

STUDIES ON MARKERS OF HEPATITIS B VIRUS  
REPLICATION IN MAN.



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**SUMMARY**

The relationship between different markers of hepatitis B virus (HBV) infection and virus replication in HBV infected human liver tissue was examined, with particular emphasis on the relationship between hepatitis B virus core antigen (HBcAg), which is the main virus capsid antigen, and replicative forms of HBV DNA.

Viral antigen detection in liver tissue

Examination of HBV infected livers by the indirect and direct immunofluorescence methods showed the classical distribution pattern of cytoplasmic hepatitis B surface antigen (HBsAg) and nuclear HBcAg respectively. However, when the livers were re-examined with a more sensitive assay, HBcAg was detected in the cytoplasm of a population of cells previously thought to contain only nuclear HBcAg and in the cytoplasm of a population of cells previously negative for HBcAg. Thus, in the samples examined, the majority of cells positive for HBcAg expressed the antigen in the cytoplasm.

The detection of viral antigens in frozen sections was compared with their detection in fixed, paraffin embedded tissue from the same liver samples. HBsAg and HBcAg, localised in the cytoplasm and nucleus respectively, were detected in the fixed tissue, but additional membraneous HBsAg and membraneous or cytoplasmic HBcAg were generally only detected in frozen sections. This problem could be overcome with respect to HBcAg by use of the

sensitive peroxidase-antiperoxidase (PAP) method, which allowed satisfactory detection of HBcAg in ethanol:acetic acid fixed, paraffin embedded tissue.

As a result of the improved histological appearance of the tissue after this method, HBcAg detected by PAP was shown to be usually restricted to foci of infected cells. Two types of foci were seen. Type 1 foci contained cells positive for nuclear and cytoplasmic HBcAg as well as cells positive for only cytoplasmic HBcAg, while Type 2 foci contained cells positive for only cytoplasmic HBcAg.

#### Correlation of replicative HBV DNA with HBcAg

The distribution of HBV DNA in different infected livers was examined by in situ hybridisation using recombinant HBV DNA probes, radiolabelled usually by nick translation. These experiments demonstrated vastly differing levels of HBV nucleic acid within different infected cells. A spectrum of HBV DNA levels, ranging from high to intermediate, was seen in hepatocytes usually in a cytoplasmic location. In contrast, low levels of HBV nucleic acid sequences were detected in the nuclei of cells that were shown to be hepatoma cells and lymphocytes.

Detailed investigation of the HBV DNA detected in the cytoplasm of hepatocytes demonstrated that this contained regions of single stranded virus DNA that was normally double stranded in mature virions, consistent with the properties of a DNA

replicative intermediate. It was concluded that the hepatocyte cytoplasm was the likely intracellular site for HBV DNA synthesis.

Analysis of the intracellular locations of HBV DNA and HBcAg (detected by direct immunofluorescence) in different infected cells showed that the presence of cytoplasmic HBV DNA was more closely correlated with cytoplasmic rather than nuclear HBcAg. These studies were extended to examine HBV DNA and HBcAg (detected by PAP) in sequential sections or simultaneously in the same tissue section. These experiments demonstrated cytoplasmic (replicative) HBV DNA in cells which were either positive or negative for nuclear HBcAg but which always contained cytoplasmic HBcAg. The levels of cytoplasmic HBV DNA and HBcAg were directly proportional; thus, cytoplasmic HBcAg was a more reliable indicator of HBV DNA replication than nuclear HBcAg at the level of the single cell.

Cells containing the above cytoplasmic replicative HBV DNA always contained HBsAg and HBcAg, while cells with low level, nuclear HBV nucleic acid sequences did not contain detectable viral antigens. In addition, HBsAg-positive, HBcAg-negative hepatocytes were frequently seen in which HBV nucleic acid could not be demonstrated with the techniques used. It is proposed that the above observations identify (i) permissively infected cells (hepatocytes) containing HBV DNA replicative intermediates and both HBsAg and HBcAg (ii) cells undergoing restricted infection, demonstrated by either the possession of HBsAg

without replicative HBV DNA (hepatocytes) or by the presence of nuclear HBV nucleic acid sequences without detectable viral antigen expression (lymphocytes and tumour cells).

#### Association of HBV with hepatocyte injury

A number of liver samples were also examined for HBV markers and these results compared with the histological diagnoses and serological markers of infection. In comparing different patients, the presence of replicative HBV DNA in liver cells was associated with liver HBcAg and usually with serum HBeAg, while absence of replicative HBV DNA was associated with the absence of liver HBcAg and usually with serum anti-HBe.

In livers containing foci of hepatocytes with high levels of cytoplasmic (replicative) HBV DNA, histological evidence of injury, viz. vacuolation and hydropic degeneration, was seen in these foci. However, in different patients, progressive liver damage (chronic active hepatitis) was seen both in those patients with or without evidence of HBV replication; in contrast, cirrhosis was strongly correlated with either HBV replication or the presence of delta-Ag. It was concluded that more than one mechanism for hepatocyte damage may operate, that one of these mechanisms was related to HBV replication, and that cirrhosis was an especially common finding in those patients with chronic active hepatitis who also had evidence of either HBV replication or delta infection.

**DECLARATION**

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

I also consent to this thesis being made available for photocopying and loan if applicable if acceptable for the award of the degree.

E.J. GOWANS

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## ABBREVIATIONS

Anti-HBc	antibody to hepatitis B core antigen
Anti-HBe	antibody to hepatitis B e antigen
Anti-HBs	antibody to hepatitis B surface antigen
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
DNAase	deoxyribonuclease 1
EDTA	ethylene diamine tetra acetic acid
IgG	immunoglobulin G
kb	kilobase
kD	kilodalton
LB medium	Luria-Bertani medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5)
mw	molecular weight
mRNA	messenger RNA
PBS	phosphate buffered saline (0.14M NaCl, 0.006M $K_2HPO_4$ , 0.002M $KH_2PO_4$ , pH 7.2)
RNAase	ribonuclease A
SSC	standard saline citrate (0.15M NaCl, 0.015M Na citrate, pH 7.2)
ssDNA	single stranded DNA
TAE	40mM Tris-acetate, pH 8.2, 20mM NaCl, 1mM EDTA.
TBE	90mM Tris, 90mM Boric acid, 2.5mM EDTA pH 8.3.
2 x TY medium	0.5% NaCl, 1.6% Tryptone, 1% yeast extract, pH 7.5

## PUBLICATIONS

1. E.J. GOWANS, C.J. BURRELL, A.R. JILBERT and B.P. MARMION. (1983). Patterns of Single- and Double-stranded Hepatitis B Virus DNA and Viral Antigen Accumulation in Infected Liver Cells. J. gen. Virol. 64: 1229-1239.
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## CHAPTER 1

### INTRODUCTION

#### PREFACE

Due to interest in hepatitis B virus from virologists, molecular biologists, histopathologists and blood transfusion workers, research into many different aspects of hepatitis B virus moves at a rapid pace. As a result, some of the observations made during the period of work towards this thesis (1982 - 1985 inclusive) were either confirmed by or differ from the results of other workers or in turn confirm the recent results of others. Consequently, in order to keep the results of this thesis in perspective, brief reference is made to my work and to the publications arising from this thesis in the appropriate sections of this chapter. These publications are listed on page x.

#### 1. SOURCES OF HEPATITIS B VIRUS INFECTED TISSUES

Hepatitis B virus (HBV) infection is restricted to man in nature, although the chimpanzee may also be infected as a result of experimental or accidental contact with man.

The virus has not been grown reliably in cell culture (Zuckerman, 1975; Hirschman, 1984) and a lymphoblastoid cell line reported to produce hepatitis B virus continuously (Romet-Lemonne et al, 1983) was not available for study.

Similarly, during this work, none of the animal hepadnavirus models was available in Australia for quarantine and other reasons. Thus, it was necessary to examine naturally infected human liver samples in order to examine aspects of HBV replication strategy.

Liver biopsy is rarely performed on patients in early acute HBV infection. Consequently, this study was based on autopsy and biopsy samples from chronically infected patients. Biopsies were carried out only where indicated for the medical management of the patients concerned, and patient data are included with the approval of the Research Review (Medical Ethics) Committee, Royal Adelaide Hospital.

## 2. POSSIBLE MECHANISMS OF PERSISTENT VIRUS INFECTION.

In general, virus infections are usually acute and self limited, with elimination of virus and recovery of the infected host as the eventual outcome. However, in a number of different situations, elimination is unsuccessful and a persistent infection results. Although viral antigen may persist passively (Mims, 1982), persistent infection is usually regarded as a state where the virus genome is present in some form, resulting in de novo synthesis of viral antigen and of complete virus in some circumstances. A number of viruses associated with persistence in man are shown in Table 1.1.

**TABLE 1.1**

**Examples of viruses associated with persistent infections in man.**

RNA Viruses

Measles

Rubella

HTLV I, II, III

DNA viruses

Papovaviruses

Herpesviruses

Adenoviruses

Non-A, Non-B hepatitis  
virus(es)

Hepatitis B virus.

Three different types of persistent virus infection have been noted (Mims, 1982). Examples of these three types are:-

1. where virus persists but no infectious virus is shed, eg SSPE/measles virus;
2. persistence with occasional reactivation, eg cold sores/HSV;
3. persistence with continuous viral replication, eg AIDS/HTLV III.

The mechanism of persistence is still unclear in many cases, but many persistent infections are associated with viruses which are considered to be non cytopathic rather than lytic (Oldstone and Fujinami, 1982). In some examples, virus infections contracted at an early age before the immune system is fully developed and/or asymptomatic infections are more likely to result in virus persistence.

In many persistent infections, viral antigen expression decreases with time from full expression in early acute infection, to little or no expression in fully differentiated tumours closely associated with long standing viral infection. A spectrum of viral antigen expression is seen between these two extremes. Loss of viral antigen expression may occur in vitro, wholly as a result of virus-cell factors, and may be

promoted in vivo by host pressure or selection. Viral persistence is more likely if viral antigen expression is limited (Mims, 1981).

Therefore, the end result of acute virus infection is dependent on the outcome of the balance between virus genome expression and the effectiveness of the immune response (Mims and White, 1984). In simple terms, viral persistence indicates that the host immune response has failed. However, the progression to persistent infection is likely to be multifactorial and Table 1.2. shows a number of virus- and host -properties that may be involved.

### **3. CLINICAL AND EPIDEMIOLOGICAL ASPECTS OF PERSISTENT HEPATITIS B VIRUS INFECTION.**

The main target organ of HBV infection is the liver; infection normally results in an acute, self limited infection with complete recovery of the infected patient. However, in a proportion of cases, the acute infection fails to resolve and the outcome is a persistent HBV infection.

It is likely that the most important predisposing factor is age, since 90-100% of neonates exposed to HBV develop chronic infections while only 20-30% of children and 1-10% of adults do so (Hoofnagle and Alter, 1984). The ratio of persistently infected males:females is approximately 2:1 in most



**TABLE 1.2**

**Situations associated with virus persistence**

Virus Related

Genome integration

Defective interfering-particles

Non immunogenic-agents

Infection of immune cells

Antigenic variation

Host Related

Antibody or cell mediated tolerance to antigens

General or specific immunosuppression

Inadequate antibody response - none

- late

- ineffective

- non neutralising

- enhancing

Interferon deficiency

Antigen inaccessibility

Restricted infection of cells.

populations. The carrier rate is higher in defined immunosuppressed sub-populations (e.g. renal dialysis patients) than in the general population. Rates of persistent HBV infection vary widely in different communities and in different parts of the world, probably due to different rates of exposure coupled with different propensities towards development of persistent infections.

#### **4. HBV PARTICLES.**

Three particle types may be detected in the serum of patients with acute or persistent HBV infection. The most numerous particle is a non infectious 22nm particle composed of the viral envelope material, hepatitis B surface antigen (HBsAg). These particles contain lipid, protein and carbohydrate but are devoid of nucleic acid. Less numerous 22nm diameter filamentous forms of HBsAg of varying length up to a few hundred nm are also found. The least common particle is the complete virion, often called the Dane particle, a 42nm spherical particle with an inner 27nm nucleocapsid comprised of hepatitis B core antigen (HBcAg), which is antigenically distinct from HBsAg, surrounded by a 14nm HBsAg envelope. The nucleocapsid or core particle contains the viral genome and an associated DNA polymerase (DNAP), as well as a protein kinase activity (Albin and Robinson, 1980).

## 5. THE HBV GENOME AND PRODUCTS.

HBV and related hepadnaviruses are unique among animal viruses since their DNA genomes are partially single-, predominantly double-stranded (Figure 1.1). The HBV genome is a relaxed circle of approximately 3200 base pairs (bp) with a nick at a defined position in the long (L) strand and a variable single stranded region (15-50% of the molecule) in the short (S) strand. This short strand, which has been shown to have a defined minimum length (Delius et al, 1983), is elongated in vitro by the virion DNA polymerase to form ds molecules. The genome is held in a circular configuration by a 200-300 bp overlap at the 5' ends of each strand; selective denaturation results in linearisation of the molecule with subsequent reannealing under appropriate conditions (Sattler and Robinson, 1979). The 5' end of the L strand has a covalently bound protein with a molecular weight of ca. 100kD (Gerlich and Robinson, 1980). The overlap region is flanked by an 11 bp direct repeat (DR1 and DR2; Figure 1.1) that may be one mechanism for specific integration of viral DNA into the host cell chromosome (DeJean et al, 1984a) and there is a hairpin sequence near the nick of the L strand (Galibert et al, 1982) that may have a role in the initiation of viral DNA replication.

Molecular cloning of the HBV genome has allowed the generation of large amounts of recombinant DNA. This has permitted detailed restriction enzyme analyses and genome sequencing to

FIGURE 1.1

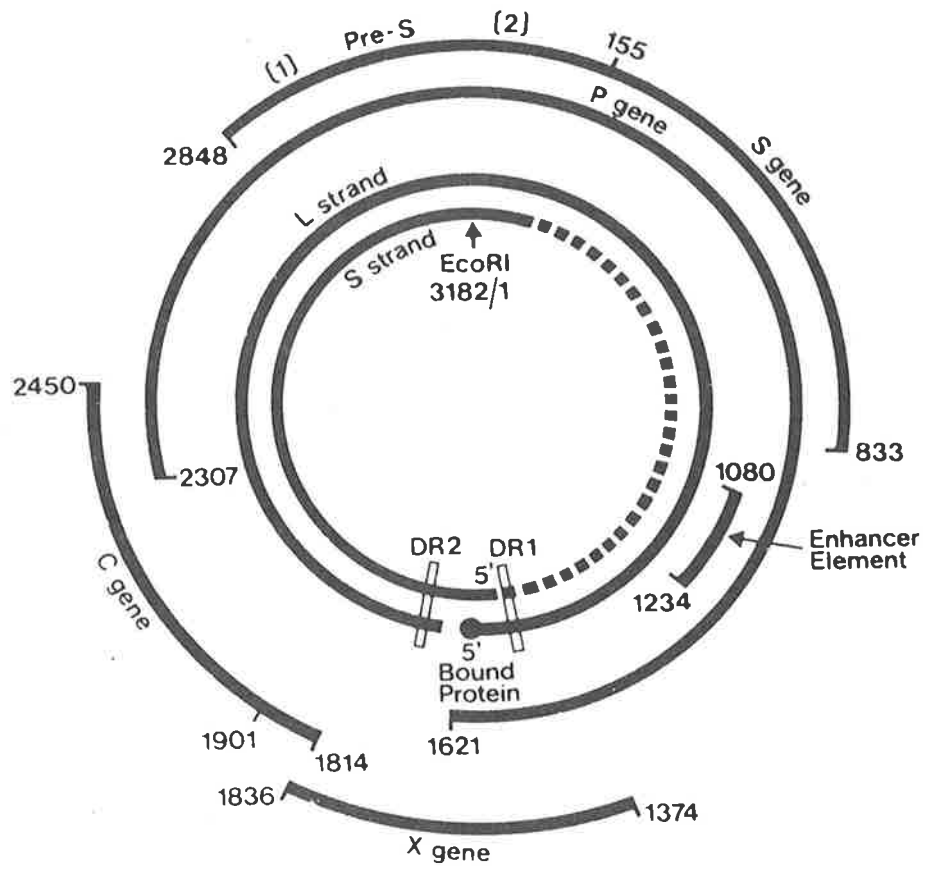


FIGURE 1.1 The genetic organisation of hepatitis B virus.

be performed, and genome expression to be studied. There are no conserved open reading frames (ORF) in potential transcripts from the short strand but there are four overlapping ORF in sequences complementary to the long strand of the genome (Figure 1.1). The long and the short strands have therefore been designated minus and plus strand polarity respectively. Two of the above ORF's have been unequivocally assigned to viral proteins.

The C gene, a 549 or 646 bp sequence depending on which of two initiation sites is used, is found in a portion of the genome that is normally ds in the complete virion. This gene codes for the HBcAg and was identified by expression of HBcAg in E.coli transfected with this specific sequence (Pasek et al, 1979). The core gene encodes a polypeptide of 21 kD that was predicted to contain a high concentration of basic amino acids and was thus considered likely to be a DNA-binding protein (Murray et al, 1981).

The S gene was identified by comparison of the genome sequence with the partial amino acid sequence published for HBsAg (Peterson et al, 1977). The S gene, 678 bp long, is located in the region of the genome that is normally ss in the virion and encodes the HBsAg major protein. Purified HBsAg was shown to contain two polypeptides, P25 and P27 (Peterson et al, 1977), which were both likely to be products of the S gene since they differed only in that P27 was glycosylated (GP27). The S gene is immediately preceded by a 522 bp ORF termed the pre-S

region with initiator codons 165 bp upstream of the S gene (pre-S[2]) and at the origin of the pre-S region (pre-S[1]). The pre-S region is conserved in a number of different HBV sequences and, together with the S gene, is able to code for a 31kD polypeptide (the middle protein) and a 45kD polypeptide (the large protein) respectively (Tiollais et al, 1984); see Figure 1.1.

The largest ORF, the P gene, covers approximately 80% of the genome and as a result overlaps the 3' end of the C gene, the entire pre-S+S gene and approximately 50% of the fourth ORF, the X gene (Figure 1.1). The P gene has not formally been assigned to a protein product, but due to its size and the partial amino acid homology between its predicted product and the reverse transcriptase of several retroviruses demonstrated by computer comparison of the sequences (Toh et al, 1983), it is considered likely to code for the HBV DNA polymerase.

The remaining ORF, the X gene, is capable of encoding a polypeptide of 145-155 amino acids. However, no known protein or function has yet been assigned to this gene, although evidence for expression of this gene in HBV infection has recently been presented (Kay et al, 1985; Moriarty et al, 1985); see part 6.b.iii below. Postulated products for the X gene include the protein which is covalently linked to the 5' end of the long strand of the genome (Figure 1.1; Gerlich and Robinson, 1980) or the HBV nuclear antigen (HBVNA) detected in human hepatoma cell lines (Wen et al, 1983).

Recently, sequences between positions 1080- 1234 (Figure 1.1) approximately 450 bp upstream of the C gene were shown to behave as a viral enhancer element (Shaul et al, 1985). This enhancer was able to operate irrespective of orientation and was shown to be much more active in hepatic (PLC/PRF/5) cells than in control (non-hepatic) cells. This was considered to account in part for the hepatotropic nature of HBV and could also provide a potential mechanism for the oncogenic activity of HBV.

## 6. HBV PRODUCTS IN INFECTED CELLS.

### 6.a. TRANSCRIPTS AND REGULATORY SIGNALS.

Due to the inability to culture HBV in tissue culture cells, studies of HBV-specific RNA transcripts have either used in vitro transcription systems or detected transcripts in host cells infected either with a viral vector or by gene transfer. Alternatively, transcripts have been detected in liver tissue from a chimpanzee infected with cloned HBV DNA. These studies have determined a number of HBV-specific transcripts which are consistent with the HBV genome map.

In HBV infected chimpanzee liver, 2 major transcripts of 3.8 kb and 2.3 kb were detected (Cattaneo et al, 1983; 1984). The 2.3 kb RNA (2.1 kb HBV sequences + 200 poly (A) sequences) was considered to represent the HBsAg transcript detected by others in heterologous systems (Gough, 1983) and in hepatoma

cells with integrated HBV DNA that secrete HBsAg (Edman et al, 1980). The initiation (start) site for this transcript was located within the pre-S gene region (Figure 1.2), while the initiation site for the 3.8 kb transcript was considered to be upstream of the HBcAg gene although it was not mapped accurately. The polyadenylation site for both transcripts was the same and was close to the 5' end of the HBcAg gene (Cattaneo et al, 1984).

A second (minor) initiation site for HBsAg mRNA was also detected in chimpanzee liver (Cattaneo et al, 1984) as shown in Figure 1.2. Similar initiation sites were also detected by Standring et al (1984) and Laub et al (1983). However, Pourcel et al (1982) detected, in transfected mouse cells and PLC/PRF/5 cells, a ca. 2.3 kb HBsAg transcript that hybridised to the pre-S region of a DNA probe. The initiation site for this transcript was around position 2848 and this was confirmed by Rall et al (1983) around position 2810 using an in vitro transcription system (Figure 1.2). This 2.3 kb transcript was considered suitable for the production of the entire pre-S+S ORF to yield a peptide of 45 kD. A HBsAg peptide of this size has recently been identified in infected cells (Heerman et al, 1984; Wong et al, 1985); see part 6.b.i.

The 3.8 kb transcript detected in chimpanzee liver (Cattaneo et al, 1984) was considered to be a candidate for the expression of HBcAg/eAg and HBV DNA polymerase as well as a possible "pregenome" involved in virus replication (see part 7



**FIGURE 1.2**

1. Initiation site for 2.3kb major HBsAg mRNA transcript (Cattaneo et al, 1983:84)
2. Alternative minor HBsAg mRNA initiation sites (Laub et al, 1983; Standring et al, 1984; Cattaneo et al, 1984)
3. Initiation site for entire pre-S+S mRNA (Pourcell et al, 1982; Rall et al, 1983)
4. Polyadenylation site (similar sites have been detected in DHBV and WHV) (Cattaneo et al, 1984; Buscher et al, 1985; Moroy et al, 1985)
5. HBcAg mRNA and pregenome (3.8kb transcript) initiation site (Rall et al, 1983)
6. HBV 700 (RNA polymerase III) initiation site (Standring et al, 1983)
7. Promotor sequence similar to SV<sub>40</sub> late promotor sequence (Cattaneo et al, 1983).

Likely mRNA initiation sites (AUG) determined from examination of the genome sequence are shown by an asterisk. The specific sites may vary depending on the HBV subtype but the general layout is as depicted.

FIGURE 1.2

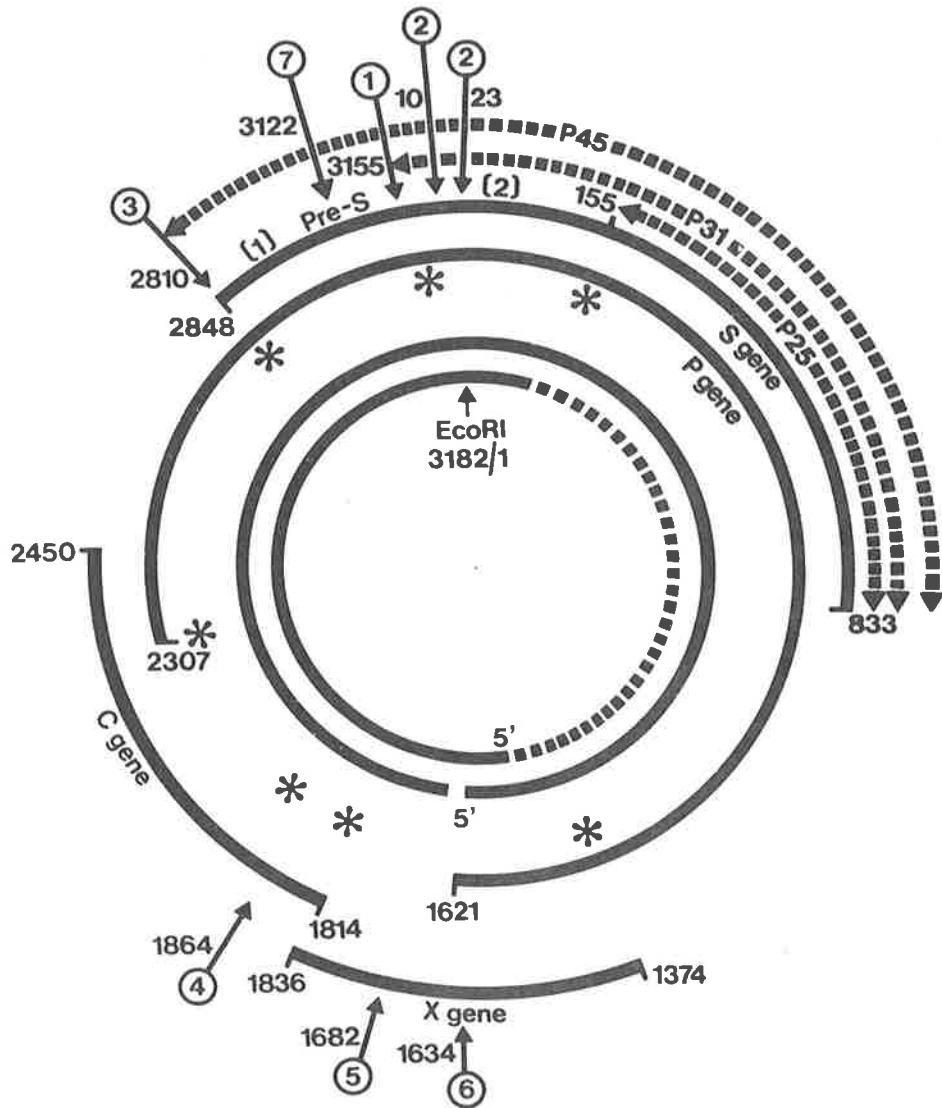


FIGURE 1.2: HBV messenger RNA control sites on the genome.

below). A similar transcript has been recently described in duck hepatitis B virus (Buscher et al, 1985) and woodchuck hepatitis virus infected hepatocytes (Moroy et al, 1985). Production of these 3.8 kb transcripts required that the single polyadenylation signal in the genome, which is located in the HBcAg gene, is processed only at the second passage. This presumed termination signal is not typical of that seen in the majority of genes (Rutter et al, 1984), suggesting that it may have a specialised function. Since the 3.8 kb transcript was only found in cells from livers supporting viral replication, it is likely that viral replication is closely linked to this transcript.

In addition, the in vitro transcription system used by Rall et al (1983) also identified the initiation site for the HBcAg gene. This was shown to be immediately upstream of the HBcAg gene ORF close to the initiation site predicted for the 3.8 kb RNA by Cattaneo et al (1984) from the chimpanzee model (Figure 1.2). Transcription from this site was considered likely to generate the "pregenome" required for HBV replication (Rall et al, 1983).

In vitro transcription of the HBV genome by RNA polymerase III produced a 700 base product (HBV 700) which was generated using the short (plus) HBV DNA strand as the template (Standring et al, 1983). The origin of this transcript was close (50bp) to the origin of the HBcAg in the overlap region. Although the HBV 700 "gene" was conserved in some HBV DNA

sequences and in the WHV DNA sequence, it was concluded that this transcript was unlikely to code for a protein, as RNA polymerase III usually transcribes short untranslated RNA species eg. tRNAs (Rutter et al, 1984).

It has been suggested that the overlap region of the genome is important in the regulation of virus replication and genome expression (Rutter et al, 1984). A sequence similar to the SV40 late promoter was identified approximately 30 nucleotides upstream of the major HBsAg mRNA initiation site. This promoter site was considered likely to direct the synthesis of the major 2.3 kb HBsAg mRNA (Cattaneo et al, 1983).

#### 6.b. PROTEINS AND POLYPEPTIDES.

##### 6.b.i. Hepatitis B Surface Antigen

Due to the scarcity of HB virions in patient material, the components of the virion envelope (HBsAg) were initially studied using purified 22nm HBsAg particles. Two major polypeptides with molecular weights (mw) of 25000 and 27000 (P25 and GP27) respectively and a number of minor higher mw polypeptides were detected (Peterson et al, 1977). A number of host contaminants, usually albumin were also detected (Burrell, 1975). GP27 was shown to be a glycosylated version of P25 and both correspond to the protein product of the S gene.

However, examination of HBsAg purified from HBeAg positive patients showed that filamentous forms of HBsAg and purified virus particle preparations contained approximately 20 times the concentration of higher mw polypeptides GP33, GP36, P39 and GP42 than did 22nm HBsAg particle preparations (Heerman et al, 1984). In each case, these polypeptides consisted of P25 (encoded by gene S) plus either an additional 55 amino acids (GP33, GP36) encoded by the pre-S[2] gene or an additional 174 amino acids (P39, GP42) encoded by the entire pre-S gene i.e., S+pre-S[1+2] (Heerman et al, 1984; Wong et al, 1985). GP42 represents a glycosylated version of P39. These polypeptides represent the major, middle and large protein products respectively of the pre-S + S gene.

Polypeptides GP31 and GP34, likely to be identical to GP33 and GP36 in other reports, harbour a receptor for polymerised human serum albumin (pHSA; Machida et al, 1983). As there may also be receptors for pHSA on hepatocytes, it has been suggested that pHSA may mediate the entry of HBV into hepatocytes (Thung and Gerber, 1984).

The higher mw polypeptides associated with HBsAg in virions are close in size to those predicted from the ORF of the HBV genome. Antibodies produced to synthetic peptides constructed to represent the pre-S protein reacted with a polypeptide (P45) considered to represent the full length translation product of the entire pre-S+S gene (Neurath et al, 1985). As varying amounts of P45 are present in different 22nm HBsAg

particle preparations, expression of the different envelope proteins of the virion is therefore achieved by variable use of the initiation codons in the pre-S ORF.

These results explain earlier observations of antibodies which were reactive with HB virions but not with 22nm HBsAg particles (Moodie et al, 1974; Alberti et al, 1978). These antibodies to the pre-S region proteins have been postulated to be important in recovery from acute hepatitis B infection (Neurath and Kent, 1985).

#### 6.b.ii HBcAg and HBeAg.

Free 27 nm HBcAg particles are not present in the serum of infected patients, but HBcAg released from virions by detergent treatment may be detected provided care is taken to remove anti-HBc, normally present in high levels in serum, that binds to the exposed HBcAg particles (Bredehorst et al, 1985). HBcAg particles purified from disrupted virions prepared from serum contained three polypeptides (P19, P70 and P80; Hruska and Robinson, 1977) or two polypeptides (P19 and P45; Takahashi et al, 1979). HBcAg particles purified from infected liver were also reported to contain one major (P19) and one minor (P43) polypeptide (Ohori et al, 1980). Despite originating from HBcAg particles, none of these polypeptides reacted with anti-HBc nor was able to generate anti-HBc in rabbits (Hruska and Robinson, 1977). They were instead reactive with anti-HBe and it was concluded that HBeAg was present in a cryptic form in HBV particles that was revealed

after disruption of the core particle structure (Ohori et al, 1980; 1984). HBe and HBe antigenicity were shown subsequently to reside on the same polypeptide by MacKay et al (1981); HBcAg synthesised in E.coli after transfection with the HBcAg gene was converted to HBeAg by proteolytic digestion, demonstrating that HBcAg and HBeAg are encoded by the C gene. A comparison of the amino acid sequences of a polypeptide (P15.5), obtained from HBeAg purified from serum, with the HBcAg polypeptide, demonstrated that P15.5 differed to the HBcAg polypeptide only through the loss of the last 34 (carboxyl terminus) amino acids (Takahashi et al, 1983). In contrast to previous reports, polypeptide P15.5 reacted with an anti-HBe monoclonal antibody.

Purified HBcAg particles contain a protein kinase activity that in vitro phosphorylates serine residues in the major core protein (Gerlich et al, 1982). Recently, Petit and Pillot (1985) confirmed that the product of this reaction was a 22kD polypeptide. This polypeptide was also shown to react with anti-HBe and anti-HBe and contained a DNA binding activity that was specific for HBV DNA, consistent with a predicted activity of this protein from the genome sequence (Murray et al, 1981). Petit and Pillot suggested that the P41 polypeptide detected as a minor component in their (and other-see above) purified HBcAg preparations represented a dimer of P22. In addition, pronase digestion and complete reduction of P40 present in purified HBcAg preparations, resulted in a single peptide of mw 15000 (P15) suggesting that the P40 polypeptides

seen in core antigen preparations were aggregates of the basic eAg component (Feitelson et al, 1982a). It has also recently been shown that P40 shared amino acids with the P19 peptide and also contained unique amino acids that may have resulted from expression and splicing of the X gene with the C gene (Feitelson et al, 1982a). Neurath and Kent (1985) have also suggested that the higher mw polypeptides in HBcAg preparations may represent translation products of spliced RNA from the C gene and from part or whole of the X gene. The major polypeptide of the duck HBV core antigen (mw 35000) is the product of the fused C+X gene seen in the duck virus genome (Mandart et al, 1984). It is possible that HBV uses a splicing mechanism to achieve a protein of similar size.

Pronase digestion of the major core Ag polypeptide (P22) resulted in a truncated form which was reactive with anti-HBc and anti-HBe, while the fully assembled form (the 22nm core particle) was only reactive with anti-HBc. P22 was considered to be a multifunctional polypeptide critical to virus assembly (Petit and Pillot,1985) and the relationship between HBc/eAg has been compared with D:C variation observed with polioviruses (Ohori et al, 1980).

In contrast to HBcAg, HBeAg is readily detected in the serum of a proportion of HBV infected patients as a soluble antigen in contrast to the particulate HBeAg associated with HBcAg particles described above. First described as an additional marker of HBV infection (Magnius and Espmark,1972), HBeAg was



shown to be closely related to viral replication in different patients (Werner et al, 1977; Burrell et al, 1984). Cessation of detectable viral replication is usually but not always accompanied by loss of HBeAg and seroconversion to anti-HBe (Hadziyannis et al, 1983).

Soluble HBeAg proved difficult to purify and characterise due to its close affinity with serum proteins (Neurath and Strick, 1977). Purified, soluble HBeAg was shown to contain a 17000 mw polypeptide and a 350000 mw polypeptide which was considered to represent HBeAg bound to dimeric IgG (Katz et al, 1980), while Howard and Zuckerman (1979) detected two major polypeptides of 17000 and 66000 in purified soluble HBeAg preparations.

6.b.iii. The X gene product.

Recently, the X gene was expressed in E.coli (Moriarty et al, 1985; Kay et al, 1985 ). The resultant peptide was recognised by human (and woodchuck) antibodies from subjects with primary hepatocellular carcinoma (Moriarty et al, 1985) and from anti-HBe carriers (Kay et al, 1985). This provided evidence for expression of the X gene in HBV infection. In addition, antibodies produced to synthetic peptide products representing defined regions of the X gene, recognised an antigen in the HBsAg secreting hepatoma cell line PLC/PRF/5 and in HBV infected liver homogenates but not in controls (Moriarty et al, 1985). As the size of the peptide from the liver homogenate that reacted with these antibodies was larger

(28kD) than could be encoded by the X gene (17kD), it was suggested that this polypeptide may represent a fusion product, although the peptide did not react with anti-HBc (Moriarty et al, 1985).

#### 6.c.i. HBV DNA in serum

HBV DNA detected in the serum of chronic carriers may be considered to be a direct measure of virus replication and infectivity. HBV DNA detection was more sensitive than the previously widely used DNA polymerase (DNAP) assay (Weller et al, 1982b), since HBV DNA was detected in a number of samples negative for HBV DNAP. Similarly, HBV DNA was detected in the serum of patients who were HBeAg negative, anti-HBe positive (Hadziyannis et al, 1983).

HBV DNA detected in serum by Southern blot was usually present as a discrete 3.2 kb band with some lower mw smearing corresponding to the degree of ssDNA present (Brechot et al, 1981). However, some sera also had either a proportion of DNA-RNA hybrid molecules (Miller et al, 1984a) or a range of lower mw bands including full length (minus) ssDNA (Scotto et al, 1985). In these two latter reports, the minor populations were considered to be replicative intermediates packaged into virions.

### 6.c.ii. HBV DNA in liver tissue

Different forms of HBV DNA have also been detected in DNA extracted from infected livers. Generally, replicative forms of HBV DNA were detected in livers from patients with active hepatitis who were positive for serum HBV DNA and liver HBcAg, while integrated, presumably non-replicative forms of HBV DNA were detected in patients with only liver HBsAg and positive for anti-HBe in the serum (Brechot et al, 1984; Shafritz and Hadziyannis, 1984). Details of these replicative and non-replicative forms are described below.

HBV DNA extracted from livers (presumably) supporting virus replication showed a concentration of circular and linear ds molecules of 3.2 kb as well as a heterologous lower mw smear seen in Southern blots that was considered to be genome length- or smaller-linear ss viral DNA (Brechot et al, 1984; Miller and Robinson, 1984). The polarity of this ss viral DNA was shown to be minus strand (This study; Blum et al, 1984a) and was localised to the cell cytoplasm either by cell fractionation (Miller and Robinson, 1984) or by in situ hybridisation studies (Gowans et al, 1983; Blum et al, 1984a). In addition to the above, a supercoiled 3.2 kb form of HBV DNA was also detected in the nuclear fraction of livers supporting virus replication (Miller and Robinson, 1984). It was suggested that this nuclear supercoiled HBV DNA was a possible template for the production of HBV RNA destined to become mRNA or "pregenomes" (see below). The term "replicative intermediates" of HBV DNA used throughout this thesis refers

to the heterologous < 3.2 kb dsDNA or linear ss minus strand DNA described above that are different from the DNA form detected in virions.

Replicative minus strand HBV DNA was detected in DNA extracted from the nuclear fraction of chimpanzee liver (Fowler et al, 1984). However, chimpanzees persistently infected with HBV never showed evidence of HBV DNA integration (Shouval et al, 1980) in contrast to human HBV carriers (see below). A small minority of virions in liver and serum of an infected chimpanzee contained similar supercoiled HBV DNA molecules to those described above (Ruiz-Opazo et al, 1982). In contrast, in livers which are not supporting virus replication (i.e. do not contain detectable HBV DNA replicative intermediates or HBcAg), HBV DNA may be detected in a form consistent with integration into the host cell chromosome (Brechot et al, 1981; Shafritz et al, 1981).

In addition to the above replicative and integrated forms of HBV DNA, a mixture of these forms present in the same liver has been described, as well as additional forms of free monomeric, dimeric and oligomeric ds viral DNA (Brechot et al, 1984). The significance of these latter forms is still unknown. The various forms of HBV DNA described above are summarised in Table 1.3.

**TABLE 1.3**

**HBV DNA FORMS AND POSSIBLE FUNCTIONS**

<u>Serum</u>	<u>Form</u>	<u>Possible Function</u>
	3.2 kb ds DNA with ss gap	Virion genome
	3.2 kb ss DNA	Replicative intermediate
	RNA-DNA hybrid	Replicative intermediate
	Smear, < 3.2 kb ss DNA	Replicative intermediate
<u>Liver</u>		
Cytoplasm	3.2 kb linear and circular ds DNA	Virion genome
	Smear, < 3.2 kb ss DNA	Replicative intermediate
Nucleus	3.2 kb Supercoiled dsDNA	Template for RNA
	High mw ds DNA	Integrated DNA
Unstated	Monomers, dimers and and oligomers of 3.2 kb ds DNA	Unknown
<u>Chimpanzee</u>		
Serum	3.2 kb ds DNA with ss gap	Virion genome
	3.2 kb supercoiled ds DNA	Unknown
Liver Nucleus	3.2 kb ss DNA	Replicative intermediate

## 7. VIRAL ANTIGEN EXPRESSION IN INFECTED TISSUES AND CELLS.

HBsAg may be detected in sections of infected human liver. In infected hepatocytes, HBsAg may be restricted to the cell membrane or completely fill the cytoplasm (Ray 1979). Membraneous HBsAg has been suggested to correlate with more active HBV replication (Ray, 1979). Although HBsAg has also occasionally been detected in Kupffer cells (Gerber and Thung, 1979), this is not considered to represent de novo synthesis; HBsAg is usually restricted to hepatocytes in infected livers.

HBcAg is usually found in the nucleus of hepatocytes, although a proportion of cells have been shown to contain varying concentrations of cytoplasmic HBcAg (Ray 1979; Huang and Neurath, 1979). Recently however, using a more sensitive method, HBcAg was detected in the cytoplasm of the majority of hepatocytes in HBV infected liver samples, while nuclear HBcAg was only present in a proportion of these cells (Gowans and Burrell, 1985; Gowans et al, 1985). HBcAg detected in liver sections has been regarded as a marker of HBV replication, and membraneous HBcAg was reported to correlate with the severity of histological injury (Huang and Neurath, 1979). Membraneous HBcAg may be masked in vivo by bound anti-HBc (Trevisan et al, 1982).

Due to the lack of an anti-HBe reagent free from anti-HBc antibody, the intracellular localisation of HBeAg remains unclear. In one report, HBeAg was localised to the cytoplasm (Trepo et al, 1976), while another study reported nuclear HBeAg (Arnold et al, 1977).

A nuclear antigen was recently detected in two different human hepatoma cell lines which secrete HBsAg (Wen et al, 1983). The antigen was considered to be analogous to the Epstein-Barr virus nuclear antigen and was detected using human antibodies from patients with persistent HBV infection. HBV nuclear antigen (HBVNA) was antigenically distinct from both HBsAg and HBcAg and it was suggested to be a possible product of the X gene.

#### **8. PERSISTENT INFECTION WITH HBV.**

A number of properties consistent with recognised mechanisms of virus persistence shown in Table 1.2 are associated with HBV persistence.

These properties are:-

1. HBV DNA integration into the host cell chromosome (Brechot et al, 1981; Shafritz et al., 1981), 2. the delayed and often low neutralising antibody response to HBsAg following acute HBV infection (Overby et al, 1982). This may be due to an intrinsic poor immunogenicity of HBsAg, although preparations of HBsAg used for vaccination elicit an effective neutralising

antibody response (Szmuness et al, 1980) and it is possible that the poor response in acute hepatitis B infection also results from virus-associated immune suppression (see point 4). 3. immune tolerance to high levels of circulating HBsAg seen in acute infection. 4. HBV infection of cells of the immune system (Lie-Injo et al, 1983; Pontisso et al, 1984). 5. restricted expression of HBV antigens in infected cells.

Patients with persistent HBV infection may be divided into two broad groups (Hoofnagle and Alter, 1984). These groups are 1. those patients with markers of viral replication (serum HBV DNA, serum HBV DNA polymerase, serum HBeAg and liver HBcAg) thought to represent an early stage of persistence, and 2. those patients with markers of non replicative infection (serum anti-HBe, integrated HBV DNA in liver cells, and absence of the other markers mentioned above). Patients in the latter group may show spontaneous reactivation of virus replication (Davis et al, 1984; 1985).

HBV DNA has been detected in a number of different tissues and secretions from persistently infected patients (Table 1.4). However, except in the case of HBV DNA detected in extracts from a bone marrow cell culture (Elfassi et al, 1984), the forms of viral DNA detected were not replicative, although HBV DNA in Kaposi sarcoma cells (Siddiqui, 1983) and in leukocytes (Pontisso et al, 1984) resembled the free monomeric and oligomeric forms described in part 6.c.ii. It is possible that contamination with or exudation of HBV DNA



**TABLE 1.4****HBV DNA detected in non-hepatocytes.**

<u>Cell/tissue type.</u>	<u>Reference.</u>
Kaposi sarcoma; skin.	Siddiqui, 1983.
Leukocytes.	Lie-Injo et al, 1983; Pontisso et al, 1984.
Bile duct epithelium, endothelial cells, smooth muscle cells.	Blum et al, 1983
Pancreas, kidney, skin.	Dejean et al, 1984b
Bone marrow culture.	Elfassi et al, 1984.
Saliva, urine, seminal fluid.	Karayiannis et al, 1985.
Urine.	Di Bisceglie et al, 1985.

positive blood could account for some of the results of Table 1.4 (Karayiannis et al, 1985). Low numbers of HBV DNA genomes were also detected by in situ hybridisation in bile duct epithelia, endothelial cells and smooth muscle cells (Blum et al, 1983) of infected liver and in lymphocytes present in infected liver (Gowans et al, 1983; this study). It is possible that HBV DNA detected in forms other than replicative intermediates represents non permissive infection.

#### 9. SEQUELAE OF PERSISTENT HBV INFECTION.

Persistently infected patients with markers of HBV replication (see part 8 above) usually show a continuing chronic hepatitis often with hepatocyte necrosis. In contrast, patients with integrated HBV DNA may show a wide spectrum of histological classifications ranging from normal liver to primary hepatocellular carcinoma (HCC). DNA extracted from tumourous and non tumourous regions of the same infected liver may not necessarily show the same pattern of HBV DNA integration (Brechot et al, 1984).

The epidemiological evidence for the association between HBV and HCC is stronger than for any other virus and human cancer (Beasley et al, 1981). A number of human hepatoma cell lines

containing integrated HBV DNA that secrete HBsAg have been cultured. The most common cell line of this type is the PLC/PRF/5 line developed by MacNab et al (1975).

HBsAg and HBVNA are produced in these cells as a result of the expression of integrated HBV DNA. On the other hand, HBcAg expression is prevented; there is evidence that this may be due, at least in part, to the high level of methylation of the C gene (Miller and Robinson, 1983).

#### 10. ANIMAL MODELS OF HBV.

HBV is the prototype for a small family of viruses that have been provisionally named Hepadnaviridae (Robinson, 1980; Gust et al, 1985). At the present time, in addition to HBV, three viruses are recognised as Hepadnaviruses. These are the Woodchuck hepatitis virus (WHV; Summers et al, 1978), the Beechey ground squirrel hepatitis virus (GSHV; Marion et al, 1980), and the Pekin duck hepatitis B virus (DHBV; Mason et al, 1980). All three have a very restricted host range, are morphologically similar, hepatotropic and may produce persistent infection. As with HBV, levels of viral replication in carriers decrease with time. Although other Hepadnavirus-like agents have been described none has been fully characterised, and these are not yet generally included in the group. A number of characteristics of the Hepadnaviruses are shown in Table 1.5.

**TABLE 1.5**

**Characteristics of hepadnaviruses**

	<u>HBV</u>	<u>WHV</u>	<u>GSHV</u>	<u>DHBV</u>
Genome size (bp)	3182	3308	3311	3021
5' linked protein	+	?	+	+
ORF	4	4	4	3
Hairpin structure	+	+	+	?
Genome overlap region (bp)	223	212	211	46
Protein Kinase	+	?	+	?
sAg/Ab	+	+	+	+
cAg/Ab	+	+	+	+
eAg/Ab	+	+	+	?
xAg/Ab	+	+	?	?
Direct repeat in genome	+	+	+	+
Associated HCC	+	+	+	+

Except for DHBV which has no filamentous forms, each of the Hepadnaviruses has three particle types similar to those described for HBV. High levels of excess envelope antigen, present as ca. 22nm surface antigen particles, are secreted into the serum of infected animals and the virions are composed of 27nm core antigen particles surrounded by the lipid-protein sAg envelope. In each case, the core particle contains the virus genome and a DNAP activity that repairs the gap in the short strand of the genome. A protein kinase activity similar to that found in HBV has been described in core particles from GSHV (Feitelson et al, 1982b) and it is likely that this is a feature of the group.

The genome structure and length of different hepadnaviruses are very similar, based on a comparison of the nucleotide sequences of HBV, WHV and DHBV (Mandart et al, 1984) or examination of the GSHV sequence (Seeger et al, 1984). However, the DHBV genome has only three ORF in the coding strand since the C and X genes seen in the other Hepadnaviruses are fused into a single ORF (Mandart et al, 1984). Similar features to those found in the HBV have also been described, viz. 1. an overlap region that maintains the circular integrity of the molecule ; 2. a 10-11 bp direct repeat region near the 5' ends of the strands (Seeger et al, 1984) and 3. a stable hairpin region considered to represent the origin of replication (Mandart et al, 1984), although this feature has not yet been described for the DHBV genome. A

similar protein to that bound to the 5' end of the long strand of the HBV genome has also been described for the GSHV and the DHBV (Ganem et al, 1982; Molnar-Kimber et al, 1984).

In addition to the surface Ag/Ab and core Ag/Ab systems, it is likely that each virus also has eAg/Ab and xAg/Ab systems since antibodies from WHV infected woodchucks reacted with HBeAg (Gerin et al, 1984) and a human X gene product synthesised in E.coli (Kay et al, 1985), while an antigen likely to be GSeAg was detected in serum by a commercial anti-HBe conjugate (Marion et al, 1983).

A comparison of the nucleotide sequences of all four viruses showed that HBV has 62-70%, 55% and 40% homology with WHV, GSHV and DHBV respectively, while WHV and GSHV shared 82% homology (Mandart et al, 1984; Seeger et al, 1984). The region with highest homology was in the P gene. These nucleotide homologies are reflected in the degree of homology of the predicted amino acids comprising the gene products. HBsAg shares 61% of amino acids with WHsAg and with GSsAg but only 35% with DHBsAg, while WHsAg and GSsAg have 90% homology. Likewise, HBcAg and WHcAg have 73% amino acid homology (Galibert et al, 1982), HBcAg and GScAg 68%, while WHcAg and GScAg have 92% homology (Seeger et al, 1984). Although there was net homology with HBcAg at the carboxylic end of the molecule, this was not quantitated for DHBcAg (Mandart et al, 1984).

The above predicted serological cross reactions were detected experimentally for the surface antigens of HBV, WHV and GSHV but not for DHBsAg (Cote and Gerin, 1983). WHsAg and GSsAg showed stronger cross reactions than did WHsAg and HBsAg and it was concluded that all three have common antigenic determinants. These common determinants permitted the detection of GSsAg by a commercial radioimmunoassay (Ausria; Abbott Laboratories) designed to detect HBsAg (Marion et al, 1983). However, tryptic peptide mapping showed less homology than predicted (Feitelson et al, 1981) and the common peptides were considered to represent the "a" determinant of HBsAg. It was considered likely that the lower than predicted cross reactions between the various surface antigens resulted from lower homology in the hydrophilic (antigenic) regions of the peptide than in the hydrophobic (non antigenic) regions (Wain-Hobson, 1984).

HBCAg and WHcAg were shown to cross react strongly in immunodiffusion reactions (Werner et al, 1979) and more recent results showed that although a commercially available assay for anti-HBc (Corab; Abbott Laboratories) detected anti-WHc very poorly (Ponzetto et al, 1985), there were partial cross reactions. In addition, peptide mapping showed up to 60% homology between HBCAg and GScAg (Feitelson et al, 1982a) and it is likely that core antigens represent a "group specific" type antigen.

Studies on the expression of hepadnavirus antigens in infected cells are limited. Surface antigens of WHV and DHBV were detected in the cytoplasm of infected cells (Frommell et al, 1984; Halpern et al, 1984) as was mature WHcAg (Ponzetto et al, 1984b; Frommell et al, 1984) and immature core particles of DHBV (Halpern et al, 1984). Only a few nuclei were shown to be weakly positive for WHcAg, in contrast to the localisation and accumulation of HBcAg.

Various forms of hepadnavirus DNA have been detected in infected cells and in each case, these were similar to the forms of HBV DNA described above in part 6.c. The major form of viral DNA was detected in the cytoplasm of DHBV, WHV and GSHV infected cells (Mason et al, 1982; Mitamura et al, 1982; and Weiser et al, 1983; respectively), and shown to be minus polarity, ss viral DNA in the case of DHBV and GSHV. In each case, supercoiled genome length virus specific DNA was also detected in the nuclei of infected cells. Furthermore, in DHBV transmission experiments, the appearance of this nuclear, supercoiled viral DNA preceded the synthesis of minus strand virion DNA and it was suggested that the supercoiled molecule could serve as a template for the transcription of viral RNA (Mason et al, 1983). In addition to the above forms of DNA, integrated viral DNA has also been detected in chronically infected woodchucks (DeJean et al, 1982), in woodchucks with WHV associated HCC (Mitamura et al, 1982) and in ducks with DHBV associated HCC (Yokosuka et al, 1985).



Thus, the mammalian hepadnaviruses are more closely related to each other than to the avian virus, with the WHV and GSHV more closely related than the others. There are sufficient similarities between all of the viruses to justify the belief that each replicates in a similar manner.

#### 11. HBV REPLICATION MECHANISMS.

Although HBV cannot be grown in cell culture systems, some aspects of viral replication have been deduced from the examination of naturally infected liver samples. However, many of these observations have only been related to virus replication as a result of the replication strategy for the Pekin duck HBV and related hepadnaviruses proposed by Summers and Mason (1982).

This proposed replication strategy is unusual, as an RNA copy of the genome is used as a replicative intermediate. Although many of the details are still unclear, the main points have been defined (Figure 1.3). The input viral DNA is probably converted to a fully ds form which migrates to the nucleus and serves as a template for RNA transcription. These transcripts are then able to serve as mRNA for protein production or, after packaging in immature cores in the cell cytoplasm, as templates (the "pregenome") for the production of full length minus strand viral DNA by a reverse transcriptase. This minus strand is in turn used as a template for the production of

### FIGURE 1.3

Input virus (1) is adsorbed to the cell membrane and internalised. The virion genome is uncoated (2) and converted to a fully ds form by the virion DNA polymerase. This may occur either in the cytoplasm followed by migration of the complete ds molecule to the nucleus or wholly in the nucleus. The ds virus DNA is then converted to a supercoiled form (4) which probably serves as a template for the production of RNA. This either behaves as mRNA for the production of virus-specified proteins (5) or is encapsidated in "immature" core particles (6); encapsidated RNA is the template for the production of negative polarity ss viral DNA by the reverse transcriptase action of the virus polymerase (7). The nascent ss DNA is in turn the template for the production of positive sense DNA (usually less than genome length) by the virion DNA polymerase (8). The "immature" core particle is then enveloped by HBsAg and the complete virion exported from the cell.

The virion polymerase is depicted as a large solid circle and the protein covalently bound to the 5' end of the genome negative strand by a smaller solid circle.

(~~~~) RNA  
(——) DNA

FIGURE 1.3

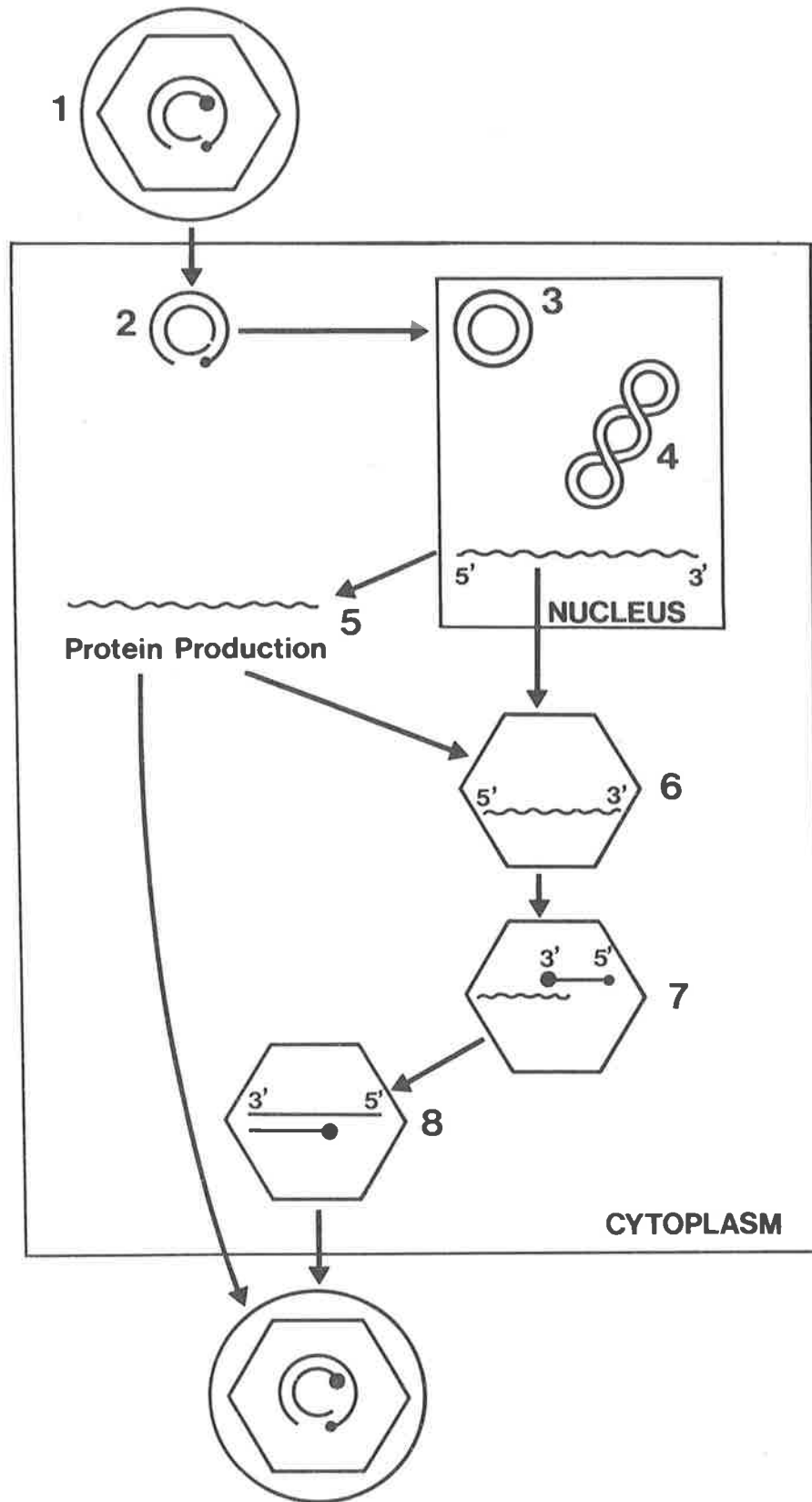


FIGURE 1.3

The proposed replication strategy of Hepadnaviruses.

(incomplete) plus strand DNA. Elongation of the minus strand by reverse transcription is accompanied by degradation of the RNA template by RNAase H-like activity, so that the product of this reaction is a full length ssDNA and not an RNA-DNA hybrid.

Since the DHBV is closely related to HBV it is likely that HBV uses a similar replication strategy. Some HBV virions which contained RNA-DNA hybrids (Miller et al, 1984b), considered to be released replicative intermediates, were shown in vitro to contain both RNA- and DNA-dependent DNA polymerase activities; it was proposed that HBV may differ from DHBV as HBV may use a strand displacement mechanism instead of the RNAase H digestion of the RNA pregenome (Miller et al, 1984b). The HBV polymerase may provide the intracellular functions required by the above general replication model. These functions are: 1. elongation of the short strand to convert the input DNA to a fully ds form. 2. a reverse transcriptase activity to produce DNA from an RNA "pregenome" template and 3. a DNA dependent DNA polymerase activity to produce (partial) ds viral DNA molecules from the minus strand DNA template.

Although the template and enzyme required for production of RNA has not been identified for either the DHBV or the HBV, supercoiled DNA molecules (Summers and Mason, 1982; Miller and Robinson, 1984) are candidate templates for this function, as they represent a form of the viral DNA with the nick in the

long strand repaired. This complete ds DNA molecule would permit expression of the X gene which normally has an internal nick in the virion DNA (Figure 1.1.). Integrated HBV DNA detected in infected livers (Brechot et al, 1981) could also act as a template. The 3.5-3.8 kb RNA transcripts detected in DHBV (Buscher et al, 1985), WHV (Moroy et al, 1985) and HBV infected livers (Cattaneo et al, 1984), may serve both as a pregenome replicative intermediate and as the functional mRNA for production of the virus core antigen and polymerase (see part 6.a).

Very short fragments (<30 bases) of nascent minus strands of DHBV DNA contained a covalently bound 5' linked protein (Molnar-Kimber et al, 1984) and the 5' end of the minus strand was considered to be the origin of reverse transcription. Thus the 5' linked protein was considered to be a primer for the replication of viral DNA. Although the origin and function of this protein in the HBV genome have still to be determined (Gerlich and Robinson, 1980), it is likely to fulfil a similar role in both viruses. However, it is also possible that the 5' linked protein provides a packaging or export signal as is possible for polioviruses (Wimmer, 1982) and the hairpin sequence close to the nick on the minus strand (Galibert et al, 1982) could provide a primer function.

Finally, a predominance of minus polarity ss HBV DNA has been detected in the cytoplasm of HBV infected hepatocytes (Gowans et al, 1983; this study; Blum et al, 1984a; Miller and Robinson, 1984) suggesting that viral DNA synthesis was ongoing in this site.

## 12. DELTA ANTIGEN.

An antigen serologically distinct from HBcAg and subsequently named Delta antigen (d-Ag) was first discovered in hepatocyte nuclei in HBsAg carriers by Rizzetto et al (1977). Since then, although nuclear localisation was shown to be prevalent, cytoplasmic localisation of d-Ag has also been described (Stocklin et al, 1981). The presence of intrahepatic d-Ag or serum d-Ag in HBsAg carriers is diagnostic of current d-agent infection.

Serum d-Ag copurified with a 36nm subpopulation of HBsAg particles which were also shown to contain a 1.7 kb RNA molecule (Bonino et al, 1984). This RNA had no poly-A tail and the particles had no reverse transcriptase activity. As was the case with HBcAg in HB virions, detergent disruption of the 36nm particle was necessary for d-Ag and d-RNA detection. However, a structure analogous to the HBcAg particle was not described. Complementary DNA made from d-RNA and cloned into

pBR322 showed no hybridisation with HBV DNA (Hoyer et al, 1983), but the d-cDNA sequence was not published and direct comparisons were not possible.

d-agent infection has only been described in HBV infected patients or in experimentally infected chimpanzee HBV carriers. Inoculation experiments with HBV carrier and HBV-immune chimpanzees demonstrated that d-agent is a defective RNA virus which requires coinfection with HBV for its replication and expression (Rizzetto, 1983). The 36nm d-associated particles contain d-Ag and d-RNA enveloped by HBsAg, although WHV may provide a similar helper function, in which case, the d-associated antigen is enveloped in WHsAg (Ponzetto et al, 1984a).

As a result of d-agent dependency on HBV, expression of d-Ag is limited temporally to the period of HBsAg expression in HBV infected patients. Thus, in patients who were simultaneously infected with HBV and d-agent, d-Ag was only expressed transiently and the acute illness was similar to that seen in patients with uncomplicated acute hepatitis B, although an increase in fulminant hepatitis was reported (Rizzetto, 1983). However, patients who were already persistently infected with HBV and were superinfected with d-agent continued to express d-Ag indefinitely. Superinfection with d-agent in asymptomatic HBV carriers (in contrast to simultaneous infection with both agents) often resulted in an initial acute hepatitis, with a decrease in the expression of HBV gene

products, followed by a severe hepatitis (Govindarajan et al, 1983). It has been suggested that d-agent has a direct cytopathic effect in contrast to the immune mediated effect seen with HBV (Popper et al, 1983).

The diagnosis of d-agent has permitted precise classification of a group of patients who were previously considered to have a non-A, non-B hepatitis superinfection.

### 13. MECHANISMS OF HEPATOCYTE INJURY.

At one time or another, many of the theoretically possible mechanisms shown in Table 1.6 have been considered in the context of the hepatocyte injury seen in hepatitis B infection. In acute hepatitis B infection, viral replication and antigen expression occur several weeks before biochemical evidence of hepatocyte damage. This is generally taken to mean that HBV is a non-cytopathic virus. However, in persistent infection, a general correlation between continuing viral replication and chronic hepatitis has recently become evident (Alberti et al, 1984; Mondelli et al, 1984). In view of the lack of a universal association between virus replication and liver injury it has been suggested that the development of a host immune response may be responsible for hepatocyte damage (Dudley et al, 1972). Recent evidence supporting this hypothesis was presented by Mondelli et al (1982) and Naumov et al (1984), who showed in vitro that HBcAg was the target of



TABLE 1.6

Possible mechanisms for hepatocyte injury.

- 1) Direct cytopathic effect of infecting virus
- 2) Immunological
  - a) effector mechanisms
    - anaphylactic (type 1)
    - antibody dependent cytolysis (type 2)
    - immune complex (type 3)
    - T cell mediated (type 4)
  - b) origin of abnormal immune reactions

<u>antigen-based</u>	<u>immune response-based</u>
virus coded antigens	- cross reacting determinants on viral and host antigens
host coded - virus induced antigens - previously masked	- reduced suppressor cell activity
	- antibody to idiootype on anti-viral antibody reacting with virus-specific receptors on cells
	- polyclonal B cell activation
	- other immunoregulatory molecules
- 3) Metabolic, hormonal etc effects as a result of impairment of higher functions normally performed by hepatocytes eg. detoxification.

a cytotoxic T-lymphocyte reaction. Membraneous and cytoplasmic HBcAg detected in a majority of hepatocytes which expressed HBcAg (Gowans and Burrell, 1985; this study) could provide the target for a similar in vivo reaction.

Nevertheless, in a proportion of persistently infected patients lacking markers of HBV replication, chronic hepatitis is evident. This is interpreted as a host immune response directed towards novel virus or cellular antigens exposed on the cell membrane (Thomas et al, 1984; this study). Antibodies to liver cell membranes have been detected in the serum of patients with chronic hepatitis (Hopf et al, 1976).

The in vivo effects of immune regulatory molecules are unknown, (Chisari, 1984) and the above in vitro observations may or may not operate in complex in vivo interactions.

#### 14. IN SITU HYBRIDISATION.

In the context of this thesis, in situ hybridisation is defined as hybridisation using a nucleic acid probe to locate a nucleic acid target in tissue sections. This technique, developed simultaneously by Gall and Pardue (1969) and John et al(1969) may also be called cytohybridisation and should not

be confused with the in situ hybridisation procedure developed by Grunstein and Hogness (1975) to detect recombinant plasmids in bacterial colonies in situ on a nitrocellulose membrane.

In situ hybridisation can provide details of intracellular location of viral nucleic acids, the distribution of infected cells within the tissue, a correlation with intracellular viral antigens and details of tissue histopathology. In contrast, Southern blot technology (Southern, 1975) examines DNA extracted from a heterogeneous cell population and provides information on the size of the viral nucleic acid detected and whether this is integrated into the host cell chromosome. The results therefore represent an average of a large number of cells. Thus, the two methods provide different but complementary information and are directly comparable with the detection of viral antigens in either intact or disrupted tissues and cells.

Most recent in situ hybridisation studies have used  $^3\text{H}$ -labelled probes (Haase et al, 1984) due to the excellent autoradiographic resolution and good signal:noise ratio afforded by this isotope. However, the short path length, which provides the high resolution, also results in low autoradiographic sensitivity and ultimately in long autoradiographic exposure times (Rogers, 1979). This time may be decreased by the use of  $^{125}\text{I}$ - or  $^{32}\text{P}$ -labelled probes. In the case of  $^{125}\text{I}$ , the autoradiographic signal is due solely to secondary B-emission and, since the average path length of

these emissions is similar to that of  $^3\text{H}$ , then  $^{125}\text{I}$  provides a similar resolution to  $^3\text{H}$ . The ultimate resolution depends on many related factors including isotope path length, tissue section thickness and the type of autoradiographic emulsion used, but for  $^3\text{H}$  and  $^{125}\text{I}$  is generally considered to be around 1-2  $\mu\text{m}$  (Rogers, 1979).  $^3\text{H}$  and  $^{125}\text{I}$  are therefore suitable for intracellular location studies. In contrast,  $^{32}\text{P}$  which has a long path length may provide higher sensitivity at the expense of poor resolution (Rogers, 1979).

The general principles of nucleic acid hybridisation apply to in situ hybridisation and the majority of reports of in situ reactions to detect viral nucleic acids have used nick translated ds DNA probes (Haase et al, 1984) which are denatured before use. Nick translated probes fulfil the requirements of high specific activity and lengths compatible with good access to tissue nucleic acids (Haase et al, 1984).

Nick translation was considered to be the most convenient, practical method of probe preparation at the time this work was commenced, although suitable alternatives, viz, M13 probe systems, synthetic probes (Richman et al, 1984) and RNA probes produced from plasmids with an SP6 RNA polymerase promoter site (Cox et al, 1984) are now generally available.

## 15. AIMS OF THESIS.

Much of the current knowledge and understanding of HBV described in this chapter accumulated during the past four years (1982-85). Therefore, it was considered appropriate to describe briefly the level of understanding of the area of interest prior to commencement of the study.

First, although all three were known, none of the animal models of HBV was well characterised. In particular, the sequence and organisation of the genomes were unknown and the elegant model for hepadnavirus replication proposed by Summers and Mason (1982) was still unpublished. Furthermore, the distribution of viral antigens in tissues infected with the animal hepadnaviruses was unknown.

Second, immunofluorescence or immunoperoxidase studies of the HBV structural antigens using assay methods available at the time, located HBsAg in the cytoplasm and HBcAg in the nucleus of infected cells. These observations, in the absence of any evidence concerning the intracellular site of HBV DNA synthesis, suggested that HBV behaved as other small DNA viruses and was therefore most likely to replicate in the infected cell nucleus. However, examination of HBV infected livers by in situ hybridisation had demonstrated viral DNA in the cytoplasm of infected hepatocytes (Gowans et al, 1981).

The significance of this report, which was the first application of in situ hybridisation to the study of HBV, was unclear.

Finally, little data were available on the molecular mechanisms of virus persistence and the relationship between virus replication and cell injury.

The aims were: 1. to study virus replication in as much detail as possible within the constraints of the chronically infected human liver model, using in situ hybridisation and viral antigen detection. 2. to correlate virus replication, viral genome expression and hepatocyte injury in natural infection.

**CHAPTER 2****MATERIALS AND METHODS****1. TISSUE COLLECTION AND STORAGE.**

A number of autopsy and biopsy human liver samples were examined. Autopsy samples were removed as soon as possible after death, cut into 1-2cm slices, frozen quickly and stored frozen at  $-70^{\circ}\text{C}$  until required. Within minutes of sampling, biopsy samples were divided into three parts; one part was embedded in OCT (Miles Scientific), snap frozen by immersion in liquid nitrogen and stored frozen at  $-70^{\circ}\text{C}$ . The second and third parts were fixed in ethanol:acetic acid and formalin respectively, and embedded in paraffin wax.

Fixation in fresh ethanol:acetic acid (3:1) was carried out for 20 minutes at room temperature (RT). The specimens were then stored in 70% ethanol (normally approximately 1 hour but occasionally overnight) and processed into paraffin wax. Alternatively, samples were fixed in 10% formalin in phosphate buffer for 24 hours at RT before paraffin embedding. The paraffin blocks were stored at RT.

**2. PREPARATION OF MICROSCOPE SLIDES AND COVERSGLIPS.**

Slides and coverslips were soaked overnight in chromic acid, rinsed in running tap water for several hours and rinsed three times in distilled water. Slides for use in

immunofluorescence, immunoperoxidase or in situ hybridisation experiments were then dipped into 0.5% gelatin, 0.5% chrome alum, (Rogers, 1979) drained and air dried in a dust free atmosphere. Slides prepared in this manner provided (a) low background for in situ hybridisation experiments; (b) good adhesion of emulsion during autoradiography; (c) good retention of frozen or fixed tissue sections. 13mm diameter coverslips used for in situ hybridisation were dried after washing, dipped into silicone solution (Ajax Chemicals), rinsed in distilled water and air dried. The coverslips were individually polished with microscope lens tissue and sterilised before use.

### **3. HBV ANTIGEN DETECTION IN LIVERS.**

#### **a. Immunofluorescence.**

Frozen sections (6µm) of livers were air dried and used either unfixed or fixed in carbon tetrachloride at 4°C for 10 minutes or in ethanol:acetic acid (3:1) for 20 minutes at RT and again air dried. The sections were washed once in PBS for 5 minutes prior to immunofluorescence (IF) staining and after each step in the reactions. Sections were stained for 1 hour at 37°C in the primary antibody and 30 minutes in the secondary antibody, or for 1 hour in the case of direct conjugates. After staining, the sections were mounted in 90% glycerol saline, 50mM Tris-HCl, pH 8.6 and examined in microscopes fitted with FITC-specific Ploem illumination.



HBsAg was detected by an indirect IF reaction using rabbit anti-HBs (Behringwerke) as the primary antibody. HBeAg was detected by a direct reaction using a fluorescein isocyanate (FITC) conjugated human anti-HBe or by an indirect reaction using rabbit anti-HBe (Dako). d-Ag was detected by a direct assay using FITC conjugated human anti-d. Primary antibodies in indirect assays were followed by an anti-rabbit IgG FITC conjugate (Wellcome). All reagents were adsorbed against a normal human liver homogenate (see below for method) and the anti-rabbit FITC conjugates were further adsorbed against a preparation of 3% normal human globulin to abolish non specific reactions against normal liver components or to human IgG bound to cells in infected livers respectively. All reagents were titrated and used at optimal dilutions. This was shown to be 1/30 for the anti-HBs, 1/200 for the anti-HBe and 1/8 for the sheep anti-rabbit FITC conjugate. The anti-d FITC conjugate was also used at 1/200. All dilutions were made in PBS.

**b. Immunoperoxidase.**

HBsAg and HBeAg were also detected by the peroxidase-antiperoxidase (PAP) method in sections cut from fixed tissue blocks. HBeAg was detected initially using a HBeAg PAP detection kit (Dako) used as recommended by the manufacturer. Individual reagents purchased from the same source were used later. HBsAg was detected by substitution of the rabbit anti-HBe with rabbit anti-HBs. A schematic diagram of the PAP method is shown in Figure 2.1.

FIGURE 2.1

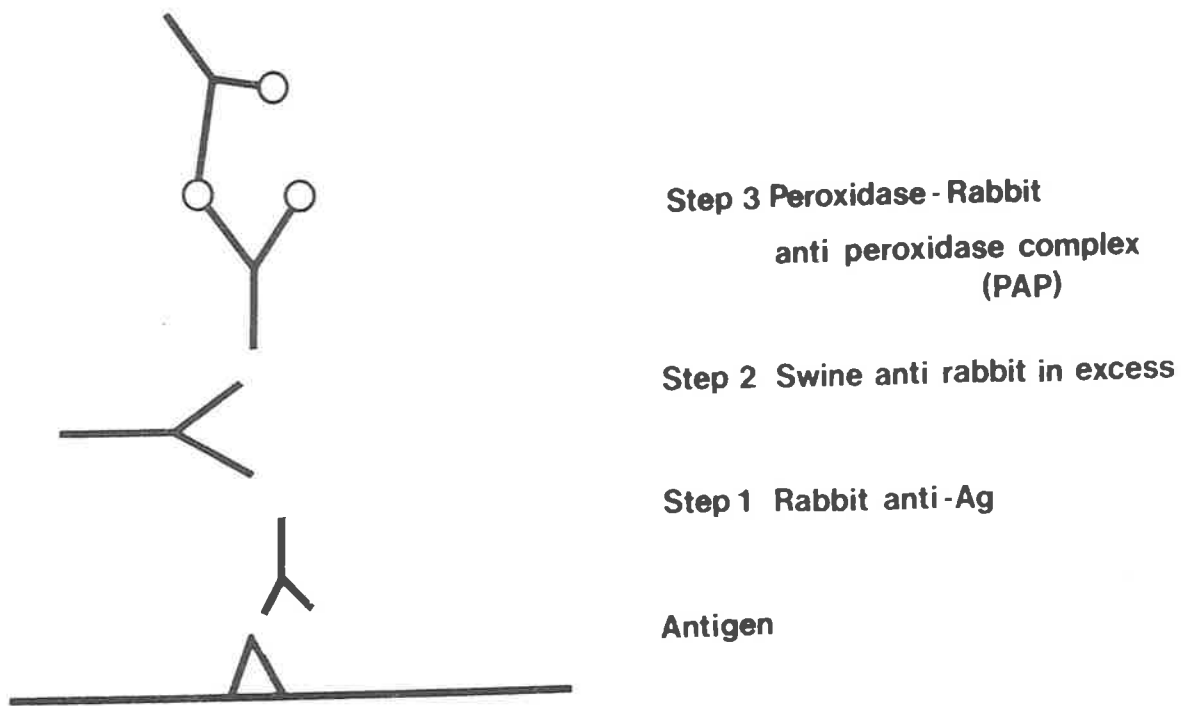


FIGURE 2.1 A schematic diagram of the peroxidase antiperoxidase (PAP) reaction.

Sections of fixed tissue blocks were dewaxed in xylene for 2 x 10 minutes and rehydrated to PBS. Endogenous peroxidase was destroyed by incubating the sections for 10 minutes at RT in 0.1% hydrogen peroxide before washing for 2 x 5 minutes in PBS. The sections were then incubated in normal swine serum diluted 1/20 for 20 minutes at RT and this reagent replaced (without any washing step) with the primary antibody. Absorbed anti-HBs was used at 1/30 as for IF while the anti-HBc was used at 1/200 without adsorption. The specificity of this reaction was ensured by prior treatment of the sections with normal swine serum and by the use of a high dilution of anti-HBc serum. The primary antibodies were incubated for 30 minutes at RT, the sections washed in PBS and incubated in swine anti-rabbit diluted 1/20 for a further 30 minutes at RT. The sections were again washed and incubated in PAP reagent diluted 1/50 for 30 minutes at RT, washed in PBS and reacted with the substrate. To increase the reaction sensitivity, for some experiments the time of the primary anti-HBs or anti-HBc incubation was increased to 1 hour at 37°C followed by 4°C overnight and the time of the PAP step was also increased to 1 hour at RT.

The substrate to detect bound PAP reagent supplied with the Dako PAP kit (aminoethyl carbazole) was used initially, but the reaction product was shown to fade with time and was consequently discarded in favour of diaminobenzidine (DAB; British Drug Houses), which produced a permanent reddish-brown reaction product. The substrate was prepared by diluting

0.5ml DAB (25mg/ml) in 100mM Tris-HCl, pH 7.6 into 25ml of 100mM Tris-HCl, pH 7.6 and adding 25ul of 30% hydrogen peroxide. This yielded a substrate solution of 500ug/ml DAB, 0.03% hydrogen peroxide as described by Graham and Karnovsky (1966).

**c. Preparation of anti-d FITC conjugate.**

A serum, positive for HBsAg and anti-d, but with a low titre of anti-HBc and negative for anti-HBs was conjugated to FITC as described by Nairn, (1976). 3ml of serum was clarified by centrifugation at 5000rpm for 15 minutes and the immunoglobulin precipitated by the dropwise addition of saturated ammonium sulphate to a final concentration of 33%. The mixture was stirred slowly at RT for 30 minutes, the precipitated globulins pelleted at 3000rpm for 10 minutes, resuspended in 1.5ml of PBS and dialysed against PBS at 4°C overnight.

The protein concentration was measured by optical density and adjusted to 2g/100ml. 0.5ml of 0.2M Na<sub>2</sub>HPO<sub>4</sub>, pH 9.0 was then added with constant stirring, followed by 1ml of 0.1M Na<sub>2</sub>HPO<sub>4</sub> containing 500ug FITC (British Drug Houses). The pH was adjusted to pH9.5 with 0.1M Na<sub>3</sub>PO<sub>4</sub>, the total volume adjusted to 4ml with 0.15M NaCl and the reaction allowed to proceed for 30 minutes at RT. The reaction vessel was then cooled rapidly to 4°C and any precipitate removed by centrifugation.

The conjugate was dialysed against PBS at 4°C with several changes for 48 hours to remove free FITC and then adsorbed against normal human liver to remove non specific reactants. The conjugate was tested against several infected and control livers, described in Chapter 7.

A direct anti-HBc conjugate prepared as above from a human serum with a high anti-HBc titre (Gowans, 1981) was used in some experiments to detect HBcAg.

**d. Specificity of immunofluorescence or immunoperoxidase reactions.**

All immunofluorescence or immunoperoxidase reactions were shown to be specific by examining control sections of normal liver, and by substitution of the primary antibodies with normal rabbit serum in indirect or multiple layer assays.

**e. Preparation of normal liver homogenate.**

An autopsy liver from a patient serologically negative for all HBV markers was used as the source. The liver was stored frozen at -70°C. While still frozen, a round 'surform' file was used to file the surface of the frozen liver and the 'shavings' collected into ice cold PBS. The homogenate was washed free of blood, the cell debris frozen and thawed twice and washed a further two times. The pellet was then stored at -20°C as packed 1ml or 2ml volumes until required and washed twice in PBS immediately before use.

Adsorption of reagents was performed by adding an equal volume of homogenate to the required reagent at 2x working dilution and the mixture incubated at 37°C for 1 hour followed by 4°C overnight. The homogenate was then removed by centrifugation at 4°C at 10.000rpm for 10 minutes, the adsorbed reagent sterilised by filtration and retitrated before use. All adsorbed reagents were stored in aliquots at -20°C at the final working dilution.

#### 4. SOURCE OF HBV DNA SEQUENCES.

##### a. pHBV114.

pHBV114 cloned by Burrell et al, (1979) was received as purified DNA from Professor K. Murray, (University of Edinburgh). In some experiments, radiolabelled fragments of this plasmid corresponding to individual HBV genes were used as gene probes (Chapter 5). These fragments were prepared by Allison Jilbert from nick translated total pHBV114 DNA as follows: the plasmid was digested with restriction enzymes (see Figure 5.2 ), the fragments separated by gel electrophoresis and the bands stained by ethidium bromide. The HBcAg and HBsAg gene probe fragments were collected by continued electrophoresis into hydroxylapatite (HAP:Biorad)-filled wells in the agarose immediately to the anode side of the respective bands. The DNA was subsequently eluted from the HAP using high molarity phosphate, passed over a Sephadex G-100 (Pharmacia) column and concentrated by freeze drying.

**b. pHBVCB.**

Plasmid pHBVCB (Burrell et al, 1979) was received in a stab culture of E.coli JM101 from Professor K. Murray. This was plated onto nutrient agar plates containing 20ug/ml tetracycline (Lederle Laboratories) and cultured overnight at 37°C. A single colony was picked and used as the inoculum for an overnight culture of nutrient broth. 50ml of this overnight culture was then used to inoculate 300ml of LB medium and the culture incubated with shaking at 37°C for 3.5 hours. Chloramphenicol (Parke Davis) was then added to a final concentration of 150ug/ml and the culture incubated overnight at 37°C with shaking (chloramphenicol was stored at -20°C in ethanol at a concentration of 50mg/ml).

The culture fluid was then centrifuged at 1000g and the pellet of bacteria resuspended in 20ml of 25mM Tris-HCl pH 7.6, 10mM EDTA, 15% sucrose and 2mg/ml lysozyme. This solution was stored on ice for 40 minutes and NaOH and SDS added to a final concentration of 0.132M and 0.1% respectively. The solution was mixed gently by inversion and stored on ice for 10 minutes. 3M sodium acetate pH 4.6 (adjusted with concentrated HCl) was added to a final concentration of 0.2M, the preparation was stored on ice for 40 minutes and then centrifuged at 20,000g for 15 minutes. The supernatant was carefully removed, RNAase A (Boehringer), previously boiled to destroy contaminating DNAase, was added to a final concentration of 2ug/ml and the preparation incubated at 37°C for 2 hours. The preparation was then extracted twice with

phenol:chloroform:isoamyl alcohol (25:24:1), the DNA was ethanol precipitated at  $-70^{\circ}\text{C}$  overnight, dried and resuspended in a minimal volume of distilled water. Sodium chloride was added to 0.4M and polyethylene glycol (PEG) 6000 (Pharmacia) to 6.5%, the preparation stored on ice for 1 hour and then centrifuged at 10,000rpm for 10 minutes. The supernatant was removed. the DNA pellet washed in 70% ethanol, dried, dissolved in DDW and stored at  $-20^{\circ}\text{C}$  after optical density (OD) evaluation of purity. The OD 260/280 ratio was 1.86.

**c. Excision of HBV insert from pHBVCB.**

Plasmid pHBVCB was digested with 1 unit of PstI (Boehringer) per  $\mu\text{g}$  plasmid DNA at  $37^{\circ}\text{C}$  overnight.  $\lambda$  DNA was similarly digested in order to check the reaction efficiency. Loading buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 3% Ficoll 400, 0.4% Bromophenol blue) was added and the digested DNA separated by electrophoresis in a 1% agarose gel in TAE for 3 hours at 150mA. The DNA bands were identified by staining the gel in ethidium bromide and the required band cut from the gel.

The excised gel strip was dialysed overnight against 0.5 x TBE in a dialysis bag containing a minimal volume of 0.5 x TBE. The dialysis bag was then placed at right angles to the current and the DNA eluted from the gel by electrophoresis at 100mA for 2 hours. The current was then reversed for 2 minutes and the eluted fragment recovered from the dialysis tube. The preparation was butanol extracted to concentrate the DNA, phenol extracted, ether extracted twice, and ethanol



precipitated. The DNA was freeze dried, resuspended in DDW and evaluated by optical density. The OD 260/280 ratio was 1.5.

Examination of the restriction enzyme map of pHBVCB shown in Fig. 2.2 (Gough and Murray, 1982), shows that digestion with Pst 1 should yield 3 fragments, a 4362bp fragment corresponding to pBR322, and 2 HBV derived fragments; a large fragment of ca.3000bp and a smaller fragment of ca.350bp. Since pHBVCB contains longer than unit genome length of HBV DNA (105%: Gough and Murray, 1982) the sum of these large and small excised fragments exceeds 3200bp (HBV genome size). Gel electrophoresis after Pst 1 digestion of pHBVCB as described above showed the expected results. The excised (large) fragment was used in experiments described in Chapters 5 and 6.

#### **d. M13 Bacteriophage.**

##### **Introduction**

Phage M13 is a single stranded DNA bacterial virus of 6407 bases. Artificial restriction enzyme cloning sites have been genetically engineered and inserted into the wild type virus to produce a number of different cloning vehicles, e.g., M13mp9 etc. During the M13 virus replication cycle, dsDNA molecules arise as replicative intermediates and these intermediates may be used as a cloning vector. Upon completion of virus replication, the ss virus genome is packaged into the M13 virion. This ssDNA is termed the plus

FIGURE 2.2

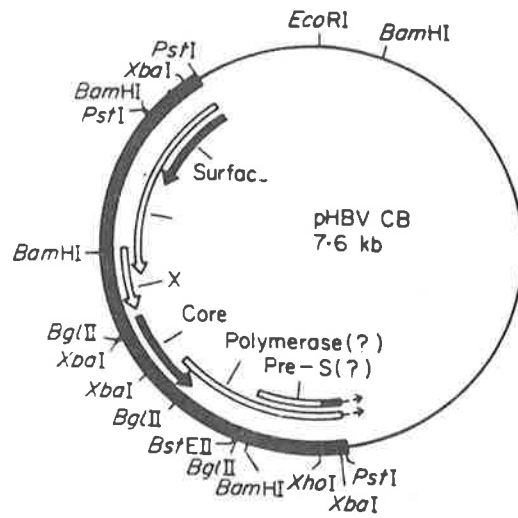


Figure 2.2: A restriction endonuclease map of pHBVCB which shows the Pst 1 sites used to excise the HBV insert. This map is printed with permission from Dr N. Gough.

strand, since it has the same polarity as mRNA. Foreign DNA inserted into any of the cloning sites is replicated with the M13 DNA. This is, in turn, termed plus or minus strand, depending on the initial orientation that occurred during cloning. Foreign DNA inserted into M13 may then be used as strand specific probes (Hu and Messing, 1982).

M13 clones containing recombinant DNA may be used either in single- or double-stranded form to transfect suitable E.coli host cells. The host cell E.coli strain JM101 has had the beta galactosidase gene removed, but contains an episome with a defective beta galactosidase gene. M13 cloning vehicles contain the remaining portion of the beta galactosidase gene and when M13 cloning vehicles infect JM101 the two defective beta galactosidase genes complement each other to produce a functional beta galactosidase gene product. This is identified by adding a lactose analogue, 5-bromo-4-chloro-3-indoyl-beta galactoside (BCIG), to the medium. This colourless compound is hydrolysed by beta-galactosidase to produce a blue dye, bromo-chloroindole (Miller, 1972).

In contrast, recombinant M13 molecules do not complement the defective episomal beta galactosidase gene because the foreign (cloned) DNA has been inserted into the incomplete M13 beta galactosidase gene and subsequent complementation is impossible. In this case, when an M13 cloning vehicle containing an inserted foreign DNA replicates in JM101, the

resultant plaque is clear. This clear plaque is picked, reinoculated into fresh JM101 in liquid culture and newly replicated M13 phage particles containing the recombinant DNA molecule are exported into the medium. After phenol extraction and ethanol precipitation, the DNA may be radiolabelled for use as a probe.

**e. Transfection and M13 replication.**

Cloned M13 DNA samples containing inserted HBV DNA sequences (received from Dr N. Gough) were used to transfect E.coli strain JM101. A single colony of JM101 was used to inoculate 20ml of 2 x TY medium and incubated at 37°C for 5 hours. The bacteria were centrifuged gently to a pellet, washed in 10ml of ice cold 50mM CaCl<sub>2</sub>, resuspended in fresh 50mM CaCl<sub>2</sub> and stored on ice for 30 minutes.

An aliquot of recombinant phage M13 DNA was added (determined empirically to produce a finite number of plaques per plate -- see below) to 200ul of the above cells and kept on ice for 40 minutes before incubation at 42°C for 2 minutes. During this two minutes, 30ul of 20mg/ml BCIG in dimethylformamide, 20ul of 24mg/ml isopropyl-B-D thio-galactopyranoside (IPTG) in DDW and 200ul of a log phase culture of JM101 were added to 3ml of 2 x TY medium containing 0.7% agarose at 42°C. IPTG was added to induce the complemented functional B-galactosidase gene. This mixture was added to the transfected JM101 cell solution and plated out by flooding on minimal agar. When the agarose solidified, the petri dish was incubated at 37°C overnight.

An isolated clear plaque from each plate was picked and inoculated into a 3ml volume of 2 x TY medium containing JM101 (this was prepared immediately before by picking 1 colony of JM101 from a minimal agar plate). The 3ml culture containing the clear plaque was incubated at 37°C for 6 hours, then centrifuged at 1000rpm to deposit the bacterial cells.

2.5M sodium chloride and 20% PEG 6000 were then added to the supernatant to a final concentration of 0.5M and 4% respectively, the mixture incubated at RT for 15 minutes and centrifuged at 4000rpm for 15 minutes. The M13 phage pellet was resuspended in 200ul of 10mM Tris-HCl pH 7.8, 0.1mM EDTA, 200ul phenol added, vortexed for ca. 10 seconds and incubated at RT for 15 minutes. This extended phenol extraction was required to remove the M13 capsid protein from the phage ss DNA. The aqueous layer was removed, ether extracted, ethanol precipitated and the dried DNA redissolved in 10mM Tris-HCl pH 7.8, 0.1mM EDTA. The OD 260:280 ratio for M13 DNA was shown to be 2.0.

## **5. PROBE PREPARATION.**

### **a. Nick translation.**

Excised HBV DNA from pHBVCB, intact pHBV plasmids and a pBR322 (Boehringer) control, were radiolabelled by nick translation. 60ng of DNA was labelled in a 20ul reaction mixture containing 120mM Tris-HCl pH 7.5, 10mM MgCl<sub>2</sub>, 10mM dithiothreitol (DTT:Sigma), 20ng/ml DNAase I (Boehringer), 200uM each dATP,

dGTP, dTTP (Boehringer), 1.6 $\mu$ M  $^{125}$ I-dCTP (1800 Ci/mmol, Amersham) or an equivalent molar concentration of  $^{32}$ P-dCTP or  $^3$ H-dNTP and 10-15 units of DNA polymerase (Kornberg enzyme; Boehringer). In order to increase the specific activity of  $^3$ H-labelled probes, these were prepared using dCTP, dTTP and dATP labelled precursors. Although 60ng of DNA in 20ul contained only 2.3 $\mu$ M of each dNTP it was found that the reaction rate was faster if the concentration of unlabelled dNTP's was in vast excess.

Reactions were carried out at 14 $^{\circ}$ C for 1-3 hours or until incorporation of isotope into TCA precipitable material ceased. The reaction was stopped by the addition of EDTA and Sarkosyl (Ciba-Geigy) to 30mM and 2.5% respectively and the labelled probe separated from free dNTP's by gel filtration through a 1ml Sepharose G-50 column (Pharmacia) equilibrated with distilled water. Probe fractions from the first peak were pooled, 60ug of sheared salmon sperm DNA added as carrier, butanol extracted to ca. 400ul if necessary, phenol extracted, ether extracted twice, ethanol precipitated and washed in 70% alcohol. The DNA precipitate was dried, redissolved in 20ul DDW, and stored at -20 $^{\circ}$ C until required. This preparation is termed "stock" probe.

Using the above protocol, it was found that pBR322 consistently labelled to a higher specific activity than the excised HBV fragment. This was probably as a result of electrophoresis of the excised HBV DNA through agarose.

Consequently, in order to prepare pBR322 probes with similar specific activity as controls for the excised HBV fragment probe, nick translations of pBR322 contained one half of the labeled dNTP concentrations described above. Probe specific activities ranged from  $2-4 \times 10^8$  dpm/ug with all isotopes used.

**b. M13 probe labelling.**

Single-stranded recombinant M13 probes may be radiolabelled by initially forming specific small ds regions using specific primers that hybridise to M13 either upstream or downstream of the inserted sequence. The ds regions are then extended using the Klenow fragment of DNA polymerase. Probes formed by these procedures will have, respectively, either an ss insert with a ds M13 tail, or a ds insert region with a continuing ds M13 tail. In each case, only the newly synthesised cDNA strand is radiolabelled. Figure 2.3 shows possible labelling strategies for recombinant M13 molecules.

Recombinant bacteriophages 2.1 and 2.11 containing HBV sequences cloned into the Bam HI site of M13 mp9 (prepared by Dr. N. Gough) were labelled using the pentadecamer sequencing primer No. 403-2 (Biolabs), as shown in Fig. 2.3. 500ng ss DNA of M13 clones 2.1 and 2.11 in 10ul of 0.1M Tris-HCl pH 7.6, 0.5M NaCl, 0.1M MgCl<sub>2</sub> and 0.14M DTT containing 10ng of primer was heated to 70°C for 5 minutes, cooled and incubated at 37°C for 5 minutes and allowed to return to RT for 15 minutes. This solution was then transferred to another Eppendorf tube with 4ul of <sup>32</sup>P-dCTP (1800 Ci/mmol:Bresa) that

FIGURE 2.3

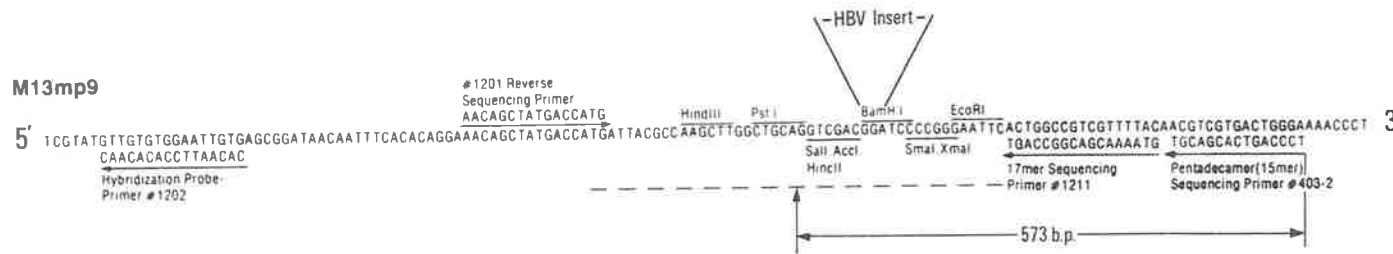


Figure 2.3: A diagram showing possible labelling strategies for M13 plasmids and the insertion site of HBV probes 2.1 and 2.11 into M13mp9.



was previously dried. 1ul of an aqueous solution of unlabelled dATP, dTTP and dGTP was added to a final concentration of 40uM and 1ul (1 unit) of Klenow large fragment DNA polymerase (Boehringer) added. The reaction was incubated at 41°C for 10 minutes, unlabelled dCTP added to 40uM and incubated at 41°C for a further 10 minutes. The reaction was stopped by cooling rapidly on ice, the probes phenol extracted, ether extracted and ethanol precipitated.

The dried DNA was redissolved in 20ul DDW and digested with 3 units of Pst 1 (Amersham)/lug DNA at 37°C overnight (Fig. 2.3), re-extracted with phenol, then ether and ethanol precipitated before use.

## 6. IN SITU HYBRIDISATION.

### a. Tissue preparation.

#### i. Frozen section fixation

Tissues were stored as described in Part 1 of this chapter. 6um frozen sections were cut, air dried and fixed in one of two ways:

- (1) by a method designed to denature ds DNA, or
- (2) under non-denaturing conditions designed to detect only DNA that was ss in its native state.

ii. Denaturation protocol: frozen sections.

Freshly cut frozen sections were air dried for 10-15 minutes, fixed in freshly prepared ethanol:acetic acid (3:1) for 20 minutes at room temperature, hydrated to 0.1 x SSC and heated to 100°C for 3 minutes in 0.1 x SSC. The sections were transferred to cold 0.1 x SSC, fixed in 0.1% glutaraldehyde in PBS at 4°C for 30 minutes, rinsed in PBS and dehydrated. The sections were then treated with 1 µg/ml Proteinase K in 30mM Tris-HCl pH 7.4, 2mM CaCl<sub>2</sub> for 20 minutes at 37°C, washed in PBS, digested in DNAase or RNAase (as described in part 6d below) for 1 hour at 37°C before further Proteinase K digestion as above. The sections were then immersed in 0.25% acetic anhydride in 0.1M triethanolamine buffer, final pH 7.2 (Hayashi et al, 1978) for 10 minutes at RT, washed in 0.1 x SSC for 2 x 5 minutes and dehydrated. The results of in situ hybridisation experiments on tissue sections fixed in this manner are described in Chapter 7.

iii. Non-denaturing protocol: frozen sections.

Air dried frozen sections were fixed in 0.1% glutaraldehyde in PBS at 4°C for 30 minutes, rinsed in PBS and dehydrated. The sections were then treated in 1 µg/ml Proteinase K as above then washed in 0.1x SSC for 15 minutes. Selected sections were then treated in DNAase, RNAase or S<sub>1</sub> nuclease and rinsed in 0.1x SSC for 3 minutes. All sections were then washed in cold 0.1x SSC, re-digested with Proteinase K, further washed in 0.1x SSC, treated with acetic anhydride and washed as described above before dehydration.

In certain experiments (Chapter 5), a direct comparison was made between denatured and undenatured sections. In these cases, a boiling step in 0.1x SSC for 3 minutes after nuclease digestions was inserted into the above non denaturing protocol.

iv. In situ hybridisation to paraffin wax embedded tissues.

Sections were dried at 37°C overnight, dewaxed in xylene and hydrated to PBS. The sections were then fixed in 0.1% glutaraldehyde at 4°C for 30 minutes, washed in PBS for 2 periods of 5 minutes, digested in 30ug/ml auto digested pronase (see part 6e, below) in 50mM Tris-HCl pH 7.6, 5mM EDTA at 37°C for 15 minutes and washed in 2mg/ml glycine, 50mM Tris-HCl pH 7.6 at RT for 5 minutes (Brigati et al, 1983). The sections were washed briefly in 0.1x SSC, denatured in 20% deionised formamide (part 6b, below), 0.1x SSC, 50mM Tris-HCl pH 7.6 at 70°C for 10 minutes then plunged into cold 0.1x SSC before dehydration and air drying. The sections were dried at RT for up to 1 hour and fixed in ethanol:acetic acid (3:1) at RT for 20 minutes, rinsed in 70% ethanol and hydrated to 0.1x SSC. The sections were then treated with DNAase or RNAase (part 6d, below), washed in 0.1x SSC, further digested in pronase as above for 5 minutes, washed in Tris/glycine, rinsed in 0.1x SSC and further fixed in 0.1% glutaraldehyde as above. They were then treated in acetic anhydride as described above, washed in 0.1x SSC, dehydrated and dried ready for in situ hybridisation.

A summary of all three in situ hybridisation fixation methods is shown in Figure 2.4.

**b. Probe mixture assembly.**

In situ hybridisation was performed in 6x SSC, 50% formamide, 50mM Tris-HCl pH 7.6, 10mM Na<sub>2</sub>HPO<sub>4</sub>, 10mM NaH<sub>2</sub>PO<sub>4</sub>, Denhardt's solution [0.02% Ficoll, 0.02% polyvinyl pyrrolidone (PVP), 1mg/ml BSA], 1mg/ml tRNA, 1mg/ml rRNA, 100ug/ml poly (A) and 100ug/ml sheared denatured salmon sperm DNA. In some experiments described in Chapter 4, the hybridisation mixture also contained dextran sulphate. <sup>125</sup>I probes also contained 2uM potassium iodide.

AR grade formamide (British Drug Houses or Merck) was deionised by the addition of Analytical Grade mixed bed resin [AG 501-X8 (D):Bio-Rad] and stirred at RT overnight. After filtration, fresh resin was added and the process continued until no decrease in the conductivity of the formamide was seen. Conductivity was measured by a conductivity meter (Radiometer:Copenhagen). Typical batches of formamide had a conductivity of 500umHo before treatment and a conductivity of 50umHo after treatment. Deionised formamide was stored at -20°C.

The above probe mixture was assembled by mixing the appropriate volumes of stock probe and formamide. This mixture (usually 70-80% formamide) was heated to 100°C for 5 minutes and cooled rapidly on ice. 20x SSC containing 4x

FIGURE 2.4

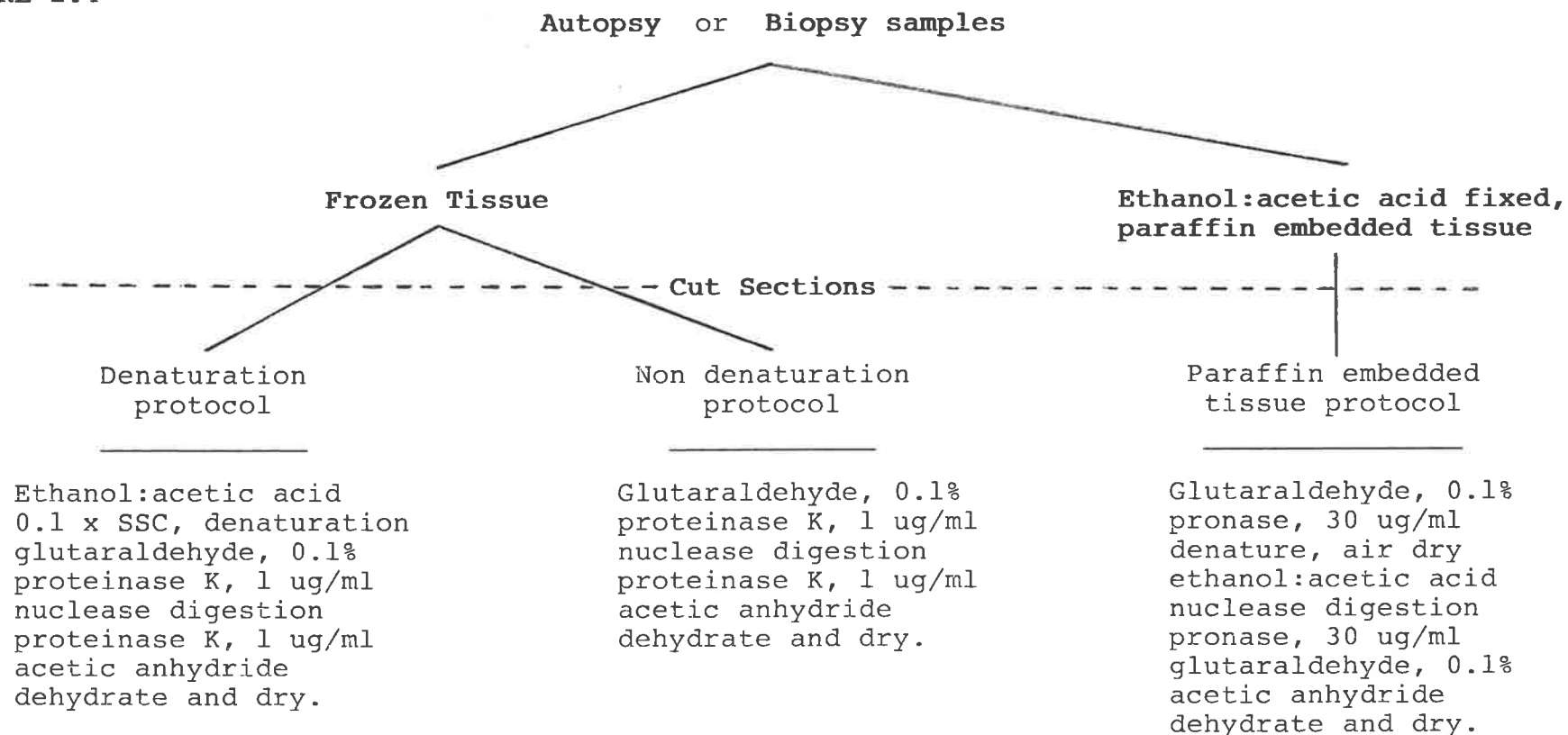


Figure 2.4: A flow chart of in situ hybridisation fixation methods.

Denhardt's solution was then added to a final concentration of 6x SSC, and 1/10 volume of 10x stock solutions of tRNA, rRNA (Boehringer) and poly(A) (Collaborative Research Inc.) were then added to the solution. If required, 50% dextran sulphate (Pharmacia) was added to a final concentration of 10%.

**c. In situ hybridisation and washing.**

2.5ul of hybridisation probe mixture was added to each tissue section, covered with a sterile siliconised 13mm diameter coverslip (Part 2, this Chapter) and hybridisation performed at 37°C for 18 hours under mineral oil to prevent evaporation of probe mixture. Early experiments used 2-4ng of complete plasmid (HBV + pBR322 sequences) per section but later experiments used 0.5ng of complete plasmid or 0.25ng of excised insert without any apparent decrease in sensitivity.

After hybridisation, the slides were removed from the mineral oil, washed for 2 x 10 minutes in chloroform to remove residual oil and air dried. The slides were then transferred to 4 litres of 2x SSC, where the coverslips fell off after a few minutes, and washed in 2x SSC for 3 x 1 hour, followed by 2 x 1 hour in 0.1x SSC. In some experiments, the slides were also washed in 0.1x SSC, 50mM Tris-HCl pH 7.2 at 45°C for 15 minutes or in 2x SSC/50% formamide at 70°C for 5 minutes as higher stringency or post-hybridisation melt (see below) washing steps respectively. Experiments performed with <sup>125</sup>I-labelled probes were washed as above but the washes also

contained 100mM potassium iodide (McAllister et al, 1983). The sections were then dehydrated and dried prior to autoradiography.

**d. Nuclease digestions.**

1. DNAase digestion:

DNAase 1, Grade 2 (Boehringer) was stored as a stock solution of 10mg/ml in DDW:glycerol (1:1) at  $-20^{\circ}\text{C}$ . For use, this solution was diluted to 100ug/ml in 50mM Tris-HCl pH 7.5, 7mM  $\text{MgCl}_2$  and sections incubated under a sterile coverslip at  $37^{\circ}\text{C}$  for 1 hour.

2. RNAase digestion:

RNAase A (Boehringer) was dissolved in 0.02M sodium acetate pH 5.0 to 3mg/ml, boiled for 3 minutes to destroy any contaminating DNAase and stored at  $-20^{\circ}\text{C}$ . For use, this stock solution was diluted to 300ug/ml in 2x SSC and the sections incubated at  $37^{\circ}\text{C}$  for 1 hour as above.

3. S<sub>1</sub> Nuclease:

S<sub>1</sub> nuclease (Sigma) was diluted to 1000U/ml in 50mM NaCl, 30mM Na acetate pH 4.6, 1mM  $\text{ZnSO}_4$ , 5% glycerol, 100ug/ml denatured salmon testis DNA (Sigma) and the sections were digested as above.

### e. Preparation and titration of pronase.

A 20mg/ml solution of pronase from Streptomyces griseus (Boehringer) was made in DDW and autodigested at 37°C for 2 hours. Dilutions of this stock (in 50mM Tris-HCl pH 7.6, 5mM EDTA) were tested on sections made from <sup>3</sup>H-thymidine labelled PLC/PRF/5 cells (MacNab et al, 1976) and on sections from a HBcAg positive liver, which was ethanol acetic acid fixed and paraffin embedded. PLC/PRF/5 cells were radiolabelled by culturing the cells in growth medium containing 5uCi/ml <sup>3</sup>H-thymidine (Amersham) for 36 hours. The cells were trypsinised, washed x 3 in PBS and the cell pellet made into a cell block by the addition of 2 drops of normal human serum followed by 1 drop of fibrin (Behringwerke) as described by Kaltenbach et al, (1973). The cell block was then fixed and embedded in paraffin wax as described in Part 1 above. Sections cut from this block were then used as the substrate to titrate the pronase in a mock in situ hybridisation fixation experiment. Likewise, the pronase was titrated against sections of the above liver and, in this case, hybridised with an HBV DNA probe (Chapter 4).

## 7. AUTORADIOGRAPHY.

### a. Emulsion preparation.

Ilford K2 emulsion (Ilford) was used throughout and procedures were performed under darkroom conditions recommended by Ilford. The emulsion was diluted 1:1 with DDW containing final concentrations of 300mM ammonium acetate (Brahic and



Haase, 1978) and 1% glycerol (Rogers 1979), and melted at 45°C for 30 minutes. The melted emulsion was then mixed carefully to avoid the formation of air bubbles. Microscope slides were dipped vertically into the liquid emulsion, drained for a few seconds, the back of the slide wiped clear of emulsion and the slide laid flat on a cold metal plate (Rogers, 1979) for 20 minutes. The slides were then removed from this plate and laid on the bench to dry at RT for 2 hours. The slides were then packed into light tight exposure boxes containing silica gel and stored at RT overnight to ensure complete drying. The silica gel was removed next day and the slides stored at 4°C without drying agent (as recommended by Ilford) for the duration of the exposure period. Complete drying was necessary to avoid latent image fading (Rogers, 1981).

**b. Development.**

The slides were brought to RT, developed in D19 (Kodak) for 5 minutes at RT and fixed in Ilford Hypam (diluted 1/4 in DDW) for 6 minutes. The slides were then washed for 5 minutes in 10% sodium sulphate, removed from the dark room and the sodium sulphate replaced slowly with running tap water. The slides were then washed for 5 minutes in running tap water and stained.

**c. Staining.**

Sections and cells were usually stained by haematoxylin and eosin (H&E), although some sections were stained by haematoxylin only or by Giemsa. It was noted that, in order

to totally retain silver grain size and number in the overlying photographic emulsion, a progressive haematoxylin staining method with a time of 1 minute was necessary. Longer staining times or the use of a regressive haematoxylin technique with the obligatory acid differentiation step resulted in etching of silver grains in the emulsion with complete loss of grains in extreme instances. After staining, slides were dehydrated and mounted in DPX (Gurr).

## 8. IN SITU HYBRIDISATION INTERPRETATION.

### a. Specificity.

The specificity of each in situ reaction was normally checked by several independent methods.

1. Experiments with radiolabelled pHBV probes were performed in parallel with a pBR322 probe of similar specific activity.
2. Duplicate sections were digested with DNAase or RNAase or in some instances,  $S_1$  nuclease before hybridisation.
3. Each experiment was performed in parallel with sections of normal human liver.
4. In a few experiments, after hybridisation, the sections were washed in 2x SSC/50% formamide at 70°C for 5 minutes. This melted any hybrids formed in situ and autoradiographic grains over cells in the section

reflected the level of background which was compared to the level of grains over cells considered to be positive in a duplicate section.

**b. Quantitation.**

Grain quantitation was performed by visually grading reactions on a + to ++++ scale or by reflected light photometry. Sections used in reflected light photometry were stained by Giemsa or haematoxylin only, as eosin caused autofluorescence when exposed to the incident light. Using a 60 x oil immersion objective and an illuminated field of 105um, the light photometer reading was corrected to zero on photographic emulsion away from the tissue section. The intensity of light reflected from silver grains over areas of tissue was then measured, since this has been shown to be proportional to the grain density (Rogers, 1979).

**9. PHOTOMICROGRAPHY.**

Most photomicrographs were taken using an Olympus microscope and camera system. However, the photomicrographs of HBcAg/HBV DNA correlation experiments in Chapter 6 were taken using a Zeiss standard microscope equipped with a Wild automatic camera.

The photomicrographs for the black and white figures were photographed using Ilford "Pan-Tech" film or Kodak "Tri-X" film for IF reactions. Kodak "Ektachrome" was used to photograph Figure 6.1 and the sequential HBcAg/HBV DNA reactions used to generate the data in Chapter 6.

Final print magnifications were calculated from the known film magnification (provided by the microscope manufacturer) and the known print magnification.

## CHAPTER 3

## DETECTION OF HBsAg and HBcAg IN FIXED TISSUES

## 1. INTRODUCTION.

The detection of HBsAg and HBcAg in liver tissue by immunostaining has resulted in a large number of publications concerning virus replication and histology e.g. Gerber and Thung, 1979; Gudat et al, 1975; Ray, 1979. In most instances, indirect IF or PAP was used to detect HBsAg but a direct conjugate was generally used to detect HBcAg, (Ray et al, 1976; Gudat et al, 1975), although some studies used human anti-HBc followed by an anti-human conjugate (Huang and Neurath, 1979; Yamada and Nakane, 1977) . HBcAg was detected with a direct assay using human antibody because high titre anti-HBc from an animal species was unavailable and because many HBV infected livers contain bound autologous IgG, which interferes with the interpretation of results obtained with human anti-HBc if an indirect reaction is used.

As a result of recombinant DNA technology, HBcAg was expressed in E.coli transfected with HBV DNA (Burrell et al, 1979; Stahl et al, 1982). This permitted the commercial production of high titre rabbit anti-HBc, which could be used in indirect IF or PAP reactions to detect HBcAg in sections of liver tissue. HBcAg from E.coli reacted with human anti-HBc similarly to

liver derived HBcAg, while the resultant rabbit anti-HBc showed a line of identity with human anti-HBc against HBcAg in an Ouchterlony gel diffusion test (Stahl et al, 1982).

The experiments described in this chapter were performed in order to compare the direct IF assay using a FITC conjugated human anti-HBc with an indirect assay using the newly available rabbit anti -HBc as the primary antibody to detect HBcAg in frozen sections. In addition, because no information was available, a comparison of the detection of HBcAg by PAP in fixed tissue with HBcAg detected by indirect IF in frozen sections was also undertaken. To complete the study, a similar comparison of the detection of HBsAg was also performed.

## **2. EXPERIMENTAL DESIGN.**

A detailed comparison of methods was performed on 5 autopsy liver samples and 10 biopsy samples were then examined using each method under optimised conditions. A summary of the methods used are shown in Figure 3.1.

## **3. RESULTS.**

### **a. Fixation of Frozen Sections.**

Although unfixed frozen sections showed very good staining reactions for HBsAg and HBcAg, histological detail was sometimes poor and the sections tended to detach from the slide. For these reasons, it was considered desirable to fix

FIGURE 3.1

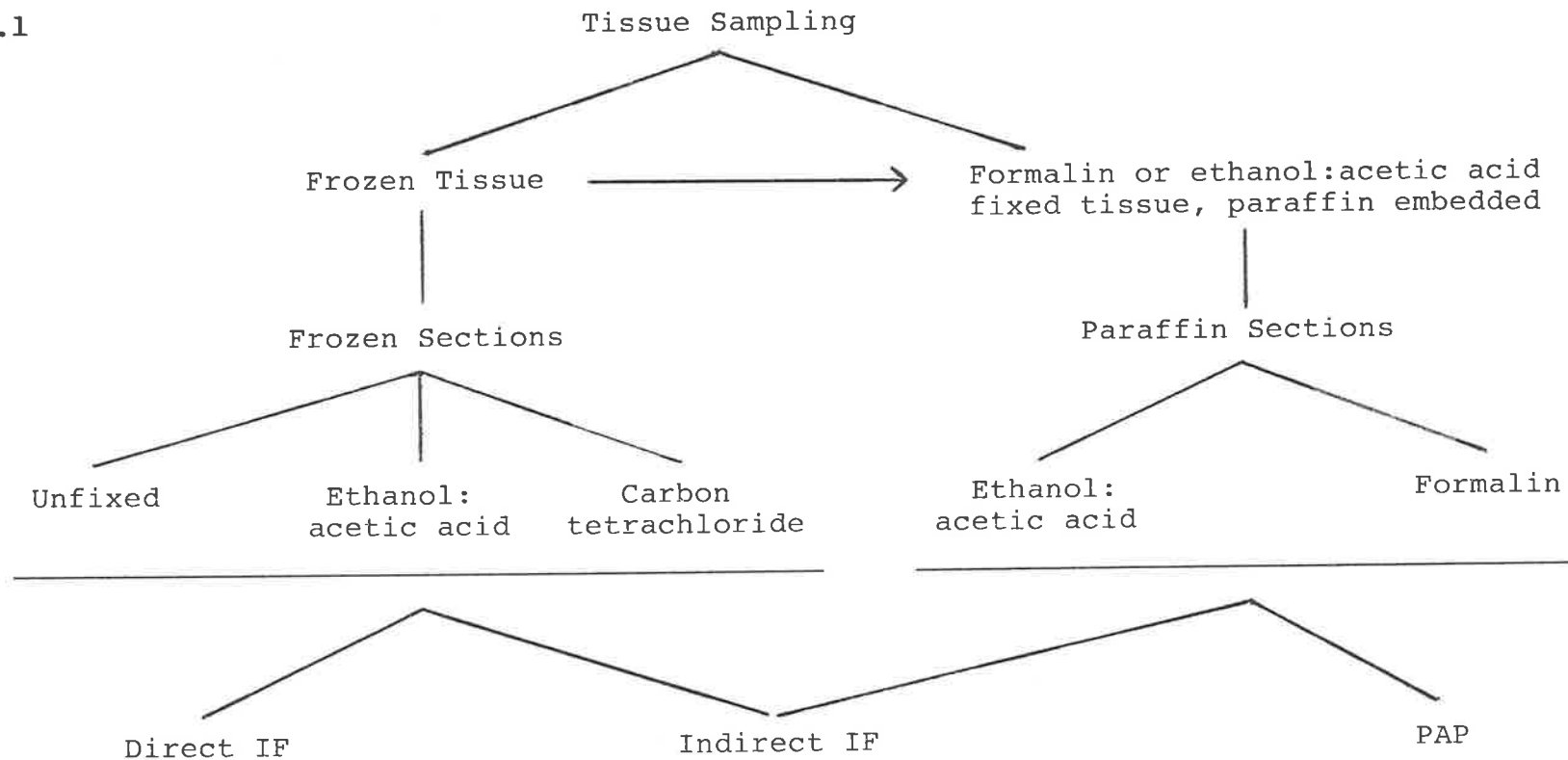


Figure 3.1: A flow chart outlining the methods used to compare HBsAg and HBcAg detection in HBV infected tissue.

frozen sections before IF examination. Ethanol:acetic acid fixation completely denatured both antigens so that only a few nuclei were faintly positive for HBcAg and HBsAg was undetected. Carbon tetrachloride fixation on the other hand resulted in similar staining for HBsAg and HBcAg to unfixed frozen sections. As both fixatives showed an improved histological appearance and better retention of sections on slides, carbon tetrachloride was chosen as a routine fixative for frozen sections. Carbon tetrachloride was previously used by Watanabe et al, (1976) as a fixative for HBsAg. Frozen sections fixed by carbon tetrachloride and stained by indirect IF to detect HBsAg and HBcAg were used as the standard to which all subsequent fixation and antigen detection was compared in this chapter.

**b. Comparison of direct and indirect IF for detection of HBcAg in frozen sections.**

Carbon tetrachloride fixed frozen sections of all five autopsy livers were then examined to compare direct and indirect IF detection of HBcAg. Nuclear HBcAg was detected equally well with either assay system, but cytoplasmic HBcAg was stained more intensely and was present in a higher number of cells using the indirect method (Figure 3.2). Owing to the large number of cells in each section, cell counts were not attempted, but an assessment of the percentage of positive cells noted. These results (Table 3.1) showed increased detection of cytoplasmic HBcAg in 4/5 livers tested, particularly in livers numbers 4 and 5; these livers, which



**FIGURE 3.2**

A comparison of the detection of HBcAg in liver number 4 (Table 3.1) in carbon tetrachloride fixed, frozen sections. Detection of HBcAg

TOP by the direct IF method, and

BOTTOM by the indirect method in a sequential section.

The bottom photograph was taken using the camera automatic exposure system, and the top photograph exposed manually for the same time.

Magnification X660

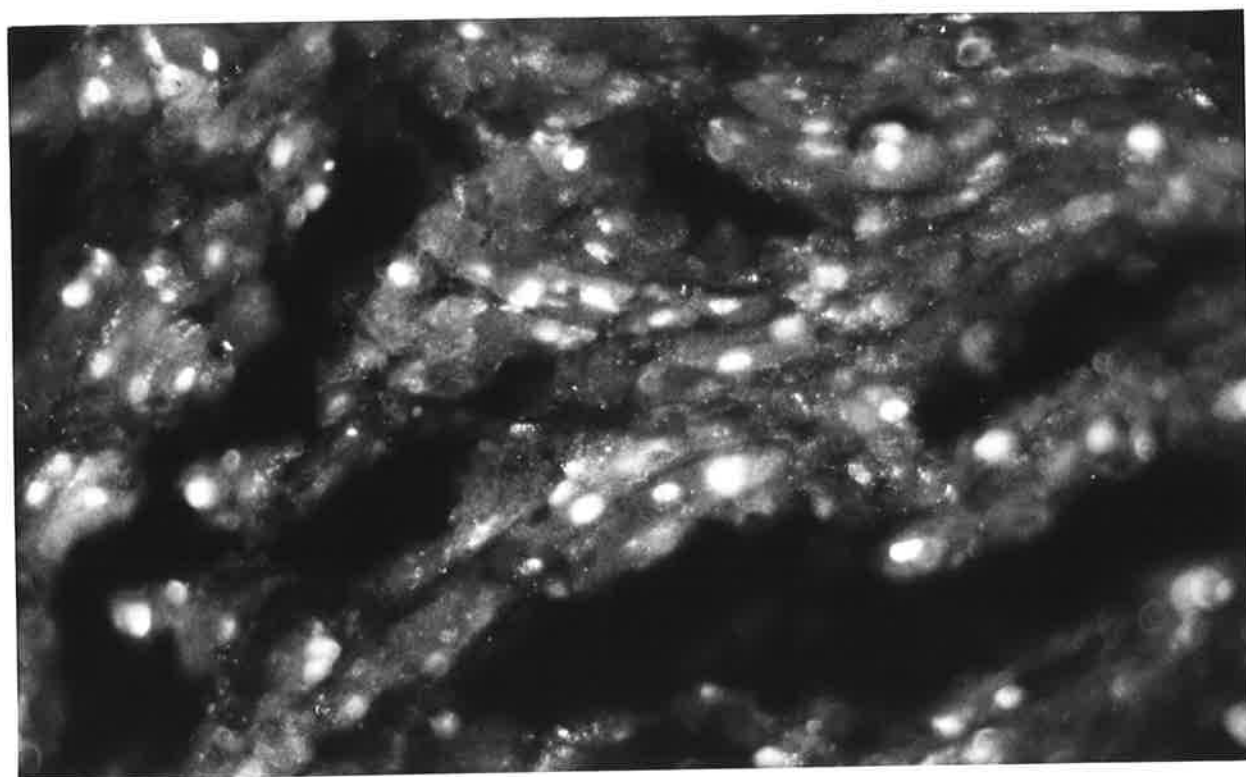
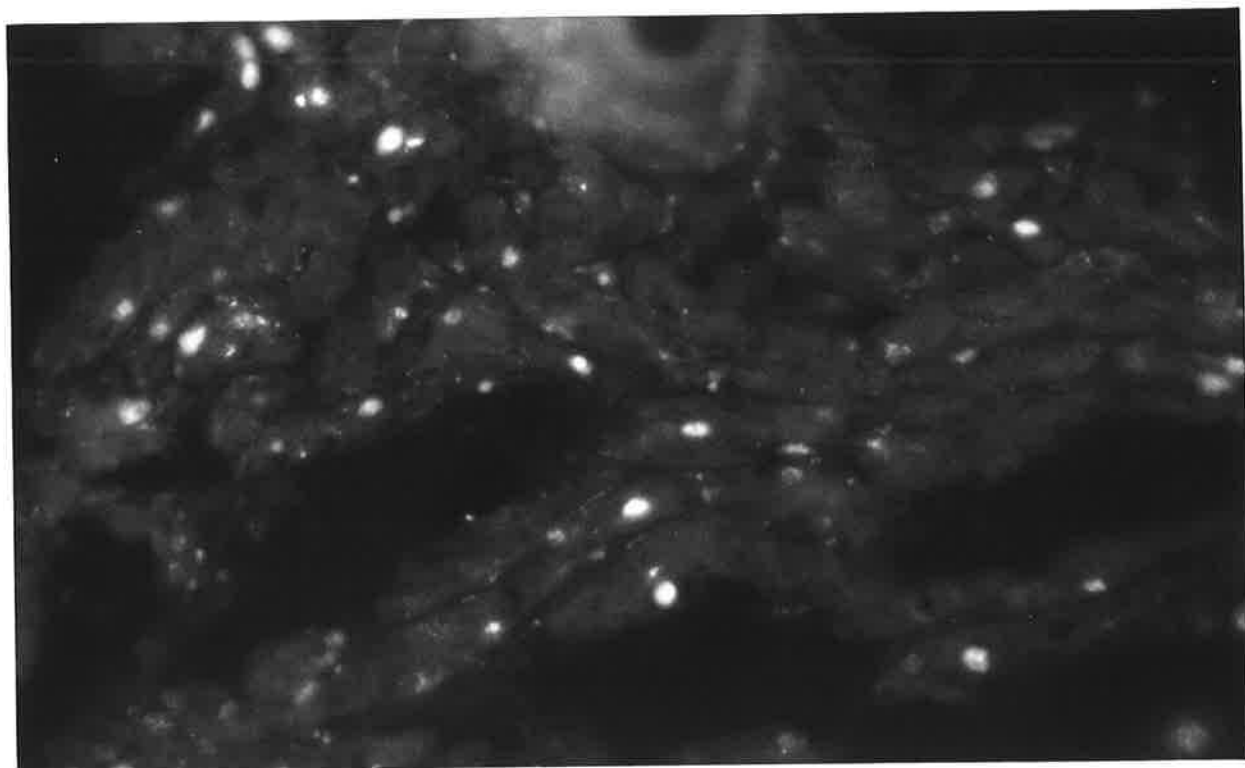


TABLE 3.1

Comparison between direct and indirect IF for the detection of HBcAg in frozen sections subsequently fixed with carbon tetrachloride.

LIVER NO.	HBcAg			
	NUCLEAR		CYTOPLASMIC#	
	DIRECT	INDIRECT	DIRECT	INDIRECT
1	***	++	++	+++
2	++	++	++	+++
3	+	+	++++	++++
4	+	+	±	++++
5	++++	++++	±	++++

# Including membranous HBcAg

\* Samples were scored on a 0 - ++++ scale, corresponding to 0, 1-25%, 25-50%, 50-75% and 75%- of total hepatocytes examined. In this instance, the 0 - ++++ scale does not represent immunofluorescence intensity.

X

showed low concentrations of cytoplasmic HBcAg in a few cells by direct IF now showed low concentrations of cytoplasmic HBcAg in virtually every hepatocyte after the indirect IF reaction. This was in contrast to the appearance of nuclear HBcAg, which was detected by both methods in a few cells in foci of liver number 4 and in every hepatocyte in liver number 5. Cytoplasmic HBcAg detected by indirect IF was present as discrete dots throughout the cell, occasionally as a large cytoplasmic inclusion or localised to the cell membrane (Figure 3.2). In each case HBcAg detection was restricted to hepatocytes.

**c. Comparison between frozen and fixed tissue sections for HBsAg detection.**

Indirect IF or PAP detected HBsAg with similar cytoplasmic staining in fixed, wax embedded tissue sections to that seen in carbon tetrachloride fixed frozen sections (Table 3.2), although the PAP method produced a relatively more intense reaction. However, membranous HBsAg shown to be present in the carbon tetrachloride fixed frozen sections was undetected by either indirect IF or PAP on the fixed tissue (Figure 3.3). Blocks fixed in formalin were slightly superior for HBsAg detection to those fixed in ethanol:acetic acid. As was the case with HBcAg, HBsAg was only detected in hepatocytes.

TABLE 3.2

The detection of HBsAg and HBcAg in (carbon tetrachloride fixed) frozen sections detected by indirect IF, compared with PAP staining in sections from fixed tissue blocks.

Liver No.	HBsAg				HBcAg			
	CYTOPLASMIC		MEMBRANE		NUCLEAR		<sup>(1)</sup> CYTOPLASMIC	
	IF	PAP	IF	PAP	IF	PAP	IF	PAP
	<u>Frozen</u>	<u>Fixed</u>	<u>Frozen</u>	<u>Fixed</u>	<u>Frozen</u>	<u>Fixed</u>	<u>Frozen</u>	<u>Fixed</u>
1	(2) +	+	+++	-	++	++	+++	+++
2	+++	+++	++	-	++	++	+++	+++
3	+	+	+++	-	+	-	++++	-
4	+	-	+++	-	+	(3) + (F-)	+++	+++ (F-)
5	+	+	+++	-	++++	+ (F-)	++++	-

(1) Includes membraneous HBcAg.

(2) Samples were scored as Table 6.1, i.e. 0 - ++++ represents cell numbers and not IF intensity

(3) (F) indicates results obtained with formalin fixed blocks when these were different to the results of ethanol:acetic acid fixed blocks.

**FIGURE 3.3**

A comparison of the detection of HBsAg by indirect IF in carbon tetrachloride fixed-frozen sections with detection by PAP in ethanol:acetic acid fixed, paraffin embedded tissue.

TOP HBsAg detected by indirect IF in carbon tetrachloride fixed frozen sections.

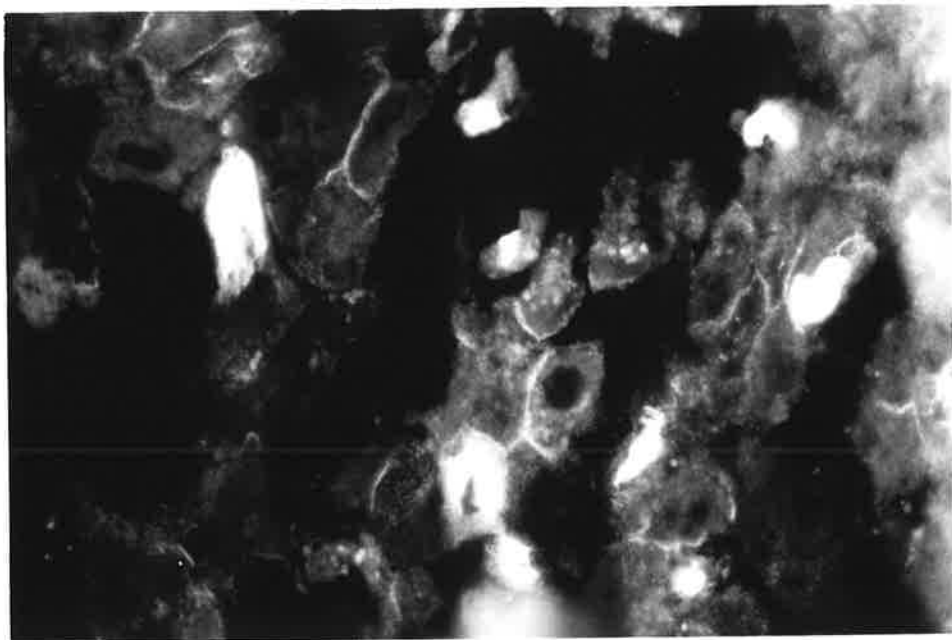
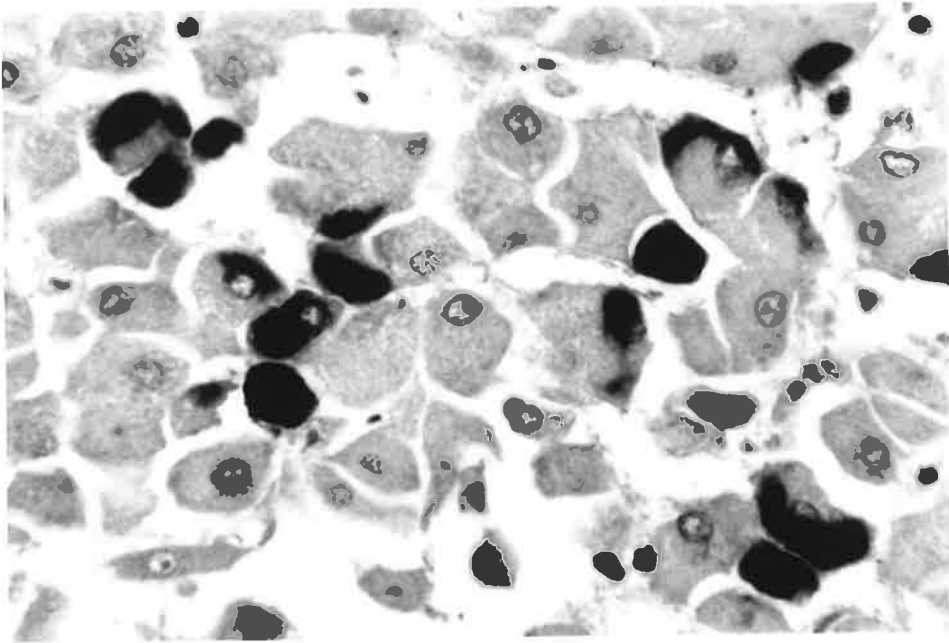
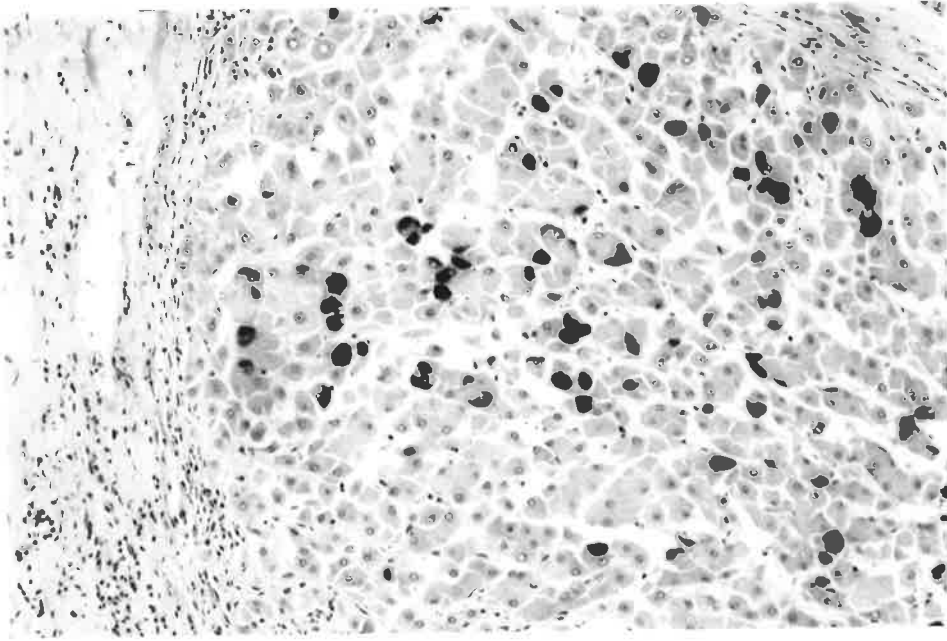
CENTRE & BOTTOM

HBsAg detected by PAP in ethanol:acetic acid fixed, paraffin embedded tissue.

Membraneous HBsAg shown in the top photograph is undetectable in the centre and bottom photographs.

Magnification top and centre X475  
bottom X120.

The area of the centre photograph is delineated in the bottom photograph. This shows the focal nature of HBsAg positive cells.



**d. Comparison between frozen and fixed tissue sections for HBcAg detection.**

The detection of HBcAg in fixed, wax embedded tissue from livers 1 and 2 was similar to that seen in the frozen tissue (Table 3.2). However, in livers 3-5 a majority of hepatocytes were HBcAg positive by indirect IF on carbon tetrachloride fixed, frozen sections but negative when fixed tissue blocks were examined. The intracytoplasmic distribution of HBcAg detected by PAP was similar to that seen by IF in carbon tetrachloride fixed frozen sections.

In contrast to the above HBsAg results, in the fixed tissue, more cells with HBcAg were detected with PAP than with indirect IF and ethanol:acetic acid in this instance was superior to formalin (Table 3.2). HBcAg present in low concentrations was not detected by either fixation method (Table 3.2).

**e. Examination of biopsy specimens.**

A larger series of biopsy samples was then examined by indirect IF on carbon tetrachloride fixed frozen sections and by PAP on formalin fixed tissue and the results compared (Table 3.3). These results showed that none of the 7 livers positive for membranous HBsAg by indirect IF was positive for HBsAg by PAP in formalin fixed tissue. This confirmed that membranous HBsAg was not detected by PAP in formalin fixed tissue.



TABLE 3.3

The detection of HBsAg and HBcAg by indirect immunofluorescence in carbon tetrachloride-fixed frozen sections compared with detection by PAP in formalin-fixed tissue.

Biopsy No.	HBsAg		HBcAg	
	IF	PAP	IF	PAP
	Frozen	Formalin	Frozen	Formalin
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	+	-	-	+
5	+	-	-	-
6	+	-	-	-
7	+	-	-	-
8	+	-	-	-
9	+	-	-	-
10	+	-	+	+

In each instance, HBsAg in the frozen sections was membraneous not cytoplasmic.

Although 2/10 biopsy samples examined showed HBcAg in the formalin fixed tissue after PAP staining, only 1/10 was positive for HBcAg by indirect IF on frozen sections. Considering the above results (section d) this probably reflected the focal distribution of HBcAg positive hepatocytes within the liver and demonstrated the sampling error associated with liver biopsy. In both cases, the formalin fixed tissue showed HBcAg within nuclei only, although cytoplasmic HBcAg was also prominent in the frozen sections of the one liver that was also positive by indirect IF.

#### 4. CONCLUSIONS AND DISCUSSION.

Generally, examination of HBV infected liver has either used fixed tissue resulting in good histological appearance and poor viral antigen detection or used frozen tissue resulting in poor histological appearance and good antigen detection. This study has defined which HBV antigens are denatured during the fixation process. There are three main findings:

- i. low levels of viral antigen (usually membraneous HBsAg and membraneous or cytoplasmic HBcAg) are often undetected in fixed tissue.
- ii. in frozen sections, the indirect IF assay detected a greater number of cells with cytoplasmic HBcAg compared with the direct IF assay.
- iii. HBsAg and HBcAg were detected only in hepatocytes.

There have been few reports of the comparative detection of HBsAg and HBcAg in frozen and fixed tissue. These antigens may be reliably detected in frozen sections and carbon tetrachloride proved a useful fixative for frozen sections. The distribution and intensity of HBsAg and HBcAg staining in frozen sections fixed by carbon tetrachloride were used as the standard to which other fixation methods were compared. Frozen sections have previously been regarded as the standard for immunohistochemical comparisons (Sternberger, 1979). The comparison of direct and indirect IF to detect HBcAg in frozen sections showed that the indirect method was more sensitive. Consequently, a new finding was the detection of HBcAg in the cytoplasm of a population of cells previously thought to be negative for HBcAg and in some cells which were thought to contain nuclear HBcAg only. It is unlikely that this additional HBcAg was previously undetected due to a less than optimal dilution of the direct anti-HBc FITC conjugate in the direct IF reaction, as the conjugate had a titre of 1/320 and was used at a dilution between 1/10 - 1/20. Thus, it is likely that the increased sensitivity was attributed to the use of an indirect rather than a direct reaction.

Using the indirect reaction, most HBcAg positive cells were shown to express the antigen in the cytoplasm, while nuclear HBcAg was only seen in a varying proportion of cytoplasmic HBcAg positive cells.

The above distribution of HBcAg was more consistent with reported antigen distribution patterns of other Hepadnaviruses; WHV core antigen was predominantly cytoplasmic in two studies (Frommell et al, 1984; Ponzetto et al, 1984b) and DHBV core antigen was cytoplasmic in a range of infected cells (Halpern et al, 1984). Studies examining membraneous HBsAg detection in formalin fixed tissue appear to be almost equally divided among those found to be positive (Huang and Neurath, 1979; Burns, 1975; Tedder et al, 1983) or negative (Tapp and Jones, 1977; Blenkinsopp and Haffenden, 1977; Ray, 1979; Kringsholm et al, 1983). It is possible that technical differences between laboratories account for these results. Other membrane antigens unrelated to HBV were also reported to be undetected after formalin fixation (Hsu et al, 1983). Of the two fixatives used in this chapter, formalin was slightly better for HBsAg detection and ethanol:acetic acid was distinctly better for HBcAg detection. The extent of antigen loss may depend on the time and degree of fixation as well as other less well defined factors and satisfactory detection of HBsAg and HBcAg should not be assumed after any type of fixation without proper evaluation. As a result of the satisfactory detection of HBcAg in livers 1 and 2 after ethanol:acetic acid fixation, it was possible to use such tissue to correlate HBcAg and HBV DNA in later experiments.

The clear demonstration of previously infrequently detected, membraneous and cytoplasmic HBcAg supports previous independent proposals relating to mechanisms of liver cell

injury. HBcAg in the nucleus and cytoplasm was shown to be the target for an in vitro complement fixation reaction in liver sections (Ray, 1979). Similarly, membranous HBcAg was assumed to be the target of bound anti-HBc present on hepatocyte membranes although its presence was not demonstrated (Trevisan et al, 1982). Subsequent lysis of these cells was a proposed mechanism of immune clearance of infected cells that could relate to the high titres of anti-HBc found in HBV infected patients. More recently, HBcAg was shown to be the target of a specific T-lymphocyte response (Mondelli et al, 1982; Naumov et al, 1984) that could account for hepatocyte damage in those cells expressing membranous or cytoplasmic HBcAg. However, in the latter report, HBcAg in isolated HBV-infected hepatocytes was only detected in nuclei, probably as a result of formalin fixation.

Results from the experiments described in this chapter indicate that future studies of HBcAg expression and hepatocyte injury should clearly distinguish between nuclear and cytoplasmic HBcAg in view of the possible differing significance of each. The present study demonstrates several compromises available in an attempt to resolve the conflicting demands on the one hand for good histological preservation and on the other hand the care required to retain viral antigenicity for maximum sensitivity of detection.

## CHAPTER 4

**THE DEVELOPMENT OF METHODS TO DETECT HBV DNA - AND HBV DNA AND VIRAL ANTIGEN SIMULTANEOUSLY - IN FIXED, PARAFFIN EMBEDDED TISSUE.****1. INTRODUCTION.**

In situ hybridisation methods to detect viral DNA in tissue samples have generally used frozen sections as the substrate. The methods used require minimum tissue manipulation and may result in a good histological appearance if the tissue is fresh, provided care is taken to avoid ice crystal damage. However, the quality of the histological appearance and the ease with which frozen sections may be cut deteriorate with the time that the tissue is stored. Furthermore, frozen sections are inconvenient to cut.

For these reasons the use of ethanol:acetic acid fixed tissue blocks which were subsequently embedded into paraffin wax was investigated because in situ hybridisation to detect HBV DNA in formalin fixed tissue yielded variable results that were difficult to control (A.R. Jilbert, personal communication). Ethanol:acetic acid fixed, paraffin embedded samples i) may be stored at room temperature without any apparent deterioration ii) provide a constant supply of sections with very good histological appearance iii) facilitate the cutting of more reproducible tissue sections than is possible with frozen sections.

The experiments described in this chapter were performed to determine if ethanol:acetic acid fixed, paraffin embedded tissue blocks were suitable for the detection of HBV DNA by in situ hybridisation. In addition, it was considered desirable to be able to detect viral antigen and DNA in the same tissue section to examine their relationship at the level of the single cell. Consequently, the simultaneous detection of these markers was also investigated in this chapter.

## 2. EXPERIMENTAL PROCEDURES

One autopsy and one biopsy sample were examined. The autopsy sample was previously frozen, and 0.5 cm<sup>3</sup> blocks from this liver, and the biopsy sample, were fixed in ethanol:acetic acid.

Subsequent steps in section preparation prior to in situ hybridisation were then optimised. Pronase digestion was used to improve the access of probe and DNAase to the tissue sections; the pronase was titrated against <sup>3</sup>H-thymidine labelled cells and against sections of the autopsy liver, in mock or genuine in situ hybridisation experiments respectively.

Simultaneous detection of HBCAg and HBV DNA in sections from ethanol:acetic acid fixed, paraffin embedded tissue was then examined.

### 3. RESULTS.

#### a. The development of in situ hybridisation with good histological detail.

The histological appearance of the frozen tissue sections (fixed as described in Chapter 2, part 6a<sub>ii</sub>) was poor after the in situ hybridisation reaction (Figure 4.1) and if the full potential of the reaction was to be realised, improved histology was imperative.

Initial experiments using <sup>3</sup>H-labelled whole pHBVCB probes on ethanol:acetic acid fixed, paraffin wax sections treated as described (Chapter 2, part 6a<sub>iv</sub>) showed a strong autoradiographic signal similar to that seen in frozen sections. The histological appearance of the tissue sections was significantly improved (Figure 4.1). DNAase digestion of these sections prior to hybridisation failed to abolish the signal in contrast to digestion of frozen sections, and it was first considered that the target of the in situ hybridisation was viral mRNA. However, previous experiments to detect HBV specific mRNA were negative (A.R. Jilbert, personal communication) and it was concluded that this effect was the result of poor DNAase access to the viral DNA contained in the cells.

It was considered that pronase digestion might aid DNAase access, as protease digestions have been shown previously to increase DNA probe access as well as reduce non specific binding of probes to tissue sections (Brahic and Haase, 1978).



**FIGURE 4.1**

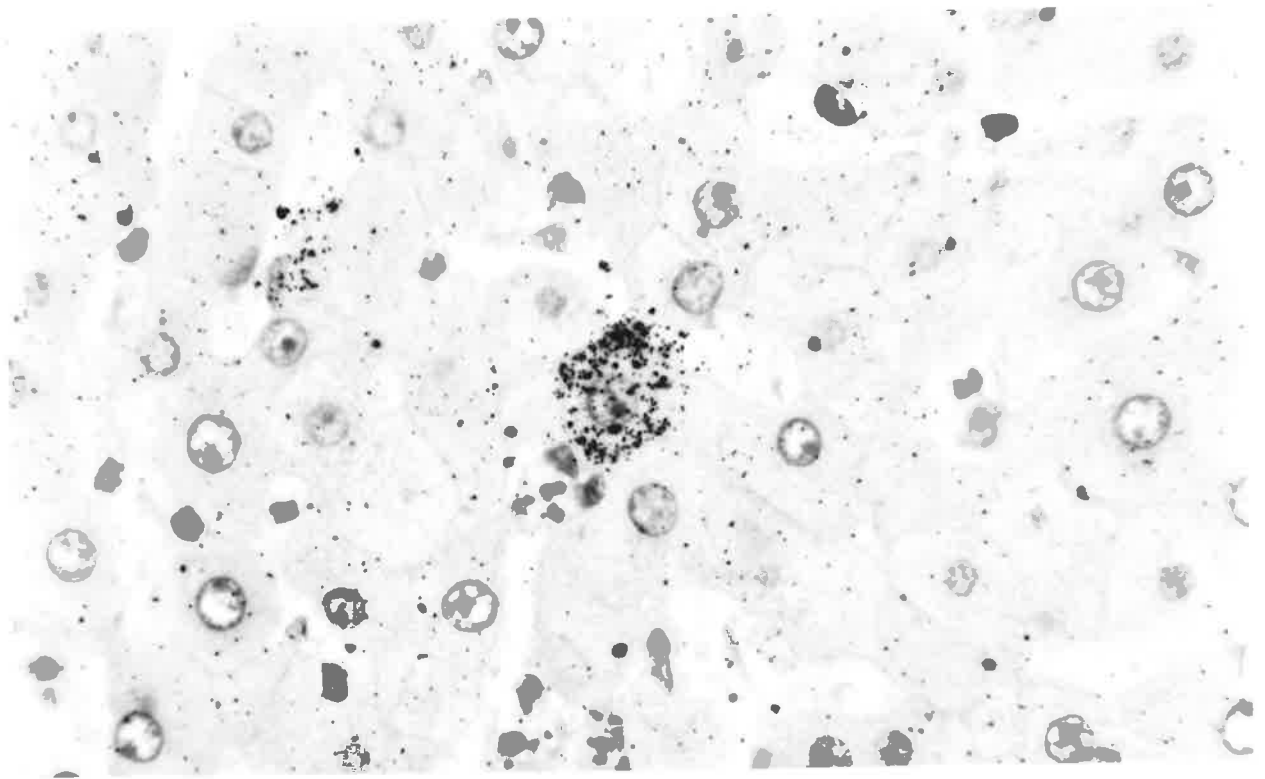
A comparison of the histological detail after in situ hybridisation to frozen sections or sections from ethanol:acetic acid fixed, paraffin embedded tissue.

TOP            Frozen sections

BOTTOM        Paraffin embedded sections

The sections were stained by H & E

Magnification X600



The pronase solution used in the fixation protocol was therefore titrated. A published method for pronase titration (Brigati et al, 1983) was considered, but was discarded in favour of the method described below, that was considered to be more suitable for tissue sections.

Varying dilutions of pronase were used in the above in situ hybridisation fixation protocol on paraffin embedded sections of the autopsy liver. The results of these experiments (Table 4.1) showed that the efficiency of DNAase digestion was dependent on the degree of pronase digestion. There was also an increase in the in situ hybridisation signal with increased pronase concentration. However, higher concentrations of pronase resulted in decreased grain density and poor histological detail. The decreased grain density was attributed to a loss of target DNA from the sections.

This was confirmed by testing dilutions of pronase on sections from an ethanol:acetic acid fixed, paraffin embedded block of <sup>3</sup>H- thymidine labelled human hepatoma cells. 5um sections cut from this block were treated by the in situ hybridisation fixation protocol used above and duplicate sections treated with differing concentrations of pronase, but the sections were not hybridised. The degree of loss of <sup>3</sup>H-labelled DNA in each section was then determined by autoradiography, by comparison with an untreated control section. The results of

**TABLE 4.1**

**The effect of differing pronase concentrations on the detection of HBV DNA by in situ hybridisation on paraffin embedded sections and on the efficiency of specific nucleases.**

<u>Pronase concentrations (ug/ml)</u>	HBV DNA in sections treated with	
	<u>RNAase</u>	<u>DNAase</u>
10 (RT)*	+	+
10	+	<u>+</u>
50	++	-
100	+	-

\* All pronase digestions were performed at 37°C for 15 minutes except in row 1 which shows results of digestion at room temperature.

this experiment showed that with higher pronase concentrations, there was a progressive loss of  $^3\text{H}$ -DNA from the sections (Table 4.2).

Thus, titration of the pronase used in in situ hybridisation experiments on paraffin embedded tissue sections was necessary to avoid overdigestion with consequent loss of histological detail, for optimum DNAase digestion and probe access, while still retaining high levels of target DNA. Although it could not be shown, it was assumed that viral DNA and cellular DNA reacted similarly under the experimental conditions. Pronase, titrated as described above, was used in all the in situ hybridisation experiments on ethanol:acetic acid fixed tissue described in later chapters. An example of the improved histological appearance of the tissue using this method is shown in Figure 4.1.

**b. The development of a method for the simultaneous detection of HBcAg and HBV DNA.**

HBcAg was detected in fixed, paraffin embedded autopsy tissue by PAP (Chapter 2, part 3b) and the sections then treated as described (Chapter 2, part 6aiv) for in situ hybridisation. At a number of different stages during fixation, the colour intensity of the horseradish peroxidase-DAB reaction product was compared with an untreated section stained for HBcAg. The colour intensity of the reaction product was unchanged throughout.

**TABLE 4.2**

**The comparison of the loss of cellular DNA with increasing pronase concentration.**

Pronase Concentration ug/ml	Total grain count*	Average grains per cell	Percent retained
no treatment **	971	32.3	100
0	862	28.7	88.7
10	908	30.2	93.5
50	1011	33.7	104.1
100	692	23.0	71.2
200	610	20.3	62.8

\* The total number of grains in 30 cells were counted to obtain this figure. The autoradiographic exposure time was 5 hours.

\*\* Control sections were simply dewaxed before autoradiography.

A section stained for HBcAg by PAP was then fixed for in situ hybridisation and hybridised with  $^{125}\text{I}$ -labelled excised HBV DNA. The in situ hybridisation results were then compared with a control section (unstained for HBcAg) that was also tested by in situ hybridisation. The results of these experiments showed that there was no loss in the colour intensity of the DAB product at this step and the distribution of autoradiographic grains was similar in both sections, although the grain density was slightly reduced in the HBcAg stained section. This decrease was considered likely as a result of impaired probe access due to the peroxidase-DAB reaction product, as a duplicate section that was treated with the various antisera in the PAP reaction but not with the DAB showed a similar grain density to the unstained control section.

Thus, it was demonstrated that, with a protocol using 5um sections from ethanol:acetic acid fixed, paraffin wax embedded tissue that were examined simultaneously for HBcAg and HBV DNA, good histology was preserved, HBcAg detection by PAP was similar to that seen with PAP alone, and HBV DNA detection by in situ hybridisation was only slightly reduced in sensitivity in comparison to that seen with in situ hybridisation alone.

#### 4. CONCLUSIONS AND DISCUSSION.

This chapter describes the development of important technical advances which enabled a number of experiments described in Chapters 5 and 6 to be performed. In situ hybridisation may be considered to be a compromise between, on the one hand, the desire for good histological preservation and on the other hand, the need to disrupt the tissue sufficiently to permit probe access. The use of fixed, paraffin embedded tissue with controlled pronase digestion provided this compromise and the resultant histological detail was much improved compared with frozen sections. Controlled pronase digestion was also important for efficient nuclease digestion used as a specificity control. Guelin et al (1985) have also recently reported the use of ethanol:acetic acid fixed tissues processed into paraffin wax to provide good histological detail in in situ hybridisation experiments.

Likewise, the simultaneous detection of viral antigen and DNA represents a technical advance which permitted the correlation of these markers of virus infection in single cells. The results of these correlation experiments are described in Chapter 6. Since the experiments of this chapter were carried out, Blum et al (1984b) and Gendelman et al (1985) have also performed similar immunochemistry/in situ hybridisation double labelling experiments.



## CHAPTER 5

DEMONSTRATION AND ANALYSIS OF HBV DNA IN THE CYTOPLASM AND  
NUCLEUS OF INFECTED CELLS.

## 1. INTRODUCTION

Initial experiments (Gowans et al, 1981) and experiments described in Chapter 4 detected large amounts of HBV DNA within the cytoplasm of infected hepatocytes. This was considered to be an unusual finding, since other small DNA viruses are known to replicate in the cell nucleus (Fenner et al, 1974). It was however possible that HBV DNA synthesis might occur in the hepatocyte nucleus followed by cytoplasmic viral DNA accumulation at a later stage in virus assembly.

The sensitivity of the above in situ hybridisation reaction was previously shown to be around 15 HBV genomes per cell (Gowans, 1981; Gowans et al, 1981). Although in these first reports, conditions for the in situ hybridisation reaction were considered to be optimal and specific, an increase in the reaction sensitivity was nevertheless considered desirable. As probes radiolabelled by nick translation were already of high specific activity and could not be readily improved, other methods of increasing the reaction sensitivity were investigated.

Therefore, the aim of the experiments described in this chapter were to determine the location, strandedness and polarity (+ or - strand) of various forms of viral DNA in different infected cells, and to distinguish viral DNA in replicative and mature forms in order to identify permissively and non-permissively infected cells. In this context, mRNA is designated + strand and viral DNA assigned + or - in relation to this convention.

## 2. EXPERIMENTAL DESIGN.

Two groups of liver samples were tested; group 1 was HBsAg- and HBCAg- positive and comprised six samples while group 2 was HBsAg- positive, HBCAg- negative and comprised three samples.

The livers were examined by in situ hybridisation to either frozen sections using fixation conditions designed to denature or not denature DNA within the cells, or to sections from ethanol:acetic acid fixed paraffin embedded tissue.

## 3. RESULTS.

In situ hybridisation showed a detectable autoradiographic signal over the cytoplasm of hepatocytes of livers in group 1 after 4-5 weeks exposure using a  $^3\text{H}$ -labelled whole pHBVCB probe on frozen sections (Figure 5.1). In three of these livers, hepatocytes positive for HBV nucleic acid

**FIGURE 5.1**

The detection of high levels of HBV DNA in the cytoplasm of infected hepatocytes of liver number 1 of group 1.

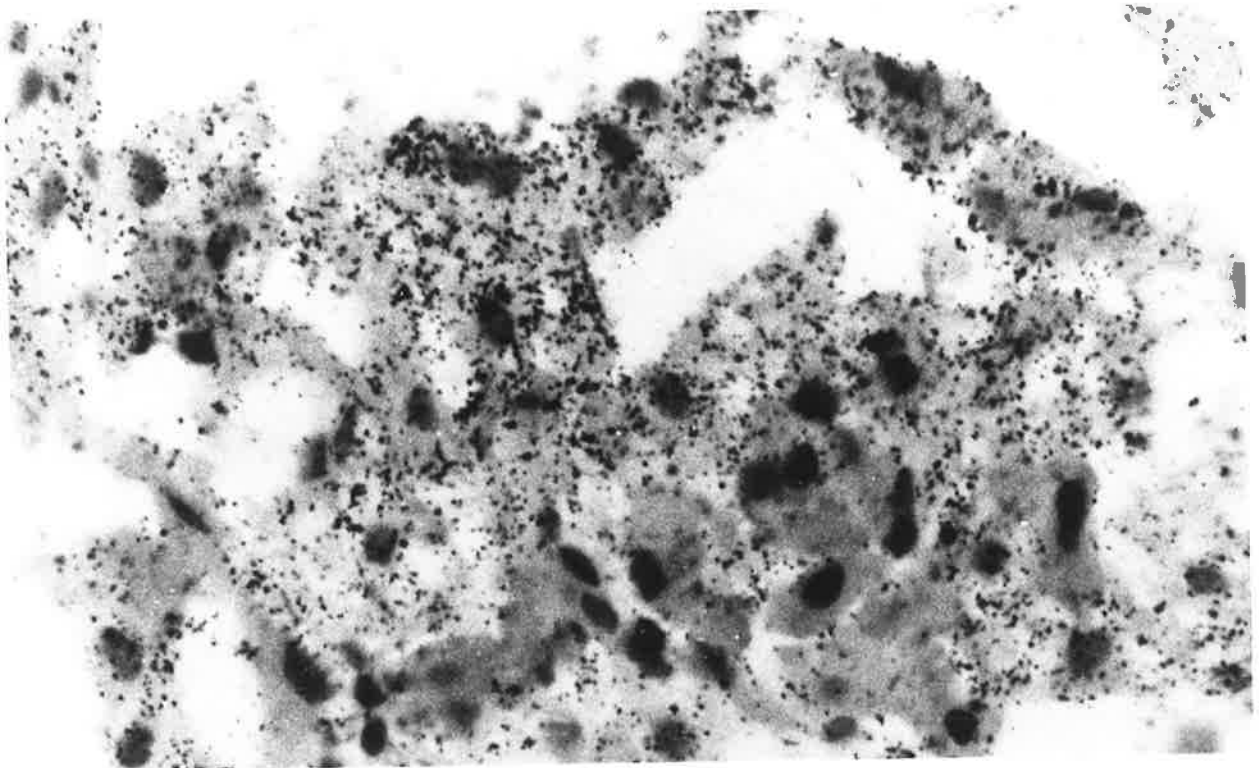
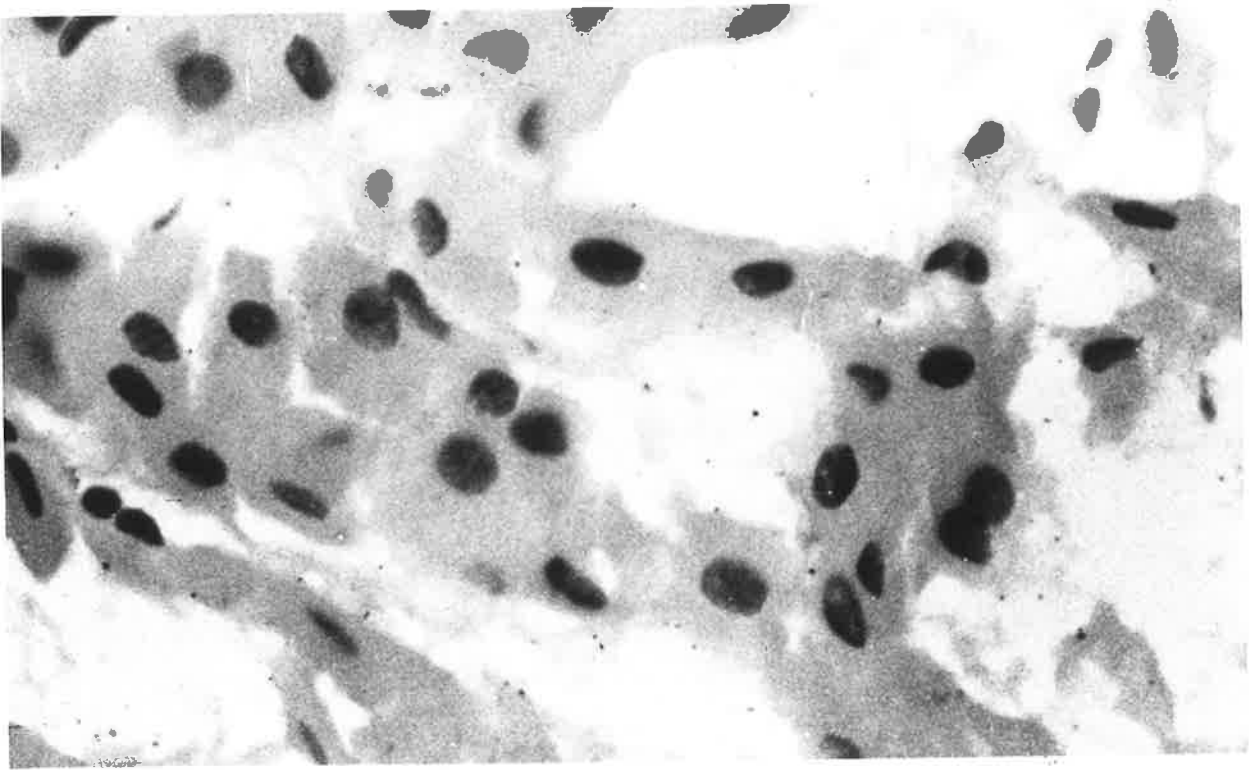
TOP            Infected hepatocytes.

BOTTOM       Non infected cells in the same tissue section.

In situ hybridisation was performed with a full length <sup>3</sup>H-labelled pHBVCB plasmid.

The autoradiographic exposure was 4 weeks and the sections were stained by H & E.

Magnification X900.



sequences were seen in foci and, in the autopsy samples, these infected foci were surrounded by bands of fibrous tissue. Livers from group 2 were negative.

**a. Characterisation of cytoplasmic HBV nucleic acid sequences.**

When tissue sections from group 1 livers were fixed using non denaturing conditions (Chapter 2, part 6a. iii.), the grain density was noticeably increased if, in addition, the sections were boiled to denature the cellular DNA prior to in situ hybridisation (Table 5.1). The levels of HBV DNA detected in each liver varied. Viral DNA in liver number 1, which showed the highest levels, was detectable in 4-5 days, while on the other hand, an exposure time of 4-5 weeks was required to unequivocally demonstrate viral DNA in liver number 5. The other livers examined contained intermediate HBV DNA levels (Table 5.1). Liver number 6 was not examined as frozen tissue was not available.

The grain density in all livers was significantly reduced if the sections were pretreated with DNAase or  $S_1$  nuclease, but RNAase had little effect (Tables 5.1 and 5.2). The grain distribution was unchanged throughout the different treatments. Similar results were seen with all five livers (nos. 1-5) tested and the results of two livers (1 and 2) were quantitated by reflected light photometry. The results of one experiment are shown in Table 5.2; the autoradiographic exposure time was 3 weeks.

TABLE 5.1

The cytoplasmic grain density in frozen sections of HBV-infected livers after in situ hybridisation with a  $^3\text{H}$ -labelled HBV DNA probe, using a basic non-denaturing protocol for section preparation.

<u>Liver No</u>	Serum <u>HBeAg/Ab</u>	Further treatments					
		<u>None</u>			<u>Boiling (denaturation)</u>		
		<u>No treatment</u>	<u>+RNAase</u>	<u>+DNAase</u>	<u>No treatment</u>	<u>+RNAase</u>	<u>+DNAase</u>
1	Ag	+++	+++	+	++++	++++	+
2	Ag	++	++	<u>+</u>	+++	+++	+
3	Ab	++	++	<u>+</u>	+++	+++	+
4	Ag	+	+	-	++	++	-
5	NT	+	+	-	+	+	-

TABLE 5.2

Effect of boiling, followed by nuclease treatment on the intensity of the autoradiographic signal obtained after in situ hybridisation using  $^3\text{H}$ -labelled pHBVCB DNA as probe on undenatured sections of livers 1 and 2 of group 1.

	LIVER NO. 1		LIVER NO. 2	
	<u>Unboiled</u>	<u>Boiled</u>	<u>Unboiled</u>	<u>Boiled</u>
No treatment	235 $\pm$ 58*	414 $\pm$ 162	205 $\pm$ 55	298 $\pm$ 70
+ RNAase	258 $\pm$ 78	419 $\pm$ 96	245 $\pm$ 83	266 $\pm$ 101
+ DNAase	87 $\pm$ 33	8 $\pm$ 19	49 $\pm$ 14	60 $\pm$ 26
+ S <sub>1</sub> nuclease	133 $\pm$ 48	114 $\pm$ 33	115 $\pm$ 43	75 $\pm$ 17

\* Mean reading in relative photometric units ( $\pm$  standard deviation). Because heavily labelled cells were often distributed in foci, a number of fields (usually 24) from each section were chosen to represent the most heavily labelled areas. The highest 15 readings from each section were averaged, the mean photometric reading from 3 fields containing unlabelled cells subtracted as a background value and the standard deviation calculated. Each microscope field contained 50 to 70 heavily labelled cells. Mean background readings ranged from 23 to 62 for different slides. Control sections using  $^3\text{H}$ -labelled pBR322 DNA as probe, or HBV probes on uninfected liver sections, showed similar background grain densities, but these were not quantified photometrically.

The results of the above experiments demonstrated that the majority of the HBV nucleic acid sequences present in the cells comprised ssDNA. The increase in the autoradiographic signal after boiling (Table 5.2) was thought to be largely due to denaturation of ds viral DNA, although it may have also resulted to a limited extent from increased probe access to ssDNA target as a result of boiling.

In order to test which regions of the HBV genome were present as ssDNA, probes from specific regions of the virus genome were constructed (by Allison Jilbert) as described in Chapter 2, part 3a. Probes representing the HBsAg and the HBcAg regions showed a good hybridisation signal when used on undenatured sections of all five livers. Only the results from liver number 1 were quantitated (Table 5.3). These results showed that virus DNA complementary to all the probes shown in Fig. 5.2 was present in partly ss form; consequently, regions of the virus genome that were normally ds in the intact virion (eg. the C gene region complementary to P3) were found to be ss within infected hepatocytes.

Furthermore, probes P3 and P4 were similar in size and specific activity; while there were similar autoradiographic signals using either the HBsAg or the HBcAg gene probes on undenatured sections (target DNA in ss form, Table 5.3), there was a 2x increase in the amount of detectable HBV DNA corresponding to only the HBcAg region (P3) in denatured sections (Table 5.3). These findings suggest that sequences



TABLE 5.3

Comparison of the autoradiographic signal after in situ hybridisation to sections of Liver No. 1 using different fragments of pHBV114 DNA as probes for different regions of the HBV genome.

	P R O B E			
	P1*	P2	P3	P4
Undenatured	193 $\pm$ 29	89 $\pm$ 10	111 $\pm$ 18	128 $\pm$ 18
Boiled	236 $\pm$ 42	139 $\pm$ 31	267 $\pm$ 28	134 $\pm$ 11

\*The location of probes P1-P4 on the HBV genome are shown in Fig. 5.2. Relative photometric units were measured as described in the legend to Table 5.2.

FIGURE 5.2

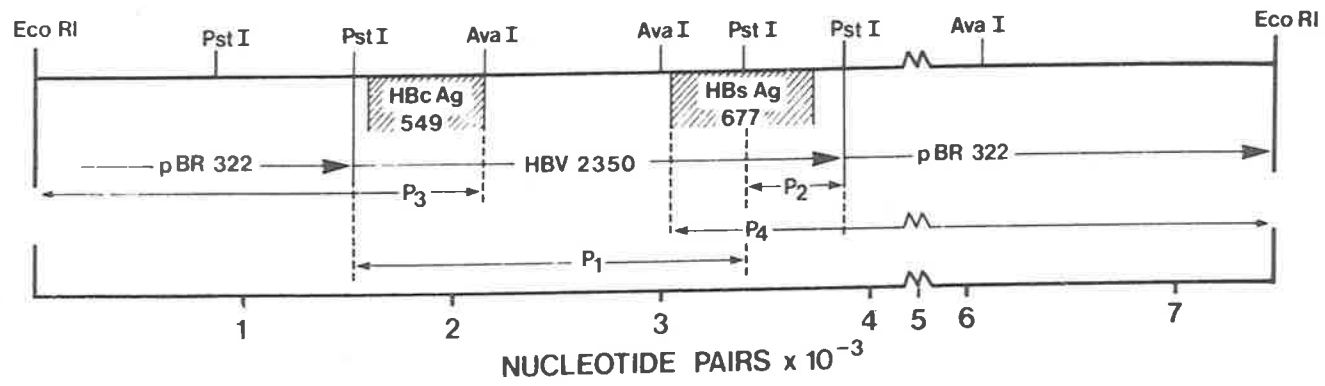


Figure 5.2 The location of subgenomic probes prepared from pHBV114.

corresponding to the HBcAg gene were present as both ss and ds forms, while sequences corresponding to the HBsAg gene were only ss ie. part of the target DNA was similar to mature virion DNA (HBcAg gene ds; HBsAg gene ss) and part was wholly ss.

To investigate the sense (+ or - polarity) of the ssDNA target described above, M13 probes 2.1 and 2.11 (Fig. 5.3) were prepared as described in Chapter 2, Part 4b. These were used in the in situ hybridisation reaction on undenatured or denatured sections of liver number 1. The strategy of radiolabelling the M13 probes by the use of primer 403-2 and subsequent synthesis of a complementary strand across the HBV insert was chosen in preference to the alternative using primer 1202 (see Figure 2.3), because it was considered that probes generated by the latter mechanism would prove to be too long for successful in situ hybridisation. Probes prepared with primer No. 1202 would be single-stranded for the length of the HBV insert (ca.500 bases) with a radiolabelled ds M13 tail (ca.7000 base pairs). In contrast, probes prepared with the 403-2 primer after digestion with the restriction enzyme Pst 1 were 573 bases long (Fig. 2.3), a size considered suitable for in situ hybridisation (Haase et al, 1984). In these experiments, probes labelled with  $^{32}\text{P}$  were used despite the poor autoradiographic resolution obtained with this isotope, since accurate cellular localisation of target

FIGURE 5.3

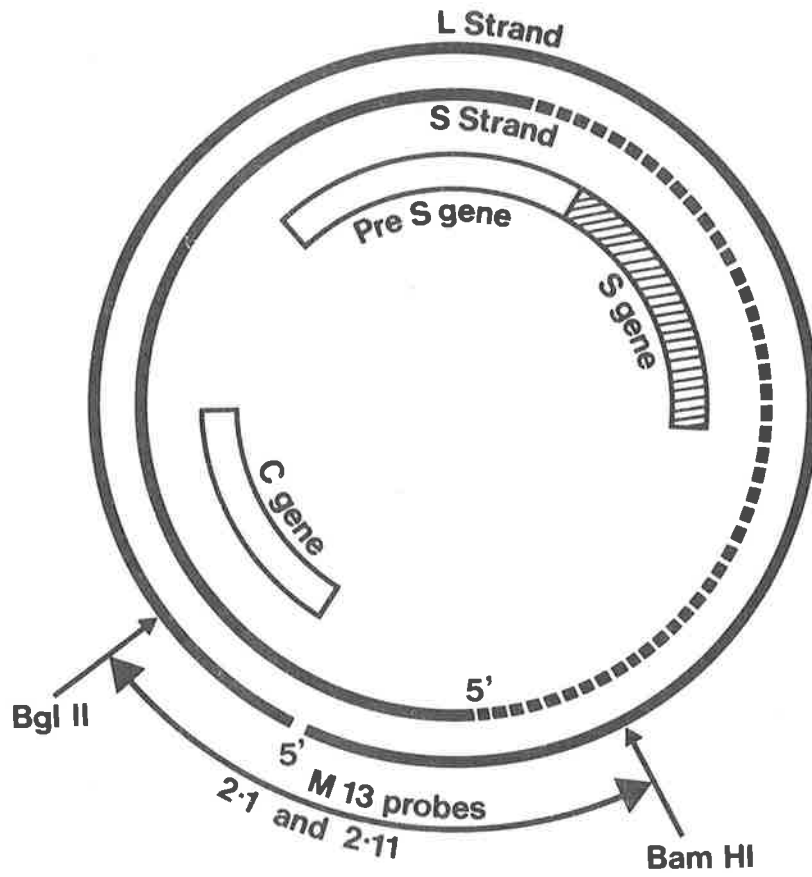


FIGURE 5.3 The location within the HBV genome of M13 probes 2.1 and 2.11.

sequences was not required in experiments which examined target polarity and since short exposure times could be used with  $^{32}\text{P}$  probes.

The results of these experiments (Table 5.4) showed that both M13 probes 2.1 and 2.11 hybridised to undenatured and denatured sections. However, probe 2.11 complementary to the long (negative sense) strand of the virion DNA (N. Gough; Personal communication) produced a more intense reaction in both undenatured and denatured tissue sections. These findings demonstrated that the target consisted of significantly more negative sense than positive sense viral DNA.

**b. Detection of nuclear HBV nucleic acid sequences.**

Three methods were used in an attempt to increase the reaction sensitivity in order to detect HBV DNA in group 2 livers and to determine if HBV DNA was present in other cells in addition to hepatocytes in group 1 livers.

(i) 10% dextran sulphate was included in the hybridisation reaction mix. Dextran sulphate has previously been shown to increase the sensitivity of in situ hybridisation reactions by the formation of probe networks (Gerhardt et al, 1981). Under the conditions of the assay, the inclusion of dextran sulphate resulted in non specific binding of the pBR322 control probe to tissue sections and was consequently discarded.

TABLE 5.4

Grain density in denatured or undenatured sections of liver No. 1 after in situ hybridisation using strand specific M13 probes.

	P2.1*	P2.11*
Undenatured	+	++
Denatured	+++	++++

P2.1 was complementary to mRNA, while P2.11 was the same sense as mRNA. The location of probes P2.1 and P2.11 on the HBV genome are shown in Fig. 5.3.

(ii) Frozen sections from the liver samples were then tested with whole pHBVCB  $^3\text{H}$ -labelled probes and the exposure period increased to 26 weeks. In addition to the high levels of cytoplasmic HBV DNA detected in hepatocytes in group 1 livers, a new population of infected cells was identified in one liver from each of groups 1 and 2.

In both cases, in contrast to the cytoplasmic grains seen in hepatocytes, the grains in this additional cell population were restricted to nuclei (Figure 5.4) and were present only in sections which were denatured. In sections which were not denatured or which were treated with  $S_1$  nuclease after denaturation, the grains were greatly reduced (Figure 5.4). This indicated that the target of the in situ hybridisation reaction in these cells was likely to be dsHBV DNA. Further confirmation by DNAase digestion was not performed in these experiments since it was very difficult to recognise nuclei in sections treated in this manner. The less than complete abolition of signal with  $S_1$  nuclease digestion was not unexpected, since in other experiments this enzyme was shown to be less efficient than DNAase in abolishing the signal after target denaturation (Table 5.2).

**FIGURE 5.4**

HBV DNA detected in the nuclei of hepatoma cells in a fully differentiated hepatocellular carcinoma after in situ hybridisation with <sup>3</sup>H-labelled pHBVCB.

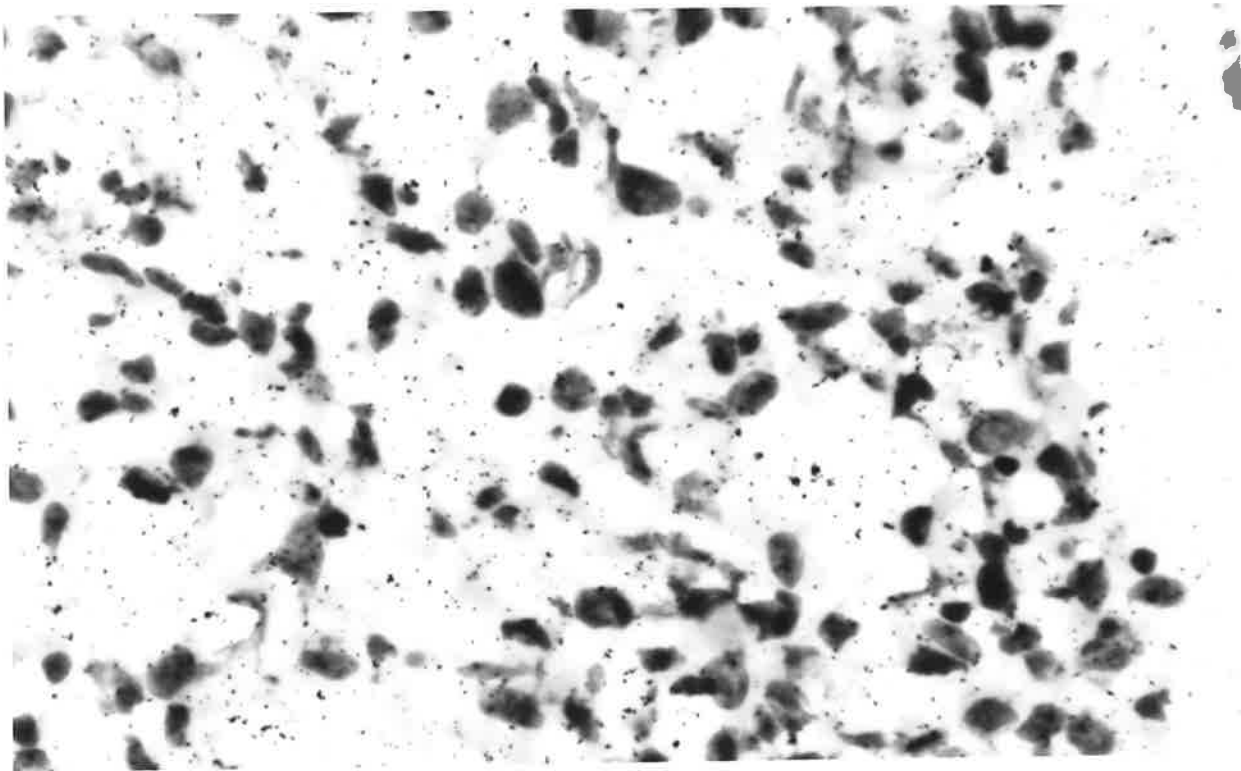
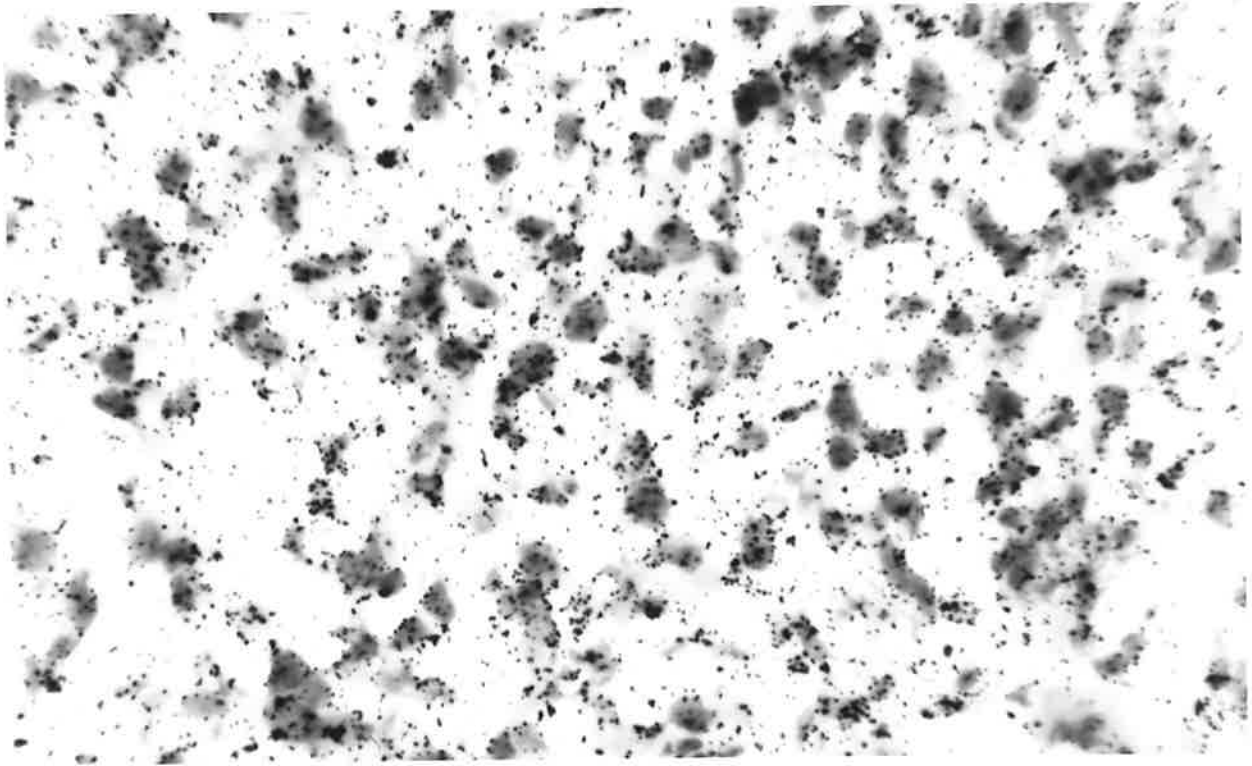
TOP shows the nuclear grain density after denaturation of cellular DNA, and

BOTTOM the grain density without denaturation.

The sections were stained by H & E and the autoradiographic exposure time was 6 months.

Magnification X600.





The number of grains overlying these nuclei varied between 20-40, but was always considerably lower than the grain numbers seen over the cytoplasm of hepatocytes. The HBV genome copy number was calculated to be approximately 5 as shown below.<sup>1</sup>

In the liver from group 2, every nucleus was positive for low genome copy numbers of HBV DNA (Figure 5.4). These cells were indentified as well differentiated hepatocellular carcinoma cells by Dr. R. Rowland (collaborating histopathologist). However, in the liver from a HBeAg-positive carrier in group 1, cells with only nuclear grains showed a different distribution to hepatocytes with high levels of cytoplasmic HBV DNA; infected hepatocytes were found in foci, whereas cells with nuclear grains were most common in portal tracts and in fibrous tissue surrounding hepatocyte foci (Figure 5.5). These cells were small with dense nuclei and were considered to be fibroblasts or mononuclear cells.

-----

1.

$$\begin{aligned}
 & 1 \text{ dalton} = 1.66 \times 10^{-24} \text{ g} \\
 \therefore & 2.1 \times 10^6 \text{ daltons} = 3.5 \times 10^{-18} \text{ g} = 1 \text{ HBV genome} \\
 & \text{Assume probe} = 2 \times 10^8 \text{ dpm/ug} \equiv 2 \times 10^{14} \text{ dpm/g} \\
 & \text{Assume target is 50\% saturated with probe}^* \\
 & \text{Then 1 HBV genome will produce} \quad 3.5 \times 10^{-18} \times \frac{2 \times 10^{14}}{2} \\
 & \qquad \qquad \qquad \qquad \qquad \qquad \qquad \qquad \qquad = 3.5 \times 10^{-4} \text{ dpm} \\
 & \qquad \qquad \qquad \qquad \qquad \qquad \qquad \qquad \qquad = 3.5 \text{ dp week} \\
 & \text{Assume autoradiography is 10\% efficient} \\
 & \text{Then 1 HBV genome will produce} \quad = 0.35 \text{ grains per week} \\
 \\
 & \therefore \text{In 26 weeks, 1 genome per cell will produce 9 grains} \\
 & \text{However, the cells contained 40 grains} \\
 & \qquad \qquad \qquad \text{HBV DNA content was } \underline{4.4 \text{ genomes/cell.}}
 \end{aligned}$$

\* Reported figures vary from 10-100%

**FIGURE 5.5**

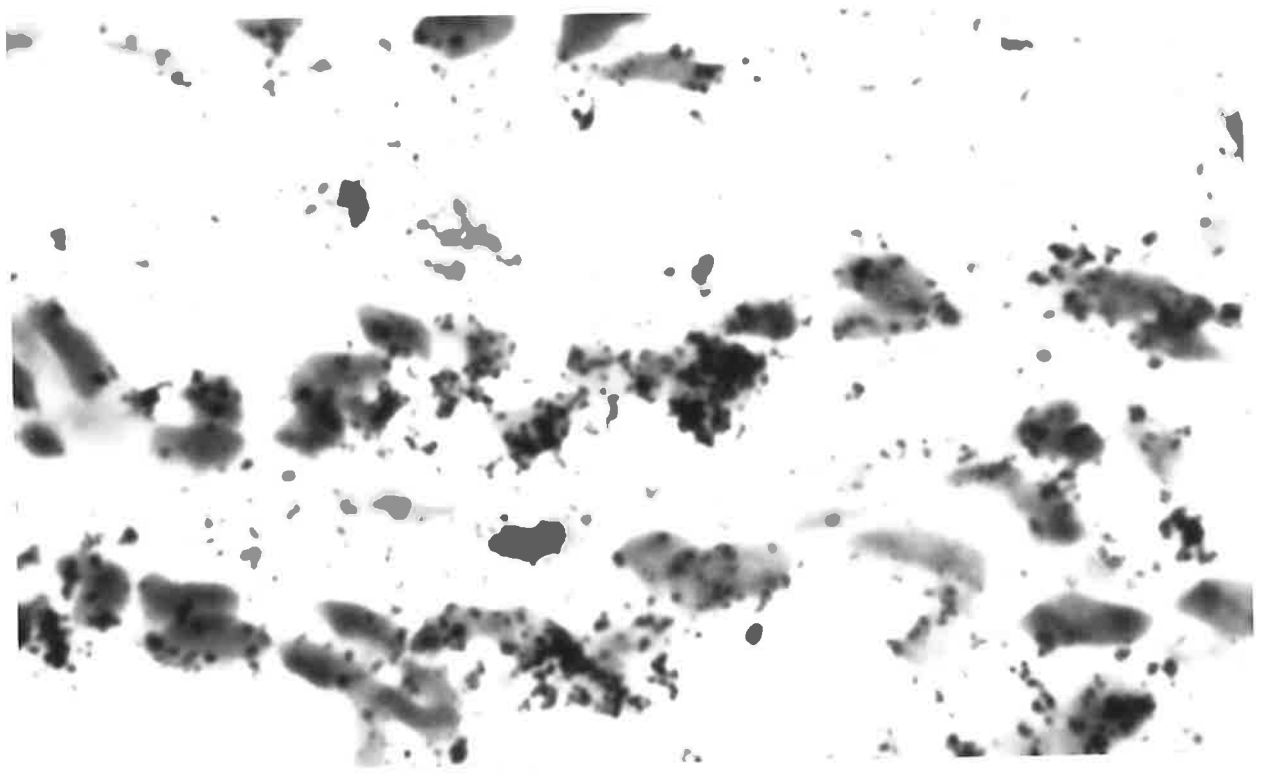
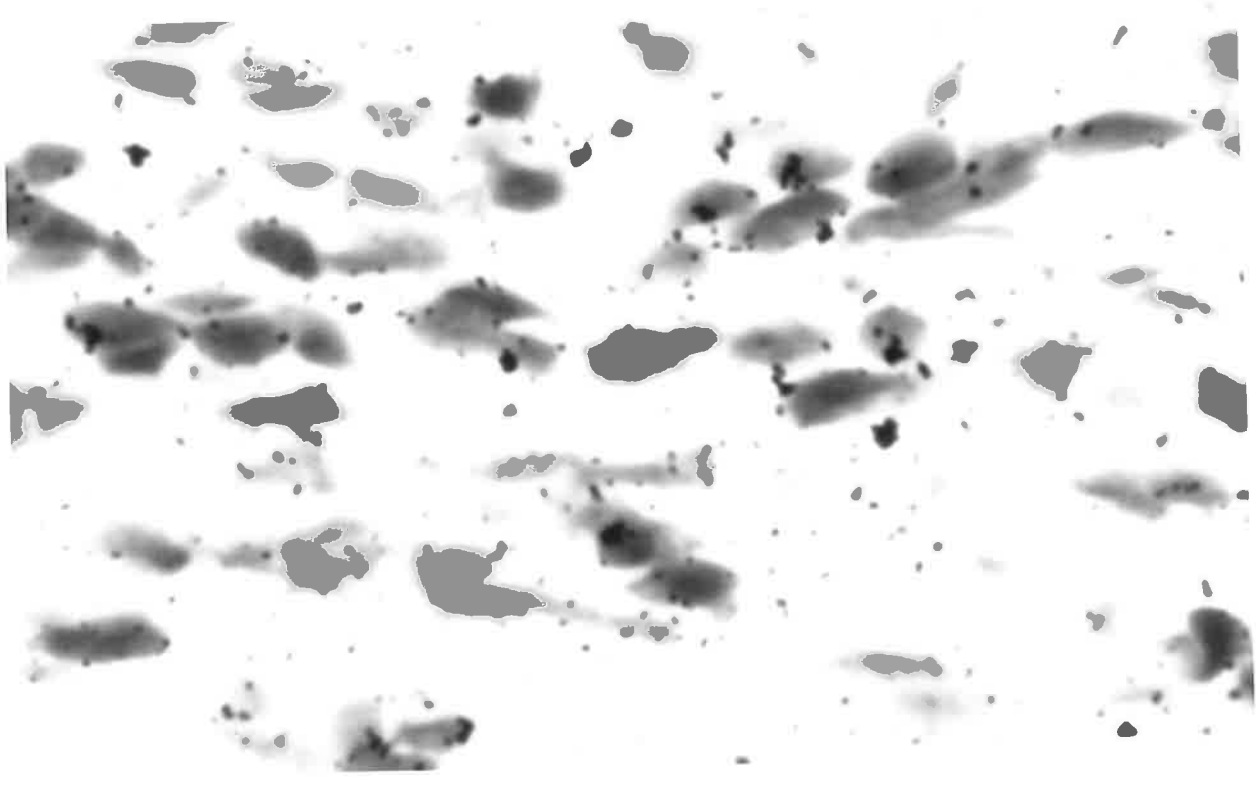
HBV DNA detected in the nuclei of small round cells after in situ hybridisation with <sup>3</sup>H-labelled pHBVCB. These cells were identified as T4 lymphocytes using specific antisera on sequential sections as described in the text.

TOP The grain density in lymphocytes which were denatured.

BOTTOM The grain density in undenatured lymphocytes.

Haematoxylin and eosin; 6 months autoradiographic exposure.

Magnification X1650.



Immunohistochemical staining of these cells in a sequential section (performed by Jim Milios, Division of Histopathology, IMVS) showed that they reacted with antibody of the T4 lineage and thus were likely to be T-helper lymphocytes. Thus, in 2 liver samples, low genome copy numbers of nuclear HBV DNA were seen in lymphocytes and hepatocellular carcinoma cells; however, nuclear HBV DNA was undetectable in typical hepatocytes which expressed only HBsAg, despite its presumed presence on theoretical grounds.

(iii) In other experiments,  $^{125}\text{I}$ -labelled probes detected cytoplasmic HBV DNA in hepatocytes after 2-3 days exposure compared with 4-5 weeks with  $^3\text{H}$ -labelled probes. However, these probes were still unable to detect HBV DNA in nuclei of either cell population described above or in hepatocytes expressing only HBsAg. Thus, the use of  $^{125}\text{I}$ -labelled probes resulted in decreased exposure times but not in increased sensitivity. Further increases in exposure times with  $^{125}\text{I}$  probes resulted in an increased background grain density and the net result was a decrease in the signal:noise ratio.

**c. Examination of ethanol:acetic acid fixed, paraffin embedded tissue.**

To extend the above observations, sections from ethanol:acetic acid fixed tissues (Chapter 2, Part 6aiv), from both groups were then examined with  $^3\text{H}$ -,  $^{32}\text{P}$ - and  $^{125}\text{I}$ -labelled probes, although the sample from the hepatocellular carcinoma was not examined. Although the histological appearance of

the tissue sections was very much improved, low levels of nuclear HBV DNA previously detected in T-lymphocytes in frozen sections were not clearly detected. A focus of cells morphologically similar to T lymphocytes in sections from a liver in group 2 (not examined in the above long exposure experiment), showed an increased grain density with the  $^{32}\text{P}$ -labelled probe (Figure 5.6). This provided independent support for the identification of lymphocytes containing HBV nucleic acid sequences in the above long exposure experiments (part b), although the intracellular location of this target could not be determined due to the poor autoradiographic resolution of  $^{32}\text{P}$ . These cells could not be identified immunologically, because the surface antigens were unreactive by PAP presumably due to ethanol:acetic acid fixation. Lymphocyte surface antigens have been reported previously to be unreactive in formalin fixed tissue (Hsu et al, 1983).

Examination of liver number 6 in group 1 confirmed that high levels of HBV DNA were localised to the cytoplasm of infected cells and emphasised the high quality of the tissue histological appearance using this procedure (Figure 5.7).

#### **4. CONCLUSIONS AND DISCUSSION.**

##### **a. Identification of replicative intermediates.**

The experiments presented in this chapter demonstrated the presence, in the cytoplasm of infected hepatocytes, of large amounts of HBV DNA that was unlikely to represent assembled

**FIGURE 5.6**

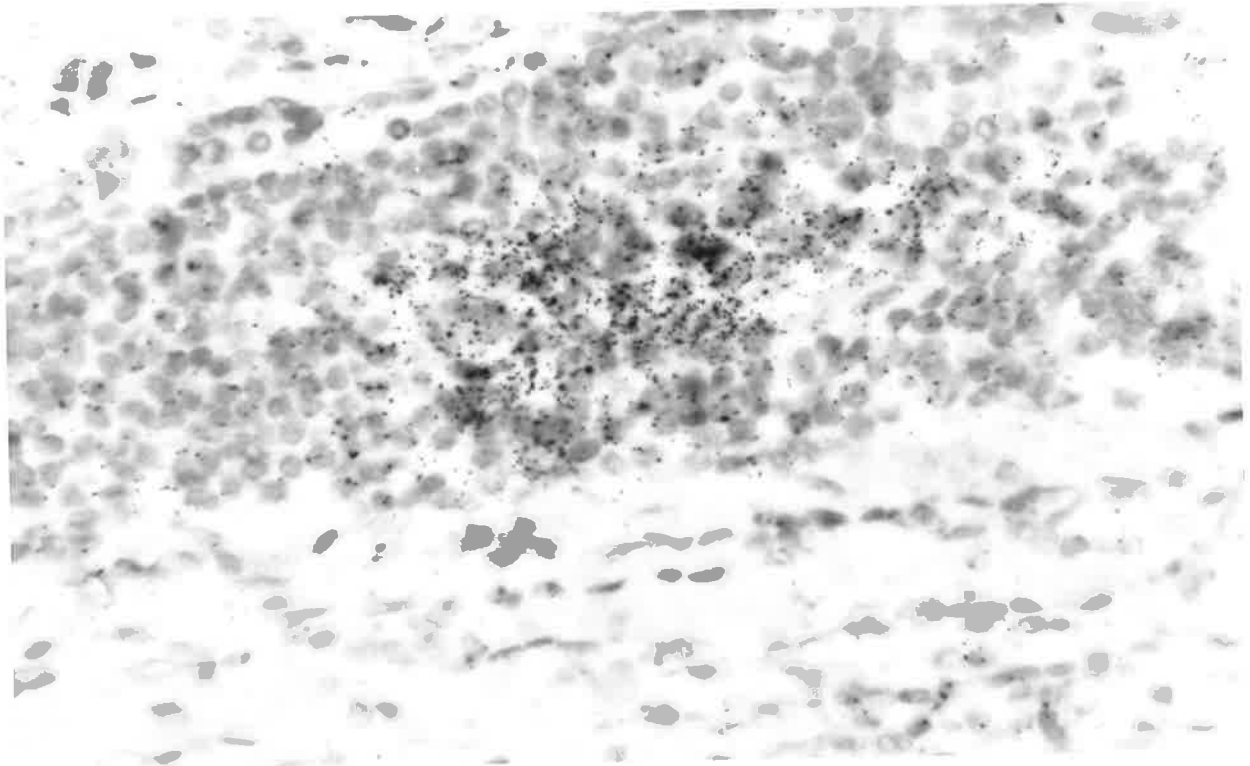
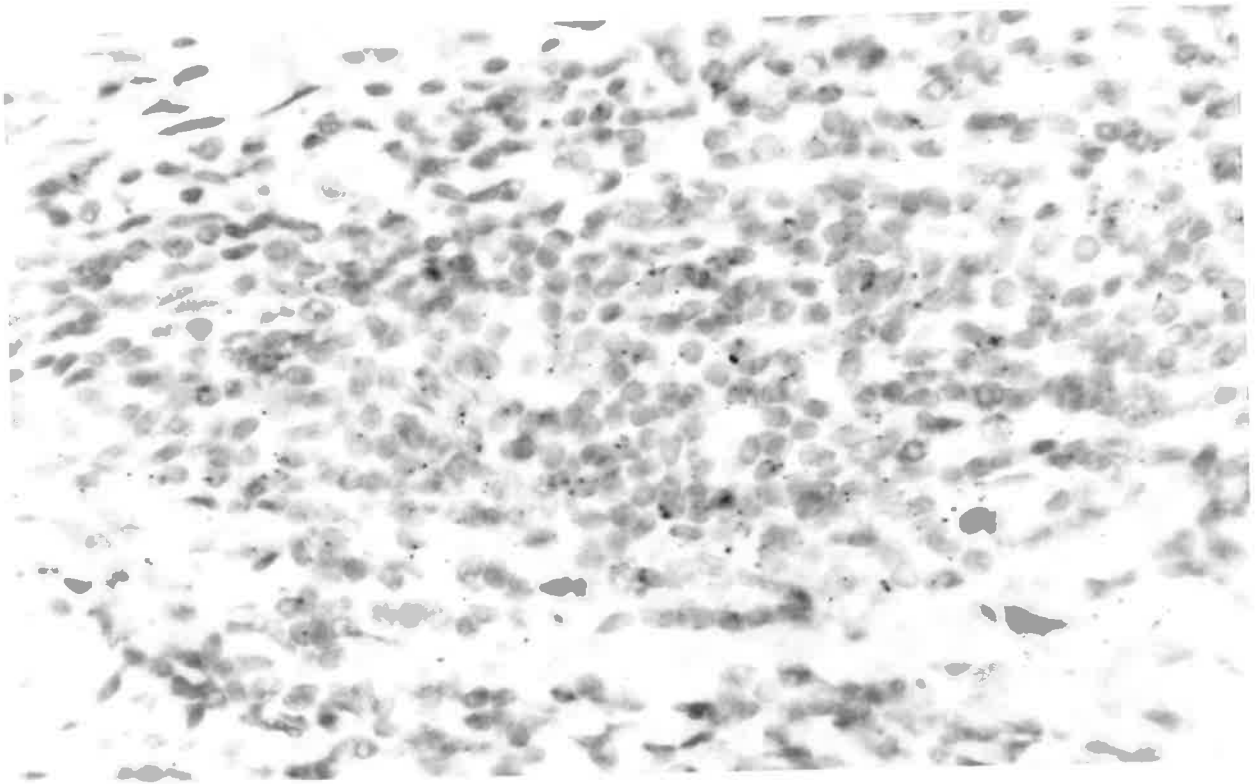
HBV DNA detected in a small focus of lymphocytes in ethanol:acetic acid fixed, paraffin embedded tissue.

TOP  $^{32}$ P-labelled-excised HBV DNA was used in in situ hybridisation to detect HBV DNA and;

BOTTOM pBR322 used as a control.

The autoradiographic exposure time was 17 days.

H & E, magnification X600.

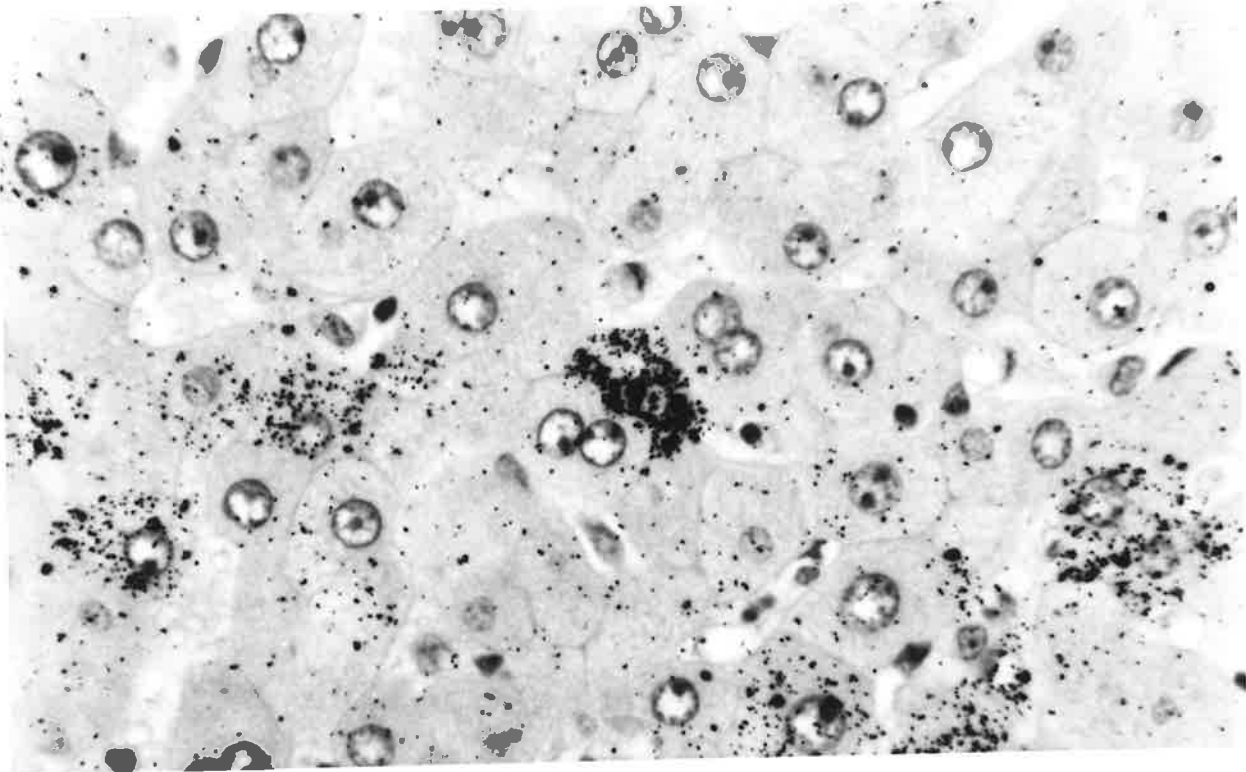
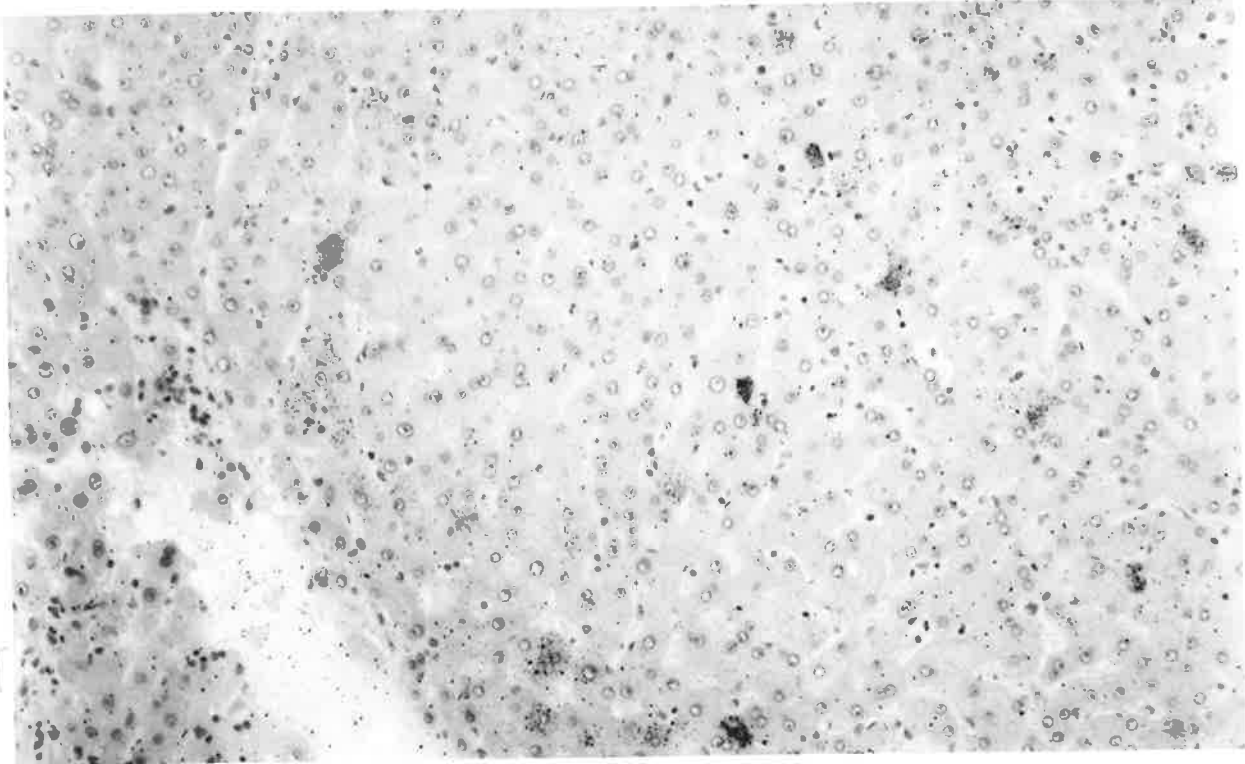




**FIGURE 5.7**

HBV DNA detected in the cytoplasm of hepatocytes in ethanol:acetic acid fixed, paraffin embedded tissue. <sup>3</sup>H-labelled pHBVCB was used in the in situ hybridisation experiment and the autoradiographic exposure time was 5 weeks, H & E.

Magnification: TOP           X150  
                  BOTTOM       X600.



virions awaiting export from the cell. This may be concluded, since negative polarity, ssDNA, from widely separated regions of the virus genome, was a principal target of the in situ hybridisation reaction. The above ss HBV DNA included a region of the genome (HBcAg gene) that was shown to be invariably ds in one study of circulating virions (Delius et al, 1983), although a minority of virions in serum have recently been shown to contain RNA-DNA hybrids (Miller et al, 1984b) or negative polarity ss DNA also considered to be replicative intermediates (Scotto et al, 1985). Thus, these results suggest that the intracellular virus DNA comprised replicative intermediate forms, at least to a significant extent. These observations indicate that virus DNA replication is probably ongoing in these hepatocytes.

Specific HBV mRNA was undetected in infected cells using the described in situ hybridisation protocol. This was probably due to the labile nature of mRNA, which requires different conditions of tissue preservation and section fixation for its detection by in situ hybridisation (Maitland et al, 1981; A.R. Jilbert, personal communication).

Although the above experiments were unable to formally prove that de novo synthesis of HBV DNA occurred in the cytoplasm, these in situ hybridisation results contrast with similar in situ hybridisation studies with papovaviruses (Dorries et al, 1979; Langelier et al, 1975; Neer et al, 1977), adenoviruses (Moar and Jones, 1975) and herpesviruses (Moar and Klein,

1978; Ross et al, 1981; Volsky et al, 1981). In each of these studies with viruses which replicate their DNA in the nucleus, autoradiographic grains representing viral DNA were confined to the nucleus. On the other hand, in situ hybridisation studies on cell cultures infected with rabbitpox virus (known to replicate the viral DNA in the cytoplasm) detected viral DNA in the cell cytoplasm (Minnigan & Moyer, 1985). Similarly, intranuclear inclusions in cells infected with parvoviruses (Kurstak, 1972), papovaviruses (Oxman, 1978), adenoviruses (Ledinko, 1978) and herpesviruses (Crouse et al, 1950) are usually Feulgen-positive, whereas those in HBV infected hepatocytes are usually Feulgen-negative (Huang et al, 1972; Bianchi and Gudat, 1976).

The cytoplasmic localisation of high levels of HBV DNA has been confirmed more recently by Blum et al (1984a) using in situ hybridisation, and the negative sense polarity of the ssDNA confirmed by Blum et al, (1984a), Miller and Robinson (1984) and Fowler et al (1984) using Southern blot and dot blot analyses.

These results are also generally compatible with a replication strategy of another Hepadnavirus, the Duck Hepatitis B Virus (DHBV) proposed by Summers and Mason (1982). In this model, discussed more fully in Chapter 6, cytoplasmic viral RNA is used as a template for the

transcription of negative strand viral DNA which is, in turn, used as the template for the (partial) transcription of the second (positive sense) DNA strand.

However, this model makes no provision for the presence of ss positive sense viral DNA detected in the experiments in this chapter. Although only one strand of the M13 probes was radiolabelled, it is possible that the complementary unlabelled strand in the probe may have hybridised to viral DNA in the sections and that the labelled strand may, in turn, have hybridised to this unlabelled probe strand. This 'network' formation is well described for probes > 500 bases long in the presence of 10% dextran sulphate (Gerhardt et al, 1981) and it is possible that even without dextran sulphate that this may have occurred to a lesser extent in the experiments in this chapter.

In summary, there is a very sharp contrast between the cytoplasmic location of replicative intermediates of HBV DNA on the one hand, and the nuclear location of the DNA of other viruses known to replicate their DNA in the nucleus on the other hand.

**b. Different forms of HBV DNA detected.**

Large amounts of HBV DNA discussed above were detected in the cytoplasm of hepatocytes and low genome copy numbers of HBV nucleic acid sequences were detected in the nucleus of hepatoma cells and T-lymphocytes. This result is consistent

with the detection of HBV DNA in peripheral blood leukocytes by Southern blot examination of extracted DNA (Lie Injo et al, 1983; Pontisso et al, 1984).

In contrast to cytoplasmic HBV DNA in hepatocytes, nuclear HBV nucleic acid sequences in T-lymphocytes were not detected consistently, probably because the viral nucleic acid levels in these nuclei were very close to the sensitivity limit of the assay.

In addition, none of the probes detected HBV DNA in HBcAg-negative hepatocytes which expressed HBsAg. Calculation of the HBV genome target copy number showed that hepatoma cells and lymphocytes contained approximately 5 genome copies per cell and it is therefore likely that HBcAg-negative, HBsAg-positive hepatocytes contained less than this genome copy number. However, the absolute sensitivity of the in situ hybridisation assay could not be tested, since cultured cells containing integrated HBV DNA bound pBR322 control probes non specifically using the above protocols, in contrast to the low level of control probe binding in liver sections.

The low genome copy number, nuclear HBV nucleotide sequences detected above may have represented the integrated HBV DNA detected by Southern blot in DNA extracts of infected liver (Brechot et al, 1981; Shafritz et al, 1981), although direct correlation was not possible. It was impossible to

determine if cells containing cytoplasmic HBV DNA also contained nuclear HBV DNA due to the grain scatter from the cytoplasm.

**c. Permissive and non permissive HBV infection.**

In the present work, only two cell types within the liver viz. hepatocytes and lymphocytes were shown to support HBV infection. This contrasted with the results of Blum et al, (1983), who detected low levels of HBV DNA in bile duct epithelia, smooth muscle cells and endothelial cells as well as high levels of HBV DNA in hepatocytes. There may be several explanations for these contrasting results; 1) hybridisation stringency in the Blum study was lower than that used in this study; this may result in hybridisation to nucleic acid sequences related to but not specific for HBV (but see next point) 2) it is possible that the Blum assay was more sensitive; however, using the Blum protocol, similar results to the above were obtained with the above liver samples (data not shown) 3) it is possible that the liver samples studied by Blum et al, but not the above liver samples, contained these additional infected cell types and the different results may represent different stages of infection. The significance of HBV infection of non hepatocytes in HBV pathogenesis is discussed in Chapter 8.

HBV DNA replicative intermediates were detected in hepatocytes in the above experiments. Independent electron microscopic (EM) studies of hepatocytes from HBcAg positive patients

demonstrated Dane particles budding into the cisternae of the endoplasmic reticulum (Kamimura et al, 1981; Sakamoto et al, 1983). In the absence of cell culture studies, the presence of Dane particles in EM studies - and of viral DNA replicative intermediates in other studies -- in infected hepatocytes, provide circumstantial evidence that these cells are capable of undergoing permissive infection.

In summary: the following different patterns of infection in different cells were identified in infected livers.

- i) hepatocytes containing high levels of cytoplasmic, replicative intermediate HBV DNA
- ii) cells (T lymphocytes and hepatocellular carcinoma cells) containing low copy number, nuclear, ds HBV nucleic acid sequences that were most likely to be DNA
- iii) hepatocytes expressing HBsAg but without detectable levels of HBV DNA
- iv) hepatocytes lacking any markers of infection

It is likely that i) above represent permissive infection, ii) and iii) represent restricted infection and iv) represent cells in which infection has not been established.



## CHAPTER 6

CORRELATION BETWEEN HBcAg EXPRESSION AND THE PRESENCE OF  
CYTOPLASMIC HBV DNA.

## 1. INTRODUCTION.

The in situ hybridisation experiments described in the previous chapter showed that replicative forms of HBV DNA were present in the cytoplasm of infected hepatocytes. This contrasted with the intracellular location of the bulk of the HBcAg, the virus capsid antigen, which was found predominantly in the nucleus when the livers were examined with the usual direct IF assay. However, when the livers were re-examined with the more sensitive indirect IF method (Chapter 3), an additional population of HBcAg positive cells was demonstrated.

The experiments described in this chapter examined in detail the relationship at the level of the single cell between gene expression and high levels of HBV DNA shown in Chapter 5 to represent replicative intermediates.

In these experiments, low genome copy numbers of nuclear HBV DNA (Chapter 5) or low levels of cytoplasmic HBV DNA would be undetected.

## 2. EXPERIMENTAL DESIGN.

Two livers (numbers 1 and 2 from Table 5.1) from HBsAg carriers were examined since the levels of HBCAg expression and HBV DNA replication in these samples were higher compared with other samples. The study used direct IF on frozen sections and PAP on sections from ethanol:acetic fixed tissue.

In the first part of the study, HBCAg was detected by direct IF in unfixed 6µm frozen sections (described in Chapter 2, Part 3a). The sections were then examined and selected areas showing strong immunofluorescence were photographed (as described in Chapter 2, Part 7) using incident u.v. illumination and the location of each field recorded accurately on the vernier scale of the microscope stage. The IF mounting medium was then removed in PBS and the section fixed for in situ hybridisation as described in Chapter 2, Part 6.a.iii, including a DNA denaturing step. In situ hybridisation was then performed with <sup>3</sup>H-labelled pHBVCB and the slides exposed to the photographic emulsion for 2 weeks. After development, the same area of the tissue was relocated and a photograph of the in situ hybridisation result recorded. Both photographs were then compared and the results of HBCAg IF and HBV DNA in situ hybridisation collated.

In the second part of the study, HBCAg was detected by the peroxidase-antiperoxidase (PAP) procedure (Chapter 2, part 3b) and in situ hybridisation performed (Chapter 2, part 6.a.iv) with excised HBV probes (Chapter 2, part 4c) radiolabelled



with  $^{125}\text{I}$ . Ethanol:acetic acid fixed, paraffin embedded tissue blocks were examined. Some tissue sections were examined sequentially by PAP and in situ hybridisation, while others were examined simultaneously by performing PAP and in situ hybridisation on the same section.  $^{125}\text{I}$  probes reduced the autoradiographic exposure time to 1-2 days.

### 3. RESULTS.

Photographs of different areas from one slide showing HBcAg IF and HBV DNA in situ hybridisation were initially examined separately without matching of individual cells, in order to determine the intracellular location of each individual marker. Thus, the cells scored to complete Table 6.1A and 6.1B included many cells in common, but did not represent identical cell populations. As many cells as possible were examined to complete Table 6.1B and an equal number examined to complete Table 6.1A. In a second analysis, cells which were identified in both matched photographs were used to complete Table 6.2.

The results of the HBcAg localisation study (Table 6.1A) showed that HBcAg was predominantly cytoplasmic in liver No. 1 [105/130 (81%) of HBcAg positive cells] and predominantly nuclear in liver No. 2 [47/66 (71%) of HBcAg positive cells]. Although direct IF usually detected cytoplasmic HBcAg poorly (Chapter 3), the levels of cytoplasmic HBcAg in liver No. 1

TABLE 6.1

The analysis of cytoplasmic and nuclear HBcAg (A) and  
HBV DNA (B) in cells of two liver samples.

## LOCATION OF HBcAg

A.

<u>Liver No.</u>	<u>Nucleus</u>	<u>Cytoplasm</u>	<u>Nucleus + Cytoplasm</u>	<u>Total</u>
1	21 (16)	105 (81)	4 (3)	130
2	47 (71)	13 (20)	6 (9)	66

## LOCATION OF HBV DNA

B.

<u>Liver No.</u>	<u>Nucleus</u>	<u>Cytoplasm</u>	<u>Nucleus + Cytoplasm</u>	<u>Total</u>
1	8 (6)	122 (94)	0 (0)	130
2	5 (8)	59 (89)	2 (3)	66

The data was collected by examination of photographs of HBcAg positive frozen sections and of the in situ hybridisation photograph correlate. Figures in parentheses represent percentages. A total of 11 microscope fields were examined.

permitted adequate detection. However, the intensity of the cytoplasmic HBcAg was always very much lower than the intensity of the nuclear HBcAg.

In contrast, HBV DNA was predominantly cytoplasmic in both livers. In liver No. 1, 122/130 (94%) of viral DNA-positive cells and in liver No. 2, 59/66 (89%) of positive cells contained high levels of cytoplasmic virus DNA (Table 6.1B).

The relationship between HBcAg and HBV DNA was then examined by selecting cells on the basis of HBcAg positivity and determining if these cells contained HBV DNA. These results (Table 6.2) showed that in both livers, the vast majority [85/90 (94%) and 22/23 (96%) respectively] of cells positive for cytoplasmic HBcAg also contained HBV DNA in the cytoplasm only. In contrast, of the cells positive for nuclear HBcAg, only 18/28 (64%) in liver No. 1 and 46/86 (53%) in liver No. 2 contained nuclear or cytoplasmic HBV DNA while 4/28 (14%) and 34/86 (40%) respectively had no detectable HBV DNA at this level of sensitivity.

However, for the converse correlation, when cells were selected on the basis of HBV DNA positivity and examined for HBcAg, 61/167 (37%) and 56/106 (53%) of DNA positive cells in livers No. 1 and 2 respectively were HBcAg negative (Table 6.3). HBcAg in the remainder of the DNA positive cells was usually cytoplasmic in liver No. 1 and nuclear in liver No. 2.

TABLE 6.2

Intracellular location of grains representing large amounts of HBV DNA in cells selected on the basis of the presence of HBcAg detected by direct IF.

<u>Location of HBcAg</u>	NO. OF CELLS CONTAINING GRAINS REPRESENTING LARGE AMOUNTS OF HBV DNA IN THE LOCATION INDICATED.				
	<u>Nucleus</u>	<u>Cytoplasm</u>	<u>Nucl.&amp;cyto.</u>	<u>Negative</u>	<u>Total</u>
Cytoplasmic					
Liver no. 1	1	85	0	4	90
Liver no. 2	0	22	0	1	23
TOTAL	1	107(95%)	0	5	113(100%)
Nuclear					
Liver no. 1	6	16	2	4	28
Liver no. 2	6	37	9	34	86
TOTAL	12(11%)	53(46%)	11(10%)	38(33)	114(100%)

TABLE 6.3

Correlation between HBV DNA detected by in situ hybridisation and HBcAg in the same cell when cells were selected on the basis of HBV DNA positivity and examined for HBcAg.

NO. OF CELLS SELECTED BY HBV DNA POSITIVITY

WITH HBcAg DETECTED IN

<u>Liver</u>	<u>Nuclear(N)</u>	<u>Cytoplasm(C)</u>	<u>N &amp; C</u>	<u>Negative</u>	<u>Total</u>
No. 1	23(14)	69(41)	14(8)	61(37)	167(100)
No. 2	31(29)	11(10)	8(8)	56(53)	106(100)
TOTAL	54(20)	80(29)	22(8)	117(43)	273(100)

In all instances, cytoplasmic HBV DNA and HBcAg were often localised to discrete intracellular foci which sometimes but not always corresponded.

The above experiments showed that cytoplasmic HBcAg, if detectable, was highly predictive of cytoplasmic HBV DNA but that detectable HBV DNA did not reliably predict the presence of HBcAg. However, as the experiments were cumbersome, only a small cell population was examined; furthermore, the experiments of Chapter 3 showed that the direct IF assay to detect HBcAg only detected a proportion of HBcAg-positive cells. For these reasons, when a PAP assay to detect HBcAg (Chapter 3) and an in situ hybridisation assay to detect HBV DNA (Chapter 4) in fixed tissue blocks were developed, these assays were used to extend the above studies.

HBcAg and HBV DNA were detected in 5µm sections cut from paraffin wax embedded, ethanol:acetic acid - fixed tissue blocks as described in Chapter 2, parts 3b and 6.a.iv respectively, either in sequential sections or simultaneously in the same section. With the improved histological appearance, within each infected liver, HBcAg positive cells were generally seen in discrete foci, within parenchymatous nodules. Two different types of infected foci were evident: Type 1 comprised a mixture of cells, those with only cytoplasmic HBcAg and those with nuclear and cytoplasmic HBcAg, while Type 2 foci contained cells almost exclusively with only cytoplasmic HBcAg (Fig. 6.1). Examination of a



**FIGURE 6.1**

HBcAg detected in foci of HBV infected cells in ethanol:acetic acid fixed, paraffin embedded tissue. HBcAg was detected by PAP.

TOP shows the focal nature of infected cells.

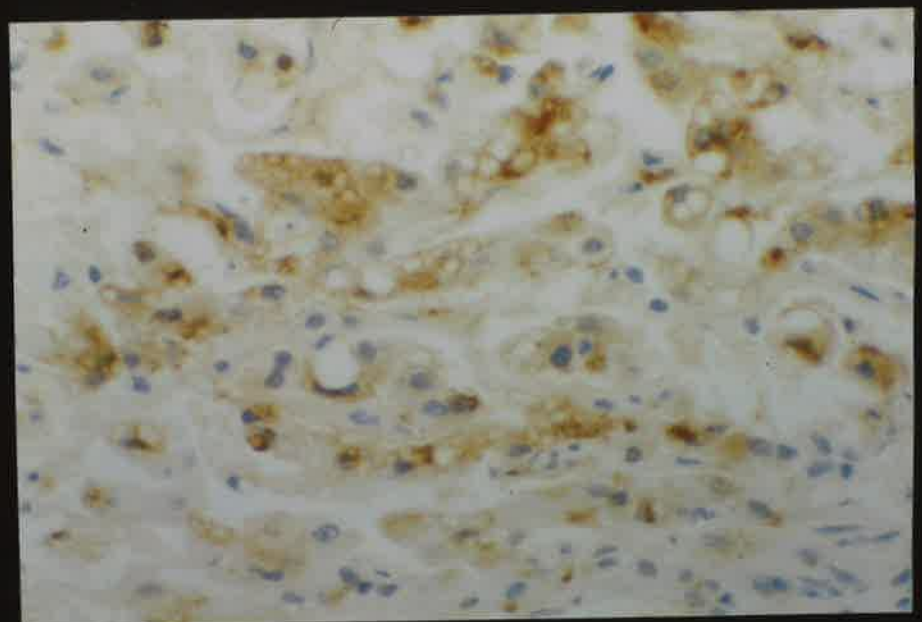
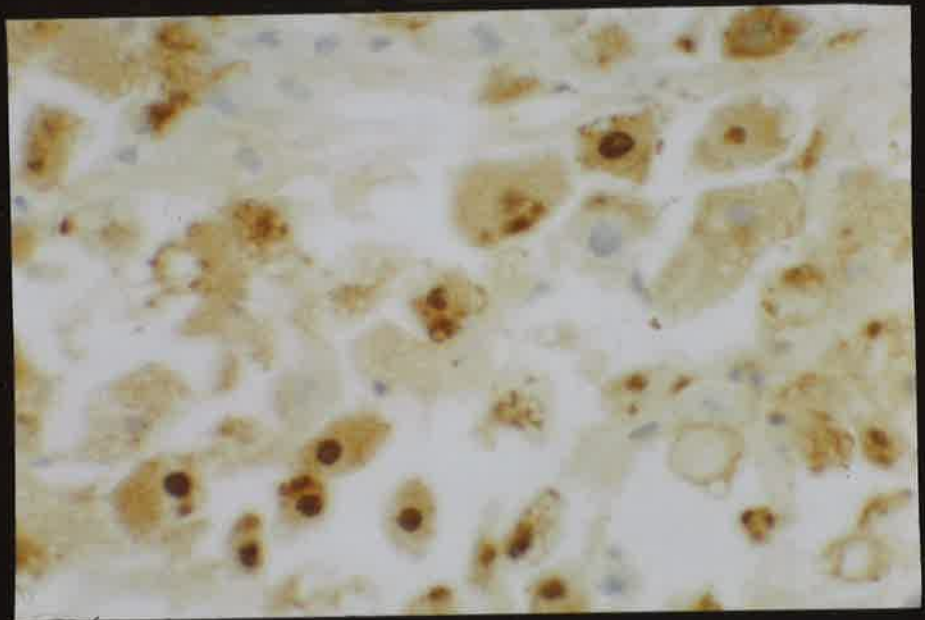
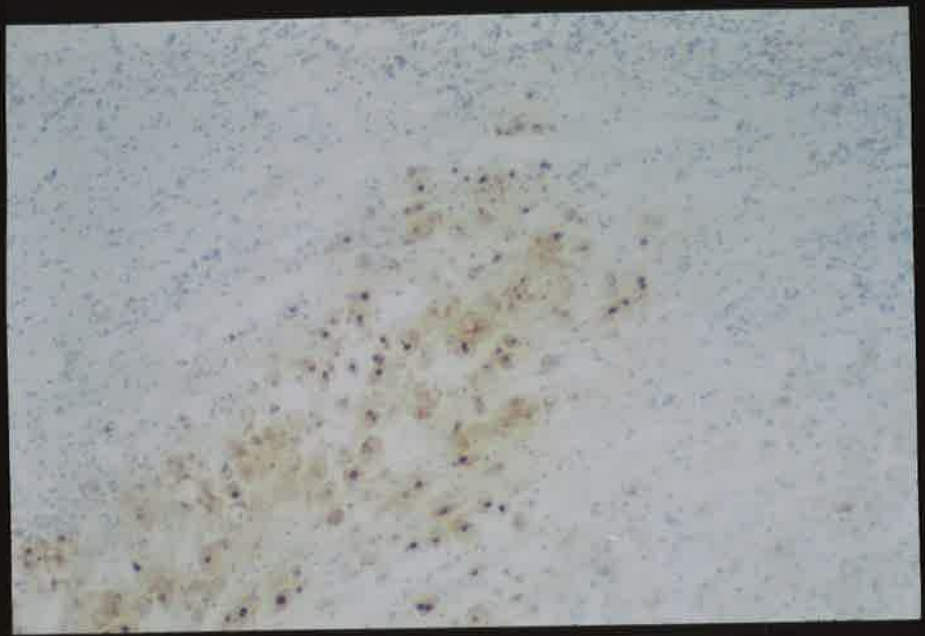
TOP & CENTRE show a Type 1 focus, and

BOTTOM shows a Type 2 focus. Note vacuolation of HBcAg positive cells (Further reference is made to this in Chapter 7)

Haematoxylin:magnification

TOP X110

CENTRE & BOTTOM X450



number of different foci, showed that the proportion of cells in Type 1 foci expressing nuclear as well as cytoplasmic HBcAg varied, but the number was always lower than the total of cells with cytoplasmic HBcAg (Table 6.4). Cells with only nuclear HBcAg were not seen.

HBcAg and HBV DNA were then correlated in single cells, both by the use of sequential sections and by simultaneous HBcAg and HBV DNA detection in the same section. In both types of foci, the extent of which was defined by the extent of the (predominantly) cytoplasmic HBcAg, cytoplasmic HBV DNA was present in most hepatocytes within the focus (Fig. 6.2).

Simultaneous PAP/in situ hybridisation demonstrated that, in different cells and in different foci, the levels of cytoplasmic HBcAg and HBV DNA ran in parallel; in contrast, the presence or absence of nuclear HBcAg was unrelated to the levels of either cytoplasmic marker (Fig. 6.3). No HBV DNA-positive, HBcAg-negative cells were seen, while the converse situation, HBV DNA negative -, HBcAg positive - cells was sometimes seen in cells that were weakly HBcAg positive in the cytoplasm.

#### 4. CONCLUSIONS AND DISCUSSION.

The above results indicated that cytoplasmic, rather than nuclear HBcAg, is closely related to HBV DNA replication. These results were obtained in two different sets of

TABLE 6.4

Analysis of the number of cells in different foci positive for only cytoplasmic HBcAg or for nuclear and cytoplasmic HBcAg using the PAP method.

<u>Focus</u>	LIVER NO. 1		LIVER NO. 2	
	HBcAg		HBcAg	
	<u>Only Cytoplasmic</u>	<u>Nuclear and Cytoplasmic</u>	<u>Only Cytoplasmic</u>	<u>Nuclear and Cytoplasmic</u>
Type 1	20 (74)	7(26)	179(64)	100(36)
	174 (54)	147(46)	29(49)	30(51)
	443 (63)	264(37)	40(28)	102(72)
	66 (47)	74(53)	73(47)	82(53)
	173 (64)	96(36)	107(60)	72(40)
			66(39)	102(61)
			129(61)	81(39)
			139(55)	114(45)
			70(65)	37(35)
Type 2	95 (86)	15(14)	181(86)	30(14)
	18(100)	0 (0)		
	78 (93)	6 (7)		
	101 (99)	1 (1)		
	135(100)	0 (0)		

Numbers in parentheses indicate percentage of total cells scored in each focus. Cells positive for only cytoplasmic HBcAg were scored only if a well defined nucleus was seen.

**FIGURE 6.2**

The detection of replicative levels of HBV DNA in the cytoplasm of infected hepatocytes in a Type 2 focus which was positive for only cytoplasmic HBcAg.

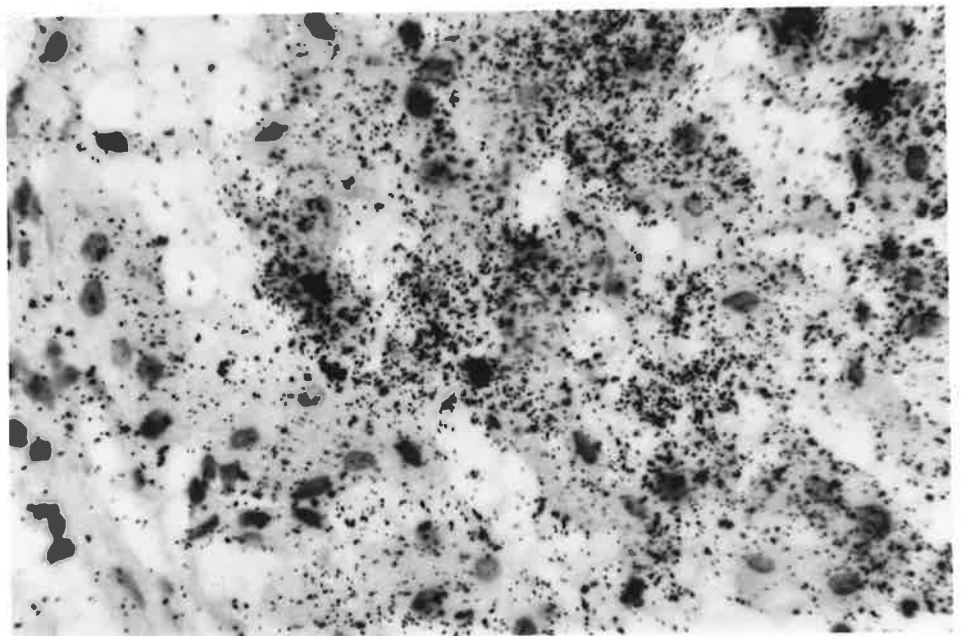
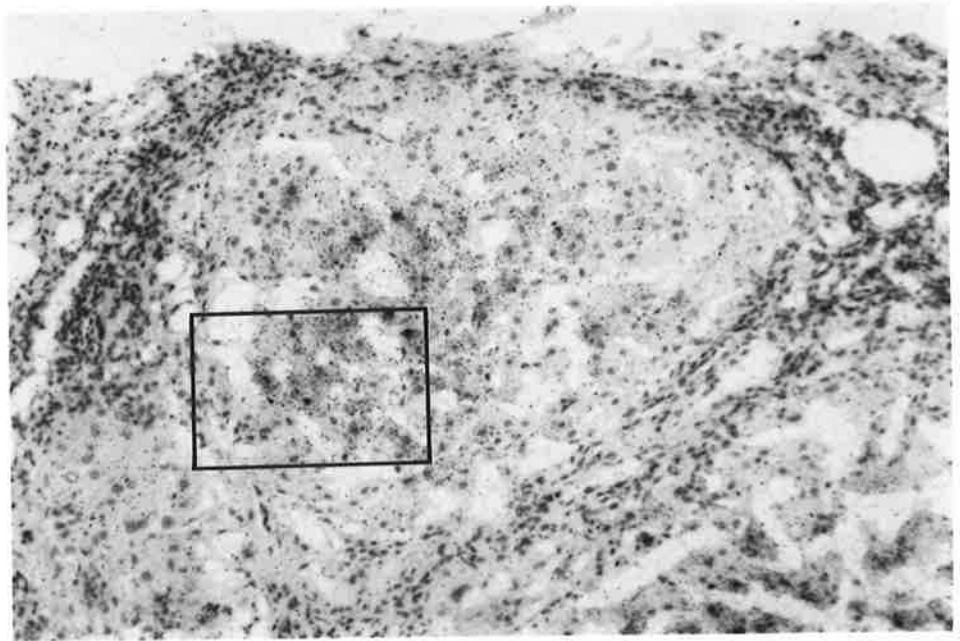
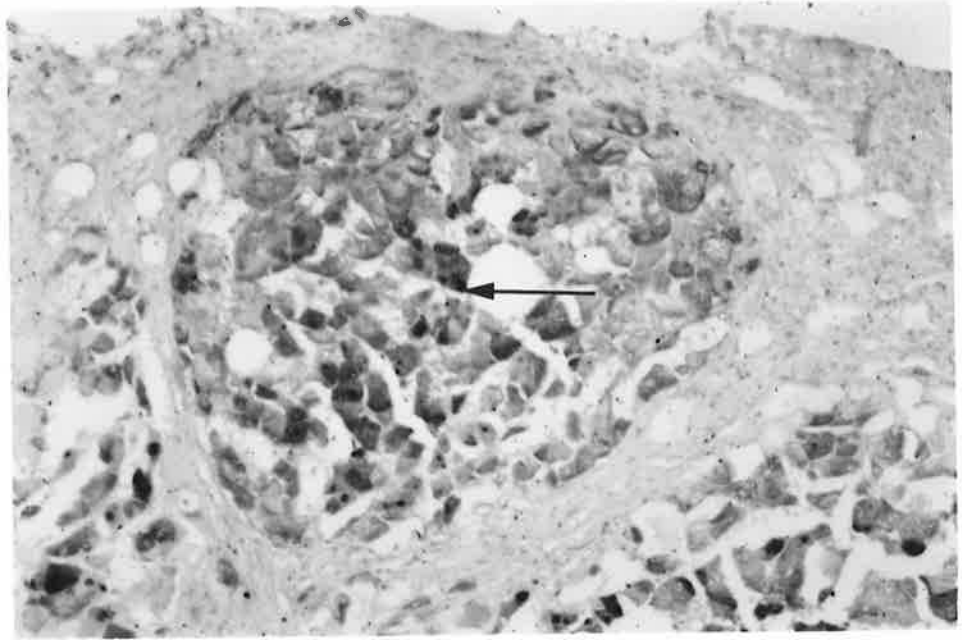
TOP A focus of HBcAg-positive cells surrounded by HBcAg-negative fibrous tissue. A Type 1 focus is seen in the lower right. HBcAg was detected by PAP.

CENTRE & BOTTOM in situ hybridisation to detect HBV DNA in a sequential section to that shown in TOP.

TOP was stained very lightly with haematoxylin. It is clearer in the original slide than the black and white photograph. This field contains only one nucleus (arrowed) positive for HBcAg. CENTRE & BOTTOM were stained normally by haematoxylin. The autoradiographic exposure after in situ hybridisation with <sup>125</sup>I-labelled excised HBV DNA was 2 days.

Magnification: TOP & CENTRE X120  
BOTTOM X475

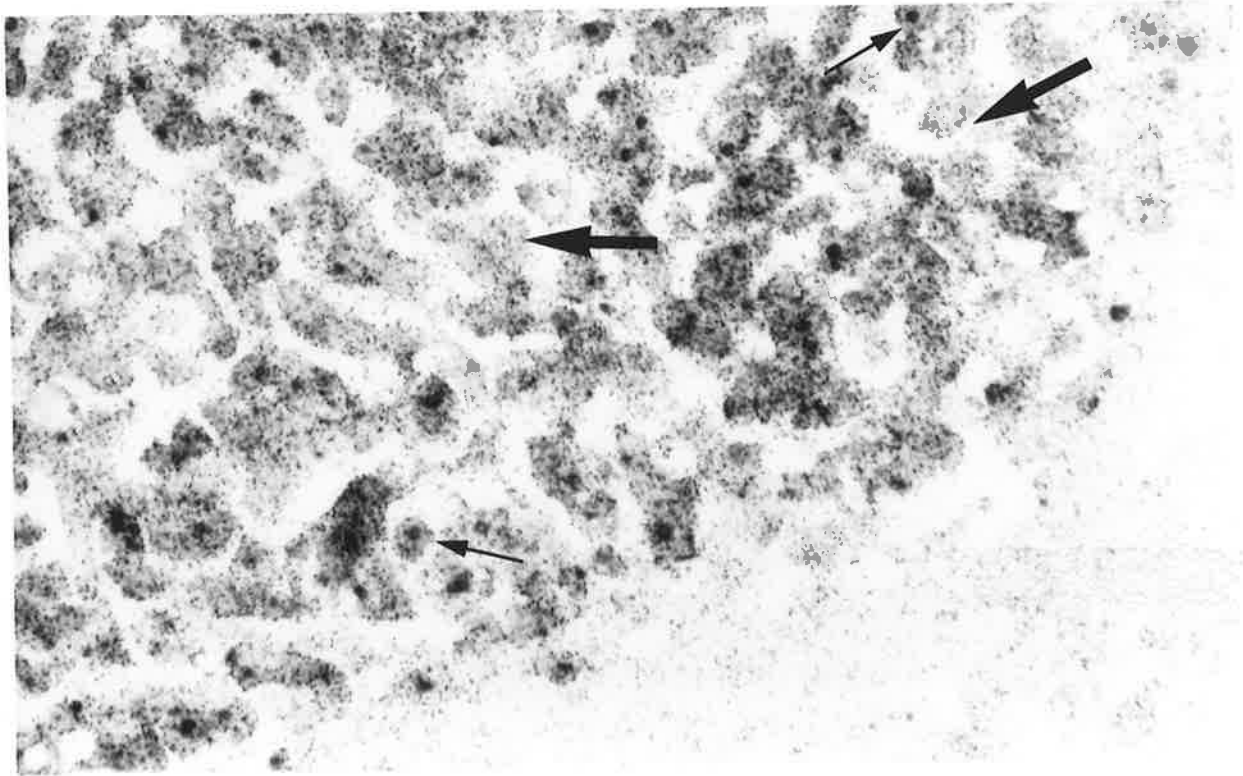
That area of CENTRE enlarged in BOTTOM is delineated.



**FIGURE 6.3**

A Type 1 focus tested simultaneously for HBcAg and HBV DNA in an ethanol:acetic acid fixed tissue section. An area negative for HBcAg and HBV DNA is shown at the upper left. Cells with only cytoplasmic HBcAg (large arrows) show similar grain densities to cells with nuclear and cytoplasmic HBcAg (small arrows). HBV DNA was detected with <sup>125</sup>I-labelled excised HBV DNA probes. The section was stained very lightly with haematoxylin and the autoradiographic exposure was 3 days.

Magnification X230.





experiments; however, two main differences between the respective data were evident. Firstly, when frozen sections were examined by direct IF, 43% of the total cells with HBV DNA were negative for HBCAg (Table 6.3), while in the experiments in sections from fixed tissue blocks examined by PAP all HBV DNA positive cells contained cytoplasmic HBCAg. This finding is consistent with results from Chapter 3 that demonstrated the increased sensitivity of the latter procedure in detecting cytoplasmic HBCAg in the two livers examined. The second major difference concerned nuclear HBCAg positive cells: in the frozen section study, 38/114 (33%) of this cell population was negative for cytoplasmic HBV DNA (Table 6.2), while all cells containing nuclear HBCAg were also positive for cytoplasmic HBV DNA in the tissues studied after ethanol:acetic acid fixation. It was considered unlikely that the in situ hybridisation reaction with  $^{125}\text{I}$ -labelled probes was more sensitive than when  $^3\text{H}$ -labelled probes were used (see Chapter 4). The possibility of inconsistencies arising from sampling different areas of the tissues was discounted because the above discrepancy held true for both livers examined.

Thus, the most likely explanation was that, in the thinner paraffin sections with good histological appearance that were examined directly under the microscope, autoradiographic grains were more easily recognised than when photographs of the sometimes distorted cells resulting from frozen sections were examined.

The observation that cytoplasmic HBcAg and cytoplasmic HBV DNA are closely related differs from the results of Blum et al (1984b), who, after similar simultaneous HBcAg/HBV DNA detection studies, reported that most cells with high levels of cytoplasmic HBV DNA did not contain HBcAg. The most likely explanation for these different results is the probable poor detection of cytoplasmic HBcAg in formalin fixed tissue (see Chapter 3) used in the Blum study.

The above experiments identified that the widespread population of infected hepatocytes which contained previously undetectable cytoplasmic HBcAg, also very frequently contained replicative cytoplasmic HBV DNA but lacked nuclear HBcAg. The proposed replication strategy of Duck HBV (DHBV), (Summers and Mason, 1982) is based on the description of immature core particles containing viral RNA which is transcribed into viral DNA. It is possible that the closely associated HBcAg and replicative HBV DNA described in the present study represent similar structures in human HBV infection. Alternatively, some of the HBcAg identified in this study may represent free HBcAg polypeptides or mature or empty assembled HBcAg particles. It was not possible to distinguish between these alternatives. Independent reports have localised WHcAg (Frommell et al, 1984) and replicative WHV DNA (Mitamura et al, 1982) to the infected cell cytoplasm.

It is possible that nuclear HBcAg is unconnected with the virus replication strategy and represents antigen produced in excess of packaging requirements that migrates to the nucleus as a result of its high concentration of basic, protamine-like amino acid sequences (Murray et al, 1981). The HBcAg peptide has recently been shown to bind HBV DNA thus confirming the predicted nature of the protein (Petit and Pillot, 1985). In addition, HBcAg polypeptides produced in E.coli by recombinant DNA technology assembled into 27nm particles containing DNA of unknown origin (Cohen and Richmond, 1982). However, it is possible that HBcAg can assemble without DNA since a subpopulation of "empty" capsids have been isolated from infected liver (Kaplan et al, 1976). The mechanisms controlling whether HBcAg either migrates to the nucleus or remains in the cytoplasm are unknown, as are mechanisms of assembly into HBcAg particles. It is possible that migration to the nucleus occurs in cells where the virus export or budding process is a rate limiting step.

In order for Hepadnaviruses to fulfil the above proposed replication strategy, certain molecular requirements are necessary. These include:

- (a) a template for viral RNA production and
- (b) a viral replicase possessing both reverse transcriptase and DNA-DNA polymerase functions.

The intracellular location of HBV mRNA and pregenome RNA synthesis has not been identified. The template requirement may be provided by supercoiled episomal (Mason et al, 1983;

Miller and Robinson, 1984) or integrated viral DNA (Brechot et al, 1981) present in the nuclei of infected hepatocytes in livers supporting virus replication. The enzyme which synthesises viral mRNA and pregenome RNA from this template has also not been identified. Host cell RNA polymerases are likely candidates since the coding capacity of the virion genome is limited and the virion polymerase is unlikely to be sufficiently versatile to perform this function in addition to those outlined below.

The viral replicase function is presumably performed by the virion polymerase. In vitro, the polymerase has the ability to function as an RNA- or DNA- dependent DNA polymerase (Miller et al, 1984a) and it is likely that it can fulfil a similar function in vivo. It is also possible that the 5' end terminal protein on the long (complete) strand of the genome acts as a primer for the reverse transcription reaction as even small nascent negative strand DNA molecules are covalently linked to this protein in the Duck HBV (Molnar-Kimber et al, 1984). The role of the 11 base pair direct repeats found on either side of the overlap region in all four Hepadnaviruses is unclear. However, since these repeats are highly conserved, it is likely that they play an important role in virus replication or control (Seeger et al, 1984) or in viral DNA integration (De Jean et al, 1984a). It is still unclear if integration is necessary for virus

replication. Thus, although the broad concept of the HBV replication strategy has been proposed, much of the fine detail has still to be determined.

In this context, the marked focal distribution of HBcAg positive cells in general and of cells that in addition contain nuclear HBcAg, requires comment. It is possible that cell to cell spread of infectious virus results either in foci of cells at the same or a similar stage of the virus replication cycle, or that there is a focal distribution of cells with intrinsically different patterns of response to the same infective virus. London and Blumberg (1982) have previously suggested that there may be different hepatocyte populations with differing responses to HBV infection. It is also possible that cells with only cytoplasmic HBcAg represent an early stage of the virus replication cycle and cells with both nuclear and cytoplasmic HBcAg represent a later stage. At an even later stage, as a result of host responses or virus strategy, virus replication and viral antigen display may be restricted, so that the eventual outcome is cells which express only HBsAg or only nuclear HBcAg (see Chapter 7), without supporting virus replication. Restricted antigen display represents one method of how virus infected cells avoid immune clearance (Mims, 1981).

Specific details of HBV replication and timing are dependent on pulse labelling experiments if and when a cell culture system becomes available. Until then, replication studies will be restricted to the study of naturally infected liver or to animal models.

## CHAPTER 7

## LIVER HISTOLOGY AND MARKERS OF HBV INFECTION.

## 1. INTRODUCTION.

This chapter examines in detail the relationship between a number of markers of virus infection and/or replication, serological status and histological diagnosis in different patients. HBV DNA was detected in liver samples by an in situ hybridisation reaction with a sensitivity of 10-15 genome copies of HBV per cell (Gowans et al, 1981). From the results of the previous chapters, it was considered likely that HBV DNA detected by this assay was likely to identify cells supporting virus replication. Consequently, any cells containing fewer HBV genome copies would appear DNA-negative, even if a low genome copy number of integrated virus DNA was present or even if a very low level of virus replication was ongoing.

In addition to the above aims, the experiments described in this chapter were also designed to investigate a possible direct link between HBV replication and histological damage. Although HBV is generally considered to be non cytopathic, previous in situ hybridisation results have shown in two liver samples that hepatocytes with large amounts of cytoplasmic HBV DNA corresponded to damaged cells with hydropic degeneration (Gowans et al, 1981). It was considered important therefore, to examine a number of liver samples in order to extend this

previous observation and determine the extent of any relationship between HBV replication and hepatocyte injury. In addition, the role of d-Ag in the disease process was also examined, since d-agent infected livers may comprise a separate group. However, it was only possible to test for d-antigen, since a cDNA probe to detect d-RNA was unavailable.

## 2. ANTI-d FITC CONJUGATE PREPARATION AND EVALUATION.

A small aliquot of anti-d FITC conjugate (prepared by Dr M. Rizzetto) was initially received from Professor Yvonne Cossart, Department of Bacteriology, University of Sydney. This conjugate was then used to screen a number of stored liver samples for d-Ag. One liver (JMcG) with intensely staining widespread d-Ag was identified. 6um frozen sections of this liver were then used as the substrate to detect anti-d by indirect IF in sera from patients attending the Infections Diseases Clinic, Royal Adelaide Hospital.

A serum sample from a HBsAg-positive carrier was found to have a high anti-d titre. As this serum was negative for anti-HBs, and had only a low titre of anti-HBc, it was used as the source of antibody for the preparation of the anti-d FITC conjugate (described in Chapter 2, Part 2c). Subsequent detection of d-Ag in liver JMcG showed that this conjugate detected d-Ag with a higher intensity than the original (Rizzetto) conjugate and showed a similar focal distribution of d-Ag positive hepatocytes. The conjugate was titrated and



further evaluated by testing against a normal liver and livers known to contain HBcAg. At a working dilution of 1/200, the conjugate showed a slight reaction with livers containing HBcAg but not with the normal liver. To investigate this reaction, an aliquot of the conjugate was adsorbed against a liver homogenate made from a HBcAg positive liver and the conjugate retested against the same and another HBcAg positive liver. These results (Table 7.1) showed that the anti-HBc activity in the anti-d FITC conjugate was completely abolished by this treatment, while the anti-d activity remained virtually unaffected. It was concluded that the anti-d FITC conjugate reacted strongly with d-Ag and weakly with HBcAg, but the conjugate was considered suitable for use since HBcAg was detected by a separate specific immunofluorescence reaction and the extent of any reactions could then be evaluated accordingly. The bulk of the anti-d conjugate was not adsorbed with a HBcAg-positive liver homogenate due to the difficulty in obtaining sufficient tissue. The anti-d FITC conjugate was then used to examine a number of liver samples and the results described below.

### 3. EXPERIMENTAL DESIGN.

Frozen sections from autopsy or biopsy liver samples were tested for HBsAg and HBcAg by indirect IF, d-Ag by direct IF as described in Chapter 2, part 3a and HBV DNA by in situ hybridisation as described in Chapter 2, part 6.a.ii. However, in some biopsy cases, HBcAg was detected by the

TABLE 7.1

The staining intensity in four HBsAg positive livers (1-4) and in a normal liver (5) after a direct immunofluorescence reaction with an anti-d FITC conjugate that had been adsorbed with normal liver, followed by no further treatment or by adsorption with a HBcAg positive liver homogenate.

Liver	Indirect IF staining for,		Staining intensity with anti-d FITC conjugate.	
	HBsAg	HBcAg	<u>With no treatment</u>	<u>Adsorbed with HBcAg positive liver</u>
1	P*	P	+	-
2	P	P	+	-
3	P	N	++++	+++
4	P	N	++++	+++
5	N	N	-	-

Livers No. 1 and 2 correspond to patients No. 1 and 2 of Table 7.2.

\* P = Positive; N = negative.

direct IF reaction only, since at the time of testing, the rabbit anti-HBc reagent was unavailable and the tissue was exhausted when it did become available. Full length plasmids pHBV114 and pHBVCB radiolabelled with  $^3\text{H}$  were used in the in situ hybridisation studies. Tests for HBsAg, HBcAg and HBV DNA were performed together or within a relatively short period on different individual livers, but IF tests to detect d-Ag were performed when the anti-d FITC conjugate was prepared some months later.

Liver samples from 21 HBsAg-positive and 12 HBsAg-negative patients were examined. These comprised:

- i. 18 consecutive biopsy or autopsy samples from acute or chronic hepatitis cases submitted to the Hepatitis Research Laboratory, IMVS,
- ii. 5 biopsy samples from HBsAg-positive renal transplant patients,
- iii. 7 cases with chronic hepatitis referred to the IMVS laboratory by other workers, and
- iv. 3 normal livers chosen randomly.

Histological diagnoses were made on tissue that was fixed in formalin either at the time of sampling, or (in 6 cases) after an interval of storage as frozen tissue. Tissue sections from formalin fixed blocks were stained by haematoxylin and eosin (H&E), coded and examined independently by Dr R. Rowland, Division of Histopathology, IMVS and Dr Pauline Hall,

Department of Histopathology, Flinders University of South Australia. The two reports, which showed good correlation, were collated to provide the histological diagnosis for each patient.

Serological markers of infection were measured where possible by the Hepatitis Diagnostic Laboratory, IMVS using Ausria, Ausab and HBeRIA to detect HBsAg, anti-HBs and HBeAg or anti-HBe respectively. However, serum samples were unavailable from some patients and HBeAg or anti-HBe had been measured by the less sensitive gel diffusion test in 6 serum samples (Table 7.2). The specificity of IF and in situ hybridisation reactions were determined as described in Chapter 2, parts 3d and 6c respectively.

#### **4. RESULTS.**

##### **a. HBV.**

Of the 21 livers from serologically HBsAg-positive patients, only 17 of these were positive in the liver for HBsAg. This discrepancy probably reflected the sampling error associated with small biopsy samples (the serologically HBsAg-positive, liver HBsAg-negative samples were biopsies). Only 9 of the 21 serologically HBsAg-positive patients were positive for HBcAg in the hepatocytes. Of these 9 samples, 7 were positive for HBcAg in both the cytoplasm and nucleus of infected hepatocytes, 1 was positive in the cytoplasm only and 1 was positive in the nucleus only. As this latter sample could

only be examined by the direct IF reaction, it is possible that low levels of HBcAg in the cytoplasm were present but undetected (Chapter 3). However, this liver may represent a group with a different HBcAg distribution, since in most other livers a proportion of the cytoplasmic HBcAg detectable by PAP was also detectable by direct IF. Only 1 of the serologically HBsAg-negative patients showed HBsAg in hepatocytes. This patient had suffered acute hepatitis 2 months before and had recently seroconverted to anti-HBs. The histology from this patient showed acute viral hepatitis. These results are summarised in Table 7.2.

HBV DNA was detected in 8 livers. In each case, the virus DNA was predominantly cytoplasmic and detection was restricted to those livers with cytoplasmic HBcAg. The one liver with only cytoplasmic HBcAg was positive for HBV DNA, while the one liver with only nuclear HBcAg (but see above) was negative for HBV DNA (Table 7.2). Since the lower limit of detection of this in situ hybridisation assay was around 15 HBV copies per cell, the high grain density over many cells in these 8 livers reflected a considerably higher HBV DNA content.

**b. Presence of d-Ag.**

The presence of d-Ag was then examined in samples from serologically HBsAg positive patients. Apart from a few normal livers (serologically HBsAg negative) that were tested as controls, it was considered unrewarding to test the remainder of these samples since d-Ag has been classically

described in HBsAg-positive, HBCAg-negative livers (Rizzetto et al, 1977). Samples from patients Nos. 13, 14 and 15 (Table 7.2) were unavailable. Four of the samples tested were positive for d-Ag. These samples were negative for HBCAg (Table 7.2).

As was the case with HBCAg detected by direct IF, the bulk of d-Ag was detected in the nucleus of infected hepatocytes although a number of cells also showed cytoplasmic staining (Fig. 7.1). These results are similar to those of Rizzetto et al (1977).

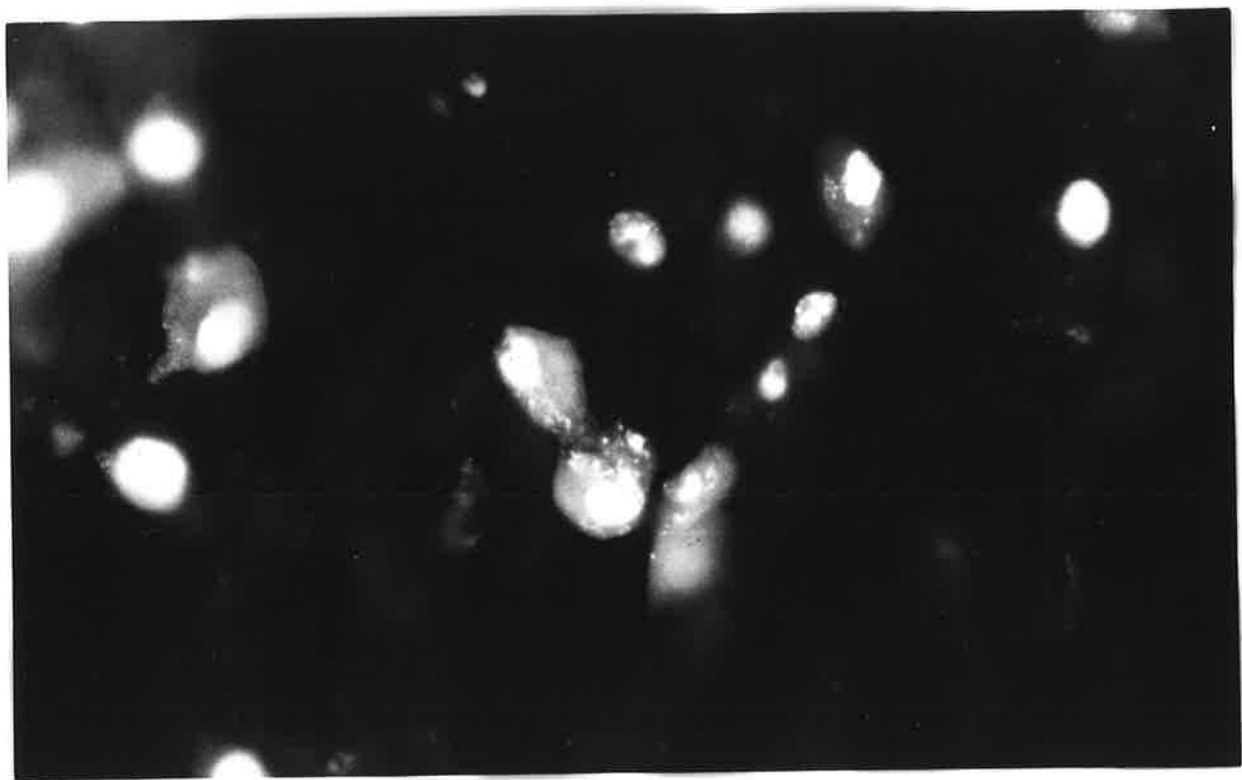
Three of 4 of the d-Ag positive patients were also positive for anti-HBe and neither HBeAg nor anti-e was detectable in the fourth patient. These results confirmed that d-Ag positive patients are generally negative for liver HBCAg and serum HBeAg and are usually associated instead with anti-HBe (Rizzetto, 1983).

**c. Cumulative results.**

As a result of these experiments, 4 distinct patient groups shown in Table 7.2 emerged:

**FIGURE 7.1** d-Ag detected in the nucleus and cytoplasm of d-agent infected cells by a direct IF reaction.

Magnification X600.





Group 1: Evidence of HBV replication.

serum HBsAg-positive, liver HBeAg-positive  
(cytoplasmic), d-Ag negative, liver HBV DNA  
positive;

Group 2: HBV infected, no detectable virus replication.

serum HBsAg-positive, liver HBeAg-negative  
(cytoplasmic), d-Ag negative, liver HBV DNA  
negative;

Group 3: HBV and d-agent coinfecting.

serum HBsAg-positive, liver HBeAg-negative  
(cytoplasmic), d-Ag positive, liver HBV DNA  
negative;

Group 4: No virus infection identified.

serum HBsAg-negative, liver HBeAg negative, d-Ag  
negative, liver HBV DNA negative.

Although the HBeAg results were incomplete, 5 of 6 patients in Group 1 were HBeAg-positive and 7 of 8 tested in Groups 2 and 3 were anti-HBe positive. This confirms a close association between HBV replication and HBeAg (Werner et al, 1977). Hepatocyte injury did not correlate with detectable HBV DNA in different patients, since histological evidence of chronic hepatitis was seen in significant numbers of livers in Groups 1, 2 and 3 despite the fact that only livers in Group 1 were identified as showing HBV DNA replication. Some HBsAg-negative livers in Group 4 also showed histological evidence of disease activity. However, in those livers that were positive for HBV DNA, the presence of viral DNA was closely

restricted to foci of cells showing cellular vacuolation and hydropic changes [evidence of hepatocyte injury (see Figure 6.1)]. A previously undescribed finding was the correlation between HBV replication and cirrhosis, as 7 of 8 patients in Group 1 but only 1 of 7 in Group 2 showed histological evidence of cirrhosis. In addition, 3 of 4 patients positive for d-Ag in Group 3 also showed evidence of cirrhosis as did 3 of the 12 patients in Group 4 (Table 7.2). Cirrhosis is usually present in d-Ag positive livers from HBsAg positive carriers (Rizzetto et al, 1983; Popper et al, 1983).

## 5. CONCLUSIONS AND DISCUSSION.

HBV DNA detected by in situ hybridisation in the cytoplasm of infected hepatocytes identified those liver samples in which HBV replication was thought to be ongoing. HBV DNA was undetected in the remainder of the HBsAg-positive livers and in HBsAg-negative livers. Patients positive for liver HBV DNA (Group 1) all showed cytoplasmic HBcAg and also tended to have serum HBeAg, which are presumed markers of virus replication (Werner et al, 1977; Gudat et al, 1975). In contrast, patients in Groups 2 and 3, negative for replicative levels of HBV DNA by in situ hybridisation, lacked liver HBcAg (with one exception, see below) and serum HBeAg and instead were anti-HBe positive. The one patient in Group 2 with liver HBcAg and serum anti-HBe was shown to have only nuclear HBcAg in contrast to Group 1 patients who either had only cytoplasmic HBcAg (1 patient) or more commonly, nuclear and

cytoplasmic HBcAg (7 patients). Livers with only nuclear HBcAg may indicate a later stage of viral replication; these results are consistent with the results of Chapter 6 and confirm that cytoplasmic HBcAg is a better marker of HBV replication than nuclear HBcAg.

Hepatocyte injury was equally common in livers with (Group 1) or without (Groups 2-4) evidence of HBV replication, but in livers of Group 1, HBV replication and histological evidence of cell damage were usually seen in the same cells. However, the results do not distinguish between HBV replication as the cause (direct or indirect) or effect of hepatocyte injury

It is possible that different mechanisms of damage in Group 1 and 2-4 were operating. Evidence for more than one mechanism for hepatocyte injury was supported by results from Weller et al (1982a), who showed that, among chronic liver disease patients, only those patients with anti-HBe but not HBeAg showed improved serum levels of liver enzymes after prednisolone/azathioprine (immunosuppression) therapy. Thus, one mechanism of damage in HBV infection may be related to HBV replication (Group 1) while other(s) unrelated to HBV replication may result from either immunological mechanisms or from infection with other agents (eg. d-agent).

The detection of d-Ag identified a group of patients (Group 3) that otherwise were indistinguishable from Group 2 patients and also showed hepatocyte damage. If d-Ag behaves like

HBcAg and is a marker of d-agent replication, then d-agent replication may be directly related to hepatocyte injury. This has previously been suggested by Popper et al (1983). However, interpretation of the above results will ultimately require proof by nucleic acid hybridisation to ascertain that d-Ag positive livers contain replicative d-RNA.

Evidence that HBV replication was associated with hepatic injury was presented by Wu et al (1982). That study showed that HBsAg-positive chronic hepatitis patients who were treated with immunosuppressants showed accelerated destruction of the hepatic limiting plate that was accompanied by increased HBV replication. On the other hand, immunosuppressed HBsAg positive renal transplant patients frequently show markers of virus replication (liver HBcAg) but remain histologically normal (Gudat et al, 1975). In relating virus replication to cell injury it is possible that virus replication per se is not directly cytopathic since the appearance or exposure of novel viral or cell antigens as a result of virus replication may present a target for immunological attack. Evidence supporting this was presented by Mondelli et al (1982) and Naumov et al (1984) who showed that HBcAg was the target of an in vitro cytotoxic T lymphocyte reaction. There have been no comparable d-Ag studies performed to date.

Thus, although the mechanism of damage is still ill-defined, one possible mechanism appears to be related to virus replication (either HBV or d-agent) while another mechanism(s) unrelated to virus replication may result from immunological attack. The ability to distinguish each of the above groups is potentially important since Group 1 patients should respond to anti-viral therapy, while Group 2 patients should respond to immunosuppressive therapy. The efficacy of this dual therapeutic approach has been demonstrated recently (Thomas and Lok, 1984). It is still unknown into which category d-Ag positive patients (Group 3) will fit.

There were 2 groups of HBsAg-positive patients who showed histological evidence of cirrhosis. One group (Group 1) was associated with HBV replication and the second group (Group 3) associated with d-Ag (and perhaps d-agent replication). Thus it is possible to speculate that cirrhosis may be a more common end result of cell damage associated with virus replication (Groups 1 and 3) than where cell damage is due to other mechanisms (Group 2).

## CHAPTER 8

## CONCLUDING REMARKS.

## 1. INTRODUCTION.

The experiments described in this thesis examined in detail the expression of HBV antigens and the occurrence of viral DNA in different cells in different infected livers, leading to a deeper understanding of the differing responses of cells to infection, the mechanism and localisation of virus replication, and the relationships of the above to histological changes seen during infection. The significance of these results is discussed more fully below.

2. IN SITU HYBRIDISATION.

The development of an in situ hybridisation technique applicable to ethanol:acetic acid fixed, wax embedded tissue sections led to greatly improved histological detail compared with previous methods. In addition, simultaneous detection of virus antigen and DNA in the same section yielded more detailed information about virus-cell interactions at the level of the single cell. In recent years, study of the mechanisms by which animal viruses spread and produce disease in the host have gained increasing emphasis. The above techniques are likely to be of particular value in studying these aspects of natural virus infection in animal and man.

The in situ hybridisation assay was able to consistently detect replicative HBV DNA in either frozen or ethanol:acetic acid fixed tissue sections. However, the detection of low genome copy numbers of HBV was inconsistent and was not improved by the use of  $^{32}\text{P}$ - or  $^{125}\text{I}$ -labelled probes, since the relative degree of non specific binding of these probes was similar or worse compared with that of  $^3\text{H}$ -labelled probes. Consequently, no improvement in the signal:noise ratio could be achieved.

The in situ hybridisation procedure used in this thesis and by other workers is likely to have an intrinsic limitation on signal:noise ratio and therefore on sensitivity, due to the use of (originally) ds probes that were denatured just prior to use. During in situ hybridisation, the rate of probe self annealing in solution is likely to be around 10X faster than the rate of annealing to the solid phase (Brahic and Haase, 1978). This means that 1) self annealed probe is unlikely to contribute to the signal but retains the capacity to bind non specifically and cause higher backgrounds and 2) even at theoretically high probe concentrations, the target may remain unsaturated by probe.

This limitation may be overcome by the use of ssRNA probes (Cox et al, 1984). These are prepared from recombinant pBR322 plasmids which are modified by insertion of a promoter site for the RNA polymerase from bacteriophage SP6. Non specifically bound RNA probes may, post hybridisation, be

digested with RNAase which has no effect on RNA-DNA or RNA-RNA hybrids, with a resultant increase in the signal:noise ratio and therefore sensitivity (Cox et al, 1984). The use of  $S_1$  nuclease to digest ssDNA target in tissue sections was inefficient (Chapter 5) and was previously shown to increase the autoradiographic background when digestion was performed post hybridisation (Godard, 1983). As high levels may also digest ds hybrids, the use of  $S_1$  nuclease to remove non specifically bound DNA probes is not recommended.

The sensitivity of in situ hybridisation using the above RNA probes was shown to be seven times that of a conventional assay to detect specific mRNA ie. a ss target (Cox et al, 1984). Although for a ds target the target concentration is effectively one half, this system may increase the sensitivity of the HBV DNA in situ hybridisation assay. Experiments similar to those above but using RNA probes should be performed in order to reliably identify and study the distribution of cells containing low copy number HBV DNA.

### 3. HBV REPLICATION.

The belief that HBV would behave like other small DNA viruses as well as the classical finding of the main capsid antigen (HBcAg) located in the nucleus promoted the view that HBV replicated in the cell nucleus (Mims and White, 1984). However, the detection of cytoplasmic replicative HBV DNA and of the simultaneous presence of widespread cytoplasmic HBcAg in many infected hepatocytes described in this study suggests



that HBV replication has an important cytoplasmic phase. These results are consistent with a proposed replication strategy for a related hepadnavirus (Summers and Mason, 1982).

It is unclear from these studies if hepadnavirus replication is wholly cytoplasmic or also has a nuclear phase. In particular, it is unknown whether RNA synthesis occurs in the cytoplasm or the nucleus and in the latter case if the template is episomal or integrated HBV DNA. If integrated viral DNA is involved, the similarities between hepadnavirus and retrovirus replication strategies would become even more striking.

Different categories of virus-cell interactions in different cells were identified, that were interpreted to represent either permissive infection or non permissive infection with or without limited antigen expression. However, the control mechanisms at the molecular level that determine whether virus-cell interactions result in permissive or non-permissive infection await clarification. Furthermore, it is not known whether progression from one category of interaction to another may occur in a single cell during the course of infection, nor whether different hepatocytes are intrinsically different in their ability to support infection. The route(s) which HBV initially gains access to hepatocytes, and the reasons why a proportion of presumably susceptible cells remains apparently uninfected, are obscure at present.

However, the striking focal nature of permissively infected hepatocytes suggests that cell-cell spread of infectious virus is one possible mode of infection.

The events and response of cells to HBV infection may differ in acute HBV infection compared with chronic infection, and the factors (thought to be immunological, see below) which influence the outcome are ill defined. In different patients, seroconversion to anti-HBe (associated with cessation of virus replication and an improvement in liver function) and the converse (reactivation of virus replication) are well recognised.

The studies in this thesis have also indicated that lymphocytes can be infected by HBV. HBV DNA has also been detected by in situ hybridisation in bile duct epithelia, endothelial and smooth muscle cells (Blum et al, 1983) and by Southern blot in leukocytes (Lie-Injo et al, 1983; Pontisso et al, 1984), cells of the pancreas and kidney (DeJean et al, 1984b) and skin cells (DeJean et al, 1984b; Siddiqui, 1983). However, replicative forms of viral DNA were not detected in any of the above cell types suggesting that virus replication was not ongoing.

Extrahepatic cells may either be capable of supporting virus replication and provide a source of infectious virus for hepatocytes or conversely, may be infected consequential to

hepatocyte infection; there is a clear need for more data regarding sites of virus replication at different stages of HBV infection.

#### 4. MECHANISMS OF LIVER INJURY.

Data reported in this thesis and elsewhere suggest that, in different HBV carriers, different mechanisms for cell damage may apply.

##### 1) Patients with evidence of virus replication.

The correlation between cell damage and virus replication may be due to either a direct cytopathic effect of virus replication, or immune mediated damage directed against virus coded antigens normally expressed only during replication. With the exception of the unexplained demonstration that PLC/PRF/5 cells that are induced to express HBcAg in in vitro cultures develop a cytopathic effect (Yoakum et al, 1983), current clinical data do not support the former possibility. In vitro immunological data suggest that HBcAg may be a likely target antigen involved in the latter mechanism. New findings in this study are also consistent with this proposal. In this situation, virus specific immune reactions are likely to be both a mediator of cell damage and also responsible for containing virus spread since virus replication is regularly more marked in immunosuppressed than untreated carriers.

2) Patients without evidence of virus replication.

Current clinical data suggest immunological mechanisms may be involved (see Introduction part 13 for a list of formal possibilities).

In addition to the above proposals at the cellular level, complex mechanisms involving hormonal, immunoregulatory and anatomical factors may apply at the level of the whole organ; for example, the association in different patients between cirrhosis and virus replication reported in this study implies that, for unknown mechanisms, hepatocyte injury may be more likely to proceed to anatomical scarring if injury is also associated with virus replication.

Clarification of the above proposals requires detailed study of appropriate animal models of disease to supplement information similar to the above that can be gained from natural infections in man.

## 5. CONCLUSION.

In contrast to many other persistent virus infections, study of HBV infection has proceeded without the benefit of cell culture assays for infective virus or, until recently, readily available animal models amenable to direct experimentation. Despite this, current knowledge of HBV molecular biology and replication strategy is at an advanced level where more

specific questions about virus-cell and virus-host interactions can now be posed with more insight than previously. Continued research will undoubtedly uncover additional subtle characteristics of this fascinating virus.

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