantification of cccDNA was not affected by the presence of low copy numbers of RC DNA .

Table 3-2: Inefficient PCR amplification of RC DNA using selective cccDNA primers.

Sample	Copies of RC DNA	Quantitation using cccDNA PCR
1	2×10^7	4.7×10^2
2	2×10^6	8×10^1
3	2×10^5	+ (<10 copies)
4	2×10^4	Negative
5	2×10^3	Negative

3.5.2.4 Quantitation and coefficient of variation

The intra-assay and inter-assay coefficients of variation were measured by repeat sampling of specimens in triplicate or quadruplicate. Intra-assay variation was 15.5 and 35% using 2 samples measuring 1141 and 195 mean copy number. Inter-assay variation was 12.7% for a sample measuring 212 mean copy number and 24.6% for a sample measuring 1787 mean copy number.

3.5.2.5 Enzymatic Digestion of DNA

In order to further increase the specificity of the assay digestion with Plasmid-Safe™ ATP-dependent DNase was used to deplete samples of non-cccDNA. The reason was to allow accurate quantitation of cccDNA in the setting of high-level infection when large amounts of RC and linear DHBV DNA are present in the liver and might contribute appreciably to measurements of cccDNA despite being inefficiently amplified.

The activity of the enzyme was tested using extracted RC DNA from serum, plasmid DNA in place of cccDNA, and extracted liver DNA. Initially the digestion was carried out using 5 units of enzyme in a volume of 20 µl incubated for 30 min at 37°C and inactivated by incubation for 30 min at 70°C. The effect on DNA was measured by quantitative PCR using primers P3 and P4.

The initial experiments, which demonstrate the activity of the enzyme, are summarized in Table 3-3. In the first experiment plasmid DNA was heated to 95 °C for 10 min and then incubated with the DNase enzyme as above. Quantitative PCR of treated and untreated plasmid DNA showed no reduction in measured plasmid DNA copy number – hence demonstrating a lack of digestion of double-stranded circular DNA with the experimental protocol.

In the second experiment duplicate samples of RC DNA were prepared and one was then pre-heated to 95°C to dissociate double-stranded DNA. Both samples were then incubated with enzyme as described above and an aliquot of each was subjected to quantitative PCR. The effect of dissociating RC DNA into single strands would be to produce template for digestion by Plasmid-Safe™ enzyme, which does not cut nicked DNA. Heating and cooling RC DNA leaves single-stranded DNA, unlike cccDNA that reanneals when cooled (Newbold *et al.* 1995). The copy number of RC DNA in samples and controls was measured by quantitative PCR after digestion +/- pre-heating.

In the third experiment 2 samples of 1 μg of extracted liver DNA were treated with pre-heating and enzyme digestion and the PCR quantitation of viral DNA compared with untreated controls. This showed no significant reduction in DHBV DNA with the protocol, despite the fact that the protocol was effective in digesting RC DNA.

Table 3-3: Effect of Plasmid-Safe™ digestion

Experiment	Sample	Copy number	Conclusion
1 Plasmid	Plasmid pBL4.8×2 heated and digested	1.99×10^{6}	No effect of digestion on plasmid DNA
	Plasmid pBL4.8×2 untreated	1.72×10^{6}	
2 RC DNA	RC DNA heated and digested (Starting copy number 1.75×10^{7})	9.32×10^{3}	Approx. 3 log reduction in RC DNA with pre-heating and digestion
	RC DNA digested alone (Starting copy number 1.75×10^{7})	2.48×10^{6}	Approx. 1 log reduction in RC DNA with digestion alone
3 Liver DNA	Liver DNA sample 1 heated and digested	4.61×10^{4}	Little or no effect of digestion
	Liver DNA sample 1 untreated	6.00×10^{4}	
	Liver DNA sample 2 heated and digested	1.56×10^{5}	Little or no effect of digestion
	Liver DNA sample 2 untreated	2.82×10^{5}	

3.5.2.6 Summary of Experiments 1-3

The combination of pre-heating DNA followed by digesting DNA with Plasmid-Safe™ enzyme did not degrade circular double-stranded DNA represented by plasmid DNA, but reduced the copy number of RC DNA by approximately 10^3-10^4-fold. Digesting without heating was ineffective in removing RC DNA. When extracted DNA containing a mixture of cccDNA, RC DNA and cellular DNA was digested after heating little or no effect was seen. This suggested that insufficient time or enzyme was used.

3.5.2.7 Titration of Template DNA in Plasmid-Safe™ DNase Digestion

In order to confirm the finding of a lack of effect of digestion on 800-1000 ng of extracted DNA another 3 samples were tested in a separate experiment. Pre-heating and digestion with 10 units of enzyme for 30 min did not decrease the copy number measured by PCR as shown in the following table.

Table 3-4: Lack of effect of digest on 800-1000 ng of extracted DNA

Sample	Treatment	Copy number (PCR)
1	Heated/digested	1.68×10^6
	Nil	8.29×10^6
2	Heated/digested	2.54×10^7
	Nil	3.67×10^7
3	Heated/digested	4.03×10^6
	Nil	4.89×10^6

It was hypothesised that cellular DNA was acting as a substrate for the Plasmid-Safe™ enzyme and that the presence of larger amounts of cellular DNA was competing with and

hence reducing the digestion of viral DNA. To improve the effectiveness of the digestion protocol the incubation time was increased to 2 hr and the effect of digestion was measured on a range of starting template quantities by taking a fixed quantity of RC DNA to which was added NDL DNA as summarized in Table 3-5. These show that maximal effectiveness of digestion of non-cccDNA occurs with up to 160 ng of added DNA. This quantity was therefore used in subsequent digests.

Table 3-5: Titration of the amount of NDL DNA in Plasmid-Safe™ digests

Sample	Copy number by PCR (P3P4)
RC DNA undigested	1.67×10^{6}
RC DNA + 800 ng NDL DNA digested	8.15×10^{4}
RC DNA + 160 ng NDL DNA digested	2.37×10^{2}
RC DNA + 40 ng NDL DNA digested	4.12×10^{2}
RC DNA + 16 ng NDL DNA digested	3.18×10^{2}

The next experiment was to confirm that the revised Plasmid-Safe™ enzyme protocol does not digest double stranded circular DNA and therefore should leave cccDNA intact. PBL4.8×2 was heated and treated with Plasmid-Safe™ DNase before measuring copy number by PCR. The untreated sample showed 3.91×10^{7} copies and the treated sample 7.36×10^{7} copies, thus demonstrating no loss of double-stranded DNA template.

3.6 Discussion

The development of PCR assays for DHBV DNA required construction of a head-to-tail dimer of DHBV DNA inserted into pBluescript IIKS+, which was to be used as a quantitative standard in the PCR assays. This also provided an infectious clone of DHBV DNA which, injected into 2-day-old ducklings, led to infection of 2 out of 4 ducklings. This rate of infection is comparable to that described by Tagawa *et al* (Tagawa *et al.* 1996) and demonstrates that the genome of this Australian DHBV strain, AusDHBV, is infectious (Triyatni *et al.* 2001).

To create a standard of known concentration a plasmid preparation of pBL 4.8×2 was prepared and its concentration measured using a fluorescent marker (Section 2.7.4) and diluted to a working stock of 90 ng/µl or 10^{10} copies/µl, which was checked by spectrophotometry. The 2 measurements showed a high degree of consistency (data not shown), which suggested that both methods provided adequate accuracy for measurement of plasmid DNA. Standards for quantitative PCR were prepared freshly from the stored working stock for each PCR run, a method which gave superior reproducibility to the use of frozen diluted aliquots in preliminary experiments (results not shown).

The purpose of the nested PCR assay described was to provide high sensitivity and specificity. The assay detected down to a single copy of cloned DHBV DNA. Rigorous techniques to minimise contamination were employed and the success of these methods documented by use of negative controls for PCR mix and template. A negative control omitting template is frequently overlooked in studies relying on PCR results, but was particularly important for work emanating from a laboratory in which high titre virus and cloned DNA were handled, although DNA extraction and preparation of PCR mix were performed in separate laboratories.

The quantitative PCR assay for DHBV DNA depended on finding a set of primers which amplified efficiently with minimal primer dimer production. Primer dimers bind Sybr Green dye and can compete with template for primer binding as well as contributing to the measured fluorescence. Since both competition for amplification and additional fluorescence are present in the samples as well as the standards this does not, in theory, prevent accurate quantitation, but in practice the effects are variable and the error in standard curves increases with some primer sets. The standard primer design software (Primer Design™) used for conventional PCR did not predict primers that did not produce excessive primer dimers, for reasons which were not entirely clear, but may relate to the relatively high primer concentrations used in the Roche Lightcycler. Satisfactory primers were found by trial and error and required adjustment of the annealing temperature

Reproducible quantitation also required careful dilution of fresh standards with every assay. The accuracy of dilution of standards may be expressed as the mean squared error in the standard curve of measured copy number vs cycle number to reach a particular level of fluorescence (the crossing point, see Section 2.8.4). The mean squared error in standard curves used was less than 0.25 in all cases and less than 0.1 in the majority, where an error of 0.6 correlates to an error of 50% in the measured copy number in a standard tube.

By inclusion of NDL DNA into the standards, the efficiency of the PCR reaction with standards should mirror that in the test samples. The amount added was titrated to ensure the reaction was not overloaded, which would lead to reduced efficiency and lower accuracy. The method described performed with satisfactory reproducibility at low template copy numbers. The coefficients of variation were higher than those described (5-10%) for high template concentration (Nitsche *et al.* 1999) but lower than some

unpublished reports when measuring low copy numbers (30-50%, Dr Warwick Fear, Roche Biochemicals Australia, personal communication).

It must be remembered, however, that measurement of the true copy number per cell depends on other factors than the PCR – i.e. DNA extraction efficiency varies from sample to sample and pipetting error effects the quantity of template and DNA loaded for PCR. Not all of these variables are readily quantified and quantitative PCR results should be interpreted accordingly. Most of these sources of error will be reduced when a single sample is tested repeatedly with one assay or with 2 different PCR assays – e.g. using 2 different primer sets.

The design of a selective PCR for cccDNA was based on work described elsewhere using conventional PCR (Kock and Schlicht 1993; Stoll-Becker *et al.* 1997; Mason *et al.* 1998). The method used here showed 10^3-10^4 fold more efficient amplification of cccDNA than RC DNA. In order to demonstrate this, plasmid DNA was used since it is covalently closed circular and can be accurately quantified. The method was specific when low copy numbers ($<10^4$) were present, but at high copy numbers inefficiently amplified non-cccDNA could be detected. This was at levels which would not be expected to compromise accuracy of a quantitative PCR, but could be of significance in qualitative PCR.

To further enhance specificity for cccDNA, enzymatic digestion of template was used to deplete samples of non-cccDNA including tissue DNA. This improved the specificity of the assay by approximately 10^3 fold and allowed further analysis of the form of residual DNA, particularly since digestion of tissue DNA would include any integrated viral DNA.

Overall if $\leq 10^{6-7}$ copies of DHBV template were present in the starting sample, the combination of selective primers and digestion of non-cccDNA means that specific detection of DHBV cccDNA was possible in the setting of high-level infection. In the experiments described in the next chapter the accuracy of the assay was tested by comparison to Southern hybridization in acute and congenital infection.

4. Tissue Localisation of DHBV in Congenital and Experimental Infection

4.1 Introduction and Aims

Previous work in this laboratory and elsewhere has defined the tissue distribution of DHBV infection in congenitally infected ducks. The findings (Section 1.13), along with data from WHV and HBV infection, show the presence of antigen in liver including hepatocytes and bile duct epithelial cells as well as spleen, kidney, pancreas and adrenal tissue (Halpern *et al.* 1983; Halpern *et al.* 1984; Halpern *et al.* 1985; Jilbert *et al.* 1987; Walter *et al.* 1991). DHBV DNA has also been detected by Southern hybridization or *in situ* hybridization in those sites, and in addition, a single report has described DHBV DNA in lung, heart, brain and intestine (Hosoda *et al.* 1990). Dot-blot hybridization of DNA extracted from PBMC showed low levels of DHBV DNA after experimental inoculation, but Southern blot hybridization of PBMC for DHBV DNA was negative (Freiman *et al.* 1988) and PBMC from ducks with congenital infection were not studied.

One purpose of studying the tissue distribution of DHBV in this project was to define the tissue distribution of DHBV antigens and DNA in the setting of congenital and experimental infection. This would include similar methods to those used previously as well as PCR detection and would be expected to show a likely maximal range of tissues infected with DHBV and allow targeting of specific tissues for detection of residual DHBV DNA.

As part of this, the distribution and quantity of DHBV DNA was assessed in the early period of active viral replication following experimental infection, for comparison with the findings for residual DHBV DNA described in Chapter 5 and with findings in congenital infection.

Most previous work involves the study of congenitally DHBV-infected ducks in which >95% of hepatocytes are infected and the ducks have high-levels of viraemia. Transient experimental infection in this study may not be as widespread within the liver and may or may not be associated with viraemia. It has been reported that DHBV appearance in extrahepatic tissues occurs later than appearance in liver (Tagawa *et al.* 1985; Jilbert *et al.* 1988; Walter *et al.* 1991; Jilbert *et al.* 1996) and this could be explained in part if initial amplification of infection in liver was required before the development of viraemia and spread to other organs. Furthermore immune responses to congenital infection are likely to be quantitatively and qualitatively different to the responses to infection at a later age. For these reasons experimental infection may set up different tissue distributions and levels of virus from congenital infection, which in turn may affect the extent of residual infection.

A third reason for assessing viral markers in both congenitally DHBV-infected ducks and in the early phase following a limited experimental DHBV infection was methodological. PCR, which is required for detection of residual DHBV DNA, has not been used in previous studies of tissue distribution of DHBV. A positive result by PCR may be subject to greater errors than a positive by Southern blot hybridization, and there may be significant differences in quantitation using the 2 methods. The work described in this chapter allowed comparison of results using the 2 methods to test the same material, and assisted in interpretation of the results found in later chapters.

The specific aims of the work outlined in this chapter were to –

1. Compare the tissue distribution of DHBsAg expression in congenitally and experimentally DHBV-infected ducks.
2. Compare the tissue distribution of DHBV DNA using Southern blot hybridization and PCR in congenitally and experimentally DHBV-infected ducks.

3. Compare quantitative PCR and Southern blot hybridization for quantitation of DHBV DNA.

4.2 Tissue Distribution of DHBsAg by Immunohistochemistry

4.2.1 Congenitally Infected Ducks

Three congenitally DHBV infected ducks were sacrificed at 26 days post-hatching and (Section 2.2.7) liver, spleen, kidney, pancreas, adrenal, heart and skeletal muscle were collected at autopsy and fixed in ethanol/acetic acid. Antigen retrieval of sections (Section 2.6.2) and immunohistochemical detection of DHBsAg with an anti-PreS monoclonal antibody (Pugh *et al.* 1995) were performed (Section 2.6.3).

All 3 ducks showed >95% of hepatocytes positive for DHBsAg. Antigen was also seen in scattered pancreatic cells in all 3 ducks, and in all kidney sections. Because of insufficient tissue, adrenal sections were available from only 2 ducks, one of which showed staining for DHBsAg although it was not clearly cellular in origin. The presence of staining which was not clearly cellular was considered to be an equivocal result. An equivocal result was also observed in myocardium in 1 out of 3 ducks, the other 2 ducks were negative, but spleen and skeletal muscle from all 3 ducks showed no DHBsAg staining. Representative sections from all tissues studied are shown in Figure 4.1.

4.2.2 Experimentally infected ducks

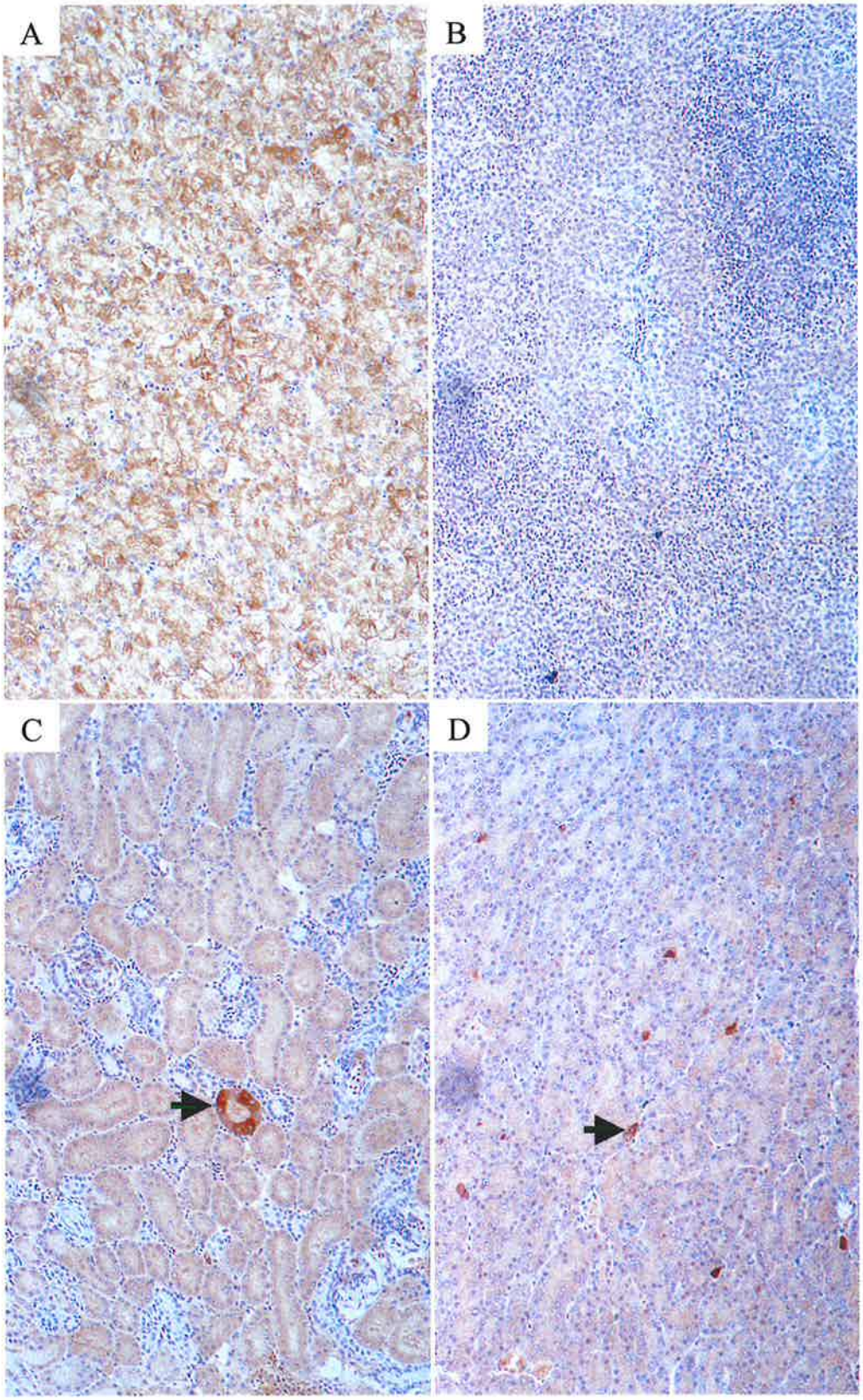
A group of three 40 day-old ducks were inoculated intravenously with 10^{10} DHBV virions. The age of the ducks and viral dose were identical to those used in chapter 5 to set up transient DHBV infection. After 3 days the ducks were sacrificed and liver, spleen,

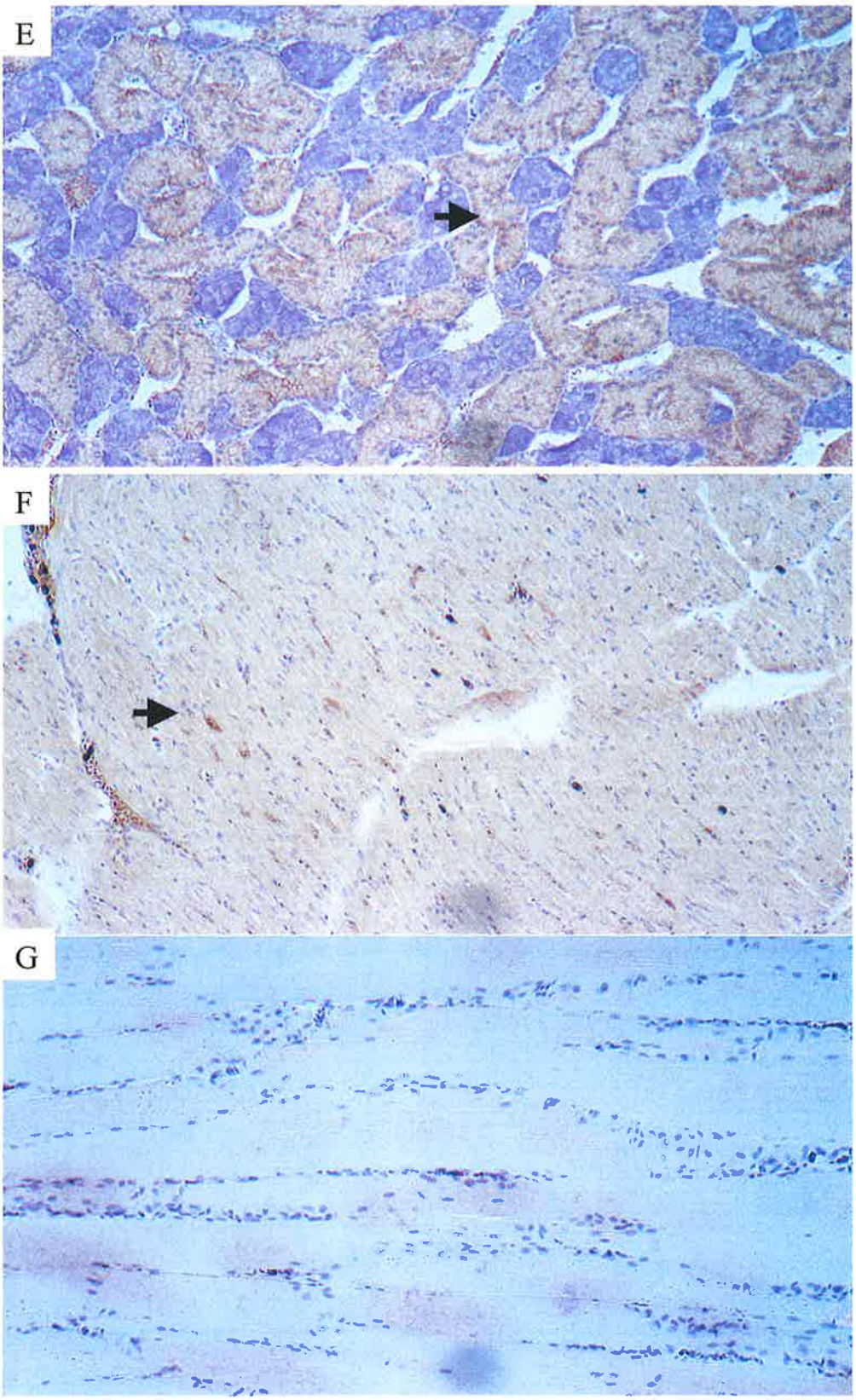
Figure 4.1: DHBsAg detection in tissues from ducks with congenital DHBV infection at 26 days post-hatching.

Immunohistochemical detection of DHBsAg was performed on ethanol:acetic acid fixed tissues using monoclonal antibodies specific for pre-S (1H1, Pugh *et al*) (Section 2.6.3). Tissues were counterstained with haematoxylin and photographed at 200x magnification.

A: Liver section from duck 11341135 showing >95% of hepatocytes staining positive for DHBsAg; B: Spleen tissue from duck 11341135 with no detectable DHBsAg staining; C: Kidney tissue from duck 11341135 with a single tubule, indicated by an arrow, staining for DHBsAg; D: Pancreatic tissue from duck 11341135 with scattered DHBsAg-positive cells.

On the following page: E: Adrenal tissue from duck 11391150 with DHBsAg-positive cortical cells; F: A section of heart tissue from duck 11391150 with staining of DHBsAg; G: Skeletal muscle tissue from duck 11341135 with no detectable DHBsAg staining.


A
B
C
D


E
F
G

kidney, pancreas, adrenal, heart and skeletal muscle were collected at autopsy, fixed and examined exactly as those in Section 4.2.1.

Liver sections from each of the 3 ducks showed DHBsAg in 2.1, 3.5 and 1.7% of cells respectively (mean 2.4%), as measured by counting antigen-positive cells and dividing by the total number of cells within a grid. One out of 3 spleen sections (1193194) showed DHBsAg in a perifollicular distribution. No other sections demonstrated any DHBsAg, although adrenal sections were only available from 2 of the 3 ducks. The results are illustrated in Figure 4.2.

4.3 Distribution of DHBV DNA Detected by Southern Blot Hybridization

4.3.1 Results: Congenital Infection

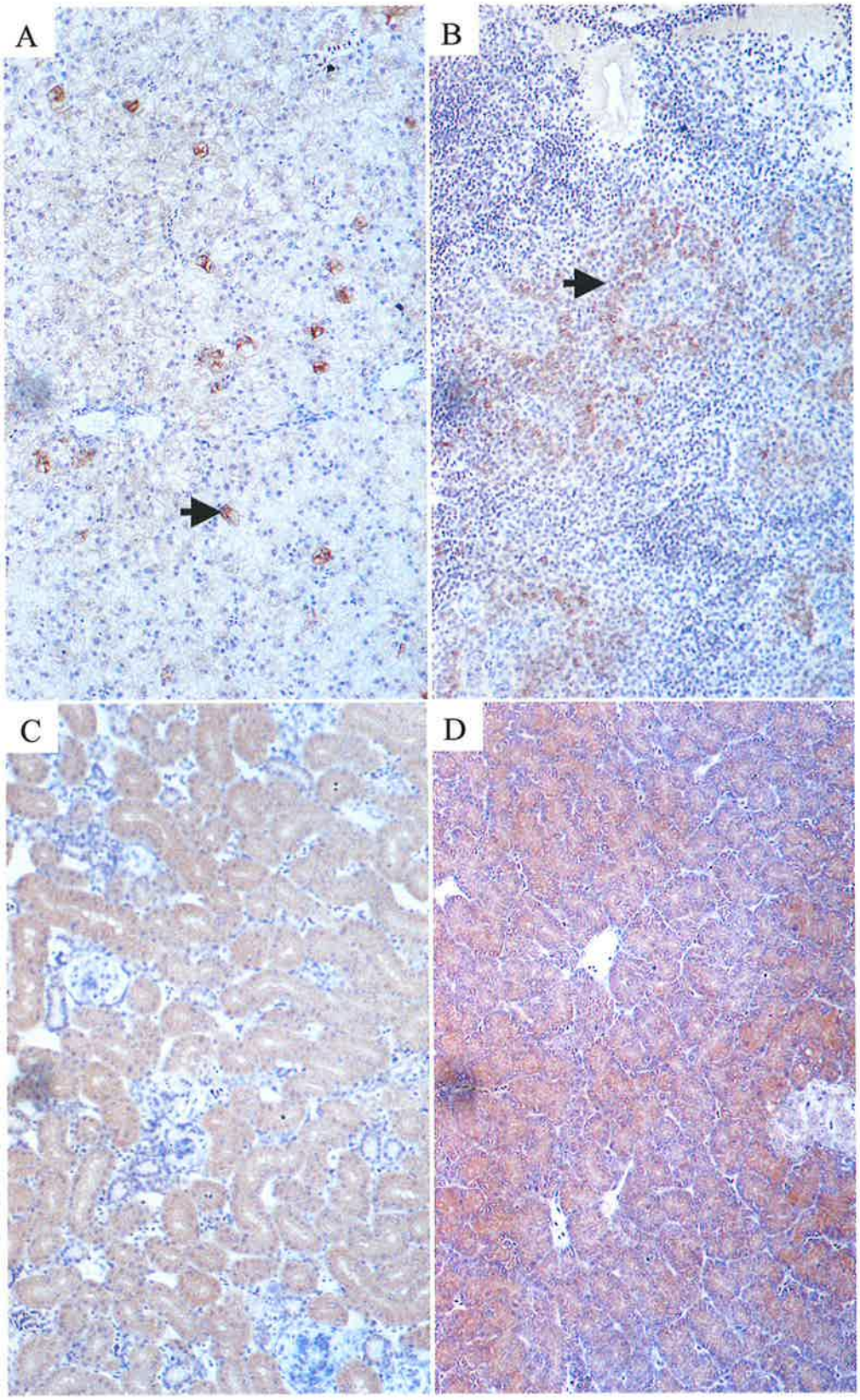
Tissues from the 3 congenitally DHBV-infected ducks were examined for total and cccDNA by Southern blot hybridization (Figure 4.3). The Southern blot hybridization for liver was repeated because of an unexpectedly low yield of DHBV cccDNA in one sample (from duck 11391150) and the repeated blot is shown in Figure 4.4. Liver, spleen, kidney, pancreas, adrenal, heart and skeletal muscle were tested. All tissues except skeletal muscle showed DHBV DNA, however cccDNA was detected only in liver and spleen samples. The quantity of DHBV DNA was expressed as average copies per cell by comparison with a plasmid DHBV DNA control. The amount of DNA loaded was measured by fluorimetry to estimate the number of cells represented in each assay, based on each duck cell containing 2.5 pg of chromosomal DNA (Vendrely 1958). The exception was liver cccDNA, for which the number of cells from which DNA was extracted was calculated from the weight of tissue. There are estimated to be 7×10^8 cells per gram of liver (Jilbert *et al.* 1992), however similar estimates are not available for other duck tissues. Results are

Figures 4.2: DHBsAg detection in tissues from ducks with limited experimental DHBV infection at day 3 p.i..

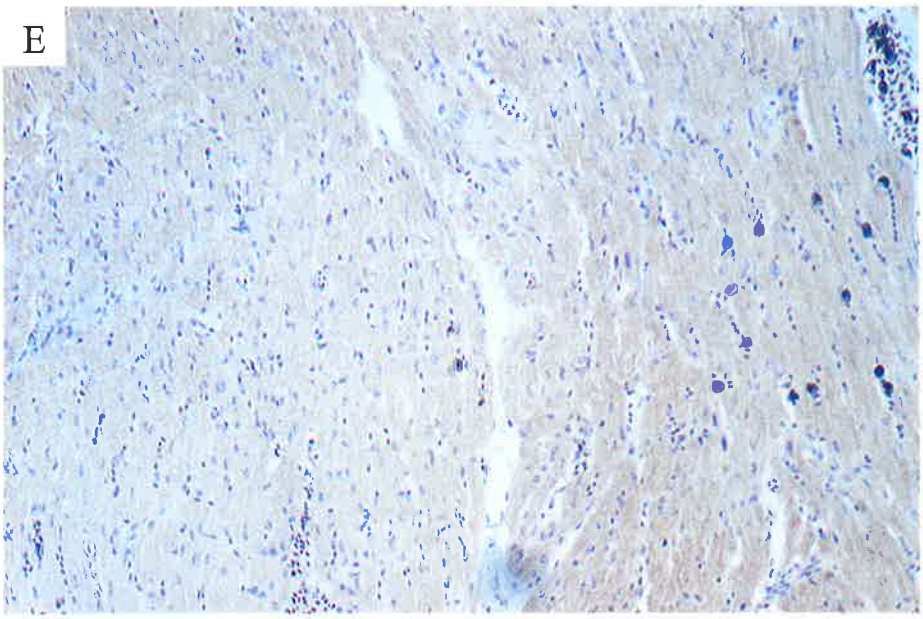
Immunohistochemical detection of DHBsAg was performed using a monoclonal antibody to pre-S1 (1H1, Pugh *et al*). Tissues were counterstained with haematoxylin and photographed at 200x magnification.

A: Liver section from duck 11261127 with 2.1% of liver cells staining positive for DHBsAg; B: Spleen from duck 11931194, the only spleen section in which DHBV antigen was detected, with DHBsAg in a perifollicular distribution; C: Kidney tissue (duck 11261127) with no detectable DHBsAg staining; D: Pancreas tissue (duck 11261127) with no detectable DHBsAg staining.

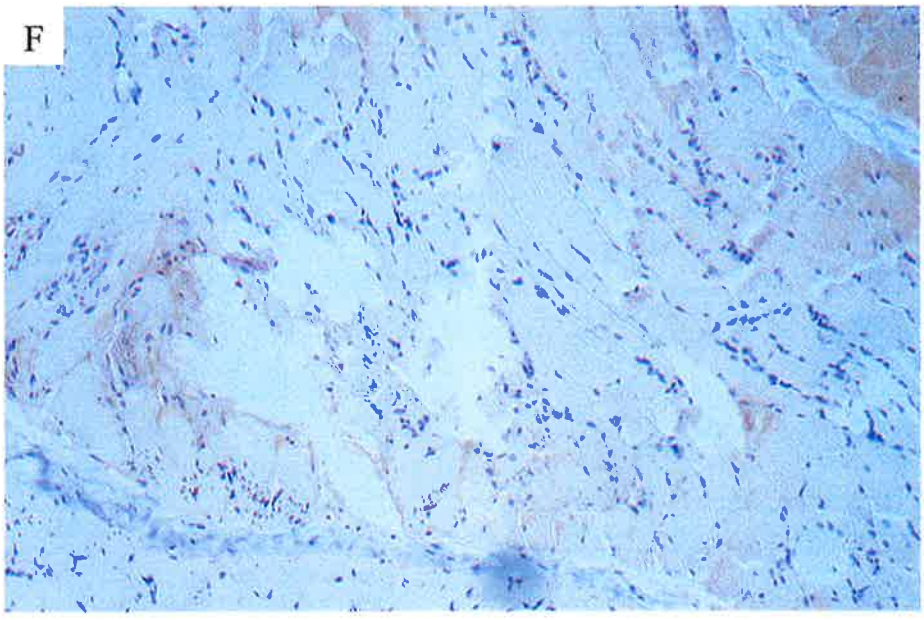
Panel E on the following page shows a heart section and panel F a skeletal muscle section, neither of which show staining for DHBsAg.


A
B
C
D

E



F



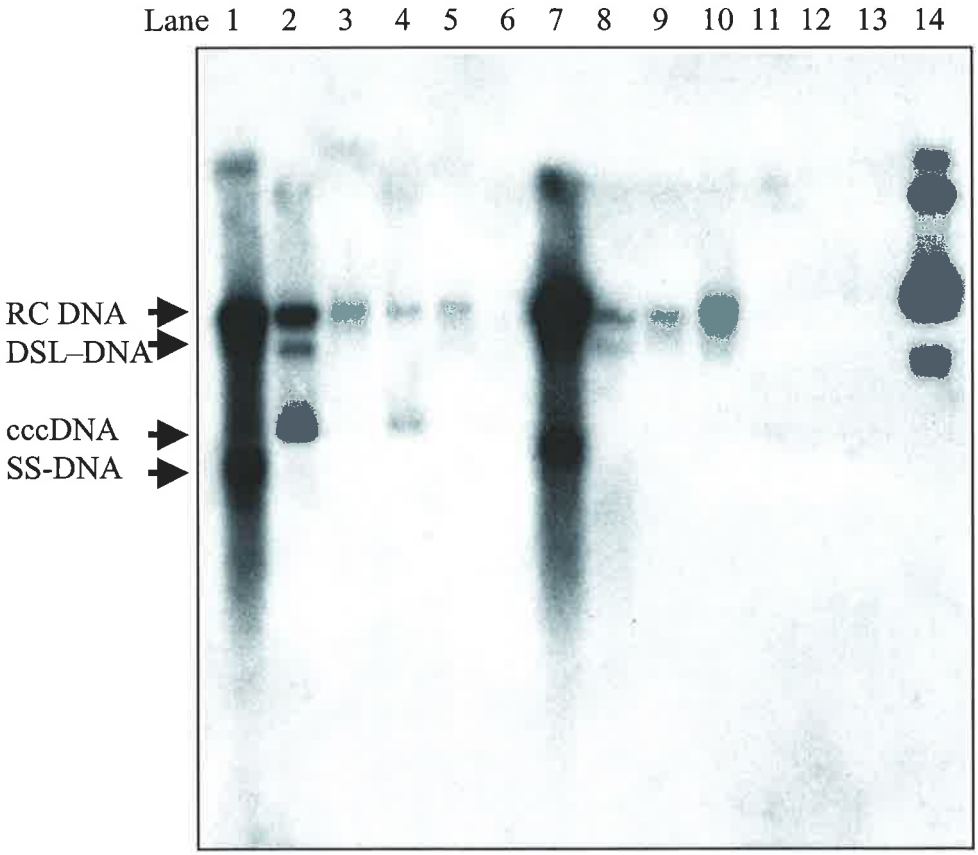


Figure 4.3: Southern blot hybridization of DHBV total and cccDNA from a congenitally DHBV-infected duck (11341135).

2µl of liver (equivalent to cccDNA from 2 mg and total DNA from 1 mg of liver), 20 µl of spleen, kidney and pancreas (equivalent to cccDNA extracted from 20 mg and total DNA from 10 mg of tissue), and 30 µl of heart, adrenal and skeletal muscle (equivalent to cccDNA extracted from 30 mg and total DNA from 15 mg of tissue), DNA samples were diluted, loaded and run on a 1.5% agarose gel at 30 mA for 16 hr, transferred and hybridized to a genome-length radiolabelled DHBV DNA probe. The blot was exposed to x-ray film for 16 hr.

Lanes 1&2 show the presence of DHBV total and cccDNA in liver and lanes 3&4 show smaller quantities in spleen. Lanes 5&6 show RC DNA but not cccDNA in samples from kidney, lanes 7&8 show pancreas with larger amounts of RC DNA and SS intermediates, but not cccDNA. Lane 9 shows RC DNA in adrenal total DNA, lanes 10&11 show heart total and cccDNA preparations with RC DNA, but not cccDNA seen, lanes 12&13 show skeletal muscle which does not demonstrate DHBV DNA and lane 14 contains 80 pg of 3.0 kb linear plasmid DHBV DNA.

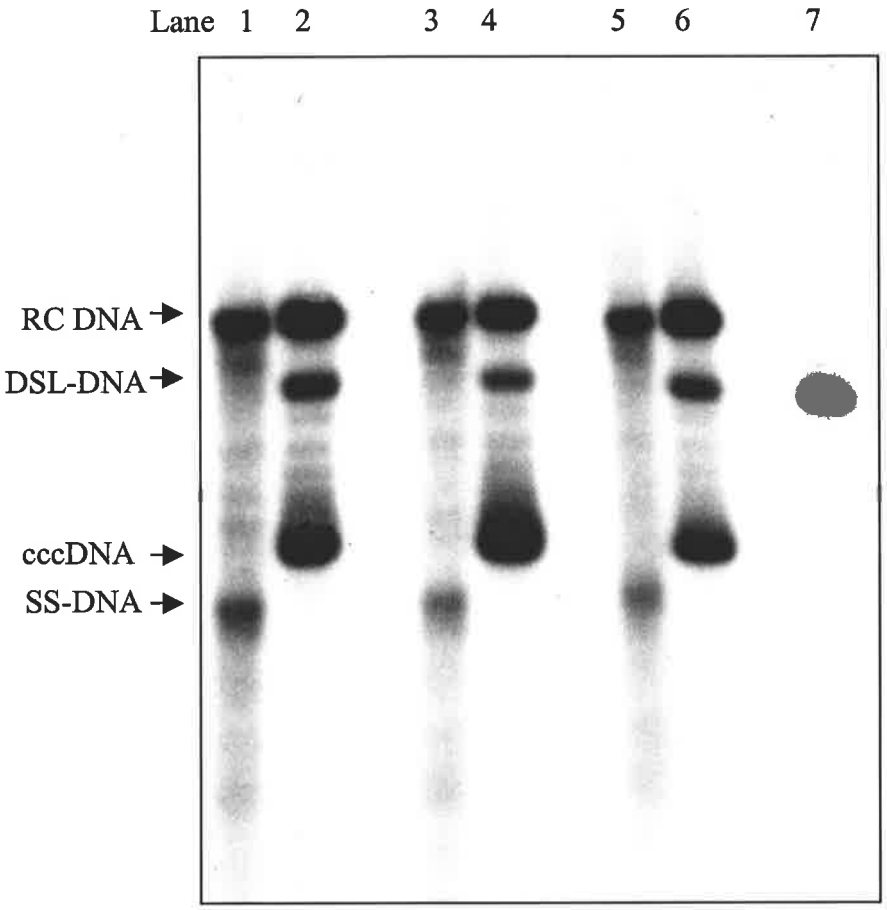


Figure 4.4: Southern blot hybridization detection of DHBV total and cccDNA in liver tissue from 3 congenitally DHBV-infected ducks.

2 µl of extracted DNA (cccDNA extracted from 1.4×10^6 liver cells and total DNA from 0.7×10^6 liver cells) was diluted and run on a 1.5% agarose gel at 30 mA for 24 hr, transferred and hybridized to a genome length radiolabelled DHBV DNA probe before autoradiography for 16 hr.

Lanes 1&2 show total and cccDNA samples from duck 11341135, lanes 3&4 show samples from duck 11361137 and lanes 5&6 show samples from duck 11391150. Lane 7 contains 80 pg of 3.0 kb linearised DHBV plasmid DNA.

summarised in Table 4-1. Similar results were found in another 4 ducks in preliminary experiments (data not shown).

Table 4-1: Quantitation of DHBV DNA by Southern Blot Hybridization: Congenital Infection

Tissue extract	Copies DHBV DNA per cell		
	Duck 11341135	Duck 11361137	Duck 11391150
Liver total DNA	615	367	270
Liver cccDNA	5.3	6.6	2.8
Spleen total DNA	4.7	3.6	0.35
Kidney total DNA	0.5	4.6	0.31
Pancreas total DNA	50.7	70	54.4
Adrenal total DNA	13.9	6.1	5.2
Heart total DNA	9.3	1.1	Not measured

4.3.2 Results: Experimental Infection

Total and cccDNA extracted from the 3 experimentally infected ducks was examined similarly (Figure 4.5). Liver, spleen and kidney (one sample only) were tested. DHBV DNA was detected in liver and spleen, but not in kidney in the single sample tested. Since other tissues had lower levels than kidney when measured by PCR, as described later, Southern blot analysis was not performed.

Since only a small percentage of liver cells (1.7-3.5%) were infected, as demonstrated by DHBsAg staining, the results in Table 4-2 were adjusted to show the measured copies of

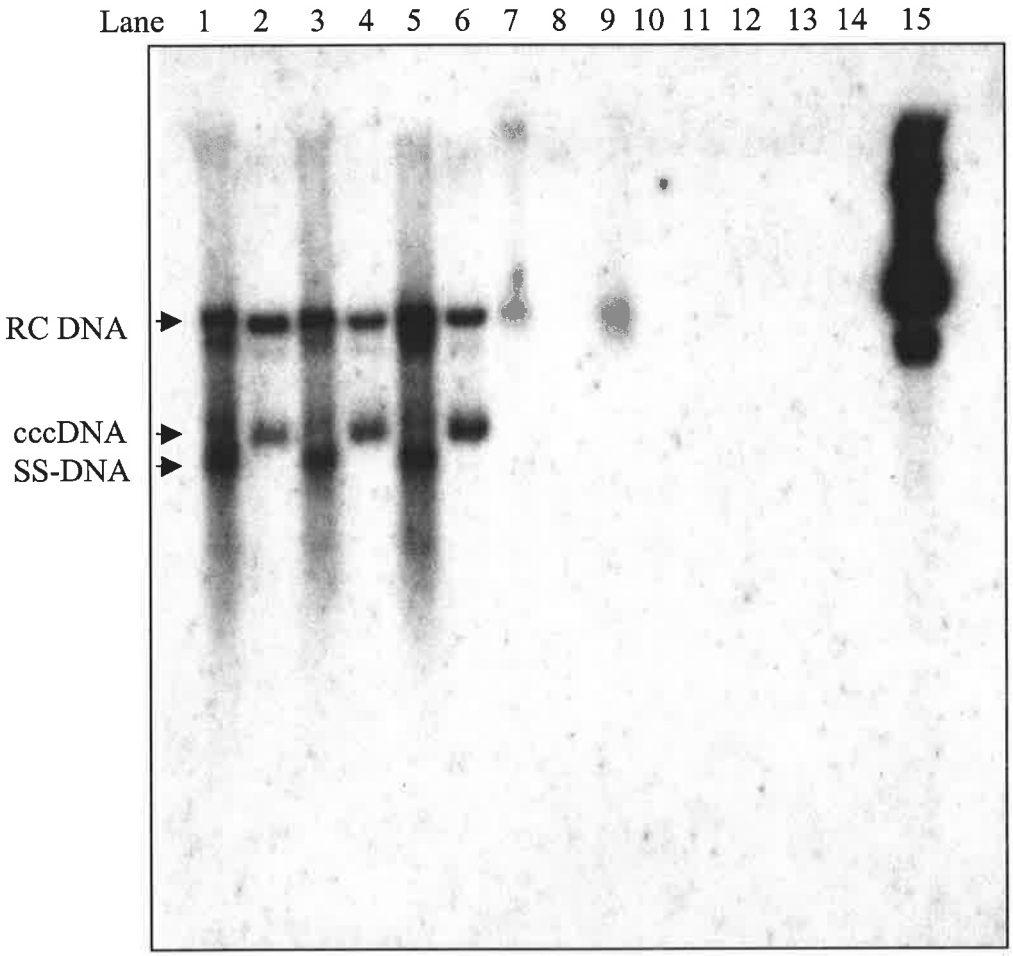


Figure 4.5: Southern blot hybridization detection of DHBV total and cccDNA in tissues collected from experimentally DHBV-infected ducks at day 3 p.i..

20 µl of DNA samples (cccDNA extracted from 1.4×10^7 liver cells and total DNA from 0.7×10^7 liver cells) were run on a 1.5% agarose gel at 30 mA for 16 hr, transferred and hybridized to a genome-length radiolabelled DHBV DNA probe before autoradiography for 72 hr.

Lanes 1&2 show DHBV total and cccDNA respectively in samples from liver from duck 11261127. Lanes 3&4 and 5&6 show the same samples from ducks 11931194 and 11991200 respectively. Lanes 7&8 shows spleen total and cccDNA samples from duck 11261127 with RC DNA but not cccDNA seen. Lanes 9&10 and 11&12 show similar spleen samples from ducks 11931194 and 11991200 with RC DNA seen in the sample from duck 11931194, but not 11991200. In lanes 13&14 kidney total and cccDNA samples from duck 11261127 are negative for DHBV DNA. Lane 15 contains 80 pg of linear 3.0 kb DHBV plasmid DNA.

DHBV per infected cell by dividing the measured amount of DNA by the proportion of cells infected (Table 4-3).

Table 4-2: Southern Blot Analysis of DHBV DNA at Day 3 p.i.: Experimental Infection

Tissue extract	Copies DHBV DNA per cell		
	Duck 11261127	Duck 11931194	Duck 11991100
Liver total DNA	6.9	5.4	11.6
Liver cccDNA	0.26	0.36	0.58
Spleen total DNA	0.085	0.083	0

Table 4-3: Southern analysis of DHBV DNA per infected cell: Experimental Infection

Tissue extract	Copies DHBV DNA per infected cell		
	Duck 11261127	Duck 11931194	Duck 11991100
% cells infected	2.1	3.5	1.7
Liver total DNA	329	154	682
Liver cccDNA	12.3	10.2	34.1

4.4 DHBV Detection by Quantitative PCR

4.4.1 Congenital DHBV Infection

Quantitative PCR was used to measure DHBV DNA in liver, spleen, kidney, pancreas, adrenal, heart, skeletal muscle and PBMC from the same ducks described above (Sections 2.8.4 and 2.8.6). 100 ng of DNA extracted by the Qiagen method and measured by fluorimetry, was used as template. PCR with primers P3 and P4 was performed to measure total DHBV DNA (Section 2.8.4). Then primers CC2 and R2 were used to selectively amplify and quantify cccDNA (Section 2.8.5). The results from PCR using

primers P3 and P4 for non-selective amplification are shown in Table 4-4 and the results of selective PCR quantitation of cccDNA using primers CC2 and R2 are shown in Table 4-5.

Table 4-4: DHBV DNA Quantitation by PCR: Congenital Infection.

Tissue	Copies of DHBV DNA per cell		
	Duck 11341135	Duck 11361137	Duck 11391150
Liver	1125	1125	1650
Spleen	11.8	5.3	13.8
Kidney	7	3	5.8
Pancreas	300	150	243
Adrenal	3.5	1.6	3
Heart	14.5	7.8	3.8
Skeletal muscle	6.1	7.8	4
PBMC	0.1	0.2	0.1

Table 4-5: DHBV cccDNA Quantitation by PCR - Congenital Infection

Tissue	Copies of DHBV cccDNA per cell		
	Duck 11341135	Duck 11361137	Duck 11391150
Liver cccDNA	11.9	6.8	3.0
Spleen	0.17	0.06	0.14
Kidney	0.02	0.01	0.03
Pancreas	NQ	NQ	NQ
Adrenal	0.01	0.05	0.05
Heart	0.06	0.02	0.01
Skeletal muscle	0.02	0.05	0.03
PBMC	<0.0003	<0.0003	<0.0003

NQ: pancreas DHBV DNA not quantified. The PCR products derived from pancreas extracts showed a pattern consistent with inefficient amplification and therefore this could not be considered a reliable method for quantitation.

4.4.2 Experimental Infection

Liver, spleen, kidney, pancreas, adrenal, heart, skeletal muscle and PBMC samples taken 3 days after experimental infection (Section 4.2.2) were then examined for DHBV DNA by PCR (Section 2.8.4) as in 4.4.1 (Table 4-6).

The results for total DHBV DNA in liver are expressed as copies per cell and copies per infected cell. All samples except those from PBMC were clearly positive although, except for liver and spleen, the levels were very low – below 200 copies in the sample of 100 ng of tissue DNA, which represents 40000 cells. In liver the levels of total DHBV DNA per infected cell ranged from 108-301 copies/infected cell.

The cccDNA PCR was done with or without pretreatment of liver DNA samples to digest non-cccDNA. The treatment, described in Section 2.8.6, consisted of heat denaturation of DNA at 95°C then incubation with a selective single-stranded DNase. It was designed to improve the specificity of the selective PCR for cccDNA, since it was previously noted that RC DNA was also amplified, albeit inefficiently, at template copy numbers of more than 10^4 (Section 3.5.2). The results in Table 4-7 show from 3.8-7.2 copies of cccDNA per infected cell using untreated DNA and 0.11-0.15 copies per infected cell using treated DNA samples. The latter result is less than a single copy of cccDNA per infected cell (an impossibility), suggesting that the treatment method removed most of the cccDNA and could not be used to improve the assay specificity. In contrast, the untreated samples yielded results closer to those from Southern blot hybridization, which showed 10.2-34.1 copies per infected cell.

Table 4-6: DHBV DNA Quantitation by PCR: Experimental Infection

Tissue	Copies of DHBV DNA per cell		
	Duck 11261127	Duck 11931194	Duck 11991200
Liver	3.9	4.1	5.1
Liver*	184*	108*	301*
Spleen	1.1	6.3	0.5
Kidney	0.009	0.005	0.002
Pancreas	0.002	0.001	<0.001
Adrenal	0.001	0.006	0.005
Heart	0.001	0.001	<0.001
Skeletal muscle	0.001	0.002	0.002
PBMC	<0.001	<0.001	<0.001

* DHBV DNA copies divided by the percentage of liver cells staining for DHBsAg to give an estimate of copies per infected cell.

Table 4-7: DHBV cccDNA Quantitation by PCR: Experimental Infection

Tissue	Copies of DHBV cccDNA per cell		
	Duck 11261127	Duck 11931194	Duck 11991200
Liver undigested	0.14	0.13	0.12
Liver undigested*	6.5*	3.8*	7.2*
Liver digested	0.002	0.004	0.003
Liver digested*	0.15*	0.11*	0.15*
Pancreas, spleen, adrenal, heart, skeletal muscle, PBMC	-	-	-

*DHBV DNA copies divided by the percentage of liver cells staining for DHBsAg to give an estimate of copies per infected cell.

4.4.3 Comparison of Southern Blot Hybridization and PCR Quantitation of DHBV DNA in Liver

Results from the above series of experiments were tabulated to compare quantitation of DHBV total and cccDNA by Southern blot hybridization and PCR (Table 4-8). For the same duck liver a variation of up to 4-fold was seen between the 2 methods although most tissue samples yielded similar results.

Table 4-8: Comparison of DHBV DNA Quantitation Methods

Sample	DHBV DNA copies per infected cell by Southern	DHBV DNA copies per infected cell by PCR
Total DNA- congenital infection	270-615	1125-1650
CccDNA – congenital infection	2.8-6.6	3.0-11.9
Total DNA – experimental infection	154-682	105-307
CccDNA – experimental infection	10.2-34.1	3.8-7.2

4.5 Discussion

The distribution of DHBsAg in tissue in congenital infection was similar to that reported in other studies (1.13). Clear-cut antigen staining in liver >95% of cells, in scattered pancreatic cells (<10%) and occasional kidney tubular cells was seen. Some equivocal staining was seen in one adrenal and one heart section. Some difficulty in distinguishing intracellular staining from that found in blood vessels lead to the classification of equivocal staining.

Surprisingly no staining was seen in spleen, in contrast to findings from a previous study from this laboratory (Jilbert *et al.* 1987) and in preliminary experiments for this study,

using older ducks with high-level infection (data not shown). This also contrasts with the finding of DHBsAg in one of 3 experimentally DHBV-infected ducks (Section 4.2.2). It is not clear what determines whether antigen localizes to the spleen, for example whether removal of antigen and virions from the bloodstream may be more efficient in older animals with less complete immune tolerance. However, infection of spleen is supported by the finding of cccDNA by Southern blot hybridization. Overall there was some correlation between antigen staining and detection of DHBV DNA in congenital infection, with the notable exception of the spleen.

In experimental infection DHBsAg was seen in 1.7-3.5% of liver cells 3 days after inoculation with 10^{10} virions. A 2060 g duck (average for a 6-week-old duck, Wendy Foster, University of Adelaide, personal communication) with 4% of body mass represented by liver (Nickel *et al.* 1977) contains 82.4 g liver or 5.8×10^{10} liver cells, calculated from a previously published figure of 7×10^{8} cells per gram of liver (Jilbert *et al.* 1992). An inoculum of 10^{10} virions could infect a maximum of 17.2% of liver cells if it localized solely in the liver and each virion infected a single cell. The observed frequency of staining suggests that some virus was cleared without reaching the liver or that the peak of infection was not at the time of the biopsy or, more likely, both occurred. As will be seen in the next chapter, repeating the same inoculation in ducks of the same age gave similar results.

In experimental infection antigen staining at day 3 p.i. was not seen outside the liver and spleen. An experimental infection with 2.4% of liver cells infected represents 1/40 of the extent of infection seen in a congenital infection (>95% of liver cells); if extrahepatic sites were to contain the same frequency of staining then antigen should be evident in the pancreas at least, and possibly even in kidney. It seems therefore that the extrahepatic

distribution of antigen is more restricted in limited experimental infection on day 3 than is predicted by the level of infection in the liver. This is consistent with a possible role for viraemia in spread of DHBV to extrahepatic sites although the data is limited. This is also consistent with other studies in which infection of extrahepatic sites occurred later than liver, possibly following amplification of infection in liver, and viraemic spread (Tagawa *et al.* 1985; Jilbert *et al.* 1988; Walter *et al.* 1991; Jilbert *et al.* 1996).

DHBV DNA levels measured by Southern blot hybridization were mostly similar to those obtained by PCR. It must be noted that the 2 methods required different DNA extraction techniques, with different efficiencies. The measurement of the host cell DNA concentration in each sample should compensate for differences in extraction efficiency although it is not known whether one method was superior in extracting viral DNA forms compared with chromosomal DNA.

There are a number of other sources of variation in measurements of DHBV DNA. In order to accurately measure the viral DNA copy number per cell it is essential to have satisfactory measurements of the host cell DNA content. Some variation between the performance of fluorimetric measurements on different sample types may have occurred and assays done on different occasions may also contribute to variation. Another source of variation is the accuracy of plasmid standards for each assay. The plasmid controls come from separate sources, which are measured fluorimetrically and diluted to the different concentrations for each method. Some inter-assay variation may result from the initial measurements and from different effects of storage. None of these sources of error have been individually measured in this study however the total effect is seen as the variation in DHBV DNA measurements outlined in this chapter.

In congenital infection analysis of DHBV DNA by Southern blot hybridization suggested infection of liver and spleen, but no evidence of infection of skeletal muscle. The remaining tissues showed DHBV RC DNA, in keeping with the presence of virions, but cccDNA was not detected. In the case of the pancreas, DHBsAg expression was seen, suggesting that there was true infection and therefore that the absence of detectable cccDNA may represent a false negative result. The reason for this is not clear but may relate to the presence of digestive enzymes, including DNases, in pancreas, which probably reduces the sensitivity of the test. Consistent with this, the ethidium bromide stained gel before transfer showed a lack of larger molecular weight chromosomal, but increased low molecular weight nucleic acid in the pancreatic DNA sample. Kidney samples also showed RC DNA but not cccDNA, and similarly showed evidence of antigen in keeping with true infection. In this case the sensitivity of Southern blot hybridization may be insufficient for detection of small amounts of cccDNA. The finding of cccDNA in the kidney by selective PCR is further evidence that the sensitivity of Southern blot was insufficient for detection of some extrahepatic cccDNA.

In experimental infection Southern blot hybridization showed DHBV DNA in liver and spleen, but cccDNA was only detectable in liver. No cccDNA was detectable in spleen using the more sensitive PCR assay. The only extrahepatic cccDNA detected was in 1 out of 3 kidney samples. The data point to more restricted distribution of DHBV infection in the limited experimental infection and are consistent with the results of immunohistochemical antigen detection. However, since DHBV DNA was detectable at low level by PCR in the full range of tissues studied more widespread extrahepatic infection cannot be ruled out.

One possible explanation is a contribution by circulating virion DNA to the tissue results, since blood is a potential source of viral DNA in extracted tissue DNA samples. The one duck examined by PCR showed approximately 1500 DNA copies/ml of serum at day 3 pi.. Hence an experimentally infected duck shows very low level viraemia compared with results seen in congenitally infected ducks ~10^{10} copies/ml (data not shown). This is a 10^7-fold difference compared with a 10^2-10^3-fold difference in levels of DHBV DNA in liver in experimentally infected ducks compared with congenitally infected ducks. It is possible to speculate that this may reflect more rapid clearance of circulating virions in limited infection, which could limit the spread outside the liver. In addition, the contribution of this level of viraemia to the copy number of DHBV DNA in tissue samples used for DNA extraction is very small. Samples for extraction were usually 20 mg of tissue and the DNA from approximately 0.2 mg was subjected to PCR. This corresponds to 0.2 μl of blood if the entire sample consisted of blood. At the observed level of viraemia only 0.3 copies of DHBV DNA would be present in that volume. A level of viraemia of 2-4 logs higher would be needed to account for the observed levels of DHBV DNA in the range of extrahepatic sites studied. This is unlikely to have occurred even allowing for differences in DNA extraction efficiency between methods.

5. Detection of Residual DHBV DNA after Transient Infection

5.1 Introduction and Aims

Transient hepadnavirus infections in the human and woodchuck are characterized by recovery and the development of anti-surface antibodies and immunity to reinfection. As summarised above (Section 1.9) transient infection leads to persistence of traces of residual viral DNA and may in certain situations lead to reactivation of high-level infection. It was not known whether DHBV also leads to recovery with persistence of residual DHBV DNA.

Also, little was known about the site(s), mechanism or form of hepadnavirus persistence after transient infection. Since the virus possesses properties allowing persistence of high-level infection in subjects infected perinatally, some of these properties may be involved in occult persistence after transient infection. The viral genome exists as relatively stable forms – cccDNA and integrated DNA, which could provide a mechanism for persistence, although integrated hepadnavirus DNA is unlikely to lead to replicative infection since it is unlikely to produce the longer than genome length RNA required. Hepadnaviruses have been found in a range of extrahepatic sites, which might provide a site where restricted expression of viral proteins could assist in evasion of immune responses.

It is likely that cells in which viral replication occurs and viral antigens are expressed will be targeted for elimination, perhaps leaving cells which either do not support efficient replication (eg perhaps in extrahepatic sites) or which are infected with virus with reduced capacity to replicate. Little is known about the DNA sequence of residual hepadnavirus DNA, although Michalak *et al* sequenced an amplified complete WHV genome and found it closely matched that of wild type virus used to experimentally infect the woodchuck (Michalak *et al.* 1999).

The residual viral DNA described by others has been in such small amounts that PCR is required for detection. However, quantitative studies are lacking, making the development of models to explain virus persistence more difficult. The recent development of real time PCR has made quantitation of residual DNA feasible and enables the monitoring of DHBV DNA levels over time.

The aims of the work included in this chapter were to -

1. Set up and monitor transient DHBV infections by inoculating 39-40-day-old ducks with different doses of DHBV.
2. Investigate persistence of DHBV DNA after transient infection.
3. Define some of the sites of residual DHBV DNA;
4. Assess the effect of different sized inocula on the amount and distribution of residual viral DNA.
5. Define the form in which residual DHBV DNA occurs, in particular whether cccDNA is present.

5.2 Transient DHBV Infection

5.2.1 Introduction and Experimental Outline

Previous work from this and other laboratories was reviewed to predict the maximum viral inoculum which, when inoculated into ducks 5-6 weeks of age, produces transient infection with recovery (see 1.12). Ducks of this age have reached approximately 60% of adult body weight and sufficient immunological maturity to clear DHBV infection after low to moderate size inoculum. Qiao *et al*, found that ducks of 4-6 weeks of age developed

transient infection in approximately 50% of cases when inoculated intravenously with 3×10^8 virions (Qiao *et al.* 1990). Vickery and Cossart reported similar findings with 2 out of 2 ducks at 26 days old inoculated with 7×10^8 virions developing persistent infection compared with 0 of 3 ducks inoculated with 7×10^7 virions (Vickery and Cossart 1996). On the basis of these previous experiments, inocula of 10^7 and 10^8 virions were chosen, aiming to produce transient infection. Later a third group was inoculated with 10^{10} virions.

Three groups of 5 39-40-day-old DHBV-negative ducks were used and inoculated intravenously with either 10^7 (Group A), 10^8 (Group B) or 10^{10} (Group C) virions. Serum was collected twice weekly for 2 weeks, weekly for 2 weeks, fortnightly for 1 month and then monthly for detection of DHBsAg and anti-DHBs antibodies by ELISA. Liver was collected by biopsy at 3 or 4 days, 1 month, 3 and 6 months p.i.. Liver, spleen, pancreas, kidney, adrenal, heart, skeletal muscle and PBMC were collected at autopsy approximately 9 months p.i.. Figure 5.1 shows a schematic outline of the experiment.

5.2.2 Results

5.2.2.1 Confirmation of DHBV Infection by DHBsAg Detection in Liver:

Liver biopsies were taken at day 4 from Groups A and B and at day 3 from Group C. Sections were cut from ethanol/acetic acid-fixed tissue and then microwave assisted antigen retrieval was performed (see 2.6.2) before immunoperoxidase staining with a monoclonal anti-pre-S antibody [1H1 (Pugh *et al.* 1995)]. Sections of DHBV-infected and uninfected duck liver were used as positive and negative controls and were stained in parallel. Cells staining for DHBsAg were counted at 200× magnification, divided by the total number of cells in the same area and multiplied by 100 to derive the percentage of infected cells. Sections of liver from all 15 inoculated ducks showed cells staining for

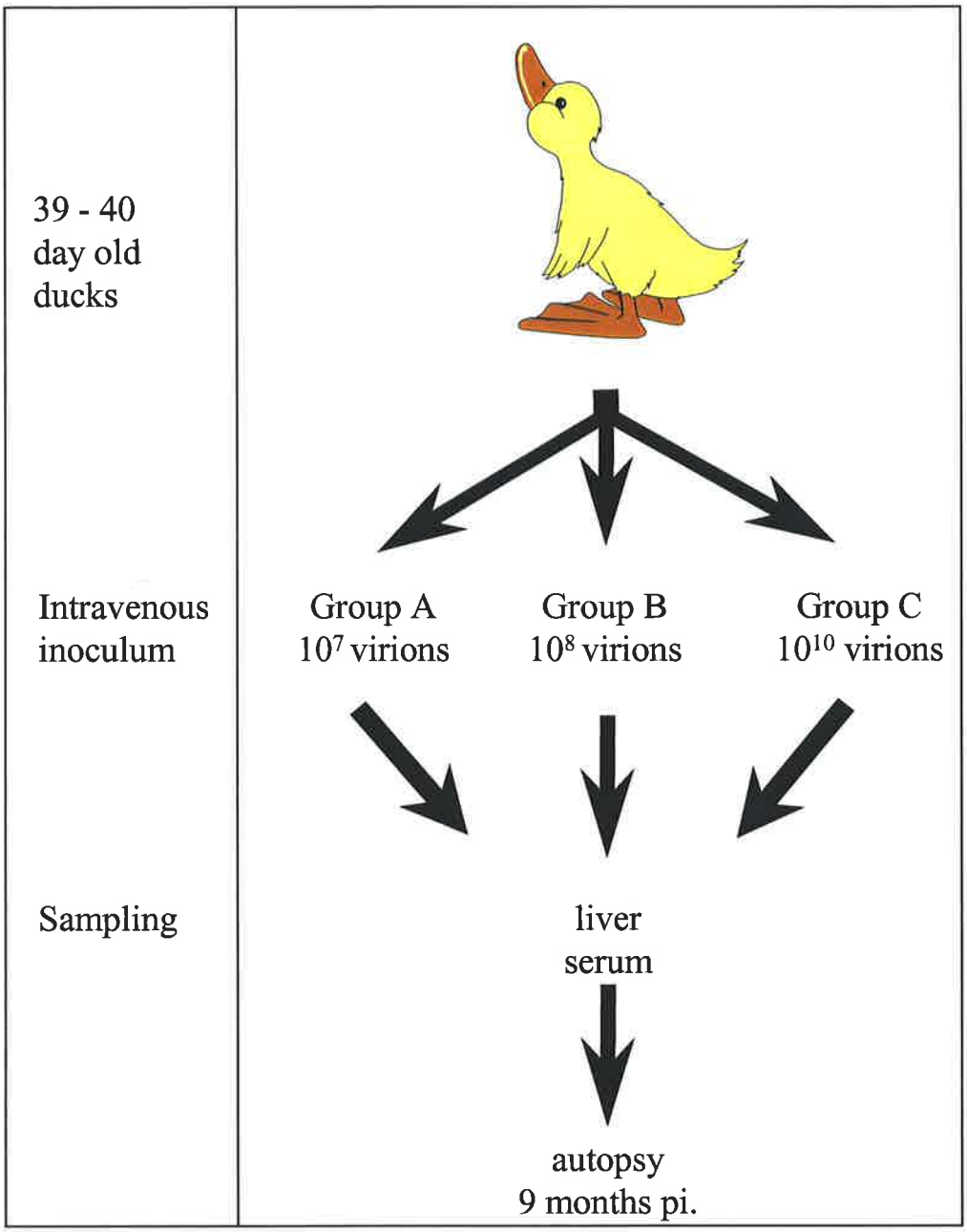


Figure 5.1: Experimental Design:
Three groups of 5 ducks were inoculated with either 10^7, 10^8 or 10^{10} DHBV virions and monitored for 9 months with regular serum and liver detection of DHBsAg, anti-DHBs antibodies and DHBV DNA. Nine months post-inoculation autopsies were performed in 11 ducks which had recovered from DHBV infection. Samples collected at autopsy were used for detection of residual DHBV DNA.

DHBsAg, with a relationship between the inoculum size and frequency of positive cells (Table 5-1). Ducks from Group A showed a mean of 0.00085% positive, Group B 0.0105% and Group C 2.64% (Figure 5.2).

Table 5-1: DHBV Surface antigen staining in liver at day 3-4 post-inoculation

Group	Duck #	Cells + per slide or per grid in Group C	Liver cells examined	% positive cells
A	223	2	317400*	0.00063
	322	3	348800	0.00086
	1112	1	263000	0.00038
	1516	2	227200	0.00088
	20	5	333200	0.00150
Mean of Group A				0.00085%
B	525	32	280800	0.0114
	821	33	248000	0.0133
	910	26	208000	0.0125
	1314	22	333200	0.0066
	1718	28	318200	0.0088
Mean of Group B				0.0105%
C	3435	42	1106	3.8
	3637	39	1114	3.5
	3839	23	1210	1.9
	4041	27	1080	2.5
	4243	18	1200	1.5
Mean of Group C				2.64%

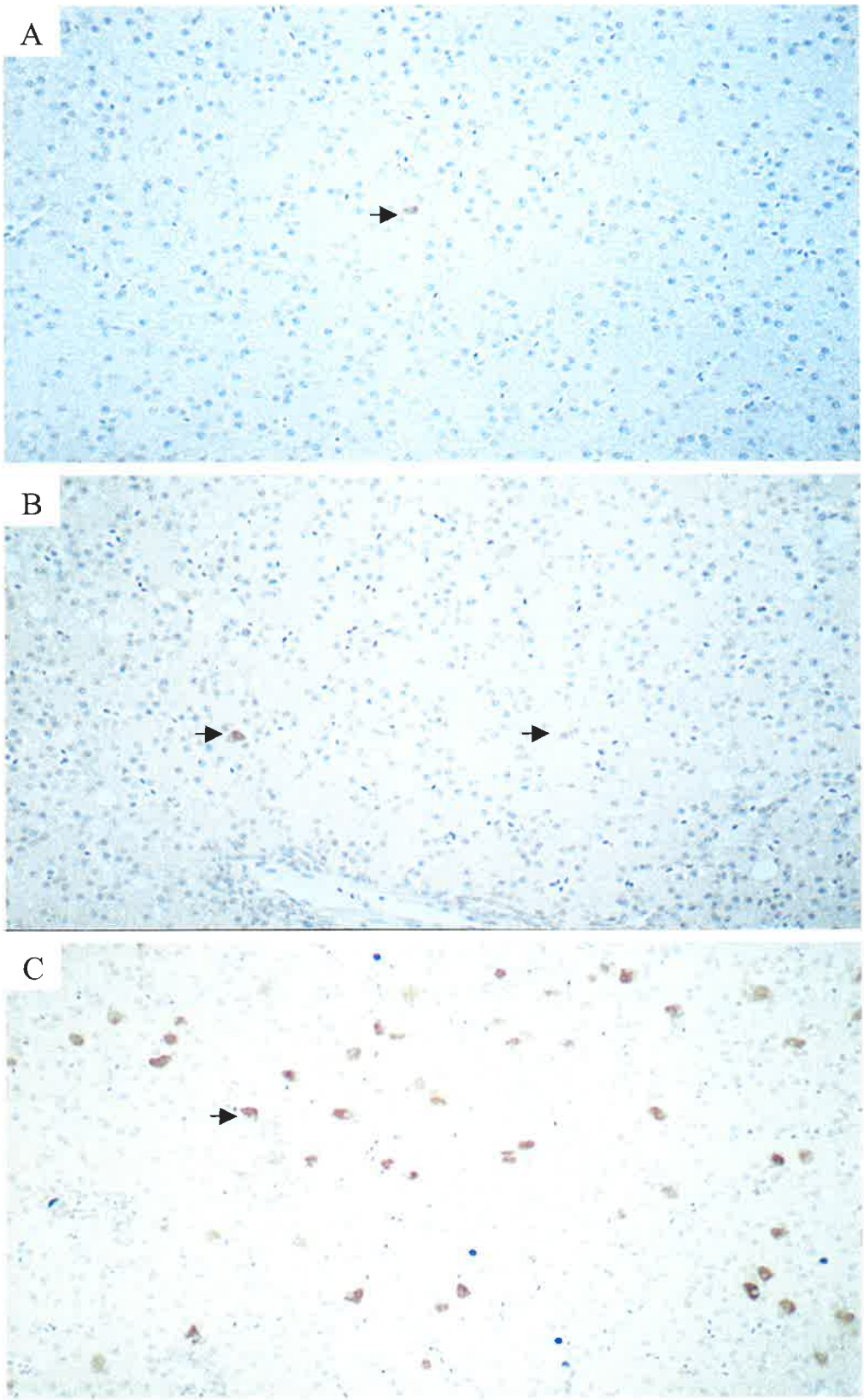
*cell numbers estimated by multiplying cells per grid by number of grids per section.

Figure 5.2: Expression of DHBsAg in Liver at Day 3-4 p.i.
Immunohistochemical detection of DHBsAg was performed using monoclonal anti-Pre-S antibodies (1H1). Sections were counterstained with H&E and photographed at 200× magnification.

A: Liver section from a duck from Group A at day 4 p.i.. 0.00085% (mean for Group A ducks) of liver cells were DHBsAg-positive (as indicated by the arrow).

B: Liver section from a Group B duck at day 4 p.i. with 2 DHBsAg-positive cells (indicated by arrows). A mean of 0.0105% of liver cells in Group B ducks were DHBsAg-positive.

C: DHBsAg-positive cells from a representative section from Group C duck liver at day 3 p.i.. A mean of 2.64% of liver cells from Group C ducks were DHBsAg-positive.


A
B
C

5.2.2.2 Detection of DHBV DNA in Liver Early after Infection

DNA was extracted by phenol-chloroform method (Section 2.7.1) from frozen liver collected at day 3 or 4 p.i. and at 1 month p.i.. Both cccDNA and total DNA were extracted using previously described methods that allow separation of non-protein bound cccDNA and total DNA sequences (Jilbert *et al.* 1992). Gel electrophoresis was performed before Southern transfer and hybridization with a full length radiolabelled AusDHBV DNA genome (Section 2.7.5). The hybridization signal intensity was measured by phosphorimager analysis and quantitation was performed by comparison of samples to a 10 or 100 pg genome-length DHBV plasmid control sample. The amount of DHBV DNA per cell was calculated by dividing the amount of DHBV DNA detected by Southern blot hybridization by the number of cells from which extracted DNA was assayed. Duck liver contains 7×10^8 cells/gram and each hepatocyte contains 2.5 pg DNA (Vendrely 1958). These measurements enable the number of cells represented in DNA extracts to be quantified according to the weight of tissue extracted (used for cccDNA) or the measured concentration of DNA in the extract by fluorimetric assay (Section 2.7.4).

DHBV DNA was not detected by Southern blot hybridization in the liver of ducks of Groups A and B, but was detectable in liver from ducks from Group C (Figure 5.3). The quantity of ccc and total DHBV DNA in liver from Group C ducks measured by phosphorimager analysis is shown in Table 5-2. DHBV DNA is shown as copies per infected cell using DHBsAg expression as a marker of infection. The ratio of cccDNA to total DHBV DNA is also shown for comparison to residual DHBV DNA (Section 5.3.5).

Figure 5.3: Southern blot hybridization of cccDNA and total DHBV DNA extracted from the liver of ducks from Group C on days 3 and 31 p.i.. The blot was hybridized to a radiolabelled genome length DHBV DNA probe and exposed to film for 16 hr. The identifiable DNA forms include relaxed circular (RC DNA), double-stranded linear DNA (DS-DNA), cccDNA and a smear of linear intermediates of variable length.

A: DHBV cccDNA from days days 3 & 31 p.i.. 30 µl of DNA extract was loaded (DNA extracted from 2.1×10^7 cells).
Lanes 1-5: DHBV cccDNA from liver collected at day 3 p.i. from ducks 3435, 3637, 3839, 4041 and 4243 respectively.
Lanes 6-10: DHBV cccDNA from liver collected at day 31 p.i. from ducks 3435, 3637, 3839, 4041 and 4243 respectively.
Lane 11: 10 pg of DHBV plasmid DNA.

B: Total DHBV DNA extracted from liver collected on days 3 and 31 p.i..
Lanes 1-5: Total DHBV DNA from liver collected at day 3 p.i. from ducks 3435, 3637, 3839, 4041 and 4243 respectively.
Lanes 6-10: Total DHBV DNA from liver collected at day 31 p.i. from ducks 3435, 3637, 3839, 4041 and 4243 respectively.
Lane 11: 100 pg DHBV plasmid DNA control.

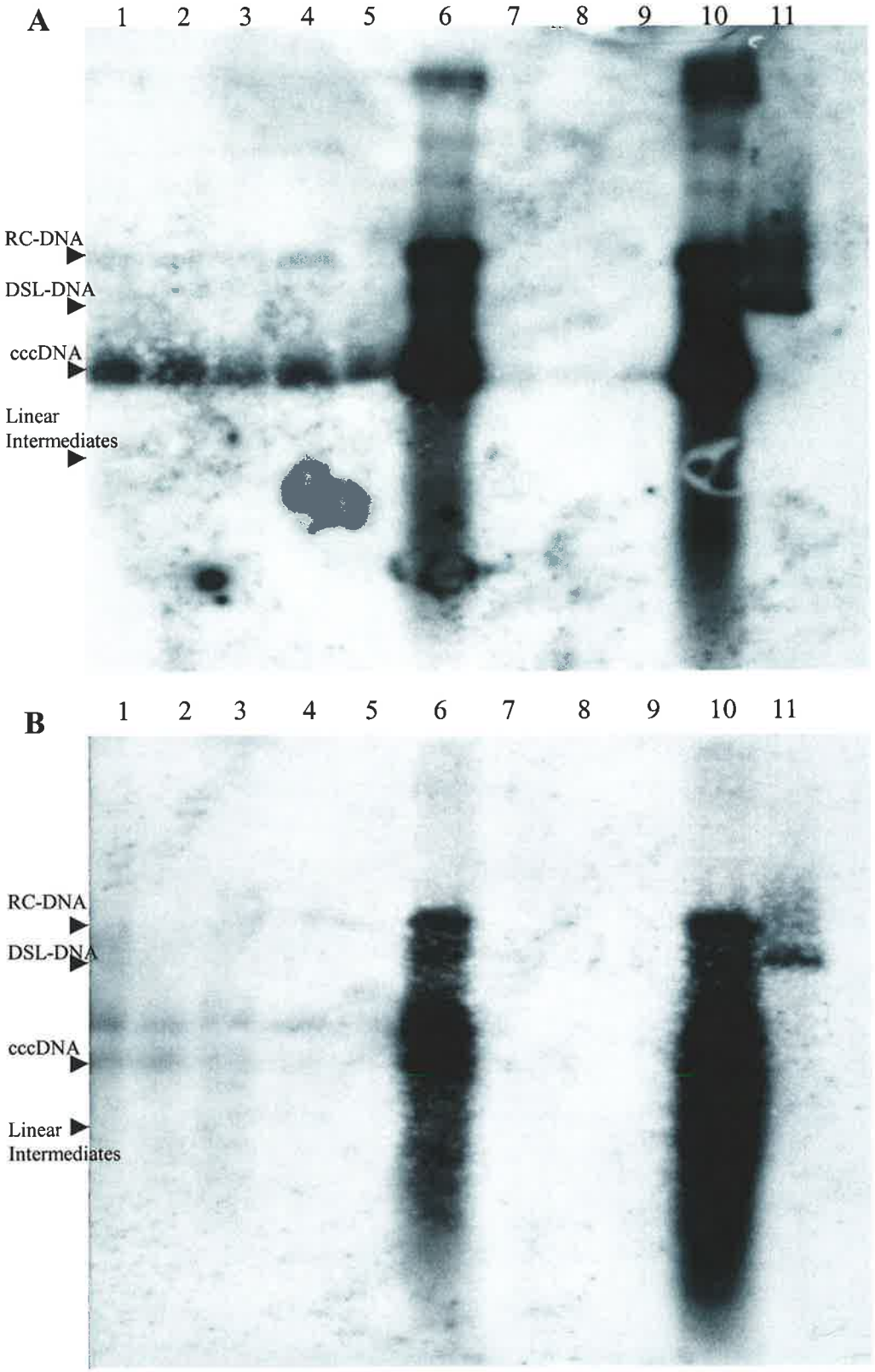

A
1 2 3 4 5 6 7 8 9 10 11
RC-DNA
DSL-DNA
cccDNA
Linear
Intermediates
B
1 2 3 4 5 6 7 8 9 10 11
RC-DNA
DSL-DNA
cccDNA
Linear
Intermediates

Table 5-2: DHBV DNA extracted from the liver of Group C ducks at day 3 p.i. and detected by Southern blot hybridization.

Duck	% liver cells sAg+ *	cccDNA		Total DHBV		cccDNA/total DNA as %
		Per cell	Per sAg+ cell	Per cell	Per sAg+ cell	
3435	3.8	0.096	2.5	4.2	111	2.3
3637	3.5	0.057	1.6	2.7	78	2.1
3839	1.9	0.035	1.8	3.8	198	0.9
4041	2.5	0.052	2.1	3.0	119	1.8
4243	1.5	0.039	2.6	5.2	347	0.7
mean	*2.64*	*0.048*	*2.1*	*3.8*	*170*	*1.2*

* From Table 5.1.

5.2.2.3 Outcome of Infection: DHBsAg staining

To assess clearance of infection liver biopsies collected one month p.i. were stained immunohistochemically for DHBsAg. Sections from all ducks in Groups A and B were negative for DHBsAg. Within Group C, 2 ducks, 3435 and 4243, showed widespread staining (>95% of hepatocytes positive) on day 31 p.i., but ducks 3637, 3839 and 4041 had no detectable DHBsAg-positive cells at 1 month p.i. and were considered to have cleared transient DHBV infection.

Ducks 3435 and 4243, which developed widespread infection, did not clear infection before autopsy at 9 months p.i. and were not included in the study of transient infection. Another 2 ducks, 233 from Group A and 1314 from Group B, died prematurely and were unavailable for autopsy, leaving 11 ducks which underwent transient infection and were autopsied at 9 months p.i..

5.2.2.4 Anti-DHBs Antibodies in Serum

Anti-surface antibodies were monitored by ELISA (Jilbert *et al.* 1998) twice weekly for the first month after inoculation and were detectable in 4 out of 5 ducks in Groups A and B as seen in Figure 5.4. In Group C, the 2 persistently infected ducks, 3435 and 4243, showed anti-surface antibodies transiently, before, in one duck, and coinciding, in the second duck, with the onset of detectable DHBsAg. The results from Group C are also shown in Figure 5.4. Anti-DHBs antibodies in ducks 3839 and 4041 from Group C persisted to at least day 90 p.i. (data not shown), but were not tested at later time points.

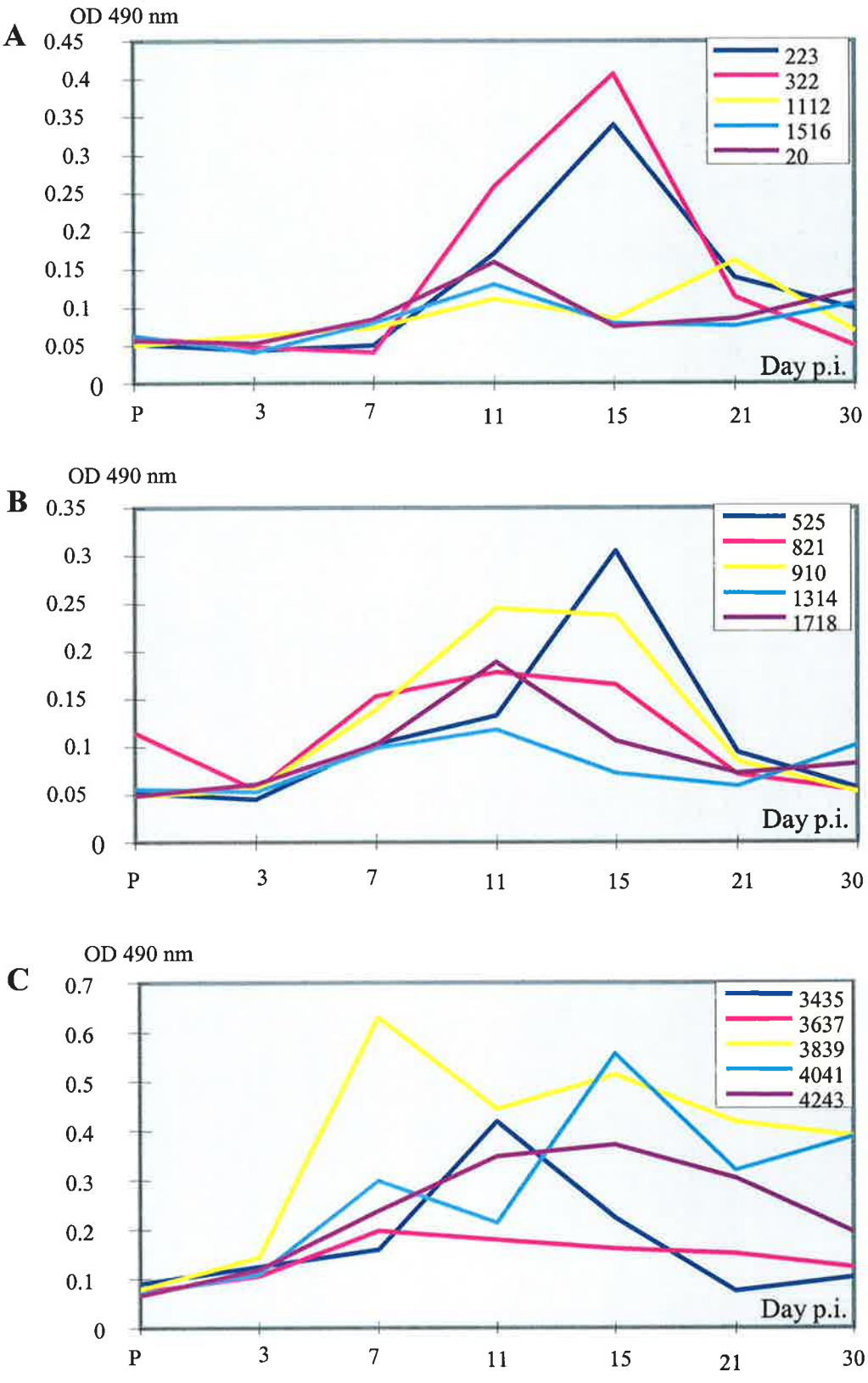
5.2.2.5 DHBsAg and DHBV DNA in Serum

Serum samples collected from all 15 ducks until day 30 p.i. were assayed for DHBsAg (Sections 2.5.1 and 2.5.2) by qualitative and quantitative ELISA. Only ducks 3435 and 4243, which failed to clear DHBV infection showed detectable DHBsAg (from day 15 and 21, data not shown).

Serum samples collected from all 15 inoculated ducks until 90 days p.i. were tested for DHBV DNA by spot blot hybridization (Section 2.7.6). As with DHBsAg, only ducks 3435 and 4243 showed detectable DHBV DNA. The sensitivity of the assay was measured using a high titre DHBV-positive serum (9.5×10^9 copies/ml) that was diluted two-fold from 1/2 to1/1024. The end-point dilutions that were positive were 1/64-1/128 dilutions corresponding to 7.4×10^7-1.5×10^8 copies/ml.

Figure 5.4 : Serum anti-DHBs antibodies measured by ELISA (Section 5.2.2.4) for each duck from before inoculation with DHBV (pre-bleed) until day 30 p.i.. Results are shown as the OD measured at 490 nm versus day p.i.. Serum samples were diluted 1/100. The OD value rose in all ducks, reaching an arbitrarily set cut-off for positive in 4 out of 5 ducks from each group.

A: Ducks from Group A inoculated with 10^7 virions.
B: Ducks from Group B inoculated with 10^8 virions.
C: Ducks from Group C inoculated with 10^{10} virions.


A
OD 490 nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
223
322
1112
1516
20
Day p.i.
P
3
7
11
15
21
30
B
OD 490 nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
525
821
910
1314
1718
Day p.i.
P
3
7
11
15
21
30
C
OD 490 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
3435
3637
3839
4041
4243
Day p.i.
P
3
7
11
15
21
30

A single series of serum samples from duck 3637 in Group C that had transient DHBV infection was assayed up to day 45 p.i. for viral DNA by real time PCR. Viral DNA was first extracted using the Roche HighPure Kit (Section 2.7.3) and then 10 µl of the final DNA extract, corresponding to 40 µl serum, was tested by PCR with primers P3 and P4 (Section 2.8.4). Samples from days 3, 8, 15 and 30 p.i. were positive, whilst those from days 11, 21 and 45 p.i. and autopsy were negative. At day 3 the estimated copy number of DHBV DNA was 61 copies in 40 µl serum or 1525 copies/ml. Quantitation was by comparison to plasmid DHBV DNA standards diluted in water and not to DNA extracts from serum. Thus these results should be considered as semi-quantitative only. The remaining positive samples were below the lowest standard (<20 copies/40 µl serum or <500 copies/ml) and therefore could not be quantitated.

5.2.3 Discussion

Inoculation of 39-40-day-old ducks with 10^7 (Group A) or 10^8 (Group B) virions led to approximately 0.00085% of hepatocytes staining positive at day 4 p.i. in Group A and 0.0105% in Group B. Thus a 10-fold increase in inoculum led to an approximately 12.4-fold increase in the frequency of hepatocytes staining positive for DHBsAg. A further 260-fold increase in the frequency of DHBsAg staining was seen in liver sections from Group C ducks which received a 100-fold higher inoculum (10^{10} virions) than Group B and were biopsied on day 3. Hence a clear dose effect on the extent of antigen expression in liver was evident early after inoculation. However, it is not known whether the infected cells seen at this time point reflect only primary infection with the initial inoculum or whether limited secondary spread between cells occurred. It is also unknown when the peak extent of infection occurs in the liver and whether the observed frequency of antigen staining was representative of the peak of infection. It is likely that the peak frequency of

antigen staining did not exceed 10-20% of hepatocytes, since surface antigenaemia is usually found with more extensive spread of DHBV in the liver (Jilbert *et al.* 1998). Surface antigenaemia was not detected by ELISA in ducks with transient infection at any time point tested.

Southern hybridization detected DHBV DNA in liver from the Group C ducks, but not in Group A or B ducks. The mean content of total DHBV DNA per infected cell (i.e. per cell expressing detectable DHBsAg) was measured as 170 copies, while cccDNA comprised 1.2 copies per infected cell. The total DHBV DNA copy number is similar to that found by Jilbert *et al* (Jilbert *et al.* 1992), who reported 204 copies per cell in 6 week old congenitally infected ducks (as used in this study) and a range of 82-816 in ducks of varying ages. The cccDNA copy number is lower than that reported by Jilbert *et al* (Jilbert *et al.* 1992), who found 2.9 copies per cell in 6-week-old congenitally infected ducks and a range of 2.9-15.8 copies per cell in ducks of varying ages. The figure of 1.2 copies of cccDNA per cell is also lower than other studies that suggest up to 50 copies of cccDNA may be present in HBV-infected cells (Miller and Robinson 1984), but overlaps with a recent study by Zhang et al showing 1-18 copies of DHBV cccDNA per infected cell (Zhang *et al.* 2002). Lower efficiency of the cccDNA extraction method is one possible explanation, since the estimate of total DHBV DNA from the same Southern blot is closer to published data. It is also possible that the low copy number of cccDNA reflects the early phase of transient infection and that cccDNA levels may rise over time. There is no experimental data to support this, however Jilbert *et al* reported a mean of 5.9 copies of DHBV cccDNA per cell were present at day 6 p.i. in transiently infected 5-month-old ducks compared with 15.9 copies per cell in 6-month-old congenitally infected ducks.

All ducks in Groups A and B and 3 out of 5 in Group C showed clearance of DHBsAg expression in liver at 1 month and anti-DHBs antibodies appeared in 10 out of 13 of these ducks during the first month p.i.. The level and duration of anti DHBs antibodies was greater in ducks given the largest inoculum, consistent with the observed higher levels of antigen staining and DHBV DNA in those ducks.

DHBsAg was not detected in serum, except ducks 3435 and 4243, which developed widespread persistent infection. This finding was consistent with previous observations from the laboratory suggesting that detectable surface antigenaemia and viraemia (by hybridization methods) are found in association with more extensive infection i.e. with >10% liver cells expressing DHBsAg (Jilbert *et al.* 1998). The sensitivity of the quantitative assay for DHBsAg (Section 2.5.2) is approximately 1-3 ng/ml, sufficient to detect DHBsAg in a 10^4 dilution of sera from congenitally DHBV infected ducks (>95% of hepatocytes infected), with a DHBsAg concentration of ~50 μg/ml (Jilbert *et al.* 1996).

It is clear from this observation that DHBsAg levels do not increase linearly as the percentage of hepatocytes expressing DHBsAg increases. This is consistent with appearance of detectable serum DHBsAg only when saturation of receptor or antibody binding occurs. Similarly, very little DHBV DNA was detected in serum. DHBV DNA was not detected by spot-blot hybridization and was detected only at low level in the one duck from Group C, which was assayed by PCR.

In summary, 3 groups of 39-40-day-old ducks were inoculated intravenously with 10^7, 10^8 or 10^{10} DHBV virions. All ducks became infected and transient infection occurred in all but 2 ducks given the highest dose. The extent of infection and serological responses were related to the size of the inoculum.

5.3 Studies of Residual DHBV DNA

5.3.1 Introduction

After monitoring the above ducks for 9-10 months p.i. autopsies were performed (277-313 days p.i.) except for duck 910 which was autopsied early, at day 159 p.i, because of an intercurrent illness. The timing was chosen to maximize the duration after recovery from infection without allowing sufficient time for the development of amyloidosis, which frequently occurs in Pekin ducks.

5.3.2 PCR Detection of Residual DHBV DNA in Liver

Initially, DHBV DNA was assayed by nested PCR (sensitivity 1 copy DHBV DNA) of DNA extracted from liver, spleen, kidney, pancreas, adrenal, heart, PBMC, skeletal muscle and serum collected at autopsy 9 months p.i.. Primers C1 and C2 were used as the outer set and C3 and C4 the nested set (Section 2.8.2); they targeted the core region of the DHBV genome yielding products of 437 bp and 267 bp respectively. The PCR was as described in Section 2.8.3 using 600 ng of extracted liver DNA (Section 2.7.2). Southern blot hybridization of the product to a ^{32}P labelled full-length DHBV DNA genome was performed for sensitive detection of any contamination of the negative controls (Figure 5.5 and Table 5.3).

Most liver and spleen samples – 10 and 7 out of 11 respectively tested positive for DHBV DNA, whereas little DHBV DNA was detected in other sites – 2 kidney samples and 1 adrenal and heart samples only. The efficiency of the extraction method varied between tissues and hence the quantity of DNA template in each PCR reaction varied, with the approximate quantity shown in Table 5-3.

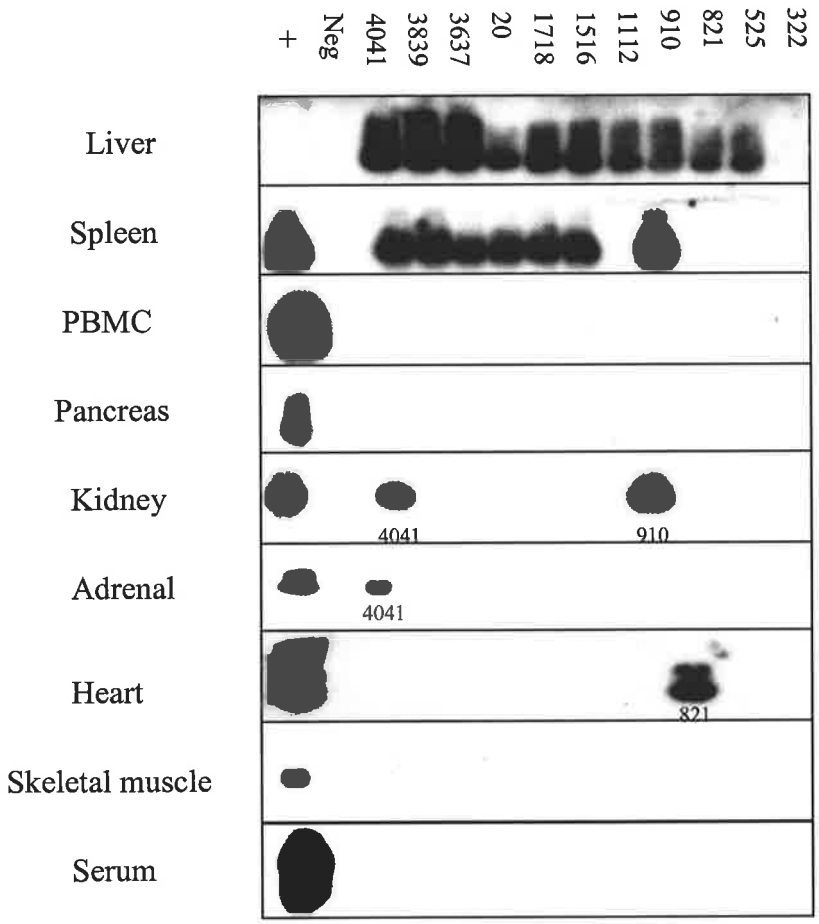


Figure 5.5: Nested PCR detection of residual DHBV DNA from autopsy tissue collected approximately 9 months p.i.. This Southern blot hybridization shows PCR products from second round of nested PCR assays. Nine separate assays, each showing a different tissue, are shown as a composite. Ten out of 11 liver, 7 out of 11 spleen, 2 out of 11 kidney and 1 each out of 11 adrenal and heart samples show DHBV DNA. Positive and negative controls are shown except for liver for which only a negative control was performed.

Table 5-3: Summary of Nested PCR Detection of Residual DHBV DNA

Tissue	DNA template (ng)	No. positive / No. tested
Liver	600	10/11
Spleen	250	7/11
Kidney	250	2/11
Pancreas	300	0/11
Adrenal	1000	1/11
Heart	200	1/11
Skeletal muscle	100	0/11
PBMC	100	0/11
Serum	DNA from 10 µl serum	0/11

5.3.3 Effect of Dose on Residual DHBV DNA

Quantitative real time PCR was performed with primers P3 and P4 (Section 2.8.2) using 200 ng of DNA extracted from the same autopsy liver and spleen samples studied by nested PCR (Sections 5.3.2) One out of 3 liver samples from Group A, 3 out of 4 from Group B and all 3 from Group C showed detectable DHBV DNA using this assay which has a sensitivity of 10 copies in 200 ng total DNA, equivalent to to DNA extracted from 8×10^4 cells. As seen in Table 5-4, liver samples from Group C showed from 190-1260 copies of DHBV DNA in 200 ng of extracted DNA, equivalent to 8×10^4 cells. This result equates to 0.002-0.016 copies/cell or a mean of 0.007 copies/cell. Thus, as inoculum size increased, the amount of residual DHBV DNA increased.

DHBV DNA in spleen was also quantitated using primers P3 and P4 with 3 out of 4 ducks in Group A and 2 out of 4 in Group B testing positive. Only one of these samples (duck 910) showed levels sufficient for quantitation, measuring 240 copies. Spleen samples from all 3 ducks tested from Group C were positive for DHBV DNA, containing from 20–70 copies/8×10^4 cells. Overall this suggests that lower levels of residual DHBV DNA were present in spleen than in liver and that there is an effect of inoculum dose, although less consistent than that found in the liver. Quantitative PCR was positive for DHBV DNA in 8/11 ducks, compared with only 7/11 that tested positive for DHBV DNA by nested PCR. Results in nested and quantitative PCR were the same for 8 and differed for 3 spleen samples. It is likely that the sensitivity of nested PCR was similar to quantitative PCR when testing spleen since a similar amount of extracted DNA was added to each reaction, unlike liver for which a larger amount was added to the nested PCR reaction.

Samples of kidney, heart and adrenal, which tested positive for DHBV DNA by nested PCR, (using primer pairs C1 and C2, then C3 and C4) were subjected to quantitative PCR. The 2 kidney, 1 heart and 1 adrenal samples were positive but at levels insufficient for quantitation, i.e. <20 copies/200 ng DNA or 8×10^4 cells.

Table 5-4: Quantitation of residual DHBV DNA in autopsy liver and spleen using PCR primers P3 and P4

Group/dose	Duck No.	Liver*	Spleen*
A 10^7 virions	322	ND	1-20
	1112	ND	1-20
	1516	1-20	1-20
	20	ND	ND
B 10^8 virions	525	1-20	ND
	821	ND	ND
	910	1-20	240
	1718	1-20	1-20
C 10^{10} virions	3637	1260	20
	3839	190	70
	4041	200	50

ND: not detected.
* Copies DHBV DNA/8×10^4 cells

5.3.4 DHBV DNA Levels: Changes over Time

Limited quantitative data were obtained from liver samples taken at day 3-4 and 1, 3 and 6 months p.i. in addition to the autopsy samples collected at 9 months p.i.. Although biopsy liver samples were collected from all ducks at each time point, quantitative data could not be obtained from all samples due to the limited amount of tissue remaining after initial phenol-chloroform extraction. For this reason some results of Southern blot hybridization assays have been used in addition to PCR as shown in Table 5-5 and the number of ducks tested and number positive is shown for each group. The results for Groups A and B were high enough for quantitation only at the first biopsy taken at day 3 p.i.; thereafter levels

were too low to be quantified and qualitative results are therefore shown in Table 5-5. Quantitative results from samples in Group C were available at all time points, showing a fall to low levels of viral DNA which occurred in the first month p.i.. Residual DHBV DNA levels remained relatively constant in the latter 6 months of the study ranging from 334-563 copies/8×10^4 cells.

Table 5-5: Time course of DHBV DNA levels measured by quantitative PCR using primers P3 and P4:

Group		Day 3-4	1 month	3 months	6 months	9 months
A	PCR Positive/ number tested	3/3	3/4	0/1	1/3	1/4
	Mean copies/ 8×10^4cells	268	*	*	*	*
B	PCR Positive/ number tested	1/1	5/5	3/3	4/4	3/4
	Mean copies/ 8×10^4cells	1380	*	*	*	*
C	PCR Positive/ number tested	5/5	3/3	1/1	3/3	3/3
	Mean copies/ 8×10^4cells	3.0×10^5 †	1.0×10^3 †	350	334	563

* insufficient for quantitation.
† measured by Southern hybridization, other samples measured by PCR using primers P3 and P4.

5.3.5 Selective Detection of cccDNA

To define the form of residual DNA, the above samples were then retested using PCR primers designed for selective detection of cccDNA (Section 2.8.5).

One out of 4 Group A, and 1 of 4 Group B samples tested positive for cccDNA, as did all 3 Group C samples (Table 5-6). Levels were insufficient for quantitation in Groups A and B but in Group C cccDNA levels ranged from 80-1110 copies/8×10^4 cells. Levels of cccDNA measured a mean of 69% of total DHBV DNA. This suggests that the most prominent form of residual DHBV DNA is cccDNA unlike what is seen in acute infection.

Table 5-6: DHBV cccDNA in liver measured by PCR using primers CC2 and R2 as proportion of total residual DNA

Group	Duck	CccDNA Copies/8×10^4 cells	Total DHBV DNA Copies/8×10^4 cells	cccDNA/total DNA as %
A 10^7 virions	322	ND	ND	
	1112	ND	ND	
	1516	ND	+	
	20	+	ND	
B 10^8 virions	525	+	+	
	821	ND	ND	
	910	ND	+	
	1718	ND	+	
C 10^{10} virions	3637	1110	1260	89
	3839	80	190	42
	4041	150	200	76

ND: not detected, +: positive but levels too low for quantitation

The selective real time PCR assay for cccDNA was also performed on all spleen samples and the remaining samples from other organs which had tested positive for total DHBV DNA. All 11 spleen samples, 2 kidney, 1 heart and 1 adrenal samples tested were negative for cccDNA by this assay (i.e. <10 copies/8×10^4 cells).

5.3.6 Discussion

The confirmation of persistence of residual DHBV DNA following recovery from transient infection is consistent with findings in studies of HBV (Blum *et al.* 1991; Michalak *et al.*

1994; Rehermann *et al.* 1995; Penna *et al.* 1996; Mason *et al.* 1998; Yotsuyanagi *et al.* 1998; Marusawa *et al.* 2000) and WHV (Michalak *et al.* 1999) and is the first report of residual DHBV DNA (Le Mire *et al.* 2001). The finding of residual DHBV DNA (i.e. in 10 out of 11 liver samples from individual ducks) approximately 9 months following inoculation and 8 months after the disappearance of detectable DHBsAg in liver, indicates that complete clearance of viral DNA is not the usual outcome of transient infection, or if so, it takes longer than 9 months. Data from studies of HBV are less uniform, probably reflecting the limited tissue available in clinical studies (Penna *et al.* 1996; Mason *et al.* 1998; Yotsuyanagi *et al.* 1998; Marusawa *et al.* 2000). In woodchucks, residual WHV DNA was found in liver and other tissues in all animals studied by Michalak *et al* (Michalak *et al.* 1999).

Residual DHBV DNA was found chiefly in liver and spleen with only 4 other samples testing positive (2 kidney, 1 heart and 1 adrenal). In addition the quantity of viral DNA was highest in liver, followed by spleen, but levels were insufficient for quantitation in samples from kidney, heart and adrenal tissue. This suggests that the liver is the most important site of residual DHBV DNA. Most other reports are consistent with this although the finding of residual WHV predominantly in lymphoid tissue, including PBMC, in some woodchucks following transient infection has led to the proposal that lymphoid tissue is an important site for residual hepadnavirus (Coffin and Michalak 1999; Michalak *et al.* 1999). The findings of this study differ significantly from those of the woodchuck study in that no DHBV DNA was found in PBMC at autopsy, although it must be remembered that the spleen is also a key lymphoid organ in which DHBV DNA was found.

Changes in levels of DHBV DNA in liver were studied over time. Although the data are more limited due to insufficient available tissue, levels fell during the first month and then persisted at a fairly constant level from 3 months p.i. until autopsy at 9 months p.i.. This observation suggests the presence of a residual steady state. This could be maintained by balanced ongoing replication and virus loss or by persistent inactive infection in long-lived cells. Studies of cell turnover are described in Chapter 6.

The presence of cccDNA is a *sine qua non* of infection of a cell with a hepadnavirus, since this is the essential template for production of progeny virus (Miller and Robinson 1984; Tuttleman *et al.* 1986). The finding of cccDNA in liver is essential for but does not necessarily prove ongoing replication. Quantitation of DHBV DNA forms found in liver suggested that most residual DHBV DNA was cccDNA (average of 69%). Applying the observed coefficients of variation of each assay of approximately 20% (see Sections 3.4.2 and 3.5.2) the level of cccDNA in duck 3637 was 1110 +/- 222, i.e. 888-1332 copies/8×10^4 cells, and the measured level of total viral DNA was 1260+/-252, i.e. 1008-1512 copies/8×10^4 cells. The latter value was assumed to represent all DHBV DNA, although additional fragments of viral DNA not containing the amplified region, if present, would not have been measured. The measured percentage of total DHBV DNA made up of cccDNA is 59-132%, meaning that most or all residual DNA may be cccDNA. This is in marked contrast to the findings in early infection after inoculation with 10^{10} virions or in the setting of congenital infection as described in Chapter 4, where cccDNA was measured as representing less than 2% of the total pool of DHBV DNA. The high proportion of DHBV DNA present as cccDNA after recovery from transient infection is consistent with a state of suppressed replication in which little DNA replication is evident despite the presence of the cccDNA template. Possible explanations for this finding include (i) defective genomes may be present that do not allow replication or (ii) residual cccDNA

may be present in cell types that do not permit replication or antigen expression or (iii) there may be endogenous or exogenous suppression of replication. In each of these cases antigen expression might not occur, thus assisting in evasion of immune responses. In order to look for evidence distinguishing these hypotheses, 2 experiments were designed. The first was to try to reactivate replication by administering immunosuppressive drugs to ducks which had recovered from DHBV infection; the second was to amplify full length genomes following recovery from transient DHBV infection and to sequence to look for mutations or deletions. These experiments are described in Chapter 6.

Residual cccDNA was not detected outside the liver, raising the possibility that the extrahepatic residual DNA does not represent true infection. In particular, the finding of >20 residual DNA copies in liver was invariably accompanied by detectable cccDNA in liver, but cccDNA was not detected in the 4 samples of spleen in which >20 copies of DHBV DNA were detected. The presence of DHBV DNA without detectable cccDNA in spleen might reflect (i) binding or phagocytosis of circulatory virus by follicular dendritic cells or other phagocytic cells within the spleen or (ii) presence of circulating virus which is detected in the vascular compartment in tissues, such as spleen and occasionally kidney. However, no residual viral DNA was detected in serum either by nested or quantitative PCR. It is likely therefore that the DHBV DNA detected in extrahepatic tissues represents tissue associated rather than circulating material, with the absence of detectable cccDNA meaning that true residual infection has not been demonstrated.

Integration of hepadnavirus DNA is well recognized and might be considered as a possible mechanism for persistence of DHBV DNA. Yang and Summers have reported integration of DHBV DNA *in vivo* (Yang and Summers 1999) and found specific DHBV DNA species which were integrated. However, none of the integrated sequences described were

continuous in the region spanned by the cccDNA PCR primers and integration is proposed to occur preferentially from within the region of the viral genome bounded by the 2 direct repeats. Hence it is unlikely that integrated DNA forms a significant part of the pool of the residual DHBV DNA measured by selective PCR for cccDNA.

Using specific PCR detection of integrated DNA Yang and Summers found an estimated rate of 1 integration in 10^3-10^4 cells 6 days after inoculation with a dose of DHBV sufficient to produce widespread infection (most hepatocytes show DHBsAg) (Yang and Summers 1999). Studies of DHBsAg and DHBV DNA in the ducks in this experiment suggest only a small percentage of cells (mean of 2.64% measured at day 3 p.i.) were infected and hence the available pool of DHBV DNA for integration may be considerably smaller. As a conservative estimate integration might be expected to occur with at least a 10-fold lower frequency i.e. 1 integration in 10^4-10^5 cells. Without expansion of a pool of integrated DNA this would yield very low levels of integrated DHBV DNA near or below the threshold for detection by the PCR assays used in this study.

In summary, DHBV DNA persists after transient infection and recovery. The liver and spleen are important sites of residual DHBV DNA. CccDNA, which suggests true infection was found only in liver where it contributed most or all the residual DHBV DNA. This is consistent with a state of suppressed replication, the explanation of which will be sought in chapter 6.

6. Further Studies of the Nature and Maintenance of Residual DHBV DNA

6.1 Introduction and Aims

The work described up to this point demonstrates the persistence of traces of DHBV in liver and spleen following recovery from transient infection. In liver most of this residual DNA is cccDNA. This suggests that the template for replication and production of virions may be present, but that significant replication is not occurring. What has not been shown thus far is whether any limited viral replication occurs, whether virions are produced. In addition the mechanism of suppression of replication and the mechanism of maintenance of residual DHBV DNA have not been determined.

6.1.1 The Role of Immune Responses

The first of the questions to be addressed in this chapter is whether viral replication is suppressed by immune mechanisms. In the setting of congenital hepadnavirus infection, leading to immune tolerance and high levels of viral replication, hepatocytes contain up to 50 copies of cccDNA and 100-1000 copies of other forms of viral replicative DNA per cell (Miller and Robinson 1984). Overall a relatively small number of copies of template cccDNA produce a large number of copies of product. When replication is suppressed with an inhibitor of viral reverse transcriptase (eg. lamivudine, entecavir or adefovir) a large reduction in copy number of viral genomes occurs within the liver. This fall is reflected in a reduction of several logs in DHBV DNA copies in serum or liver. However, the copy number of cccDNA within the liver is relatively stable (Nicoll *et al.* 1998; Zhu *et al.* 2001), leading to an increase in the ratio of copies of cccDNA to total viral DNA. Similar changes in the ratios of total to cccDNA were found in this study during recovery from transient DHBV infection (see chapter 5). In Group C ducks overall levels of DHBV DNA in the liver fell approximately 3 logs and levels of cccDNA fell 1 log during

recovery, such that the measured ratio of cccDNA/total DHBV DNA increased from 1.2% to 69 %. Indeed it is possible, when assay error is taken into account, that all residual viral DNA was in the form of cccDNA.

In order to test the hypothesis that immune mechanisms are responsible for maintaining suppression of replication a group of ducks was treated with immunosuppressive drugs and to document any changes in levels of DHBV DNA in liver. Immunosuppressive drug treatment in humans has been associated with reactivation of HBV in patients initially positive for anti-HBs antibodies. The frequency of reactivation is not known but is probably less than 5% (Lok *et al.* 1991; Markovic *et al.* 1999). It is not known whether subclinical increases in viral replication occur in a larger proportion of such patients. A study of woodchucks (Michalak 2001) showed a measurable transient increase in levels of WHV DNA in the liver after 2 weeks of immunosuppression, with 30 mg/kg/day of intramuscular cyclosporin, but this did not lead to development of overt markers of reactivation such as surface antigenaemia.

Little data are available relating to the use of immunosuppressive drugs in ducks. The effect on ducks of commonly used immunosuppressive drugs, such as corticosteroids, azathioprine and cyclosporin A is unknown. Corticosteroid treatment was associated with an increase in DHBV DNA polymerase activity in chronically infected ducks in one study (Hirota *et al.* 1986). Cyclosporin use is not reported in ducks, but has been used in chickens (Nowak *et al.* 1982). It has been effective in suppressing autoimmune disease (Wick *et al.* 1982; Pardue *et al.* 1987) and led to increased morbidity from avian polyomavirus infection (Fitzgerald *et al.* 1996). Decreases in the weights of bursa, spleen and thymus have been reported in chickens treated with cyclosporin (Nowak *et al.* 1982). Because of the uncertain efficacy of both corticosteroids and cyclosporin it was decided to

use both in an immunosuppressive regimen, and to assess its efficiency by monitoring lymphocyte proliferation before and during treatment and histological changes in the spleen.

6.1.2 Are Defective DHBV Genomes Present?

Another possible explanation for the apparently limited or absent DHBV replication observed after recovery from transient infection is that the residual viral genomes are defective and incapable of replication. Defective genomes might not permit antigen production, thereby assisting in evading immune clearance. Mutants with altered or reduced antigen expression are found in HBV infection. Mutations in the preS-S ORF develop as vaccine escape variants and are found when immune globulin is used as prophylaxis against reinfection of liver allografts after transplantation. Mutations in the pre-core region leading to defective production of HBe are also found under immune pressure and appear to have reduced replication efficiency (Cabrerizo *et al.* 1998; Lindh *et al.* 1999), although the findings vary (Scaglione *et al.* 1997) and it has been suggested that mutations in the precore translation initation codon in some HBV strains allows for increased replication (Laras *et al.* 1998). It is conceivable that residual viral genomes include other variants that may have reduced antigen expression and/or replication. To examine the nature of residual DHBV genomes, residual DHBV DNA was examined by Southern blot hybridization and by PCR amplification of full-length genomes (to identify major size changes) and by sequencing of a portion, the DR region (to identify smaller deletions or sequence changes)

6.1.3 Are Infectious Virions Produced?

A third question is whether any virions are produced from the residual DHBV DNA. The apparent lack of measurable RC DNA in serum does not indicate that none is produced. A

reduced half-life of virions due e.g. to neutralizing antibody could explain a marked reduction in levels of RC DNA. The DHBV model provides a sensitive test for infectious virions since a single virion has been shown to be infectious in 2-3-day-old ducklings (Jilbert *et al.* 1996) inoculated intravenously with the Australian strain of DHBV used in this study. Approximately 10^2 copies inoculated intraperitoneally have also led to infection in ducklings (Vickery and Cossart 1996). Hence small numbers of infectious virions may be detectable by IV and intraperitoneal inoculation into ducklings. Previous demonstration of infectivity of residual hepadnavirus has been performed in humans and woodchucks as outlined in sections 1.9.4 and 1.9.5. In humans infection is transmissible by liver transplants from donors with past HBV infection, and in woodchucks, following transient WHV infection, peripheral blood mononuclear cells yielded infectious virions after culture (Michalak *et al.* 1999). Blood transfusion, however does not appear to be associated with transmission of HBV from blood donors with markers of past HBV infection. This is despite reports of detectable serum HBV DNA many years after transient HBV infection. It is possible that any virions present are bound to neutralizing antibodies and are hence rendered inactive.

6.1.4 Maintenance of Residual Viral Genomes

The final question to be addressed in this study relates to the maintenance of residual DHBV DNA. The relatively stable levels of residual DHBV DNA found from 3-9 months after inoculation may be readily explained in 2 ways. In the first, replication may lead to replenishment of DHBV DNA with the level of residual DNA reflecting a balance between production and clearance. In the second, DHBV DNA may be inactive but levels remain stable because cell turnover is very low. Discrimination between these alternatives may be assisted by comparing the rate of hepatocyte turnover with changes in levels of intrahepatic residual viral DNA.

Hepatocyte turnover in 3-6 month-old congenitally DHBV-infected ducks was measured by BrdU labeling in a study by Fourel *et al* (Fourel *et al.* 1994) and estimated to be 0.75-1.5% per day. These workers also estimated a turnover of 0.05-0.1% of cells per day in uninfected ducks. Mason *et al* (Mason *et al.* 1994) performed BrdU labeling in 8-month-old congenitally DHBV infected ducks and reported a lower limit estimate of 1.5% cell turnover per day.

Models for the possible fate of cccDNA after cell division have been proposed and tested by Zhu *et al* (Zhu *et al.* 2001) by correlating levels of DHBV cccDNA with cell division measured by 5-Bromo-2'-deoxyuridine (BrdU) labeling. Levels of cccDNA are expected to decline during antiviral therapy (to block synthesis of cccDNA) at a rate which is related to cell turnover. Cell death is taken to mean loss of the cccDNA content of that cell. Cell division may or may not mean loss of cccDNA depending on whether cccDNA can be passed on to daughter cells. Mathematical models were used by Zhu *et al* (Zhu *et al.* 2001) to predict the expected level of hepadnavirus cccDNA in the setting of antiviral therapy at a given time point. The results suggested that cccDNA may be distributed to daughter cells after cell division (Zhu *et al.* 2001). In other words, cccDNA is lost with cell death, but survives cell division.

In this study, the observed fall in cccDNA levels during recovery from transient infection was compared with levels predicted by the mathematical model of Zhu *et al.* If predicted levels of cccDNA were lower than actual observed levels, then cccDNA must be maintained by replenishment or exist in a stable subset of cells.

The aims of the work described in this chapter were to –

1. Attempt to reactivate viral replication with immunosuppressive therapy after recovery from transient DHBV infection.
2. Investigate the presence of full length residual cccDNA by
    - amplification of genome length DHBV DNA by long range PCR,
    - sequencing across the DR region of the genome and
    - Southern blot analysis of residual DHBV DNA.
3. Test for the presence of infectious virus after recovery from transient infection.
4. Correlate levels of residual DHBV DNA with cell death and division in order to determine possible mechanisms of genome persistence.

6.2 Immunosuppression Study

6.2.1 Experimental Outline

Seven 40-day-old ducks (Group G) were inoculated intravenously with 10^{10} DHBV virions. Serum DHBsAg was assayed on days 14 and 27 p.i. and was detected in only 1 of the 7 ducks. This duck was excluded from the study, since it had developed widespread DHBV infection and viraemia and not cleared infection. Four months later the experiment was commenced with 5 of the remaining 6 ducks, 3 of which were treated with immunosuppressive drugs and 2 with placebo (water).

Cyclosporin A (50 mg/kg/day) and dexamethasone (0.1 mg/kg/day) were administered as single oral daily doses via a gavage tube (Section 2.2.3) for 28 days to 3 ducks and water was administered as placebo to 2 ducks. The cyclosporin dose was similar to doses used in published experiments with chickens (Nowak *et al.* 1982; Wick *et al.* 1982; Thomson *et al.* 1984; Pardue *et al.* 1987; Fitzgerald *et al.* 1996) and the dexamethasone dose was based on a report on its use in ducks (Hirota *et al.* 1986). Liver and serum were collected from all ducks before, after 2 weeks and at the end of 4 weeks of treatment and used for detection

of DHBV DNA. Liver and spleen samples were collected at autopsy (5 months p.i. and after 4 weeks of immunosuppressive treatment) and were used to inoculate 2-day-old ducklings (as described in Section 6.4) as well as for detection of DHBV DNA. PBMC were collected before and 2 weeks into the period of immunosuppressive therapy and assayed *in vitro* for lymphocyte proliferation in response to recombinant DHBcAg and DHBV. The physical activity levels of the ducks was monitored daily, they were weighed 3 times per week and a single serum cyclosporin level was assayed prior to dosing near the end of the treatment.

6.2.2 Results

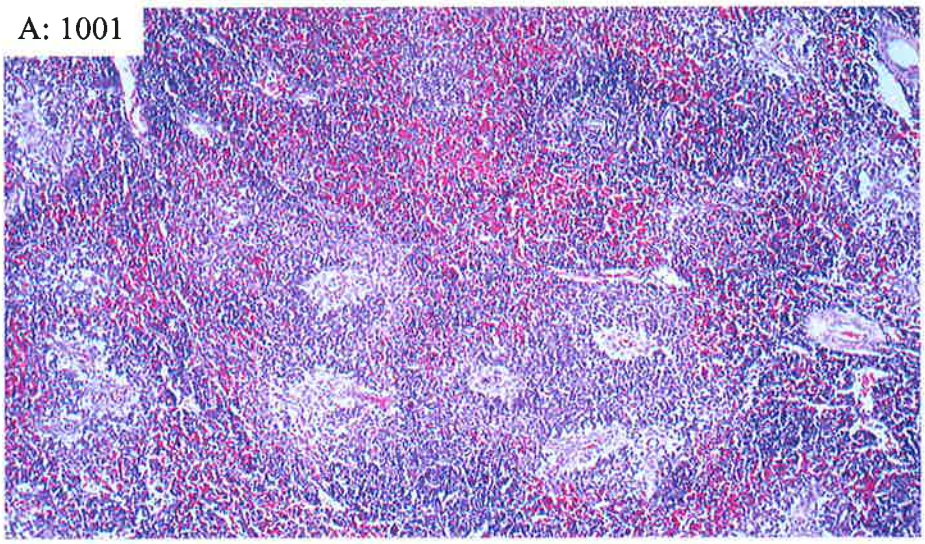
The ducks tolerated the treatment. Physical activity levels appeared normal. Weight fell by a mean of 400 g in treated ducks vs. 100 g in those given placebo over the 4 week study (data not shown). Levels of cyclosporin taken as trough (i.e. immediately before dosing) were 58, 134 and 244 μg/L (therapeutic range in humans: 80-250 μg/L). At autopsy, histological depletion of spleen cells was noted in 2 out of 3 ducks treated ducks, but not in those given placebo as seen in Figure 6.1. This was assessed by examination of H&E stained sections by an experienced histopathologist (Dr James Nolan, Institute of Medical and Veterinary Science, Adelaide), who was not aware of the treatment status of each duck.

Lymphocyte proliferation gave inconsistent results. At week 1 prior to immunosuppression only 1 of the 3 ducks (1001) showed mitogen induced proliferation, and all 3 ducks showed minimal response to antigen compared to no antigen. Two weeks into the period of immunosuppression, some tendency to lower proliferation was seen but with the exception of duck 1001 treated with mitogen, the assays were too variable to show

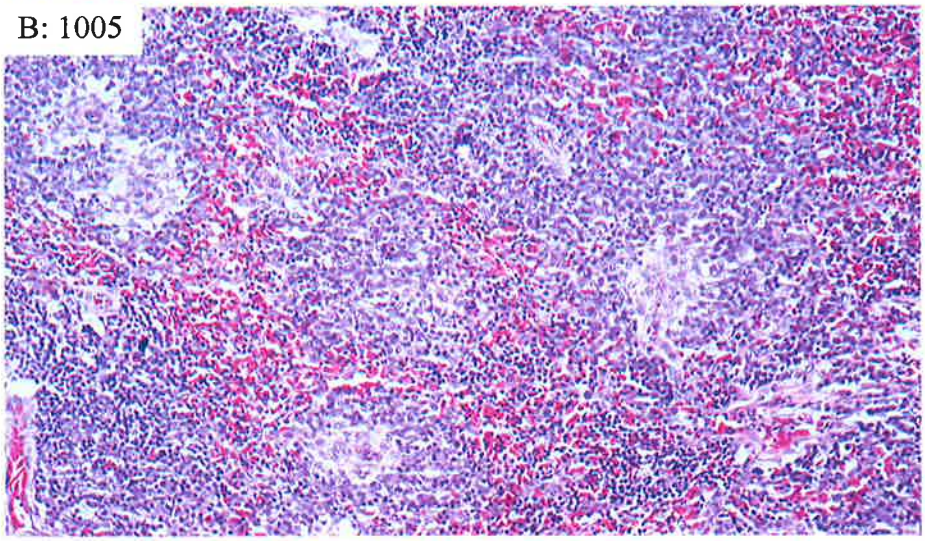
Figure 6.1: Effect of immunosuppressive treatment on spleen histology.

Ducks 1001, 1005 and 1006 received 4 weeks of cyclosporin A (50 mg/kg/day) and dexamethasone (0.5 mg/kg/day) before autopsy and collection of spleen samples as shown in panels A-C. Sections are labeled with the duck number, stained with H&E and photographed at 200× magnification. Sections from ducks 1001 and 1005 show reduced cellularity. This is less marked in the section from 1006. On panels D-F the 3 control duck spleen samples are shown – 1003, 1004, 1007. These show mild or no depletion of cellularity in the spleen (Section 6.2.2).

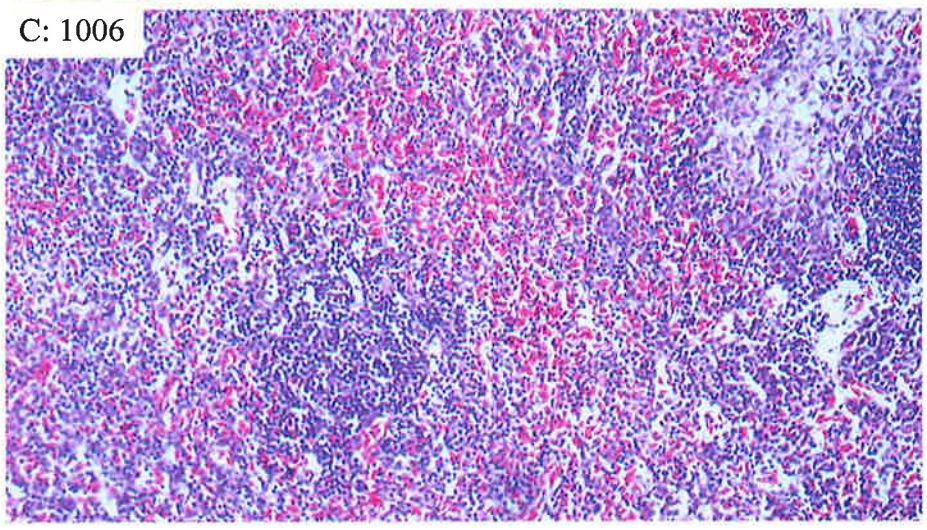
A: 1001



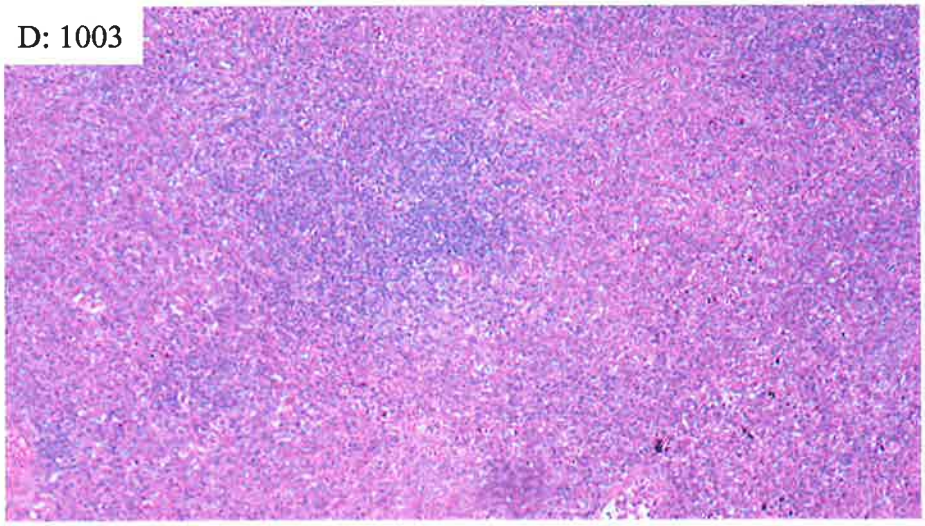
B: 1005



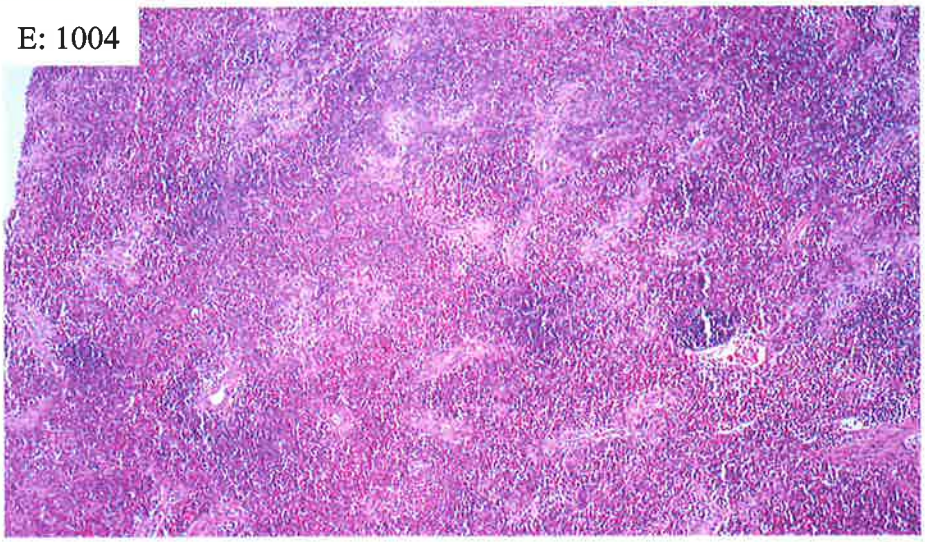
C: 1006



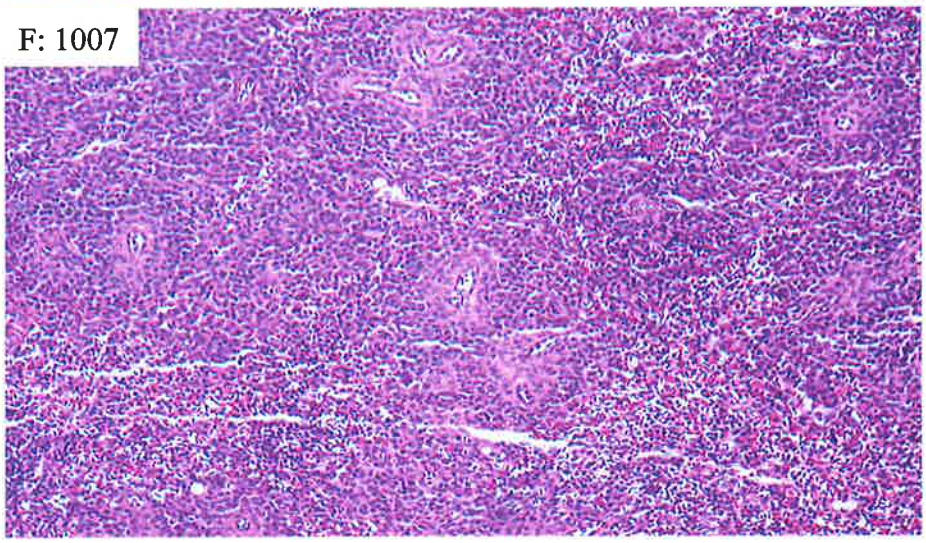
D: 1003



E: 1004



F: 1007



significant evidence that immunosuppression had been achieved. Results are summarized in Table 6-1.

Table 6-1: Lymphocyte Proliferation of PBMC collected from ducks with immunosuppressive treatment

Duck	Assay Antigen*	Pre-treatment Counts/min	During treatment Counts/min	Result
1001	No antigen	1444+/-328	265+/-145	
	DHBV	2935+/-2697	368+/-234	No antigen stimulated proliferation
	DHBc	2162+/-2355	568+/-225	No antigen stimulated proliferation
	PHA	219704+/-12239	675+/-473	Reduced mitogen stimulated proliferation with treatment
1005	No antigen	277+/-245	231+/-180	
	DHBV	382+/-124	648+/-490	
	DHBc	1543+/-1511	2376+/-2126	
	PHA	1073+/-248	5602+/-5110	No mitogen stimulated proliferation pre-treatment
1006	No antigen	328+/-159	111+/-35	
	DHBV	1094+/-1859	117+/-52	
	DHBc	863+/-798	578+/-321	
	PHA	1208+/-1242	473+/-667	No mitogen stimulated proliferation pre-treatment

* see Section 2.3.2 for a description of the assays.

DHBV DNA was measured in liver DNA extracts by performing quantitative PCR (Section 2.8.4). No significant change in levels of DHBV DNA was found at 2 or 4 weeks

after commencing immunosuppressive treatment (see Table 6-2). Similar results (data not shown) were seen in ducks given placebo.

Table 6-2: DHBV DNA Levels under Immunosuppression

Duck	DHBV DNA copies per 200 ng liver DNA*		
	Pre-treatment	2 weeks into treatment	4 weeks into treatment
1001	210	130	110
1005	200	60	140
1006	170	170	150

* 200 ng liver DNA is equivalent to 80000 cells.

6.3 Further analysis of residual DHBV DNA Genomes

6.3.1 Experimental Outline

Three experiments were performed: (i) to show that full-length DHBV genomes were present, (ii) to show that the DR region of some residual DHBV DNA was intact and (iii) to confirm using Southern hybridization that the residual DHBV DNA detected using the cccDNA PCR was present in a characteristic cccDNA form.

6.3.2 Results

In the first experiment, PCR amplification of genome-length DHBV DNA was performed using 1 μg of liver DNA extracted (Qiagen method) from ducks 3637, 3839 and 4041 9 months p.i. (Section 2.8.7). Extracted DNA was amplified using primers FL1 and FL2 (corresponding to nucleotide positions 1669-1688 and 1668-1649 on the DHBV genome). These primers were designed to amplify full-length DHBV DNA and should amplify a cccDNA template with a greater efficiency than RC DNA, since RC DNA is not

continuous between the primers. PCR products were analyzed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide (Figure 6.2 A). A 3 kb product was seen in all 3 autopsy samples as well as in a positive control sample that consisted of extracted liver DNA from a duck with high-level DHBV infection (lane 2). Southern blot hybridization using a genome-length radiolabelled DHBV DNA probe demonstrated that the 3 kb product contains DHBV sequences (Figure 6.2 B). The quantity of full-length product appears greatest for ducks 3839 and 4041. Also shown were smaller than genome-length products, which also hybridize to the DHBV probe.

In the second experiment, sequencing of the DR region of cccDNA was performed to determine whether this region was preserved intact. The DR region was amplified by PCR (primers CC2 and R2, Section 2.8.2) using 500 ng of liver DNA extracted from duck 3334, a duck from a separate experiment (Group W, see below). This duck was chosen because higher levels of residual DHBV were present, reducing the technical problems of sequencing a small amount of PCR product. The duck had been experimentally infected with 10^6 DHBV virions at 2 weeks of age and then developed widespread infection in liver before clearance of liver and serum DHBsAg (serum after day 132) and development of anti-DHBs antibodies. Liver was collected at autopsy at day 182 and DNA was extracted for PCR. PCR amplification yielded a 618 bp product, which was sequenced using the same primers as for the PCR reaction (Section 2.9). A 503 bp segment of the PCR product was sequenced using primer CC2 and showed a single bp substitution at nt 2525. The predicted amino acid sequence showed no alteration from the wild-type virus [AusDHBV (Triyatni *et al.* 2001)] used to inoculate the duck. Hence the DR region of DHBV DNA from a duck which had previously cleared DHBV infection showed no significant changes from wild-type DHBV.

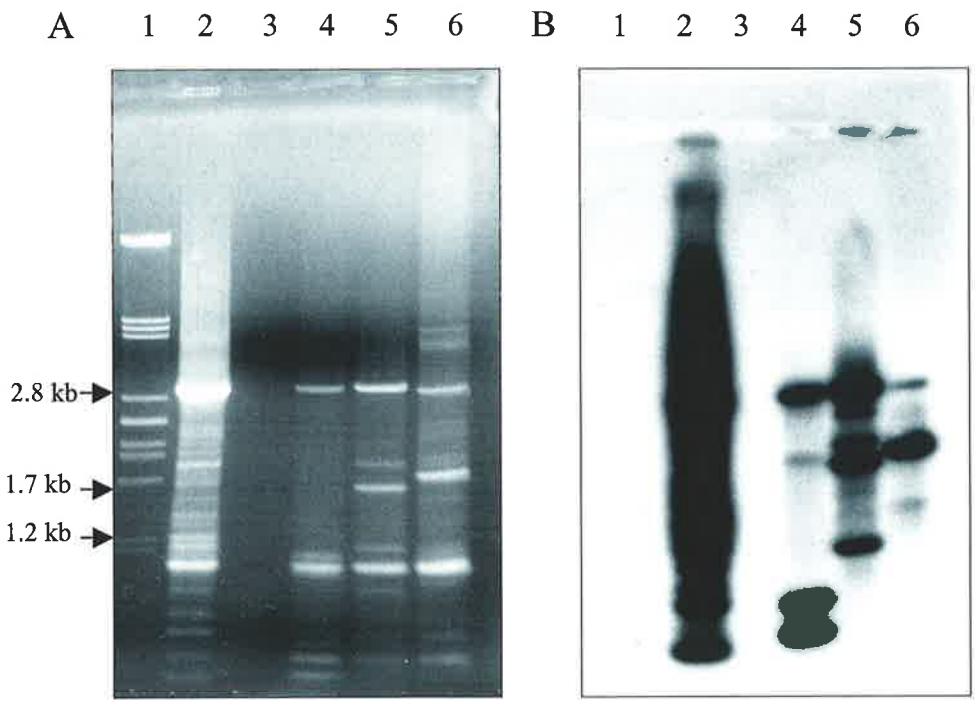


Figure 6.2: Detection of full-length DHBV DNA by PCR using liver DNA extracted from Group C ducks at 9 months p.i.:

Panel A: An ethidium bromide stained gel of the products of a full length PCR reaction (Section 6.3.2)

Panel B: A Southern blot hybridization of the same samples. After Southern transfer the membrane was hybridized with a genome-length radiolabelled DHBV DNA probe before autoradiographic exposure for 30 min.

Lane 1 shows λ-pst molecular weight marker; Lane 2 shows a positive control sample; lane 3 is empty; Lanes 4-6 show products from full-length PCR using autopsy liver from ducks 4041, 3839 and 3637 respectively. The positive control and 3 Group C samples show an approximately 3 kb product and several smaller products including from 1-1.8 kb in length.

In the third experiment DNA was extracted from samples of liver from ducks 3637, 3839 and 4041 taken 3 days and 1, 6 and 9 months p.i. for Southern blot hybridization. A sample from 3 months p.i was available from duck 3637 only. In order to increase the sensitivity of the assay, a larger than usual quantity of cccDNA extract was loaded onto the agarose gel. This was made possible by dissolving extracted cccDNA in one quarter of the usual volume of TE buffer and loading a larger than usual volume onto the gel. The samples were extracted using the standard protocol to isolate non-protein bound DNA (Section 2.7.10). DNA was extracted from 150 mg of liver from samples taken at 3, 6 and 9 months p.i., and 20 mg of liver from the samples taken at day 3 and 1 month p.i. due in part to the available quantity of remaining tissue.

The samples taken at day 3 and 1 month p.i. were previously analyzed by Southern blot hybridization and are shown in Figure 5.3. This demonstrated both RC DNA and cccDNA at day 3 when DHBsAg was detectable in liver in 1.9-3.5% of liver cells (Section 5.2.2). At 1 month p.i. RC DNA was not detectable but a cccDNA band was still visible. When these and the later samples were analyzed together by Southern blot as shown in Figure 6.3, cccDNA was seen to persist beyond 1 month in all ducks. In duck 3637 cccDNA was seen at 3 months, but not at 6 or 9 months p.i.. Samples from ducks 3839 and 4041 showed cccDNA at all time points analyzed. This Southern blot analysis confirmed the persistence of DHBV cccDNA up to 9 months p.i in 2 out of 3 ducks and 3 months p.i. in the remaining duck.

Sensitivity was not accurately assessed but the background signal produced was estimated at equivalent to 0.003 genomes per cell. Signal at or below this level cannot be reliably distinguished from background and therefore sensitivity is near this level. This compares to a sensitivity of 0.0001 genomes/cell using PCR.

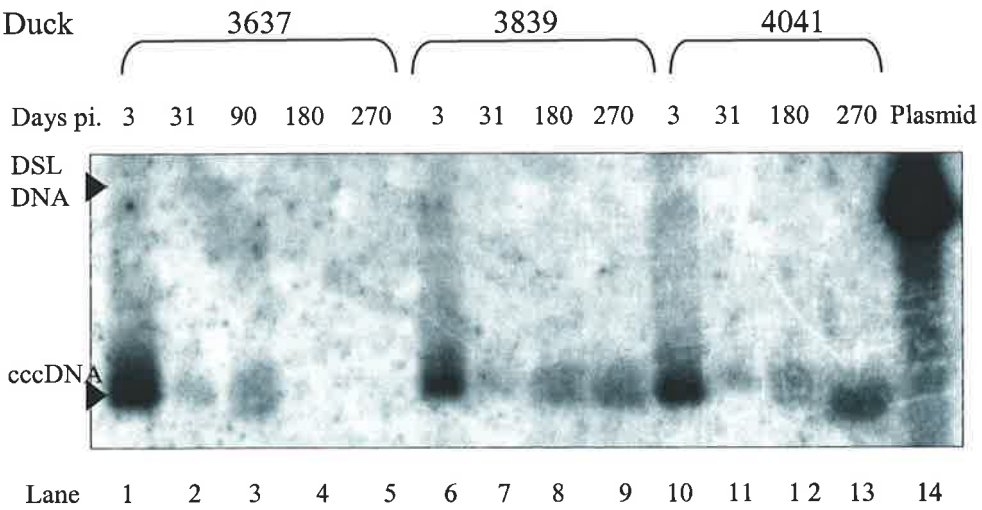


Figure 6.3: Southern blot hybridization detection of cccDNA extracted from autopsy liver from 3 Group C ducks. Samples from different time points are shown, illustrating that cccDNA is detectable in liver from duck 3637 until 3 months pi and in ducks 3839 and 4041 until 9 months pi.. A plasmid control of 80 pg DHBV DNA is shown in lane 14.

DNA extracted from 20 mg of liver was used for days 3 and 31 and DNA extracted from 150 mg of liver was used at later time points.

Samples were loaded onto a 1.5% agarose gel and electrophoresis performed overnight at 30 mA. After Southern transfer the membrane was hybridized to a genome-length radiolabelled DHBV DNA probe and autoradiography performed for 64 hr.

Quantitation is only approximate when the signal intensity is similar to the background as was the case. DHBV cccDNA in liver 9 months p.i. was measured as ≤0.003, 0.008 and 0.009 copies per cell by Southern blot hybridization compared with 0.014, 0.001 and 0.002 copies per cell by selective PCR. This confirms that cccDNA was measurable in some samples by Southern hybridization, an assay with sensitivity approximating the levels measured by PCR.

6.4 Studies of Infectivity

6.4.1 Experimental Outline

Several strategies were used to attempt to demonstrate the presence of infectious virions in tissues with residual hepadnavirus infection. All involved inoculation into 2-day-old ducklings of material derived from ducks which had recovered from transient DHBV infection. Fresh and frozen liver homogenates, cultured spleen cells (Section 2.4) and the culture supernatant and a sucrose cushion purified fraction of serum were used.

1-2-day-old ducklings can be infected with a single virion when diluted serum from ducks with congenital DHBV infection is used (Jilbert *et al.* 1996). When longer than genome-length DHBV plasmid DNA is delivered intravenously or intrahepatically a much greater number of genomes is needed to produce infection. In this study (see Section 3.2.4) 5.5×10^{12} genome equivalents produced infection in 2 out of 4 ducks. Another study also reported infection in a proportion of ducklings inoculated with plasmid DNA equivalent to more than 10^{12} genomes (Tagawa *et al.* 1996).

Inoculation was either by i.p. or i.v. injection. Serum DHBsAg was used as the marker of infection in the newly inoculated duckling and was assayed by ELISA, before and weekly for 4 weeks p.i., at which time the experiments were terminated.

6.4.2 Results

Infection (defined by detectable serum DHBsAg) was not transmitted by inocula prepared from material from ducks with residual DHBV DNA. Infection was, however, transmitted using liver homogenate prepared from a congenitally DHBV-infected duck. The results of a total of 6 experiments are summarised in Table 6-3.

Table 6-3: Infectivity studies using inocula from ducks with residual DHBV DNA

Experiment	Material and quantity inoculated	Route of Inoculation*	Number of ducks studied	Result of DHBsAg testing (No. DHBsAg+/No. tested)
1 Group D	100 mg liver (7×10^7 cells) from a congenitally DHBV-infected duck	IP	2	2/2 (from day 6 p.i.)
2-4 Groups D, E, H	100 mg liver (7×10^7 cells) from a duck with residual DHBVDNA	IP	9 (3 experiments)	0/5
5 Group L	100 mg liver (7×10^7 cells) from immunosuppressed ducks (see 6.2)	IP	3	0/3
6 Group I	50 mg liver (3.5×10^7 cells) from a duck with residual DHBV DNA	IV	2	0/2
	Serum (sucrose cushion purified)†	IV	1	0/1
	Cultured spleen cells	IV	1	0/1
	Spleen cell culture supernatant (sucrose cushion purified)†	IV	3	0/3

* IV, intravenous; IP, intraperitoneal.
† Ultracentrifugation using a sucrose cushion (Section 2.5.4).

6.5 *Comparison of Levels of Residual DHBV DNA with Rates of Cell Turnover*

6.5.1 Experimental Outline

Parallel studies of liver cell turnover, described in this chapter, and levels of cccDNA, described in Chapter 5 (Section 5.3.4) were considered together. The outlines are shown schematically in Figure 6.4. The studies of cell turnover were used as described below to predict changes in levels of cccDNA over time for comparison to observed levels of cccDNA.

BrdU labeling was used to estimate the rate of cell division in the liver of ducks of 6 weeks of age and 3 days after inoculation with 10^{10} DHBV virions (Group J, Table 2-1) and in ducks with residual DHBV DNA infection at 8 months of age and 6 months after infection (Group F, Table 2-1) as outlined in Figure 6.4. The times were chosen to provide estimates of cell turnover in liver in the early phase of infection and during a period of rapid growth of the duck, and later at a time after clearance of infection after attainment of stable adult body weight.

BrdU is taken up by dividing cells during S phase which has been estimated in other studies of hepatocytes using this technique to last 8 hours (Fourel *et al.* 1994; Mason *et al.* 1994). By injecting BrdU 8 hr before harvesting tissues, cells in or entering S phase over a 16 hr period will be labeled and hence approximately 2/3 of cells which divide in a 24 hr period will be labeled, assuming that S phase lasts 8 hr and that uptake and detection of BrdU are 100% efficient. Hence, used in this way, BrdU provides only an approximate measurement of cell division. Cell death is assumed to equal replacement by cell division whilst liver size is stable. During growth, cell division exceeds cell death, although only slightly as discussed later.

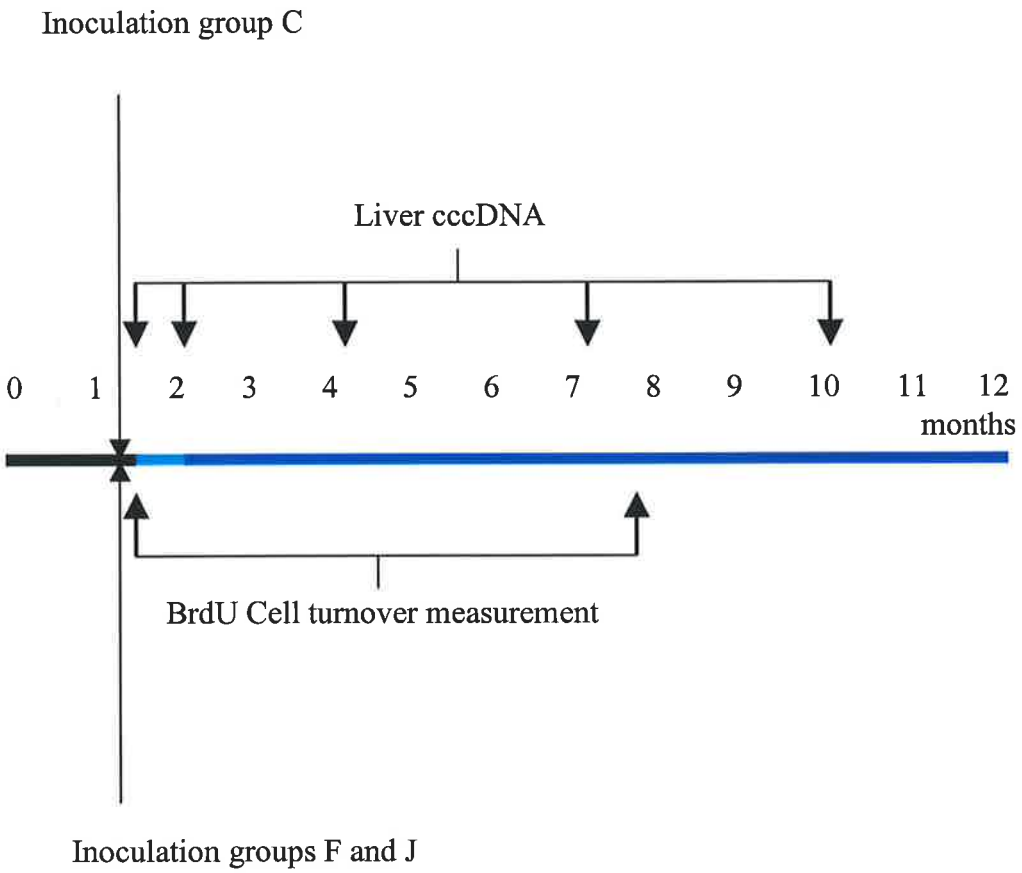


Figure 6.4: Modeling of cccDNA levels vs. cell turnover (Section 6.5.1): The time line shows the time points when samples were taken for measurement of cccDNA above the line and cell turnover measurements below the line. Group C ducks were used for monitoring of liver cccDNA by Southern blot hybridization and PCR. Groups F and J were used for measurements of cell turnover. Time points and ducks were chosen so that cell turnover measurement was done in ducks of similar age and time post inoculation to ducks used for DHBV cccDNA measurement.

For the early time point 3 ducks (Group J) referred to in studies of experimental infection (Chapter 4, ducks 2627, 9394 and 9900) were inoculated intravenously with 10^{10} DHBV virions at 40 days old. Three days later BrdU was injected (Section 2.6.4) and autopsy harvesting of liver and other organs was carried out 8 hr later. Similarly, 3 ducks of ~8 months old and 6 months p.i. (Group F) were injected with BrdU and liver and spleen was harvested 8 hours later. Liver was used for immunohistochemical detection of BrdU and also for DNA extraction and quantitation of DHBV DNA in the case of the material collected 3 days p.i..

6.5.2 Results: Cell Turnover

In 3 6-week-old ducks, 3 days p.i., 9.4+/-7.1% (mean+/-SD) of cells in the liver showed staining for BrdU. In sections of spleen tissue that were included as a positive control from all 3 ducks since high cell turnover is expected, 15.8% +/-5.2% of cells stained for BrdU. Examples of specific animals are shown in Figure 6.5. Staining occurred evenly throughout the liver lobules.

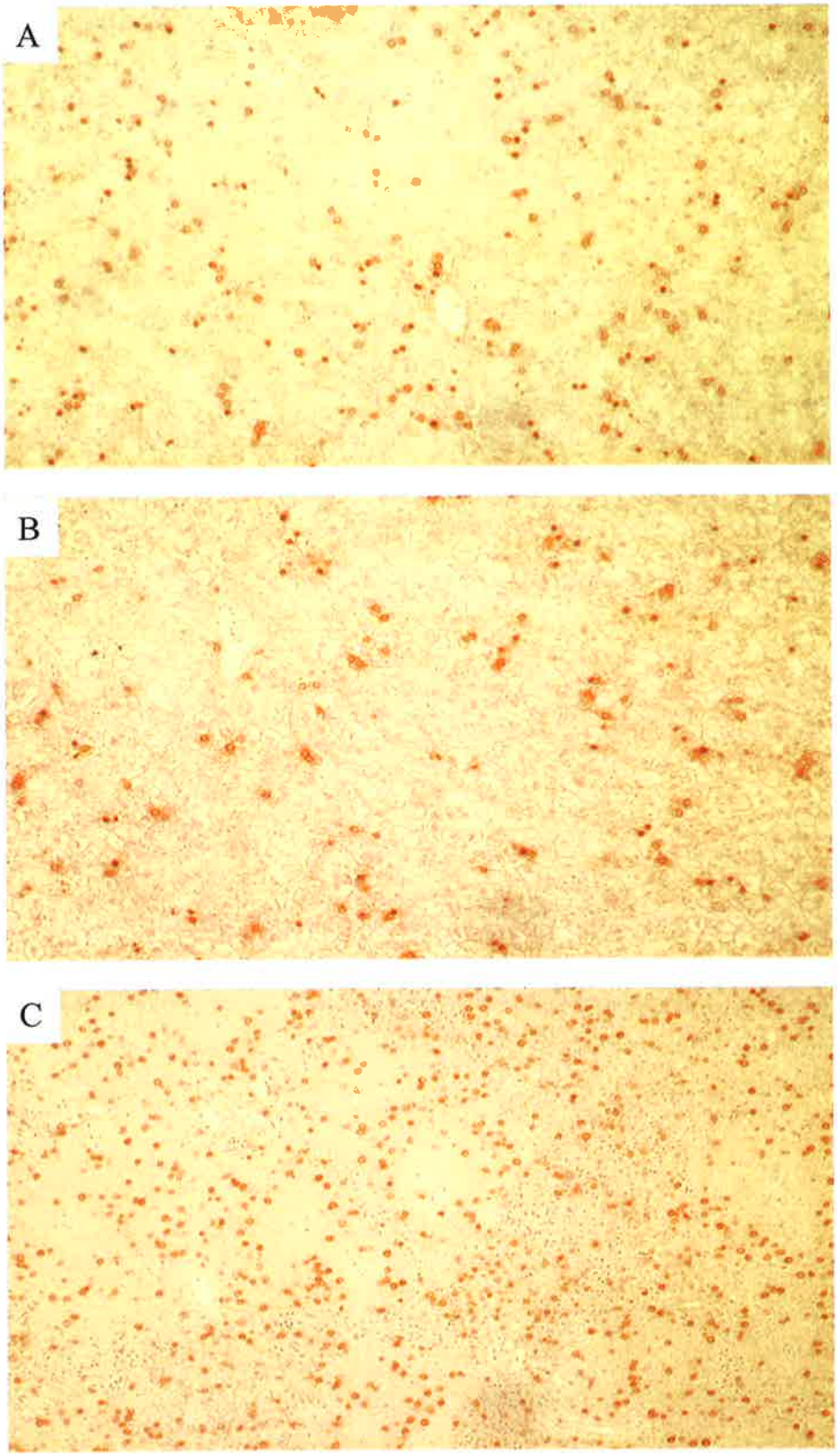
In 8-month-old ducks, ~6 months p.i., an average of 1.4+/-0.5% of liver cells stained for BrdU. In some lobules staining was seen more frequently in portal tracts than in lobules, reflecting the presence of mononuclear cells. Counts of BrdU-positive cells included only lobular cells (assumed to include mostly hepatocytes). Examples of staining are seen in Figure 6.5. Staining in spleen occurred in 8.3+/-1.4% of cells in the 8-month-old ducks.

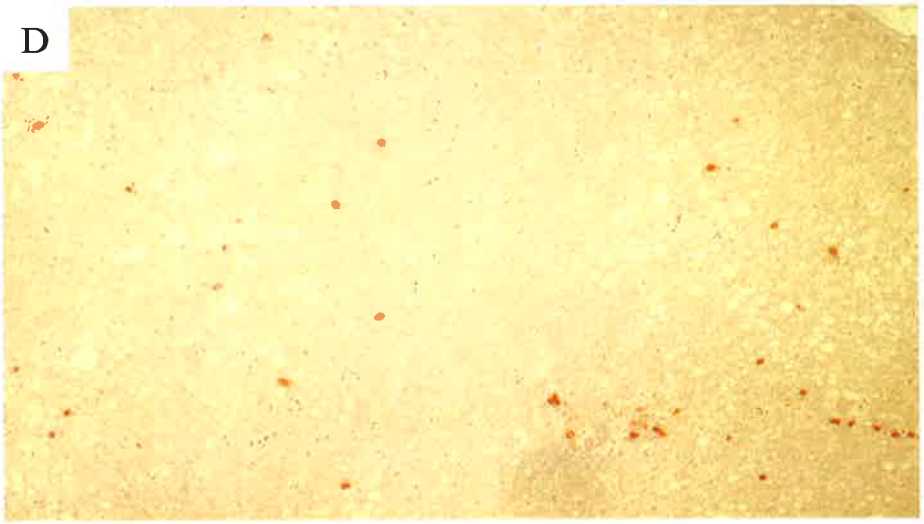
The daily cell turnover, expressed as a percentage, is the percentage of cell staining multiplied by 1.5, since, as explained above, 2/3 of cells which divide in a 24 hr period are estimated to take up BrdU. Daily turnover was therefore approximately 14% at 6 weeks

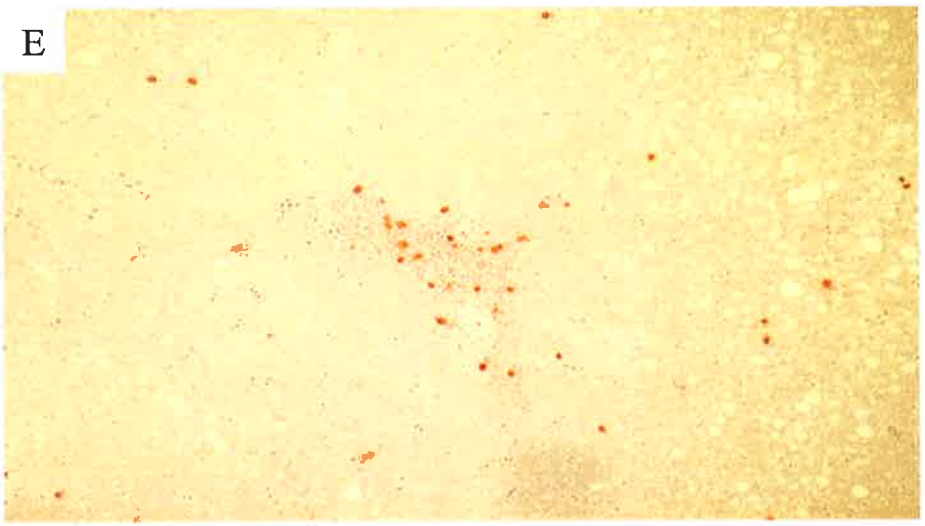
Figure 6.5: Estimation of cell turnover by bromodeoxyuridine (BrdU) labeling of dividing cells. BrdU was injected i.p. 8 hr before autopsy collection of liver, spleen and other tissues. EAA fixed and wax-embedded tissue sections were incubated with monoclonal antibodies to BrdU allowing immunohistochemical detection (Section 6.5.2).

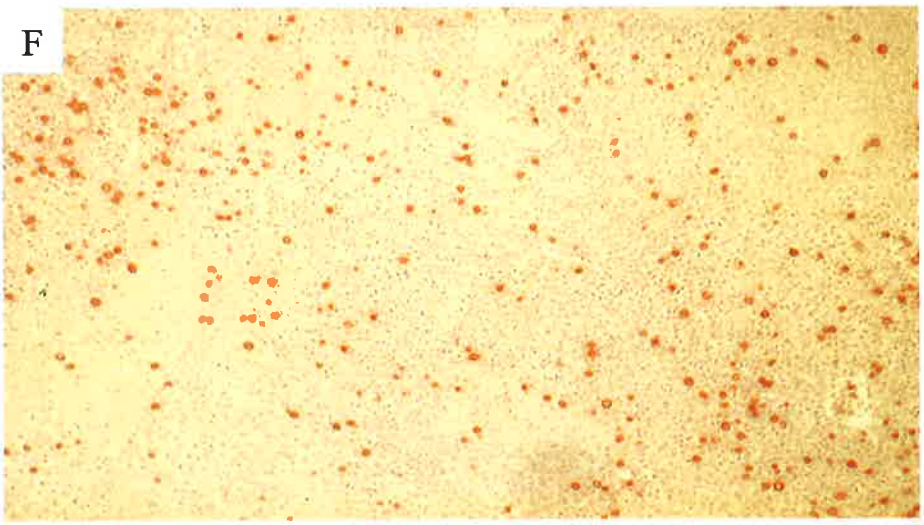
A,B,C: Liver sections from 6 week-old ducks 3 days p.i. with 10^{10} virions (Group J). A: Liver from duck 11261127 photographed at 100× magnification. 16.3% of liver cells were labeled. B: Liver from duck 11931194 photographed at 200× magnification. This section showed 9.8% of liver cells labeled. C: For comparison a spleen section from duck 11931194. This section showed 21.4% labeling, reflecting the high cell turnover in spleen.

D,E,F: Sections from Group F ducks taken approximately 6 months p.i. and 8-months-old and photographed at 200× magnification. D: Liver section from duck 163, in which 1.4% of cells were labeled. E: Section from duck 180, with 1.8% of liver cells labeled. F: Spleen from duck 158 with 9.6% of cells BrdU labeled.


A
B
C


D


E


F

and 2% at 8 months. Assuming all cells turn over at the same rate, the time for replacement of all liver cells was calculated as 100 divided by percentage daily turnover. The mean time for "complete turnover" of hepatocytes in 6-week-old ducks was thus estimated to be 7.1 days and in 8-month-old ducks was estimated to be 48.8 days. For spleen cell turnover the results are 4.2 days in 6-week-old ducks and 8.0 days in 8-month-old ducks. These estimates assume that all cells are turning over at the same rate.

6.5.3 Results: Modeling of decline of cccDNA levels

Virological data required for modeling were obtained from Group C ducks (see Chapter 5). The initial number of cells infected was taken as the fraction of cells staining for DHBsAg at day 3 p.i.. The initial cccDNA copy number used in modeling was also from the Group C duck liver biopsies collected at day 3 p.i. and DHBV DNA levels were measured by Southern blot hybridization.

In order to predict the decline of cccDNA a computer simulation of the model of Zhu *et al* was used (Zhu *et al.* 2001). The software and advice were kindly provided by Dr Samuel Litwin and Dr William Mason (Fox Chase Cancer Center, Philadelphia, PA, USA). CccDNA was assumed to be in a truncated Poisson distribution, such that from 1-3 copies were present in each infected cell, with a mean of 2, approximating the actual measurement of 2.1 copies per infected cell that was determined by PCR in Chapter 5 (Section 5.2.2.2). The mathematical model is one where cccDNA is lost with cell death and distributed randomly between daughter cells with cell division. This means that cccDNA declines at a rate determined by the rate of cell death, which is equal to cell division when the weight is stable. The decline in cccDNA per cell is accelerated because the number of cells increases as the duck grows – hence. Growth of the liver was allowed for in the model. The weight of the liver was estimated as 3% of total body weight (Nickel *et al.* 1977).

Measurements of body weight were obtained from records of ducks from the same breeding stock, from previous experiments in the laboratory conducted by Wendy Foster (see Appendix 8.1).

The initial fraction of cells infected was 0.0264 (Section 5.2.2.1). The daily fraction of cells dying was 0.14 (Section 6.5.2). Based on these parameters the model was used to predict cccDNA levels for a period of 28 days until the next biopsy. This 28 day time period was chosen assuming that replication and spread of infected cells did not occur. It is almost certain that some replication did occur and hence the model provides only a simplistic characterization of the early phase of infection and clearance. The rapid turnover of liver cells in this period was associated with a rapid decline in predicted cccDNA levels. The initial cccDNA copy number per cell was 5.28×10^{-2}, falling to 6.89×10^{-4} after 28 days according to the model (Figure 6.6). At the end of the 28 day period the predicted fraction of cells infected was 6.522×10^{-4} .

The cccDNA copy number per cell and fraction of infected cells generated by modeling for the first 28 days were used as parameters for the following 8 months of the study. In this period the fraction of cells dying per day was taken as 0.02 (see Section 6.5.2). The slower turnover of liver cells predicted a slower decline in cccDNA levels to 1.032×10^{-5} at 310 days old. The predicted decline in cccDNA is shown in Figure 6.6 along with the observed cccDNA levels for Group C at 5 time points. The observed cccDNA decline is slower than that predicted by the model and is maintained at a stable level of $4\text{-}6\times10^{-3}$ copies per cell from 3-9 months p.i. (Table 5-5 shows DHBV DNA copies/80000 cells).

If the rate of cell turnover is lower than that which was observed then the decline in cccDNA will be slower. This could apply if not all labeled cells were hepatocytes, which

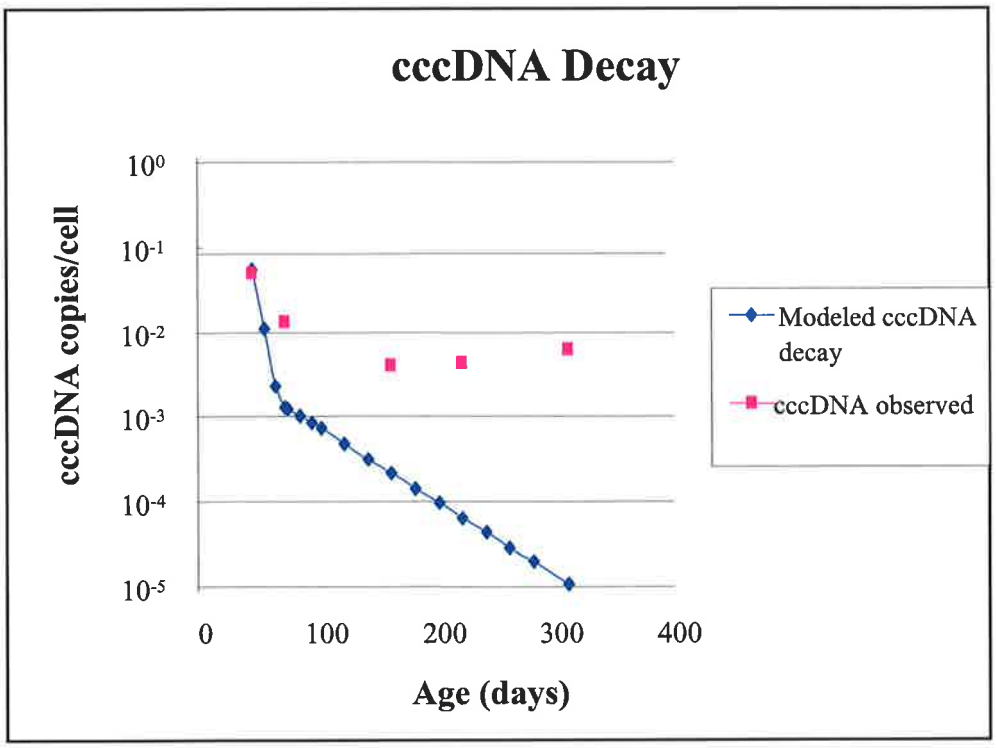


Figure 6.6: DHBV cccDNA changes over time.

Levels of DHBV cccDNA/cell predicted (blue data points) and observed in Group C ducks (pink data points).

Predicted cccDNA levels are based on an initial measurement and then simulated according to a model where cccDNA is lost with cell death. Cell death is assumed to equal replacement, which is measured by BrdU labeling of dividing cells. In the first month cccDNA levels are predicted to decline rapidly then more slowly for the remaining 8 months. The slope of the curve depends on the rate of cell turnover and shows a predicted decline to undetectable levels (<0.0001 copies/cell). The observed cccDNA levels are stable after an initial decline in the first month, suggesting a lower rate of turnover of infected cells or that replication and replenishment of cccDNA is leading to a steady state of production and loss.

cannot be ruled out in this experiment since the cell morphology cannot be easily assessed in some sections. Fourel *et al* (Fourel *et al.* 1994) found cell turnover in uninfected ducks to be approximately 0.05-0.1% per day. This is the lowest of the published estimates of liver cell turnover which were reviewed. Using this figure (a fractional turnover of 0.0005 per day) a second simulation based on the model of cccDNA decay was performed. This predicts little change in levels of cccDNA over the period of the experiment as seen in Figure 6.7.

6.6 Discussion

The experiments described in this chapter confirm the persistence of cccDNA following apparent recovery from DHBV infection, at levels that appear to be relatively stable over 6 months of observation. Issues relating to residual DHBV DNA which were not resolved include whether ongoing replication occurs, whether replication can be induced or enhanced by reduced immune control, and whether DHBV infection is transmissible from such tissues.

The first experiment involved immunosuppression of ducks carrying residual DHBV DNA. Levels of DHBV DNA in liver remained stable during 4 weeks of immunosuppressive treatment. This may be explained in several ways, either because the immunosuppressive treatment was ineffective, because reactivation of DHBV is a rare event or because reactivation of DHBV does not occur. These alternatives could not be distinguished by the current work.

The effectiveness of immunosuppressive treatment was assessed by lymphocyte proliferation assay and indirectly by spleen histology. Studies of the immune system of ducks have been hampered by very limited reagents and in the case of lymphocyte

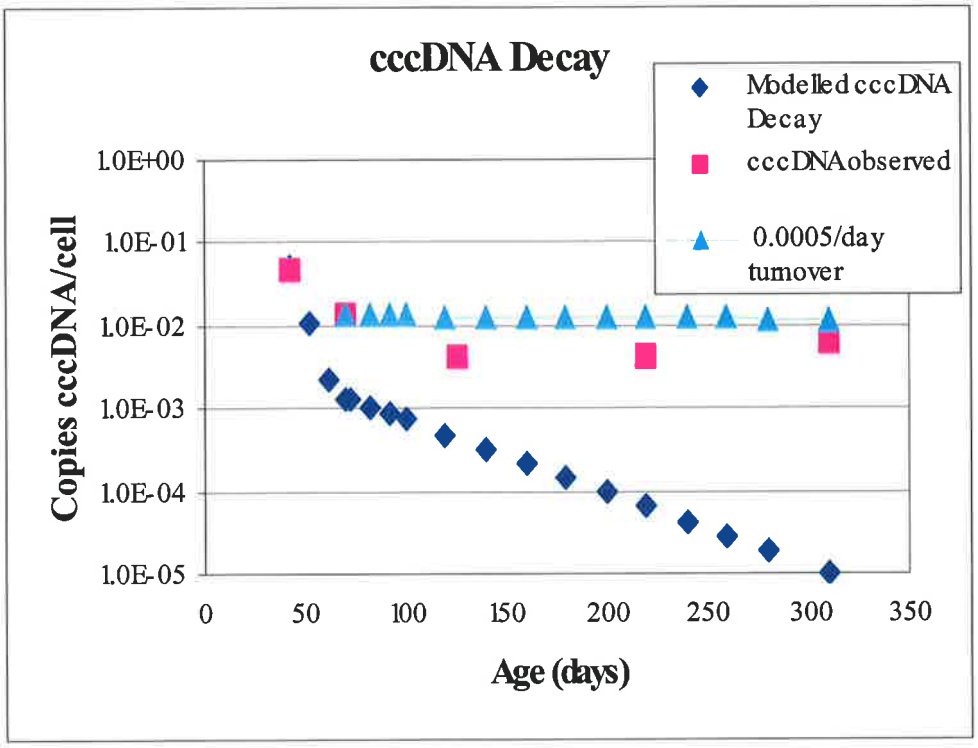


Figure 6.7: DHBV cccDNA changes over time: comparison with a projection of cccDNA decay based on low cell turnover.

The dark blue data points are the levels of cccDNA based on the cell turnover measured by BrdU labeling of dividing cells as seen in Figure 6.5. The light blue data points show the projected levels of cccDNA if cell turnover fraction is 0.0005/day (Fourel *et al*). In pink the observed cccDNA levels are shown and appear similar to those based on very low cell turnover.

proliferation assays, by a lack of consistent results (Bertram 1997). Baseline lymphocyte proliferation was variable and there was no consistent response in the 3 ducks to exogenously added mitogen or antigen. In previous experiments in the laboratory, uptake of tritiated thymidine signifying proliferation of lymphocytes yielded counts ranging from approximately 10^4 to 10^5/well (Bertram 1997). Proliferation of lymphocytes during treatment was not increased significantly by antigen or mitogen. One duck (1001) responded to mitogen before, but not during treatment. This single result is insufficient to draw conclusions. That leaves the spleen histology as the only marker of a potentially meaningful biological effect of treatment. Depletion of spleen cellularity was noted in 2 out of 3 treated ducks. Interestingly, the 2 ducks with reduced cellularity in spleen sections showed higher blood cyclosporin levels. Levels were 134.3 and 243.6 µg/L in the ducks with apparent depletion of spleen cells, but only 57.5 µg/L in the remaining treated duck (1006). Because there is little or no reported experience with cyclosporin in ducks it is only possible to speculate about the possible significance of this observation.

A negative result can be explained if reactivation of hepadnaviral replication is relatively rare in the setting of immunosuppression. The few prospective studies in humans suggest that overt reactivation is uncommon, with observed rates of less than 5% (Lok *et al.* 1991; Markovic *et al.* 1999). Because of this possibility the experiment was designed to show smaller changes in replication than would be clinically evident. In summary, the experiment does not confirm the presence of replication competent virus or suggest an immune mechanism for control, however the negative result does not rule out these possibilities.

The second set of experiments shows the persistence of cccDNA after transient infection and apparent clearance in 2 ways. First is the amplification of full-length genomes with

primers which favour amplification of cccDNA but not RC DNA or linear DNA. Previous use of strategies to amplify full-length hepadnavirus genomes have been directed at RC DNA in serum and therefore target the sequences adjacent to the nick in the minus strand (Netter *et al.* 1997). Primers targeting the nick and gap region were tried but were not used since the performance of PCR with these primers was suboptimal (data not shown) and the amount of starting template was small. A second set of PCR primers designed to produce full-length DHBV DNA products yielded PCR products of ~3 kb, which hybridized to a DHBV DNA probe. The PCR products were considered to represent full length DNA and several shorter products ranging from less than 1 kb to 1.8 kb. The shorter than full-length products could be as result of the nature of the template or an unintended product of the PCR reaction. Deletions within residual DHBV DNA which preserve the primer binding sequences could produce smaller bands. Evidence against this possibility was the presence of similar products in the positive control lane. In this case the template was liver DNA from a duck with experimental infection involving approximately 2% of liver cells. In this situation it is unlikely that defective genomes would contribute more than a small proportion of the total and would not be expected to form a significant proportion of PCR products.

The sequence of a cccDNA PCR product was determined and shown to be similar to the wild-type AusDHBV. Although duck liver DNA from a separate experiment (Group W) was used as template, the high proportion of residual DNA in cccDNA form in this duck appeared similar to the proportion of cccDNA seen in the Group C ducks. Strictly speaking the efficient amplification of DNA using the selective cccDNA primers does not prove the presence of cccDNA, but indicates that the template is continuous between the primers and therefore is not RC DNA , or known forms of linear DNA or linear precursors of integrated DNA described elsewhere (Yang and Summers 1999). In order to provide

further evidence that residual DNA was present as intact cccDNA, sequence analysis was first performed and later, Southern blot hybridization. The sequence analysis showed the expected close similarity to the sequence of wild -type cccDNA. Since the sequence is of a PCR product it represents a significant proportion of residual genomes, but does not reveal the presence or absence of variants, insufficient in number to form a detectable proportion of PCR products.

The Southern analysis of residual DNA relied on a method of DNA extraction similar to that described by Hirt (Hirt 1976). This excludes most protein bound DNA, i.e. chromosomal DNA and DHBV RC DNA , which has viral polymerase protein attached, by precipitation in KCl, centrifugation and removal of the pellet and retention of the supernatant containing cccDNA. Since most chromosomal DNA is removed a much larger liver sample can be extracted and the cccDNA loaded onto an agarose gel for Southern blot hybridization. This permitted a marked increase in the sensitivity of the method into the range required for detection of residual DHBV DNA following limited transient infection and allowed independent confirmation that significant quantities of residual cccDNA were present. However there was a poor correlation between Southern blot hybridization and PCR when quantifying low copy numbers of DHBV cccDNA. The high ratio of background to sample signal intensity showed that Southern hybridization was not satisfactory for quantitation of very low copy numbers. However, detection of DHBV cccDNA using the less sensitive assay supported the PCR data showing that cccDNA contributes a major proportion of residual DHBV DNA.

Unlike observations with other hepadnaviruses, transmission experiments using inocula from ducks with residual DHBV DNA failed to show the presence of infectious virus. HBV has been transmitted by liver transplantation using grafts from donors with past HBV

infection (Wachs *et al.* 1995; Dickson and Everhart 1996; Delmonico and Snydman 1998; Michalak *et al.* 1999). The current study could not replicate this process, which effectively involves placing and maintaining a large number of infected hepatocytes in a new host with inadequate immune responses.

Mouse hepatocytes transplanted intraperitoneally appear necrotic within 3 days (Vessey and Hall 2001) so i.v. inoculation was also attempted. In the current work, small numbers of hepatocytes, ~50 mg or 3.5×10^6 cells were injected intravenously, it is not known whether they engrafted or survived long enough to allow production of virions and infection of the duckling. Given that 0.001-0.01 copies of residual DHBV DNA per cell were found in autopsy liver it can be estimated that from $3.5\times10^3 - 7.0\times10^4$ genome copies would be delivered within homogenized tissue inocula. If these were in the form of virions, unbound by neutralizing antibody, infection should result, however if these were in the form of cccDNA or other DNA forms not contained within virions, then the inoculum would not be expected to lead to infection. In other studies with plasmid DNA (Section 3.2.4), 5.5×10^{12} genome equivalents inoculated iv or intrahepatically were required to infect 2 out of 4 ducklings (Triyatni *et al.* 2001).

The alternative strategy of attempting to propagate virus outside the host before inoculating into ducklings was also tried. This is a similar strategy to that used successfully by Michalak *et al* (Michalak *et al.* 1999) to infect woodchucks. In that experiment peripheral blood lymphocytes were cultured and the supernatant was used to infect young woodchucks, which went on to develop typical WHV infection. In the current experiment PBMC did not contain detectable levels of residual DHBV DNA so spleen cells were used in attempts to transmit infection. Also, unlike the obvious alternative of hepatocytes, spleen cells are readily culturable in the laboratory. After this experiment was shown to be

unsuccessful, it was noted that spleen and other extrahepatic sites did not contain detectable cccDNA. Hence it is possible that spleen does not contain replication competent virus, but viral remnants undergoing immune clearance e.g. as immune complexes associated with follicular dendritic cells (Jilbert *et al.* 1987). Hence it can be seen that the optimal conditions for transmission of residual HBV and WHV may not have been replicated in these experiments. An alternative strategy which could allow for the rescue of infectious virions might involve collagenase perfusion of liver and culture of cells *in vitro* for several days to allow time for production of virus in the absence of immune control. In a possibly analogous system herpes simplex virus can be induced to reactivate from latently infected ganglia when these are maintained as explant cultures.

Overall the failure to infect ducklings suggests that few or no free virions are available in the setting of residual DHBV DNA. What is not clear is whether virions are produced but cleared or inactivated rapidly by neutralizing anti-surface antibodies or whether production of virions does not occur. The lack of transmission of HBV by blood transfusion using blood from donors with past infection combined with the finding of HBV DNA in the serum of similar subjects supports the presence of replication and effective neutralization of virions.

In the final experiment, levels of cell turnover were correlated with levels of cccDNA. CccDNA levels appeared stable, consistent with a balanced steady state of replication and clearance, or with persistence of DHBV DNA within cells with very low turnover. If the observation recorded here of significant turnover (~2%) in a similar group of ducks is an accurate reflection of turnover of infected cells then the notion of a steady state of replication and clearance is supported. Alternatively if the cell turnover of infected cells is lower, such as reported in uninfected control ducks by Fourel *et al* (Fourel *et al.* 1994) then

the maintenance of cccDNA levels would not require replication. The inclusion of a group of uninfected control ducks in the experiment may have helped strengthen the data if a lower rate of turnover was seen. This would suggest that the observed rate of cell turnover was a consequence of clearance of infected cells. Even with such data the possible presence of a subset of long-lived cells could not be excluded. The BrdU-labeled sections show an even distribution of staining throughout the lobule, however the cell types are not easily confirmed as hepatocytes because of poor morphological definition with this method.

In summary, the persistence of apparently stable levels of residual DHBV DNA despite cell turnover is consistent with the existence of a steady state of viral DNA production and clearance. However, the possibility that DHBV DNA persists in a subset of long-lived cells has not been excluded. This will be discussed in the final chapter.

7. Concluding Remarks

7.1 Introduction

HBV has infected an estimated 2 billion people worldwide with 350 million currently infected (WHO 2001). The outcome of HBV infection acquired as an infant is usually persistent high-level infection. However, infection acquired in adulthood is usually cleared with the development of neutralising anti-surface antibodies and immunity to reinfection. Despite this apparent clearance of infection, HBV DNA can be detected in serum and liver, months or years later (Blum *et al.* 1991; Mason *et al.* 1998; Yotsuyanagi *et al.* 1998; Michalak *et al.* 1999), cytotoxic T-cell responses also persist (Rehermann *et al.* 1995) and reactivation of infection can occur following immunosuppression or after liver transplantation (Lok *et al.* 1991; Dickson and Everhart 1996).

The significance of residual hepadnavirus DNA, apart from the problems of transmission with liver transplantation and reactivation with immunosuppression, is not fully understood. There may be effects on the natural history of other liver diseases. There is some evidence that past HBV infection may be linked to a higher rate of cirrhosis from hepatitis C (Cacciola *et al.* 1999) and that hepatocellular carcinoma (HCC) may be more common in those with hepatitis C who also have markers of past hepatitis B and HBV DNA sequences in liver (Koike *et al.* 1998; Shibata *et al.* 1999). An epidemiological study of sporadic HCC, i.e. not related to active hepatitis B or C, showed anti-HBs antibodies were associated with a 4.7 fold increased risk of HCC (Yu *et al.* 1997).

Probably of greater significance is the fact that the existence of residual hepadnavirus DNA after acute infection is consistent with a host-parasite relationship in which a non-cytopathic virus escapes eradication by an apparently effective immune response. This poses challenges for the development of therapeutic strategies for improved treatment of

chronic hepatitis B, where the immune response is inadequate. Current therapy does not eradicate HBV and reactivation of high-level replication post-therapy will occur unless an augmented immune response can be generated.

At the commencement of this study the phenomenon of hepadnavirus DNA persistence after transient infection was established, infectivity was proven and extrahepatic sites were shown to be involved. Less well understood was the molecular basis for persistence, the relative importance of extrahepatic sites, and the contribution of viral replication to persistence and the quantity of residual viral DNA. The study has examined these aspects using the duck model.

7.2 Method Development

The study of traces of residual viral DNA required sensitive and specific assays to detect and quantitate DHBV DNA. As a preliminary step a dimer of the Australian strain of DHBV was cloned for use as a plasmid control and found to be infectious (Triyatni *et al.* 2001). The careful use of this plasmid provided an accurate baseline measurement of sensitivity and a reliable standard for quantitative PCR. The initial screening of autopsy tissues for residual DHBV DNA was done with a nested PCR, which allowed a relatively large amount of tissue to be assayed. The high sensitivity required rigorous prevention of PCR contamination. Monitoring for contamination included negative control samples for the tissue extraction process (extracted NDL DNA) as well as the PCR reaction (water in place of sample).

Quantitative assays for DHBV DNA and DHBV cccDNA were established using the Roche Lightcycler real time PCR format with SYBR green detection of product. This was found to be reasonably accurate even at low copy numbers when repeat testing of samples

was performed. Primer design was of greater importance than conventional PCR due to the need for reliable and efficient amplification for quantitation. Detection of cccDNA appeared highly specific at low copy numbers, but inefficient amplification of RC DNA occurred at high template copy numbers as reported elsewhere (Kock and Schlicht 1993). This problem was circumvented by use of quantitation rather than enzymatic digestion of RC DNA because the latter reduced assay sensitivity. It is possible that the lack of a gap in the plus strand, noted in 85% of DHBV virions (Lien *et al.* 1987), unlike in mammalian hepadnaviruses, makes the use of such strategies less satisfactory. Further development of selective PCR detection of DHBV cccDNA could make use of alternative DNase enzymes, but the accuracy of quantitation is likely to be reduced by adding treatment steps to the assay.

7.3 DHBV DNA in Congenital and Early Experimental Infection

"High level" infection was studied in 2 groups – congenitally infected ducks with >95% of hepatocytes infected and high level viraemia (>10^8 copies/ml) and experimentally infected ducks with approximately 2.5% of hepatocytes infected (i.e. DHBsAg-positive) and very low level viraemia (<10^4copies/ml detected).

DHBV DNA detection was widespread in high-level infection, although antigen was seen only in liver, kidney and pancreas. DHBV DNA distribution appeared more restricted during experimental infection. This could reflect a need for viraemia to distribute DHBV to extrahepatic sites. Lymphoid infection has been found in studies of HBV and WHV, but little evidence for the same was found in this study. This may reflect significant differences in the organization of the avian immune system compared with mammals and caution is need in interpretation of these findings.

The results demonstrated, as expected, the increased sensitivity of PCR compared with Southern blot hybridization and also showed a reasonable correlation between quantitative results obtained by PCR with those obtained by Southern blot hybridization. The high sensitivity of PCR raised the possibility that some positive results may not be due to true infection of a particular tissue, but to DNA from circulating virions, which have not established replicative infection. The use of cccDNA detection by PCR improved the specificity of detection of infected tissues although there was no alternative method to verify the finding of low levels of cccDNA and so it cannot be confirmed whether or not the increase in sensitivity of PCR comes at the expense of a loss of specificity in defining sites of DHBV infection.

7.4 Residual DHBV DNA after Transient Infection

The finding of DHBV DNA 9 months after inoculation and 8 months after apparent clearance of infection is the first report of residual DHBV DNA (Le Mire *et al.* 2001). It confirmed the feasibility of studying this phenomenon using DHBV instead of the mammalian hepadnaviruses. The use of DHBV allowed manipulation of the dose of inoculum with variation in levels of infection and serological response in the weeks after inoculation and the amount of residual viral DNA found at autopsy 9 months after inoculation. The dose effect suggests that a sub-population of infected cells is maintained after apparent recovery from transient infection. It is understood that many infected cells are killed during clearance of acute hepadnavirus infection and it is not clear whether there are particular characteristics of the remaining cells or of the virus which allow for persistence. Possibilities include infection of long-lived cells and/or reduced expression of antigen caused by altered viral genomes, however it is also possible that residual hepadnavirus persists by efficient cell-to-cell transmission from cells which are rapidly cleared following the appearance of viral antigens on the cell surface.

The study findings suggest that the liver is the principal site of residual DHBV DNA, both quantitatively and since the liver was the only site of detectable residual cccDNA. The experience of solid organ transplantation is consistent with this in that the liver appears to be the only organ, which, when transplanted after HBV infection, frequently transmits HBV (Dickson and Everhart 1996). The specific cell type which harbours residual DHBV DNA is not clear from this study. Hepatocytes, Kupffer cells and bile duct epithelial cells are among the candidates. Since hepatocytes were clearly infected in the early phase of infection, attempts to show the cell type containing residual viral DNA should initially involve hepatocytes. Experimental strategies could include laser microdissection of individual cells, before PCR targeting viral DNA. This has been used to detect mutant HBV in ground glass hepatocytes, hence cells with a large copy number of viral genomes, so infrequently infected cells or low copy numbers of viral DNA per cell might present difficulties (Liang 2001).

The finding that most residual DHBV DNA amplified as cccDNA is the first such report in studies of hepadnaviruses, although residual cccDNA has been detected using qualitative PCR (Mason *et al.* 1998; Marusawa *et al.* 2000). This finding is consistent with the known relative stability of cccDNA and its place in the viral replication cycle in which it functions as the template for synthesis of pregenomic RNA and mRNA. The apparent depletion of RC DNA is consistent with suppression of viral replication and also with rapid removal of cells which express viral antigens.

7.5 Further Studies of Residual DHBV DNA

The remaining experiments examined issues raised in the preceding work. The apparent suppression of replication was hypothesized to be due to immune mechanisms, however

immunosuppressive drug treatment did not reverse this. Interpretation of this negative finding was made difficult by the limited understanding of duck immunity and lack of experience with immunosuppressive drugs in ducks. As a preliminary to further studies of immunosuppression in ducks the effect of drug therapy requires further understanding. In particular, demonstration of impaired clearance of infection with DHBV or another organism would be helpful. However, interpretation of a negative finding in such experiments will remain difficult.

The nature of residual DHBV genomes was studied by PCR designed to amplify full-length cccDNA and Southern blot hybridization. These experiments, in particular, the Southern blot hybridization, confirmed that residual DHBV DNA included significant amounts of cccDNA. The method of cccDNA extraction is readily applicable to studies of HBV and could be performed on operative or autopsy samples from patients as an extension of this work. This study demonstrates the utility of Southern blot hybridization in studies of residual hepadnavirus DNA, the need for which was discussed in a recent review of the subject (Brechot *et al.* 2001).

The lack of demonstrated infectivity using material derived from ducks with residual DHBV DNA may have reflected the strategies used rather than absence of replication competent virus. This possibility is raised by the observations with HBV and WHV, where infected cells were removed from the host and either placed in the new host (liver transplantation) or cultured *in vitro* to produce virions. Removal of duck hepatocytes and maintenance in cell culture medium could allow removal of immune suppression or neutralizing antibody and might have a greater chance of successful transmission of infection. Since infectivity has already been shown with other hepadnaviruses, this aspect was not pursued.

The study of cell turnover and modeling of cccDNA levels provides some interesting insights into possible mechanisms of maintenance of viral genomes, but does not resolve the issue. Of note is that the level of cccDNA appears relatively constant over time, within the limitations of assay accuracy. At the observed levels of cell turnover levels of cccDNA would be expected to decline to undetectable within the study period. Ongoing replication or persistence in a subset of long-lived cells could explain the findings. Suppression of viral replication and hence replenishment of cccDNA with an inhibitor of viral reverse transcriptase (e.g. lamivudine) could help resolve this question if cccDNA declined in the presence of drug, but not placebo.

7.6 Final Comments

Recent refinements in methods of detection of small quantities of viral nucleic acids have shown persistence of viral genomes and, in the case of hepadnaviruses, of virus after apparent clearance of infection (Blum *et al.* 1991; Michalak *et al.* 1994; Lanford *et al.* 1995; Dickson and Everhart 1996; Mason *et al.* 1998; Yotsuyanagi *et al.* 1998; Cacciola *et al.* 1999; Coffin and Michalak 1999; Michalak *et al.* 1999; Marusawa *et al.* 2000). A number of viruses have been known to persist after an apparently acute infection because of the occurrence of morbidity, e.g. herpes viruses, measles virus (Ahmed *et al.* 1996). The addition of other organisms, with only occult persistence, to this list means that a re-evaluation of the host-parasite relationship is needed for some viral infections. Is virus persistence the norm rather than the exception and is immunological memory stimulated by persistent traces of antigen? The phenomenon of virus persistence may involve more than just DNA viruses. In a study of long-term immunity to lymphocytic choriomeningitis virus, an RNA virus, Klenerman *et al* found traces of viral nucleic acid, persist as cDNA

and speculated on a role for such nucleic acid in immunological memory for RNA viruses (Klenerman *et al.* 1997).

Of more immediate concern is the significance of the ability of hepadnaviruses to persist on strategies to treat subjects with chronic hepatitis B. The current strategy of virus suppression with a nucleoside analogue sometimes leads to a stable state of lower replication associated with e-antigen to e-antibody seroconversion. In other cases, however, immune responses remain weak and termination of the drug is followed by return to pre-treatment levels of viral replication. The finding of persistent cccDNA indistinguishable in size from wild-type viral cccDNA and with relatively stable levels 9 months p.i. suggests that HBV genomes will remain regardless of the duration of treatment and that a combined approach of virus suppression and stimulation of immune responses needs further study despite limited success to date.

8. Appendix

8.1 Duck weights

The following weights are mean weights of 24 DHBV negative ducks measured by Wendy Foster.

Age (days)	Weight (grams)
20	633
22	755
27	1226
34	1716
41	2061
48	2638
55	3001
62	3211
69	3307
76	3407
83	3589
90	3588
97	3639
104	3635
111	3518
118	3622
125	3630
153	3434
160	3449
167	3498
174	3483
181	3542
188	3540
195	3550
202	3612
209	3598
216	3647
223	3595
230	3614
237	3720

8.2 Reagents and Solutions

8.2.1 PBS

0.13 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4 and 0.002 M KH_2PO_4, pH 7.4.

Sterilised by autoclaving.

8.2.2 1 × SSC

0.15 M NaCl

0.015 M Na_3Citrate

pH 7.0

NB. Stock solution of 20 × SSC.

Sterilise by autoclaving

8.2.3 TE buffer 10:10 pH 8

10 mM Tris HCl pH 8, 10 mM EDTA.

8.2.4 TE buffer 10:2 pH 8

10 mM Tris HCl pH 8, 2 mM EDTA.

8.2.5 Phenol

200 ml of phenol (BDH) was melted at 56°C and, in a fume hood, added to 200 ml of 0.05 M Tris HCl pH 8 in a glass bottle. After vigorous shaking the mixture was left to settle at RT. The upper layer of Tris HCl solution was then removed and replaced with another 200 ml of 0.05 M Tris HCl pH 8 which was mixed and allowed to settle before the removal and replacement was repeated. After mixing again the saturated phenol was then left to settle before use or storage in the dark at RT.

8.2.6 Phenol/Cloroform

100 ml of phenol melted at 56°C, 100 ml of chloroform and 200 ml of 0.5 M Tris HCl pH 8 were mixed vigorously in a glass bottle in a fume hood. The mixture was left to settle at

RT and the upper layer of Tris HCl was removed and replaced with another 200 ml of 0.5 M Tris HCl pH 8. Mixing, removal and replacement of Tris HCl were repeated and the mixture was allowed to settle before use or storage in the dark at RT.

8.2.7 Pre-hybridization Mix:

50% deionised formamide

500 µg/ml salmon sperm DNA

0.1% Polyvinylpyrollidone (PVP)

0.02% Ficoll 400

50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 6.5

1 mg/ml BSA

5 × SSC

8.2.8 Hybridization Mix

50% deionised formamide

100 µg/ml salmon sperm DNA

0.2% PVP

0.02% Ficoll 400

25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 6.5

0.2 mg/ml BSA

5 × SSC

10% dextran sulphate

8.2.9 TAE Buffer

40 mM Tris Acetate

1 mM EDTA

made as 50 × solution and adjust to pH 7.5-7.8 with glacial acetic acid

8.2.10 10 × TAE Loading Buffer

60% sucrose

1% Sarkosyl

1 × TAE buffer

0.1% Bromophenol blue

0.1% Xylene cyanol

water

8.2.11 Borate Buffer

121 g Tris

61.7 g boric acid

7.44 g EDTA

Make up to 1 litre in DW, adjust to pH 8.5 with boric acid

8.2.12 Nutrient Broth and Agar

To make 1000 ml:

10 g Peptone (Oxoid)

10 g Lab Lemco (Oxoid)

5 g NaCl

water

Sterilise by autoclaving. For agar add 15 g agar (Oxoid Bacteriological Agar No. 1)

8.2.13 Luria Bertani (LB) Broth and Agar

To make 1000 ml:

10 g Bacto tryptone (Difco)

5 g Bacto yeast extract (Difco)

10 g NaCl

water

pH 7.0

For agar add 20 g Bacto agar (Difco).

Sterilise by autoclaving.

8.2.14 1 × SSC

0.15 M NaCl

0.015 M Na_3Citrate

pH 7.0

NB. Stock solution of 20 × SSC.

Sterilise by autoclaving

8.2.15 DNA Size Markers

8.2.16 SPP-1 bacteriophage *Eco* R1 fragments

Fragment size (kb)	Quantity per 300 ng digest (ng)
8.51	58.4
7.35	50.4
6.11	41.9
4.84	33.2
3.59	24.6
2.81	19.3
1.95	13.4
1.86	12.8
1.51	10.4
1.39	9.5
1.16	8.0
0.98	6.7
0.72	4.9
0.48	3.3
0.36	2.5
0.09	0.6

8.2.17 Lambda bacteriophage-*Pst* I fragments

Fragment size (kb)	Quantity per 250 ng digest (ng)
11.50	60
5.08	26
4.75	25
4.51	22.5
2.84	14.8
2.56} 2.46} 2.44}	38.5
2.14	11
1.96	10
1.70	8.8
1.16	6
1.09	5.6
0.81	4.3
0.51	2.8
0.47} 0.45}	4.8
0.34	1.8
0.26	1.3
0.25	1.3
0.22} 0.21} 0.20}	3.0
0.16	0.8
0.15	0.8
0.10	0.5
0.09	0.5
0.087	0.50
0.015	0.08

9. References

Ahmed, R., L. Morrison and D. Knipe (1996). Persistence of Viruses. Fundamental Virology. B. Feilds, D. Knipe and P. Howley. Philadelphia, Lippincott-Raven: 207-229.

Anonymous. Lightcycler Operator's Manual, Roche Molecular Biochemicals.

Beck, J. and M. Nassal (1997). "Sequence- and Structure-Specific Determinants in the Interaction between the RNA Encapsidation Signal and Reverse Transcriptase of Avian Hepatitis B Viruses." Journal of Virology **71**(7): 4971-1980.

Bertram, E. (1997). Characterisation of duck lymphoid cell populations and their role in immunity to duck hepatitis B virus. Department of Microbiology and Immunology. Adelaide, The University of Adelaide: 218.

Blum, H., T. Liang, E. Galun and J. Wands (1991). "Persistence of Hepatitis B Viral DNA After Serological Recovery from Hepatitis B Virus Infection." Hepatology **14**: 56-63.

Blumberg, B., B. Gerstley, D. Hungerford, W. London and A. Sutnick (1967). "A serum antigen (Australia Antigen) in Down's syndrome, leukemia and hepatitis." Annals of Internal Medicine **66**: 924-931.

Boni, C., A. Bertoletti, A. Penna, A. Cavalli, M. Pilli, S. Urbani, P. Scognamiglio, R. Boehme, R. Panebianco, F. Fiaccadori and C. Ferrari (1998). "Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B." Journal of Clinical Investigation **102**(5): 968-975.

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

Bowden, S. (2000). Personal communication. M. Le Mire.

Brechot, C., V. Thiers, D. Kremsdorf, B. Nalpas, S. Pol and P. Paterlini-Brechot (2001). "Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: clinically significant or purely "occult"." Hepatology **34**(1): 194-203.

Bruss, V. and K. Vieluf (1995). "Functions of the Internal Pre-S Domain of the Large Surface Protein in Hepatitis B Virus Particle Morphogenesis." Journal of Virology **69**: 6652-6657.

Cabrerizo, M., J. Bartolome, E. Inigo, J. Lopez-Alcorocho, T. Cotonat and V. Carreno (1998). "Analysis of the hepatitis B virus precore and ORF-X sequences in patients with antibody to hepatitis B e antigen with and without normal ALT levels." Journal of Medical Virology **56**(4): 294-9.

Cacciola, I., T. Pollicino, G. Squadrito, G. Cerenzia, M. Orlando and G. Raimondo (1999). "Occult hepatits B virus infection in patients with chronic hepatitis C liver disease." The New England Journal of Medicine **341**(1): 22-26.

Calvert, J. and J. Summers (1994). "Two regions of an avian hepadnavirus RNA pregenome are required in cis for encapsidation." Journal of Virology **68**(4): 2084-2090.

Chang, S.-F., H. Netter, E. Hildt, R. Schuster, S. Schaefer, Y. Hsu, A. Rang and H. Will (2001). "Duck hepatitis B virus expresses a regulatory HBx-like protein from a hidden open reading frame." Journal of Virology **75**(1): 161-170.

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

Coffin, C. and T. Michalak (1999). "Persistence of infectious hepadnavirus in the offspring of woodchuck mothers recovered from viral hepatitis." The Journal of Clinical Investigation **104**(2): 203-212.

Conway, J., N. Cheng, A. Ziotnick and e. al (1997). "Visualization of a 4-helix bundle in the hepatitis B virus capsid by cryo-electron microscopy." Nature **386**: 91-94.

Dane, D., C. Cameron and M. Briggs (1970). "Virus-like particles in serum of patients with Australian-antigen associated hepatitis." Lancet(1): 695-698.

Delmonico, F. and D. Snydman (1998). "Organ donor screening for infectious diseases." Transplantation **65**(5): 603-610.

Dickson, R. and J. Everhart (1996). "Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen." Gastroenterology **110**: A1182.

Ferrell, L., N. Theise and P. Scheuer (2002). Acute and chronic viral hepatitis. Pathology of the Liver. R. MacSween, A. Burt, B. Portmannet al, Churchill Livingstone: 313-362.

Fitzgerald, S., S. Williams and W. Reed (1996). "Development of a Chicken Model for Studying Avian Polyomavirus Infection." Avian Diseases **40**: 377-381.

Fourel, I., J. Saputelli, P. Schaffer and W. Mason (1994). "The Carboxylic Analog of 2'-Deoxyguanosine Induces a Prolonged Inhibition of Duck Hepatitis B Virus DNA Synthesis in Primary Hepatocyte Cultures and in the Liver." Journal of Virology **68**(2): 1059-1065.

Freiman, J., A. Jilbert, M. Holmes *et al* (1988). Duck Hepatitis B Virus: Sequence and Significance of Lymphoid System Infection. Viral Hepatitis and Liver Disease, Alan R. Liss Inc: 501-502.

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

Guidotti, L., S. Guilhot and F. Chisari (1994). "Interleukin-2 and Apha/Beta Interferon Down-Regulate Hepatitis B Virus Gene Expression In Vivo by Tumor Necrosis Factor-Dependent and -Independent Pathways." Journal of Virology **68**(3): 1265-1270.

Guidotti, L., T. Ishikawa, M. Hobbs, B. Matzke, R. Shreiber and F. Chisari (1996). "Intracellular Inactivation of the Hepatitis B Virus by Cytotoxic T Lymphocytes." Immunity **4**: 25-36.

Guidotti, L., R. Rochford, J. Chung, M. Shapiro, R. Purcell and F. Chisari (1999). "Viral clearance without destruction of infected cells during acute HBV infection." Science **284**: 825-829.

Gunther, S., G. Sommer, F. von Breunig, A. Iwanska, T. Kalinina, M. Sternbeck and H. Will (1998). "Amplification of Full-length Hepatitis B Virus Genomes from Samples from Patients with Low Levels of Viraemia: Frequency and Functional Consequences fo PCR-Introduced Mutations." Journal of Clinical Microbiology **36**(2): 531-538.

Gust, I. (1992). "Control of hepatitis B in Australia." The Medical Journal of Australia **156**: 819-821.

Halpern, M., J. Egan, W. Mason and J. England (1984). "Viral antigen in endocrine cells of the pancreatic islets and adrenal cortex of Pekin ducks infected with duck hepatitis B virus." Virus Research **1**: 213-223.

Halpern, M., J. Egan, S. McMahon and D. Ewert (1985). "Duck Hepatitis B Virus is Tropic for Exocrine Cells of the Pancreas." Virology **146**: 157-161.

Halpern, M., J. England, D. Deery and e. al (1983). "Viral nucleic acid synthesis and antigen accumulation in pancreas and kidney of Pekin ducks infected with duck hepatitis B virus." Proceedings of the National Academy of Science of the United States of America **80**: 4865-4869.

Hirota, K., A. Sherker, M. Omata, O. Yokusuka and K. Okuda (1986). "Effects of Adenine Arabinoside and Corticosteroid on Replication of Duck Hepatitis B Virus DNA in the Liver." Annals of the Academy of Medicine of Singapore **15**(2): 227-231.

Hirt, B. (1976). "Selective extraction of polyoma DNA from infected mouse cell cultures." Journal of Molecular Biology **26**: 365-369.

Hollinger, F. (1990). Hepatitis B Virus. Fields Virology. New York, Raven Press. **2:** 2171-2236.

Hoofnagle, J., L. Seef, Z. Buskell-Bales and H. Zimmerman (1978). "Type B Hepatitis after transfusion with blood containing antibody to Hepatitis B core antigen." The New England Journal of Medicine **298**: 1379-1383.

Hoofnagle, J., D. Shafritz and H. Popper (1987). "Chronic type B hepatitis and the "healthy" HBsAg carrier state." Hepatology **7**(4): 758-763.

Hosoda, K., M. Omata, K. Uchiumi *et al* (1990). "Extrahepatic Replication of Duck Hepatitis B Virus: More than Expected." Hepatology **11**(1): 44-48.

Jilbert, A., J. Botten, D. Miller, E. Bertram *et al* (1998). "Characterisation of age and dose related outcomes of duck hepatitis B virus infection." Virology **244**(2): 273-282.

Jilbert, A., J. Freiman, C. Burrell, M. Holmes, E. Gowans, R. Rowland, P. Hall and Y. Cossart (1988). "Virus-liver cell interactions in Duck Hepatitis B Virus infection." Gastroenterology **95**: 1375-82.

Jilbert, A., J. Freiman, C. Burrell, M. Holmes, E. Gowans, R. Rowland, P. d. l. M. Hall and Y. Cossart (1988). Early virus-cell interactions in duck hepatitis B virus infection: a time course study of tissue tropism and sites of virus production. Viral Hepatitis and Liver Disease. A. Zuckerman. New York, Alan R Liss**:** 497-500.

Jilbert, A., J. Freiman, E. Gowans *et al* (1987). "Duck hepatitis B DNA in liver, spleen, and pancreas: analysis by in situ and Southern blot hybridisation." Virology **158**: 330-338.

Jilbert, A, I. Kotlarski (2000). "Immune responses to duck hepatitis B virus infection." Developmental & Comparative Immunology **24**: 285-302.

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

Jilbert, A., T.-T. Wu, J. England, P. d. l. M. Hall , N. Carp, A. O'Connell and W. Mason (1992). "Rapid resolution of duck hepatitis B virus infection occurs after massive hepatocellular involvement." Journal of Virology **66**(3): 1377-1388.

Kajino, K., A. Jilbert, J. Saputelli, C. Aldrich, J. Cullen and W. Mason (1994). "Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viraemia and infection of virtually every hepatocyte." Journal of Virology **68**(9): 5792-5803.

Klenerman, P., H. Hengarter and R. Zinkernagel (1997). "A non-retroviral RNA virus persists in DNA form." Nature **390**: 298-301.

Klingmüller, U. and H. Schaller (1993). "Hepadnavirus Infection Requires Interaction between the Viral Pre-S Domain and a Specific Hepatocellular Receptor." Journal of Virology **67**: 7414-7422.

Kock, J., M. Nassal, S. MacNelly, T. Baumert, H. Blum and F. von Weizsacker (2001). "Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus." Journal of Virology **75**(11): 5084-9.

Kock, J. and H. Schlicht (1993). "Analysis of the earliest steps of hepadnavirus replication: genome repair after infectious entry into hepatocytes does not depend on viral polymerase activity." Journal of Virology **67**: 4867-4874.

Koike, K., M. Kobayashi, M. Gondo, I. Hayashi, T. Osuga and S. Takada (1998). "Hepatitis B virus DNA is frequently found in liver biopsy samples from hepatitis C virus-infected chronic hepatitis patients." Journal of Medical Virology **54**(4): 249-55.

Korba, B., E. Gowans, F. Wells, B. Tennant, R. Clarke and J. Gerin (1988). "Systemic distribution of woodchuck hepatitis virus in the tissues of experimentally infected woodchucks." Virology **165**: 172-181.

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

Laras, A., J. Koskinas, K. Avgidis and S. Hadzidiyannis (1998). "Incidence and clinical significance of hepatitis B virus precore gene translation initiation mutations in e antigen-negative patients." Journal of Viral Hepatitis **5**(4): 241-8.

Le Mire, M., D. Miller, C. Burrell and A. Jilbert (2001). "Persistence of Duck Hepatitis B Virus DNA after Transient Infection." Journal of Gastroenterology and Hepatology **16**(Supplement): A78.

Liang, J. (2001). Personal Communication. A. Jilbert.

Lien, J.-M., C. Aldrich and W. Mason (1986). "Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B virus plus-strand DNA synthesis." Journal of Virology **57**: 229-236.

Lien, J.-M., D. Petcu, C. Aldrich and W. Mason (1987). "Initiation and termination of Duck Hepatitis B Virus DNA synthesis during virus maturation." Journal of Virology **61**: 3832-3840.

Lindh, M., C. Hannoun, A. Dhillon, G. Norktrans and P. Horal (1999). "Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers." Journal of Infectious Diseases **179**(4): 775-82.

Lok, A., R. Liang, E. Chiu, K.-L. Wong, T.-K. Chan and D. Todd (1991). "Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy: Report of a prospective study." Gastroenterology **100**(1): 182-188.

Luciv, P. (1996). Human immunodeficiency viruses and their replication. Fundamental Virology. B. Fields, D. Knipe, P. Howley and e. al. Philadelphia, Lippincott-Raven: 845-916.

Mandart, E., A. Kay and F. Galibert (1984). "Nucleotide sequence of a cloned duck hepatitis B genome: comparison with woodchuck and human hepatitis B virus sequences." Journal of Virology **49**(3): 782-792.

Marion, P., S. Knight, Y.-Y. Ho, W. Robinson and H. Popper (1984). "Liver disease associated with duck hepatitis B virus infection of domestic ducks." Proceedings of the National Academy of Science of the United States of America **81**: 898-902.

Markovic, S., G. Drozina, M. Vovk and Fidler-Jenko (1999). "Reactivation of hepatitis B but not hepatitis C in patients with malignant lymphoma and immunosuppressive therapy. A prospective study in 305 patients." Hepato-Gastroenterology **46**: 2925-2930.

Marusawa, H., S. Uemoto, M. Hijikata, Y. Ueda, K. Tanaka, K. Shimotohno and T. Chiba (2000). "Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen." Hepatology **31**(2): 488-95.

Mason, A., L. Xu, L. Guo, M. Kuhns and R. Perrillo (1998). "Molecular Basis for Persistent Hepatits B Virus Infection In the Liver After Clearance of Serum Hepatitis B Surface Antigen." Hepatology **27**(6): 1736-1742.

Mason, A., B. Yoffe, C. Noonan, M. Mearns, C. Campbell, A. Kelley and R. Perrilo (1992). "Hepatitis B Virus DNA in Peripheral-blood Mononuclear Cells in Chronic Hepatitis B After HBsAg Clearance." Hepatology **16**(1): 36-41.

Mason, W., J. Cullen, J. Saputelli, T.-T. Wu, C. Liu, W. London, E. Lustbader, P. Schaffer, A. O'Connell, I. Fourel, C. Aldrich and A. Jilbert (1994). "Characterisation of the Antiviral Effects of 2' Carbodeoxyguanosine in Ducks Chronically Infected with Duck Hepatitis B Virus." Hepatology **19**(2): 398-411.

Mason, W., G. Seal and J. Summers (1980). "Virus of Peking Ducks with Structural and Biological Relatedness to Human Hepatitis B Virus." Journal of Virology **36**(3): 829-836.

McQuillan, G. e. a. (1999). "Prevalence of Hepatitis B virus infection in the United States: The National Health and Nutrition Examination Surveys, 1976 through 1994." The American Journal of Public Health **89**: 14-8.

Michalak, T. (2001). Personal communication. M. Le Mire.

Michalak, T., I. Pardoe, C. Coffin, N. Churchill, D. Freake, P. Smith and C. Trelegan (1999). "Occult Lifelong Persistence of Infectious Hepadnavirus and Residual Liver Inflammation in Woodchucks Convalescent from Acute Viral Hepatitis." Hepatology **29**(3): 928-938.

Michalak, T., C. Pasquinelli, S. Guilhot and F. Chisari (1994). "Hepatitis B Virus Persistence after Recovery from Acute Viral Hepatitis." The Journal of Clinical Investigation **93**: 230-239.

Milich, D. (1997). "Influence of T-helper cell subsets and crossregulation in hepatitis B virus infection." Journal of Viral Hepatitis **4**(Suppl): 48-59.

Milich, D. (1997). "Pathobiology of acute and chronic hepatitis B infection: an introduction." Journal of Viral Hepatitis **4**(Suppl): 25-30.

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

Monjardino, J. (1998). Molecular Biology of Human Hepatitis Viruses. London, Imerial College Press.

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

Netter, H., S. Chassot, S.-F. Chang, L. Cova and H. Will (1997). "Sequence heterogeneity of heron hepatitis B virus genomes determined by full-length DNA amplification and direct sequencing reveals novel and unique features." Journal of General Virology **78**: 1707-1718.

Newbold, J., H. Xin and M. Tencza (1995). "The covalently closed duplex form of the hepadnavirus genome exists *in situ* as a heterogeneous population of minichromosomes." Journal of Virology **69**: 3350-3357.

Nickel, R., A. Schummer and E. Seiferle (1977). Anatomy of the domestic birds. Berlin, Parey.

Nicoll, A., D. Colledge, J. Toole, P. Angus, R. Smallwood and S. Locarnini (1998). "Inhibition of Duck Hepatitis B Virus Replication by 9-(2-Phosphonylmethoxyethyl)adenine, an Acyclic Phosphonate Nucleoside Analogue." Antimicrobial Agents and Chemotherapy **42**(12): 3130-3135.

Nitsche, A., N. Steuer, C. Schmidt, O. Landt and W. Siegert (1999). "Different Real-Time PCR Formats Compared for the Quantitative detection of Human Cytomegalovirus." Clinical Chemistry **45**(11): 1932-1937.

Nowak, J., O. Kai, R. Peck and R. Franklin (1982). "The effects of cyclosporin A on the chicken immune system." European Journal of Immunology **12**: 867-876.

Omata, M. (1990). "Significance of extrahepatic replication of hepatitis B virus." Hepatology **12**(2): 364.

Pardue, S., K. Fite, L. Bengston, S. Lamont, M. Boyle and R. Smyth (1987). "Enhanced Integumental and Ocular Amelanosis Following Termination of Cyclosporine Administration." The Journal of Investigative Dermatology **88**(6): 758-761.

Penna, A., M. Artini, A. Cavalli, M. Levrero, A. Bertoletti, M. Pilli, F. Chisari, B. Rehermann, G. Del Prete, F. Fiaccadori and C. Ferrari (1996). "Long lasting memory T cell responses following self-limited acute hepatitis B." The Journal of Clinical Investigation **98**(5): 1185-1194.

Peters, M., J. Vierling, M. Gershwin, D. Milich, F. Chisari and J. Hoofnagle (1991). "Immunology and the Liver." Hepatology **13**(5): 977-994.

Pugh, J., Q. Di, W. 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." Journal of Virology **69**(8): 4814-4822.

Qiao, M., E. Gowans, S. Bailey, A. Jilbert and C. Burrell (1990). "Serological analysis of duck hepatitis B infection." Virus Research **17**: 3-4.

Rehermann, B. and F. Chisari (1998). The Immunology of Chronic Hepatitis B. in Molecular Mechanisms in Disease and Novel Strategies for Therapy. R. Koshy and W. Caselmann. London, Imperial College Press: 111-132.

Rehermann, B., P. Fowler, J. Sidney, J. Person, A. Redeker, M. Brown, B. Moss, A. Sette and F. Chisari (1995). "The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis." The Journal of Experimental Medicine **181**: 1047-1058.

Robinson, W. (1990). Hepatitis B Virus and Hepatitis Delta Virus. Principles and Practice of Infectious Diseases. G. Mandell, R. Gordon Douglas and J. Bennett. New York, Churchill Livingstone: 1204-1230.

Robinson, W. (1995). Hepadnaviridae. Principles and Practice of Infectious Diseases. G. Mandell, J. Bennett and R. Dolin. New York, Churchill Livingstone. **2:** 1406-1439.

Sambrook, J., E. Fritsch and T. Maniatis (1989). Molecular Cloning: a laboratory manual, Cold Spring Harbour Laboratory Press.

Scaglione, P., M. Melegari and J. Wands (1997). "Biologic properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame." Virology **233**(2): 374-81.

Schaefer, S., T. Tolle, S. Lottmann and W. Gerlich (1998). Animal models and experimental systems in Hepatitis B virus research. Hepatitis B Virus. R. Koshy and W. Caselmann. London, Imperial College Press. **1:** 51-74.

Seeger, C. and W. Mason (1998). Replication of the Hepatitis B Genome. London, Imperial College Press.

Shibata, Y., K. Nakata, S. Tsuruta, K. Hamasaki, Y. Hayashida, Y. Kato, K. Nakao and K. Eguchi (1999). "Detection of hepatitis B virus X-region DNA in liver tissue from patients with hepatitis C virus-assocated cirrhosis who subsequently developed hepatocellular carcinoma." The International Journal of Oncology **14**: 1153-1156.

Sprengel, R., C. Kuhn, C. Manso and H. Will (1984). "Cloned Duck Hepatitis B Virus DNA Is Infectious in Pekin Ducks." Journal of Virology **52**(3): 932-937.

Stoll-Becker, S., R. Repp, D. Glebe, S. Schaefer, J. Kreuder, M. Kann, F. Lampert and W. Gerlich (1997). "Transcription of Hepatitis B Virus in Peripheral Blood Mononuclear Cells from Persistently Infected Patients." Journal of Virology **71**(7): 5399-5407.

Summers, J., P. Smith and A. Horwich (1990). "Hepadnavirus Envelope Proteins Regulate Covalently Closed Circular DNA Amplification." Journal of Virology **64**(6): 2819-2824.

Summers, J., J. Smolec and R. Snyder (1978). "A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks." Proceedings of the National Academy of Science of the United States of America **75**(9): 4533-4537.

Tagawa, M., M. Omata, O. Yokusuka, K. Uchiumi, F. Imazeki and K. Okuda (1985). "Early events in duck hepatitis B virus infection." Gastroenterology **89**: 1224-1229.

Tagawa, M., O. Yokusuka, F. Imazeki, M. Ohto and M. Omata (1996). "Gene expression and active virus replication in the liver after injection of duck hepatitis B virus DNA into the peripheral vein of ducklings." Journal of Hepatology **24**: 328-334.

Thomson, A., P. Whiting and J. Simpson (1984). "Cyclosporine: Immunology, Toxicity and Pharmacology in Experimental Animals." Agents and Actions **15**(3/4): 306-327.

Triyatni, M. (1998). Studies on the protective and therapeutic efficacy of duck hepatitis B virus vaccines. Dept of Microbiology and Immunology. Adelaide, The University of Adelaide: 187.

Triyatni, M., P. Ey, T. Tran, M. Le Mire, M. Qiao, C. Burrell and A. Jilbert (2001). "Sequence comparison of an Australian duck hepatitis B virus strain with other avian hepadnaviruses." Journal of General Virology **82**: 373-378.

Triyatni, M., M. Qaio, P. Ey, C. Burrell and A. Jilbert (1998). Cloning and sequencing of an Australian Duck hepatitis B virus isolate, EMBL Accession No. AJ006350.

Tuttleman, J., C. Pourcel and J. Summers (1986). "Formation of the pool of covalently closed circlar DNA in hepadnavirus-infected cells." Cell **47**: 451-460.

Vendrely, R. (1958). "La notion d'espece a travers quelques donnes biochimiques recentes et le cycle." Annals de L'Institut Pasteur (Paris) **94**: 142-166.

Vessey, C. and P. d. l. M. Hall (2001). "Hepatic Stem Cells." Pathology **33**: 130-141.

Vickery, K. and Y. Cossart (1996). "DHBV manipulation and prediction of the outcome of infection." Journal of Hepatology **25**: 504-509.

Wachs, M., W. Amend, N. Ascher and e. al (1995). "The risk of transmission of Hepatitis B from HBsAg(-), HBcAb(+), HBIgM(-) organ donors." Transplantation **59**: 230-234.

Walter, E., K. Teubner, H. Blum and e. al (1991). "Duck Hepatitis B virus infection of non-hepatocytes." Liver **11**: 53-62.

Wang, G.-H. and C. Seeger (1993). "Novel Mechanism for Reverse Transcription in Hepatitis Viruses." Journal of Virology **67**(11): 6507-6512.

White, D. and F. Fenner (1994). Medical Virology. London, Academic Press.

WHO (2001). Vaccines and Biologicals - Hepatitis B, WHO. **2001**.

Wick, G., P.-U. Muller and S. Schwarz (1982). "Effect of cyclosporin A on spontaneous autoimmune thyroiditis of Obese strain (OS) chickens." European Journal of Immunology **12**: 877-881.

Wingfield, P., S. Stahl, R. Williams and A. Stevens (1995). "Hepatitis core antigen produced in *Escherichia coli*: subunit composition, conformational analysis, and in vitro capsid assembly." Biochemistry **34**: 4919-4932.

Yang, W. and J. Summers (1999). "Integration of hepadnavirus DNA in infected liver: evidence for a linear precursor." Journal of Virology **73**(12): 9710-7.

Yoffe, B., D. Burns, H. Bhatt and B. Combes (1990). "Extrahepatic Hepatitis B Virus DNA Sequences in Patients with Acute Hepatitis B Infection." Hepatology **12**: 187-192.

Yoffe, B., C. Noonan, J. Melnick and e. al (1986). "Hepatitis B virus DNA in mononuclear cells and analysis of cell subsets for the presence of replicative intermediates of viral DNA." The Journal of Infectious Diseases **153**(3): 471-477.

Yotsuyanagi, H., K. Yasuda, S. Iino, K. Moriya, Y. Shintani, H. Fujie, T. Tsutsumi, S. Kimura and K. Koike (1998). "Persistent Viremia After Recovery From Self-Limited Acute Hepatitis B." Hepatology **27**(5): 1377-1381.

Yu, M., J. Yuan, R. Ross and S. Govindarajan (1997). "Presence of antibodies to the hepatitis B surface antigen is associated with an excess risk for hepatocellular carcinoma among non-Asians in Los Angeles County, California." Hepatology **25**(1): 226-228.

Zhang, Y.-Y., D. Theele, S. Litwin and J. Summers (2002). Single cell analysis of mixed DHBV-infected ducks. Personal communication to A Jilbert, Asilomar.

Zhu, Y., T. Yamamoto, J. Cullen, J. Saputtelli, C. Aldrich, D. Miller, S. Litwin, P. Furman, A. Jilbert and W. Mason (2001). "Kinetics of Hepadnavirus Loss from the Liver during Inhibition of Viral DNA Synthesis." Journal of Virology **75**(1): 311-322.