STUDIES ON THE REPLICATION OF HEPADNAVIRUSES AND HEPATITIS DELTA VIRUS.

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CONTENTS.

SUMMARY.

Hepadnavirus and HDV replication and gene expression were examined, with particular emphasis on the block(s) preventing HBV infection in vitro, the extent of the helper function provided to HDV by HBV and the mechanism of HDV RNA replication.

ATTEMPTS TO CULTURE HBV IN CONTINUOUS CELL LINES.

A total of 23 primary and continuous cell lines of human and animal origin, including 5 derived from human hepatomas, were evaluated for their ability to support HBV infection in vitro. Infection protocols included the manipulation of classic variables, viz. virus concentration, and adsorption time and temperature, and examined the effects of hormones and substances that promote gene deregulation or cellular differentiation. However, despite these attempts, in vitro HBV infection was not achieved.

Consequently, factors which regulate HBV protein synthesis were then examined in the hope that this might in itself provide clues to HBV cultivation in vitro. These studies showed that steroid hormones stimulated HBsAg production, low calcium medium increased levels of secreted HBsAg and HBV, and 5-azacytidine treatment induced HBcAg synthesis. However manipulation of these factors still did not lead to successful cultivation of HBV.

SYNTHESIS OF INFECTIOUS DHBV IN A HUMAN HEPATOMA CELL LINE.

To examine the possibility that the HepG2 cell line, known to support HBV replication after transfection of HBV DNA could also support replication of a related hepadnavirus, a dimeric construct of DHBV DNA contained in the vector pSV2Neo was transfected into this cell line.

All five clones that were successfully expanded expressed DHBsAg, and one (G2 DHBV-3) was also positive for DHB pre-S and DHBcAg and synthesised infectious DHBV. Nevertheless, despite the G2 DHBV-3 cell line displaying a homogeneous pattern of integrated DHBV DNA, only a low

percentage of virus antigen-positive cells were detected at passage #5 or greater, and approximately one tenth of the mature virus produced by HepG2 cells after transfection with HBV DNA was synthesised. Thus, although the hepadnavirus species barrier can be breached, efficient transcription and/or translation of DHBV mRNA was likely to depend on both hepatocyte- and species-specific factors.

EXPRESSION AND PROPERTIES OF RECOMBINANT HDAg.

The gene encoding the hepatitis delta virus structural antigen was linked to a neomycin resistance gene in a retrovirus expression vector, and human HepG2 and HeLa cells transfected with the recombinant plasmid.

Several clones showed varying percentages of recombinant HDAg (rHDAg)-positive cells but most of these were lost in culture suggesting that HDAg may be cytotoxic. Thus only one cell line (A3) derived from HepG2 cells that expressed rHDAg in the nuclei of 100% of cells was expanded successfully. The HDAg staining pattern in the A3 cell line indicated a close relationship with cell nucleoli.

Analysis of partially-purified rHDAg by HPLC showed a molecular weight in the range of $7x10^2$-$2x10^3$kD that appeared to contain conformational-dependent epitopes, while the density of the antigen was 1.19g/cm^3 by equilibrium centrifugation in caesium chloride, and in rate zonal centrifugation the antigen sedimented with a value of 50S, close to that of particulate HBsAg. Immunoblotting demonstrated a single polypeptide with a molecular weight of 24kD that corresponded to the smaller of the two HDAg-specific polypeptides present in infected sera. The rHDAg polypeptide was shown to be a RNA-binding protein with specificity for both genomic- and antigenomic-species of hepatitis delta virus RNA.

The A3 cell line was also used successfully in an indirect immunofluorescence assay to detect anti-HD, and rHDAg extracted from these cells permitted the substitution of human liver-derived HDAg in a competitive anti-HD radioimmunoassay.

HDV RNA REPLICATION AND GENE EXPRESSION.

Multimeric constructs of HDV cDNA were assembled and introduced into the same retrovirus expression vector used in the experiments described above. A recombinant plasmid, designed to produce trimeric-length HDV RNA of antigenomic-sense, was transfected into a subclone of the HBsAg-positive PLC/PRF/5 hepatoma cell line, and two stable cell lines (H1δ8 and H1δ9) were selected and clonally amplified. These cells supported the synthesis of both genomic and antigenomic sense HDV RNA, and also expressed HDAg in cell nuclei in three distinct morphological patterns, including patterns typically seen in HDV-infected livers. HDAg expression was restricted to the smaller (p24) of the two HDAg-associated polypeptides in early passages of the H1δ8 and H1δ9 cell lines, but continuous passage of the cells resulted in increasing expression of the larger (p27) HDAg-specific polypeptide. Passage of the H1δ9 cell line also led to sustained expression of monomeric HDV RNA and a loss of dimeric- and trimeric-HDV RNA. These changes were accompanied by an attenuation of virus-related cytotoxicity which was a feature of early cell passage numbers. HDV RNA replication in the H1δ9 cells was resistant to actinomycin D but sensitive to 1μg/ml α-amanitin suggesting that HDV RNA-RNA replication was performed by host cell RNA polymerase II.

In an extension of these studies, HDV RNA replication was dependent on HDAg in intact cells but was independent of HDAg in nuclear extracts. It was concluded that a primary role of HDAg in HDV infection was nuclear targeting of the input RNA.

HBV HELPER FUNCTION.

Transfection of the HBV-negative cell line HepG2 with a recombinant plasmid designed to produce dimeric, genomic-sense HDV RNA also resulted in HDV RNA replication and HDAg synthesis indicating that HBV helper functions are not required for these steps of HDV replication. Nevertheless, despite the presence of replicative intermediates of HDV RNA, HDAg (p24 and p27) and

all three HBsAg envelope proteins in the H1δ8 and H1δ9 cell lines, HDV was not secreted into the culture fluid, suggesting that additional HBV helper functions are required for HDV packaging and secretion.

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.

Tom Macnaughton

19/12/90

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ABBREVIATIONS

Anti-HD	antibody to hepatitis delta antigen
ATCC	American Type Culture Collection.
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
dsRNA	double stranded RNA
DTT	dithiothreitol
gp	glycoprotein
HAT	0.1mM hypoxanthine, 0.4μM aminopterin, 16μM thymidine
IgG	immunoglobulin G
kb	kilobase
kD	kilodalton
MAb	monoclonal antibody
mRNA	messenger RNA
mw	molecular weight
NET	100mM NaCl, 10mM EDTA, 10mM Tris-HCl pH 7.4.
PBS	phosphate buffered saline; 150mM NaCl, 6mM K_2HPO_4, 2mM KH_2PO_4, pH 7.2.
rATP	riboadenosine triphosphate
rCTP	ribocytidine triphosphate
rGTP	riboguanosine triphosphate
rUTP	ribouridine triphosphate
rNTP	ribonucleotide triphosphate
rHDAg	recombinant hepatitis delta antigen
RT	room temperature.
SSC	standard saline citrate; 150mM NaCl, 15mM Na citrate, pH 7.1

ssDNA	single stranded DNA
SSPE	80mM Nacl, 10mM NaH_2PO_4, 1mM EDTA, pH 7.4.
ssRNA	single stranded RNA
STET	5% Triton X-100, 50mM EDTA, 50mM Tris-HCl pH 8.0, 50mM sucrose.
TAE	40mM Tris-HCl pH 8.0, 40mM acetic acid, 1mM EDTA.
TBE	50mM Tris-HCl pH 8.0, 50mM boric acid, 1mM EDTA.
TCA	trichloro acetic acid
TE8	10mM Tris-HCl, 1mM EDTA, pH 8.0.
TN	10mM Tris-HCl pH 7.5, 100mM NaCl
TNE	10mM Tris-HCl pH 7.4, 100mM NaCl, 1mM EDTA
TNT	10mM Tris-HCl pH 7.4, 150mM NaCl, 0.05% (v/v) Tween 20
vge	viral genome equivalents.

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CHAPTER 1

INTRODUCTION

PREFACE.

Due to the wide interest in the biology of hepadnavirus- and hepatitis delta virus-replication, rapid progress was made during the period of work towards this thesis (1983-1990 inclusive). As a result, some of the observations presented in this thesis were either confirmed by or differ from the results of other workers or in turn confirm the recent results of others. Consequently, to put the results in this thesis in perspective, reference is made in the appropriate sections of this chapter to the publications arising from this work. These publications are listed on page XIV.

1.1 OVERVIEW.

1.1A HEPATITIS B VIRUS: HISTORICAL PERSPECTIVES.

Epidemic jaundice has been known since Greek and Roman times (Krugman, 1978; Zuckerman and Howard, 1979; Purcell, 1981) and was described by Hipprocrates more than two thousand years ago. Later, during the middle ages, epidemics referred to as "campaign jaundice" were common in wartime, particularly amongst military personnel. Outbreaks were recorded in the British army both in Flanders in 1743 and in Germany from 1761-1763, jaundice afflicted Napoleon's army in Egypt (Zuckerman and Howard, 1979) and during the American Civil War an estimated 70,000 Union troops were disabled with hepatitis (Krugman, 1978). While such incidents were probably due to infection with hepatitis A virus (HAV) as a consequence of poor hygiene, an outbreak, reported as an "epidemic of icterus" in 1885 (Krugman, 1978; Zuckerman and Howard, 1979) represents the first recognised outbreak of hepatitis B virus (HBV). This occurred in shipyard workers inoculated with a batch of smallpox vaccine that contained human lymph. Over the following 70

years a causal link with hepatitis infections and contaminated syringes, needles, blood and blood products was established, with jaundice-associated illnesses observed (i) in diabetic clinics and venereal disease clinics where intravenous arsenic compounds were employed, (ii) in patients who had received blood transfusions, (iii) in children inoculated with measles or mumps convalescent serum and (iv) during the second world war in soldiers inoculated with a yellow fever (YF) vaccine containing YF virus stabilised in pooled human serum. The volunteer transmission experiments performed between 1939 and 1948 not only established that hepatitis had a viral etiology (Krugman, 1978; Zuckerman and Howard, 1979; Purcell, 1981) but also that at least two different types of viruses were responsible. In 1947 MacCallum proposed "that the virus giving rise to epidemic hepatitis should be known as hepatitis virus A, and that giving rise to homologous serum jaundice (as serum hepatitis was then known) as hepatitis virus B." (MacCallum, 1947). These terms (albeit slightly rearranged) were generally adopted in 1973 by the Scientific Group on Viral Hepatitis of the World Health Organisation (Zuckerman and Howard, 1979) and are still in use today.

For HBV the next major advance occurred when a circulating antigen (designated "Australian antigen") was discovered in an Australian Aborigine (Blumberg et al. 1965) that reacted with serum from a patient with haemophilia. This antigen, which was subsequently shown to be associated with HBV (Blumberg et al, 1967; Prince, 1968), was found to be particulate by electron microscopic examination, consisting predominantly of 19-22nm spherical particles and filamentous forms with a diameter of 20nm and lengths varying from 50-230nm (Bayer et al, 1968). Two years later Dane et al (1970) detected additional double-shelled particles of 42nm in Australian antigen positive serum, and suggested that this might represent the complete virus and the smaller particles, surplus envelope proteins. This conclusion was not universally accepted then, as many believed Australian antigen to be the infectious agent. Moreover it was even suggested that the above 42nm

particles were merely coat proteins that had undergone self-assembly in vitro (Jenson et al, 1970; Cossart and Field, 1970). However, it soon became apparent that purified Australian antigen (22nm particles) did not contain any genetic material (Millman et al, 1970; Gerin et al, 1971) whereas the larger particles were associated with DNA (Robinson et al, 1974). This observation together with the finding that infectivity titres correlated closely with the level of circulating 42nm particles (Barker et al, 1975; Robinson and Lutwick, 1976) led to the conclusion that these particles were indeed the transmissible agent associated with HBV.

In 1974 the "Committee on Viral Hepatitis" (1974) in the United States recommended the present terminology; HBsAg for the surface component or Australian antigen, HBcAg for the internal or core component (see below) and HBV for the 42nm particle. However the latter are still commonly called Dane particles after their discoverer.

1.1B PATHOGENESIS OF HBV INFECTION.

Transmission of HBV can occur both horizontally (sexual transmission, needle stick, blood transfusion) or perinatally (mother to baby) and results in a primary infection of the liver which leads to acute clinical or subclinical hepatitis. Primary infections usually resolve with clearance of virus antigens from the liver and blood, and the development of lasting immunity to re-infection. However a variable proportion of those infected develop a chronic infection and become carriers of the virus. The most prejudicial factor for this appears to be age, as of those exposed to HBV, 90-100% of neonates, 20-30% of children and 1-10% of adults develop chronic infections (Hoofnagle and Alter, 1984). World wide, there are approximately 200 million HBV carriers who provide a large reservoir from which the virus can be spread to others. In acute HBV infection, despite a preceding stage of virus replication, hepatitis is only seen after the generation of an immune response, and it has been suggested that hepatocyte injury is immune mediated (Dudley et al, 1972). More recently the mechanism for this injury has been suggested

to be cytotoxic T cells directed against membraneous expression of HBcAg (Mondelli et al, 1982; 1988).

Although the virus is hepatotropic, HBV or HB-specific antigens have been detected in a number of extrahepatic sites in chronic carriers; these include the pancreas (Shimoda et al, 1981, Dejean et al, 1984a) and pancreatic secretions (Hoefs et al, 1980), saliva (Villarejos et al, 1974; Macaya, et al,1979; Karayiannis et al, 1985), semen (Karayiannis et al, 1985), bone marrow (Romet-Lemonne et al, 1983, Elfassi et al, 1984) and peripheral blood mononuclear cells (Pontisso et al, 1984; Lie-Injo et al, 1983; Morichika et al, 1985). Apart from a potential role in HBV transmission, the significance (if any) of these extrahepatic sites in the pathogenesis of HBV is unknown. However, complete viral replication may not necessary occur at these sites, as, apart from two reports (Romet-Lemonne et al, 1983; Elfassi et al, 1984), the forms of viral DNA differed to the pattern of replicative intermediates (see section 1.4) detected in productively-infected hepatocytes.

Most of the fatalities attributed to HBV occur during chronic rather than acute infections (Ganem, 1982) as a direct result of liver failure. However, a significant proportion of carriers develop primary hepatocellular carcinoma (HCC) which is often fatal. In this context, HBV was one of the first viruses to be linked with an human cancer and the risk factor for the development of HCC in long term carriers is more than 200 times that of aged-matched controls (Beasley et al, 1981). The mechanism of HBV-induced carcinogenesis is not fully understood but the finding of integrated HBV DNA sequences in both tumour tissue and tumour cell lines (DeJean et al, 1983; 1984b), as well as in some livers from long term chronically-infected patients (Shafritz et al, 1981; Takada et al, 1990), suggests that integration precedes cancer.

1.1C ANIMAL MODELS OF HBV.

HBV is the prototype of a small group of viruses that collectively are called the Hepadnaviridae (hepatropic deoxyribonucleic acid viruses; Gust et

al, 1986). So far five viruses have been assigned to this group, three of which infect mammals viz. HBV, woodchuck hepatitis virus (WHV; Summers et al, 1978) and ground squirrel hepatitis virus (GSHV; Marion et al, 1980a) and two that infect birds viz duck hepatitis B virus (DHBV; Mason et al, 1980) and heron hepatitis B virus (HHBV; Sprengel et al, 1988). All hepadnaviruses identified to date share many common properties including morphology, replication strategy, and genome structure and organisation. Furthermore, all have a restricted host range, are principally hepatotropic and can cause chronic infections. The core and surface antigens of the three mammalian hepadnaviruses cross react to some degree and may cross-protect since chimpanzees "vaccinated" with woodchuck hepatitis surface antigen (WHsAg) were protected from subsequent challenge with HBV (Cote et al, 1986). In contrast, the two avian hepadnaviruses diverge somewhat from the mammalian species and their genomes contain three rather than four open reading frames (see below). A comparison of some of the properties of the hepadnaviruses is shown in Table 1.1.

In addition to HBV other mammalian hepadnaviruses are associated with the development of HCC; the strongest link has been established between woodchuck HCC and WHV under both natural (Summers et al, 1978) and experimental conditions. One study in particular showed that virtually all neonates infected with WHV became chronic carriers and subsequently developed HCC (Popper et al, 1987). Moreover, like HBV, chromosomal integration of WHV DNA was detected in both non-tumour (Rogler and Summers, 1984) and tumour (Ogston et al, 1982) tissue of chronically-infected woodchuck liver. HCC has also been detected in ground squirrels chronically-infected with GSHV (Marion et al, 1986) but a causal role has not yet been established. In contrast, DHBV does not appear to promote tumour development, as long-term carrier ducks held in an experimentally controlled environment do not develop HCC (Locarnini; personal communication). The only reported incidence of HCC occurred in 1 of 24 DHBV-carrier ducks in a

TABLE 1.1 Comparison of the properties of the Hepadnaviridae.

	HBV	WHV	GSHV	DHBV	HHBV
VIRION FEATURES					
Virion diam. (nm)	42	47	47	40	40-60
Nucleocapsid diam. (nm)	27	27	27	35	?
Density (gm/cm^3)	1.25	1.25	1.25	1.16	?
GENOME					
Size (bp)	3182	3308	3311	3021	3027
5' Overlap (bp)	223	212	211	46	45
5' Linked Protein (-strand)	+	+	+	+	?
5' oligoRNA (+strand)	+	+	+	+	?
Open Reading Frames	4	4	4	3	3
% Sequence Homology					
to HBV	100	70 <___> 55		40 <___>	?
to each other		83.6		78.5	
ANTIGENS					
sAg	+	+	+	+	+
cAg	+	+	+	+	+?
eAg	+	+	+	+	?
xAg	+	+	?	-	-
MISCELLANEOUS					
Genome Integration	+	+	rare	-?[1]	-
Associated HCC	+	+	+?	-?[1]	-
Experimental Host Range	Chimp Gibbon	Marmot	Woodchuck[2] Chipmunk	Goose	-

1 Only one report (Yokosuka et al. 1985)
2 Woodchucks are susceptible to GSHV but ground squirrels are not susceptible to WHV (Seeger et al, 1987).

REFS

Wain-Hobson, (1984)
Sprengel et al, 1988)
Schodel et al, (1989)

domestic flock in China (Yokosuka et al, 1985) while, in the only study to date, HHBV was not associated with an increased incidence of HCC in herons (Sprengel et al, 1988).

1.1D *IN VITRO* MODELS OF HEPADNAVIRUS INFECTION.

1.1D(a) Cell culture.

In the twelve years that followed the discovery of the HB virion (Dane et al, 1970) many attempts were made to cultivate HBV in vitro using a variety of different cell and tissue types. At best these studies resulted in the transient production of HBsAg, and less frequently in HBcAg expression (Zuckerman, 1975; Hirschman, 1984). The first successful in vitro hepadnavirus culture was established with DHBV, using primary duck hepatocytes isolated by liver perfusion (Tuttleman et al, 1986a). Ironically, many groups had attempted previously to infect (without success) primary hepatocyte cultures with HBV. However Tuttleman et al (1986a) were the first to incorporate the perfusion technique for this purpose. It was known for some time that liver perfusion gave the highest yields of functionally active hepatocytes (Berry and Friend, 1969), but this technology could not be used until the advent of the animal models of hepadnavirus infection.

The primary duck hepatocytes could only be infected with DHBV within the first four days after plating, despite the fact that cells infected within this time continued to secrete virus for the three week life of the cultures (Tuttleman et al, 1986a). The resistance to infection was attributed to a change in differentiation, a view that has been confirmed in subsequent in vitro cultivation studies of DHBV (Galle et al, 1989) , HBV (Gripon et al, 1988; Ochiya et al, 1989) and WHV (Aldrich et al, 1989).

Thus the only successful in vitro cultivation studies, to date, have depended on primary hepatocyte cultures. This approach is expensive, labour intensive and, due to the scarcity of fresh liver tissue, difficult to apply to HBV. Consequently, a need still exists for a more convenient system. Attempts to establish this are the subject of Chapter 3.

1.1D(b) HCC-Derived Cell Lines.

Although integrated HBV DNA is often a feature of HBV-induced tumours, markers of viral replication are rarely detected in the tumour tissue. Nevertheless the first in vitro models developed to study aspects of HBV replication used cell lines expanded from HBV-induced tumours. Of these cell lines, PLC/PRF/5 (Macnab et al, 1976) and Hep3B (Aden et al, 1979; Knowles et al, 1980) have been the most widely used.

PLC/PRF/5 cells contain between 4-8 copies of HBV DNA (Marion et al, 1980b; Knowles et al, 1984) that is integrated into chromosomal DNA at three different sites and includes all regions of the virus genome (Marion et al, 1980b). In contrast, the Hep3B cells only contain a single copy of HBV DNA, integrated at a single site (Knowles et al, 1984). Both these cell lines synthesise and secrete substantial quantities of HBsAg (Macnab et al, 1976; Aden et al, 1979) which is similar in size, morphology and buoyant density to the 22nm particles of HBsAg detected in HBV-infected serum (Skelly et al, 1979). In addition to HBsAg, PLC/PRF/5 can also be induced to synthesise HBcAg (see Chapter 4). Since, episomal HBV DNA was not detected in either cell line, HBV-specific mRNA synthesis must rely on integrated sequences. This is in contrast to the template used during the normal replication mechanism (see section I.4 C).

Although PLC/PRF/5 and Hep3B cells have been studied extensively and have contributed substantially to our understanding of the kinetics and pathway of HBsAg synthesis and secretion (Aden et al, 1979; Aoki et al, 1982; Patzer et al, 1984) it is perhaps ironic that the differentiated hepatocyte cell lines, HepG2, HuH6 and HuH7, derived from non-HBV-induced hepatomas have proved more useful in recent years (see below).

1.1D(c) Transfection.

The refractory nature of members of the hepadnavirus group to cultivation in vitro has led to the application of a variety of different techniques to study these viruses. Of these, transfection has become one of the most

widely used. This technique has permitted not only examination of the intracellular events of HBV replication in cell lines resistant to infection, but also the effect of introduced mutations on genome replication and gene expression.

The first transfections with HBV DNA, performed shortly after molecularly cloned DNA became available, were into continuous rodent epithelial or fibroblast cultures, or non-hepatocyte human cultures (Hirschman et al, 1980; Dubois et al, 1980; Gough and Murray, 1982; Stenlund et al, 1983). Although frequent HBsAg and occasional HBcAg expression were noted in these experiments, infectious virus was never detected despite the use of full- or greater than genome-length HBV DNA, which in similar constructs, in one study, was infectious when inoculated intrahepatically into chimpanzees (Will et al, 1982; 1985).

The synthesis of infectious HB virions was not achieved until differentiated hepatoma cell lines of human (HepG2, HuH6 and HuH7; Sureau et al, 1986; Sells et al, 1987; Tsurimoto et al, 1987; Yaginuma et al 1987) or rat origin (Q7; Shih et al, 1989) were used as the transfection targets. Similarly, for DHBV, efficient virion synthesis was only observed after transfection into the differentiated chicken liver tumour cell line, LMH (Condreay et al, 1990). However, the requirement for differentiated hepatocytes can be overcome if transcription of the large 3.5kb ("pregenomic") RNA (see Section 1.3C) is placed under the control of a foreign promoter. In this way HBV was expressed in Hela cells (Junker et al, 1987) and WHV in murine and avian fibroblasts (Seeger et al, 1989). Although the above studies demonstrate that the hepadnavirus replication cycle is dependent on hepatocyte-specific factors, episomal covalently closed circular DNA [cccDNA; the proposed template for virus-specific RNA transcription (see Section 1.4 C)] was only detected in two of these experimental systems (Sells et al, 1987; Condreay et al, 1990). While the reason for this is still unknown, it implies that a transcriptional template for virion production can also be

provided by integrated DNA in a manner similar to retroviruses.

1.2 PARTICLES ASSOCIATED WITH HBV INFECTION.

A diagram of the virus-specific products associated with acute or chronic HBV infection is shown in Fig 1.1. As well as the three classical types of particles described below, additional HBV-related products, hepatitis B e antigen (HBeAg), hepatitis B x antigen (HBxAg) and large- and middle-HBsAg have also been identified, that will be described in section 1.3 B below.

1.2A FILAMENTS AND SPHERES.

The non-infectious spheres and filaments described in section 1.1 A above are derived from viral-coded envelope protein, hepatitis B surface antigen (HBsAg) and also contain host cell-derived lipid and carbohydrate. These particles are by far the most numerous of the HBV-related particles and outnumber Dane particles by ca. 10^3-10^5 (Bond and Hall, 1972; Ganem and Varmus, 1987). The reason for such synthetic expenditure on a non-infectious product is unknown although it has been hypothesised (Ganem and Varmus, 1987) that these particles may act as "dummy" targets, adsorbing antibodies that would otherwise neutralise the virus particle. However, since these subviral particles are themselves potent immunogens and elicit the production of neutralizing antibodies which are also active against the virion this interpretation is somewhat inconsistent. The first generation HBV vaccines utilised this property and were prepared entirely from 22nm HBsAg particles purified from carrier serum (Szmuness et al, 1980).

1.2B HEPATITIS B VIRION.

Dane particles consist of an outer envelope of HBsAg and an immunologically distinct internal component, hepatitis B core antigen (HBcAg), which is exposed when Dane particles are incubated with a solution containing 0.5% Tween-80 (Almeida et al, 1971). The internal core particle has a diameter of 27nm and an icosahedral symmetry. The core of the Dane particle has been shown to contain a DNA-dependent DNA polymerase

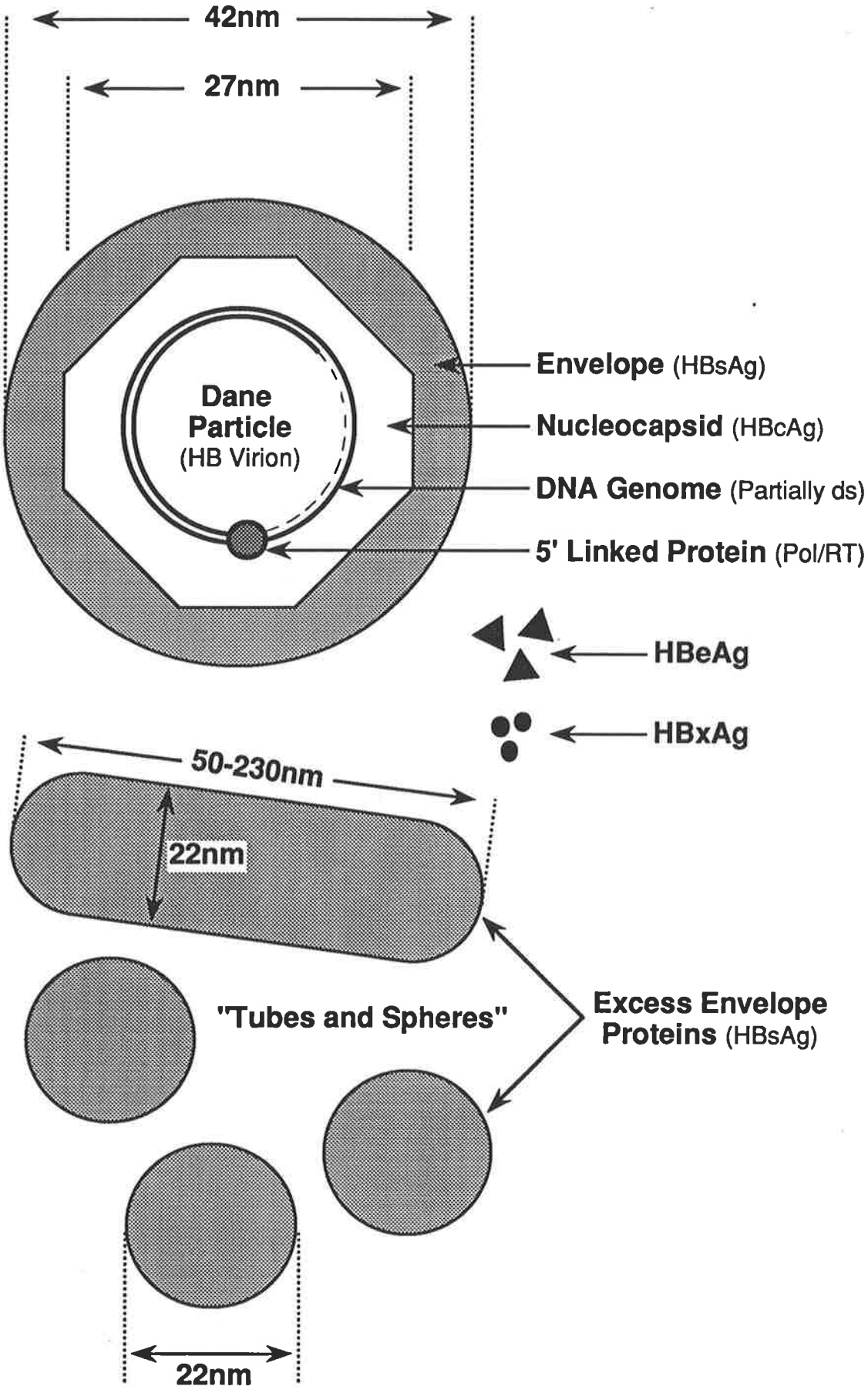


Fig 1.1. PRODUCTS ASSOCIATED WITH HBV INFECTION.

activity (Kaplan et al, 1973; Robinson and Greenman, 1974), a protein kinase activity (Albin and Robinson, 1980) and a circular partially-double stranded DNA molecule (Robinson et al, 1974; Summers et al, 1975; Robinson, 1977). Particles identical to the core of Dane particles are also found in liver homogenates from HBV-infected patients (Hirschman, 1979). Protease digestion of HBcAg produces HBeAg (Mackay et al, 1981) indicating that these two antigens are related. The molecular mechanism for HBeAg production is described in Section 1.3 B(b).

1.3 HEPADNAVIRUS GENOME ORGANISATION.

The genomes of all the identified Hepadnaviridae have been molecularly cloned and sequenced; HBV (e.g. Pasek et al, 1979; Galibert et al, 1979; Ono et al, 1983), WHV (Galibert et al, 1982; Kodama et al, 1985; Cohen et al, 1988; Girones et al, 1989), DHBV (Mandart et al, 1984), GSHV (Seeger et al, 1984) and HHBV (Sprengel et al, 1988). The genomes, which range in size from 3.0 to 3.3kbp, are the smallest of the replication-competent DNA-containing animal viruses. Nevertheless, these genomes are both compact and efficiently organised and the size belies a remarkable complexity. The structure and genome organisation of HBV (Fig 1.2) is the best understood and will be used to illustrate the features of the hepadnavirus genome. However, where HBV-specific data are unavailable or if significant differences exist between HBV and the other hepadnaviruses, then specific hepadnavirus data are included in the text.

1.3A GENOME STRUCTURE.

The HBV genome consists of a relaxed circular DNA molecule with a single-stranded gap 15-50% of genome length. The long strand [L strand or minus (-)strand] is of constant length with a nick at a defined position with respect to the 5' end of the short strand [S strand or positive (+)strand]. The S strand has a defined minimum length (Delius et al, 1983) of 1700-2800 bases with a fixed 5' end and a variable 3' end according to the length of the gap. The genome is held in a circular configuration by a ca. 200 bp overlap of the

Fig 1.2 A The structure and genome organisation of HBV DNA, illustrating positions of the direct repeats (DR1 and DR2), the protein and RNA species (∿∿) detected at the 5' ends of the (-) and (+)strands respectively, enhancer elements E1 and E2 and the glucocorticoid responsive element (GRE). The thin line of the inside circle indicates the position of the single-stranded gap. The open reading frames (ORFs) are shown with arrowheads to indicate the direction of transcription and translation. The viral RNA transcripts together with their common polyadenylation site are shown in the outermost portion of the figure.

Fig 1.2 B&C Microheterogeneity at the 5' ends of the 2.1kb subgenomic (Fig 1.2B) and 3.5kb genomic (Fig 1.2C) RNA transcripts relative to coding regions.

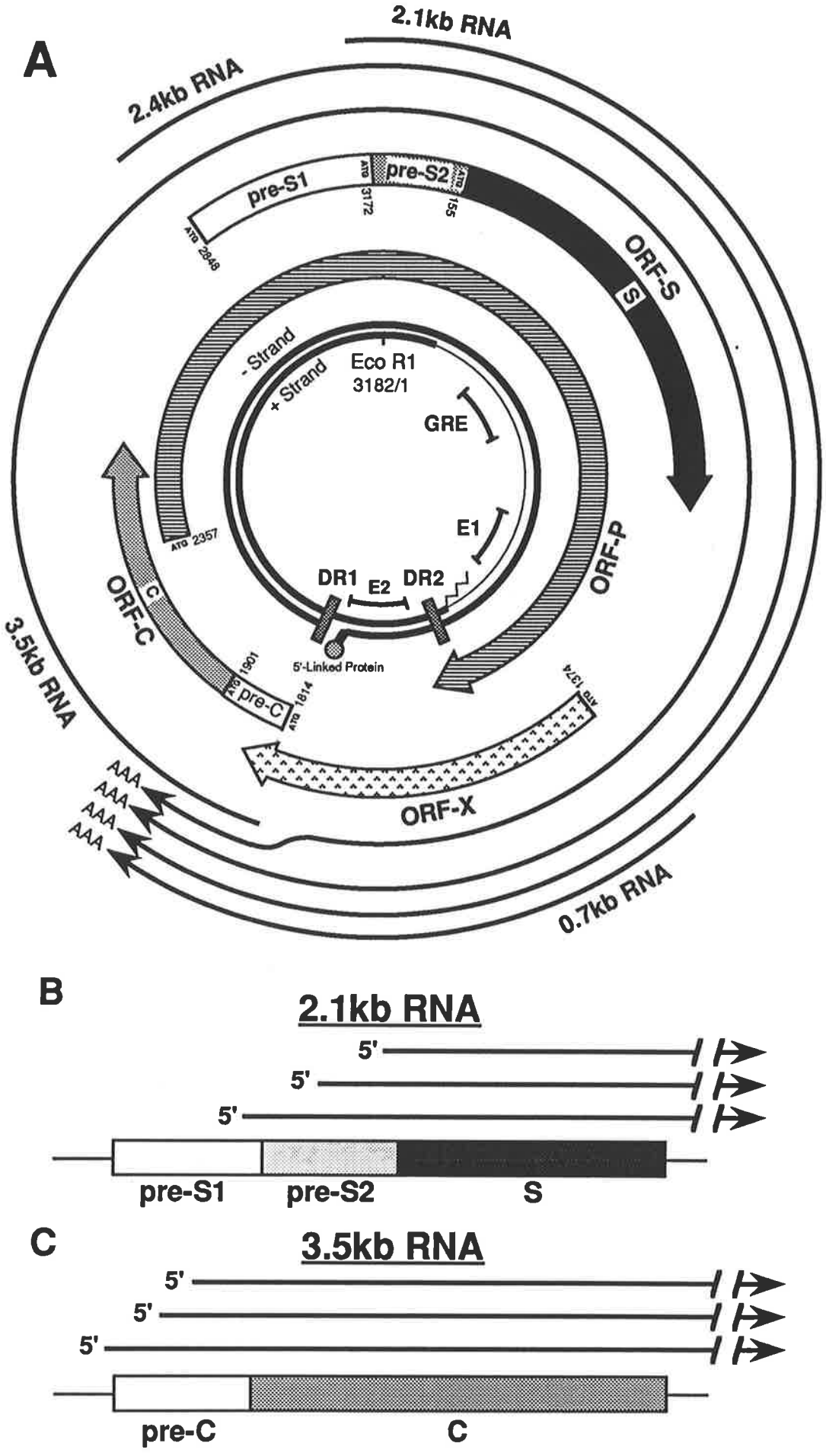


Fig 1.2 STRUCTURE AND GENOME ORGANISATION OF HBV DNA.

5' ends (Sattler and Robinson, 1979) for the mammalian hepadnaviruses or by a ca. 50 bp overlap in the case of the avian species (Sprengel et al, 1988). This overlap region is flanked by two 11bp direct repeats (shown as DR1 and DR2 in Fig 1.2A) that are vital for genome replication (see Section 1.4D and Fig 1.3) and have also been implicated in the mechanism for HBV genome integration (DeJean et al, 1984b). However, similar sequences in DHBV and GSHV do not appear to lead to the latter phenomenon. The L strand has a 9 bp terminal redundancy (Will et al, 1987) and a protein with a MW of ca. 100kD (Gerlich and Robinson, 1980) covalently linked to its 5' end that serves as the primer for L strand synthesis (Molnar-Kimber et al, 1983; Mason et al, 1987; Seeger and Maragos, 1990; see Section 1.4D and Fig 1.3). Finally, there is a capped short oligoribonucleotide sequence which is attached to the 5' end of the S strand that serves as the primer for the synthesis of this strand during genome replication (Seeger et al, 1986; Will et al, 1987; see Section 1.4D and Fig 1.3).

1.3B OPEN READING FRAMES AND THEIR PROTEIN PRODUCTS.

A comparison of cloned HBV nucleotide sequences revealed no conserved open reading frames (ORFs) in the S strand whereas four highly conserved regions (ORFs -C, -P, -S and -X; Fig 1.2A) were found on the L strand (Tiollais et al, 1981; Tiollais and Wain-Hobson, 1984; Tiollais et al, 1985). The L strand therefore carries the protein coding capacity of the genome and will henceforth be referred to as the (-)strand while the S strand will be referred to as the (+)strand. Regions corresponding to the above ORFs are also found in the other hepadnaviruses, although the two avian species lack ORF-X (see below).

All four ORFs possess a potential initiator ATG codon and each region overlaps at least one other region while ORF-P overlaps all three of the other regions. By the use of these overlapping ORFs the (-)strand of the HBV

genome can be read ca. 1.5 times thereby extending considerably its protein coding capacity.

1.3B(a) ORF-S.

ORF-S which spans ca. 1200bp contains three, in-frame, initiation codons that subdivide the region into three domains (pre-S1, pre-S2 and S; Tiollais et al, 1985). ORF-S was shown to be the coding region for the envelope proteins by aligning the translated HBV DNA sequence with the partial amino acid sequence of HBsAg (Valenzuela et al, 1979). Since then, envelope-specific polypeptide products have been detected for each of the three domains (Stibbe and Gerlich, 1983; Heerman et al, 1984). The large HBsAg envelope protein is initiated at the first AUG (pre-S1 domain) from which translation continues through all three domains. This polypeptide occurs in a glycosylated (p39) and non-glycosylated form (gp42). Initiation at the second AUG (pre-S2 domain) generates the middle HBsAg protein which, like the large HBsAg protein, contains the polypeptide encoded by the S domain at its carboxy-terminus and occurs in two glycosylated forms (gp33 and gp36). The major HBsAg protein species is initiated from the third AUG (S domain) and also appears in a glycosylated (gp27) and non-glycosylated form (p24). The structure of ORF-S is conserved in all hepadnaviruses and corresponding polypeptides have been detected in WHV and GSHV infections. In DHBV however, only a single pre-S polypeptide of ca. 36kD has been identified (Schödel et al, 1989).

In HBV and other hepadnavirus infections, the large and middle HBsAg proteins are only a minor component of circulating HBsAg [usually less than 1-10% of the total (Ganem and Varmus, 1987)], whereas the most abundant species in all cases is the major protein. The 22nm subviral particles are composed primarily of the major protein plus a small but variable percentage of middle and barely detectable levels of large HBsAg proteins. In contrast, Dane and filamentous particles are composed of almost equimolar quantities of all three proteins (Heerman et al, 1984).

1.3B(b) ORF-C.

ORF-C spans ca. 600 nucleotides and like ORF-S contains multiple (two) in-frame initiation codons. Initiation at the first AUG produces a "precore" polypeptide which contains a signal peptide sequence at the aminoterminus that leads to the translocation of the polypeptide into the endoplasmic reticulum. From there the protein enters the secretory pathway prior to cleavage of part of the carboxy-terminal domain that leads to secretion of HBeAg (Ou et al, 1986; Standring et al, 1988; Schlicht and Schaller, 1989). Thus HBeAg contains 10 additional amino acids at the amino-terminus but lacks 34 amino acids from the carboxy-terminus of the core protein described below.

HBeAg is found as a soluble antigen in the serum of some HBV-infected patients (Magnius and Espmark, 1972) and has long been considered a surrogate marker of active viral replication, as HBeAg levels in the bloodstream often correlate with infectivity and high titres of circulating Dane particles (Okada et al, 1976; Ganem, 1982). Conversely, seroconversion to anti-HBe, especially after an acute HBV infection, has been taken as a marker of reduced virus replication (Miyakawa and Mayumi, 1981). However, this relationship is not entirely diagnostic as a significant proportion of anti-HBe-positive patients can still have high levels of circulating virus (Gowans, 1986).

The function(s) of the precore region (and HBeAg) in virus replication are unknown although DHBV mutants defective in this region are viable (Schlicht et al, 1987). Nevertheless, the precore sequence has been preserved in all hepadnaviruses identified to date (Schödel et al, 1989) and recent evidence suggests that HBeAg may induce immunologic tolerance in utero to the products of ORF-C (Milich et al, 1990). Thus, the precore region may promote persistence after perinatal infection rather than having a direct role in viral replication.

The major nucleocapsid protein (HBcAg) originates from the second

AUG, as translation from this codon produces a protein that co-migrates with the 21.5kD core polypeptide detected in HBV-infected livers (Weimer et al, 1987; Schlicht and Schaller, 1989). Unlike HBeAg, HBcAg is not secreted but accumulates in the cytoplasm and nucleus of infected cells. HBcAg contains a highly basic arginine-rich carboxy-terminus which is presumed to interact with the HBV genome within the nucleocapsid (Tiollais et al, 1985). HBcAg has also has been shown to be phosphorylated, a modification that may be important during virus maturation (Pugh et al, 1989; see Section 1.4E).

1.3B(c) ORF-P

ORF-P is by far the largest open reading frame, spanning almost the entire genome, that overlaps the entire ORF-S, the 5' region of ORF-C and the 3' region of ORF-X. Although a protein of the predicted mw (ca. 90kd) has not been detected in vivo, ORF-P is believed to code for a virion polymerase/reverse transcriptase/RNaseH due to its size, the need for these enzyme activities in the replication cycle (see Section 1.3 D) and the observation that the proposed product of this gene shares homologies with certain retroviral reverse transcriptases (Toh et al, 1983) and E. coli RNaseH (Khudyakov and Makhov, 1989). Recently, more direct evidence from the DHBV model shows that the insertion of frame shift mutants or translational stops at different locations within ORF-P abolishes polymerase activity in the resultant core particles (Schlicht et al, 1989).

The product of ORF-P is now known to contain four regions which is expressed as a single functional polypeptide (Bosch et al, 1988; Bartenschlager and Schaller, 1988; Radziwill et al, 1990) as mutations in all regions except the spacer region (see below) result in a non-functional polymerase gene product that can not be rescued in trans (Radziwill et al, 1990). In order from the amino-terminus, these regions are; the 5'-linked protein, spacer region, reverse transcriptase/DNA polymerase and RNaseH.

1.3B(d) ORF-X

ORF-X represents one of the enigmas of HBV replication, and the role of the gene product (HBxAg) in the replication cycle is unclear, as it is derived from the least conserved region of the mammalian hepadnaviruses and has been deleted from the genomes of the avian hepadnaviruses (Schödel et al, 1989). Furthermore HBV DNA containing an inserted transcriptional stop codon within ORF-X, still induced normal replication in the human hepatoma cell line HuH7 (Yaginuma et al, 1987) indicating that an intact X gene product may be dispensable for HBV replication.

Proof for the expression of this antigen during natural infection was provided by the detection of antibodies in patient sera that were reactive against HBxAg epitopes expressed as prokaryotic fusion proteins (Kay et al, 1985; Elfassi et al, 1986) or synthetic peptides (Moriarty et al, 1985). Furthermore, antibodies to synthetic HBxAg peptides detected proteins in HBV-infected liver homogenates, in the livers of some HCC patients, and in the human hepatoma cell line PLC/PRF/5 (Moriarty et al, 1985). The ORF-X product has the ability to transactivate the transcriptional regulatory sequences of both HBV and other viruses (Spandau and Lee, 1988) as well as those of various cellular genes (Twu and Schloemer, 1987; Treinin and Laub, 1987). It is possible that this transactivation may play a role in carcinogenesis, as integrated HBV DNA sequences in HCC and chronically infected liver tissue often retain most of ORF-X (Takada et al, 1990; Takada and Koike, 1990) and fusion products resulting from an integrated 3'-truncated X gene also retained transactivating activity (Takada and Koike, 1990).

1.3C CONTROL ELEMENTS AND RNA TRANSCRIPTION.

Three (3.5, 2.4 and 2.1kb) of the four predicted RNA transcripts shown in Fig 1.2A have been identified in HBV infected cells, while the fourth transcript (0.7kb) has been detected in cells transfected with ORF-X (Saito et al, 1986) and in WHV-infected liver extracts (Kaneko and Miller, 1988). All the identified transcripts have been assigned protein coding functions, are of +ve polarity

and coterminate just after a common polyadenylation signal within ORF-C. In addition the 3.5kb transcript serves as the template for the reverse transcription of (-)strand DNA (see Section 1.4 D).

1.3C(a) 2.1 and 2.4kb RNA Transcripts.

Primer extension and nuclease protection analyses have shown that the 5' ends of the 2.1kb and 2.4kb RNA transcripts map close to the respective putative initiation sites in ORF-S (Will et al, 1987) and therefore are likely to encode all three of the envelope proteins. The 2.1kb RNA transcript is highly abundant and shows microheterogeneity at the 5' ends such that it initiates on either side of the AUG specifying the middle protein (Cattaneo et al, 1984; Standring et al, 1984; see Fig 1.2B). This arrangement enables the synthesis of two proteins from one mRNA species without the need for internal initiation. The 2.4kb RNA transcript , which encodes the large protein, is present in very low quantities in infected liver (Will et al, 1987) which may account for the low concentration of this protein in serum.

In the ORF-S promoter regions TATA-like elements [sequence motifs implicated in the initiation of RNA transcription (Alberts et al, 1989)] have only been detected upstream of the pre-S1 domain (Will and Rapicetta, 1988). In contrast, sequences resembling the simian virus 40 (SV40) late promoter are located upstream of the 2.1kb RNA initiation site (Cattaneo et al, 1983). As this SV40 promoter also lacks a TATA element and induces mRNA initiation at three alternate sites (Brady et al, 1982), the lack of a TATA promoter is likely to account for the observed 5' heterogeneity of the 2.1kb RNA transcript of HBV. The only regulatory sequence in ORF-S to be identified so far is a binding site for nuclear factor 1 that is essential for optimal S promoter activity (Shaul et al, 1986).

1.3C(b) 3.5kb RNA Transcript.

A promoter for the transcription of the 3.5kb RNA species has been identified upstream of ORF-C (Roossinck et al, 1986), that also lacks a conventional TATA-like element, like the promoter for the 2.1kb RNA

described above. Moreover, the 3.5kb RNA species also shows 5' microheterogeneity to permit the translation of two proteins from one RNA species. The most abundant 3.5kb species initiates within the pre-C domain, thereby positioning the internal AUG of ORF-C as the first initiation codon on the transcript (Will et al, 1987; Yaginuma et al, 1987; see Fig 1.2C). This species specifies the synthesis of HBcAg, while the less common 3.5kb RNA species, which initiates upstream of the pre-C AUG (Will et al, 1987; Yaginuma et al, 1987) encodes the precore plus core protein which is post-translationally cleaved to HBeAg.

An RNA transcript encompassing ORF-P that initiates immediately upstream of the polymerase gene initiation codon has not been identified in any hepadnaviral infection. Products of the ORF-P are therefore also likely to be encoded by the 3.5kb RNA species and recent work using the DHBV model suggests that the polymerase gene product is synthesized by initiation of translation at the internal AUG at position 2357 (Fig 1.2A); recombinant DHBV DNA containing a mutation within this codon was non-infectious when inoculated intrahepatically into ducks (Chang et al, 1989) and led to a profound reduction of polymerase activity in core particles and DHBV produced after transient transfection into HepG2 cells (Chang et al, 1989; 1990; Schlicht et al, 1989). Consistent with internal initiation of the polymerase gene is the fact that a ORF-P-specific promoter has not been detected.

Transcription of the 3.5kb RNA species requires readthrough of the single polyadenylation processing site on the first pass, but efficient recognition on the second pass. The mechanism for this is unknown, but may depend on the secondary structure of the RNA (Will and Rapicetta, 1988).

1.3C(c) 0.7kb RNA Transcript.

Although all three of the above RNA transcripts could, by internal initiation or ribosomal frameshifting, potentially encode the product(s) of ORF-X, the identification of a transcriptional promoter sequence upstream of

this region (Treinin and Laub, 1987) and the demonstration that in vitro gene expression generates a 0.7kb transcript (Saito et al, 1986), argues against these possibilities. However, no RNA transcript initiating upstream of the ORF-X AUG codon has been identified in HBV-infected liver. Recently however, a 0.65kb transcript was detected in woodchuck liver from both acutely- and chronically-infected animals that was specific for ORF-X (Kaneko and Miller, 1988). Remarkably, the majority of these transcripts were not polyadenylated and were localized within the nucleus of infected cells.

1.3C(d) Additional Control Elements.

Two transcriptional enhancer elements have been identified within the HBV genome. The first (E1, see Fig 1.2A) is situated between the 3' end of ORF-S and the 5' end of ORF-X (Shaul et al, 1985) and the second (E2) is situated in the core promoter region immediately upstream of pre-C (Yee, 1989). Like other known enhancer elements, both sequences act in an orientation-independent manner and are host and tissue specific, showing the greatest activity in human liver cells (Shaul et al, 1985; Roossinck et al, 1986; Yee, 1989).

Finally, a region located upstream of the enhancer element E1 contains an element that is responsive to glucocorticoid hormones (GRE; Fig 1.2A) but unlike the enhancer elements shows no species- or tissue- specificity (Tur-Kaspa et al, 1986). Nevertheless the presence of this GRE may explain the stimulatory effect glucocorticoid hormones have on the synthesis of HBsAg (see Chapter 4).

1.4 REPLICATION STRATEGY OF HEPADNAVIRUSES.

Much of our present understanding of the hepadnavirus replication cycle stems from work with DHBV (Summers and Mason, 1982). This and subsequent studies revealed that the Hepadnaviridae replicate by a unique strategy that includes reverse transcription of an RNA intermediate. While this is unusual for DNA-containing viruses, it has analogies with retroviruses and cauliflower mosaic virus (Ganem and Varmus, 1987). The replication strategy

shown in Fig 1.3 is based on available data from all Hepadnaviridae but it is unlikely that significant differences exist between the five known species. The cycle can be divided into six stages; (A) adsorption and penetration, (B) conversion of input asymmetric DNA to covalently closed circular DNA [cccDNA], (C) synthesis of mRNA and pregenome, (D) DNA synthesis, (E) virus maturation and secretion and (F) intracellular amplification of cccDNA.

1.4A ADSORPTION.

The mechanism used by the hepadnaviruses to gain entry to the cell may account for both the species specificity and hepatotropism shown by this virus group, and this remains one of the less well understood aspects of the replication cycle. However, although little is known about the virus receptor on the cell, progress has been made to identify the cell-binding protein present on the virus.

Since the cell-binding protein will logically reside within the envelope proteins (HBsAg), early work examined the binding of purified 22nm particles to liver sections (Lutwick et al, 1982). However it is unlikely that the principal protein component of these particles (major protein) can confer species specificity as the gene for this protein is well conserved in all the mammalian viruses to the point that the resultant polypeptides show immunological cross-reactions. Furthermore Seeger et al (1987) demonstrated that recombinant GSHV DNA containing the S domain of WHV was infectious (by intrahepatic inoculation of DNA) to ground squirrels whereas wild type WHV DNA was not, suggesting that the middle and large proteins were likely to impart host specificity. In contrast, the pre-S domain of different hepadnaviruses, shows considerable variability at both the nucleic acid sequence and amino acid level, consistent with species specificity and a putative receptor role (Schödel et al, 1989). The middle HBsAg binds specifically to polymerised serum albumen (pHSA; Machida et al, 1984; Swan et al, 1984) and this mechanism was believed to mediate the attachment of the virus to the hepatocyte membrane. However, a receptor for pHSA has not

Fig 1.3 Hepadnavirus replication strategy. Input virus adsorbs to the cell membrane and is internalised and uncoated (A). Viral DNA then migrates to the nucleus and is converted (B) to covalently closed circular DNA (cccDNA) which probably serves as the template for viral RNA synthesis (C). Transcribed RNA then either behaves as mRNA for the production of virus-specific proteins (C) or is encapsidated with the virus polymerase gene product into immature core particles (D). Reverse transcription of the encapsidated RNA leads to the production of the DNA (-)strand which in turn serves as the template for the synthesis of the DNA (+)strand. The core particle is then either enveloped with HBsAg and the complete virion exported from the cell (E) or enters the intracellular pathway that leads to amplification of the cccDNA copy number (F).

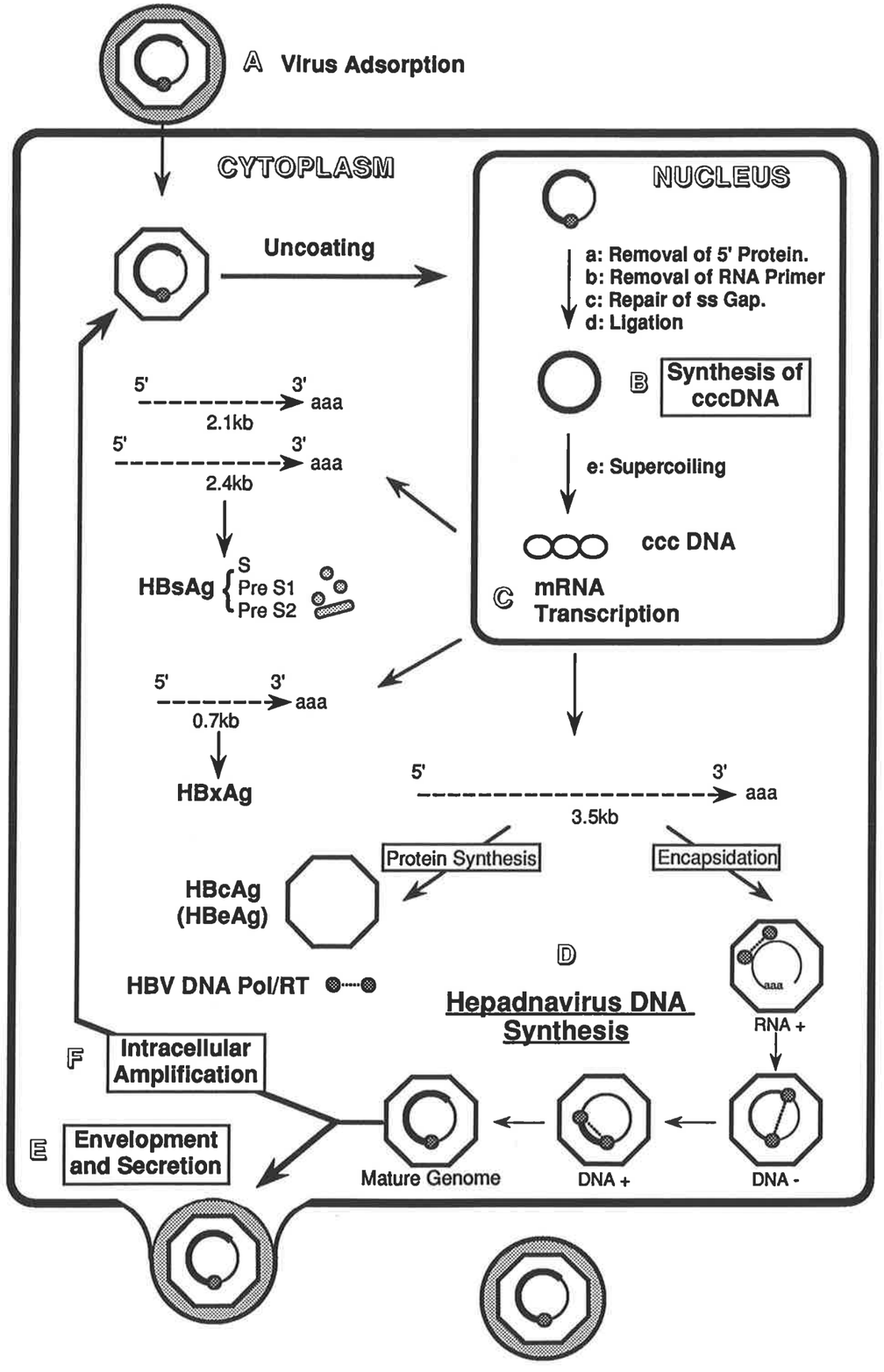


Fig 1.3 a HEPADNAVIRUS REPLICATION STRATEGY

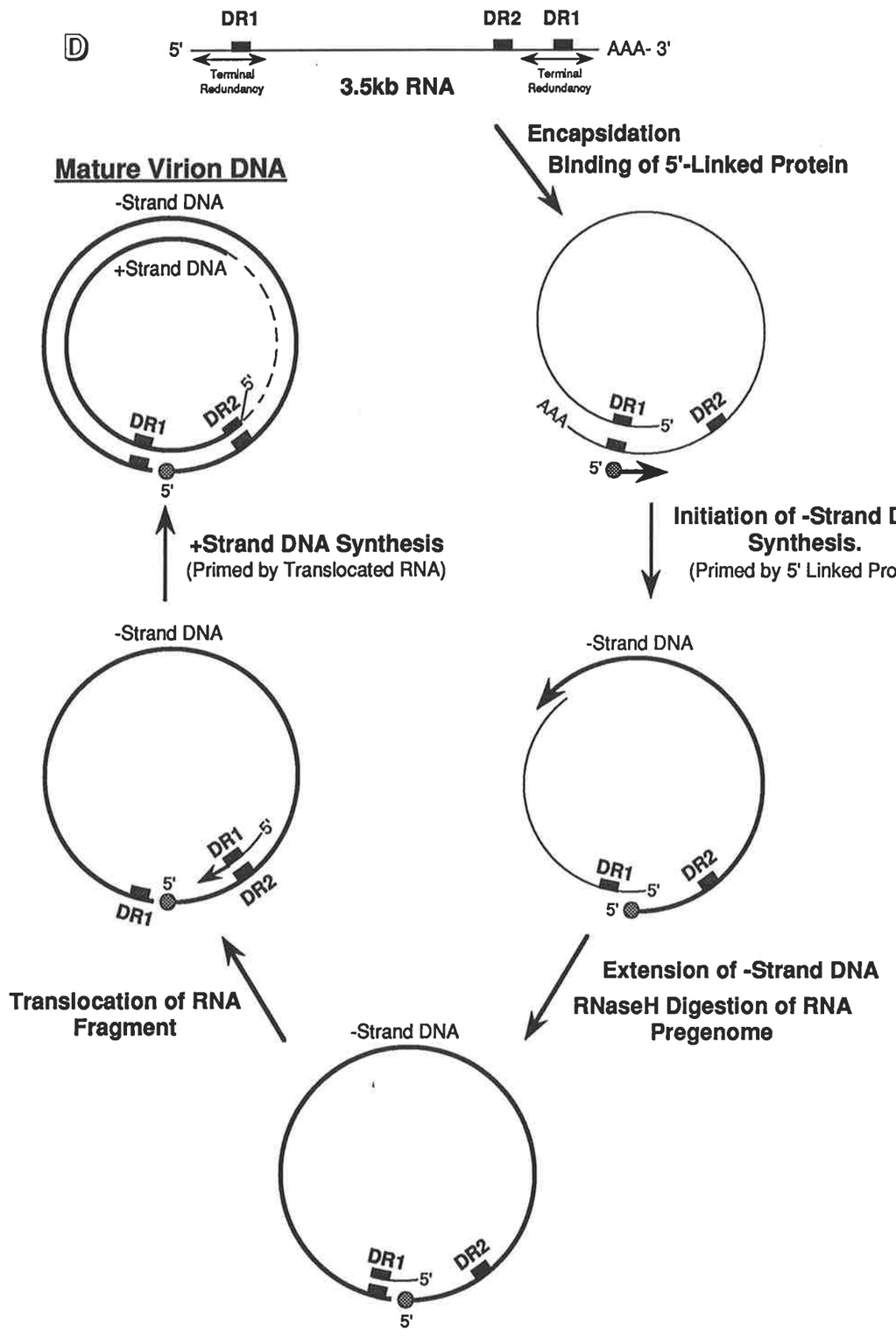


Fig 1.3 b HEPADNAVIRUS DNA SYNTHESIS.

been identified on liver cells and as a similar binding region could not identified within the corresponding domain of WHV (Pohl et al, 1986), this mechanism of binding now seems unlikely.

Recent evidence using recombinant or serum-derived HBsAg has shown that the large protein contains a site which binds to human liver plasma membranes (Pontisso et al, 1989a; 1989b) and to some cells of non-hepatic origin (Neurath et al, 1990) including peripheral blood lymphocytes, a known extrahepatic target of HBV infection (Section 1.1B). Furthermore, analysis with peptide-specific monoclonal antibodies revealed that the binding site was contained within amino acid residues 27-49 of this domain (Pontisso et al, 1989b). As this region is highly conserved in specific hepadnaviruses it is highly likely that the large protein mediates hepatocyte attachment.

1.4B UNCOATING TO SYNTHESIS OF cccDNA.

Following entry and uncoating the single stranded region in the virion DNA is repaired to form fully dsDNA which in turn is converted to cccDNA. This conversion is rapid as cccDNA can be identified very early after infection (Mason et al, 1983). In Fig 1.3 this conversion is shown to be a nuclear event. This is likely since (i) cccDNA is detected exclusively in the nucleus of HBV-infected hepatocytes (Miller and Robinson, 1984) and (ii) host cell DNA polymerases are responsible in vivo for repair of the (+)strand DNA, as the repair reaction was resistant to phosphonoformic acid, in contrast to the late DNA synthesis which is performed by the virion polymerase (Mason et al, 1987).

The enzymes responsible for the other steps in the conversion of the input viral DNA to the ccc molecule are unknown at present. These events are removal of the 5'-linked protein and the 9 bp terminal repeat from the (-)strand DNA, cleavage of the oligoribonucleotide from the 5' end of the (+)strand DNA, ligation of the DNA ends and finally supercoiling.

1.4C RNA SYNTHESIS.

Viral RNA in experimentally-transmitted DHBV infection was only

detected after the appearance of cccDNA (Mason et al, 1983; Tagawa et al, 1986). Thus it is likely that cccDNA is the template for RNA transcription catalysed by the host-derived enzyme RNA polymerase II (Rall et al, 1983). In Fig 1.3 (part C) the four RNA species predicted to occur in mammalian hepadnavirus infections are shown, although all four have not been detected unequivocally in individual infections.

Although the promoters for RNA transcription and the expected products from each individual RNA species are well established and are described in Section 1.3, little is known about the temporal relationship of these events. Recently in experimentally-transmitted DHBV infection (Yokosuka et al,1988), DHBcAg was detected as early as 24 hours after infection, consistent with a role in nucleic acid packaging (see below) whereas large DHBsAg was not detected until 3 days post infection. The late appearance of large DHBsAg was proposed as a mechanism for regulation of the replication cycle (Yokosuka et al,1988), allowing initial amplification of cccDNA (see part F below) prior to virus envelopment and export.

1.4D DNA SYNTHESIS.

The DNA synthesis cycle can be subdivided into three steps; (a) encapsidation of pregenomic RNA (b) synthesis of (-)strand DNA by reverse transcriptase and (c) synthesis of (+)strand DNA by DNA polymerase. The diagrammatic representation in Fig 1.3 is based on the model proposed by Summers and Mason (1982) and refined by Seeger et al (1986) and Will et al (1987), but, while Fig 1.3 suggests that circularisation of nascent (-)strand DNA is an early event, this is for convenience only, as it is probable that this does not occur until the synthesis of (+)strand DNA begins.

1.4D(a) Encapsidation.

As the 3.5kb RNA is the only transcript with complete genomic coding capacity suitable for reverse transcription, the first step requires this RNA species (pregenome) to be packaged into immature core particles along with the polymerase gene product. Packaging is highly selective as only

pregenomic length- but not subgenomic-RNAs are encapsidated (Enders et al, 1987; Hirsch et al, 1990). Furthermore only the shortest species of pregenomic RNAs that differ by as little as 31 nucleotides (due to microheterogeneity at the 5' end; Section 1.3 C) are packaged (Enders et al, 1987; Hirsch et al, 1990). It is unlikely that the mechanism for this is based solely on sequence as the large RNAs contain all the information in the shorter species. It is also unlikely that the core protein plays an active role in the encapsidation process, as core polypeptides expressed in vitro are able to assemble into nucleocapsids, identical to those of the virion, in the absence of other viral proteins and nucleic acids (Cohen and Richmond, 1982; Gallina et al, 1989). In contrast, recent evidence suggests that the polymerase gene product is essential for encapsidation as infection with viruses containing mutations within the polymerase gene produce nucleocapsids which do not contain viral RNA (Hirsch et al, 1990). Although the mechanism for this is unknown, when polymerase-mutant genomes were complemented in trans with wild type DHBV genomes, encapsidated progeny genomes were predominantly of wild type origin (Lavine et al, 1989; Hirsch et al, 1990). This suggests that nascent polymerase polypeptides may interact with and ultimately become packaged with their own mRNAs (Hirsch et al, 1990).

1.4D(b) Synthesis of Minus Strand DNA.

The site for the initiation of the DNA (-)strand has been mapped to within the DR1 region (Molnar-Kimber et al, 1984; Seeger et al, 1986; Will et al, 1987). This sequence lies in the terminally redundant region of the pregenomic RNA, which contains two copies of DR1. Until recently the exact site of DNA initiation was ambiguous as either copy of DR1 could be employed. However, as only initiation at the 3' copy of DR1 would allow uninterrupted synthesis of the DNA (-)strand, this was the expected site. This view has recently been confirmed for WHV (Seeger and Maragos, 1990; see Fig 1.3).

Although the exact mechanism of priming of the DNA (-)strand is

unknown, it is probable that the 5'-linked protein performs this function as it is found attached to nascent DNA (-)strands of less than 30 nucleotides (Molnar-Kimber et al, 1983). Synthesis of the (-)strand DNA then proceeds by reverse transcription of the RNA pregenome concomitant with degradation of the RNA template by the RNase H-like activity of the virus polymerase (Summers and Mason, 1982). By a mechanism that is still unclear, a small capped oligoribonucleotide of ca.17 nucleotides derived from the 5' end of the RNA pregenome and containing the DR1 sequence (Lien et al, 1986; 1987) escapes this degradation (see Fig 1.3).

1.4D(c) Synthesis of Plus Strand DNA.

Following reverse transcription, the nascent viral (-)strand is used as the template for the synthesis of viral (+)strand DNA. This reaction is performed by the viral polymerase and primed by the short capped oligoribonucleotide described above following translocation from DR1 to DR2 (Lien et al, 1986; Seeger et al, 1986; Will et al, 1987). The mechanism of translocation of this primer is unknown, but may be aided by the high A+T ratio within this sequence.

In the mammalian hepadnaviruses synthesis of (+)strand DNA fails to reach completion, accounting for the asymmetric DNA species found in the virion. This may be due to the (premature) process of capsid envelopment by HBsAg that inhibits the supply of nucleotides to the growing (+)strand. For DHBV the majority of virions contain virtually complete dsDNA and in this case (+)strand synthesis terminates just upstream of DR2. The reason for this termination site is unknown but may be due to the inability of the viral DNA polymerase to displace the RNA primer from the (-)strand DNA template (Lien et al ,1987).

1.4E ENVELOPMENT AND SECRETION.

Hepadnavirus envelopment is likely to be associated with intracellular membranes as this is the site where the three envelope proteins usually accumulate (Ou and Rutter, 1987). Electron microscopic studies support this

view (Kanimura et al, 1981; Yamada et al, 1982), as core particles budding into the cisternae of the endoplasmic reticulum were observed. However, the secretory pathway for both virions and excess surface antigen particles is not well understood although the latter particles are processed on route via the Golgi complex (Patzer et al, 1984).

Virion formation may be regulated by intracellular levels of the large HBsAg as coexpression or over-expression of this protein with the middle and major HBsAg proteins inhibits secretion of all three envelope proteins (Chisari et al, 1986; Ou and Rutter, 1987). The mechanism for this is unknown but may be related to the myristic acid group added post-translationally to the amino terminal of the large polypeptide (Persing et al, 1987) that is likely to anchor this protein in the lipid bilayer of the endoplasmic reticulum. Although secreted virions generally contain mature core particles, the intracellular selection mechanism which ensures this is unknown. Recent studies by Pugh et al (1989) showed that DHBV virion-derived cores were underphosphorylated compared to liver-derived cores. This implies that dephosphorylation may either have a role in core selection or perhaps be a consequence of envelopment.

1.4F INTRACELLULAR AMPLIFICATION OF cccDNA.

Shortly after the development of in vitro cultivation techniques for DHBV (see Section 1.1D), Tuttleman et al (1986b) observed amplification of cccDNA prior to significant virion release that apparently occurred via an intracellular pathway (intracellular cycling). Confirmation of this proposition was obtained recently by Wu et al (1990a), who demonstrated cccDNA amplification in the presence of suramin or neutralising antibodies that would prevent infection from without contributing to the cccDNA pool.

While the intracellular mature core particles are presumably the source of hepadnaviral DNA for cccDNA amplification, the pathway selection mechanism (intracellular cycling or envelopment and secretion) is unknown. Regulation is potentially important as insufficient production of cccDNA may

lead to cessation of virus replication whereas over-production is associated with cytotoxicity (Summers et al, 1990). A role for precore protein in the process of selection and/or regulation can now be excluded as cccDNA amplification still occurred after infection of primary duck hepatocytes with DHBV containing a mutation in the precore region (Wu et al, 1990b). Furthermore, cccDNA accumulation ceased after five days, at the same time as occurred with wild-type genomes (Tuttleman et al, 1986b) In contrast, infection with viral mutants unable to synthesize the major or large DHBsAg proteins led to extraordinarily high levels of cccDNA (Summers et al, 1990) suggesting that viral envelope proteins may have a regulatory role in this process. Since the large proteins appear late in infection (Yokosuka et al,1988), it seems likely that these polypeptides could be responsible for the regulation of cccDNA amplification. The above observations may be incorporated into the following proposed mechanism to account for this process:

(a) Virus infection leading to the formation of cccDNA and subsequently RNA transcription.

(b) The transcript for the large HBsAg polypeptide is of low abundance (Will et al, 1987), consequently this protein may be poorly expressed initially thereby allowing the intracellular cycling of mature core particles to amplify cccDNA.

(c) Higher cccDNA copy numbers enable increased large protein production which in turn inhibits the secretion of middle and major proteins.

(d) The intracellular build up of envelope proteins switches off intracellular cycling of mature core particles in favour of virus envelopment and secretion.

(e) As the infection continues the cccDNA copy number may fall slightly. This leads to a decrease in the production of the large HBsAg to permit a proportion of the mature core particles to enter the intracellular pathway to replenish cccDNA levels.

1.5 HEPATITIS DELTA VIRUS.

1.5A. OVERVIEW.

The hepatitis delta agent was discovered following the detection of a novel nuclear antigen, distinct from HBcAg, in the hepatocytes of some HBV carriers (Rizzetto et al, 1977). Following transmission studies, this antigen, Hepatitis delta antigen (HDAg), was shown to be a structural antigen of an infectious agent, the hepatitis delta virus (HDV), a defective virus which is dependent for its replication and expression on helper functions provided by HBV (Rizzetto et al, 1980b) or by WHV (Ponzetto et al, 1984). While the full extent of this helper function is unknown, HBV (and WHV) provides HDV with an envelope of HBsAg (or WHsAg) that has a polypeptide composition similar, but not identical, to the 22nm particles described in section 1.3 B(a) (Bonino et al, 1986). HDV can only infect individuals who are infected simultaneously with HBV (co-infection) or individuals who are pre-existing carriers of HBV (super-infection), and expression of HDV is limited temporally to the period of HBsAg expression. In both cases there is interference with HBV (or WHV) replication by a mechanism that is yet to be clarified. Patients co-infected with HBV and HDV usually present with acute hepatitis which resolves in ca. 95% of cases, although a minority of these patients show an increased incidence of fulminant hepatitis (Govindarajan et al, 1984). Similarly, HBsAg carriers who become superinfected usually become HDV carriers and often develop severe chronic liver disease (Govindarajan et al, 1986). Curiously, despite the detection of HBV and WHV nucleic acids at extrahepatic sites (see Section 1.1B; Negro et al, 1989a), HDV replication has only ever been detected in liver and HCC tissue (Negro et al, 1989a).

The HD virion consists of a 36nm particle that, unlike HBV, has no discrete capsid and the virion structure is still unknown. The internal components, exposed by detergent disruption of the HBsAg envelope, comprise HDAg and a small circular, single stranded, poly $(A)^-$ RNA of ca. 1.7kb (Rizzetto et al, 1980b; Bonino et al, 1981,1984; Hoyer et al, 1983;

Chen et al, 1986). HDV RNA of virion-polarity (genomic sense) is the principal species detected in HDV-infected liver (Gowans et al, 1987; 1988) occurring at 10-20 fold higher levels than HDV RNA of the opposite polarity (antigenomic sense). In contrast to the cytoplasmic localization of the majority of HBV-associated nucleic acids, the bulk of genomic and antigenomic HDV RNA is found in the nucleus of infected cells (Gowans et al, 1987; 1988).

1.5B HDV RNA.

HDV RNA has been molecularly cloned from virus passaged in chimpanzees (Wang et al, 1986), woodchucks (Kuo et al,1988) and directly from human serum (Makino et al, 1987), and the complete sequences determined. Analysis of these clones revealed five conserved ORFs distributed over both genomic and antigenomic strands. One of these (ORF-5; Wang et al, 1986) on the antigenomic strand is the gene coding for HDAg (Wang et al, 1986; Makino et al, 1987; Kuo et al , 1988) and, to date, is the only ORF for which a protein product has been assigned.

Sequence analysis also predicts intramolecular base-pairing between ca. 70% of the nucleotides within the HDV RNA molecule leading to the formation of an unbranched rod-like structure (see Fig 1.4A) similar to that observed for viroids (Wang et al, 1986; Makino et al, 1987; Kuo et al, 1988). However, while such structures have been observed following electron microscopic analysis of purified preparations of undenatured HDV RNA (Kos et al, 1986), they may not occur in vivo, as little difference was observed in detectable levels of genomic sense HDV RNA in sequential denatured and non-denatured liver sections by in situ hybridization (Gowans et al, 1987; 1988). Other similarities between viroid RNA and HDV RNA have been noted including the identification of regions on both sense strands that can undergo autocatalytic self cleavage and ligation in vitro (Hutchins et al, 1986; Sharmeen et al, 1988; 1989; Wu and Lai, 1989; Wu et al, 1989) and the likelihood that both agents replicate by a similar mechanism (see below). Significant differences also exist; HDV RNA is much larger (5-10X) and in

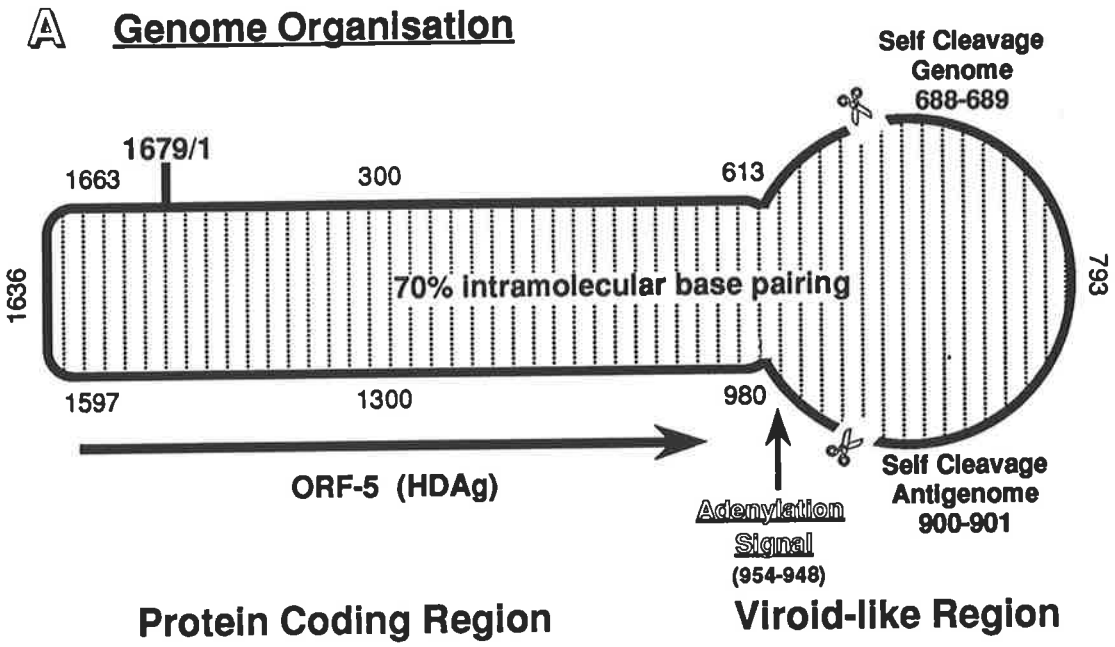


FIG 1.4 A Structure and Genome Organisation of HDV RNA according to the model proposed by Branch et al, 1989.

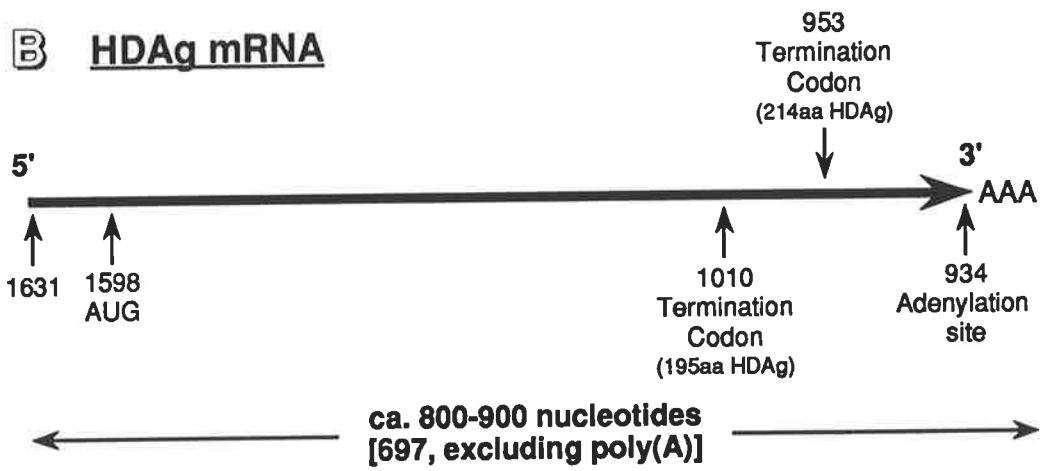


FIG 1.4 B Putative HDAg mRNA as described by Hsieh et al, 1990

contrast to viroid RNA, encodes at least one protein. These data have been incorporated recently into a structural model for HDV RNA (Branch et al, 1989; Fig 1.4A.) that divides the putative unbranched rod structure into protein coding and viroid-like domains. Shown also in Fig 1.4A is the position of the only polyadenylation signal identified within HDV RNA (Sharmeen et al, 1988). This occurs on the antigenomic sense strand and, significantly, is immediately downstream of the coding region for HDAg (see below).

1.5C HEPATITIS DELTA ANTIGEN.

The sequence of the HDAg gene of different cDNA clones of chimpanzee-derived HDV RNA predicts HDAg to be either 195 or 214 amino acids (aa) depending on the position of the stop codon (Wang et al, 1986), while a human-derived sequence predicts 214 aa (Makino et al, 1987) and a woodchuck-derived sequence predicts 195 aa (Kuo et al,1988). The predicted molecular mass is ca. 24 kD and 21kD for the 214 and 195 aa proteins respectively. However, in HDAg extracted from infected liver or serum two major proteins with mws of 27 and 24 kD (Bergmann and Gerin, 1986; Macnaughton et al, 1990a; 1990b) or 29 and 27 kD (Bonino et al, 1986; Roggendorf et al, 1987) were detected. While the variations in the reported mws are likely to result from differences in mw standards, both these major polypeptides react with a human monoclonal antibody to HDAg (Pohl et al, 1987) and therefore contain at least one similar antigenic epitope. Although the mechanism for the production of these two polypeptides is not well understood, a chimpanzee-derived ORF-5 sequence which predicted the 195 aa form of HDAg was used to express both 24 and 27 kD polypeptides in E. coli by suppression of an amber termination codon (Weiner et al, 1988). Furthermore, in systems where stable HDV RNA replication occurred (Sureau et al, 1989; Luo et al, 1990; Macnaughton et al, 1990b; Chapter 7) both HDAg protein species eventually appeared from a sequence that only encoded a protein of 195 aa. This effect was shown to be probably due to a specific mutation in the termination codon for the 195 aa polypeptide that enabled

translation to continue to the downstream stop codon thereby producing a HDAg polypeptide of 214 aa (Luo et al, 1990). The mechanism for this is unknown but HDV RNA replication appears to be essential as this mutation failed to appear in cell lines that contain and express only the HDAg gene even after extended periods in culture (Macnaughton et al, 1990a).

The 214 aa form of HDAg was shown to be a RNA-binding phosphoprotein (Chang et al, 1988) and recently this observation was extended to the 195 aa form of HDAg in a reaction that was specific for HDV RNA (Macnaughton et al, 1990a; Chapter 6). The 195 aa form of HDAg has also been shown to capable of self aggregation and possesses conformational-dependent epitopes (Macnaughton et al, 1990a; Chapter 6).

The circular nature and nuclear localization of full-length HDV RNA suggest that it is unlikely to act as messenger RNA for the expression of HDAg (Kozak, 1979), yet an mRNA species specific for HDAg production has not been identified unequivocally. However, a recently characterized (Hsieh et al, 1990) subgenomic poly(A)$^+$ HDV-specific RNA species of 800-900 nucleotides in length, detected in infected liver and in cell lines transfected with HDV cDNA (Chen et al, 1986; Jilbert et al, 1990; Macnaughton et al, 1990b, Hsieh et al, 1990) is a likely candidate (see Fig 1.4B). In the latter report, the 5' end of this RNA species was mapped to a site immediately upstream of the putative initiation codon for HDAg, while the 3' end, minus the poly(A)$^+$ tail, was positioned 15 nucleotides downstream of the polyadenylation site. How this RNA species arises is unknown although an attractive hypothesis suggests that it is synthesized as a "by-product" of RNA replication (see below).

1.5D HDV RNA REPLICATION.

Although HDV RNA is thought to replicate by a double rolling circle mechanism similar to that proposed for viroids (Branch and Robertson, 1984), at present the only evidence to support this is circumstantial and therefore the details of this strategy, as shown in Fig 1.5, remain highly speculative.

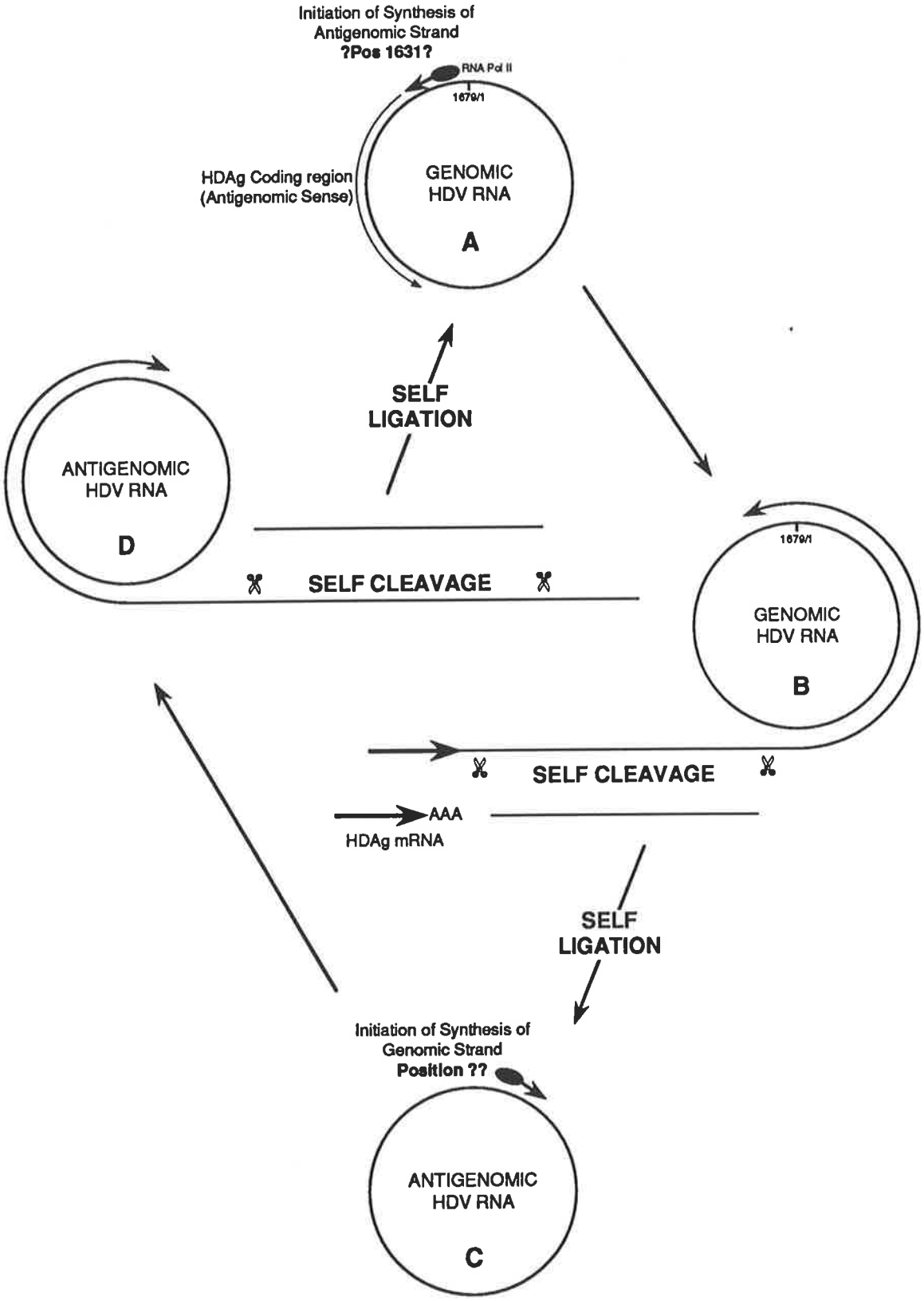


Fig 1.5 HDV GENOME REPLICATION

Transcription of antigenomic sense HDV RNA from input virion RNA is thought to initiate at the recently mapped 5' end of the putative HDAg mRNA (position 1631; Hsieh et al, 1990), although no recognised promoter element has been identified upstream of this site as yet. Continued transcription from the circular template leads to the production of multimeric length antigenomic HDV RNA which is subsequently self-cleaved into monomers and a ca. 700 nucleotide species that could be processed into the mRNA species described above. According to the model shown in Fig 1.5 the latter RNA species can only be synthesised once during transcription, a prediction that may account for its low abundance in vivo (ca. 500 times less abundant than full-length genomic RNA; Hsieh et al, 1990). The antigenomic sense monomers are then thought to self-ligate into a circular species which serves as the template for transcription of genomic sense HDV RNA. This leads, in a similar manner to that described above, to the production of a multimeric intermediate that self cleaves and self ligates to form new monomeric circular genomic sense HDV RNA. Evidence for this strategy comes from the detection of circular and linear forms of monomers and multimers of both polarities in infected and transfected cells (Chen et al, 1986; Negro et al, 1989a; Kuo et al, 1989; Macnaughton et al, 1990b; Glenn et al, 1990) and the in vitro self-cleavage and self-ligation activities described above for HDV RNA.

The replication strategy shown in Fig 1.5 requires RNA directed RNA synthesis which, in the absence of a gene for this activity, is likely to be performed by host derived RNA polymerase II (Gowans et al, 1990b; see Chapter 7). How the template specificity of this enzyme is altered to enable it to perform this function is unknown although this may be related to the fact that HDAg is essential for HDV RNA replication (Kuo et al, 1989; Glenn et al, 1990).

1.6 AIMS OF THESIS.

The aims of this thesis were to establish suitable in vitro models for the replication and pathogenesis of both HBV and HDV, and to examine details of the helper function/defective nature of the relationship between these two viruses. However, most of the details of HBV and HDV replication described in this chapter emerged during the period of this study (1983-1990) and it was considered desirable to describe the level of understanding in these areas at the time of commencement of this study.

Firstly although three of the four animal models of HBV had been identified, none had been well characterised and the nucleotide sequence of only one, WHV, had been determined. Early in situ hybridisation studies (Gowans et al, 1981) and work with DHBV had established that unlike most other small DNA-containing viruses, hepadnavirus replication was likely to occur in the cytoplasm of the hepatocyte. Nevertheless no in vitro model for virus production (either infection or transfection) existed and little data was available on the molecular mechanisms of virus replication.

The HDV section of this study was performed from 1987-1990 soon after cloned HDVcDNA became available (Wang et al, 1986). At this stage although the antigen and RNA species present in infected liver and serum had been identified the replication mechanisms and the nature of the hepadnavirus helper function were largely unknown.

Thus the specific aims for HBV were to determine: (a) the conditions required to infect cultured cells with HBV, (b) the factors controlling the level of antigen and virus expression, and (c) the potential blocks to hepadnaviral infection in vitro. For HDV the aims were: (d) to express the HDAg gene in transfected cells, determine the properties of the resultant product and compare these to that of antigen produced in natural infections, (e) to establish an in vitro model for HDV replication by transfection of a multimeric construct of HDV cDNA and (f) use this model to investigate HDV pathogenesis and replication, and the extent of the HBV helper function.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 EUKARYOTIC CELL CULTURES.

2.1A MEDIA.

The majority of cell culture work was performed using Dulbecco's Modified Eagles Medium (DMEM; Flow or Gibco) or CMRL 1640 (Gibco) supplemented with 5-10% non-inactivated foetal bovine serum (FBS; Flow, CSL, Gibco), 100 units/ml penicillin and 40 µg/ml Gentamycin. RPMI 1640 (Flow) with the above supplements was used for MLA144 cultures and 199 medium (Flow) was used for endothelial cultures. MCDB 151 (Sigma) and its derivative LHC-4 supplemented with the above antibiotics plus or minus 5% non-inactivated FBS was employed in many HBV infection experiments and studies of viral antigen expression (see Appendix 1 for the formulation of DMEM, CMRL, 199 and MCDB 151).

MCDB 151 was prepared according to the manufacturers directions and sterilized by membrane filtration. LHC-4 medium (Lechner et al, 1982; 1983) was prepared by the addition of hormones, hormone precursors, trace elements and salts (see Appendix 2). The additive, bovine pituitary extract, was prepared as described by Tsao et al (1982); 8 mixed-sex pituitary glands were removed from animals shortly after slaughter and transported to the laboratory on ice. Connective tissue was removed, cold 150mM NaCl added and the mix homogenized on ice for 10 minutes then stirred at 4°C for a further 90 minutes. The homogenate was centrifuged at 9,800g for 10 minutes and the supernatant sterilized by filtration. Following measurement of total protein and haemoglobin (Biuret method and model IV Coulter counter respectively) the non-haemoglobin protein concentration of the filtrate was adjusted to 3.5 mg/ml, divided into 5ml aliquots and stored at -70°C.

Other media and cell culture reagents such as trypsin, PBS and antibiotics were used as supplied by the IMVS media kitchen.

2.1B CULTURE METHODS.

2.1B(a) Routine Maintenance.

All continuous cell lines except human vascular endothelial cells (HUVE) and MLA144 were maintained in 75 cm^2 flasks (Flow, Costar, Corning, Nunc and Falcon) in DMEM or CMRL supplemented with antibiotics and 5% FBS. Media were changed twice per week and the cells subcultured every 7-10 days. For long term storage, cells were stored in DMEM containing 20% FBS and 10% dimethylsulphoxide (DMSO; Univar) under liquid nitrogen.

2.1B(b) Coverslip Cultures.

Coverslip cultures were used for immunofluorescence assays and other cytological staining techniques. 13mm diameter glass coverslips (Lomb; Arncliffe, NSW) were soaked overnight in chromic acid , washed for 4 hours in running tap water, rinsed 5 times in distilled water (DDW) and stored in methanol. For use, the coverslips were separated on filter paper with fine forceps, dipped in methanol, flame sterilized, transferred to the 16mm wells of a 24 well multiplate (Costar), then seeded with freshly trypsinised cell cultures.

2.1B(c) Endothelial Cell Culture.

2.1B(c)(i) Isolation.

The methods used were largely as described by Grimbrone (1976) with the medium modifications suggested by Thornton et al (1983).

Human umbilical cords were collected soon after birth, immersed in a transport medium consisting of 199 medium supplemented with 100units/ml Penicillin, 40units/ml Gentamycin and 50µg/ml heparin, and sent to the laboratory on ice. A section of cord, free of clamp marks, was then excised, transferred to a sterile petri dish containing PBS, 500units/ml Penicillin, 200units/ml Gentamycin and 20 µg/ml fungizone and incubated for 10 minutes at room temperature (RT). Both ends of the umbilical vein were

cannulated and the cannula secured with string. A 50 ml syringe was attached to each cannula and 200 ml of PBS was flushed through the cord in both directions. The cord was then filled with a pre-warmed solution of collagenase (125 units/ml; Sigma) in Ca^{++}, Mg^{++} Earles Salts (see Appendix 3) and incubated in a prewarmed PBS bath at 37°C for 15 minutes. The digest was flushed from the cord with Ca^{++}, Mg^{++} free Hanks Balanced Salt solution (Hanks BSS; Appendix 3) and collected into a 50 ml centrifuge tube. The cells were pelleted at 250g for 5 minutes and washed once with Hanks BSS. Care was taken not to break up cell clumps as this was known to reduce plating efficiency. The pellet was finally resuspended in endothelial culture medium (ECM) consisting of 199 medium supplemented with 20% heat inactivated (56°C, 60 minutes) FBS, 90µg/ml heparin, 20µg/ml endothelial cell growth factor (Sigma), 100 units/ml penicillin, 40units/ml gentamycin and 2µg/ml fungizone, and seeded into uncoated 25 cm^2 flasks (Falcon, Costar) or fibronectin-coated glass coverslips at 5-10x10^5 cells/flask or 1x10^5 cells/well respectively.

2.1B(c)(ii) Flask and Coverslip Coatings.

While uncoated flasks provided an adequate substrata for primary cultures of HUVE, the cells lost adherence after 2-3 weeks. For longer term cultures and serial passages it was necessary to use fibronectin- (Knauer and Cunningham, 1983) or 1% gelatin- (Thornton et al, 1983) coated culture vessels. Fibronectin-coated coverslips were prepared by the addition of 200µl of a 100µg/ml solution of human fibronectin (Sigma) in serum free ECM to each coverslip-containing well of 24 well multiplates and allowed to absorb for 10 minutes at RT. Unabsorbed material was then aspirated and the wells seeded immediately with an HUVE cell suspension. Gelatin was used to coat all plastic culture vessels. A 1% gelatin solution was prepared by dissolving powdered gelatin (BDH Chemicals) in boiling DDW and 0.2 ml/cm^2 of this solution was added immediately to the flasks and agitated to ensure the surfaces were fully covered. Excess solution was then aspirated and the

uncapped flasks dried under ultraviolet light in a cell culture safety cabinet. The flasks could be used for up to 6 months after coating.

2.1B(d) Other cell cultures.

Twenty five continous and semi-continous cell lines were used in this study and are listed, along with their mycoplasma status and usual seeding rates in Table 2.1.

2.1C NUCLEOTIDE INCORPORATION ASSAYS.

^{3}H-thymidine and ^{3}H-uridine incorporation assays were performed on confluent or subconfluent cell monolayers grown on 13mm glass coverslips contained in the wells of 24 well multiplates. The cells were rinsed with PBS then duplicate wells incubated for 1-6 hours at 37°C in 5% CO_2 in DMEM containing 2µCi/ml of ^{3}H-thymidine or ^{3}H-uridine (123 and 37 Ci/mMole respectively; Amersham). The coverslips were harvested and, without drying, fixed in Carnoys solution (methanol : glacial acetic acid, 3:1) for 10 minutes at RT, washed 2x5 minutes in ice cold 10% TCA, rinsed in DDW, air dried and counted in a toluene-based scintillation fluid in a liquid scintillation counter.

2.2 PROKARYOTIC CELL CULTURE.

2.2A MEDIA AND CELLS.

Initially L broth and L agar were used but later, better results were obtained with a combination of 2YT broth, 2YT agar, and Super Broth. All recombinant plasmids and vectors in this study carried an ampicillin gene and selection of transformed cells was made in media or agar containing 100µg/ml ampicillin. The recA$^-$ E coli strain DH-5, used throughout this work, was obtained from Ray Harris (South Australian Institute of Technology). The preparation of culture media for bacterial work is described in Appendix 4. For long term storage, transformed and non-transformed DH-5 were stored at -20°C or -70°C as a 50:50 mixture of overnight broth culture and glycerol.

2.2B PREPARATION OF COMPETENT CELLS.

A single colony from a 2YT agar plate, containing an overnight culture of DH-5, was transferred to 25 ml of 2YT broth and incubated on a shaker at

Table 2.1 Cell Lines

CELL LINE	ORIGIN	SPLIT RATIO	MYCO PLASMA	REFERENCE
HUMAN				
HepG2	Hepatoma	1:4	-	Knowles el al (1980)
Hep3B	Hepatoma	1:6	-	Knowles el al (1980)
HH1	Hepatoma	1:10	-	Fowler et al (1983)
Mahlavu	Hepatoma	1:10	+	Prozesky et al (1973)
PLC/PRF/5	Hepatoma	1:7	+	Macnab et al (1976)
Endothelial	Umbilical Cord	1:2	-	See Section 2.1 B(c)
FS Fibroblast	Foreskin	1:3	-	prepared at IMVS
Graham-293	Transformed Embryonic Kidney (Ad-5)	1:4	+	Graham et al (1977)
Hep-2	Epidermal Carcinoma	1:8	-	ATCC CCL 23
Hela	Cervical Tumour	1:8	-	ATCC CCL 2.1
Vitus Schroder	Submandibular Gland Tumour	1:3	+	Zenner et al (1979)
MONKEY				
BGM	Kidney	1:5	-	Barron et al (1970)
BSC-1-4	Kidney	1:5	+	Hopps et al (1963)
LLC MK2	Kidney	1:4	-	ATCC CCL 7
Vero	Kidney	1:6	-	ATCC CCL 81
MLA-144	T Lymphocyte	1:4	-	Kawakami et al (1972)
OTHER				
3T3	Mouse Fibroblast	1:4	-	ATCC CCL 92
EHMG118	Transformed Avian CNS (Retrovirus)	1:7	+	Unknown
GSM	Transformed Goat Synovial Membrane (Retrovirus)	1:4	+	Prepared at the IMVS
MDCK	Canine Kidney	1:4	-	ATCC CCL 34
Rat 2	TK^- Rat Epithelial	1:4	-	ATCC CRL 1764
RK13	Rabbit Kidney	1:5	-	ATCC CCL 37
TRANSFECTED				
2.2.15	HepG2 Transfected with HBV DNA	1:5	-	Sells et al (1987)
TK4	Rat 2 Transfected with HBV DNA	1:5	+	Gough et al (1982)

37°C for approximately 6 hours [until the optical density (OD) at 550nm was 0.4-0.6]. The broth was then centrifuged (2,500g, 5 minutes, 4°C) and the pellet resuspended in 12 ml of ice cold 100mM $CaCl_2$ in DDW. After incubation on ice for 30 minutes the cells were repelleted under the same conditions and finally resuspended in 2.0 ml of cold 100 mM $CaCl_2$. Where high transformation efficiency was expected the cells were used immediately; in most cases, however, transformation sensitivity was optimised by storage of the cells at 4°C for 18-48 hrs before use.

2.3 CLONING.

2.3A VECTORS AND PLASMIDS.

2.3A(a) pGEM-3. (Fig 2.1)

pGem-3, one of the Gemini series of vectors, was obtained from Promega Biotechnology and used in the construction of most of the recombinant plasmids described in this work. This vector is comprised of a pBR322 origin of replication, an ampicillin resistance gene and a M13 multiple cloning site (MCS) consisting of 12 unique restriction enzyme sites flanked by SP6- and T7-RNA polymerase promoters. These can be used to generate strand-specific RNA probes from any DNA species inserted into the MCS.

2.3A(b) pSV2neo. (Fig 2.2)

pSV2neo, an eukaryotic expression vector (Southern and Berg, 1982), was obtained from Alan Robins (Dept of Biochemistry, University of Adelaide). It contains a pBR322 origin of replication, an ampicillin resistance gene and a 1.4 kb neomycin (neo) coding sequence from the bacterial transposon Tn5. The latter has been arranged in a mammalian transcription unit by sandwiching between the SV40 early region promoter and small t antigen splice/polyadenylation sites. As the product of the neo gene inactivates the cytotoxic aminoglycoside antibiotic G418, transfection leading to stable integration of pSV2neo will confer resistance to this antibiotic to any eukaryotic cell type. Foreign DNA can be inserted into the EcoR1 and BamH1

Fig 2.1 pGem-3

pGem-3 (Promega) was used for the construction of recombinant plasmids and for the preparation of riboprobes. pGem-3 contains a pBR322 origin of replication, an ampicillin resistance gene and SP6 and T7 RNA polymerase promoters that flank a M13 multiple cloning site (MCS).

Fig 2.2 pSV2Neo

pSV2Neo was developed by Southern and Berg (1982), and contains a pBR322 origin of replication, an ampicillin resistance gene and a bacterial neomycin-resistance gene under the control of a SV40 early region promoter. This plasmid was used for the stable expression of viral genes in mammalian cells.

Fig 2.3 pRSV009.

pRSV009 was also used for the stable expression of viral genes in mammalian cells and contains the same origin of replication and selection systems as pSV2Neo. In addition, pRSV009 has a RSV LTR promoter, and RNA cap and polyadenylation sites that enable mRNA to be synthesised from DNA inserted into its BamHI site.

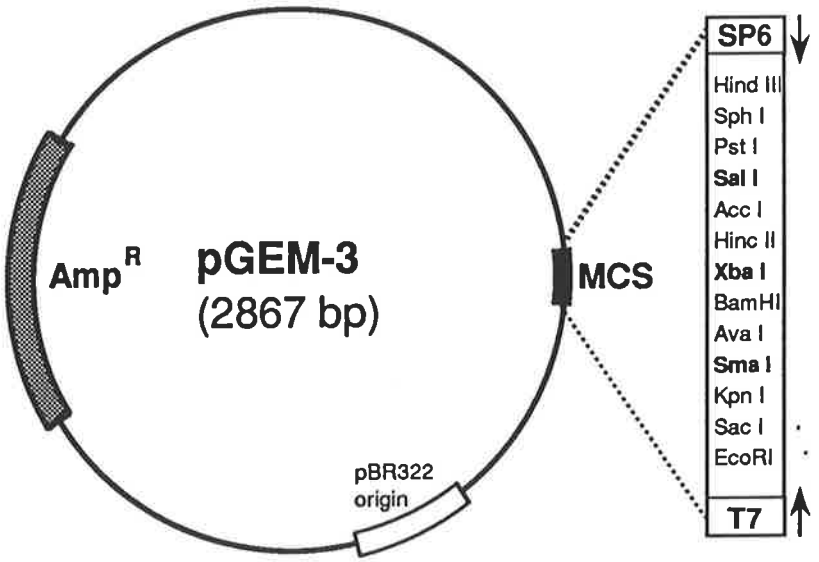


FIG 2.1

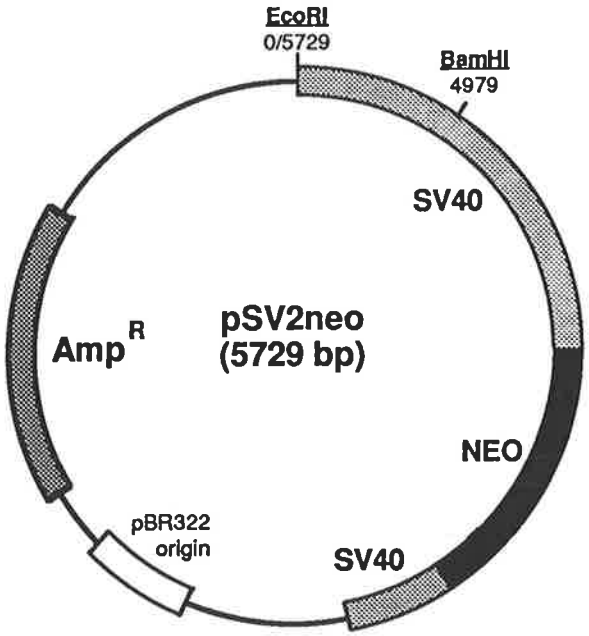


FIG 2.2

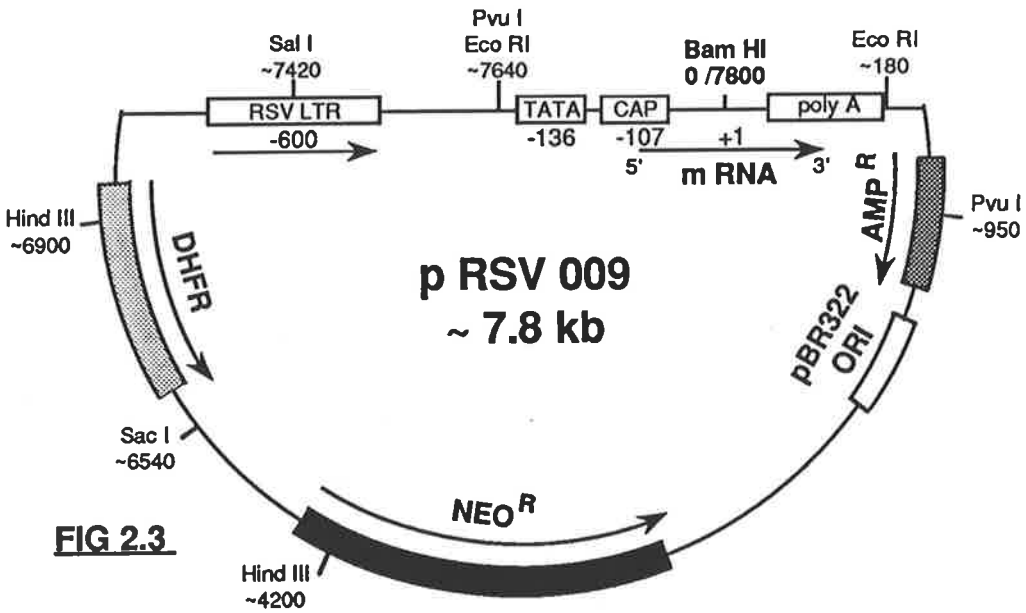


FIG 2.3

restriction enzyme sites in pSV2neo, but, must include its own promoter and polyadenylation signals for gene expression.

2.3A(c) pRSV009. (Fig 2.3)

pRSV009, another eukaryotic expression vector (also obtained from Alan Robins), also integrates into eukaryotic chromosomal DNA and carries the same cellular selection systems as pSV2neo. Unlike pSV2neo however, pRSV009 contains appropriate signals for gene expression, comprising a Rous sarcoma virus long terminal repeat (RSV LTR) promoter coupled to a RNA capping site, and a polyadenylation signal, and is able to generate mRNA from any DNA inserted into the unique BamH1 site. pRSV009 also carries a dihydrofolate reductase (DHFR) gene that permits methotrexate selection and gene amplification.

2.3A(d) pSPDHBV 5.1 and 5.2. (See Fig 5.1)

pSPDHBV 5.1 and 5.2 (Tuttleman et al, 1986b) were obtained from John Pugh (Fox Chase Cancer Center) and consist of a full length copy of DHBV DNA inserted, in either orientation, into the EcoR1 site of SP65. After linearization with restriction enzyme Sac1, SP6 RNA polymerase was used to generate riboprobes to detect short and long strand DHBV DNA sequences respectively. pSPDHBV 5.1 was also used as a source of DHBV sequences for the cloning work described in Chapter 5.

2.3A(e) pTKHH2.

pTKHH2 was supplied by Hans Will (University of Heidelberg) and contains a tandem arrangement of HBV DNA that was infectious when inoculated intrahepatically into chimpanzees (Will et al, 1982; 1985). Full length HBV DNA was removed from pTKHH2 by EcoR1 digestion and gel purification. The excised HBV DNA was recircularized with T4 DNA ligase (Boehringer) and used in the co-transfection experiments described in Chapter 4.

2.3A(f) pHBVCB.

Plasmid pHBVCB (Burrell et al, 1979) was obtained from Professor Ken

Murray (University of Edinburgh) and used as a source of HBV DNA for preparing nick translated probes. HBV DNA was isolated from pHBVCB after digestion with restriction enzyme Pst1 followed by gel purification and electroelution. This was performed by Allison Jilbert.

2.3A(g) BMB37 and BMB104. (see Fig 6.2)

BMB37 and BMB104 (Gowans et al, 1988) were obtained from John Gerin (Georgetown University). They were prepared (by Bahige Baroudy) by inserting the Sma1 fragments from HDV cDNA clones δ4 and δ115 (Wang et al, 1986) respectively into the Sma1 site of vector pGem3. These plasmids were used as the starting material for all the HDV cloning described in this thesis and were also used to generate strand-specific HDV riboprobes to detect genomic and antigenomic HDV RNA. Later this task was performed with pTMδ3 (see Chapter 6) using the same promoters and restriction enzymes.

2.3B PHENOL EXTRACTION AND ETHANOL PRECIPITATION OF NUCLEIC ACIDS.

Phenol for use in extractions was prepared according to Maniatis et al (1982). Crystalised phenol (BDH) was melted at 68°C then 8-hydroxyquinoline added to a final concentration of 0.1%. The melted phenol was then extracted three times in 1M Tris (pH8.0) followed by repeated washes in 0.1M Tris (pH8.0) until the pH of the aqueous phase was ≥7.6. Phenol was stored under 0.1M Tris (pH8.0) at 4°C until required.

Crude nucleic acid samples were extracted by adding an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol. The mixture was then vortexed, centrifuged at ca. 10,000g for 5 minutes and the aqueous phase collected into a clean tube. Nucleic acids were precipitated by the addition of a 1/10 volume of 3M Na Acetate and either a 1.2 volume of isopropanol or 2 volumes of 100% ethanol, followed by incubation at -20°C overnight. The nucleic acid was pelleted by centrifugation (15,000g, -20°C, 30 minutes) and the pellets washed 2-3 times in 70% ethanol prior to freeze drying.

2.3C DNA MODIFYING ENZYMES.

2.3C(a) Restriction enzymes.

Restriction enzymes were purchased from several manufacturers and digestions were performed at 37°C for 60 - 180 minutes. Enzyme : DNA ratios varied according to the enzyme type and the desired outcome (ie. partial or complete digestion). All restriction enzyme reactions utilized the three buffer system described in Maniatis et al (1982, Appendix 5) except SmaI, which used the buffer recommended by the manufacturer (Boehringer, Appendix 5). The optimum enzyme concentrations for partial DNA digestions (a technique used in the construction of full length and multimeric length HDV cDNA and dimeric DHBV DNA) was determined empirically.

2.3C(b) Blunt Ending.

After restriction enzyme digestion, 5' overhangs were end filled in a reaction mixture containing 7mM Tris-HCl pH 7.4, 50mM NaCl, 7mM $MgCl_2$, 1mM DTT, 0.5 mM of each dNTP, 1µg/µl DNA and 0.05 units/ml Klenow (Boehringer), that was incubated at 37°C for 15 minutes. The enzyme was then inactivated by incubation at 70°C for 10 minutes and the DNA phenol-chloroform extracted and ethanol precipitated.

The procedure to end fill 3' overhangs was similar, however in this case the enzyme T4 DNA polymerase (Promega) was used in a mixture containing 70mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 5mM DTT, 0.5 mM of each dNTP, 1.0 µg/µl DNA and 0.5 units/µl enzyme.

2.3C(c) Alkaline Phosphatase.

After cleavage with restriction enzymes, vector DNA was prevented from self ligation by digestion with alkaline phosphatase (37°C, 30 minutes) in a mixture containing 50mM Tris-HCl pH9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 100-200ng DNA/µl and 0.1 units of alkaline phosphatase (Boehringer) per µg of DNA.

2.3C(d) Ligation.

Ligation reactions were performed using T4 DNA ligase (Boehringer) in

a buffer containing 50mM Tris-HCl (pH 7.4), 10mM $MgCl_2$, 10mM DTT, 1mM adenosine triphosphate and 0.1mg/ml bovine serum albumin (BSA, Boehringer). Cohesive end ligation mixtures were incubated at RT for 2-16 hours and contained 0.5 units of ligase/µg DNA with final DNA concentrations of 5-10ng/µl and vector : insert molar ratios of 1 : 1 to 1 : 3. Blunt end ligations were incubated at RT for 16-24 hours and utilized 1-3 units of enzyme/µg DNA with final DNA concentrations of 100-200ng/µl and vector : insert ratios of 1 : 3 to 1 : 20. The DNA products of all ligation reactions were ethanol precipitated prior to transformation into E. coli.

2.3D AGAROSE GEL ELECTROPHORESIS.

DNA was separated in horizontal 1% agarose gels using a BRL model H3 electrophoresis system and a Bio-Rad model 3000xi computerized power supply. The gels were prepared by heating powdered agarose (Biorad) in TAE buffer until dissolved. The gel mixture was then cooled to ca. 50°C, poured into the gel forming trays, a 1-2mm thick comb inserted and the gel left to set for at least 20 minutes at RT. DNA samples were mixed with loading buffer [final concentrations 10mM Tris-HCl pH 8.0, 1mM EDTA, 3% (w/v) Ficoll, 0.05% Bromophenol blue (BPB; Sigma) and 0.05% Xylene cyanol (Ajax chemicals, Sydney)], loaded onto the gel and electrophoresed in TAE buffer at 5-35 watts until the BPB dye front neared the end of the gel. The gel was then stained for 20 minutes at RT in a 10µg/ml solution (in DDW) of ethidium bromide (Sigma), rinsed in DDW and the DNA visualized using a UVP untra violet (UV) transilluminator (Gelman, NSW) and photographed on Polaroid Type 665 or 667 film. DNA fragment sizes were determined by comparison to the bands generated from λ DNA digested with restriction enzyme Pst1 (Table 2.2).

DNA was recovered from agarose gels by electroelution. The gel slice containing the desired DNA fragment was inserted into a 2.5 cm diameter dialysis bag (Selby Scientific, Adelaide) containing 3-4mls of 0.2xTBE. The DNA was electroeluted for 20-40 minutes (depending on the size of the DNA

TABLE 2.2 λ Pst DNA STANDARD

λ DNA (48502 bp) Digested with PstI

Fragment Size (bp)	% of Total	ng DNA/Fragment/Total λ DNA Loaded			
		125	250	375	500
11502	24	30	60	90	120
5077	10.5	13	26	39	53
4749	10	12.5	25	37.5	50
4507	9	11.3	22.5	33.8	45
2838	5.9	7.4	14.8	22.1	29.5
2556 } 2459 } 2443 }	15.4	19.3	38.5	57.8	77
2140	4.4	5.5	11	16.5	22
1959	4.0	5	10	15	20
1700	3.5	4.4	8.8	13.1	17.5
1159	2.4	3	6	9	12
1093	2.3	2.9	5.6	8.6	11.5
805	1.7	2.1	4.3	6.4	8.5
514	1.1	1.4	2.8	4.1	5.5
468 } 448 }	1.9	2.4	4.8	7.1	9.5
339	0.7	0.9	1.8	2.6	3.5
264	0.5	0.6	1.3	1.9	2.5
247	0.5	0.6	1.3	1.9	2.5
216 } 211 } 200 }	1.2	1.5	3.0	4.5	6.0
164	0.3	0.4	0.8	1.1	1.5
150	0.3	0.4	0.8	1.1	1.5
99	0.2	0.3	0.5	0.8	1.0
94	0.2	0.3	0.5	0.8	1.0
87	0.2	0.3	0.5	0.8	1.0
15	0.03	0.04	0.08	0.11	0.15

fragment) at 200 volts then the current was reversed for 1 minute (to dislodge the DNA from the sides of the bag). The DNA-containing buffer was then collected from the bag, extracted with butanol to ca. 300µl, phenol extracted, ethanol precipitated, freeze dried and finally redissolved in 50-100µl of TE8. The concentration of the DNA was determined by comparison of band intensity with the λ DNA standard above (Table 2.2).

2.3E TRANSFORMATION AND PLASMID AMPLIFICATION.

2.3E(a) Transformation and Screening.

Up to 1.5µg DNA, dissolved in 1-20µl of TE8 or DDW, was gently mixed with a 200µl aliquot of competent DH5 cells and incubated on ice for 40 minutes. The mix was then heat shocked for 150 seconds at 42°C, 0.8 ml of 2YT broth added and incubated for a further 60 minutes at 37°C with constant shaking. Aliquots were then spread over 2YT agar plates containing 100µg/ml ampicillin and incubated at 37°C until antibiotic-resistant colonies had developed (usually 16-24 hours). The colonies were screened by colony transfer to two nitrocelluose filters (Gelman) previously overlaid on L agar plates containing 100µg/ml ampicillin. After incubation overnight at 37°C, one plate was wrapped in plastic film and stored at 4°C while the filter from the other was removed and floated on top of a 0.5M NaOH bath for 10 minutes at room temperature. This filter was then neutralised by transferring in succession to 3MM paper (Whatman) soaked with 1M Tris-HCl pH 7.2 (3 x 30 seconds) then 1M Tris-HCl pH 7.2, 1.5M NaCl (10 minutes, RT). The filter was then air dried and baked in a vacuum oven at 80°C for 2 hours. Prior to hybridisation and exposure to X-ray film [as described below; part 2.5, section E (a)], the filters were first washed in a solution of 50mM Tris-HCl (pH 7.6), 1M NaCl, 1mM EDTA and 0.1% SDS for 1-2 hours at 42°C in a shaking water bath.

Hybridisation-positive colonies were further analyzed by mini preparations; 4ml of 2YT broth containing 100µg/ml ampicillin was inoculated with a single colony and incubated overnight with shaking at 37°C. 1.5ml of

this overnight broth was transferred to an Eppendorf tube and centrifuged for 5 minutes at 13,000 rpm. The cell pellet was resuspended in 100µl of lysis solution A [50mM Glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA and 5mg/ml lysozyme (Boehringer)], incubated at RT for 5 minutes then 200µl of lysis solution B (0.2 M NaOH,1% SDS) was added and incubated on ice for a further 5 minutes. Finally 150 µl of 3M sodium acetate pH 5.2 was added and the cellular DNA/protein precipitate was removed by centrifugation. The supernatant was then transferred to a fresh Eppendorf tube, mixed with two volumes of 100% ethanol, stored on ice for 5 minutes, centrifuged and the recombinant plasmid DNA pellet washed once in 70% ethanol. After removal of all residual fluid with a micropipette the pellet was redissolved in 50-100µl of TE8 containing 300µg/ml of preboiled RNase A (Boehringer) and analysed, without further purification, by restriction enzyme mapping and Southern blot hybridisation.

2.3E(b) Large Scale Plasmid Amplification.

20 ml of an overnight broth culture of transformed DH5 cells was transferred to 500 ml of Super Broth containing 100µg/ml ampicillin and incubated in half-filled 500ml glass bottles on a cell shaker for 6 hours at 37°C. Chloramphenicol (Parke Davis) was then added to a final concentration of 150µg/ml and incubation continued overnight. The cells were pelleted by centrifugation (7000g, 10 minutes, 4°C), resuspended in 100ml STET, transferred to a 500ml volumetric flask, 50mg lysozyme added and the mixture swirled on a boiling water bath for 5 minutes. The mixture was then transferred to a 250ml polypropylene centrifuge tube, cooled on ice and centrifuged for 15 minutes at 20,000g and 4°C. The supernatant was transferred to a clean 250ml polypropylene centrifuge tube, an equal volume of very cold (-70°C) isopropanol added and the precipitate collected immediately by centrifugation (20,000g, 30 minutes, -20°C). After removal of all visible supernatant, the pellet was dissolved in 17 ml of TE8 and transferred to a 40 ml polypropylene tube containing 17.9 gm of CsCl and 0.9

ml of a 10mg/ml solution of ethidium bromide. When fully dissolved (incubation at 37°C helped) the mixture was clarified by centrifugation (16,500g, 10 minutes, 20°C) and the supernatant transferred to 2 Beckman 13.5ml heat seal tubes. The DNA was banded by centrifugation in a Beckman L8 ultracentrifuge in a 70.1Ti rotor using a gradient relaxation protocol (49,700 rpm, 24 hours, 20°C then 39,700, 2 hours, 20°C).

The DNA was visualized under UV light and the band corresponding to plasmid DNA collected through the side of the tube using a 19 gauge needle. Ethidium bromide was removed by 3-4 extractions with H_2O-saturated butanol and the DNA precipitated by the addition of 2.5 volumes of 100% ethanol and storage on ice for 10 minutes. After centrifugation (20,000g, 4°C, 15 minutes) the DNA pellet was redisolved in TE8, reprecipitated in ethanol, washed x2 in 70% ethanol, freeze dried and finally redissolved in 500-1000 µl of TE8 or DDW. The DNA concentration was determined by adsorption spectrophometry at 260 nm (where 1 OD unit at A^{260} = 50µg/ml), adjusted to a final concentration of 1mg/ml, aliquotted in 100µl volumes then stored at -70°C. This technique yielded up to 4mg of plasmid DNA per 500ml of Super Broth.

2.4 TRANSFECTION.

DNA transfection experiments were performed using the polybrene technique (Kawai and Nishzawa, 1984) coupled with Geneticin (G418; Gibco) selection. The optimum polybrene concentrations required for transfection of the cell lines used in these experiments was either obtained from the literature or, like the optimum G418 concentration, determined empirically (These concentrations are shown in Table 2.3). Two variations of the polybrene technique were used.

2.4A ADHERENT CELL TECHNIQUE.

This method was used for HepG2, Hep3B and HH1 cells and is based on the methods described by Kawai and Nishzawa (1984) and Morgan et al (1986); 30% confluent 25 cm^2 flasks of cells (seeded the previous day at

TABLE 2.3 Polybrene and G418 concentrations used in the transfection experiments.

CELL LINE	[POLYBRENE] optimum for transfection (μg/ml)	[G418] optimum for selection (μg/ml)
HeLa	25	450
Hep3B	10	200
HepG2	5	350
HH1	25	200
Vero	40	400

$2.5x10^6$, $1x10^6$ and $8x10^5$ cells/flask respectively) were incubated briefly at RT with 10ml of DMEM containing 5% FBS and polybrene. This was replaced with 1.0ml of medium containing 5-10µg of recombinant DNA (no carrier) and the flasks incubated, with occasional agitation, for 6 hours at 37°C. The medium was removed and the cells shocked for 4 minutes at RT with 10 ml of 30%DMSO in DMEM. The cells were then washed once in DMEM, DMEM+5% FBS added and the cells incubated for 48 hours at 37°C in 5%CO_2. Finally the cells were trypsinised and seeded into 48 well multiplates (Costar) in DMEM+5% FBS containing G418. This medium was replaced twice weekly, and resistant colonies were detected after 2-3 weeks. The use of 48 well plates simplified colony isolation as rarely did more than single colony arise in each well. Full G418 selection was maintained until liquid nitrogen stocks were established (6-10 weeks) when the G418 concentration was reduced by 50% or in some cases removed completely.

2.4B SUSPENSION CELL TECHNIQUE.

This method was used for Hela and Vero cells; subconfluent $75cm^2$ flasks of cells (seeded the previous day) were trypsinised to the point where the cells just detached (excessive trypsinisation reduced transfection efficiency) and resuspended in 10 ml of DMEM+5% FBS. The cells were counted and $5x10^5$ cells transferred to a 10ml centrifuge tube and pelleted at 800g for 5 minutes. The cells were then resuspended in 300µl of DMEM containing 5% FBS, polybrene and 5-10µg DNA (no carrier), and incubated at 37°C for 1hour 40 minutes with gentle agitation every 20 minutes. A further 1.5 ml of polybrene medium without DNA was then added, incubated for an additional 20 minutes, the suspension transferred to a $25cm^2$ flask and incubated at 37°C in 5% CO_2 until the cells adhered (generally 4-5 hours). The cells were then shocked with DMSO as described above except, due to the high transformation efficiency obtained with this technique, the cells were seeded during the initial selection step into 96 well plates.

2.4C RNA TRANSFECTION.

The RNA transfections described in Chapter 7 were mediated by lipofectin reagent (BRL) using the protocol recommended by the manufacturer. RNA for use in this technique was prepared as described in section 2.5 D(b).

2.4D SUBCLONING.

Subcloning was performed by reseeding the cells into 96 well plates at the rate of ca. 0.8 cells/well. The plates were then monitored at regular intervals and cultures were expanded only from the wells in which single colonies had arisen. For most cell types the plates were used directly, although for some Hep G2-derived cultures, feeder layers were also required. These were prepared in the Radiotherapy unit of the Royal Adelaide Hospital by irradiating Swiss 3T3 cells with 6,000 Rads of X-rays generated from a linear accelerator.

2.4E G418 TITRATION.

This was performed for each cell type and repeated with each new batch of G418. Replicate cultures were seeded and grown in medium containing increasing concentrations of G418. The optimum G418 concentration for selection was taken as the minimum that caused all cells to die and detach by 3 weeks for HepG2 and 2 weeks for other cell lines.

2.5 NUCLEIC ACID HYBRIDISATION.

2.5A SAMPLE PREPARATION.

2.5A(a) DNA Extraction from Cultured Cells.

Cell monolayers in 150cm^2 flasks (ca. 2-10x10^7 cells depending on cell line) were trypsinised, pelleted by centrifugation, washed twice in PBS and resuspended in 1ml of a solution containing 100mM Tris-HCl (pH 7.5), 20mM EDTA, 1% SDS and 1.0 mg/ml Proteinase K (Boehringer). The mixture was incubated for 18 hours at 37°C, then extracted twice with phenol, ethanol precipitated, freeze dried and redissolved (by incubation overnight at 4°C) in 500µl of TE8. The DNA concentration was determined by OD as described in

section 2.3 E(b) above.

2.5A(b) RNA Extraction from Cultured Cells

2.5A(b)(I) Cytoplasmic RNA.

Cytoplasmic RNA was extracted as described Wilkinson (1988). A 75cm^2 flask containing ca. 1-5x10^7 cells (depending on cell line) was washed twice with cold PBS, drained, 2.0 ml of NDD [0.2% Nonidet P40 (NP40), 0.1% deoxycholate and 0.002% dextran sulphate in Tris-HCl-saline pH 7.4) added, the flask incubated for 5 minutes at RT and the contents centrifuged in a microfuge for 30 seconds at 6500rpm. The supernatant was transferred to a clean tube together with an equal volume of phenol:chloroform:isoamylalcohol, then SDS and NaCl added to 0.5% and 75mM respectively. The mix was then vortexed and centrifuged in a microfuge at 13,000rpm for 10 minutes. RNA was precipitated from the supernatant with isopropanol, washed twice in 80% ethanol and stored under 80% ethanol at -20°C until required. For use the RNA was freeze dried, redissolved in 0.05%SDS by incubation at 65°C for 10 minutes and the concentration determined by OD where 1 A^{260} unit = 40µg/ml RNA.

2.5A(b)(ii) Total RNA.

Total RNA was extracted by a published method (Chromczynski and Sacchi, 1987). A 150 cm^2 flask containing 2-10x10^7 cells was washed twice in PBS, drained , 8ml GIT buffer added [4M Guanidium isothiocynate (ultrapure; IBI), 25mM Na citrate pH 7.0, 0.5% Sarkosyl and 0.1M 2-mercaptoethanol] and the flask incubated at RT for 5 minutes. The contents of the flask (that had become viscous), were transferred to a 30ml centrifuge tube and the following ingredients were added in the order listed with mixing between each addition; 1ml 2M NaOAc pH 4.6, 10ml Phenol (H_2O saturated) and 2.0ml of chloroform:isoamylalcohol (49:1). The final mix was incubated on ice for 15 minutes then centrifuged at 10,000g for 15 minutes at 4°C. RNA was precipitated from the supernatant as described above. The pellet was redissolved in 2.0ml of GIT buffer, reprecipitated and stored under 80%

ethanol at -70°C until required.

2.5A(b)(iii) Nuclear RNA.

Trypsinised cells were were washed twice in PBS, resuspended in lysis buffer (10mM Tris-HCl pH 7.4, 3mM $MgCl_2$, 1mM KCl, 0.25%NP40), incubated on ice for 3 minutes then centrifuged for 5 minutes at 500g. The pellet (containing isolated nuclei) was washed twice in PBS then the RNA was extracted in GIT buffer (as above).

2.5A(c) HDV RNA Extraction from Serum and Cell Culture Fluid.

1ml of serum or 8ml of cell culture fluid were overlaid on a 9ml or 2ml cushion respectively of 20%sucrose containing 20mM Hepes pH7.4, 0.1%BSA, 10mM $CaCl_2$ and 10mM $MgCl_2$ in a 10ml centrifuge tube and centrifuged in a IEC ultracentrifuge in a Type 494 fixed angle rotor at 340,000g for 4 hours at 4°C. The supernatant was removed, the sides of the tube dried with tissue paper and the pellet resuspended in 400µl of lysing solution (50mM Hepes pH7.4, 0.2M NaCl, 20mM EDTA, 2%SDS, 500µg/ml Proteinase K and 50µg/ml yeast tRNA). The mixture was then incubated at 37°C overnight, phenol extracted, ethanol precipitated and stored under 80% ethanol at -70°C until required.

2.5A(d) Purification of Poly(A)$^+$ and Poly(A)$^-$ RNA.

Poly (A)$^+$ and poly(A)$^-$ RNA was isolated using oligo(dT) cellulose as described by Davis et al (1986). 500mg of oligo(dT) cellulose powder (BRL) was swollen in 20mM Tris-HCl (pH7.4), 0.1M NaCl, 1mM EDTA, 0.1%SDS (Buffer A) then poured into the body of a 5ml syringe (plugged at the neck with sterile glass wool) to give a packed volume of ca.2.0ml. Prior to the first use and between different RNA isolations, the column was washed with 5.0ml of 0.1M NaOH, 5mM EDTA, then sterile DDW until the pH of the effluent was less than pH8.0, and finally equilibrated by washing with 5ml of Buffer B [40mM Tris (pH7.4), 1M NaCl, 1mM EDTA, 0.1%SDS].

1-5mg of total RNA [extracted from cultured cells as described in Section 2.5 A(b)(ii) above] was redissolved in ca 0.5-1.0ml of DDW then mixed with

an equal volume of Buffer B. The sample was denatured at 65°C for 5 minutes, cooled to RT, and applied to the column. The (first) filtrate was then collected, reheated, cooled and reapplied to the column. Poly(A)$^-$ RNA was obtained by ethanol precipitation of the second filtrate. The column was then washed in ca. 5.0ml of Buffer A and the bound poly(A)$^+$ RNA eluted from the column by washing with 10mM Tris-HCl (pH7.4), 1mM EDTA, 0.05%SDS and 10 drop fractions collected. Poly(A)$^+$ RNA-positive fractions were identified on an 1% agarose gel containing 1µg/ml ethidium bromide, pooled, ethanol precipitated and stored at -70°C. Between uses the column was stored at 4°C in Buffer A containing 0.02% NaN_3.

2.5B TRANSFER OF NUCLEIC ACIDS TO NITROCELLUOSE.

2.5B(a) DNA.

DNA for Southern blot hybridisation analysis was separated by agarose gel electrophoresis (section 2.3 C) then transferred to Zeta-probe membrane (Bio-Rad) using an alkaline transfer protocol (Reid, personal communication); the gel was treated for 5 minutes in 0.25M HCl, rinsed briefly in DDW and assembled into a BRL DNA Blot Transfer System. DNA was transferred to the membrane for 5-16 hours by capillary diffusion, with 0.4M NaOH, then the membrane rinsed in 2XSSC and air dried. The membrane was either hybridised immediately or baked at 80°C for 2 hours and stored at RT for later processing.

2.5B(b) RNA.

Samples were prepared and electrophoresed as described by Thomas (1980). Formamide, formaldehyde and 20xE buffer (1x; 18mM Na_2HPO_4, 2mM NaH_2PO_4) were added to the redissolved RNA samples to a final concentration of 50%, 6% and 1x respectively. Immediately prior to electrophoresis, the RNA mixture was denatured by boiling for 10 minutes, quenched on wet ice, then a 1/4 volume of 4x loading buffer (LB) added (1xLB; 10 % formamide, 5% glycerol, 0.02% BPB, 0.05% xylene cyanol) and finally loaded onto the gel.

1% agarose gels in 1xE buffer containing 6% formaldehyde were used to analyse the RNA. Agarose was added to DDW and heated in a microwave oven until dissolved, cooled to 60°C, the remaining ingredients added and poured into a gel forming tray (BRL) and allowed to set for at least 20 minutes. The gels were electrophoresed at 10-15 watts for 3-5 hours.

The nitrocellulose membrane was wet successively in 2xSSC and 20xSSC then assembled with the gel in a BRL DNA Blot Transfer System. Transfer was performed overnight in 20xSSC, the membrane rinsed in 2xSSC, air dried and baked in a vacuum oven at 80°C for 2 hours.

2.5C DOT BLOTS HYBRIDISATION.

2.5C(a) Spot Method.

Ten microlitre volumes of neat untreated serum or control plasmid DNA (in DDW) were spotted directly onto dry nitrocelluose membrane (Schleicher and Schuell). When the samples were dry the filter was treated as described above for colony blots [section 2.3 E(a)].

2.5C(b) Filtration Method.

25 µl aliquots of neat serum or resuspended cell culture fluid pellets or 200µl aliquots of neat cell culture fluid were mixed with an equal volume of 2M NaCl and two volumes of 1M NaOH and incubated at RT 10 minutes. Meanwhile, nitrocellulose membrane that had been presoaked successively in DDW (2 minutes) and 10xSSC (5 minutes) was inserted into a 96 well dot blot apparatus (Bio-Rad) and a light vacuum applied. The treated samples were filtered through duplicate wells followed by 100µl of neutralising solution (1 M NaCl, 0.5 M Tris-HCl, pH 7.2). The membrane was then removed from the apparatus, rinsed in 10xSSC and dried at RT. As pure DNA binds poorly to nitrocelluose under filtration (Scotto et al, 1983), control, plasmid-derived, DNA was boiled for 5 minutes, cooled rapidly on wet ice and loaded directly (in 1-2µl volumes) onto the dried membrane, which was then baked in a vacuum oven at 80°C for 2 hours.

2.5D NUCLEIC ACID PROBES.

Nick translated probes were used in all the early hybridisation experiments, but were later replaced with RNA probes except in some Southern blot hybridisation experiments.

2.5D(a) Nick Translation of DNA Probes.

100μCi each of ethanolic ^{32}P-labelled dCTP and dATP (~3000Ci/mM. Bresatec; Adelaide, South Australia) were dried down in a Eppendorf tube then resuspended in a 30μl reaction mixture containing 100mM Tris-HCl (pH 7.5), 12mM $MgCl_2$, 10mM DTT, 20μM cold dGTP and dTTP, 2.5pg/μl DNase 1 (Boehringer), 0.5 units/μl DNA polymerase 1 (Boehringer) and 60-100ng of template DNA. The reaction was incubated for 2 hours at 14°C then stopped by the addition of 8μl of 100mM EDTA and 2μl of 25% Sarkosyl. Initially, labelled DNA was separated from unincorporated nucleotides by gel filtration through a Sepharose-G50 column (Pharmacia), but later this was performed by ethanol precipitation followed by 3-4 washes in 70% ethanol. The probes were freeze dried and finally redissolved in 30μl of 20mM DTT. To monitor the reaction and calculate the specific activity of the product, 1μl samples were taken, and precipitated in 10% TCA, at the beginning (T_0) and at the end (T_{120}) of the two hour incubation period. The probe specific activity was then determined from the formula: specific activity = total TCA precipitable cpm at T_{120} divided by the counting efficiency (~100% for ^{32}P; Gowans, personal communication) and the amount of DNA added. Probes with specific activities of 5-9x10^8 dpm/μg DNA were usual.

2.5D(b) RNA probes.

RNA probes (riboprobes) were prepared from template DNA inserted into the cloning vector pSP65 or pGEM-3 (see Fig 2.1). These vectors, through the SP6 and T7 RNA transcriptional promoters that flank inserted DNA, enable strand-specific riboprobes to be generated from the same recombinant plasmid. Later, to achieve more consistent levels of nucleotide incorporation, probes of both sense were prepared from the T7 promoter

using two separate recombinant plasmids that contained a flipped insert of the same DNA species.

Riboprobe reactions were performed, by the recommended protocol, using commercial kits (Promega) and ^{32}P-UTP (3,000ci/mM, Bresatec; Adelaide, South Australia), and resulted in probes with a specific activity of ca. 6-8x10^8 dpm/µg RNA. The latter was calculated from the following formula:

$$\textbf{Specific Activity} = \frac{^{32}\text{P-labelled UTP}}{\text{cold UTP}} \times 765 \times \text{specific activity of the } ^{32}\text{P-labelled UTP}$$

where 1µCi = 2.22x10^6 dpm and, assuming equal amounts of each nucleotide in the RNA product, 1µg RNA contains 765 pM of each base.

Unlabelled RNA for use in transfection and a number of the nuclear run-off transcription assays described in Chapter 7 was prepared by a recommended modification (Promega) of the usual protocol using the same riboprobe kits. The concentration of the product was determined from the OD at A^{260}. Yields of ca. 20µg RNA/µg DNA template were usual.

2.5E HYBRIDISATION.

2.5E(a) Dot Blot Hybridisation.

Prehybridisation and hybridisation reactions were performed for 4 hours and 16-20 hours respectively at 42°C (DNA probes) or 47°C (Riboprobes) in 50% deionized formamide, 4xSSC, 50mM Na_2HPO_4, 50mM NaH_2PO_4, 50mM Tris-HCl (pH 7.1), 8x Denhardts (0.16% Ficoll + 0.16% PVP), 500µg/ml sheared salmon sperm DNA (ssDNA; Boehringer), 50µg/ml yeast tRNA (Boehringer) and 0.1% SDS. These solutions were assembled by first boiling the formamide with the ssDNA and tRNA and, for hybridisation solutions, ^{32}P-labelled-riboprobes or -nick translated probes (5-10x10^6 cpm/ml or 2-5x10^6 cpm/ml respectively), for 5 minutes. This was then quenched on wet ice, the other ingredients added and the mixture sealed in polythene bags together with the baked nitrocellulose membranes. After hybridisation, the membranes were washed in 2xSSC, 0.1%SDS at room temperature for 4x5

minutes, then 2xSSC, 0.1%SDS at 68°C or 70°C (nick translated probes or riboprobes) for 2x30 minutes and finally 0.2xSSC, 0.1%SDS at 68°C or 70°C for 2x30 minutes.

The washed membranes were wrapped in plastic film and exposed at -70°C to X-Omat RP or AR X-ray film (Kodak Australasia) in cassettes with intensifying screens (Kronex; Dupont, USA). After exposure the cassette was brought to RT and the film processed in an Ilfospeed 2240 (Ilford) X-ray processor. Virus DNA concentrations, calculated as vge, were determined by comparison to plasmid DNA standards.

2.5E(b) Southern Blot Hybridisations.

The prehybridisation solution for Southern blots consisted of 0.5% Blotto (dry skim milk powder; Johnson et al, 1984), 4X SSPE, 500µg/ml ssDNA, 50% deionized formamide and 100µg/ml yeast tRNA. The hybridisation solution was essentially identical except that 3XSSPE replaced 4X and the mix also contained either 2-5x10^6dpm/ml of ^{32}P-labelled nick-translated probe or 4-10x10^6dpm/ml of ^{32}P-labelled riboprobe. Both mixtures were assembled as described above and wash and exposure conditions were also identical to those above [Section 2.5 E(a)].

2.5E(c) Northern Blot Hybridisations.

Hybridisation and washes were performed as previously described [Section 2.5 E(a)], with the exceptions that riboprobes were used throughout and hybridisation and wash temperatures were 55°C and 75-80°C respectively.

2.5E(d) Hybridisation Reagents.

Formamide (AR grade, BDH) was deionized by the addition of 100gm of mixed bed resin [AG 501-X8 (D); Bio-Rad] per litre of formamide and stirring until a 100x drop in conductivity occurred (from ~200 to 2µS/cm measured on a Activon PT1-18 conductivity meter). The ssDNA was dissolved overnight in 0.4M NaOH to a final concentration of 10mg/ml, heated to 100°C for 45 minutes, chilled on ice and neutralised with glacial acetic acid. The DNA was

then precipitated with ethanol, freeze dried and redissolved in TE8 to a final concentration of 10mg/ml (1 A^{260} unit = 40µg/ml for denatured DNA). Deionized formamide and ssDNA were stored at -20°C.

2.6 ANTIGEN DETECTION.

2.6A IMMUNOFLUORESCENCE.

2.6A(a) Staining.

The source, species and usual titres of antisera used in this thesis are shown in Table 2.4. Titres were determined by titration on positive and negative tissue or cultured cells and was the highest dilution that gave the optimal signal to noise ratio.

Coverslips containing monolayers of cells were rinsed in PBS, air dried, fixed in acetone at 4°C for 10-15 minutes and rehydrated in PBS for at least 5 minutes prior to staining. Incubation with primary antibodies, secondary antibodies or direct conjugates was performed at 37°C for 40-60 minutes with 2x5 minute washes in PBS after each incubation step. After staining, the coverslips were mounted in 90% glycerol containing PBS and 50 mM Tris-HCl pH 8.5 and examined with a model BH-2 UV microscope (Olympus) and photographed using a PM-10ADS camera system and PM-CBSP exposure control unit (Olympus).

All direct and indirect immunofluorescence reactions were shown to be specific by examining coverslips containing control cultures and/or by substituting primary antibodies and direct conjugates respectively with normal serum from the same species. Direct conjugates to alternative antigens were also used to confirm the specificities of reactions using direct conjugates.

2.6A(b) Conjugation with FITC.

Most labelled antibodies were purchased from commercial suppliers; however fluorescein isothiocynate (FITC) conjugates of human anti-HD and human anti-HBc were unavailable and were prepared in the laboratory.

The IgG fraction of serum was purified by adsorption to a Protein-A-Sepharose column (1x20cm; Pharmacia) connected to a UV-2 dual

TABLE 2.4 Antibodies used in immunofluorescence experiments.

PRIMARY

ANTIBODY	SPECIES	TYPE	USUAL TITRE	SOURCE
Anti-DHBc	Rabbit	polyclonal	1:20	Bill Mason
Anti-DHB pre-s	Mouse	monoclonal	1:20	Anna O'Connell
Anti-DHBs	Rabbit	polyclonal	1:15-1:30	Ming Qiao
Anti-Factor VIII	Rabbit	polyclonal	1:10	Nordic
Anti-dsRNA	Rabbit	polyclonal	1:20	Richard Francki
Anti-HBc	Rabbit	polyclonal	1:25	Dako
Anti-HB pre-S1	Mouse	monoclonal	1:20	Steve Kent
Anti-HB pre-S2	Mouse	monoclonal	1:40	Steve Kent
Anti-HBs	Rabbit	polyclonal	1:15-1:25	Behring
Anti-HBc-FITC	Human	polyclonal	1:200	Tom Macnaughton
Anti-HD-FITC	Human	polyclonal	1:200	Tom Macnaughton

SECONDARY

ANTIBODY	SPECIES	TYPE	USUAL TITRE	SOURCE
Anti-Rabbit-FITC	Sheep	Polyclonal	1:15-1:30	Wellcome
Anti-Mouse-FITC	Sheep	Polyclonal	1:10	Silenus

path monitor and a chart recorder (Pharmacia). The serum was pumped through the column at 0.5ml/min and the column then washed with PBS at 1ml/min until the OD at 280nm returned to baseline. IgG was eluted from the Protein-A with 0.58% acetic acid in 150mM NaCl (0.7 ml/min) and the peak OD fractions were collected, pooled and the pH adjusted to 7.2 with NaOH. IgG was concentrated by ultrafiltration through a YM-30 membrane (exclusion limit 30kD), in a stirred cell (Amicon; Lexington, Mass), the concentration determined (Biuret) and adjusted to a final concentration of 20mg/ml with PBS and stored at -20°C.

The conjugation technique was performed as described by Nairn, (1976). 1.5ml (30mg) of the above IgG fraction was mixed, with constant stirring, with 0.5ml of 0.2M Na_2HPO_4 followed by 1ml of 0.1M Na_2HPO_4 containing 500µg FITC (BDH Chemicals). The pH was adjusted to 9.5 with 0.1M Na_3PO_4, the total volume adjusted to 4.0ml with 150mM NaCl and the mixture incubated in an end over end mixer for 40 minutes at RT. The mixture was then chilled on ice and any precipitate removed by centrifugation. Finally the conjugate was dialyzed against 4 changes of PBS (over a 26 hour period) to remove unbound FITC, sterilized by filtration and titrated as described above.

The unlabelled primary and FITC-conjugated antibodies were adsorbed against normal liver homogenate to reduce non-specific reactions. An autopsy liver from a patient serologically free of markers for HBV and HDV was frozen in liquid nitrogen and pulverized with a hammer. The frozen powder was passed through a kitchen sieve and collected in a beaker containing ice cold PBS. The homogenate was washed with cold PBS until the supernatant was clear and stored at -20°C in 5ml packed volumes until required.

For use, the homogenate was thawed, washed twice with PBS then mixed with an equal volume of antibody at 2x the expected working dilution and incubated for 1 hour at 37°C, then 4°C overnight. The mixture was

centrifuged at 4°C at 10,000g for 10 minutes to remove the liver homogenate then the supernatant was collected, filter sterilized, titrated and stored at -20°C in small aliquots at the working dilution.

2.6B ELISA.

Serological assays for markers of HBV infection and assays for HBsAg in cell culture fluids and cell homogenates were performed using commercial kits according to the manufacturers instructions (Abbott Laboratories).

2.6C RADIO-IMMUNO ASSAYS (RIA).

HDAg and anti-HD were assayed by a solid phase RIA, performed essentially as described by Rizzetto et al (1980a). Initially, liver-derived HDAg extracted by Eric Gowans was used in the formulation of the anti-HD RIA, but in later assays this reagent was replaced with partially-purified rHDAg (section 2.9 A) produced in transfected HepG2 cells (see Chapter 6).

2.6C(a) Direct HDAg RIA.

Antibodies from a patient with a high titre of anti-HD were Protein-A-Sepharose purified [Section 2.6 A (b)], and the IgG concentration adjusted to 1µg/ml. "Remove a well" strips (Immulon) were coated overnight at RT with 100µl/well of the above antibody preparation diluted 1/800 in PBS containing 40 mM Tris-HCl pH 9.6. The wells were then washed three times with PBS and unoccupied binding sites blocked by incubation for 2 hours at 37°C with 100µl/well of 2% BSA in PBS. The blocking solution was aspirated and 80µl of test sample was added to each well and incubated for 3 hours at 37°C or overnight at RT. The plates were again washed three times with PBS then 600,000cpm/100µl/well of ^{125}I-labelled anti-HD [prepared by Allison Jilbert using the chloramine-T method (Greenwood et al, 1963)] was added and incubated for 3 hours at 37°C. The wells were then washed three times in PBS containing 0.1% Tween-20, three times in PBS only, air dried and counted on a Packard Crystal 400 series gamma counter. Samples were considered positive for HDAg if the positive : negative (P/N) ratio was >2.

2.6C(b) Competitive RIA to Detect Anti-HD.

This test was performed as described above except that liver-derived or rHDAg replaced the "test sample" (at a concentration that gave ca. 20,000 bound counts in the above direct RIA) and following this step, patient serum samples were incubated for 2 hours at 37°C prior to the addition of ^{125}I-labelled anti-HD. Samples were considered positive for anti-HD if the ^{125}I counts bound were reduced by ≥50%.

2.6D IMMUNOBLOTTING.

Polyacrylamide gels (Laemmli, 1970) containing 5% (stacking gel) and 15% (separating gel) acrylamide were prepared from a stock solution of 50% (w/v) acrylamide and 1.3% (w/v) N, N'-methylene-bis-acrylamide. The separating gel was prepared in 0.375M Tris-HCl pH8.8, 0.1%SDS, 0.0625% (v/v) tetramethylenediamine (TEMED) and 0.034% (w/v) ammonium persulphate (APS), and the stacking gel in 0.125M Tris-HCl pH 6.8, 0.1%SDS, 0.1% TEMED and 0.0525% APS. Separating and stacking gels of 15cms and 5 cms lengths respectively were poured successively and polymerized (each for 1 hour at RT) into the 200x200x2 mm gel former (assembled according to the manufacturers instructions) of a Protean II PAGE System (Bio-Rad).

The samples were mixed with loading buffer (50mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2%SDS, a trace of BPB plus, for reducing gels, 1% (v/v) 2-mercaptoethanol). For samples that were too dilute or contained high concentrations of salts the protein fraction was first precipitated by the addition of 4 volumes of ice cold acetone. The precipitate was then collected by centrifugation (5000g, 5 minutes, 4°C), washed twice in 90% acetone at 4°C and finally resuspended in loading buffer as above.

Electrophoresis was performed in 25mM Tris-HCl, 192mM glycine and 0.1%SDS, at 200 volts for ca. 5 hours until the band containing a dye linked protein of approximately 14kD was 1-2 cms from the bottom of the gel. Dye-linked protein standards (Rainbow markers; Amersham) were used to

monitor running conditions and to determine the approximate molecular sizes of proteins in stained gels and immunoblots. In some gels the protein bands were visualized by staining the gel for 1 hour, on a rocking platform, with 0.2% (w/v) Coomassie brilliant blue (Sigma) in 10% (v/v) acetic acid, 50% (v/v) methanol, 40% DDW. The gel was then destained in the above solution (without dye), rinsed in DDW, sandwiched between moistened sheets of cellophane and placed in a fume hood to dry.

Electrophoretically separated proteins were transferred from the gel to Immobilon membrane (Millipore) at 80 volts for 2 hours or 50 volts for 6 hours in a buffer comprised of 25mM Tris-HCl, 192mM glycine and 20% (v/v) methanol. The dye standards were used to monitor the transfer efficiency. After transfer, additional binding sites on the membrane were blocked by incubation in 3% Blotto in PBS at 4°C overnight. The membrane was then incubated for 4 hours at RT, in a 1/1000 dilution (in PBS) of a high titre human anti-HD serum, washed 4x5 minutes in TNT, then incubated for 3 hours at RT with 10^5 cpm/ml (in TNT) of ^{125}I-labelled Protein A prepared by the chloramine-T method. After another 4x5minute washes in TNT the membrane was dried and exposed to X-ray film as described previously [Section 2.5 E(a)].

2.7 INFECTION AND SECRETION EXPERIMENTS.

2.7A SCREENING OF INOCULA (HBV).

Sera positive for both HBsAg and HBeAg were screened for HBV DNA, by dot blot hybridisation [Section 2.5 E(a)], and for HBV DNA polymerase levels using an assay described by Hirschman and Garfinkel (1977).

Sera were mixed with an equal volume of TNE and clarified at 5000g for 10 minutes. 3.0 ml of the supernatant was then layered on a 0.5ml cushion of 20% sucrose in TNE and centrifuged in an IEC ultracentrifuge in a Type 498 swing rotor at 340,000g for 3 hours at 5°C. The supernatant was then aspirated, the sides of the centrifuge tube dried with tissue paper and the pellet resuspended in 40µl of TN. HBV DNA polymerase activity was assayed

in a 33µl reaction mixture containing 20µl of resuspended pellet, 200mM Tris-HCl pH 7.5, 80mM $MgCl_2$, 230mM NH_4Cl, 0.3% NP40, 0.3% 2-mercaptoethanol, and 0.033 mM each of GTP, CTP, ^{3}H-ATP (60Ci/mM, Amersham) and ^{3}H-TTP (109Ci/mM, Amersham) that was incubated at 37°C for 3 hours. 2µl samples were collected at the start (T_0), after 30 minutes (T_{30}), after 60 minutes (T_{60}) and at the end (T_{180}) of this incubation period, spotted onto Whatmann 541 filter paper, washed twice in ice cold 10%TCA and counted in a liquid scintillation counter. Sera that showed T_{180}:T_0 ratios ≥ 2 were considered positive for HBV DNA polymerase.

Sera that gave strong responses in both dot blot and DNA polymerase assays were further screened by electron microscopy (EM). For this, a formvar-carbon coated 400 mesh EM grid was inverted on a 10µl aliquot of the above resuspended pellet and incubated for 5 minutes at RT. The grid was then rinsed in DDW and stained with 3% phosphotungstic acid. Excess fluid was removed with filter paper and the grids dried at RT for at least 1 hour prior to examination in a Jeol electron microscope. Final selection was made on the basis of the proportion of HB virions to the two types of HBsAg particles (filaments and 22nm spheres) and the proportion of empty to full virions.

Of the three sera chosen for this study, two were from long term carriers of HBV that, based on dot blot analysis, had ca. $5x10^8$ vge/ml of HBV DNA and the third from a patient with acute HBV infection (ca. $5x10^7$ vge/ml of HBV DNA). All the infection experiments described in Chapter 3 were performed using these sera either individually or as a pool.

2.7B PURIFICATION OF HB VIRIONS.

For most experiments, the inocula consisted of the above sera. However in other experiments, the HB virions were first partially purified on a sucrose gradient. A HBV pellet derived as above from 10mls of serum was overlayed on a 10ml 5-20% linear sucrose gradient (in TNE) and centrifuged in an IEC ultracentrifuge using a 488 rotor at 270,000g for 30 minutes at 4°C. The bottom of the tube was then punctured and 44 drop fractions collected.

Following EM examination, fractions rich in HB virions (and low in HBsAg particles) were pooled and dialyzed overnight against PBS containing 0.1%BSA. The volume was then adjusted to 5ml with PBS, BSA added to 0.1%, the preparation sterilized by filtration through a 0.2µm membrane and stored in 0.5ml aliquots at -70°C. The HBV concentration of these preparations (estimated from dot blot hybridisation) was usually ca. 2-5x10^8 vge/ml.

2.7C INFECTION OF CULTURED CELLS.

While many variations were tried (see Chapter 3), all cell types listed in Table 2.1 with the exception of the transfected line 2.2.15. were, at least, subjected to the following infectivity assay. Semi-confluent to confluent monolayers of cells were incubated with HBV-infected sera (at a multiplicity of infection ranging from 1-500 vge/cell), for 24 hours at 37°C in 5%CO_2. The inoculum was then removed, the cells washed three times with PBS, and fresh medium added. At various intervals, the cells were harvested, fixed and examined by immunofluorescence for HBsAg and HBcAg. Similarly, cell culture fluids were harvested and tested for HBsAg by Ausria or Auzyme II (Abbott Laboratories) and HBV DNA by dot blot hybridisation.

2.7D HBsAg SECRETION ASSAYS.

Cells for HBsAg secretion assays (see Chapter 4) were seeded from the same flask into replicate wells of six-well cluster plates (Costar) in DMEM containing 5%FBS. Two days after achieving confluence, the cells were washed three times with PBS, drained, 5 ml of medium (containing the agent(s) for testing) added and incubated for 24 hours. The cultures were then washed with PBS, 2.0 ml of similar medium added and incubated for a further 24 hours. After this second incubation period the culture fluids were harvested and the cells counted using a haemocytometer. The culture fluids were assayed for HBV DNA by dot blot hybridisation or for HBsAg by Auzyme II (Abbott Laboratories). The HBsAg concentration in the culture fluids was calculated by comparison of OD at 492nm with that of a serially-diluted

HBsAg-positive control (concentration ca. 10ng/ml).

2.7E DUCK INOCULATION.

1-7 day old Pekin-Aylesbury cross ducks, generously donated by Tegel (NSW) were housed and maintained by the staff of the IMVS animal house. After collection of 0.5 ml of blood (pre-bleed) the birds were inoculated intravenously (i.v.) or intraperitoneally (i.p.) with 0.5-1ml of DHBV-infected sera or cell culture fluid derived from a cell line transfected with DHBV DNA (see Chapter 5). At weekly intervals after inoculation, blood was collected from the neck or wing vein and tested for DHBV DNA by dot blot hybridisation.

2.8 RNA TRANSCRIPTION INHIBITION ASSAYS.

2.8A ACTINOMYCIN D.

The effect of actinomycin D on in vivo HBV- and HDV-RNA transcription was assessed on 80% confluent monolayers of H1δ9 cells (see Chapter 7) grown in 150cm^2 flasks. The cells were washed twice with PBS then incubated for 2 hours at 37°C in 50 ml of phosphate-free DMEM (prepared according to the formulae shown in Appendix 1 but without sodium phosphate) supplemented with 5% dialyzed FBS. The medium was then replaced with 20 ml of similar medium containing 80μCi/ml ^{32}P-orthophosphate (Amersham) and either 0, 400ng/ml or 2000ng/ml actinomycin D (Boehringer) and incubated for a further 4 hours. Total RNA was then extracted as described previously [section 2.5 A(b)(ii)] and reverse hybridised to nitrocellulose membrane containing 1μg and 5μg spots of HBV DNA and HDV cDNA, prepared by spotting plasmid-derived DNA (after prior denaturation) on nitrocellulose which was then baked in a vacuum oven for 2 hours at 80°C. Prehybridisation, hybridisation, washes and exposure were performed as described in section 2.5 E(a), except that the hybridisation step was extended to 72 hours.

2.8B α-AMANITIN.

The sensitivity of in vitro RNA transcription to α-amanitin was measured in a nuclear run-off assay performed by a modification of a published method

(Marzluff and Huang, 1984). Each assay used the nuclei derived from 1x150 cm^2 flask of H1δ9 cells (ca. $2x10^7$ cells). The cells were washed twice with ice-cold PBS then incubated in 10ml of lysis buffer [10mM Tris-HCl pH 7.4, 3mM $MgCl_2$, 1mM KCl, 0.25%NP40 and 0.1mM Phenylmethylsulfonyl fluoride (PMSF; Boehringer)] for 2 minutes at 4°C. The cells were then scraped from the flask with a cell scraper (Costar), layered over 10ml of ice cold 30% sucrose (w/v) in lysis buffer without NP40 and centrifuged at 200g for 5 minutes at 4°C. The nuclear pellet was washed once in storage buffer (40% glycerol, 5mM $MgCl_2$, 0.1mM EDTA and 50mM Tris-HCl pH 8.3) then resuspended in 250µl of the same buffer. To determine the purity and concentration of nuclei, an aliquot was removed, mixed with an equal volume of 0.1% Trypan blue and examined microscopically. The remaining suspension (ca. 350µl) was added to a transcription mixture (final volume 500µl) of 5mM Tris-HCl pH 8.0, 125mM KCl, 2.5mM $MgCl_2$, 1mM DTT, 1 unit/µl RNasin (Promega), 0.1mM each of CTP, GTP and ATP, 250µCi ^{32}P-UTP (3000Ci/mM, Bresatec, Adelaide) and varying concentrations of α-amanitin (Sigma), and incubated at 25°C for 25 minutes. The ^{32}P-incorporation was determined from 5µl samples taken at the beginning and end of the incubation period. These were mixed with 19 volumes of GIT buffer [Section 2.5 A (b)(ii)], spotted onto Whatmann 541 filter paper, TCA precipitated and counted. After completion of the reaction, total RNA was then extracted from the nuclei [Section 2.5 A (b)(iii)] and reverse hybridised, as described above, to nitrocellulose membrane containing 1µg and 5µg spots of HBV DNA, HDV cDNA, pGEM-3 DNA and total cellular DNA (from HepG2 or HH1 cells). However, unlike the actinomycin D study above, hybridization mixtures were adjusted to contain the same level of TCA-precipitable ^{32}P counts irrespective of the concentration of α-amanitin added to the transcription reaction.

2.8C TRANSCRIPTION FROM EXOGENOUSLY-ADDED RNA TEMPLATES.

The ability of exogenously-added RNAs to act as templates for RNA transcription was assessed in a procedure similar to the above α-amanitin assay, with the exception that extracted nuclei were homogenised by 30 strokes of a tissue grinder (Wheaton) prior to use in the assay. Transcription mixtures were essentially identical to those described above excèpt that α-amanitin was ommited and 15μg of either HDV RNA transcribed in vitro [see section 2.5 D(b)] or tRNA was included.

2.9 ANALYSIS OF RECOMBINANT HDAg.

2.9A PURIFICATION OF RECOMBINANT HDAg.

Crude rHDAg was prepared by resuspending a pellet of trypsinized A3 cells (see Chapter 6) in 6M urea, freeze thawing three times then clarifying by centrifugation (20,000g, 15 minutes, 20°C).

Alternatively, partially-purified HDAg was prepared as follows; packed A3 or H1δ9 cells, (from freshly trypsinised confluent monolayers or from cells stored at -70°C) were resuspended in four volumes of PBS containing 50mM Tris-HCl pH8.0 and Triton X-100 added to 1%. 2mm glass balls were added and the mixture vortexed vigorously for 2 minutes, followed by two freeze-thaw cycles and further vortexing. The preparation was centrifuged at 27,000g at 4°C for 10 minutes, the pellet re-extracted as above with twice the original volume of PBS and all supernatants pooled. Ammonium sulphate powder was added slowly, with stirring, to a final concentration of 25% and the precipitate removed by centrifugation (20,000g, 20°C, 10 minutes). Ammonium sulphate was then added to 65% and the precipitate recovered by centrifugation as above. The pellet was redissolved in PBS, chilled on ice and reprecipitated by the addition of cold acetone to 40%. The precipitate was collected by centrifugation (500g, 5 minutes, 4°C), washed twice in cold

90% acetone, freeze dried (to remove residual acetone) and finally redissolved in PBS.

In some experiments liver-derived HDAg (see section 2.6 C) was used as a standard.

2.9B HPLC FRACTIONATION.

HPLC fractionation of rHDAg was performed by Betty Reinboth (Department of Pathology, University of Adelaide) by gel permeation through Varian TSK G4000 SW in a 7.5mmx50cm column (Varian Instrument Group, Walnut Group, Ca) with a Biosil TSK (7.5mmx75mm) guard column (Biorad Laboratories, Richmond, Ca). A Waters (Waters Assoc. Milford, MA) solvent delivery system equipped with a Wisp 701B injector fitted with a 200µl loop and linked to a model 440 fixed wavelength detector and Pharmacia Frac-100 fraction collector, was also employed. 200µl of crude or partially-purified rHDAg was injected and the buffer (PBS, 6M Urea or 4M guanidine-HCl) flow rate adjusted to 0.8ml/min. 0.8ml fractions were collected and in most cases tested neat for HDAg by RIA, while fractions containing 4M guanidine-HCl were tested at 1/4 dilution and neat after dialysis against PBS. The running buffers were degassed before use and the column equilibrated for a least 30 minutes in buffer prior to loading the samples. Calibration of the column was performed with each buffer, using high molecular weight protein standards (Pharmacia).

2.9C ULTRACENTRIFUGATION.

2.9C(a) Determination of sedimentation coefficient.

Two hundred microlitre samples of either serum-derived HBsAg 22nm particles, liver-derived HDAg or partially-purified rHDAg were overlaid on 10ml linear 5-20% sucrose gradients and centrifuged in an IEC ultracentrifuge using a Type 488 rotor at 208,000g for 60 minutes at 4°C. Half mililitre fractions were then collected from the bottom, the sucrose concentration estimated from the refractive index and the HDAg or HBsAg content measured by RIA and Auzyme respectively. Sedimentation coefficients (S values) were

determined using a computer programme (Young, 1978).

2.9C(b) Determination of density.

Similarly, HBsAg and HDAg samples were overlaid on 10ml preformed caesium chloride gradients (1.1-1.5 g/cm^3) and centrifuged at 120,000g for 25 hours at 20°C in an IEC 488 rotor. Fractions were collected and analyzed as described above.

CHAPTER 3.

IN VITRO CULTURE OF HBV.

3.1 INTRODUCTION.

All the experiments described in this chapter, with the exception of those using DMSO, were performed from 1983 to 1986. This period saw many advances in hepadnavirus cell culture, principally in the area of transfection using HBV DNA, but also included the development of an in vitro culture system for an hepadnavirus in which primary duck hepatocytes were infected sucessfully with DHBV (Tuttleman et al, 1986a), Subsequently, primary hepatocyte culture studies have been extended to other members of the hepadnavirus group; WHV and GSHV were cultured in woodchuck hepatocytes (Aldrich et al, 1989) and HBV in human hepatocytes (Shimizu et al, 1986; Gripon et al, 1988; Rijntjes et al, 1988; Ochiya et al, 1989). These systems will continue to provide useful models for the study of the hepatocyte-specific factors required in hepadnavirus replication. However these culture systems are expensive to produce and maintain, cumbersome, short lived, only permissive to hepadnavirus infection for a short time after plating and, in the case of human hepatocytes, difficult to obtain. Thus an urgent need exists to develop a convenient culture system employing either easily maintained primary or continuous cell lines. This need was equally pertinent when this part of the thesis was initiated, and in the work described in this chapter numerous cell lines were screened for their ability to support HBV replication in vitro using a number of different infection conditions.

3.2 EXPERIMENTAL DESIGN.

3.2A CELL LINES.

Using the previously outlined protocol (section 2.7 C) every cell type in Table 2.1 (except 2.2.15) was evaluated for its ability to support HBV

replication in vitro. In addition, a number of variations to the infection protocol were investigated (see below) with a reduced range of cell types; only the human hepatoma cell line HepG2 was tested with every variation. This cell line was chosen for extensive investigation as it has properties consistent with well differentiated hepatocytes and was derived from a non-HBV induced hepatoma (Knowles et al. 1980). This selection has been vindicated by recent studies, as Neurath et al (1986) demonstrated that a synthetic peptide derived from the sequence of the pre-S1 region binds to these cells and Sells et al (1987) showed that HepG2 cells supported HBV replication after stable transfection with a multimeric construct of HBV DNA (which gave rise to the 2.2.15. cell line).

Other human hepatoma cell lines (Mahlavu, Hep 3B, HH1 and PLC/PRF/5) were chosen as most likely to be permissive to HBV infection in vitro. Moreover the HBV-induced lines HH1, Hep 3B and PLC/PRF/5 and in addition the HBV DNA-transfected rat cell line TK4 contain integrated HBV DNA, synthesize HBsAg and may therefore have offered possible complementation events to assist in vitro HBV replication. Potential complementation was also the principal reason for selection of the retrovirus-producing lines (MLA144, EHMG118 and GSM) that might supply reverse transcriptase. Other cell lines were chosen for their ability to grow unusual and difficult-to-cultivate viral agents [Graham-293: Adeno viruses 40 and 41 (De Jong et al, 1983); BSC-1-4: Hepatitis A Virus (Lemon et al, 1983); RK-13: Rubella] and others for their wide susceptibility to a number of different virus types (LLCMK2, BGM, Vero, FS, HeLa, Hep-2 and MDCK). The selection of the human submandibular gland cell line (VS) was made on the basis of studies that demonstrated the presence HBV particles in saliva in the absence of detectable blood contamination and, thus, implicated salivary glands as a site of extra-hepatic HBV replication (Villarejos et al, 1974; Macaya et al, 1979). The remaining cell lines (Swiss 3T3 and Rat2) were

chosen for no other reason than that they were available and in regular use in the laboratory.

3.3 RESULTS.

3.3A SELECTION OF HBV DNA-POSITIVE SERA AS INOCULA.

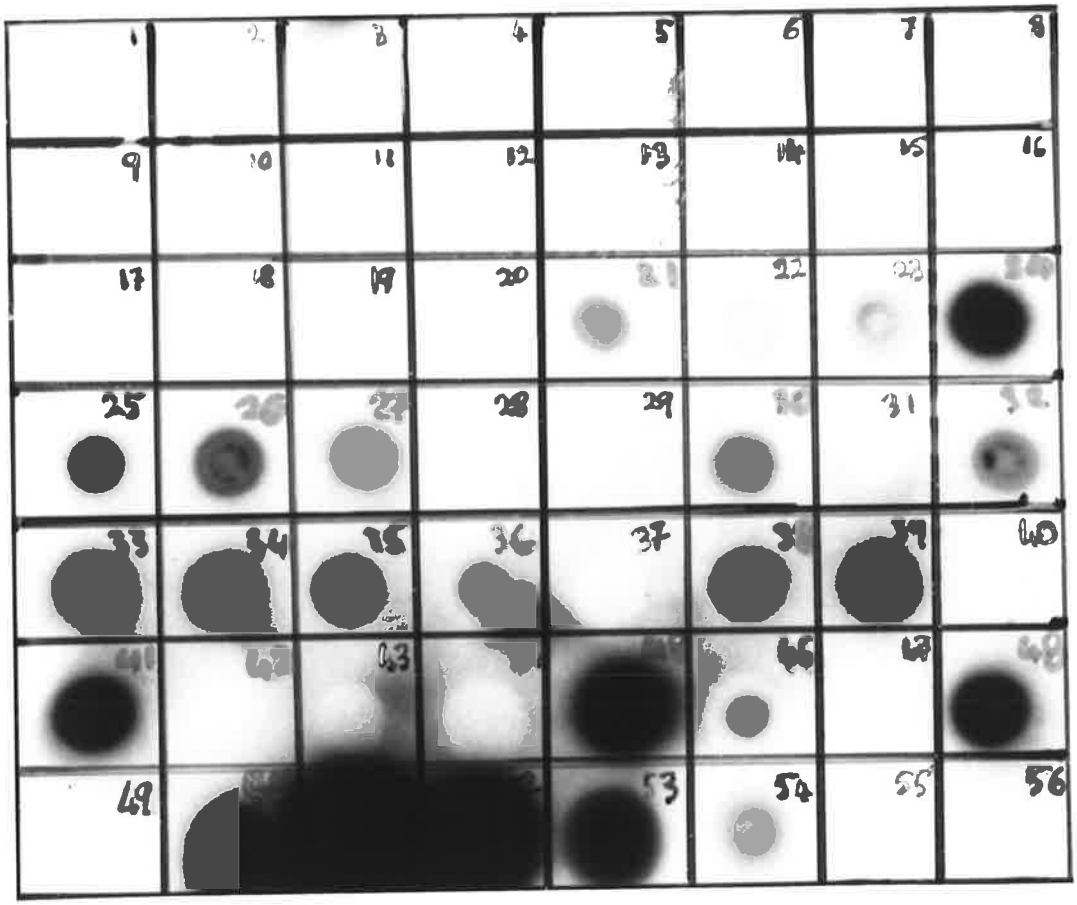
3.3A(a) Detection of HBV by Dot Blot Hybridisation and HBV DNA Polymerase Assays.

Levels of HBV in serum samples were compared using spot blot hybridisation and DNA polymerase assays. Twenty serum specimens (nos 1-20) serologically negative for HBV and thirty serum specimens (nos 21-50) serologically positive for both HBsAg and HBeAg were tested. The spot blot hybridisation assay appeared to be more sensitive for the detection of HBV DNA as 20 (66%) were positive compared to 17 (56%) positive by HBV DNA polymerase (Fig 3.1) in the HBsAg positive group. No positives were detected in the negative control group. As the spot and dot blot hybridisation assays required a smaller sample, and were much simpler and cheaper to perform, they were used exclusively for the detection of HBV in serum and cell culture fluids in subsequent experiments.

3.3A(b) Selection.

One acute and two chronic sera were selected from the results of similar dot blot hybridisation experiments and were used as the inoculum throughout the infection experiments. However, in some experiments, to overcome potential blocking of cellular receptors by 22nm HBsAg particles, virion-enriched inocula (see Chapter 2 section 2.7 B) were employed. These were used directly or after preincubation with nucleotide precursors in an attempt to repair the single stranded gap in the virion DNA. Similarly, based on observations by Miller et al (1984) suggesting that certain activities of the endogenous DNA polymerase of GSHV were abolished after storage at 4°C or below, "fresh, unfrozen" sera were used in some experiments.

Fig 3.1 Comparison of spot blot hybridisation and HBV DNA polymerase assays for the detection of HBV in patient serum.
5µl serum samples spotted onto nitrocellulose were hybridised with a ^{32}P-labelled nick-translated DNA probe (derived from pHBVCB) to detect HBV DNA. Serum specimens 1-20 were serologically negative for HBV, serum specimens 21-50 were serologically positive for both HBsAg and HBeAg and samples 51-54, and 55-56 contain 1000, 100, 10 and 1pg of plasmid-derived HBV DNA, and 1000 and 100pg of λ DNA respectively. Dark and pale numbers indicate serum samples that were negative or positive, respectively, for HBV DNA polymerase.
Sample 41 was one of the three sera used in the infection experiments.



3.3B INFECTION EXPERIMENTS.

The duration of most infection experiments ranged from 5-30 days after inoculation, although several experiments employing HepG2, BSC-1-4 and endothelial cells were extended for up to 6 months with repeat inoculation of virus every month. However, apart from the detection of HBsAg in cells and cell culture fluid of the HBV-induced hepatoma lines (HH1, PLC/PRF/5 and Hep3B) and the HBV-transfected line (TK4), HBV products were never detected after inoculation with HBV using a number of variables. Nevertheless, for the sake of completeness and because much of the expertise gained was used later in the experiments described in Chapters 4-7, these variables and the reasoning behind the experiments are presented in a brief form below.

3.3B(a) Adsorption.

Various changes to the standard infection procedure were assessed. These included variations in time and temperature during the virus adsorption step (1hour-5days and 4-37°C respectively), the addition of DEAE-dextran to assist virus uptake and centrifugation of virus directly onto the cell cultures.

3.3B(b) Cell Confluency.

Variations in the degree of cell confluence and time in culture (without serial passage to try to mimic hepatocytes in vivo) at the time of infection were performed to allow for effects of the cell cycle on HBV replication. Co-cultivation experiments were also attempted in which HepG2 cells were mixed with each of the three retrovirus-producing cell lines prior to infection.

3.3B(c) FBS Concentration.

Animal-derived sera have been reported to occasionally show weak levels of anti-HBs (Hoofnagle et al, 1983). As this could potentially lead to virus neutralisation, the adsorption step in some experiments was performed either without or in low concentrations of FBS often in conjunction with LHC-4 medium (a complete medium designed for serum-free applications; Lechner et al, 1982; 1983). However few cultures survived for long in low serum

conditions, and normal FBS levels were usually reintroduced after the adsorption step.

3.3B(d) Hormones and Differentiation-inducing Agents

In an attempt to duplicate in vivo conditions or induce cell differentiation, DMSO, cholera toxin (an agent that induces differentiation by activation of cyclic-AMP) or individual hormones (such as testosterone, dexamethazone and glucagon) or mixtures of hormones (such as those contained in LHC-4 medium; see Appendix 2) were added to cell culture media prior to, during and after virus adsorption. The rationale for these experiments was prompted by the results of Tuttleman et al (1986a) who observed that primary duck hepatocytes could only be infected within the first four days after plating, despite the fact that cells infected during this period synthesised virus for the three week life of the culture. The authors attributed this to a change in the state of differentiation of the cells in culture. More recent experiments have been able to extend the "infective window" of primary hepatocytes to DHBV (Galle et al, 1989) and HBV (Gripon et al, 1988) by the addition of low concentrations (0.5-2%) of DMSO to the culture fluid. This agent has been shown to maintain differentiation in cultured primary rat liver cells (Isom et al, 1985).

3.3B(e) Gene deregulatory substances and media.

An indirect approach to duplicate some hormone effects is by the use of substances that lead to gene deregulation. To test the hypothesis that the nonpermissive nature of cells to HBV cultivation was due to methylation of chromosomal or input (HBV) DNA, many infection experiments were performed in which the cells were pretreated with 5-azacytidine and/or grown in LHC-4 medium prior to, during and after inoculation. The possibility that HBV replication could be affected by gene deregulatory substances was suggested from experiments performed using HBV-induced hepatoma lines such as PLC/PRF/5 (Miller and Robinson, 1983) and human epithelial cultures transfected with a HBV DNA fragment containing the HBcAg gene

(Yoakum et al ,1983; Korba et al ,1985) in which the authors were able to "turn on" HBcAg synthesis with 5-azacytidine, an inhibitor of cell methylases (reviewed by Doerfler, 1983; Jones, 1985; Vesely, 1985). Methylation of a single HpaII site 280bp upstream from the core structural gene was shown to regulate HBcAg expression suggesting that methylation may play an important role in the pathogenesis of HBV (Korba et al ,1985). HBcAg expression was also induced in PLC/PRF/5 and HBcAg gene-transfected human epithelial cultures by LHC-4 medium (Yoakum et al ,1983) by an unknown mechanism that did not appear to be mediated by demethylation. In contrast, in this study, limited HBcAg expression was detected, but this was non reproducible and was limited to the TK4 cell line after treatment with 5-azacytidine.

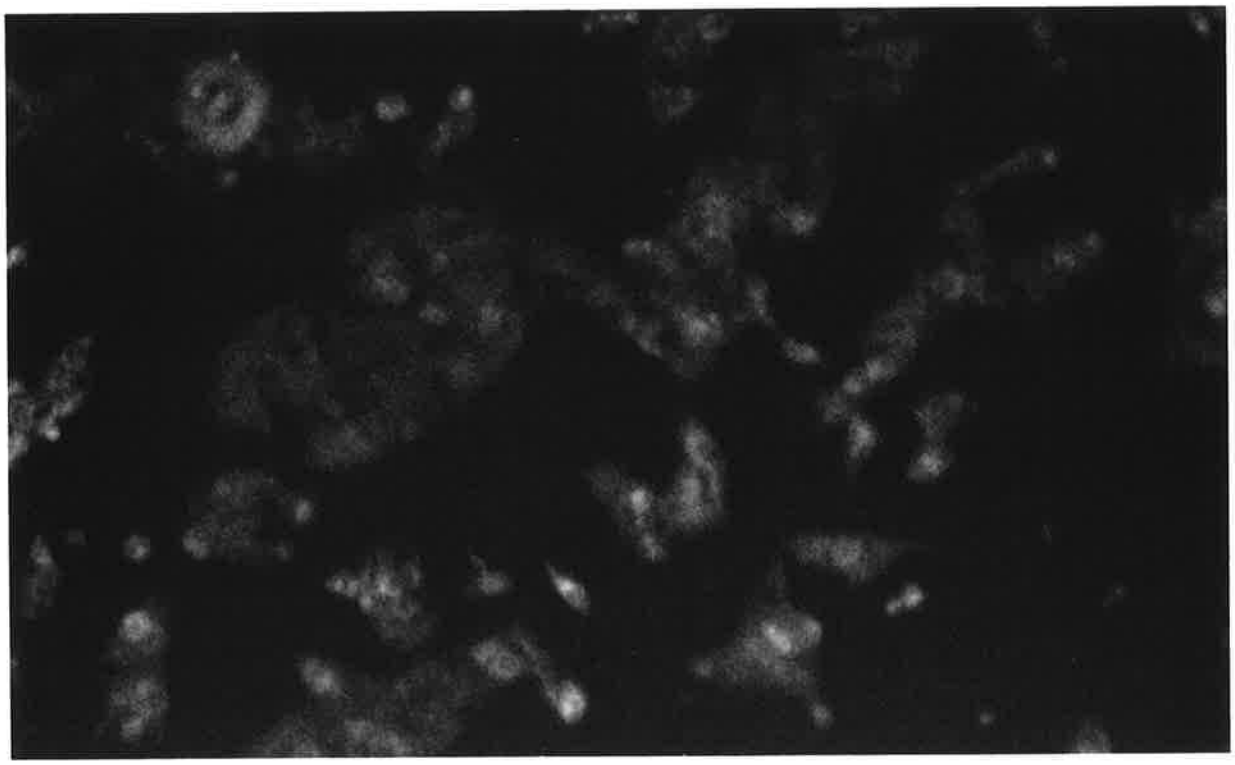
3.3B(f) Endothelial Cell Cultures.

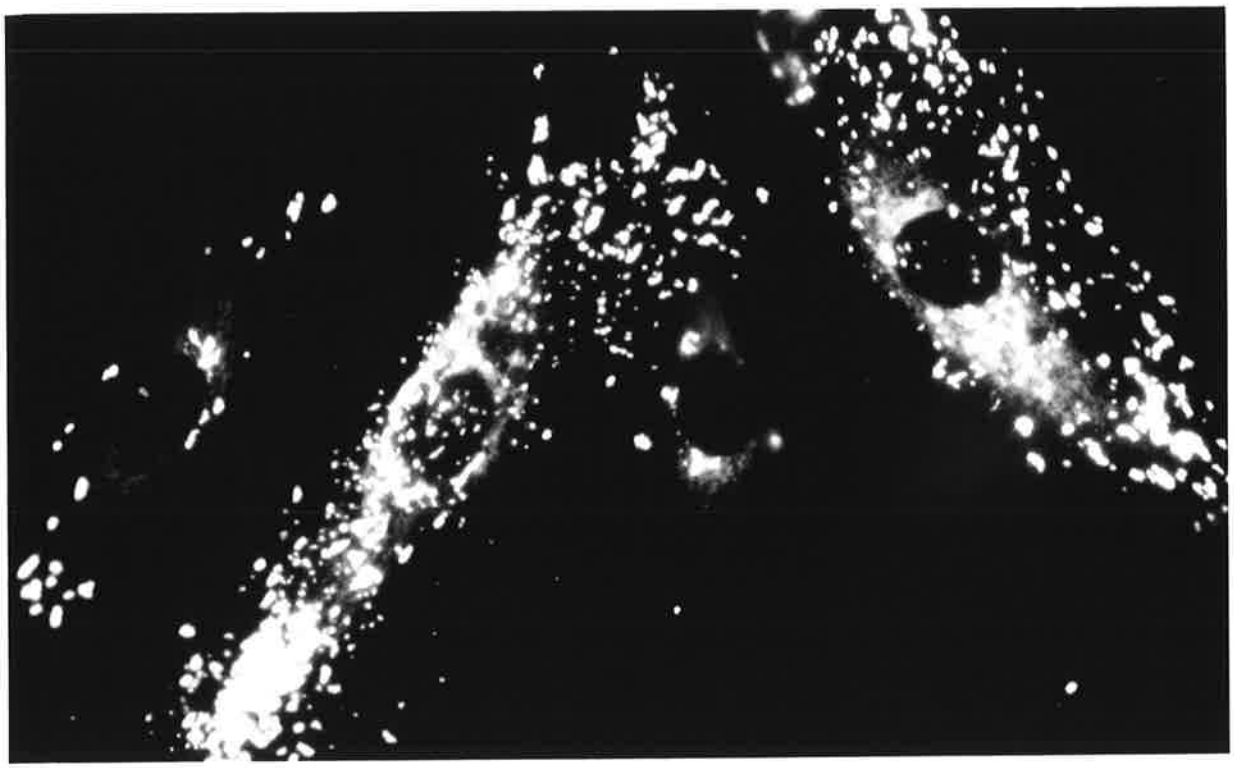
Endothelial cells uniquely synthesize the antihaemophilic agent, Factor VIII (Jaffe, 1977). To confirm the authenticity of these cells, immunofluorescence assays were performed that localized the protein to the cytoplasm (Fig 3.2). Furthermore, using coated culture vessels and endothelial cell media [see section 2.1 B(c)], endothelial cells were maintained in continuous culture for more than 6 months and 25 serial passages without signs of senescence or loss of factor VIII synthesis.

One unconfirmed report detected HBV DNA in liver endothelial cells (Blum et al, 1983). As these cells line the portal tracts, a hypothesis was proposed that HBV must first cross this endothelial cell layer in order to gain access to the hepatocyte. This could be achieved either by passing through fenestrations within the endothelial cell layer or by cell to cell spread from the endothelial cell layer to the hepatocyte (Jilbert et al, 1988). For these reasons endothelial cells were considered to be prime candidates for extrahepatic HBV replication and were included in several infection experiments (some of which were extended for six months). However the outcomes of these experiments, and experiments with primary HUVEs, cultured from a

Fig 3.2 Detection of Factor VIII by indirect immunofluorescence.

Top HeLa cells.
Bottom Human umbilical cord endothelial cells (passage #5).
Magnification: top x500; bottom X2000.





HBV-carrier mother, were negative for HBV DNA and antigens.

3.4 DISCUSSION.

3.4A. BLOCK IN EVENTS LEADING TO HBV INFECTION *IN VITRO*.

Despite numerous attempts, no evidence was obtained that in vitro HBV replication occurred in any of the continuous or primary cell lines tested. It is unlikely that the negative results in these experiments were due to the inocula, as sera containing high levels of HBV DNA from three different patients representing both chronic and acute stages of HBV infection were employed. From a number of studies, it is now clear that HBV replication in vitro requires primary hepatocyte cultures and although it was planned to perform these experiments a suitable source could not be found locally. The ability of primary hepatocyte cultures to support HBV replication suggest that the obstacle(s) encountered with continous cell lines is probably of cellular origin. Therefore, the key to the HBV replication is probably a factor(s) present in primary hepatocytes that is either supplied from the milieu of the functioning liver or synthesised in vivo in hepatocytes. These factors are probably present transiently when hepatocytes are cultured in vitro.

The hepadnavirus replication cycle (Fig 1.3) offers many potential steps that could be dependent on specific cellular factors. However, as the HepG2 cells support virus replication after transfection of virus DNA, this suggests that a block in earlier events causes the resistance to infection in these cells. Recent evidence suggests that two steps may be responsible viz. virus adsorption and viral RNA transcription (see below). These same mechanisms may account for the observed hepadnavirus hepatropism and species specificity.

3.4B FACTORS THAT INFLUENCE ADSORPTION OF HBV TO CULTURED CELLS.

The identity of the cellular receptor for HBV is unknown although the binding site on the virus probably lies within the large HBsAg, as an amino

acid sequence in this region has been identified that binds to HepG2 cells (Neurath et al, 1986) and liver cell membranes (Pontisso et al, 1989a; 1989b). This suggests that the block in replication occurs at a later stage, although it is possible that preS1 peptides may bind even if whole virions do not. Thus this point should be clarified. Although DMSO was reported to extend the period of time that primary hepatocyte cultures were permissive to hepadnavirus infection (Gripon et al, 1988; Galle et al, 1989), an effect that may result from the prolonged expression and/or exposure of cellular receptors on cultured hepatocytes (Galle et al, 1989), in the experiments described in this chapter, HepG2 cells treated with DMSO remained resistant to infection. Thus DMSO appears unable to restore the early block in infection. Nevertheless it is still possible that this function may be restored by other agents which induce cell differentiation.

3.4C HBV RNA TRANSCRIPTION.

The 2.1 kb mRNA species that codes for HBsAg has been detected in a wide range of transfected cell lines of diverse tissue and species origin (discussed in Chapter 4). However in vitro production of the pregenomic RNA species (which also codes for HBcAg and reverse transcriptase/DNA polymerase) has only been detected in hepadnavirus-infected primary hepatocyte cultures and in differentiated hepatoma cell lines following transfection with HBV DNA (Sureau et al, 1986; Sells et al, 1987; Yaginuma et al, 1987; Tsurimotto et al, 1987; Shih et al, 1989). A recent report from Seeger et al (1989) in which murine and avian fibroblasts were transfected with WHV DNA showed that infectious virus was only produced in these cells when the production of WHV pregenomic RNA was placed under the control of the cytomegalovirus immediate-early promoter.

These results show that some of the natural hepadnavirus promoters are not active in non-hepatocytes and further that hepatocyte-specific factors are required for transcriptional but not for the posttranscriptional phases of hepadnavirus replication. The transcriptional block could be mediated by a

methylation-induced repression of the pregenomic promoter, as suggested by work described in section 3.3 B(e) above, or, alternatively, the HBV enhancer or pregenomic promoter may require host factors, present only in certain differentiated target tissues. To investigate these issues, factors which affect the expression of integrated HBV sequences were examined in Chapter 4.

CHAPTER 4.

HBV ANTIGEN EXPRESSION IN HEPATOMA-DERIVED AND TRANSFECTED CELL LINES.

4.1 INTRODUCTION.

Since the key to successful in vitro HBV replication in continuous cell lines may lie in the determination of the factors which are able to regulate HBV gene expression, this chapter examines the effects of a range of agents on HBV antigen synthesis . These experiments were performed independently but at the same time as a number of other experiments reported in the literature.

A number of diverse agents have been shown to stimulate HBsAg expression; these include corticosteroids (Oefinger et al, 1981; Clementi et al, 1983; Marshall et al, 1983; Tur-Kaspa et al, 1986;), sex steroids in the transgenic mouse model (Farza et al ,1987; DeLoia et al, 1989), caffeine and low cultivation temperatures on hepatoma cell cultures (McAleer et al, 1983). Of these studies, only those of Farza et al (1987) and DeLoia et al (1989) were performed in vivo. In contrast the demethylation agent 5-azacytidine and/or LHC-4 medium (Yoakum et al, 1983; Korba et al, 1985) are the only agents, described to date, that have been reported to modulate HBcAg expression in cultured cells.

In this chapter some of the above and a number of other agents were examined for their effect on HBsAg, HBcAg and HBV expression in a number of HBV-associated cell lines either held or raised in the laboratory as part of the study.

4.2 EXPERIMENTAL METHODS AND DESIGN.

4.2A CELL LINES.

4.2A(a) Development of the 22/B2 Cell Line.

22/B2 cells were derived from the African Green monkey kidney cell line,

Vero, after co-transfection with full length recircularised HBV DNA and the plasmid pSV2neo [Southern and Berg, 1982; Section 2.3 A(b)] which confers resistance to the antibiotic analog G418. The HBV DNA was prepared by ligation of the gel-purified 3.2kb EcoR1 fragments from the plasmid pTKHH2 [Section 2.3 A(e)]. This DNA, either in the dimeric form (in pTKHH2) or as a recircularised monomer, induced a HBV infection when inoculated intrahepatically into chimpanzees (Will et al, 1982; 1985).

Although twenty-four single-cell clones were isolated after G418 selection and twenty-two initially secreted HBsAg into the culture fluid, by passage #4 only one clone, 22/B2, continued to secrete HBsAg. This clone continued to produce HBsAg in a stable pattern for over 24 passages. Many of the other clones were also positive for HBsAg by immunofluorescence, but expression in these clones was transient and closely reflected the above results obtained for secreted HBsAg. In some clones, but not in the 22/B2 clone, transient weak HBcAg staining was also seen.

4.2A(b) Source and Characteristics of Other Lines.

4.2A(b)(i) TK4 Cells.

TK4 cells (Gough and Murray, 1982), kindly provided by Nick Gough, are Rat-2 cells which contain 4 tandem repeats (to allow full-length pregenomic HBV mRNA transcripts to be made) of the entire HBV genome and a second plasmid containing the herpes simplex virus thymidine kinase gene (to permit cell selection in HAT medium). TK4 cells were reported initially to synthesise HBsAg, HBeAg and HBcAg, but during the infection experiments described in Chapter 3, it was noted that only about 20% of cells were HBsAg-positive and HBcAg was only detected (non-reproducably) after 5-azacytidine induction in a small proportion (max. 5%). As these results suggested that a degree of clonal heterogeneity had arisen since the original isolation, the cells were recloned prior to the experiments described in this chapter. Two of the resultant subclones, A1 and B4, were selected on the basis of negative or high HBsAg expression respectively, and were studied in detail.

In contrast to the HBV DNA used to prepare the 22/B2 cells, HBV DNA sequences used in the preparation of TK4 cells were derived from the plasmid pHBV130 (Burrell et al, 1979) which was not infectious in a dimeric form when inoculated intrahepatically into chimpanzees (Will et al, 1985).

4.2A(b)(ii) 2.2.15 Cells.

2.2.15 cells, developed by Sells et al (1987) and generously provided by George Acs, are HepG2 cells containing a plasmid with a Neomycin resistance gene and two opposing (3'-3') head-tail dimers of HBV DNA. These cells were reported to synthesize and secrete HBsAg and HBeAg as well as HBV particles which were subsequently shown to be infectious to chimpanzees (Acs et al, 1987).

4.2A(b)(iii) HBsAg-Positive Hepatoma lines.

The HBV-induced human hepatoma lines HH1 [a mycoplasma free subclone of PLC/PRF/5 (Fowler et al, 1983)], PLC/PRF/5 (Macnab et al, 1976) and Hep 3B (Knowles et al, 1980) were also examined. These cell lines are of hepatocyte origin and contain integrated HBV DNA which was acquired during natural HBV infection. PLC/PRF/5 and Hep3B cells have been reported to synthesize and secrete HBsAg constitutively (Macnab et al, 1976; Knowles et al, 1980), but HBcAg is synthesised only after 5-azacytidine induction (Yoakum et al, 1983).

4.2B DETECTION OF HBV-SPECIFIC MARKERS.

4.2B(a) Nucleic Acid Analysis.

In eukaryotic systems, methylation of cytosine residues in chromosomal DNA can influence gene regulation (reviewed by Doerfler, 1983); in this manner, HBcAg expression from integrated HBV DNA was induced by 5-azacytidine treatment (Miller and Robinson, 1983; Yoakum et al, 1983; Korba et al, 1985) particularly if the methylated residues occurred in the core promoter region (Korba et al, 1985). In this thesis, the restriction enzyme isoschizomer pair, HpaII/MspI, was used to study variations in the methylation patterns of TK4 cell DNA. These enzymes normally recognise and cleave the

sequence CCGG, but HpaII does not cleave if either cytosine residue is methylated (ie. C^mCGG, CC^mGG and C^mC^mGG) while MspI cleaves C^mCGG but not CC^mGG (Doerfler, 1983).

4.2B(b) Immunofluorescence.

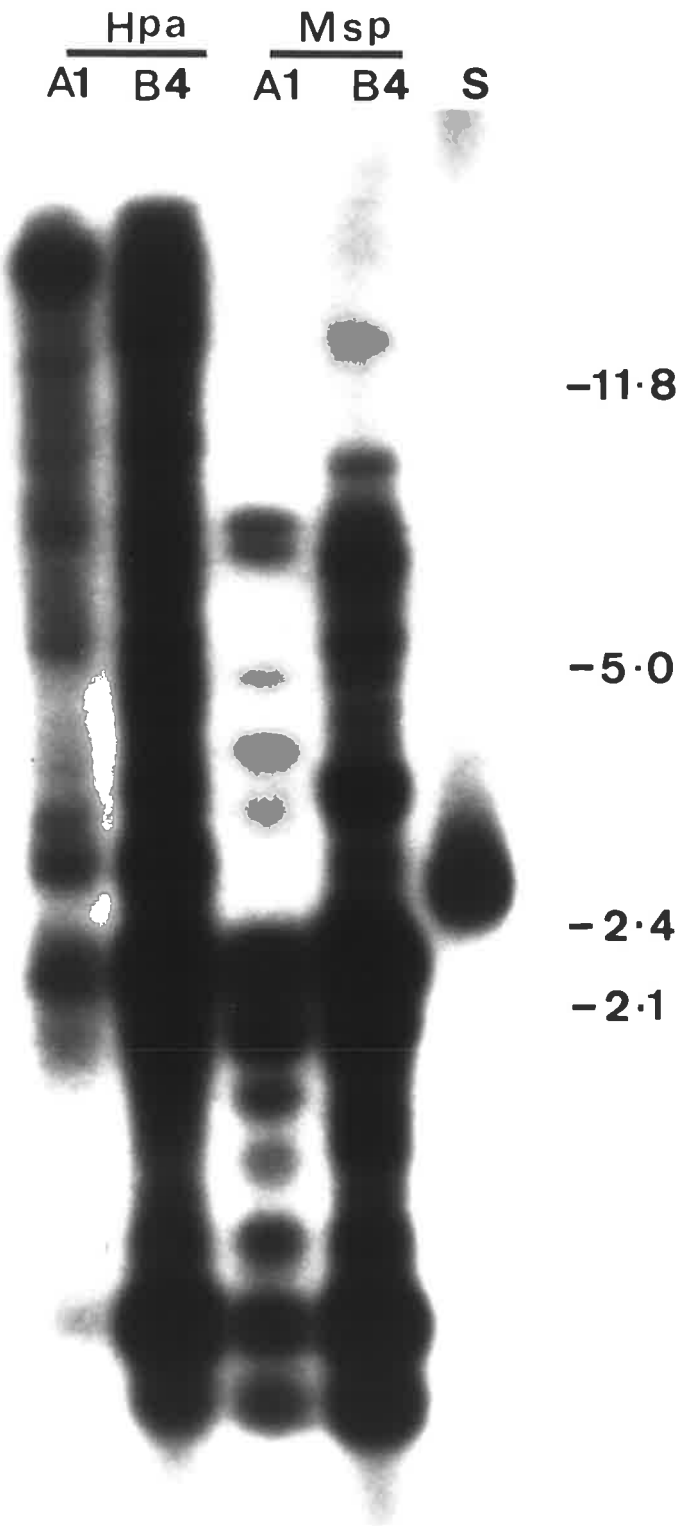
A number of different cell lines were examined for major-, middle- and large-HBsAg, and HBcAg by immunofluorescence after culture in normal and calcium-free DMEM (supplemented, respectively,with 5% non-dialysed and 5% dialysed FBS). The "pre-S1" and "pre-S2" antibodies (a kind gift from Steve Kent), were polyclonal rabbit antisera directed against synthetic pre-S peptides whereas the antibody for the detection of major HBsAg was a commercial rabbit antiserum (Behring), raised against serum-derived HBsAg, and is therefore likely to react with the major HBsAg species and also with the large and middle HBsAg polypeptides.

4.3 RESULTS.

4.3A METHYLATION PATTERNS OF INTEGRATED HBV DNA IN TK4 CLONES A1 AND B4.

Total cellular DNA was extracted, as described in Chapter 2 [section 2.5 A(a)], from TK4 clones A1 and B4 (HBsAg -negative and -positive respectively), digested with restriction enzymes HpaII and MspI and analyzed by Southern blot hybridisation (Fig 4.1). Although both clones contained integrated HBV DNA, the overall level was much higher (ca. 4X) in the B4 clone compared to A1 clone. Furthermore, based on the HBV DNA sequence (Burrell et al, 1979) and the construct used for transfection (Gough and Murray, 1982), the two intense bands of HBV DNA detected at ca. 2,300 bp and ca. 750 bp in DNA extracted from both clones were exactly as predicted. In both clones the pattern of HBV DNA fragments generated after digestion with HpaII and MspI, particularly in the higher molecular weight range, showed considerable variability (Fig 4.1), indicating that methylation of cytosine residues at HpaII and MspI restriction sites had occurred.

Fig 4.1 Southern blot hybridisation analysis of DNA extracted from TK4 subclones A1 and B4 after hybridisation with ^{32}P-labelled nick-translated probes to detect HBV DNA. Total DNA was analysed after digestion with restriction enzymes HpaII (Hpa) or MspI (Msp). Lane S contains 100pg of the (2.9kb) PstI fragment of HBV DNA from pHBVCB. The size markers on the right correspond to PstI digested λ DNA fragments.


Hpa
Msp
A1
B4
A1
B4
S
-11·8
-5·0
-2·4
-2·1

4.3B HBsAg SECRETION.

As the degree of cell confluence has been shown to influence the level of HBsAg secretion (Aden et al, 1979; Gough and Murray, 1982), the HBsAg secretion assays presented in this section were not performed until at least two days after the cells had become fully confluent.

4.3B(a) Hormones.

The glucocorticoid hormone dexamethasone induced a small increase (40%) in HBsAg secretion from TK4 B4 cells and a larger increase (ca. 3X) from PLC/PRF/5 cells (Fig 4.2). This increase was reproducable and was observed with 1 and 10μg/ml dexamethasone ($2.5x10^{-5}$ M and $2.5x10^{-6}$ M respectively) consistent with other studies which reported similar enhancement of HBsAg secretion (Marshall et al,1983; Tur-Kaspa et al, 1986). In contrast, the sex steroid hormones, Progesterone, ß-estradiol, Testosterone and Dihydrotestosterone, all failed to induce a significant increase in HBsAg secretion from the the same two cell lines. These results differ to the in vivo results obtained in HBV-transgenic mice (Farza et al ,1987; DeLoia et al, 1989) where Testosterone and Dihydrotestosterone strongly enhanced serum HBsAg levels.

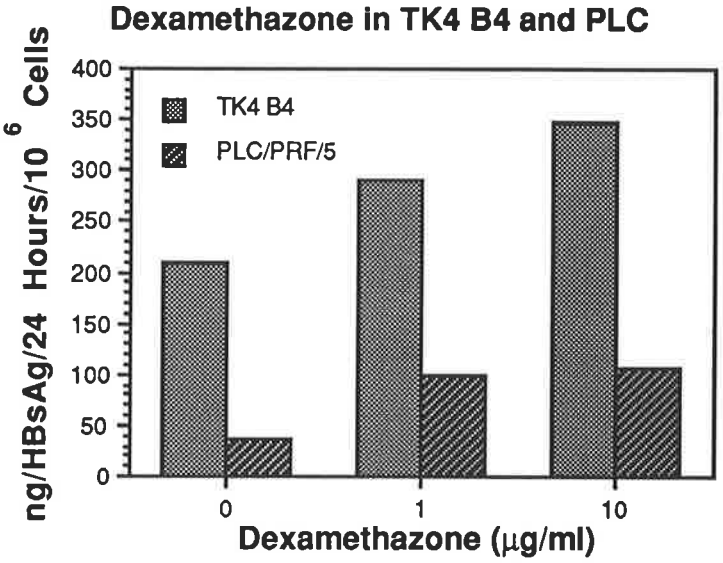
The hormone glucagon was also tested for its effect on secreted HBsAg in 22/B2, HH1, PLC/PRF/5 and TK4 B4 cells (Fig 4.3). Curiously this hormone, at a concentration (10μg/ml) recommended for the cultivation of primary hepatocytes (Reid and Jefferson, 1984), had no effect when the cells were grown in DMEM and no significant effect in LHC-4 medium except on the TK4 B4 cells where HBsAg secretion increased threefold (Fig 4.3).

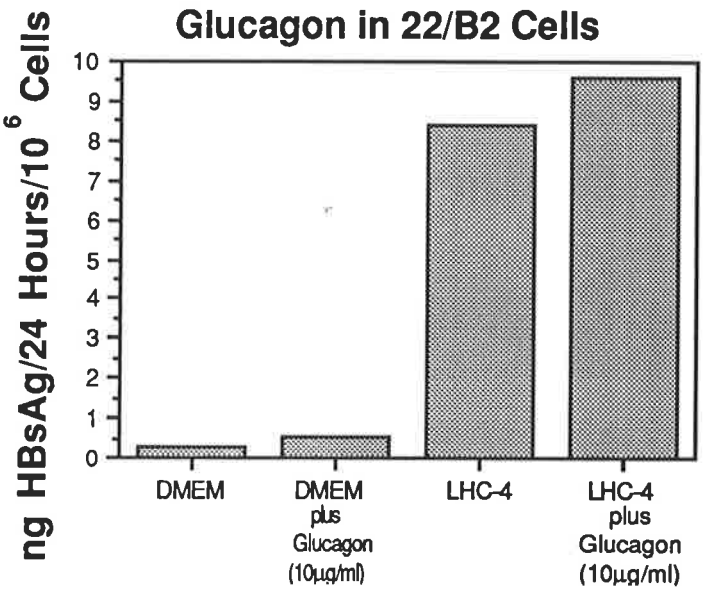
4.3B(b) Comparison of DMEM and LHC-4 Media.

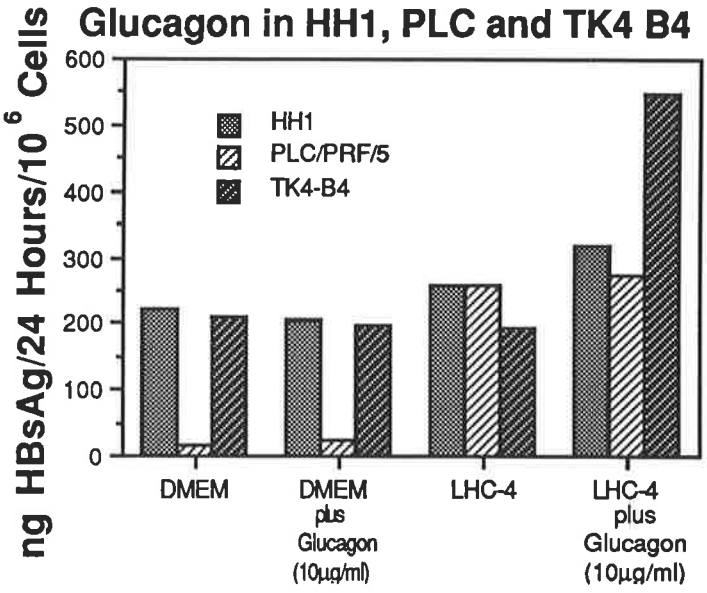
In the above glucagon experiments the largest increase in HBsAg secretion was observed when 22/B2 and PLC/PRF/5 cells were switched from cultivation in DMEM to LHC-4 medium (Fig 4.3). This effect was first observed during the infection experiments of Chapter 3 and was assumed to be dependent on the hormone component(s) of the latter medium (see

FIG 4.2 (Top) The effect of Dexamethazone on levels of secreted HBsAg from TK4 B4 and PLC/PRF/5 cells.

Fig 4.3 (Middle and bottom) The effect of Glucagon on levels of secreted HBsAg from 22/B2, HH1, PLC/PRF/5 and TK4 B4 cells.


Dexamethazone in TK4 B4 and PLC
ng/HBsAg/24 Hours/10 6 Cells
400
350
300
250
200
150
100
50
0
TK4 B4
PLC/PRF/5
0
1
10
Dexamethazone (µg/ml)


Glucagon in 22/B2 Cells
ng HBsAg/24 Hours/10 6 Cells
10
9
8
7
6
5
4
3
2
1
0
DMEM
DMEM plus Glucagon (10µg/ml)
LHC-4
LHC-4 plus Glucagon (10µg/ml)


Glucagon in HH1, PLC and TK4 B4
ng HBsAg/24 Hours/10 6 Cells
600
500
400
300
200
100
0
HH1
PLC/PRF/5
TK4-B4
DMEM
DMEM plus Glucagon (10µg/ml)
LHC-4
LHC-4 plus Glucagon (10µg/ml)

Appendix 2).

To further investigate this enhancement, the level of HBsAg secretion from a number of cell lines cultured in DMEM or LHC-4 (both supplemented with 5% FBS) was compared. These results are summarised in Table 4.1. The HBV DNA-transfected lines TK4 B4 and 2.2.15 remained unchanged, while the other cell lines showed increased levels of HBsAg secretion in LHC-4 medium compared to DMEM, ranging from a small increase in HH1 cells (40%) to a 23-fold increase in 22/B2 cells (Table 4.1).

The level of HBsAg secreted from PLC/PRF/5 cells cultured in DMEM was within the range of published values (Stratowa et al,1983; Aspinall and Alexander,1988), whereas secretion from the subclone HH1 was nearly four times higher in the same media. In contrast, while HH1 and PLC/PRF/5 cell lines both secreted similar levels of HBsAg when cultured in LHC-4 medium, these levels were 1.4x and 4x (respectively) higher than that detected in DMEM media (Table 4.1).

To determine the active ingredient(s) in LHC-4 medium that induced the 23-fold increase of HBsAg secretion from the 22/B2 cells, substitution and supplementation experiments were then performed and the results are shown in Fig 4.4. Unexpectedly the HBsAg secretion-enhancing activity of the LHC-4 medium was found to reside with the base medium (MCDB151) used in its preparation, as addition of the hormone supplements of LHC-4, including the corticosteroid hydrocortisone, either individually or collectively, had no effect in either DMEM or MCDB151 media (Fig 4.4A). In a second series of experiments, 22/B2 cells were grown in different mixtures of LHC-4 and DMEM; low percentages of DMEM in the mixture (10 or 25%) reduced dramatically (45 and 85% respectively) the level of HBsAg secreted into the culture fluid compared to that from 22/B2 cells grown in LHC-4 medium alone (Fig 4.4B). These results suggested that a component(s) of DMEM inhibited HBsAg secretion. To determine which component(s) was responsible, the concentration of several of the reagents in LHC-4 medium was raised to the

CELL LINE	DMEM	LHC-4	LHC-4/DMEM
TK4 A1	<cut off	<cut off	NA
TK4 B4	211±24	216±27	1.02
2.2.15	78±11	67±13	0.85
22/B2	0.362±.08	8.40±1.18	23.1
Hep3B	45.2±6.4	195±22	4.31
HH1	209±32	293±23	1.40
PLC/PRF/5	60.6±18	236±16	3.93

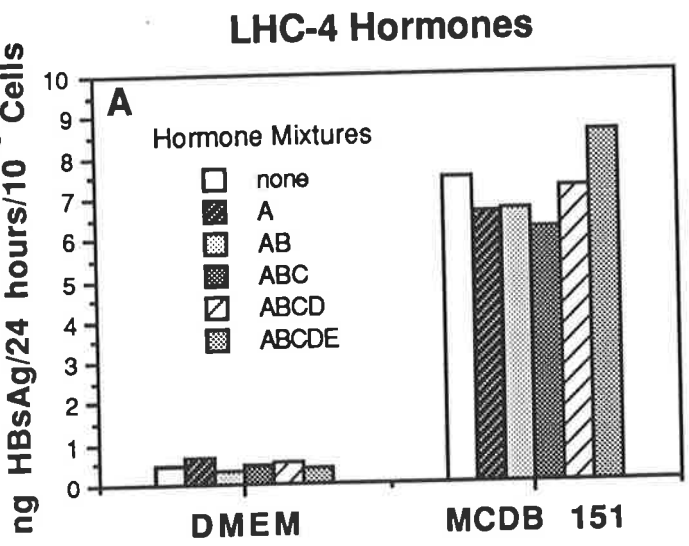
Table 4.1. Levels of secreted HBsAg from cells cultured in DMEM, LHC-4. Expressed as ngHBsAg/24 hours/10^6 cells. NA = Not applicable.

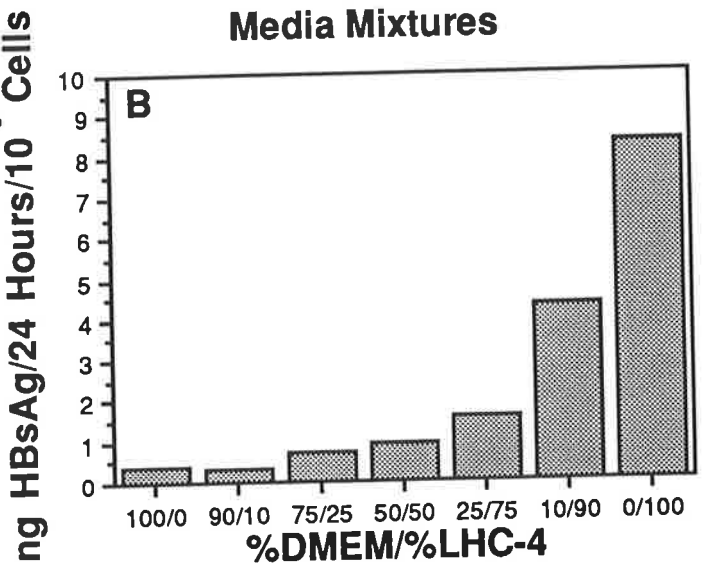
Fig 4.4A. The effect of the hormone components of LHC-4 medium (see appendix 2) on HBsAg secretion from 22/B2 cells.

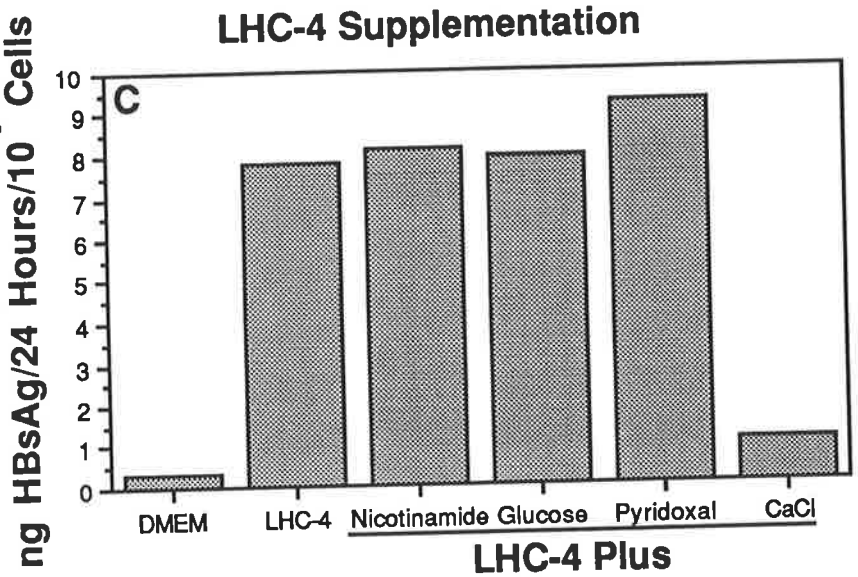
Hormones A = Phosphoethanolamine + Ethanolamine + Hydrocortizone.
Hormones B = Epidermal Growth Factor (EGF).
Hormones C = Transferrin + Insulin.
Hormones D = 3,3',5-Triiodothyronine
Hormones E = Bovine Pituitary Extract

Fig4.4B. The effect of mixtures of DMEM and LHC-4 on secretion of HBsAg from 22/B2 cells.

Fig 4.4C. The effect of supplementation of LHC-4 media with various reagents (to achieve the same concentrations as found in DMEM) on levels of HBsAg secretion from 22/B2 cells.


LHC-4 Hormones
A
Hormone Mixtures
none
A
AB
ABC
ABCD
ABCDE
ng HBsAg/24 hours/10 6 Cells
0
1
2
3
4
5
6
7
8
9
10
DMEM
MCDB 151


Media Mixtures
B
ng HBsAg/24 Hours/10 6 Cells
0
1
2
3
4
5
6
7
8
9
10
100/0
90/10
75/25
50/50
25/75
10/90
0/100
%DMEM/%LHC-4


LHC-4 Supplementation
C
ng HBsAg/24 Hours/10 6 Cells
0
1
2
3
4
5
6
7
8
9
10
DMEM
LHC-4
Nicotinamide
Glucose
Pyridoxal
CaCl
LHC-4 Plus

level found in DMEM, and secreted HBsAg measured (Fig 4.4C). No effect was observed with nicotinamide, glucose or pyridoxal, while in contrast, supplementation with $CaCl_2$ reduced the level of HBsAg secretion close to that found in cells grown in DMEM (Fig 4.4C).

4.3B(c) Effect of Calcium on HBsAg Secretion.

To examine exclusively the effect of calcium on HBsAg secretion, modified DMEM was prepared (Appendix 1) in which calcium was omitted. Following supplementation of this medium with 5% dialyzed FBS (vs PBS) and 0-500μg/ml $CaCl_2$, the level of secreted HBsAg from a number of different cell lines was determined (Figs 4.5 and 4.6). Calcium levels below 10 and above 200 μg/ml led to a reduction in cell numbers with some cell lines (particularly 22/B2 and TK4 B4). To account for this, secreted HBsAg levels were measured as ng HBsAg/24 hours/10^6 cells and ng HBsAg/24 hours/cm^2 of culture area to represent individual cell- and total culture-secretion respectively.

HBsAg secretion from 22/B2 cells (Fig 4.5) was inversely proportional to $CaCl_2$ concentration over the entire range examined (0-500μg/ml), with a maximum secretion in calcium-free medium; compared with normal DMEM, HBsAg secretion in calcium-free DMEM was enhanced 40-fold. Similarly, differing levels of enhancement (1-10 fold) of HBsAg secretion from the HH1, TK4 B4 and Hep 3B cell lines were also observed (per cell and total) when the $CaCl_2$ level was lowered to ≤50 μg/ml (Fig 4.6 shows per cell results; total values are not shown as these were virtually identical). No increase in HBsAg secretion was seen from 2.2.15 cells. Thus, with the exception of 2.2.15 cells, all the cell lines tested showed increased secretion of HBsAg when cultured in calcium-free medium compared with medium containing 200μg/ml $CaCl_2$ (the concentration found in normal DMEM). In one experiment using calcium-free DMEM, the HH1 cell line secreted over 800ng HBsAg/24 hours/10^6cells (Fig 4.6), four times higher than the previously recorded value for a non-transfected cell line (Aspinall and Alexander,1988).

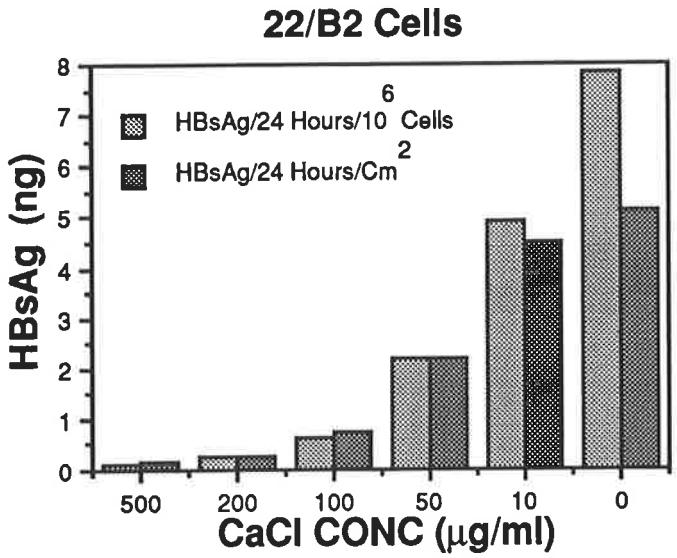


FIG 4.5 The effect of calcium chloride addition to calcium-free DMEM medium on levels of HBsAg secretion from Vero 22/B2 cells.

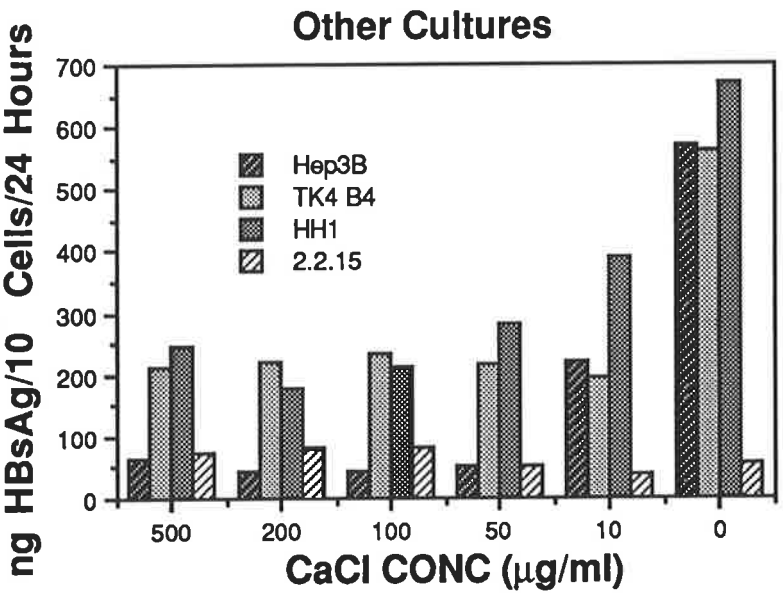


Fig 4.6. The effect of the addition of calcium chloride to calcium-free DMEM on levels of HBsAg secretion from Hep3B, TK4 B4, HH1 and 2.2.15 cells.

The effect of calcium-free medium could not be duplicated by chelating the calcium from normal DMEM with EDTA. However, this agent was found to be cytotoxic at the concentration required to remove all Ca^{++} ions from the medium.

4.3C EFFECT OF DEMETHYLATION.

4.3C(a) TK4 Clone A1.

Normally this clone was found to be both HBsAg- and HBcAg-negative by immunofluorescence. However when these cells were treated with 5-azacytidine then cultured in LHC-4 medium supplemented with 5% FBS, foci of HBsAg-positive cells were detected occasionally (Fig 4.7 top). As these foci were considered to be too large to have arisen by clonal division of one derepressed cell, it is possible that cell-cell transfer of an infective agent or promoter substance from a single induced cell accounted for these observations. This may then represent an example of HBsAg gene induction, as distinct from enhancement of gene expression, an observation not described previously. In contrast, after the same induction step, HBcAg was still not observed and HBV DNA was undetected in the cell culture fluid.

4.3C(b) TK4 Clone B4.

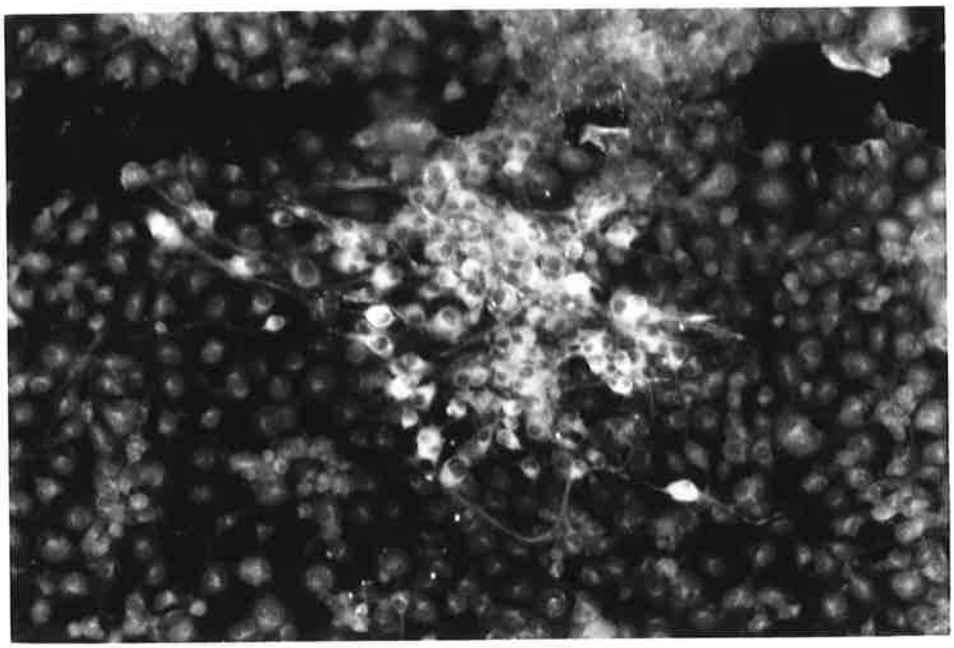
HBsAg but not HBcAg was normally detectable (Fig 4.7 middle) in all cells of clone B4. After 5-azacytidine induction in LHC-4 medium, the proportion of HBcAg-positive cells varied between 2-50% of the total cells (Fig 4.7 bottom). As alteration in gene expression patterns due to 5-azacytidine-induced demethylation normally requires a minimum of 2-3 cell divisions to be detected (Jones and Taylor, 1980), these induced changes (48 hours) occurred sooner than expected if demethylation was the sole mediating event (doubling time of TK4 cells in LHC-4 medium was ca. 40 hours). Furthermore, as HBcAg was not detected when these cells were cultured either in LHC-4 medium alone or in DMEM after 5-azacytidine induction, it is likely that HBcAg expression was dependent on both 5-azacytidine and the hormone constituents of LHC-4 media. Despite the

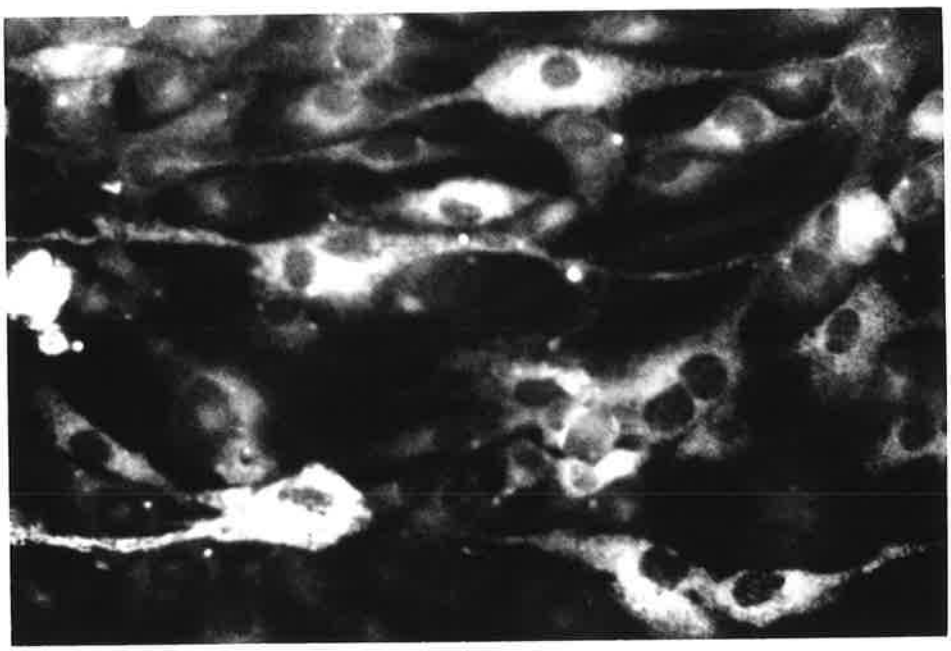
Fig 4.7 Detection of HBV antigens in TK4 subclones A1 and B4 by indirect immunofluorescence. Magnification x400 (top); (middle and bottom); x1600.

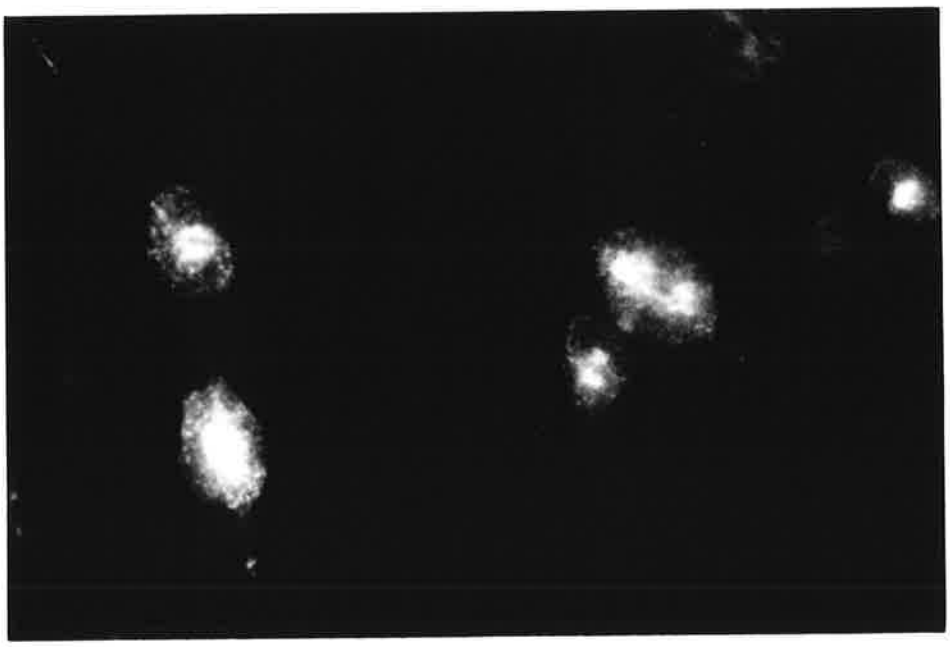
Top: TK4 A1 stained for HBsAg (following 5-azacytidine treatment and culture in LHC-4 medium)

Middle: TK4 B4 stained for HBsAg (cultured in DMEM alone)

Bottom: TK4 B4 stained for HBcAg (following 5-azacytidine treatment and culture in LHC-4 medium).







induction of HBcAg expression in these cells, simultaneous production of HBV, as measured by dot blot hybridisation of the culture fluid, was not observed.

4.3C(c) Other Cell lines.

Despite 5-azacytidine induction in the cells described above, HBcAg was never detected in HH1, 22/B2 and PLC/PRF/5 cells. This is in contrast to the results of Yoakum et al (1983), who reported HBcAg synthesis in PLC/PRF/5 cells after similar 5-azacytidine treatment.

4.3D THE EFFECT OF CALCIUM ON HBV GENE EXPRESSION.

To complement the above studies on secretion of HBsAg, the intra- and extra-cellular expression of several HBV genes were examined.

4.3D(a) HBsAg-Specific Proteins.

All cell lines tested (HH1, 22/B2, TK4 B4 and 2.2.15) revealed slightly enhanced intracellular levels of major HBsAg by immunofluorescence when the cells were grown in calcium-free compared to normal DMEM. In contrast, intracellular levels of the large and middle proteins, which were only detected in HH1 and 2.2.15 cells (Fig 4.8) were increased markedly when these cells were grown in calcium-free DMEM.

4.3D(b) HBcAg.

Attempts to increase the levels of HBcAg expression described above [section 4.3 C(b)] by the use of calcium-free DMEM were unsuccessful, and although HBcAg was detected in 2.2.15 cells (as weak cytoplasmic staining), the level was unaffected by the calcium content of the medium.

4.3D(c) HBV Production.

Culture fluids obtained after cultivation of TK4 B4, 22/B2, HH1, Hep3B and 2.2.15 cells in DMEM containing varying levels of $CaCl_2$ were assayed for HBV DNA by dot blot hybridisation (Fig 4.9). HBV DNA was not detected in the culture fluids from TK4 B4, 22/B2, HH1 or Hep3B at any of the concentrations of calcium tested. However, the level of HBV DNA in 2.2.15 culture supernatants increased from an estimated 3vge/cell/day in DMEM

Fig 4.8 Detection of large HBsAg by indirect immunofluorescence in HH1 and 2.2.15 cells cultured in calcium-modified DMEM.

A HH1 cells cultured in normal DMEM (containing 200g/ml $CaCl_2$).
B HH1 cells cultured in calcium-free DMEM.
C 2.2.15 cells cultured in normal DMEM (containing 200g/ml $CaCl_2$).
D 2.2.15 cells cultured in calcium-free DMEM.

Magnification x1800.

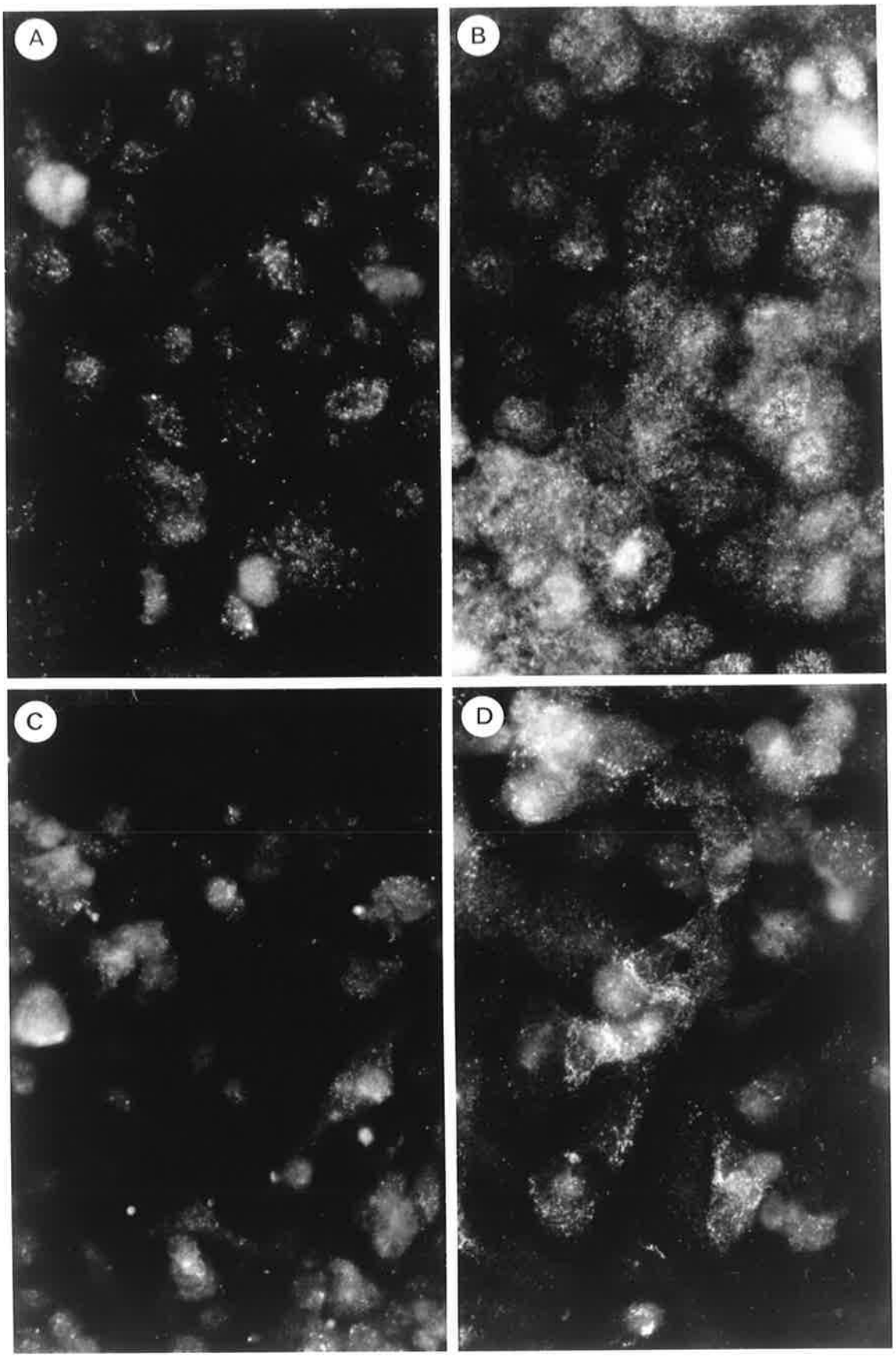
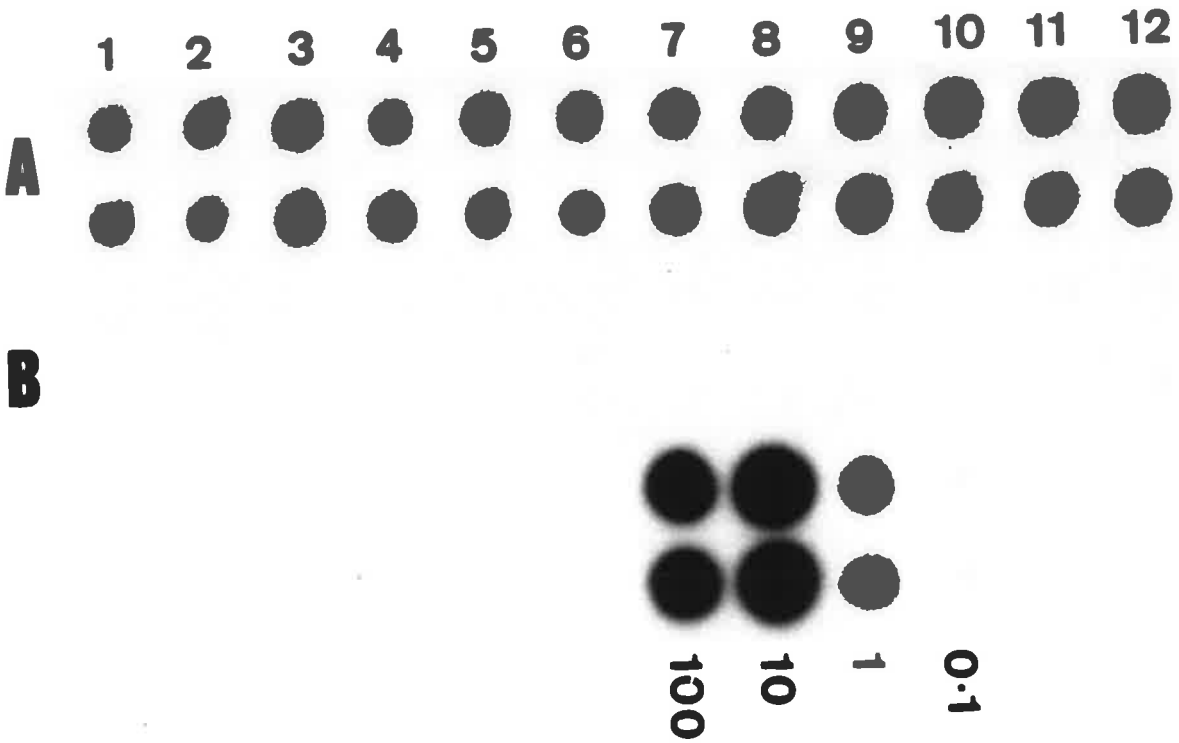

A
B
C
D

Fig 4.9 Dot blot hybridisation assay to detect HBV DNA in culture fluids obtained after cultivation of various cell lines in calcium-modified DMEM. All samples were tested in duplicate.

Row A columns 1-12 culture fluids obtained from 2.2.15 cells grown in DMEM containing 1000, 500, 200, 150, 100, 50, 40, 30, 20, 10, 5 and 0µg/ml $CaCl_2$ respectively.

Row B culture fluids from: columns 1 and 2, HepG2-; columns 5 and 6, HH1-; columns 7 and 8, 22/B2-; columns 9 and 10, Hep3B-; columns 11 and 12, TK4 B4-cells grown in normal and calcium-free DMEM respectively.

DNA standards at bottom represent 100, 10, 1 or 0.1pg of cloned HBV DNA derived from pHBVCB.


1
2
3
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11
12
A
B
100
10
1
0.1

containing 500µg/ml $CaCl_2$ to nearly 25vge/cell/day in DMEM containing from 0-20µg/ml $CaCl_2$ (Fig 4.9).

4.3E HBV INFECTION.

Although Chapter 3 described attempts to infect cells with HBV, it was considered appropriate to report the results of infection experiments using HepG2 cells cultured in calcium-free medium in this section of the thesis. These cells were infected and analysed as described in Chapter 2 (section 2.7 C). Despite the fact that calcium-free medium increased HBsAg secretion, HepG2 cells grown in calcium-free DMEM remained refractile to infection with HBV. Thus although it appears that (low) calcium exerts an effect on the HBsAg gene expression, the preexisting blocks to HBV infection in vitro were not overcome.

4.4 DISCUSSION.

4.4A REGULATORY ELEMENTS OF THE HBsAg GENE.

Regulation of the HBsAg gene promoter is largely unknown although a binding site for nuclear factor 1, that was essential for maximal S promoter activity, was mapped to a site ca. 200 base pairs upstream (Shaul et al, 1986). In addition a glucocorticoid-responsive element has been identified upstream of the HBsAg gene (Tur-Kaspa et al, 1986) that could account for the in vitro enhancement of HBsAg synthesis observed with dexamethasone in this and other studies as well as the activation of latent infection in patients undergoing corticosteroid therapy (Scullard et al, 1981).

4.4B CYTOSINE METHYLATION.

The above experiments showed that the HpaII and MspI restriction enzyme sites in integrated HBV DNA in TK4 clones A1 and B4 contained methylated cytosine bases. Unfortunately, although three HpaII and MspI sites were predicted in the HBV DNA clone used for the preparation of TK4 cells [map positions 430 (S gene region) 2791 and 2852 (pre-S1 region)], none of these regions correspond to recognised HBV promoter or enhancer sequences. Nevertheless it is equally likely that other cytosine residues, in

regions not cleaved by these enzymes, were also methylated and could therefore partly account for the ability of 5-azacytidine to induce surface and core gene expression observed in this study. However the possibility that other media-based factors contained in LHC-4 medium contributed to this expression cannot be discounted.

4.4C EFFECT OF CALCIUM.

With the exception of 2.2.15 cells, all cell lines tested demonstrated a moderate to strong enhancement of HBsAg secretion when calcium was removed from the medium. This effect has not been noted previously and the exact mechanism is unclear, although calcium is known to play an important role as an intracellular secondary messenger for many hormone actions (Alberts et al, 1989). This role is dependant on the maintenance of a large calcium gradient across the plasma membrane, where the resting intracellular cytosol Ca^{++} concentration can be as low as 10^{-7}M compared with an extracellular concentration of ca. 10^{-3}M (≈100µg/ml $CaCl_2$). A Ca^{++} influx into the cytosol, either from the external environment or from the intracellular "calcium-sequestering compartment" (Alberts et al, 1989), then functions as a secondary messenger. Alteration of the extracellular Ca^{++} concentration may affect the normal equilibrium which may lead to the stimulation of HBsAg production described in this chapter.

The reason for the failure of 2.2.15 cells to show the same enhancement of HBsAg synthesis shown by other cell lines cultivated in calcium-free media was unclear. However, this may be related to the concomitant stimulation of the large HBsAg protein, as this has been shown to inhibit the secretion of the middle and major HBsAg proteins (Chisari et al, 1986; Molnar-Kimber et al, 1988). Nevertheless, an intracellular accumulation of HBsAg was not noted in the 2.2.15 cells nor in the HH1 cells, which did demonstrate a calcium-related stimulation of all three HBsAg proteins. The highest stimulation of HBsAg secretion (ca. 40 fold) was seen in the 22/B2 cell line in which the large and middle proteins were not detected. Thus the contrasting response in

calcium-free DMEM of the large HBsAg-positive cell lines, HH1 and 2.2.15 (enhanced and non-enhanced HBsAg secretion respectively), may be related to the ability of these cell lines to synthesize complete HBV particles (produced by 2.2.15 but not HH1 cells) or may reflect the more differentiated, hepatocyte-specific, nature of HepG2 cells (Knowles et al, 1980), the parent line for 2.2.15 cells.

As enhancement of HBV production in 2.2.15 cells grown in calcium-free DMEM was not associated with a similar increase in HBcAg production, then synthesis of the large HBsAg protein may be the rate limiting step in virus replication in these cells. This is consistent with recent data which suggests that this protein may act as the signal for core particle encapsidation (Yokosuka et al,1988; Summers et al, 1990; see Chapter 1, Section 1.4 F).

4.4D THE EFFECT OF GLUCAGON.

The effect of glucagon, which showed moderate stimulation of HBsAg synthesis, was of particular interest, as this hormone exerts its principal effect on hepatocytes, to stimulate glycogen catalysis and release glucose into the bloodstream. The mechanism of stimulation of HBsAg synthesis was unclear, but may be related to the calcium effect described above, as glucagon treatment is known to decrease the calcium ion content of cultured hepatocytes (Kraus-Friedmann, 1986), due possibly to a specific inhibition of the calcium pump in hepatocyte plasma membranes (Mallat et al, 1987).

4.4E CONCLUSION.

In this thesis, many agents which have been reported to show increased expression of HBV antigens showed some effect but, with the exception of dexamethasone, this was transient and/or not reproducable. In contrast, the results in this chapter have shown for the first time that the level of HBsAg and HBV expression in a number of HBV-induced hepatoma and transfected cell lines may be influenced by the calcium concentration in the culture media. This effect may prove useful in further studies which, coupled with the effect of glucagon, may be relevant to HBV replication in vivo.

CHAPTER 5.

HEPADNAVIRUS TRANSFECTION.

5.1 INTRODUCTION AND EXPERIMENTAL DESIGN.

The five hepadnaviruses share similar structure, replication strategy and genome organisation (see Chapter 1, Sections 1.3, 1.4) and also interact with their hosts in a characteristic manner, including hepatotropism and species specificity. These latter two properties may result from the same mechanisms ie. cellular expression of virus receptors and also intracellular "factors" required for transcription of pregenomic-RNA.

Although the differentiated human hepatoma cell lines HuH6, HuH7 and HepG2 cannot be infected with HBV, these cells permit HBV replication from transfected viral DNA (Sureau et al, 1986, Tsurimoto et al, 1987, Sells et al, 1987, Yaginuma et al, 1987) and therefore possess the appropiate cellular "factors" for the transcription of HBV genes. However, at the commencement of the work described in this Chapter it was unknown if these cellular transcription factors were species specific or common to all hepadnaviruses. To examine this possibility the HepG2 cell line, known to support HBV replication after transfection of HBV DNA, was transfected with a dimeric construct of DHBV DNA contained in the vector pSV2Neo. Transfected cells were examined for DHBV antigens by immunofluorescence and the culture fluids tested for DHBV DNA by dot blot hybridisation. Finally, to determine whether infectious virus was produced, culture fluids from cells at various passages after transfection were inoculated into 1-7 day old ducks and the birds monitored by dot blot hybridisation for the appearance DHBV in the serum.

5.2 RESULTS.

5.2A PLASMID CONSTRUCTION.

The construction of the DHBV expression vector used in this work is shown in Fig 5.1. Full length DHBV DNA, excised from plasmid pSPDHBV-5.1 [Chapter 2 Section 2.3 A(d)] by EcoRI digestion, was inserted into the eukaryotic expression vector pSV2neo [Chapter 2 Section 2.3 A(b)]. Then using a process of partial digestion and religation, an additional full-length fragment was added to create a head-tail dimer of DHBV DNA The final recombinant vector was renamed pTMDHBVneo2. This plasmid is designed to integrate into chromosomal DNA, contains a neomycin selection marker and relies on endogenous DHBV promoters for transcription of DHBV-specific mRNA. The dimer construct was designed to produce the DHBV mRNA transcripts associated with DHBV replication (Fig 5.2). In particular two regions are capable of encoding the subgenomic DHBsAg mRNAs of 1.8 and 2.1kb and one region can potentially encode the large 3.3kb pregenomic mRNA (analogous to the 2.1, 2.4 and 3.5 kb RNAs of HBV).

5.2B PRODUCTION OF STABLE CELL LINES.

5.2B(a) DHBV Antigen Expression.

Following transfection of plasmid pTMDHBVneo2 into HepG2 cells as described in Chapter 2 section 2.4A, five G418-resistant clones, were isolated and expanded. These were screened by immunofluorescence for the presence of DHBV-specific polypeptides DHBsAg and DHBV pre-S (Table 5.1). All five clones expressed DHBsAg, but only one clone, G2 DHBV-3, also expressed DHBV pre-S. This clone was then studied in more detail.

5.2B(b) DHBV Antigen staining in G2 DHBV-3 Cells.

Initially (passage#2), approximately 50% of cells were positive for DHBsAg and 10% for DHBV pre-S. Both antigens were visualised as speckled cytoplasmic staining of variable intensity. However, by passage #5 and in all subsequent passages, the proportion of positive cells had dropped

Fig 5.1 Strategy for construction of recombinant plasmid pTMDHBVneo2, which contains a dimer of DHBV DNA inserted into the eukaryotic expression vector pSV2neo.

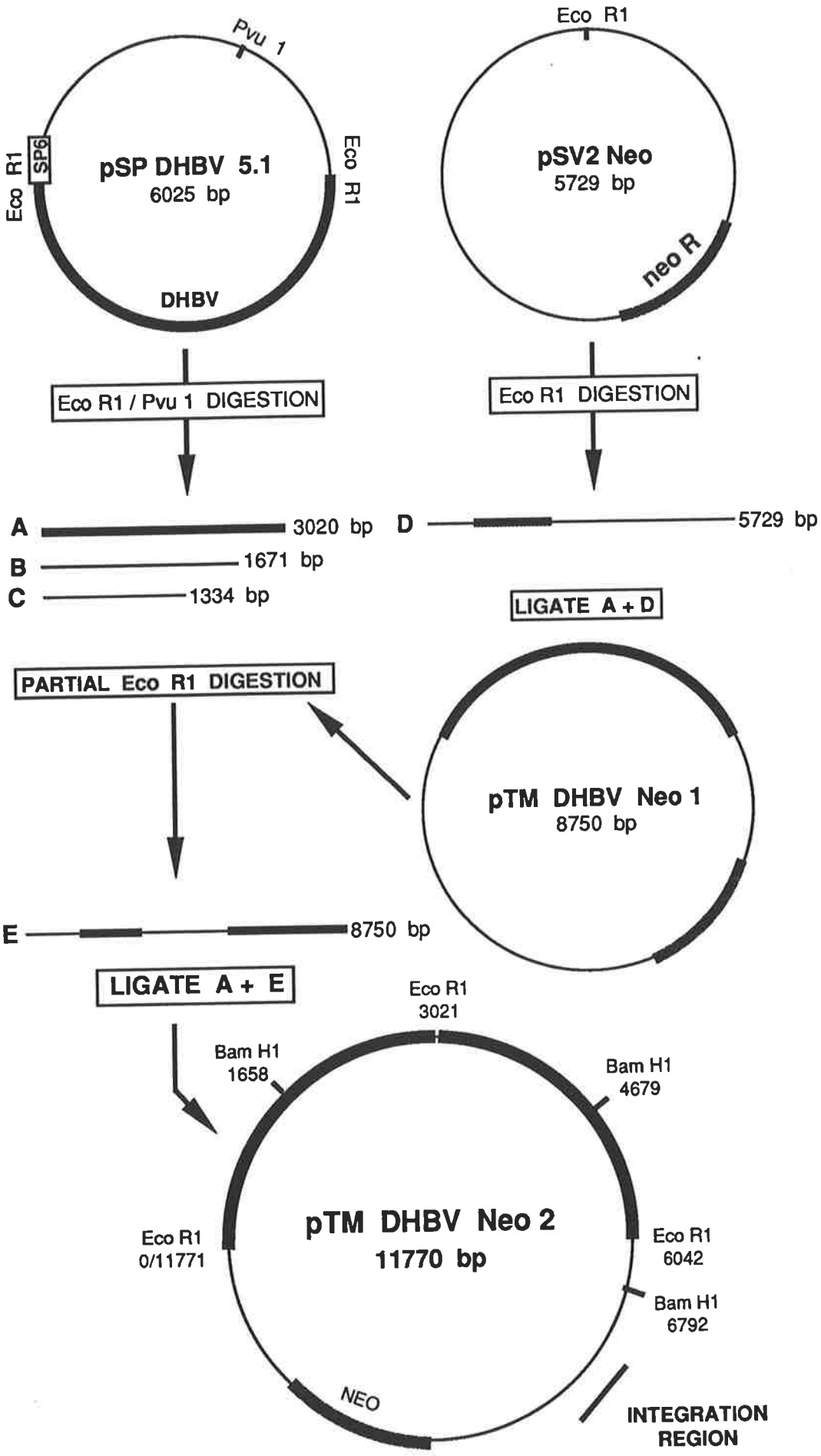


Fig 5.1 Construction of DHBV Expression Vector

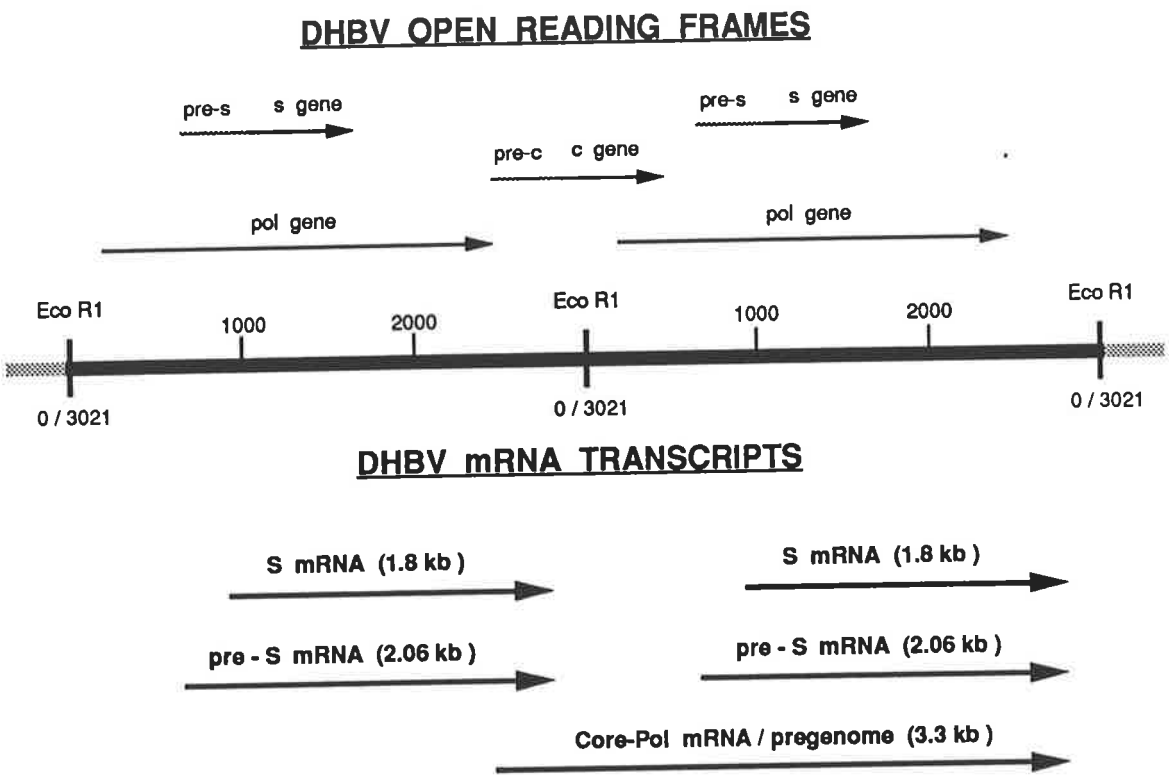


Fig 5.2 Configuration of the DHBV DNA dimer insert in pTM DHBV Neo 2 showing the location of the open reading frames and the RNA transcripts that can potentially be synthesised.

Table 5.1: Summary of results following Transfection of HepG2 Cells with the DHBV expression vector pTMDHBVneo2.

CELL LINE	DHBVsAg (IF)	PRE -S (IF)	VIRUS
G2 DHBV 1	+	-	-
G2 DHBV 2	+	-	-
G2 DHBV 3	+	+	+
G2 DHBV 4	+	-	-
G2 DHBV 5	+	-	-

to 5% and 2% respectively (Fig 5.3 top and middle). A rabbit antiserum specific for DHBcAg (a gift from Bill Mason), that became available later was used to examine G2 DHBV-3 cultures from passage #7 onwards. The DHBcAg distribution in these cells showed a similar staining pattern to DHDsAg and DHBV pre-S, with 2% of cells consistently showing cytoplasmic staining (Fig 5.3 bottom). The level of expression and distribution of DHBV antigens in G2 DHBV-3 cells was unaffected by the degree of cell confluence, in contrast to the results obtained in Hep3B cells and HBV DNA-transfected Rat2 cells (Aden et al, 1979; Gough and Murray, 1982)

5.2B(c) Replicative intermediates in G2 DHBV-3 Cells.

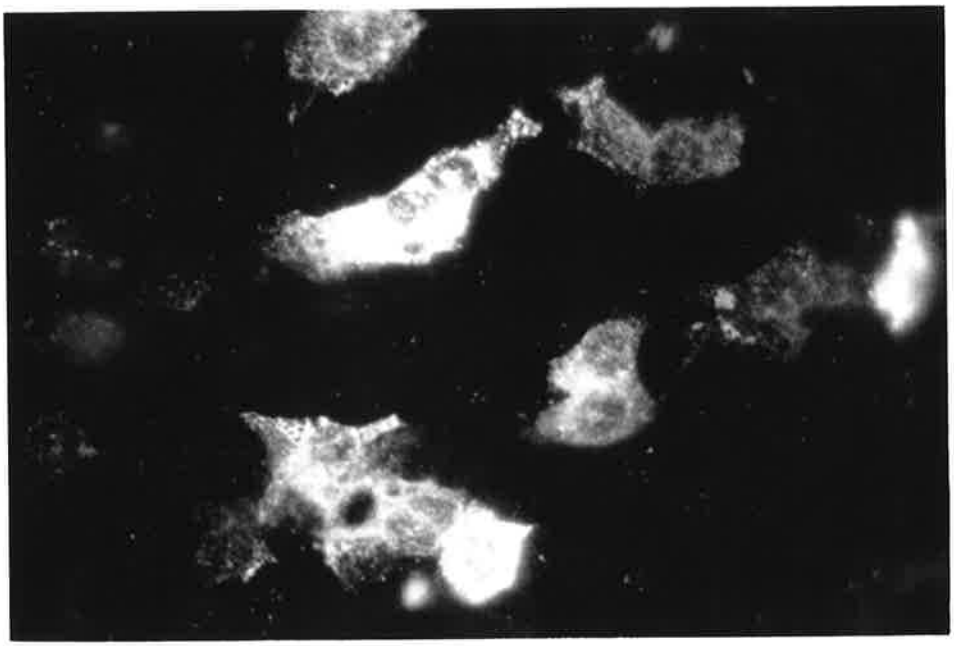
Total DNA was extracted from G2 DHBV-3 cells and analysed by Southern blot hybridisation as described in Chapter 2 section 2.5E(b). The DNA was examined before and after digestion with the restriction enzymes BamH1 or EcoR1, both of which digest monomeric DHBV DNA once only (Fig 5.4 group1). Although DHBV DNA was detected in high molecular weight (chromosomal) DNA, a monomeric or subgenomic species of DHBV DNA indicative of replicative intermediates was not detected in undigested DNA. This suggested that the DHBV mRNA necessary for the synthesis of the DHBV products detected above was likely to be transcribed from integrated DHBV DNA and not from covalently closed circular DNA (ccc DNA), the more usual template for hepadnavirus mRNA transcription. This was supported by digestion of extracted DNA with BamHI and EcoRI, as a major reactive band of ca. 3000bp was then observed indicating that G2 DHBV-3 cells contained a major integrated DHBV DNA species of greater than unit length (Fig 5.4 lanes B and E).

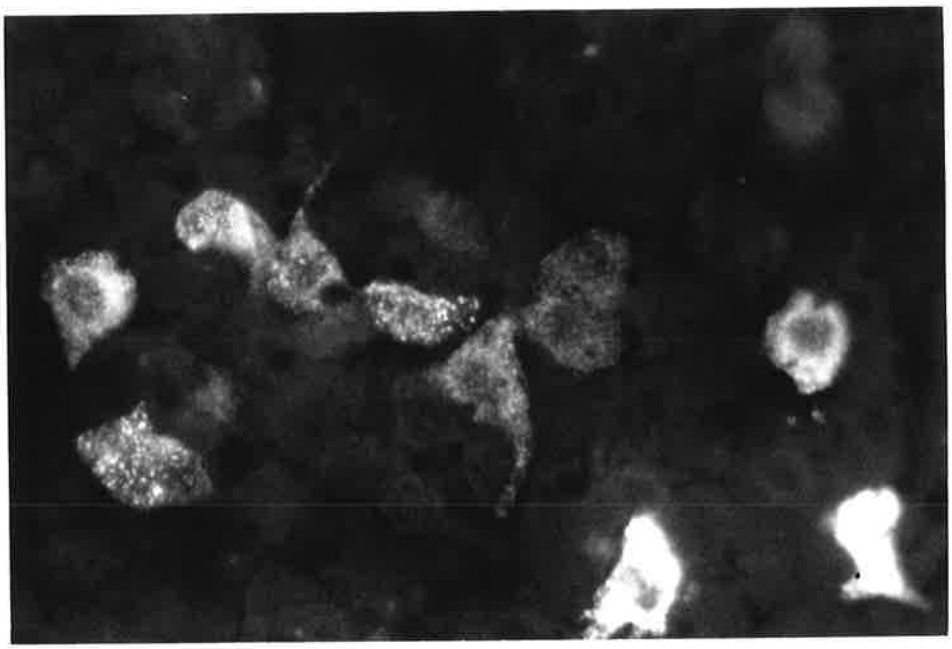
5.2B(d) Recloning of G2 DHBV-3 Cells.

The initial decrease in DHBsAg and DHB pre-S expression in G2 DHBV-3 cells between passage #2 to passage #5 may have occurred due to the loss of transient expression from non-integrated pTMDHBVneo2. Nevertheless, to investigate the possibility that the G2 DHBV-3 clone had

Fig 5.3 Detection of DHBV antigens in G2 DHBV-3 cells by indirect immunofluorescence. Magnification x2000

Top: Stained for DHBsAg.
Middle: Stained for DHBV pre-S.
Bottom: Stained for DHBcAg.





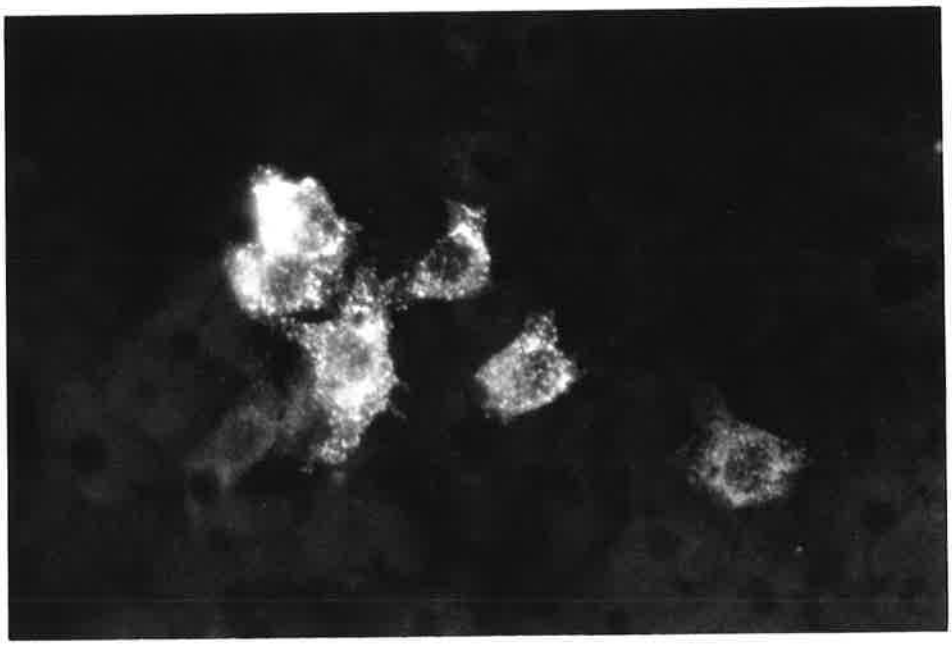
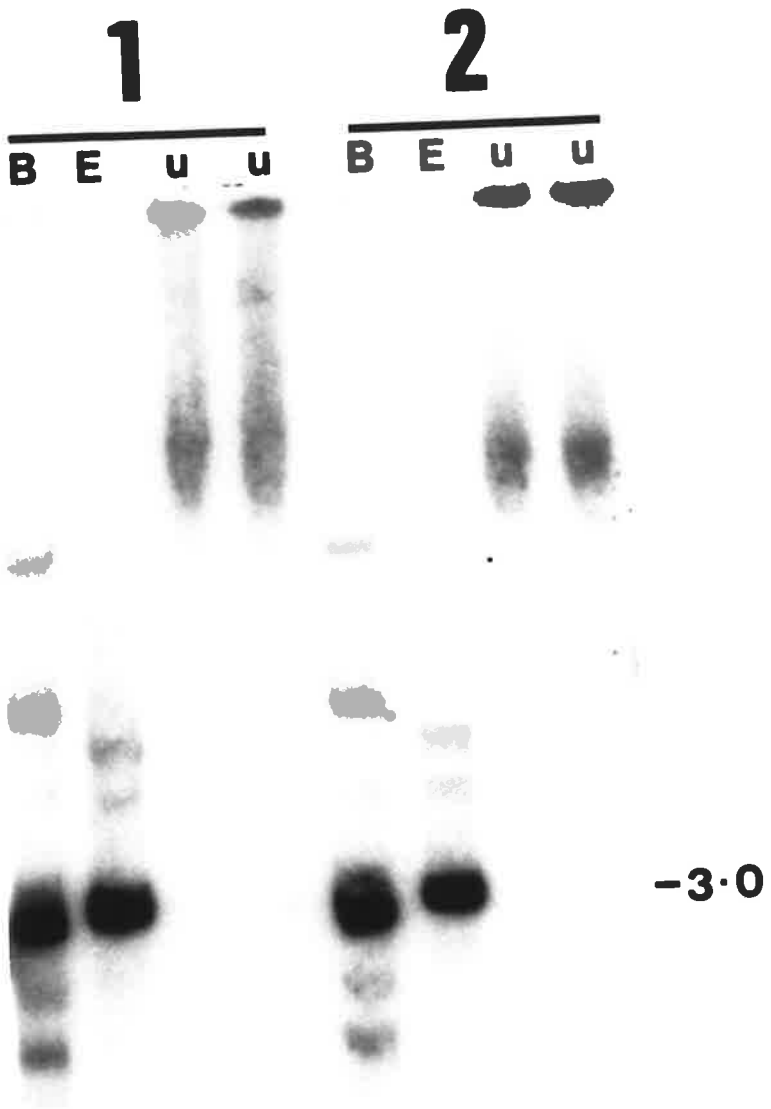


Fig 5.4 Southern blot hybridisation analysis of DNA extracted from G2 DHBV-3 cells (group 1) and subclone B11 (group 2) after hybridisation with ^{32}P-labelled riboprobes (transcribed from pSPDHBV-5.1) to detect DHBV DNA. Total DNA was analysed uncut (u) or after digestion with restriction enzymes BamHI (B) or EcoRI (E). The size marker on the right shows the position of full-length DHBV DNA (derived from pSPDHBV-5.1).


1
2
B E u u
B E u u
-3·0

become heterogeneous and also enhance the proportion of DHBV-positive cells, the G2 DHBV-3 cell line was recloned. However, although seven single-cell clones were expanded after this procedure, all showed similar percentages of DHBsAg, pre-S and DHBcAg expression (5%, 2% and 2% respectively) to the parent G2 DHBV-3 cells. Furthermore, Southern blot hybridisation analysis of DNA extracted from one of these subclones [B11 (Fig 5.4 group 2)] also showed identical results to those obtained for the parent line. Attempts to enhance the levels of DHBV products using low calcium medium or 5-azacytidine induction as described in Chapter 4 were unsuccessful.

5.2C *IN VITRO* SYNTHESIS OF INFECTIOUS DHBV.

Neat cell culture fluids from G2 DHBV clones -1, -2, -4 and -5 at passage #9 as well as neat and 100X concentrated (by ultracentrifugation) culture fluids from G2 DHBV-3 cell line at passage #9 and two subclones (F11 and B11) at passage #18 were examined by dot blot hybridisation to detect DHBV DNA.

DHBV DNA was only detected in concentrated culture fluid from the G2 DHBV-3 cells and its subclones (Fig 5.5). The level of DHBV synthesis in these cells was equivalent to 0.2 virus particles/cell/day, lower than the basal level of HBV produced by the HepG2-derived 2.2.15 cell line described in Chapter 4 (ca. 3 virus particles/cell/ day).

To examine if infectious DHBV was produced in the above experiments, cell culture fluids from all G2 DHBV clones at passage #2 as well as neat and 100X concentrated culture fluids from two G2 DHBV-3 subclones (F11 and B11) at passage #18 were inoculated iv or ip into 1-7 day old Peking-Aylesbury ducks. Sera collected before and at various intervals after inoculation were examined by dot blot hybridisation to detect DHBV DNA (an example of the resultant dot blot hybridisations is shown in Fig 5.5). Variable levels of DHBV DNA were detected but only in sera from ducks inoculated with neat or concentrated cell culture fluids from G2 DHBV-3 cells and its

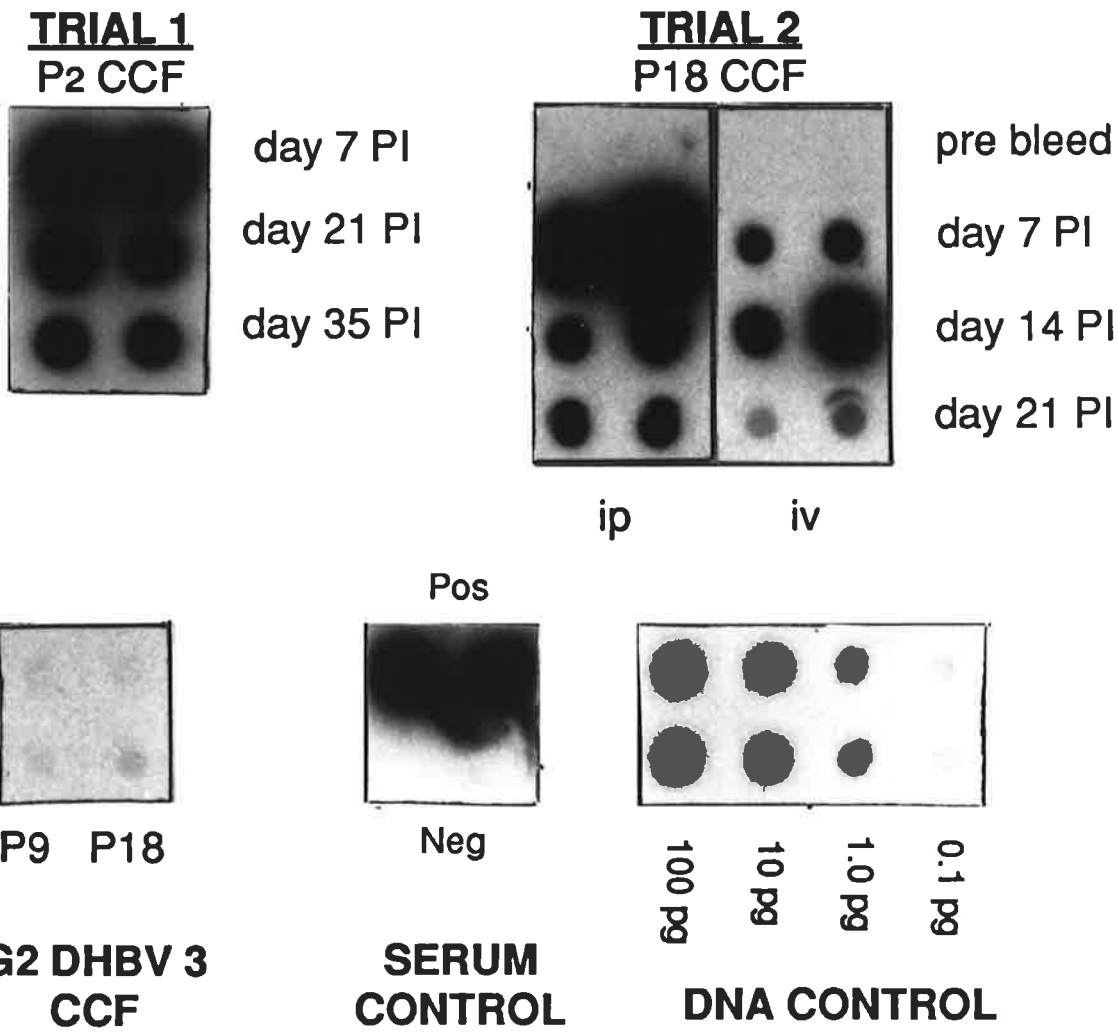
Fig 5.5 Dot blot hybridisation assay of culture fluids and duck sera after hybridisation with ^{32}P-labelled riboprobes (transcribed from pSPDHBV-5.1) to detect DHBV DNA. All samples were tested in duplicate.

Trial 1, sera from ducks 7-35 days post inoculation (PI) with neat culture fluid from G2 DHBV-3 cells at passage #2.

Trial 2, sera from ducks 0-21 days after intravenous (iv) or intraperitoneal (ip) inoculation with 100x concentrated culture fluids from G2 DHBV 3 subclones B11 and F11 (passage #18) respectively.

Also shown are 100x concentrated culture fluids from G2 DHBV-3 cells (passage #9) and subclone B11 (passage #18), positive and negative duck serum controls and a DHBV DNA standard comprised of serially-diluted DHBV DNA insert from pSPDHBV-5.1.

DHBV DNA DOT BLOT



subclones (Fig 5.5). In these ducks, DHBV DNA appeared from 4-14 days after inoculation and persisted throughout the period of study. Due to the small numbers of birds inoculated, it was impossible to determine whether these variations were due to differences either in the inoculum or in the susceptibility of different ducks to DHBV infection. However, as previous studies using in vivo-derived DHBV have consistently shown an incubation period of 3-4 days (Qiao et al, 1990) the prolonged incubation period was most likely to result from a low dose inoculum. In contrast, DHBV DNA was not detected in sera from ducks inoculated with culture fluids from G2 DHBV clones -1, -2, -4 and -5 irrespective of passage number. The level of DHBV DNA in the resultant positive sera was at least 10-fold higher than that detected in 100X concentrated cell culture fluids from G2 DHBV-3 cells at passage #9 or subclones F11 or B11 at passage #18 (Fig 5.5) and indicates that de novo DHBV DNA synthesis, resulting from DHBV replication, had occurred in these ducks.

5.3 DISCUSSION.

The above experiments have established that a human hepatoma cell line can synthesise DHBsAg, DHB pre-S and DHBcAg as well as infectious DHBV after transfection with DHBV DNA and thus show that the hepadnavirus species barrier can be breached, at least in part . During the course of this study a number of other researchers also demonstrated the production of infectious DHBV in the human hepatoma cell lines HepG2 and HuH7 (Pugh et al, 1988; Hirsch et al, 1988; Galle et al, 1988). However, as these latter studies only showed transient DHBV production, the above results have also established that infectious DHBV can be produced stably by HepG2 cells since the G2 DHBV-3 cell line has continually secreted infectious DHBV into the culture fluid for 9 months (18 passages). In contrast to the results obtained for HDV (see Chapters 6 and 7), no evidence of cytotoxicity was observed in any of the DHBV DNA-transfected cell lines. Of the five G418-resistant clones isolated, only the G2 DHBV-3 cells expressed pre-S. As this was also the

only cell line which secreted DHBV, then pre-S expression was a marker for DHBV replication in this system. This interpretation was confirmed by the detection of DHBcAg, a recognised marker of hepadnavirus replication (Gowans et al, 1983), in a similar proportion of the G2 DHBV-3 cells that showed pre-S positivity.

Nevertheless, these cells only produced approximately one tenth of the mature virus produced by HepG2 cells after transfection with HBV DNA (i.e. the 2.2.15 cell line; see Chapter 4) suggesting that other cell-specific factors may enhance replication levels. Since the G2 DHBV-3 cell line displayed a homogeneous pattern of integrated DHBV DNA, the low percentage of antigen-positive cells at passage #5 or greater, and low level of DHBV production may reflect inefficient transcription or translation of DHBV mRNAs in HepG2 cells. Condreay et al (1990) demonstrated recently that an avian hepatoma cell line synthesised 20-fold higher levels of DHBV compared with the HepG2 cell line, confirming the above interpretation. Thus, possession of the appropriate virus receptor is not the only factor which affects hepadnavirus species specificity.

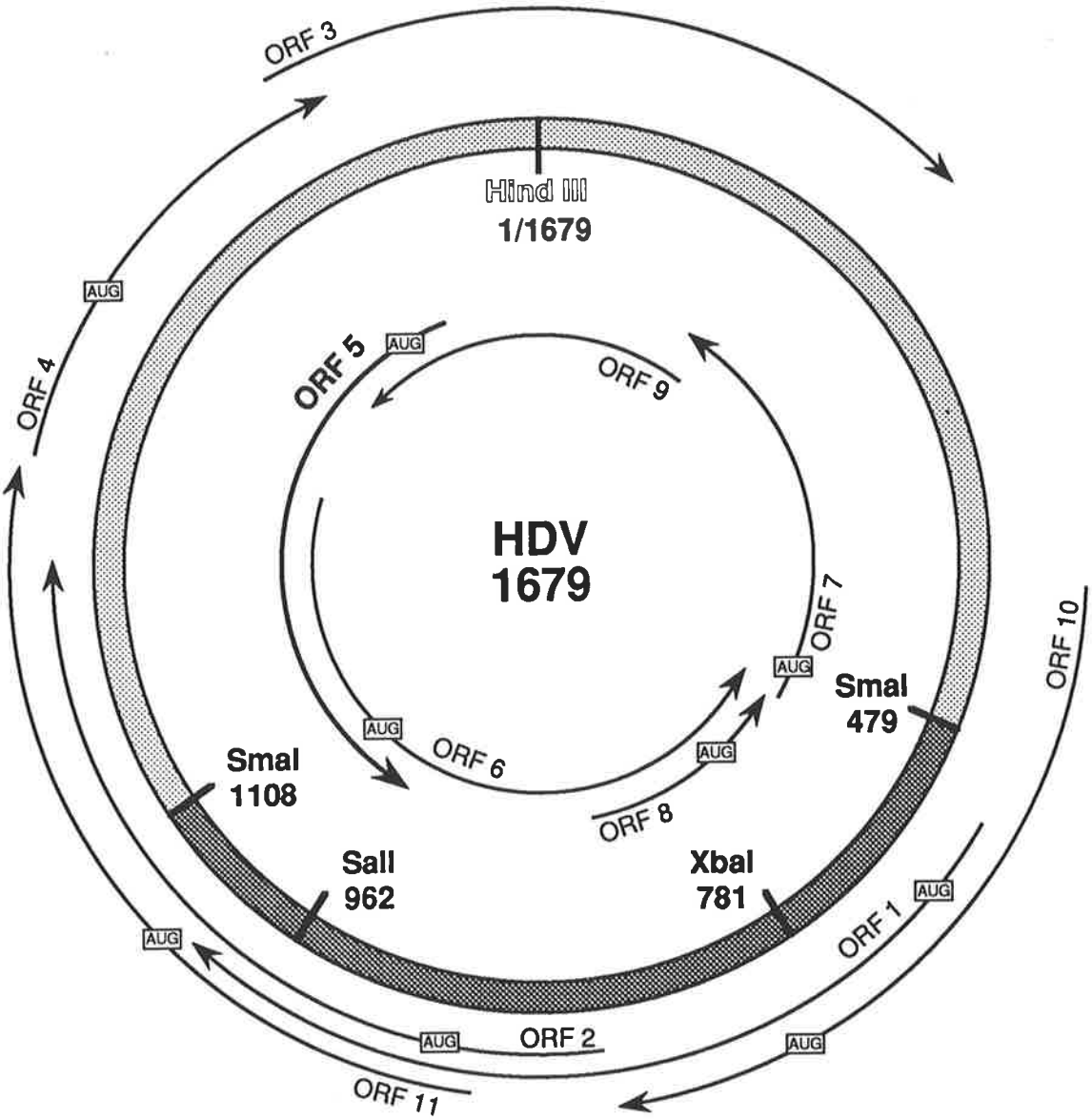
CHAPTER 6.

EXPRESSION, PROPERTIES AND APPLICATIONS OF RECOMBINANT HDAg.

6.1 INTRODUCTION.

This and the next chapter of this thesis examine aspects of hepatitis delta virus (HDV) gene expression and replication. This chapter describes the expression of the hepatitis delta antigen (HDAg) gene in HepG2 and Hela cells, while Chapter 7 focuses on the expression and replication of the whole HDV genome.

When the work described in this chapter was commenced only one complete sequence of HDV cDNA (isolated from HDV passaged five times in chimpanzees) was published (Wang et al, 1986; Fig 6.1). Analysis of this sequence revealed 11 potential open reading frames (ORFs) that could code for protein products. However only one protein, derived from ORF-5, on the antigenomic-sense strand, reacted with HDV-carrier sera. This finding was confirmed in more recent studies which analysed full length HDV cDNA isolates prepared from HDV-infected human (Makino et al, 1987) and woodchuck sera (Kuo et al, 1988), and it is now generally accepted that ORF-5 is the coding region for the virus-coded internal structural antigen, HDAg (Rizzetto et al, 1980b). Although analysis of the HDAg gene in these different isolates predicted HDAg to be either 195aa (p24) or 214aa (p27), in extracts from infected liver or serum, two HDAg-related polypeptides were detected (Bergmann and Gerin, 1986; Bonino et al, 1986; Roggendorf et al, 1987). Later two HDAg-specific polypeptide species of 24 and 27 kD were detected when a cloned HDAg gene [coding for a 195aa protein (p24)], was expressed in an amber suppression E. coli mutant (Weiner et al, 1988). While this is an unlikely mechanism for the in vivo expression of HDAg p27, other



GENOMIC SEQUENCE

ORF	aa	Frame
1	152	2
2	121	1
3	0 (no AUG)	1
4	68	3
10	33	1
11	86	3

ANTIGENOMIC SEQUENCE

ORF	aa	Frame
5	214/195	1
6	179	3
7	148	1
8	26	1
9	0 (no AUG)	1

FIG 6.1 Potential open reading frames (ORFs) and the number of amino acids (aa) encoded on the genomic and antigenomic strands of HDV RNA, as predicted from the HDV cDNA sequence published by Wang et al, (1986). Also shown are the regions of the genome contained in plasmids BMB-37 and BMB-104, and the locations of the restriction enzyme sites used in the cloning work described in Chapters 6 and 7.

proteins are expressed in this manner by read through of a stop codon (Parker, 1989).

The general aims of this and the next chapter were to study the expression of HDAg, and to investigate HDV RNA replication. However, when this study commenced, no complete full-length copy of HDVcDNA was available. Thus an initial aim of this study was to produce a full length copy of HDV cDNA for further cloning and for the generation of strand-specific riboprobes.

For the sake of expediency in a highly competitive field some of the results described in this chapter are derived from experiments performed by others. In particular, Eric Gowans performed the immunoblot presented in section 6.3 B(c) and the RNA binding assay presented in section 6.3 D, the anti-HD assays in section 6.3 F were performed by the staff of the hepatitis diagnosis laboratory (IMVS) and Betty Reinboth (Department of Pathology, University of Adelaide) gave expert technical assistance with the HPLC analysis of HDAg described in section 6.3 C(a).

Most of the data presented in this Chapter have been published (Gowans et al, 1990a; Macnaughton et al, 1990a).

6.2 EXPERIMENTAL METHODS.

6.2A SOURCE OF HDV cDNA FOR CLONING.

The cloning strategy to produce a full-length HDV cDNA used plasmids BMB37 and BMB104, which contain contiguous sequences of HDV cDNA that together comprise the whole genome [Gowans et al, 1988; see Chapter 2 section 2.3 A(g)]. BMB37 and BMB104 were derived from clones δ4 and δ115 respectively (Wang et al, 1986) and were gifts from Drs. John Gerin and Bahige Baroudy. This HDV cDNA sequence contains an amber stop codon at aa position 195 in the HDAg gene (Weiner et al, 1988).

6.2B EXPRESSION STUDIES.

HepG2 and HeLa cells were transfected as described in Chapter 2 section 2.4 A and B with a stable expression vector containing the HDAg

gene; the construction of this vector is described below in Results. HDAg expression was monitored initially by immunofluorescence and subsequently by radioimmunoassay and immunoblotting. Physical properties of the expressed antigen were analysed by HPLC, isopycnic and rate zonal centrifugation and RNA-binding activities determined by incubation with ^{32}P-labelled RNA. Finally the potential of the expressed antigen to replace liver-derived HDAg in diagnostic assays was assessed.

6.3 RESULTS.

6.3A CONSTRUCTION OF PLASMIDS.

The strategy used to construct the HDAg expression vector is shown in Fig 6.2. The HDV cDNA fragment from BMB104 was excised by digestion with the restriction enzyme Sma1 and ligated into BMB37 which was linearised previously by partial digestion with the same enzyme. The resultant recombinant plasmid, named pTMδ3, contains a full length copy of HDV cDNA in vector pGEM3 [see Chapter 2 section 2.3A(a)]. Thus the SP6 and T7 promoters of pTMδ3 could be utilised to generate strand-specific riboprobes to detect genomic and antigenomic sense HDV RNA respectively .

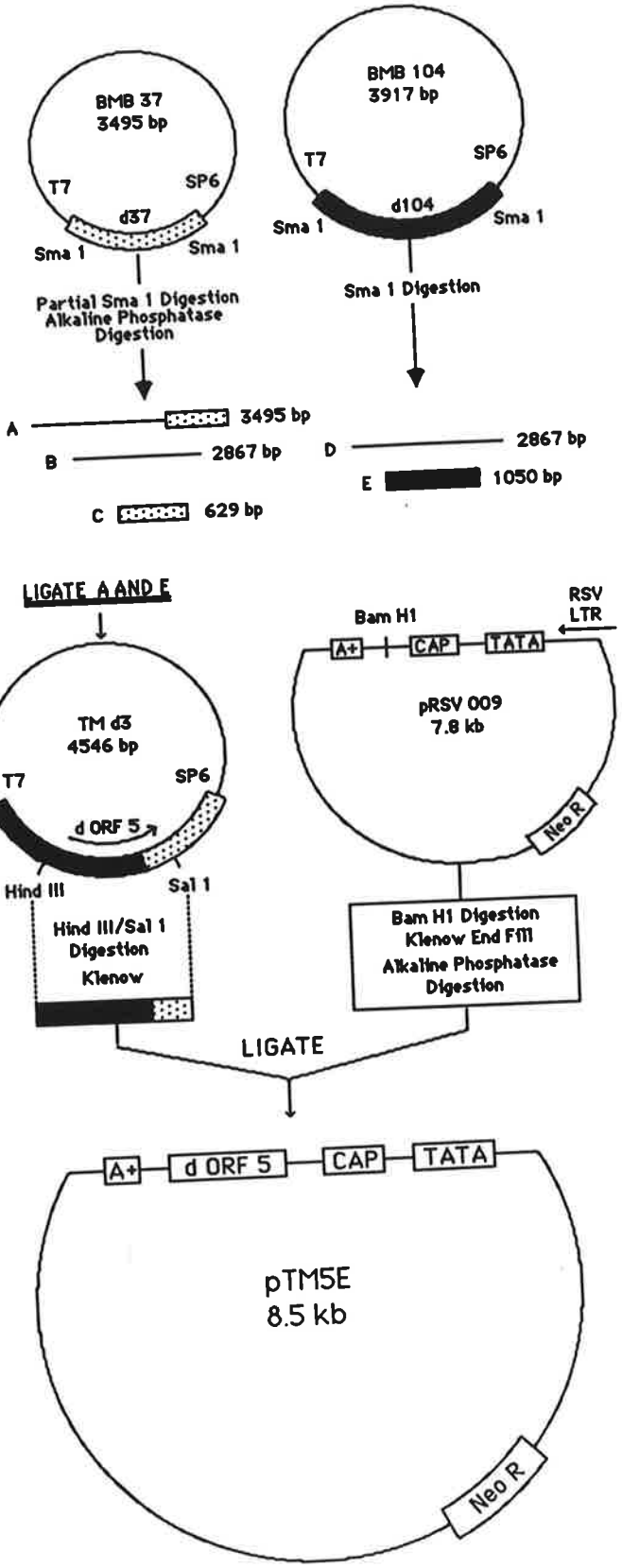
A region encompassing nucleotides 966-1679 (Wang et al, 1986) representing HDV ORF-5 (minus 10bp at the 3' end) and including 81bp upstream was subsequently excised from pTMδ3 by double digestion with HindIII and Sal1, end-filled using the Klenow fragment of DNA polymerase I and inserted into the unique BamI site of the retrovirus expression vector pRSV009 [Chapter 2 section 2.3 A(c)] to produce plasmid pTM5E. This plasmid is designed to integrate into chromosomal DNA and contains a neomycin resistance gene to permit cell selection. Expression of the HDV cDNA from this plasmid is controlled by a RSV LTR promoter.

6.3B DETECTION OF RECOMBINANT HDAg IN TRANSFECTED CELLS.

6.3B(a) Immunofluorescence.

Following transfection of pTM5E into HeLa and HepG2 cells,

Fig 6.2 Strategy for construction of recombinant plasmid pTM5E, which expresses HDAg under the control of the Rous sarcoma virus promoter.


BMB 37
3495 bp
T7
SP6
d37
Sma 1
Sma 1
Partial Sma 1 Digestion
Alkaline Phosphatase
Digestion
BMB 104
3917 bp
T7
SP6
d104
Sma 1
Sma 1
Sma 1 Digestion
A
3495 bp
B
2867 bp
C
629 bp
D
2867 bp
E
1050 bp
LIGATE A AND E
TM d3
4546 bp
T7
SP6
d ORF 5
Hind III
Sal 1
Hind III/Sal 1
Digestion
Klenow
Bam H1
RSV
LTR
A+
CAP
TATA
pRSV 009
7.8 kb
Neo R
Bam H1 Digestion
Klenow End Fill
Alkaline Phosphatase
Digestion
LIGATE
A+
d ORF 5
CAP
TATA
pTM5E
8.5 kb
Neo R

G418-resistant clones were examined for HDAg expression by immunofluorescence when the cells in individual wells had reached confluence. Several clones showing varying percentages of rHDAg-positive cells were detected, but a progressive loss of cells that expressed high levels of rHDAg was noted and these could not be clonally amplified. This was particularly apparent for the HeLa cells (Fig 6.3A) and no rHDAg-positive HeLa cells could be expanded. However it was possible to expand three HepG2 clones; one of these (A3) showed rHDAg in every cell examined (Fig 6.3B), while a second (D2) clone only showed rHDAg in 5% of total cells (Fig 6.3C) and the third clone (C1) showed no rHDAg expression (Fig 6.3D).

A3 cells were selected for further study; the bulk of the rHDAg was detected in the nucleus of positive cells, and was closely associated with the nucleolus (Fig 6.3B). This distribution differs from the diffuse nucleoplasmic pattern for HDAg seen in naturally infected hepatocytes (Rizzetto et al, 1977). In contrast to the in vitro expression of HBV antigens (Gough and Murray, 1982) expression of rHDAg was independent of the degree of cell confluency.

6.3B(b) Radioimmunoassay.

Crude cell homogenates and partially purified rHDAg (prepared as described in Chapter 2 section 2.9A) from A3 cells were examined by RIA to detect HDAg, as described in Chapter 2 section 2.6 C(a). C1 and HepG2 cells were treated in the same way and represented control preparations. Only extracts from A3 cells were reactive, but neat and 20-fold concentrated cell culture fluid was non-reactive, suggesting that rHDAg was not secreted.

6.3B(c) Immunoblotting.

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting experiments were performed as described in Chapter 2 section 2.6 D to examine the mw of rHDAg from A3 cells and also of serum-derived HDAg. In several different experiments, a single band with a mw of ca. 24kD was detected in A3, but not in HepG2 or C1 cells (Fig 6.4) and the electrophoretic mobility of the rHDAg was similar to that of the smaller of the two

Fig 6.3 Detection of HDAg by direct immunofluorescence in HeLa cells (A), A3 cells (B) and D2 cells (C) showing the nucleolar localisation of HDAg characteristic of these cell lines. The control cell line C1 (D) is also shown. Magnification x1800.

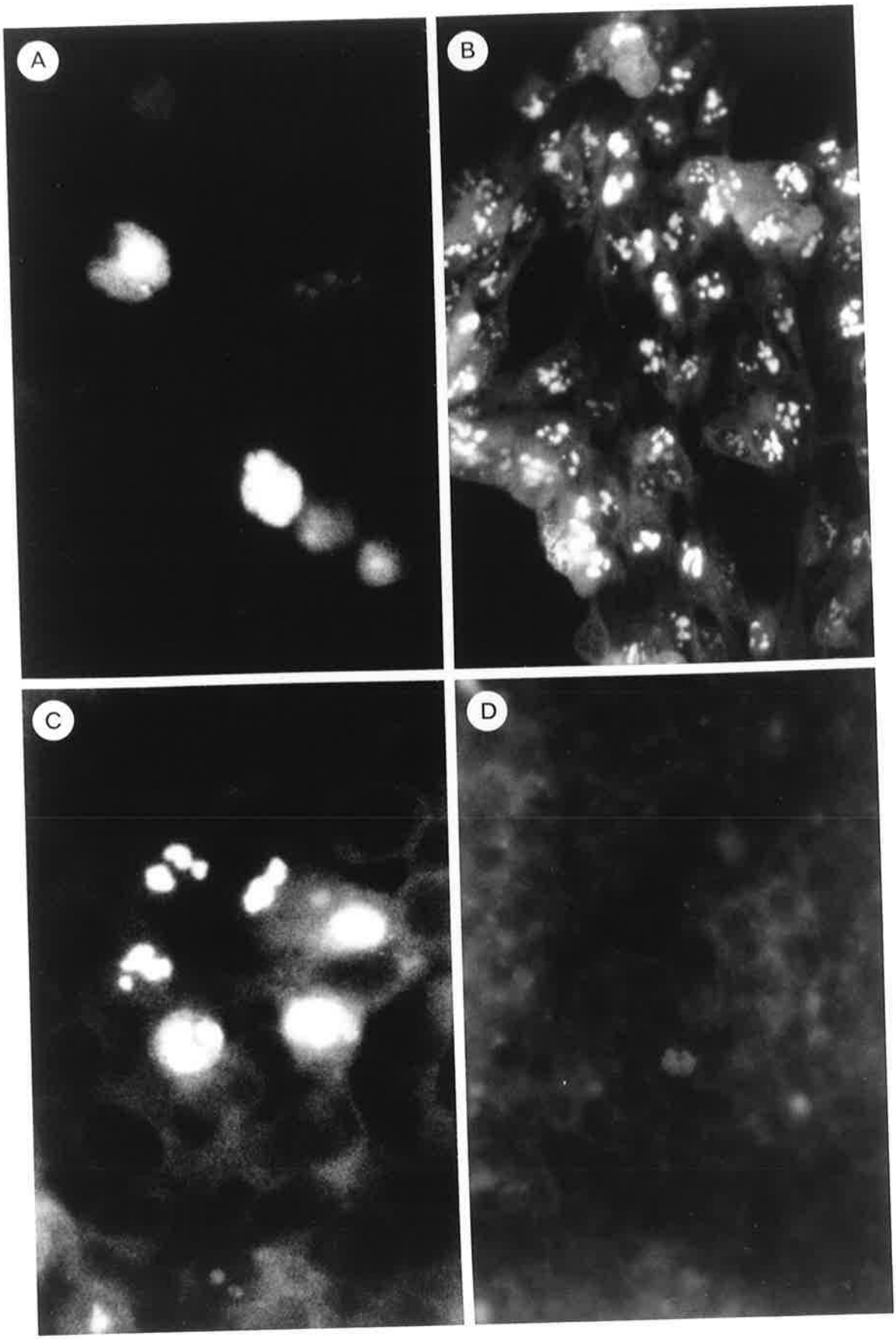
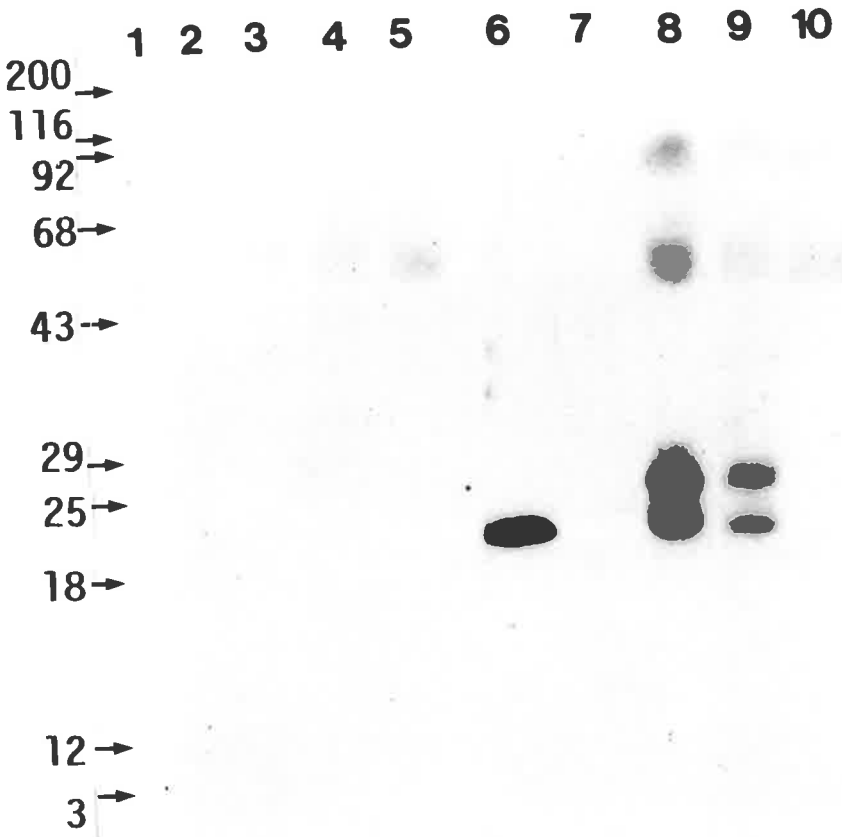

A
B
C
D

Fig 6.4 Western blot analysis of rHDAg and serum-derived HDAg. Lanes 1-5 were tested with normal human serum and lanes 6-10 were tested with human anti-HD as the primary antibody. Lanes 1 and 6 contain the A3 cell homogenate and lanes 2 and 7 contain homogenate from the C1 control cell line. Lanes 3 and 8, and 4 and 9 contain HDV pelleted from two positive serum samples respectively, and lanes 5 and 10 represent normal human serum treated the same way. The smeared bands in the upper part of the gel are likely to correspond to human globulin. Molecular weight markers are indicated on the left of the gel.


1 2 3 4 5 6 7 8 9 10
200
116
92
68
43
29
25
18
12
3

HDAg-specific polypeptides detected in HDV-positive serum. The mw of the serum-derived HDAg polypeptides was determined as 27 and 24kD, as reported previously (Bergmann and Gerin, 1986). The mw values for both rHDAg and serum-derived HDAg were unchanged when 2-mercaptoethanol was omitted from the PAGE denaturation buffer. Consequently, HDAg p24 and p27 also do not appear to form disulphide bonds. This data was noted previously by Bergmann and Gerin (1986).

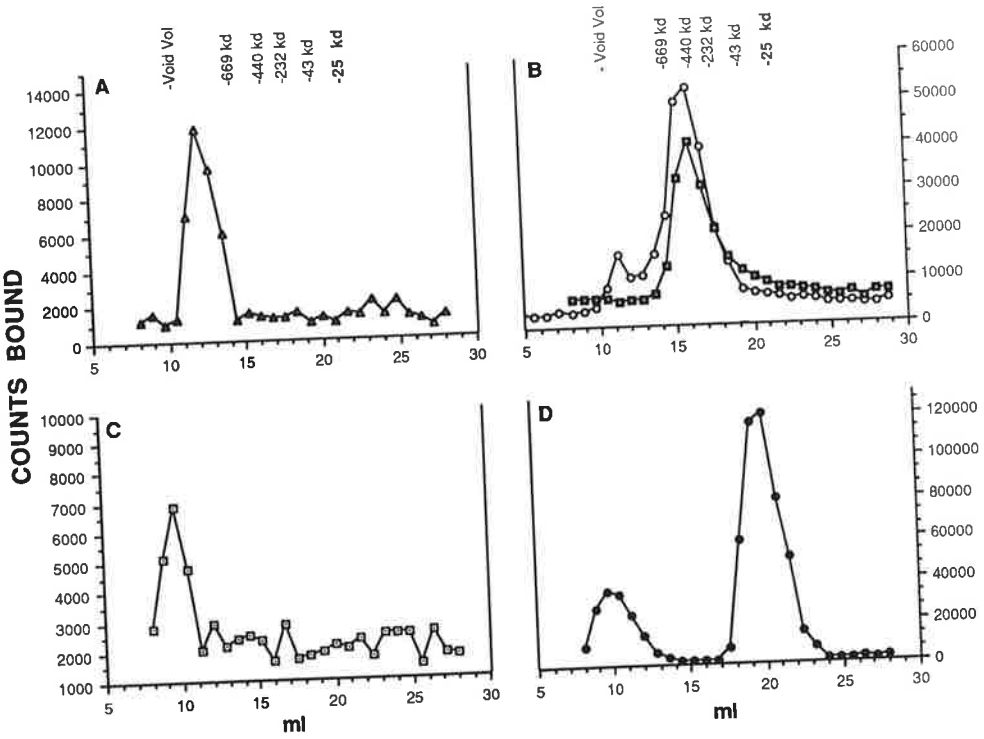
6.3C PHYSICAL PROPERTIES OF rHDAg.

6.3C(a) HPLC Analysis.

To further characterise rHDAg, crude cell homogenates and partially-purified rHDAg was analysed by exclusion HPLC as described in Chapter 2 section 2.9 B and the fractions were assayed by RIA (Fig 6.5). The rHDAg was prepared and analysed in 6M urea or 4M guanidine hydrochloride.

Crude rHDAg showed one discrete peak (Fig 6.5A) which appeared just behind the void volume (exclusion limit $2x10^6$kD). It is likely that this represents the natural form of rHDAg because this preparation was prepared in urea (by freeze-thaw cycles) without any precipitation steps that might lead to aggregation, and analysed by HPLC in 6M urea. However, owing to the low levels of HDAg present, further HPLC analysis of this type of preparation was not possible. Analysis of partially-purified, concentrated, rHDAg confirmed a small peak at $2X10^6$kD, with a dominant peak at $5x10^6$kD (Fig 6.5B). In preparations frozen prior to chromatography, the peak at $2X10^6$kD, was undetected (Fig 6.5B). HDAg activity was never detected in fractions corresponding to 24kD unless the preparations were previously denatured and analysed in 4M guanidine hydrochloride. Nevertheless, when the fractions were diluted 1:4 in PBS and analysed by RIA in the presence of (1M) guanidine hydrochloride (4M guanidine hydrochloride inhibited the RIA), rHDAg was detected only in high molecular weight fractions (Fig 6.5C). In contrast, after dialysis to remove the guanidine hydrochloride (thus

Fig 6.5 The detection of HDAg by RIA in fractions collected after HPLC analysis of rHDAg: (A) rHDAg freshly extracted and analysed in 6M urea; (B) rHDAg partially purified by ammonium sulphate followed by acetone precipitation and either stored at -20°C for 24 hours [o] or frozen and thawed five times [■] prior to HPLC; (C) and (D), rHDAg denatured and analysed in 4M GuHCl either before [diluted 1:4 with PBS (C)] or after (D) dialysis of the fractions against PBS.


A
B
C
D
-Void Vol
-669 kd
-440 kd
-232 kd
-43 kd
-25 kd
COUNTS BOUND
ml

permitting refolding of the polypeptides) the bulk of the rHDAg activity was shown to be present in fractions corresponding to 24kD (Fig 6.5D). This indicates that rHDAg contains conformational-dependent epitopes which are recognised by human anti-HD.

6.3C(b) Density of rHDAg.

The buoyant density of rHDAg was determined by isopycnic centrifugation in caesium chloride as described in Chapter 2 section 2.9 C(b). The peak rHDAg activity corresponded to a density of 1.19g/ml (Fig 6.6 top) which was nearly identical to liver-derived HDAg (1.20g/ml) extracted in guanidine hydrochloride and analysed under identical conditions (Fig 6.6 top). While these densities are low compared with other reports of 1.28g/ml (Bonino et al, 1984; Rizzetto et al, 1980) it was clear that the rHDAg and the liver-derived HDAg showed similar densities. In comparison, serum-derived HBsAg corresponded to a density of 1.22g/ml (Fig 6.6 top) which is close to a previously reported value (Gerin et al, 1969). The buoyant density of rHDAg was unaltered after extraction with either guanidine hydrochloride or chloroform indicating that the low density was not due to the presence of lipid.

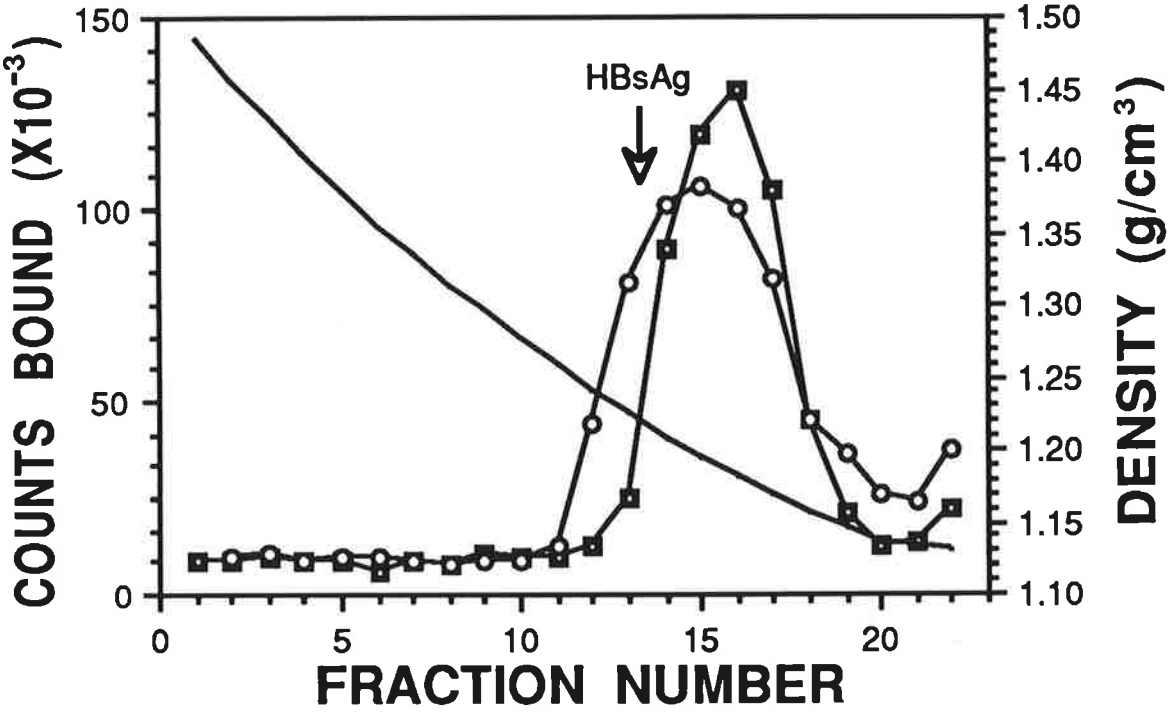
6.3C(c) Sedimentation value of rHDAg.

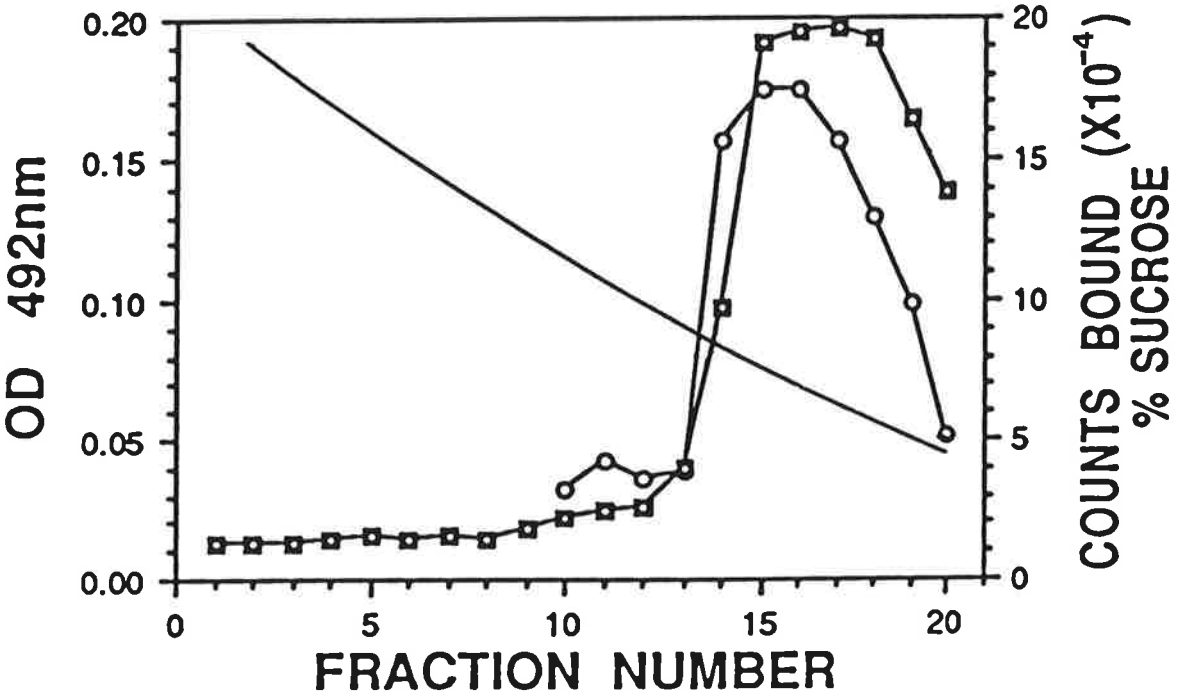
Analysis by rate zonal centrifugation, as described in Chapter 2 section 2.9 C(a), showed that rHDAg sedimented through sucrose only slightly slower than serum-derived HBsAg 22nm particles suggesting that, in its native state, rHDAg is of similar size (Fig 6.6 bottom). Analysis of the sedimentation coefficient (S value) using a computer programme (Young, 1978) determined the S values of rHDAg and HBsAg to range between 35 and 72 (peak 50S) and 46 and 83 (peak 58S) respectively. The value for HBsAg is close to that of 54S reported previously (Gerin et al, 1971).

6.3D RNA-BINDING PROPERTIES OF rHDAg.

Recombinant HDAg has been shown previously to bind HDV RNA (Chang et al, 1988), although the specificity of this reaction was not

Fig 6.6 Isopycnic and rate zonal centrifugation analysis of rHDAg. 200µl of ammonium sulphate-precipitated rHDAg was centrifuged at 120,000g for 25 hours on a preformed CsCl gradient (top) or at 208,000g for 45 minutes in a 5-20% sucrose gradient (bottom). In each case, HBsAg was analysed, and liver-derived HDAg was also analysed by isopycnic centrifugation. HDAg and HBsAg were detected in the gradient fractions by RIA and ELISA respectively. The density of the CsCl was estimated from the refractive index. (top) rHDAg [■], liver HDAg [o]; (bottom) rHDAg [■], HBsAg [o].


HBsAg
COUNTS BOUND (X10^{-3})
DENSITY (g/cm^3)
FRACTION NUMBER
150
100
50
0
1.50
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
0
5
10
15
20


OD 492nm
COUNTS BOUND (X10^{-4})
% SUCROSE
FRACTION NUMBER
0.20
0.15
0.10
0.05
0.00
20
15
10
5
0
0
5
10
15
20

determined. To examine this, concentrated, HPLC-purified rHDAg from A3 cells was separated by PAGE and the gel stained with Coomassie blue or the protein transferred to nitrocelluose. Strips of the latter were then analysed by immunoblotting as described above or incubated with ^{32}P-labelled RNA.

Coomassie blue staining revealed that the HPLC-purified rHDAg preparation contained a number of proteins (Fig 6.7 track 1). Of these, the strongest band and a band that comigrated with the dye front both reacted with anti-HD (Fig 6.7 track 2). The same two bands also bound strongly both genomic- and antigenomic-HDV RNA (Fig 6.7 tracks 3-6) transcribed from the SP6- and T7-RNA promoters of plasmids BMB37 and BMB104 respectively (Fig 6.2). In contrast, negative- and positive-polarity human papilloma virus RNA prepared by Geoff Higgins and labelled to the same specific activity, bound weakly (Fig 6.7 tracks 7-8), showing that the binding favoured HDV RNA selectively. The lower mw HDAg-specific polypeptide (ca. 20kD), seen in all tracks, probably resulted from degradation due to prior storage of the rHDAg at -70°C. Similar degradation products have been reported for liver-derived HDAg (Roggendorf et al, 1987).

6.3E ACTINOMYCIN D STUDIES.

A3 cells were treated with 50-5000ng/ml of actinomycin D for 24 hours. At all concentrations of actinomycin D used, the intranuclear distribution of rHDAg altered from the nucleolar localisation pattern described above to a diffuse nuclear pattern (Fig 6.8). In contrast, toluidine blue staining revealed that the nucleolar structures within the cell were only disrupted at the highest concentration of actinomycin D used (5000ng/ml). Furthermore, a comparison of immunofluorescence staining intensity revealed that the level of rHDAg in the actinomycin D-treated A3 cells was only marginally less than that detected in untreated cells. These results suggest that the HDAg distribution seen in A3 cells may be a result of an interaction of native rHDAg with ribosomal RNA (the major RNA species) contained within the nucleolus. The

Fig 6.7 RNA binding assay to demonstrate that HDAg binds HDV RNA specifically. HPLC-purified rHDAg was denatured in loading buffer lacking 2-mercaptoethanol and, after electrophoresis, transferred to nitrocelluose and incubated with ^{32}P-labelled RNA. Lane 1 shows Coomassie blue staining for total protein and lane 2 represents an immunoblot to identify HDAg polypeptides. Lanes 3 and 4 were incubated with anti-genomic HDV RNA and lanes 5 and 6 with genomic HDV RNA transcribed from plasmids BMB104 and BMB37 respectively. Lanes 7 and 8 were incubated with positive- and negative-sense human papilloma virus RNA respectively, labelled to the same specific activity.

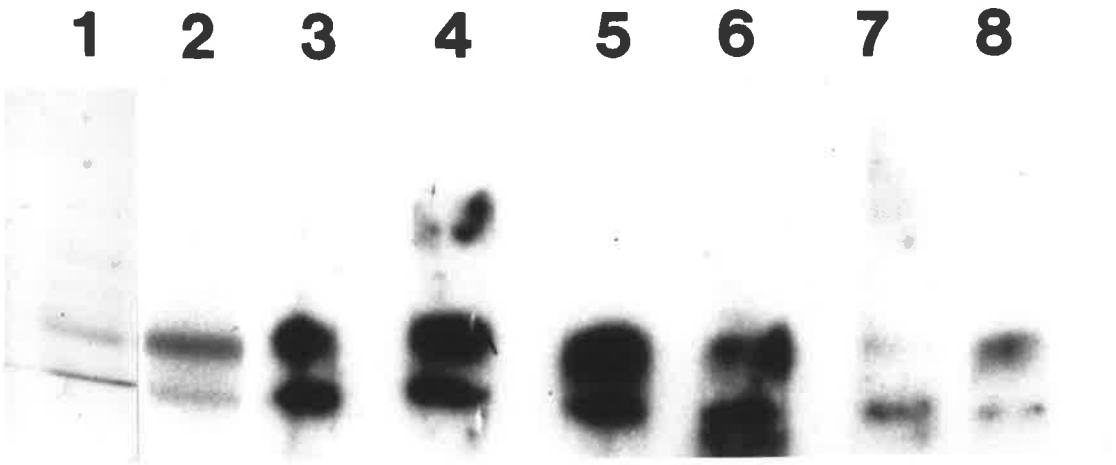
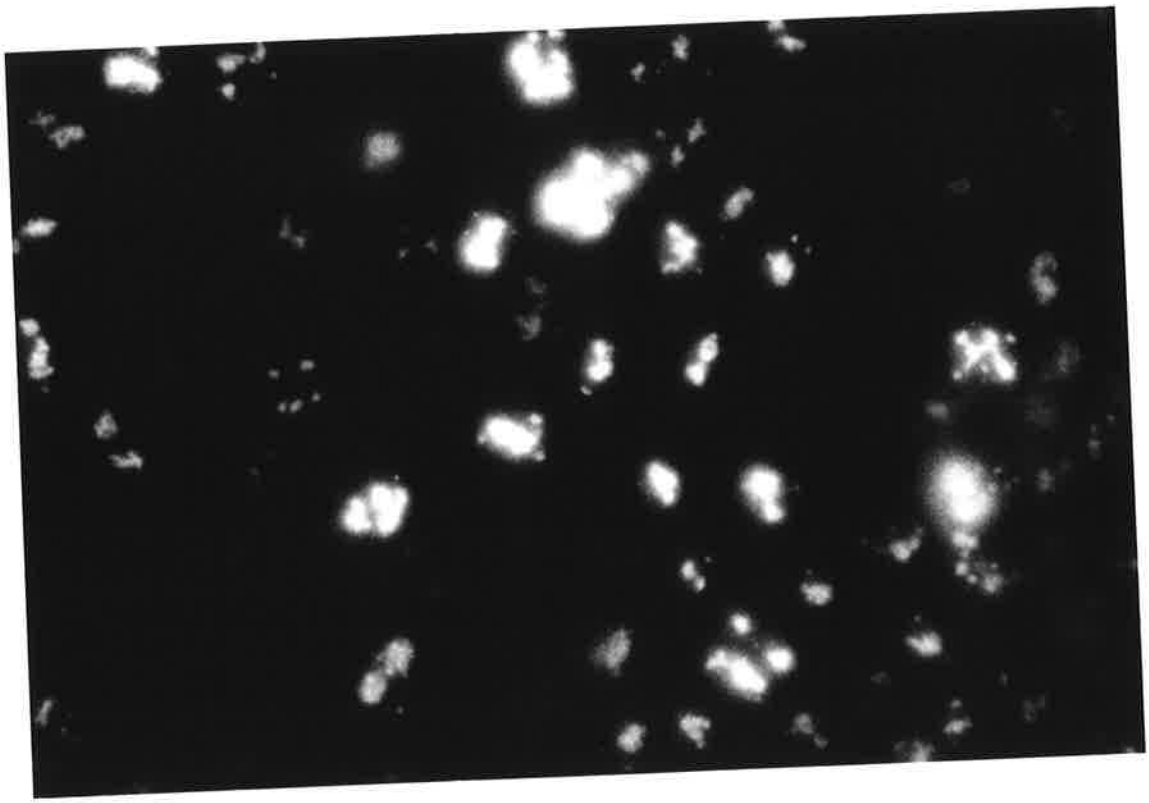
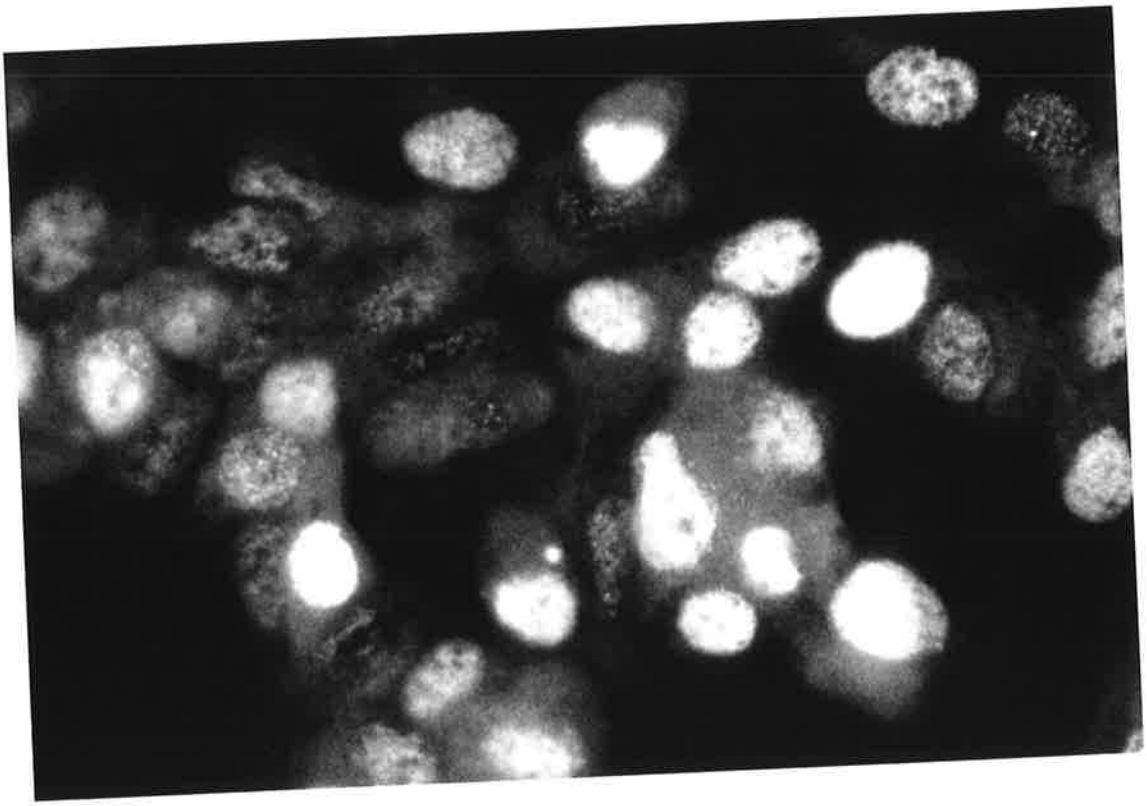

1
2
3
4
5
6
7
8

Fig 6.8 The effect of actinomycin D on the intranuclear distribution of rHDAg. A3 cells were incubated for 24 hours in DMEM containing 0 (top) or 50ng/ml (bottom) actinomycin D, then tested for HDAg by direct immunofluorescence. Magnification top and bottom x2000





results also suggest that rHDAg and/or its mRNA is very stable within the A3 cells.

6.3F DIAGNOSTIC APPLICATIONS OF rHDAg.

The most reliable, non-invasive method for the diagnosis of current HDV infection is the detection of anti-HD in patients serum by RIA or enzyme immunoassay (Gowans et al, 1990a). These tests use HDAg which in the past was either extracted from HDV-infected human and woodchuck liver or human serum. As HDAg from these sources is limited in availability and preparations are highly variable in HDAg content, standardisation of diagnostic anti-HD assays has not been possible. Consequently, the suitablity of acetone-fixed A3 cells as a target for the detection of anti-HD by indirect immunofluorescence and the rHDAg produced in these cells as a substitute for liver-derived HDAg in anti-HD RIA was investigated. However, as this was a joint study (Gowans et al,1990a), a condensed form of the results are presented. A3 cell-derived rHDAg was found to be equally suitable to the liver-derived reagent for diagnostic purposes in anti-HD RIA for the detection of both low- and high-titre anti-HD (Fig 6.9). Similarly, fixed A3 cells provided a sensitive substrate for an indirect immunofluoresence anti-HD assay (Fig 6.10). Identical samples showed slightly higher titres to those in the above RIA. In future, it is hoped to also use the immunofluoresence assay for the selective detection of both IgG and IgM class anti-HD.

6.4 DISCUSSION.

In this Chapter, HDAg was expressed in HepG2 and HeLa cells by placing the HDAg gene under the control of a RSV LTR promoter in a vector designed to integrate into host cell chromosome. All of the positive HeLa clones were lost in culture whereas a proportion of HDAg-positive HepG2 clones were expanded successfully. These results imply that HDAg may itself be cytotoxic and may explain the hepatocyte injury attributed to HDV which was suggested previously to be directly cytopathic (Popper et al, 1983). Although rHDAg synthesised in A3 cells possessed similar antigenic reactivity

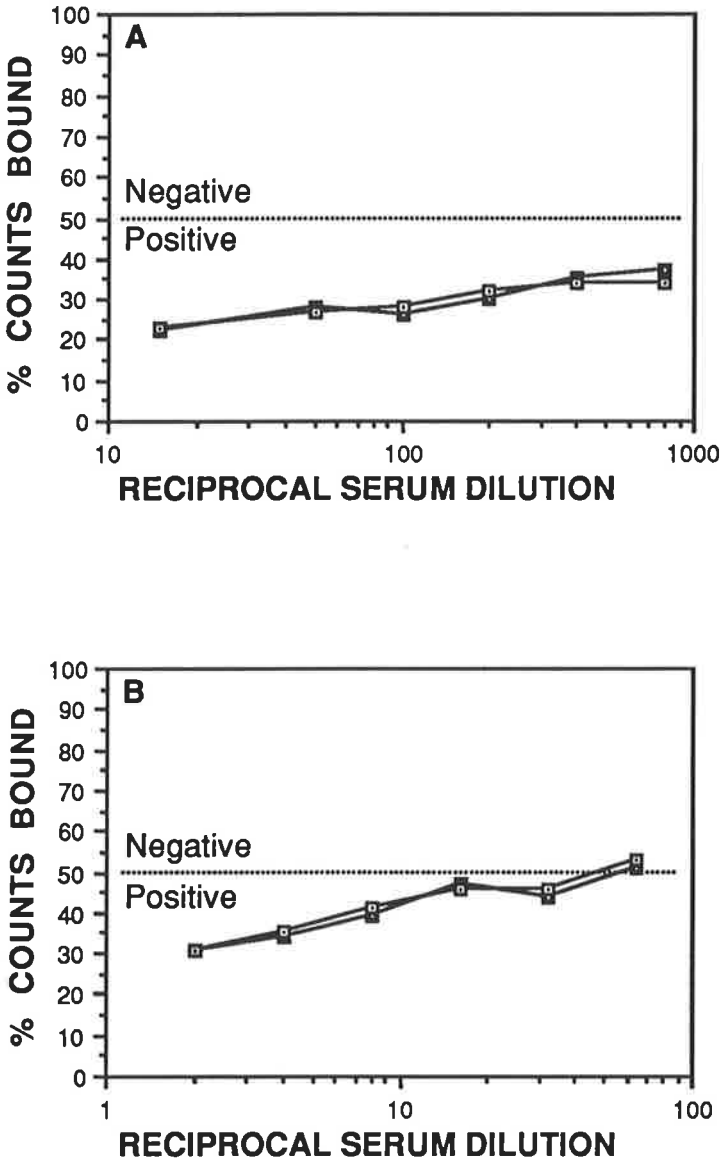
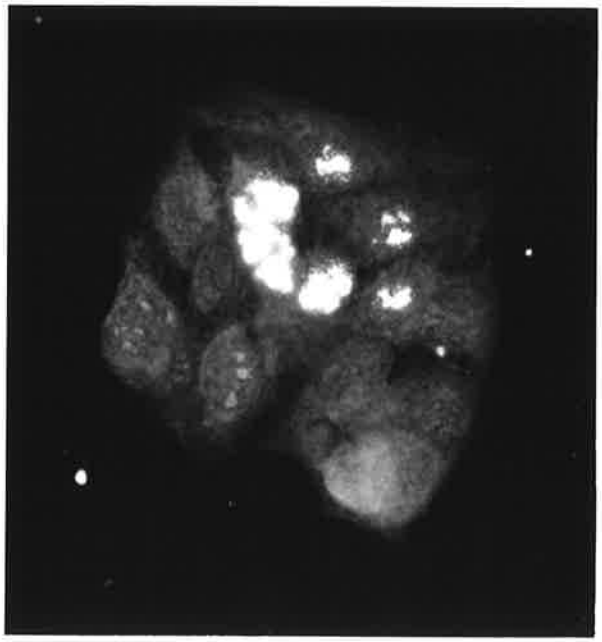
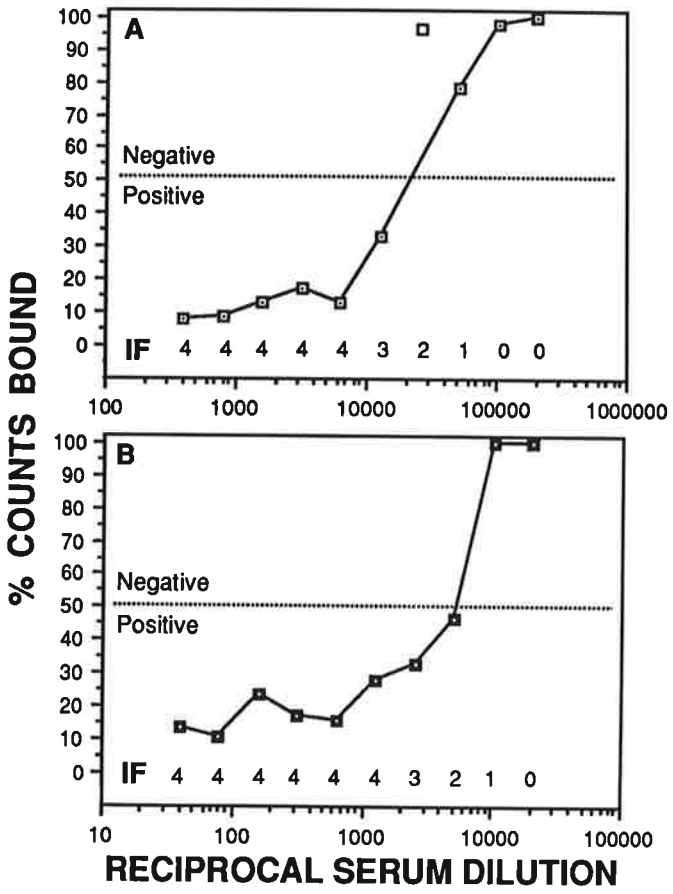


Fig 6.9 Titration of human sera containing high (A), or low (B) levels of anti-HD against human liver-derived HDAg and rHDAg extracted from A3 cells. Human liver-derived HDAg (■); rHDAg (□).

Fig 6.10 Top. Detection of anti-HD by indirect immunofluorescence in the serum from a patient with chronic hepatitis.

Fig 6.10 Middle and Bottom. Dilutions of two (A and B) anti-HD positive sera were tested in parallel by RIA and by indirect immunofluorescence. Immunofluorescence staining intensity was scored 0 (negative) to 4+ (intense)




A
B
Negative
Positive
IF 4 4 4 4 4 3 2 1 0 0
IF 4 4 4 4 4 4 3 2 1 0
% COUNTS BOUND
RECIPROCAL SERUM DILUTION
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70
60
50
40
30
20
10
0
10
100
1000
10000
100000
1000000

to HDAg derived from in vivo infected livers, the A3 cell model differed from the in vivo production of HDAg in several ways: the rHDAg was composed of the smaller (24kD) polypeptide only; rHDAg was not secreted from the cells; rHDAg present in the A3 cells showed a nucleolar distribution compared to the nucleoplasmic distribution in infected hepatocytes unless the cells were treated with actinomycin D; there was no HDV genome replication from native virus; finally, there was an absence of HBV superinfection.

The data presented above show that the single polypeptide produced in A3 cells formed a HDAg-reactive particle with a density of 1.19 g/ml and an approximate S value of 50. As gentle methods, avoiding any precipitation steps that might result in aggregation, were used to prepare the rHDAg used for HPLC analysis, the high mw form of rHDAg detected is likely to represent its natural state within the nuclei of the A3 cells. A possible mechanism for the production of this particle could be the formation of a ribonucleoprotein by the interaction of HDAg with cellular RNA. This is consistent with the observed nucleolar distribution of rHDAg and the disruption of this pattern when the cells were treated with actinomycin D [the nucleolus is responsible for ribosome synthesis and therefore contains a high concentration of ribosomal RNA (Alberts et al, 1989)]. However, the density of the particle in caesium chloride suggests that a ribonucleoprotein complex was unlikely, although a weak interaction of rHDAg with cellular RNA might be disrupted in the high concentration of caesium chloride used in this type of analysis leading to a reduced density. The data presented above also shows that rHDAg contains conformational epitopes which were detected with human anti-HD produced in response to infection, and thus it is likely that native HDAg also shows a degree of conformational specificity.

The 214 aa form of HDAg was reported to be an RNA-binding phosphoprotein (Chang et al, 1988). The results presented above extend this observation to the 195 aa form of HDAg and, since other proteins were present in the gel, have shown that this binding is specific to both the protein

and RNA components of the reaction. Furthermore, rHDAg binds both genomic and antigenomic RNA with equal efficiency. This finding is not surprising since HDV RNA shows a high degree of intramolecular base-pairing (Wang et al, 1986; Kuo et al, 1988) and consequently, there must be regions in the genomic and antigenomic RNA with similar (as well as complementary) base sequences. As two contiguous sequences of RNA (that constitute the complete HDV genome) bound to rHDAg with equal efficiency then at least two regions of the genome must be involved. The RNA binding properties of native HDAg could not be examined due to lack of sufficient quantities of native antigen.

Although the rHDAg expressed in A3 cells only corresponds to the smaller of the two liver- and serum-derived polypeptides, this product successfully replaced the liver-derived reagent in anti-HD diagnostic assays. Thus, a useful future application of A3 cell rHDAg may be in the formulation and standardisation of RIA, ELISA and immunofluorescent assays for the detection of anti-HD in patient sera.

Finally, as p27 was not detected in this system, p27 is unlikely to be expressed in vivo from a read through of the amber stop codon of the HDAg mRNA as has been described for some other proteins (Parker, 1989). Furthermore, since the results published by Chang et al (1988) suggest that p27 is not cleaved to p24, it is possible that HDAg p24 and p27 are expressed from unique mRNA molecules. This is discussed further in Chapter 7.

CHAPTER 7.

HEPATITIS DELTA VIRUS RNA REPLICATION.

7.1 INTRODUCTION.

The study of HDV has been hampered by the lack of convenient cell culture systems and also by a highly restricted range of animal hosts. These problems have been further exacerbated in many laboratories including our own by the scarcity of high titre HDV serum particularly from the acute stage of HDV infection. Since many of these constraints also applied to the study of HBV, and because a highly rewarding alternative approach has been the transfection of molecularly cloned HBV DNA, it was decided to attempt similar experiments with HDV. When the work described in this chapter was commenced, it was unknown whether HDV cDNA was capable of initiating HDV replication. Nevertheless Racaniello and Baltimore (1981) established a precedent for the use of cDNA to study RNA virus replication when they demonstrated that full-length poliovirus cDNA was infectious in cell cultures. Similarly cDNA derived from the circular RNA genome of viroids was also shown to be infectious providing a multimeric construct was employed (Diener, 1986).

As in Chapter 6 some of the work described in this chaper was the work of others. In particular, the RNA transfections shown in Fig 7.9 were performed by Sue McNamara, and the in situ cytohybridisations [section 7.3D(a)] and the Northern blot hybridisation shown in Fig 7.5 were performed by Allison Jilbert.

Most of the observations described in this chapter have been published (Macnaughton et al, 1990 b, 1990 c; Gowans et al, 1990 b, 1990 c; Jilbert et al, 1990).

7.2 AIMS AND EXPERIMENTAL METHODS.

The HepG2, HH1 and Hep3B cells lines were transfected, as described in Chapter 2 section 2.4A, with expression plasmids containing multimeric copies of HDV cDNA (constructed as described in section 7.3A below). These cell lines were chosen as they were all derived from human hepatomas and possess functions characteristic of differentiated hepatocytes (Knowles et al, 1980). Comparison of these cell lines may have permitted the effect of some of the putative HBV helper functions of HDV replication to be assessed, and in the case of the HBsAg-positive cell lines, HH1 and Hep3B, offered the prospect of HDV virion morphogenesis and secretion.

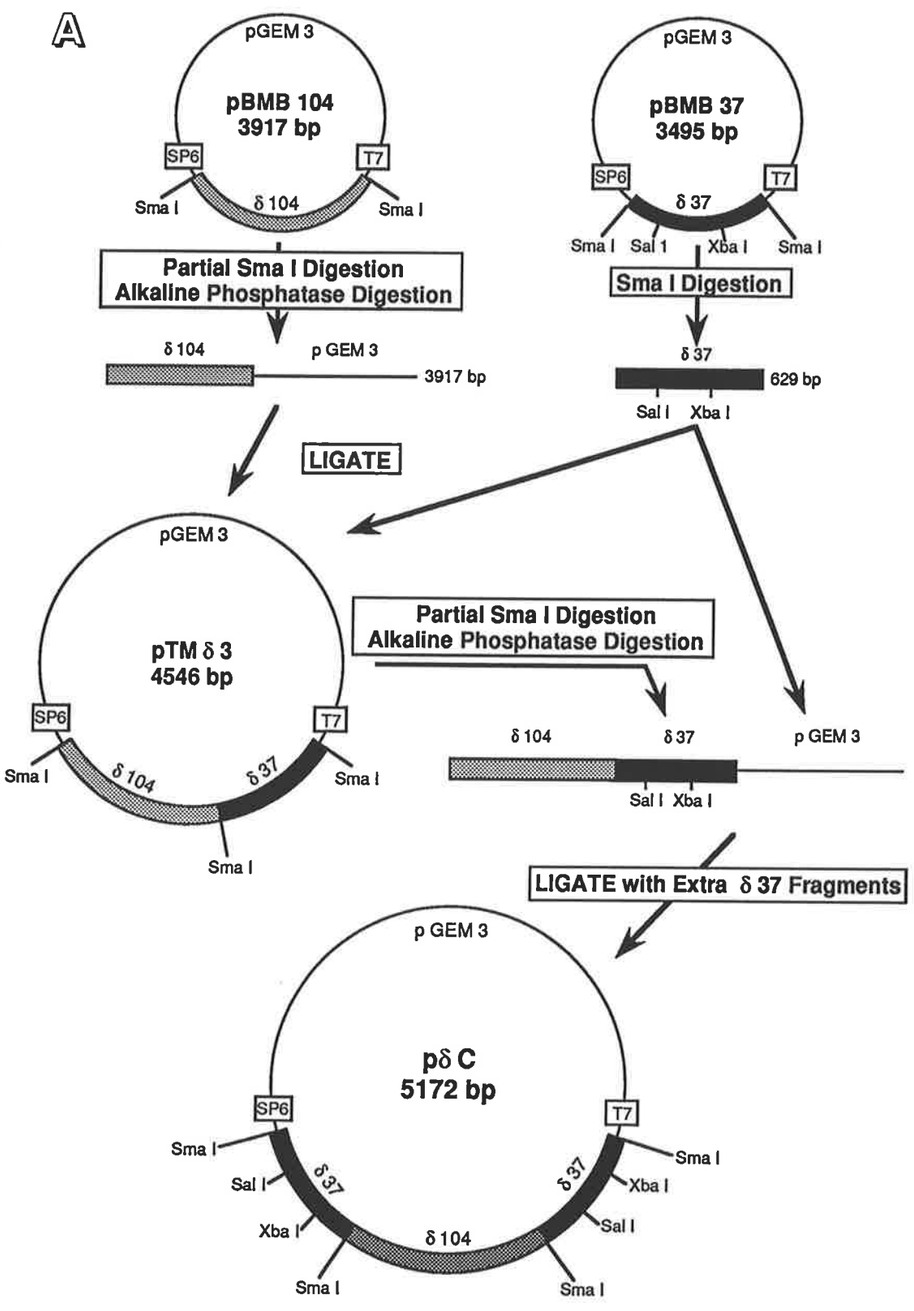
Following transfection and G418 selection, amplified clones were examined for HDAg by immunofluorescence. Despite the fact that the transfection plasmids employed in these experiments were not expected to be capable of directly coding for this antigen (see section 7.3 A below), HDAg was used as a marker of HDV expression as this was the most convenient and because no other protein products of the HDV genome have been detected. HDAg-positive clones were further examined for HDV RNA by in situ cytohybridisation and Northern blot hybridisation, and for HDV-specific proteins by immunoblotting. To determine the mechanism of HDV genome replication, transfected cells were treated with inhibitors of RNA synthesis or stained with an antibody specific for dsRNA.

The methods used for in situ cytohybridisation have been described previously (Gowans et al, 1989) but are not outlined in Chapter 2 as this part of the work was performed almost entirely by Allison Jilbert.

7.3 RESULTS.

7.3A CONSTRUCTION OF PLASMIDS.

The transfection studies described in this chapter required the development of expression plasmids containing both dimeric and trimeric copies of HDV cDNA. The strategy used to construct these plasmids is shown in Fig 7.1.



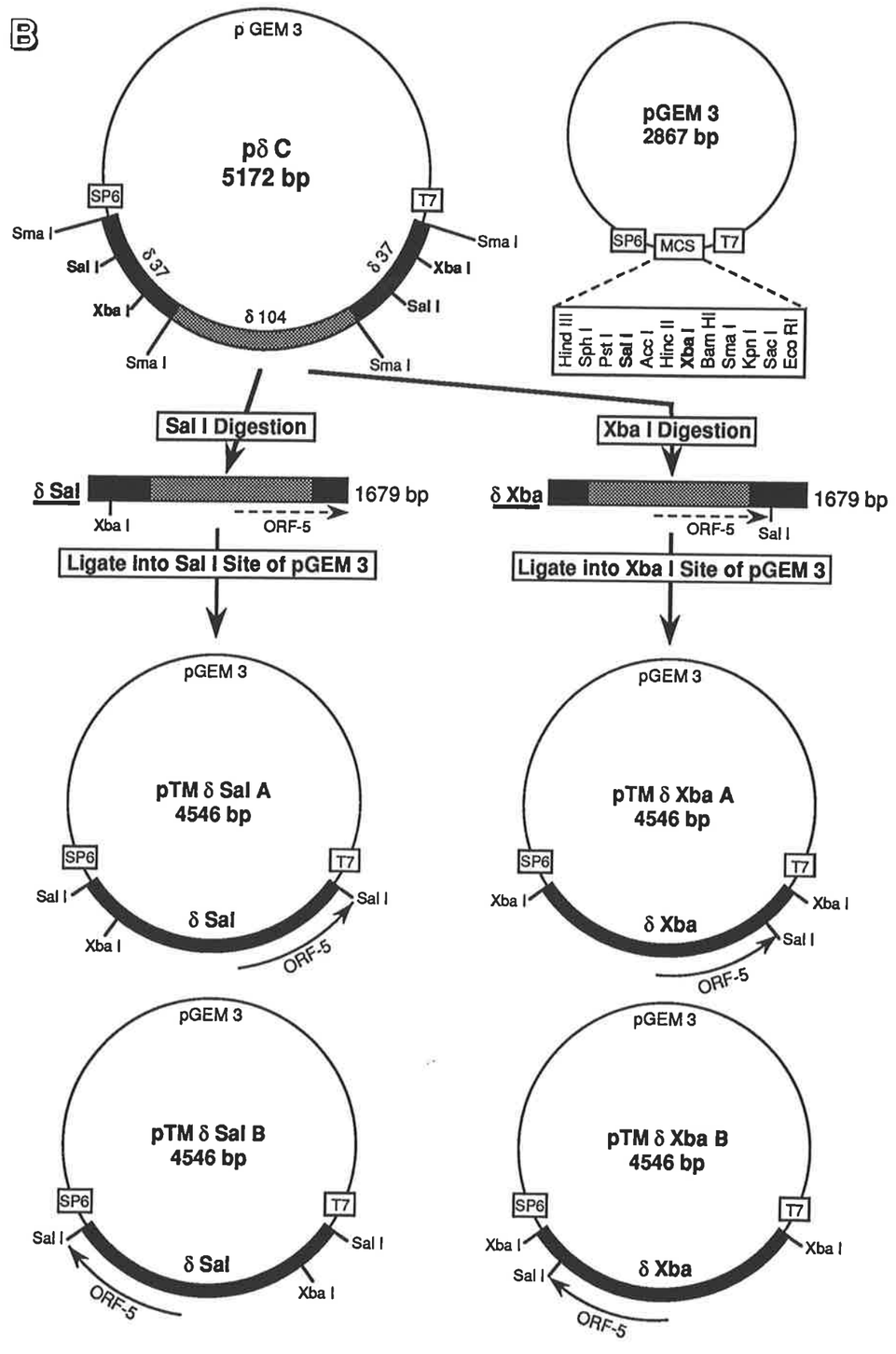
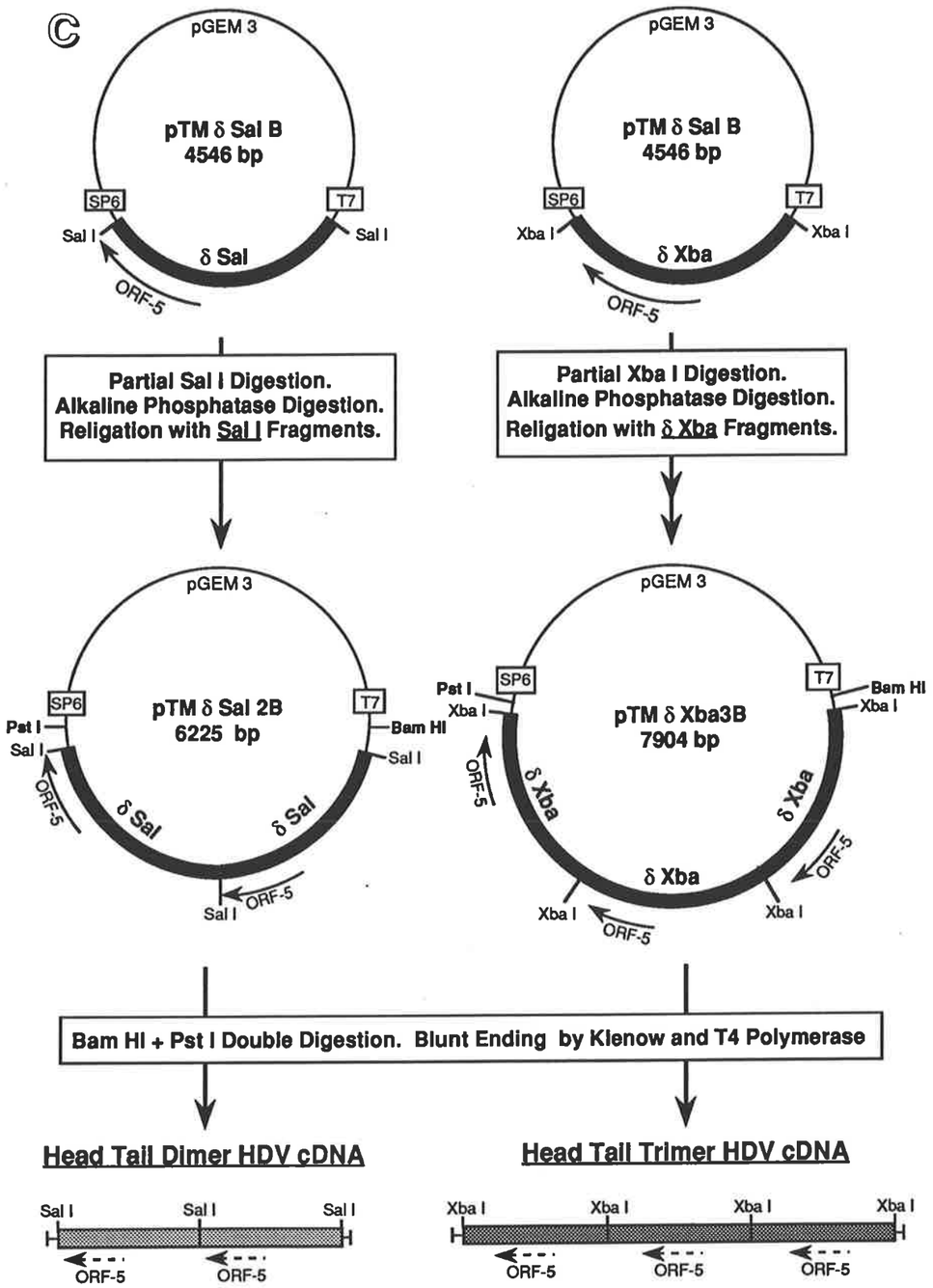


Fig 7.1 Construction of HDV cDNA Expression Plasmids used in Chapter 7
Pages A&B: Preparation of HDV cDNA monomers and insertion into pGem 3



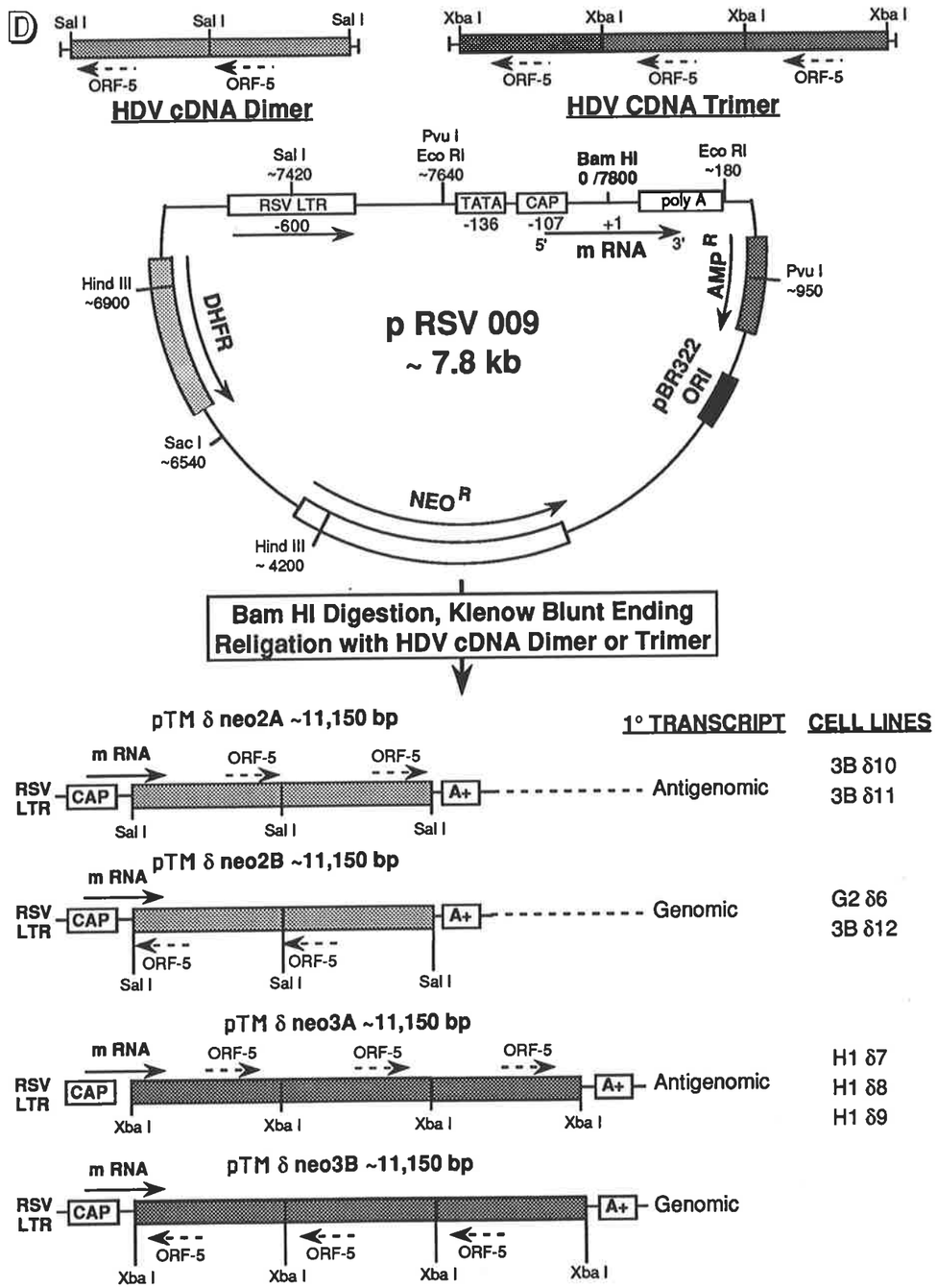


Fig 7.1 Construction of HDV cDNA Expression Plasmids used in Chapter 7 Pages C&D: Preparation of HDV cDNA multimers, insertion into pRSV 009, expected polarity of primary HDV RNA transcript and resultant transfected cell lines.

As a starting point, the cloning strategy used the recombinant plasmid pTMδ3 which was described in Chapter 6 section 6.3A. To simplify further cloning, a plasmid in which full length HDV cDNA (with cohesive ends) could be excised by digestion with a single restriction enzyme was constructed. This was achieved by excision of the 629 bp HDV cDNA fragment from plasmid BMB37 [Chapter 2 section 2.3 A(g)] by digestion with restriction enzyme SmaI and subsequent ligation into pTMδ3 which was previously partially digested with the same enzyme. The resultant plasmid pδC was used as the source of HDV cDNA sequences for all further sub-cloning.

To construct the HDVcDNA dimer, full length HDVcDNA was excised from pδC by digestion with restriction enzyme Sal1 and inserted into the Sal1 site of pGEM3 [Chapter 2 section 2.3A(a)]. The resultant recombinant plasmids, pTMδSalA and pTMδSalB (depending on the orientation of the insert), were then partially digested with Sal1 and religated with additional full-length Sal1 HDVcDNA fragments from pδC to create plasmids pTMδSal2A and pTMδSal2B (Fig 7.1).

Plasmids pTMδSalA and pTMδSalB replaced pTMδ3 as the templates for the preparation of HDV riboprobes. These plasmids permitted antigenomic and genomic strand-specific probes (from pTMδSalA and pTMδSalB respectively) to be prepared using the more reliable and efficient T7 RNA polymerase.

The strategy used to create the HDVcDNA trimer was similar to that used for the dimer. Full length HDVcDNA was excised from pδC by digestion with XbaI and inserted into pGem3 to create pTMδXbaA and pTMδXbaB. Then using a series of partial digestions and religations with additional XbaI HDV cDNA fragments the total insert was raised to a trimer and the plasmid renamed pTMδXba3B (Fig 7.1).

To construct the expression plasmids, dimeric and trimeric HDVcDNA was excised from plasmids pTMδSal2B and pTMδXba3B respectively by double digestion with restriction enzymes BamHI and PstI. The fragments

were then end-filled using a combination of the Klenow fragment of DNA polymerase I (to fill the 5' BamHI overhang) and T4 DNA polymerase (to fill the 3' PstI overhang) and finally blunt end ligated into the BamHI site of pRSV009 [Chapter 2 section 2.3 A(c)] to create the HDV expression plasmids pTMδNeo2A, pTMδNeo2B, pTMδNeo3A and pTMδNeo3B (Fig 7.1). All constructs were verified by restriction enzyme mapping and Southern blot hybridisation.

The orientation and position of the HDV cDNA inserts in these plasmids, relative to the expression control elements, and the expected polarity of the primary mRNA transcripts from each is shown at the bottom of Fig 7.1 part D. Also shown is the relative positions of the HDAg gene (ORF-5). It should be noted that HDAg expression was not expected from any of these plasmids as the predicted primary mRNA transcripts were either of the wrong sense (pTMδNeo2B, pTMδNeo3B) or commenced well upstream of the HDAg gene (pTMδNeo2A, pTMδNeo3A). Finally in the lower right hand corner of Fig 7.1 part D are the stable cell lines derived after transfection with these expression plasmids (see part B below). However, as the cell lines 3Bδ10, 3Bδ11 and 3Bδ12 (obtained after transfection of Hep3B cells with either pTMδNeo2A or pTMδNeo2B) had similar properties to the H1δ8 and H1δ9 cell lines described later in this chapter, experiments employing these cells have not been described in this thesis.

7.3B EXPRESSION AND LOCALISATION OF HDAg IN TRANSFECTED HH1 CELLS.

Following transfection of HH1 cells with plasmid pTMδNeo3A, ten G418-resistant clones were isolated. Three of these clones were also positive for HDAg by immunofluorescence and two, H1δ8 and H1δ9, were studied in detail for more than 50 passages.

The broad pattern of HDAg localisation in the H1δ8 and H1δ9 cells was similar to that described in the A3 cell line in Chapter 6; HDAg was detected principally in the nucleus with low levels also in the cytoplasm. In contrast to

A3 cells, and despite the fact that the H1δ8 andH1δ9 cell lines were cloned from single cells, three distinct patterns of nuclear HDAg expression were observed; (i) nucleolar staining only, (ii) nucleoplasmic staining only, (iii) both nucleolar and nucleoplasmic staining. Examples of these patterns in H1δ9 cells at passage 45 are shown in Fig 7. 2. Pattern (i) is similar to that seen after transient transfection with multimeric HDV cDNA (Kuo et al, 1989) and in the A3 cell line in which only the HDAg gene is expressed (see Chapter 6 Fig 6.3B), whereas patterns (ii) and (iii) are similar to those described in sections from livers supporting HDV replication (Rizzetto, 1983). With continuous passage, the proportion of cells showing each pattern varied (Table 7.1), but in general the proportion showing pattern (i) decreased while the proportion showing pattern (ii) increased with increasing cell passage number. It is possible that these changes were related to the appearance of HDAg p27 [see part 7.3 G(b) below].

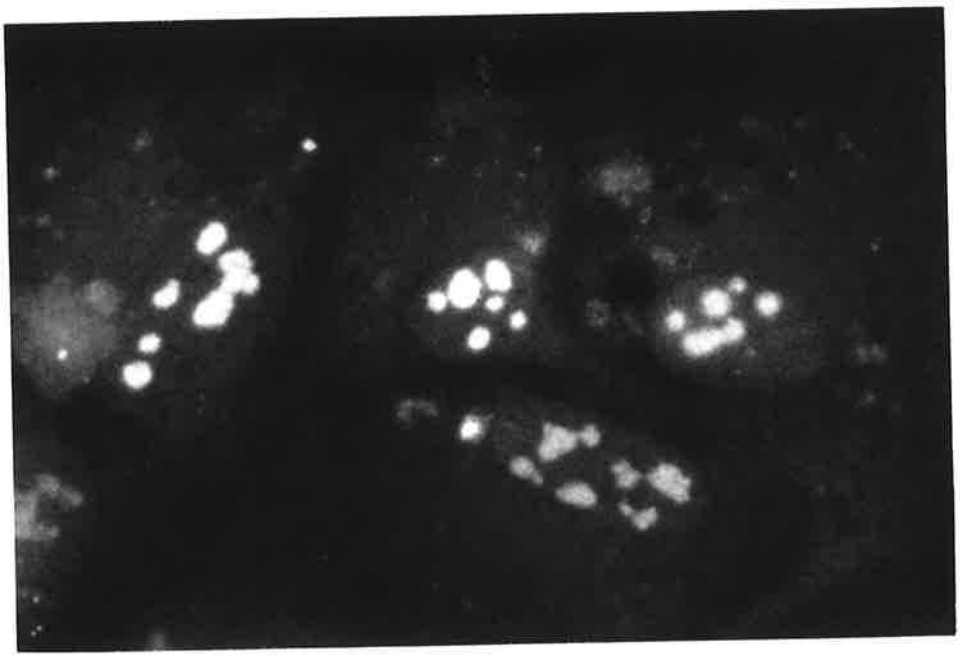
7.3C INTEGRATION OF HDV cDNA.

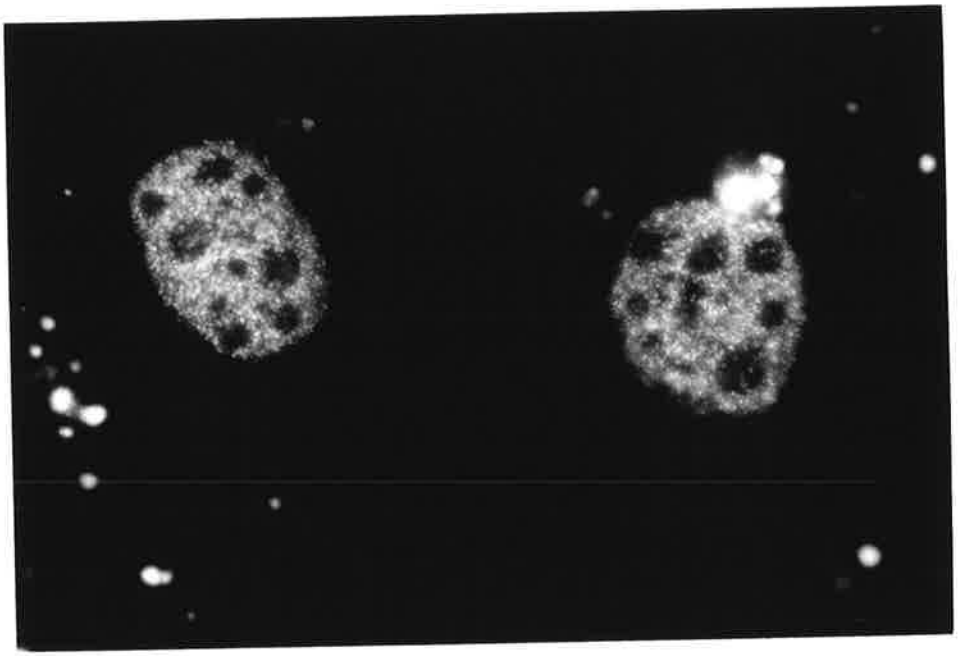
To examine the state of HDVcDNA in the above HDAg-positive cell lines, total DNA was extracted from H1δ8 cells at passage #11 and from H1δ9 cells at passage #12 and #41 and analysed by Southern blot hybridisation (Fig 7.3).

No HDV-specific bands were detected in any of the undigested DNA samples indicating that episomal HDV cDNA was not present. In contrast, multiple HDV-specific bands were detected when the total DNA was examined after digestion with the restriction enzymes PstI [three sites in the transfection plasmid (pTMδNeo3A) but not within the HDV cDNA insert] and XbaI (which digests the HDV cDNA insert of the transfection plasmid into monomers). The banding pattern of restriction enzyme digested DNA extracted from the H1δ9 cells was similar at both passages indicating that the HDV cDNA was integrated stably. PstI digestion revealed that at least 3 and 2 integration events had occurred in the H1δ8 and H1δ9 cells respectively. These must include monomeric or greater length HDV cDNA as a

Fig 7.2 Patterns of HDAg expression in H1δ9 cells passage #45. The cells were fixed and stained with a FITC-conjugated human anti-HD.
Magnification x2400

Top: nucleolar staining only
Middle: nucleoplasmic staining only
Bottom: both nucleolar and nucleoplasmic staining.





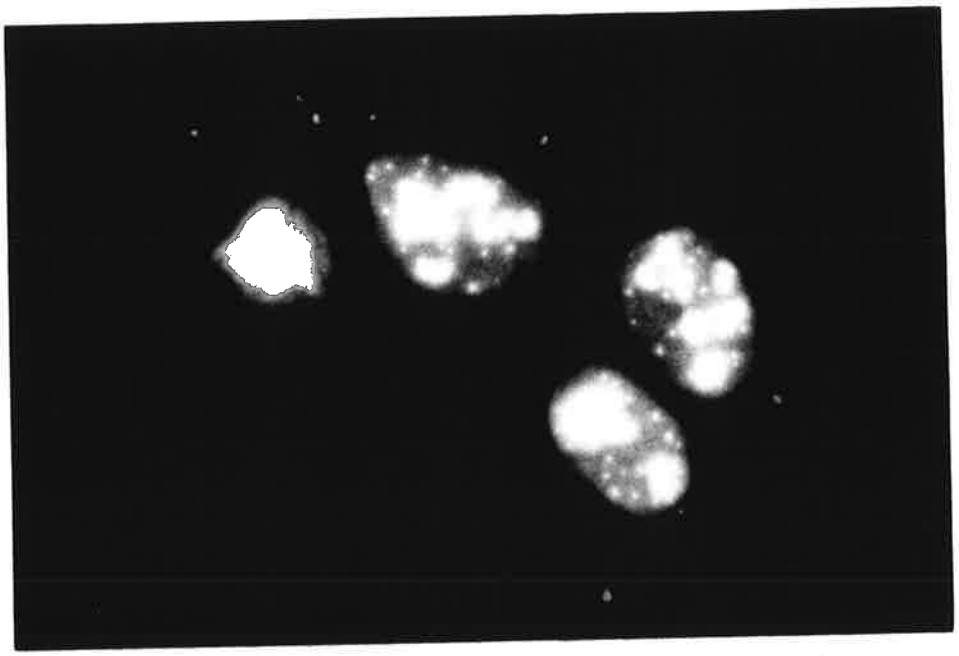
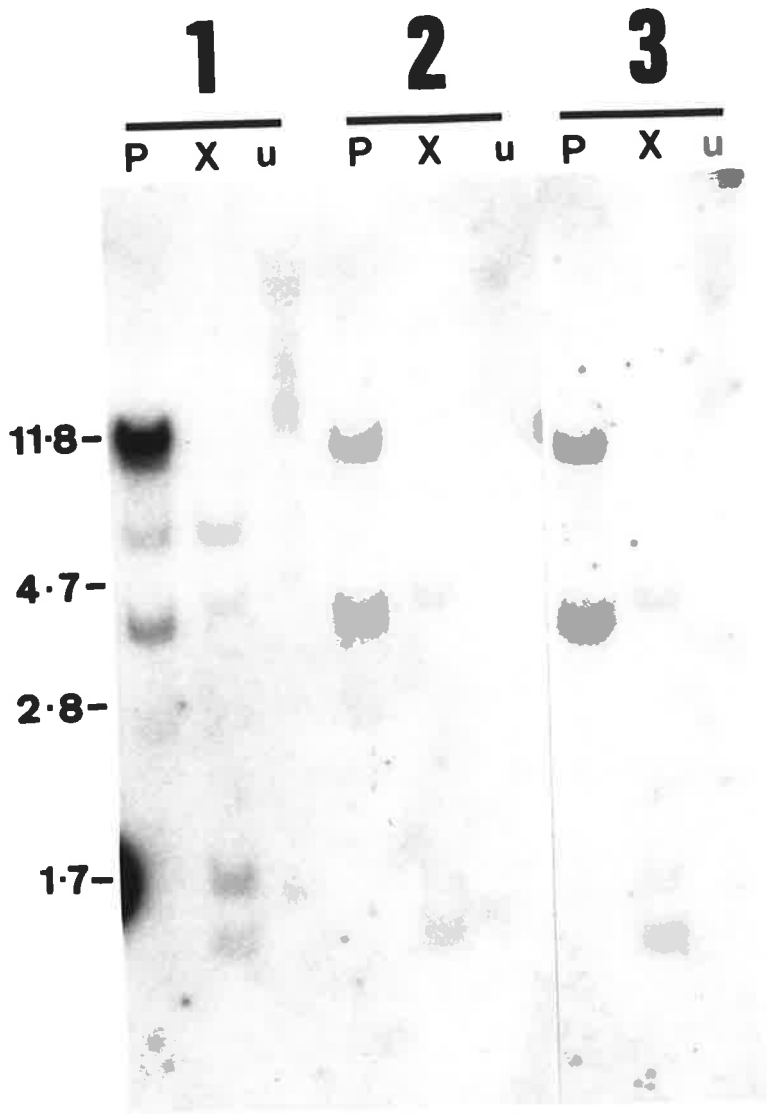


Table 7.1: The effect of increasing passage number on the localisation of HDAg in the A3 and H1δ9 cell line.

Cell Line	Passage Number	Nucleolar	Nucleoplasmic	Nucleolar +Nucleoplasmic
A3	12	100%	-	-
A3	38	100%	-	-
H1δ9	10	80%	5%	15%
H1δ9	18	70%	20%	10%
H1δ9	45	5%	80%	15%

Fig 7.3 Southern blot hybridisation analysis of DNA extracted from H1δ8 cells at passage 11 (group 1), and H1δ9 cells at passages 12 (group 2) and 41 (group 3) after hybridisation with ^{32}P-labelled riboprobes to detect HDV cDNA. Total DNA was analysed uncut (u) or after digestion with restriction enzymes PstI (P), which cleaves the transfection plasmid pTMδNeo3A at three sites but not in the HDV cDNA insert, or XbaI (X) which digests the HDV cDNA insert into monomers. The size markers on the left correspond to PstI digested λ DNA fragments.


1
2
3
P X u P X u P X u
11·8–
4·7–
2·8–
1·7–

HDV-reactive band at ca. 1.7kb was detected in all DNA samples after digestion with XbaI.

7.3D LOCALISATION AND CONFIGURATION OF HDV RNA IN THE H1δ8 AND H1δ9 CELLS.

The HDAg expression seen in the H1δ8 and H1δ9 cells was quite unexpected as, although the primary mRNA transcript from pTMδNeo3A had the correct polarity, two complete in frame ORFs (ORF-7 and ORF-8, see Chapter 6 Fig 6.1) were upstream of the translational (AUG) start site of ORF-5. Furthermore, Allison Jilbert showed previously that a RNA species similar to that expected from pTMδNeo3A was not translated in vitro (Jilbert et al, 1990) indicating that internal initiation was unlikely to account for the observed HDAg expression. Thus, although a splicing event could not be ruled out it was likely that HDV RNA processing similar to that occurring in the natural infection was ongoing within the H1δ8 and H1δ9 cell lines. To investigate these possibilities the localisation and configuration of HDV RNA in the H1δ8 and H1δ9 cells was examined.

7.3D(a) In Situ Cytohybridisation.

H1δ9 and the parent line HH1 cells were grown on glass microscope slides and examined by in situ cytohybridisation using strand-specific riboprobes that were generated from the T7- and SP6-RNA promoters of plasmid pTMδ3. Despite the fact that the plasmid pTMδNeo3A was designed to synthesise antigenomic HDV RNA only, both genomic and antigenomic HDV RNA were detected predominantly in the nuclei of the cells, with lower levels in the cytoplasm (Fig 7.4). Antigenomic HDV RNA was detected in 100% of the cells with small clusters (ca. 10%) showing high levels. In contrast, only about 20% of cells were positive for genomic HDV RNA although small clusters of strongly reactive cells were again observed. No HDV-specific sequences were detected in the HH1 cells.

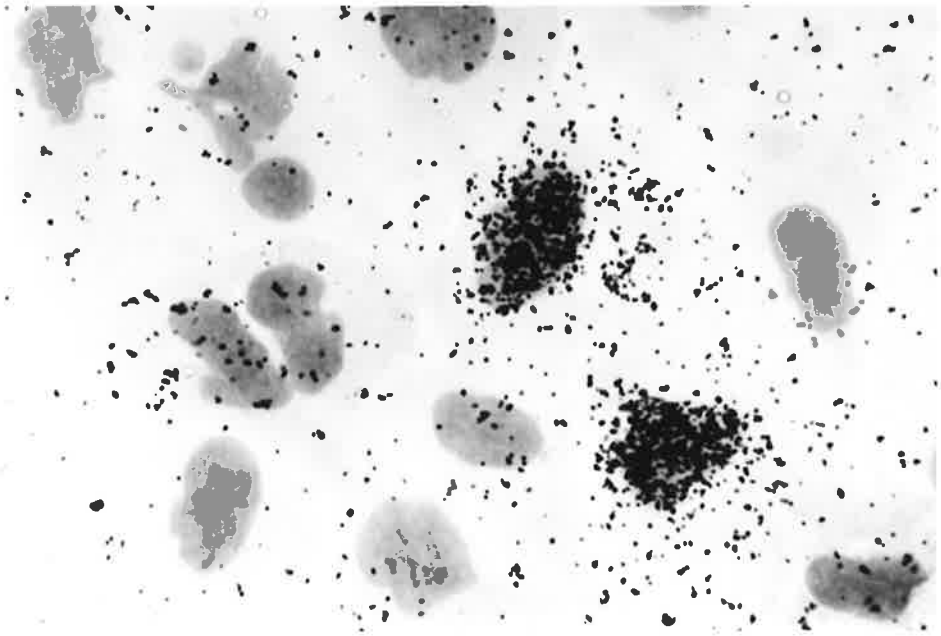
7.3D(b) Northern Blot.

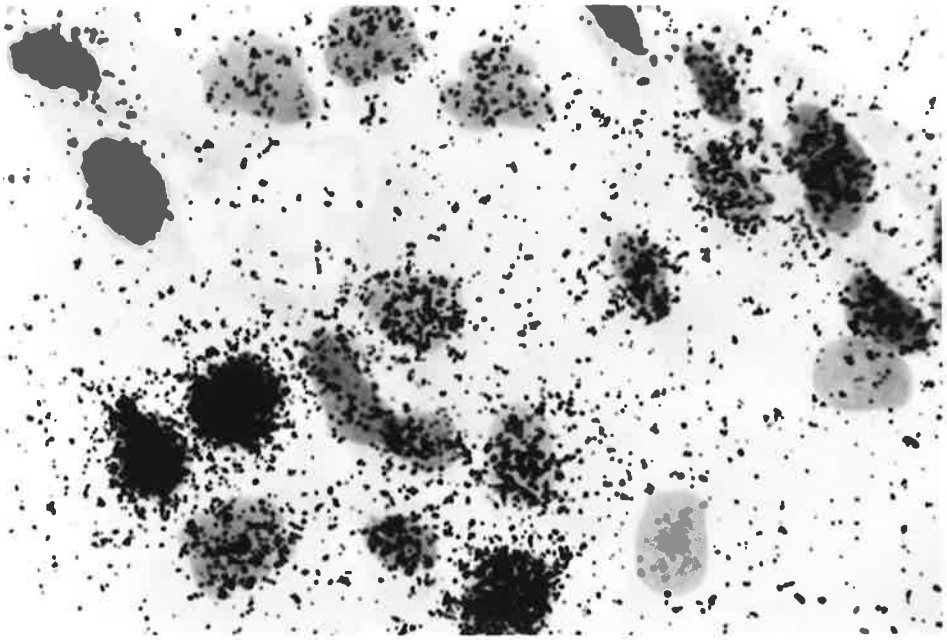
Total and cytoplasmic RNA from H1δ8 and H1δ9 cells (at passage #10

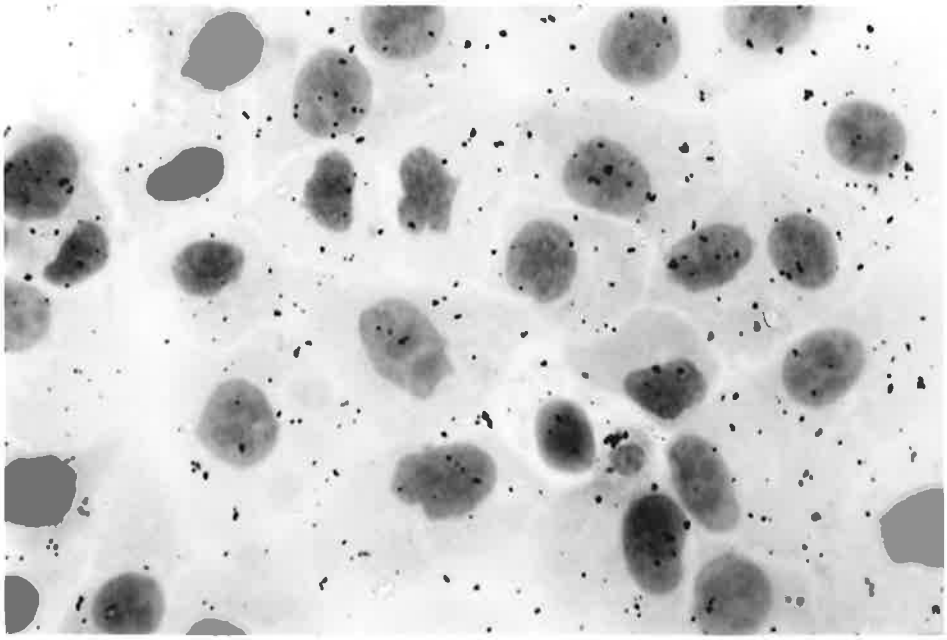
Fig 7.4 Detection of HDV RNA by in situ cytohybridisation.

Top HH1 cells.
Middle H1δ9, antigenomic HDV RNA.
Bottom H1δ9, genomic HDV RNA.

The HH1 cells were hybridised with a probe to detect antigenomic HDV RNA and showed a similar result with the complementary probe. The cells were fixed in paraformaldehyde and hybridised with ^{125}I-labelled strand-specific riboprobes transcribed from pTMδ3. The autoradiographic exposure was 24 hours and the cells were stained by hematoxylin and eosin. This experiment was performed by Allison Jilbert.
Magnification x2000.







and #22 respectively) and total RNA from HH1 and A3 cells were analysed by Northern blot hybridisation (Fig 7.5).

HDV RNA was not detected in the HH1 or A3 cells despite the fact that the latter cell line produced HDAg. In contrast, high levels of both genomic and antigenomic HDV RNA were detected in the H1δ8 and H1δ9 cells. In total and cytoplasmic extracts from the H1δ8 cells, and in cytoplasmic extracts from the H1δ9 cells, the vast bulk of this RNA was of genome length (1.7 kb), whereas in total extracts from the H1δ9 cells, dimeric and trimeric forms were also detected that constituted 10-20% of total HDV RNA. In a repeat experiment which examined a small amount of total RNA from the H1δ9 cells, a typical RNA doublet was noted in the monomer and dimer but not in the trimer position (see Fig 7.11 below). These results suggested that HDV RNA replication was ongoing in these cells.

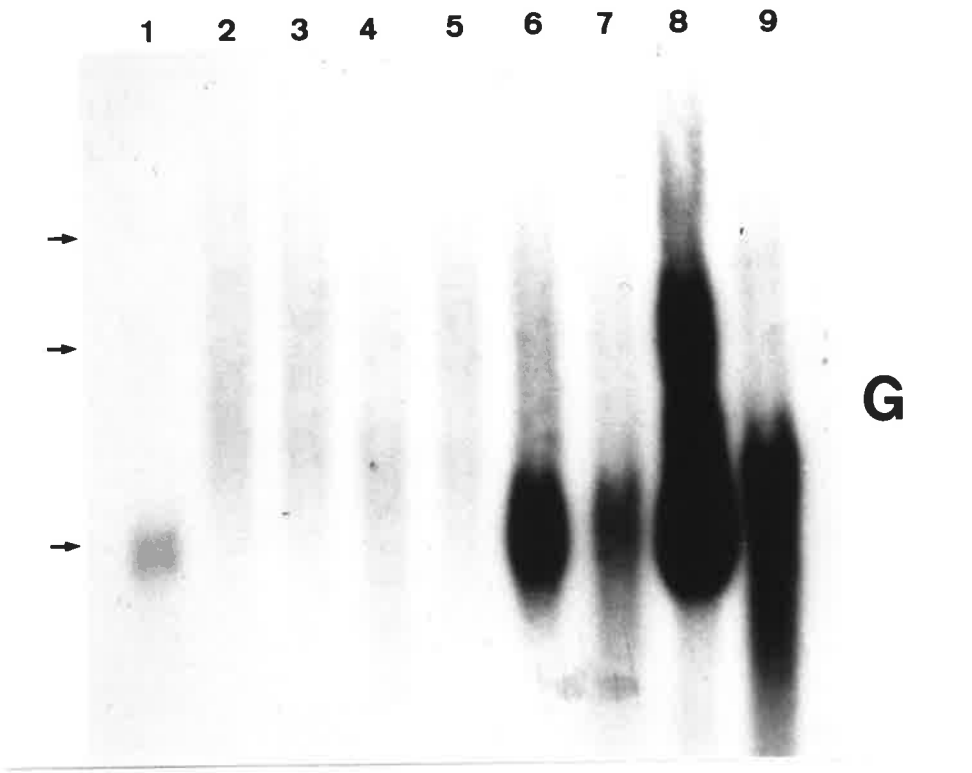
7.3D(c) Polyadenylated Species of HDV RNA.

In addition to the production of high levels of genomic and antigenomic HDV RNA, the H1δ8 and H1δ9 cell lines were shown to unexpectedly synthesise HDAg (part B above). In this connection, since a subgenomic RNA species was undetected in the above experiment, the poly(A)$^+$ fraction of the RNA was examined for HDV RNA content.

Total RNA was extracted from the H1δ8 and H1δ9 cells at passage #15 and #18 respectively, and following a single oligo(dT)-purification step, poly(A)$^+$ and poly(A)$^-$ RNA fractions were examined by Northern blot hybridisation (Fig 7.6). A small amount of full length monomeric HDV RNA of both genomic and antigenomic polarity was detected that was considered to represent contamination of the poly(A)$^+$ fraction by poly(A)$^-$ RNA. In addition, two subgenomic species representing antigenomic RNA only of ca. 900 and 600 bases were detected in both the H1δ8 and H1δ9 cells. No subgenomic species were detected in the poly(A)$^-$ RNA (Fig 7.6).

The larger subgenomic species has been detected previously and may represent HDAg mRNA (Chen et al, 1986; Jilbert et al, 1990; Hsieh et al,

Fig 7.5 Northern blot analysis of total and cytoplasmic RNA extracted from cells after hybridisation with ^{32}P-labelled riboprobes to detect genomic (G) or antigenomic (AG) HDV RNA that were transcribed from pTMδ3. Lane 1, HDV RNA purified from serum; lane 2 and 5, total RNA extracted from HepG2 and HH1 cells respectively; lanes 3, 6 and 8, total- and lanes 4, 7 and 9, cytoplasmic-RNA extracted from A3, H1δ8 (passage #10) and H1δ9 (passage #22) cells respectively. The arrows show the position of monomeric, dimeric and trimeric single-stranded HDV cDNA.


1 2 3 4 5 6 7 8 9
G

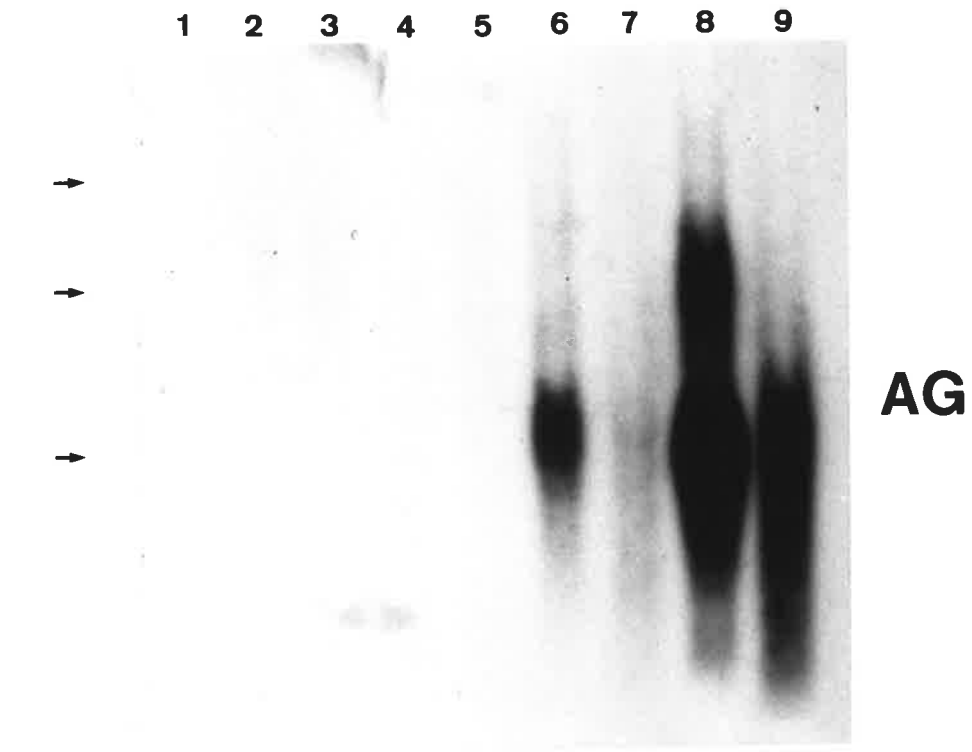
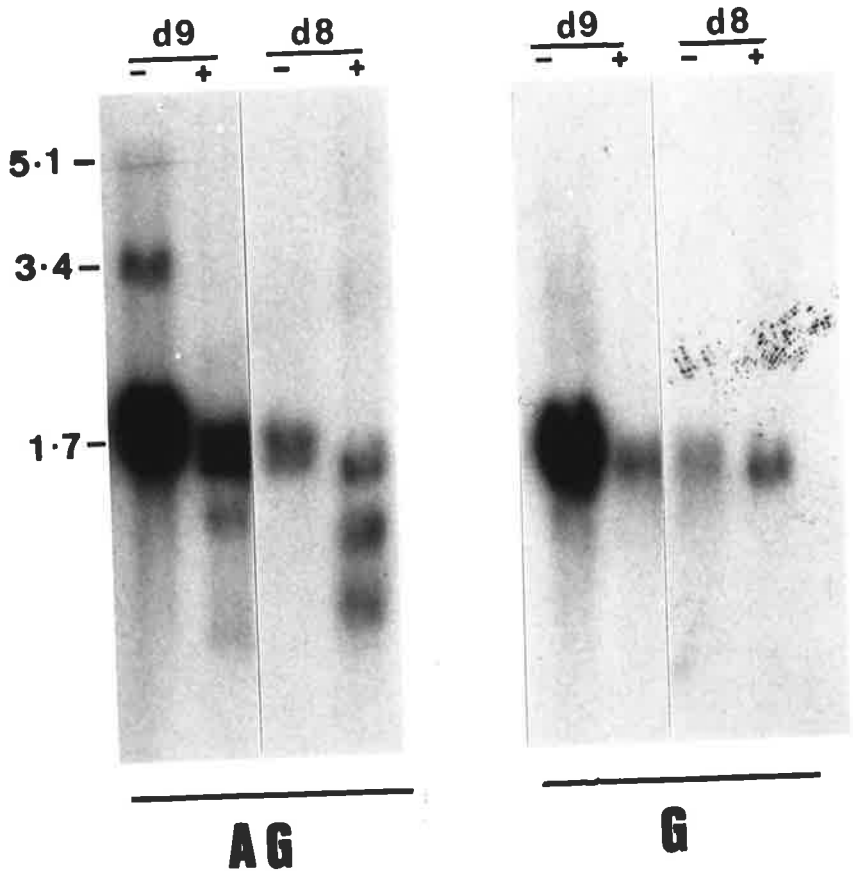

1 2 3 4 5 6 7 8 9
AG

Fig 7.6 Northern blot analysis of poly(A)$^+$ and poly(A)$^-$ RNA purified from H1δ8 and H1δ9 cells, at passage # 11 and 18 respectively, after hybridisation with ^{32}P-labelled riboprobes (transcribed from pTMδSalA and pTMδSalB) to detect antigenomic (AG) or genomic (G) HDV RNA. Markers on the left correspond to monomeric (1.7kb), dimeric (3.4kb) and trimeric (5.1kb) single-stranded HDV cDNA.


d9
d8
d9
d8
- +
- +
- +
- +
5·1 –
3·4 –
1·7 –
AG
G

1990), while the function of the smaller species is unknown and its existence has not been described previously. However, since this smaller species is polyadenylated it may be functional, coding for an additional HDV-specific protein.

7.3D(d) Double stranded HDV RNA.

Like viroid RNA, HDV RNA has the potential to form an unbranched rod structure by intramolecular base pairing (Wang et al, 1986; Makino et al, 1987; Kuo et al, 1988). While such structures have been detected by electron microscopy in HDV RNA extracted from serum (Kos et al, 1986), the bulk of HDV RNA detected in naturally infected hepatocytes was shown to be single-stranded (Gowans et al, 1988). Consequently, it remains unclear if intramolecular base-pairing occurs naturally or is an artifact of the RNA extraction procedure. Since the formation of this rod structure produces dsHDV RNA, the H1δ9 cells were examined by immunofluorescence for the presence of dsHDV RNA using a rabbit antibody (a gift from Richard Francki, Waite Institute, Adelaide) known to be specific for dsRNA (Anderson et al, 1988). HeLa and H1δ9 cells infected with poliovirus were included as positive controls.

Coverslip cultures of HeLa or H1δ9 cells showed intense cytoplasmic staining four hours after infection with poliovirus type III (Fig 7.7) indicating the presence of poliovirus dsRNA replicative forms. In contrast, dsRNA was not detected in either the uninfected HeLa or H1δ9 cells (Fig 7.7).

The above result suggests that HDV RNA in the H1δ9 cells was largely single stranded and is consistent with published in situ hybridisation data (Gowans et al, 1988) in which the level of detectable genomic HDV RNA in liver sections was not appreciably affected by prior denaturation (in contrast to the expected result if the target was double stranded).

Fig 7.7 Detection of dsRNA in HeLa and H1δ9 cells.
Coverslip cultures of Hela or H1δ9 cells were examined by immunofluorescence using a rabbit antibody specific for dsRNA

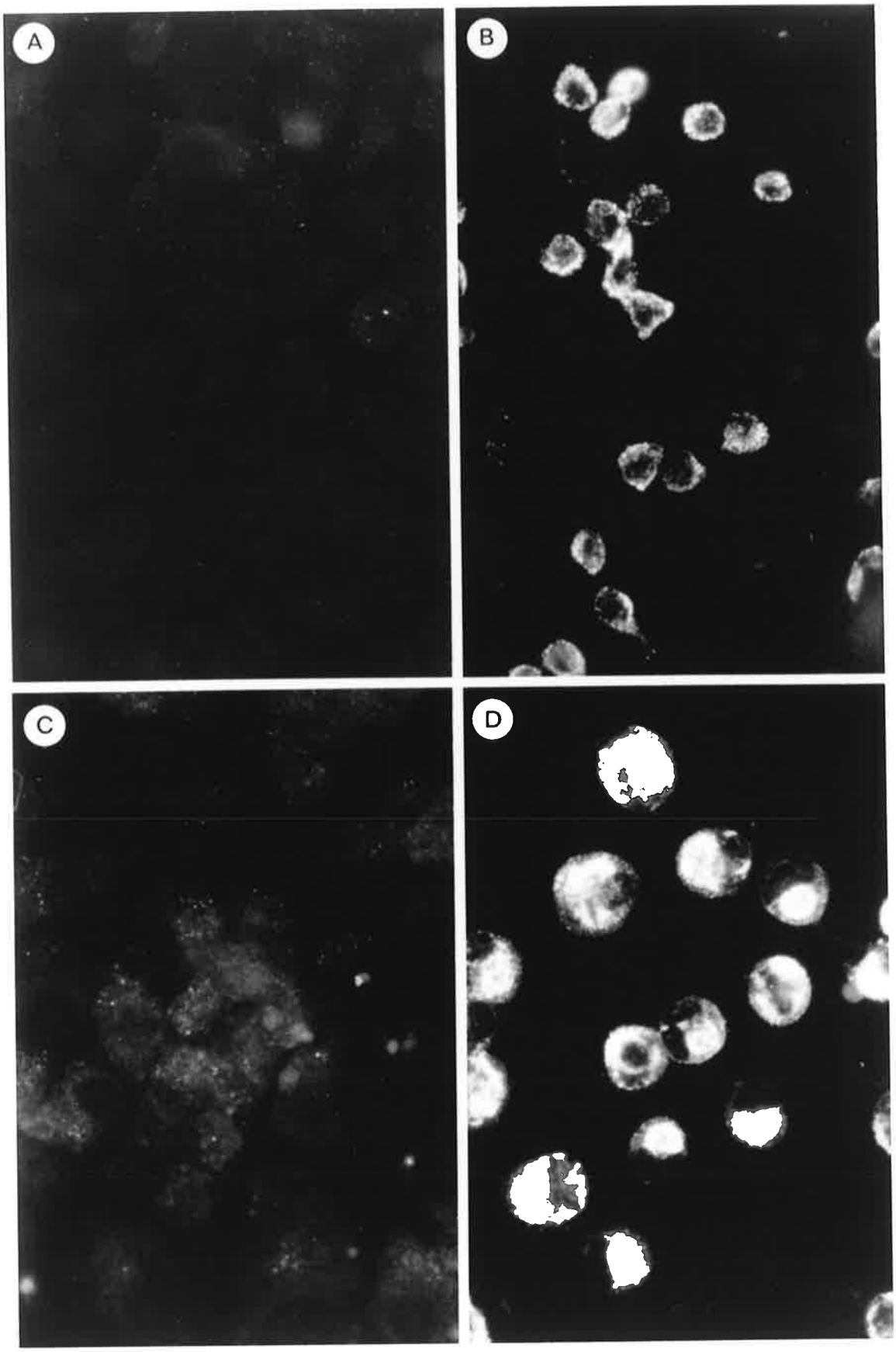
A HeLa cells, uninfected.

B HeLa cells, four hours after infection with poliovirus type III.

C H1δ9 cells, uninfected.

D H1δ9 cells, four hours after infection with poliovirus type III.

Magnification x1800


A
B
C
D

7.3E MECHANISM OF HDV RNA SYNTHESIS.

To investigate the mechanism of HDV RNA replication described above, the effect of actinomycin D and α-amanitin on RNA synthesis in the H1δ9 cells was examined.

7.3E(a) Actinomycin D.

In a preliminary experiment, the effect of a range of concentrations of actinomycin D on the level of ^{3}H-uridine incorporation by the H1δ9 cells was determined (Fig 7.8A). This titration showed that H1δ9 cells were very sensitive to this inhibitor since concentrations as low as 25ng/ml caused greater than 50% reduction in ^{3}H-uridine incorporation. Two concentrations of actinomycin D, 400 and 2,000ng/ml, that caused a 90% and 99% reduction respectively in ^{3}H-uridine incorporation were chosen for use in a large scale in vivo labelling experiment (performed as described in Chapter 2 section 2.8A) in which the H1δ9 cells were incubated in the presence of ^{32}P-orthophosphate and actinomycin D.

The results of this experiment showed that HDV RNA in the H1δ9 cells was synthesised and labelled with the ^{32}P-orthophosphate to comparable levels irrespective of the concentration of actinomycin D added to the cell culture medium (Fig 7.8B). In contrast, HBV RNA transcription in these cells was markedly reduced by the action of actinomycin D. These results confirmed that dsDNA was the template for transcription of HBV- but not HDV-RNA. Thus it is likely that RNA constituted the template for HDV RNA transcription, and in view of the fact that these cells have continued to express high levels of HDV RNA for nearly 18 months, the RNA-RNA transcription is likely to be self sustaining.

7.3E(b) α-Amanitin.

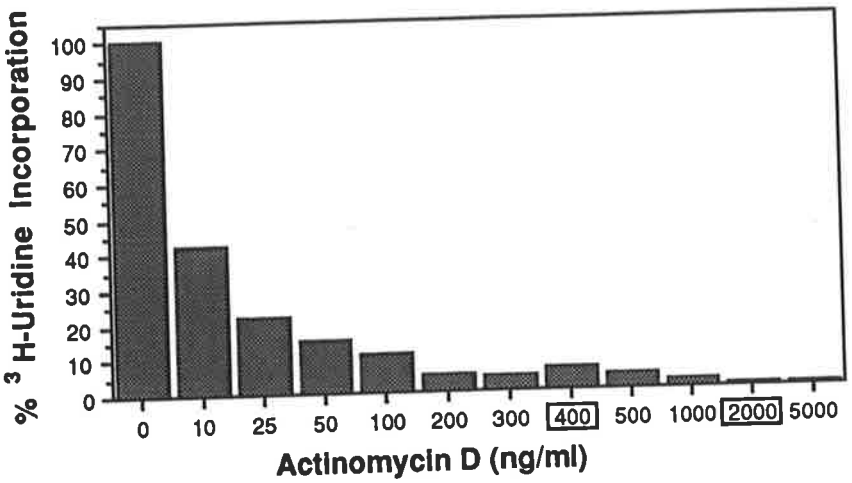
Nuclear transcription run-off assays using isolated H1δ9 nuclei were performed either with or without the inclusion of α-amanitin as described in Chapter 2 section 2.8 B. Both HBV- and HDV-RNA transcription was strongly inhibited by 1μg/ml α-amanitin (Fig 7.8C), a level that inhibits RNA

Fig 7.8A The effect of increasing concentrations of actinomycin D on the level of ^{3}H-uridine incorporation by coverslip cultures of H1δ9 cells.

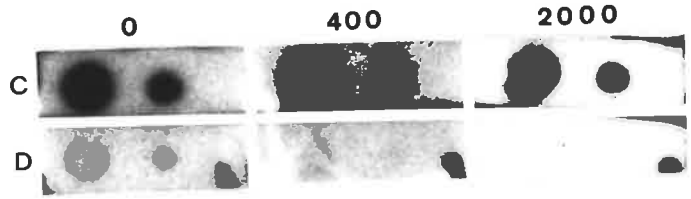
Fig 7.8B The effect of different concentrations of actinomycin D on the in vivo synthesis of HDV- and HBV-RNA. H1δ9 cells were incubated for 2 hours in phosphate-free medium then in medium containing 0, 400 or 2000ng/ml actinomycin D, and 80μCi/ml ^{32}P-orthophosphate, and incubated for a further 4 hours. Total RNA was extracted and hybridised against HDV cDNA (C) or HBV DNA (D) previously bound to the nitrocelluose. The DNA target was 5 and 1μg in the left and right positions respectively of each panel.

Fig 7.8C The effect of different concentrations of α-amanitin on the in vitro synthesis of RNA. H1δ9 were incubated in a transcription solution containing ^{32}P-UTP with 0, 1 or 10 μg/ml α-amanitin. Control HH1 cell nuclei incubated in a similar transcription mixture but without α-amanitin were also tested. The RNA was then extracted and hybridised against (A) total DNA extracted from HH1 cells, (B) pGEM-3 DNA, (C) HDV cDNA or (D) HBV DNA, as described above in the legend to Fig 7.8B. A-D exposed for 16 hours, D° exposed for 4 days.

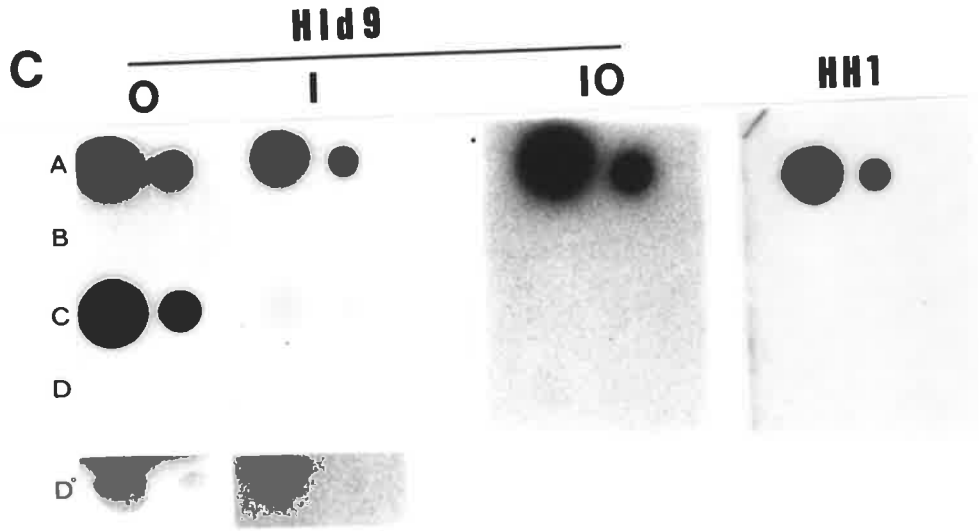
A


% 3H-Uridine Incorporation
100
90
80
70
60
50
40
30
20
10
0
0
10
25
50
100
200
300
400
500
1000
2000
5000
Actinomycin D (ng/ml)

B


0
400
2000
C
D

C


Hld9
O
I
IO
HH1
A
B
C
D
D°

Polymerase II only (Marzluff and Huang, 1984). In contrast, although reduced by approximately 35%, total cellular RNA transcription still took place in the presence of ≥10μg/ml α-amanitin, consistent with the expected involvement of RNA Polymerase I and III in total RNA synthesis.

Thus, HDV RNA replication in the H1δ9 cells was performed by RNA Polymerase II or by an enzyme with a similar sensivity to α-amanitin, and was likely to use HDV RNA as a template as shown by the resistance of the reaction to actinomycin D.

7.3F HDV RNA SYNTHESIS: THE ROLE OF HDAg.

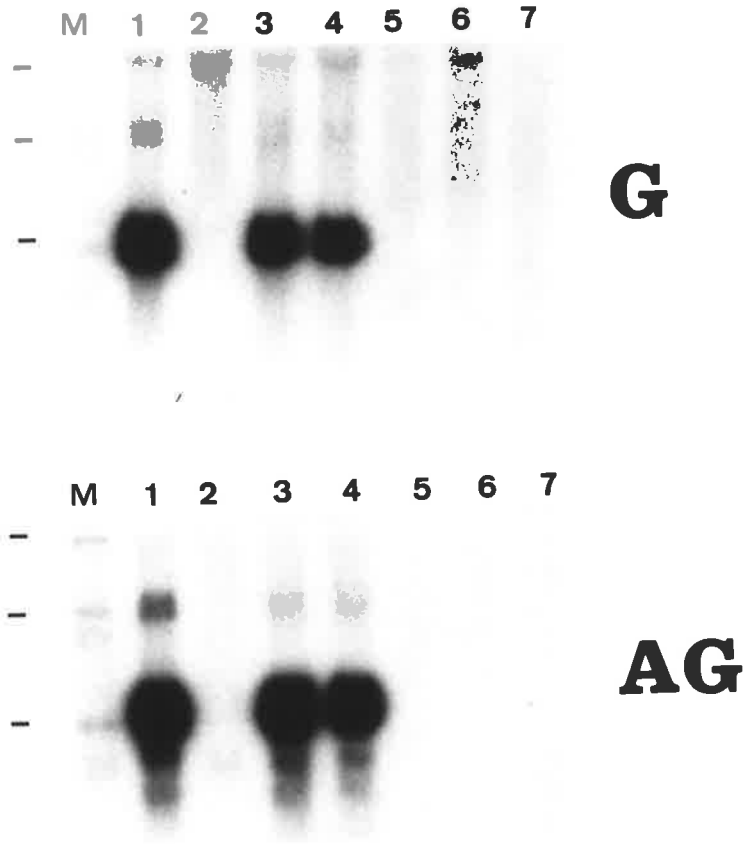
Recent studies have shown that HDAg is essential for HDV RNA replication (Kuo et al, 1989, Glenn et al, 1990). Since H1δ9 cells produce HDAg it could not be discounted that HDAg in these cells had formed part of a novel replicase which had an α-amanitin sensitivity similar to that of RNA Polymerase II. Thus, to determine the role of HDAg in HDV RNA replication, the ability of exogenous HDV RNA to initiate HDV RNA replication in vivo and in vitro was assessed, with or without the inclusion of HDAg.

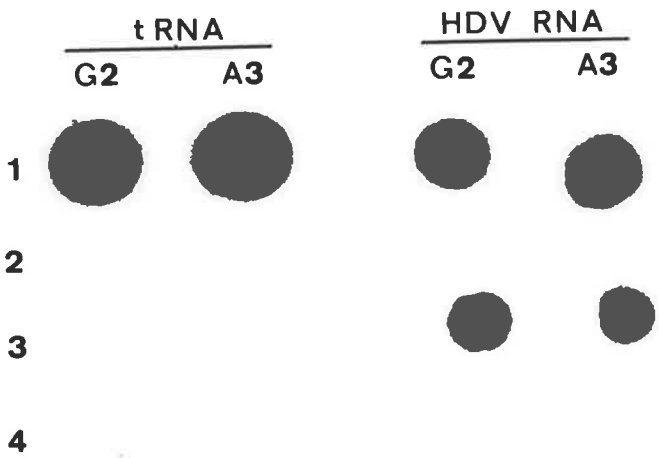
Genomic-sense, trimeric-length HDV RNA transcribed from pTMδXba3B was transfected into HepG2- and A3-cells and 4 days later total RNA was extracted and analysed by Northern blot hybridisation (Fig 7.9). Monomeric, dimeric and trimeric HDV RNA of both polarities, and two subgenomic antigenomic-sense species similar to those described above for the H1δ9 cell line were only detected in the A3 cells. Thus antigenomic HDV RNA was produced from a genomic template only in HDAg-containing cells, confirming previous reports that HDAg is essential for HDV RNA replication in vivo.

To confirm the above in vivo results, an in vitro study was performed; trimeric, genomic-sense HDV RNA identical to that used above or control tRNA was added to crude nuclear extracts from isolated HepG2 or A3 cell nuclei, prepared as described in Chapter 2 section 2.8 C (Fig 7.10). HDV-specific RNA transcription was only detected in extracts containing exogenous HDV RNA. However, the level of transcription was identical in

Fig 7.9 Northern blot analysis of RNA extracted from HepG2 and A3 cells 4 days after transfection with genomic-sense, trimeric HDV RNA transcribed from pTMδXba3B. RNA was transferred to nitrocelluose and hybridised with ^{32}P-labelled riboprobes to detect genomic (G) or antigenomic (AG) HDV RNA. Lane 1, monomeric, dimeric and trimeric markers of HDV RNA (H1δ9 cell extract); lanes 2 and 5, total RNA extracted from A3 and HepG2 cells respectively after transfection with tRNA; lanes 3 and 4, and 6 and 7, duplicate samples of total RNA extracted from A3 and HepG2 cells respectively after transfection with trimeric HDV RNA; lane M, size marker containing monomeric, dimeric and trimeric single-stranded HDV cDNA.

Fig 7.10 Hybridisation analysis of ^{32}P-labelled RNA synthesised in transcriptionally active nuclear homogenates of A3 and HepG2 (G2) cells containing exogeneous tRNA or trimeric genomic HDV RNA (from pTMδXba3B). The RNA was then extracted and hybridised against 1μg targets of (1) total DNA extracted from HH1 cells, (2) pGEM-3 DNA, (3) HDV cDNA or (4) HBV DNA that was bound previously to the nitrocelluose.


M 1 2 3 4 5 6 7
G
M 1 2 3 4 5 6 7
AG


t RNA
G2
A3
HDV RNA
G2
A3
1
2
3
4

extracts from both cell lines, indicating that HDAg was not required for HDV RNA transcription per se.

7.3G H1δ8 AND H1δ9 CELLS AS A MODEL FOR PERSISTENT HDV INFECTION.

The intracellular distribution of HDAg in the H1δ8 and H1δ9 cells particularly at higher passage numbers (described in section B above) was similar to that described in naturally infected livers (Rizzetto, 1983). In this context, the H1δ8 and H1δ9 cell lines were further assessed as a model system for acute and persistent HDV infection.

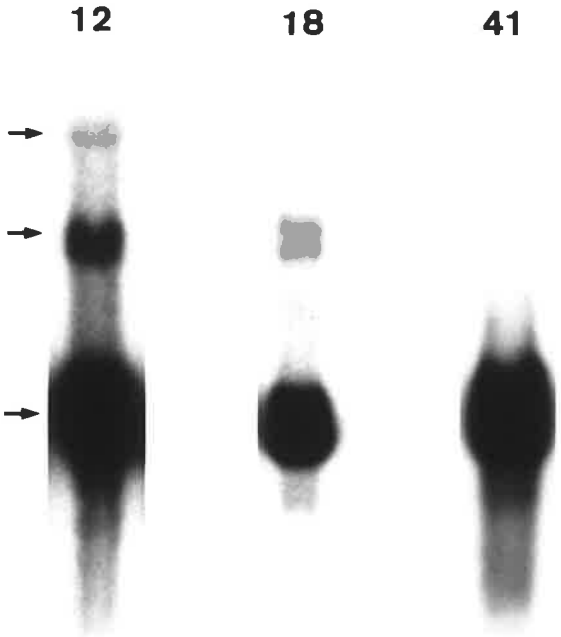
7.3G(a) Effect of H1δ9 cell passage number on HDV RNA synthesis and expression.

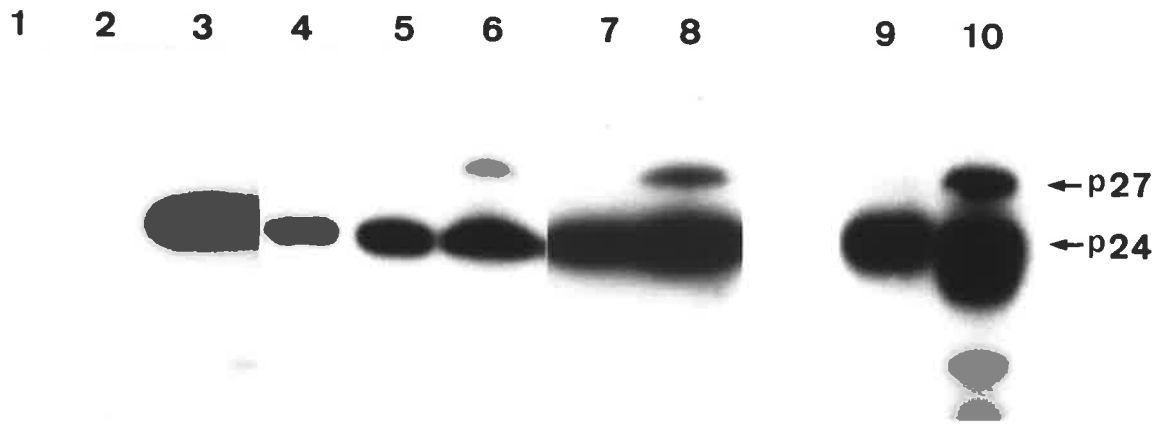
Initally both the H1δ8 and H1δ9 cell lines grew slowly and continually produced detached cells which stained positive with trypan blue. Eventually the culture medium no longer contained dead cells and the doubling time for the H1δ9 cells decreased from 50 hours at passage #11 to 28 hours at passage #41. As the cell death seen in early passage numbers might have been HDV-related, a number of experiments to examine this relationship were performed. The increased cell growth rate was (i) not related to the removal of the selection antibiotic nor (ii) to the loss of integrated HDV cDNA as Southern blot hybridisation analysis of total DNA extracted from the H1δ9 cells showed identical results at both passages. HDV RNA from the H1δ9 cells and HDAg from both the H1δ8 and H1δ9 cells (see below) were then examined at various intervals after transfection. Total RNA was extracted from the H1δ9 cells at passages 12, 18 and 41 (4, 6 and 11 months respectively post-transfection) and analysed by Northern blot hybridisation (results for antigenomic-sense HDV RNA are shown in Fig 7.11).

Monomeric HDV RNA of both polarities was detected as the predominant species at each passage number and in #12 and #18 significant levels of dimeric and trimeric forms were also seen. In contrast, by passage #41 the concentration of these larger forms was markedly reduced, a consistent

Fig 7.11 Northern blot analysis of total RNA extracted from H1δ9 cells at passage numbers 12, 18 and 41 after hybridisation with ^{32}P-labelled riboprobes (transcribed from pTMδSalA) to detect antigenomic-sense HDV RNA. The arrows show the position of monomeric, dimeric and trimeric single-stranded HDV cDNA.

Fig 7.12 The polypeptide profile of HDAg detected by immunoblot in cell and tissue extracts using human anti-HD as the primary antiboby. Lanes 1 and 2, HepG2 and HH1 cell extracts respectively; lane 3, A3 cell extract from passage #27; lanes 4, 5 and 6, H1δ9 cell extracts from passage #7, 22 and 37 respectively; lanes 7 and 8, H1δ8 cell extracts from passage #4 and 12 respectively; lane 9, acute phase chimpanzee liver; lane 10, chronically infected human liver. The arrows show the position of polypeptides of 24 and 27kD.


12
18
41


1
2
3
4
5
6
7
8
9
10
p27
p24

finding in three independent experiments.

7.3G(b) Comparison of the HDAg Polypeptide Profiles in the A3, H1δ8 and H1δ9 Cell Lines.

The polypeptide profile of HDAg present in the A3, H1δ8 and H1δ9 cell lines at various passages after transfection were examined by immunoblotting; the parent HepG2 and HH1 cell lines were also examined as controls.

In the A3 cells, only the 24 kD form (p24) of HDAg was ever detected [Fig 7.12 lane 3 (passage #27)] despite regular examination up to passage #43 (ca. 12 months in continuous culture post transfection). In contrast, the polypeptide profile of HDAg in both the H1δ8 and H1δ9 cell lines changed as the passage number increased. At passage #7, only HDAg-p24 was detected in the H1δ9 cells (Fig 7.12 lane 4). However, by passage #22 a faint second band, corresponding to the 27kD form HDAg (p27) was detected (Fig 7.12 lane 5) which by passage #37 had become quite distinct (Fig 7.12 lane 6). Similar changes were detected in the H1δ8 cell line between passage #4 and #12 (Fig 7.12 lane 7 and 8 respectively). No HDAg-reactive species were detected in the HepG2 or HH1 cells (Fig 7.12 lanes 1 and 2 respectively)

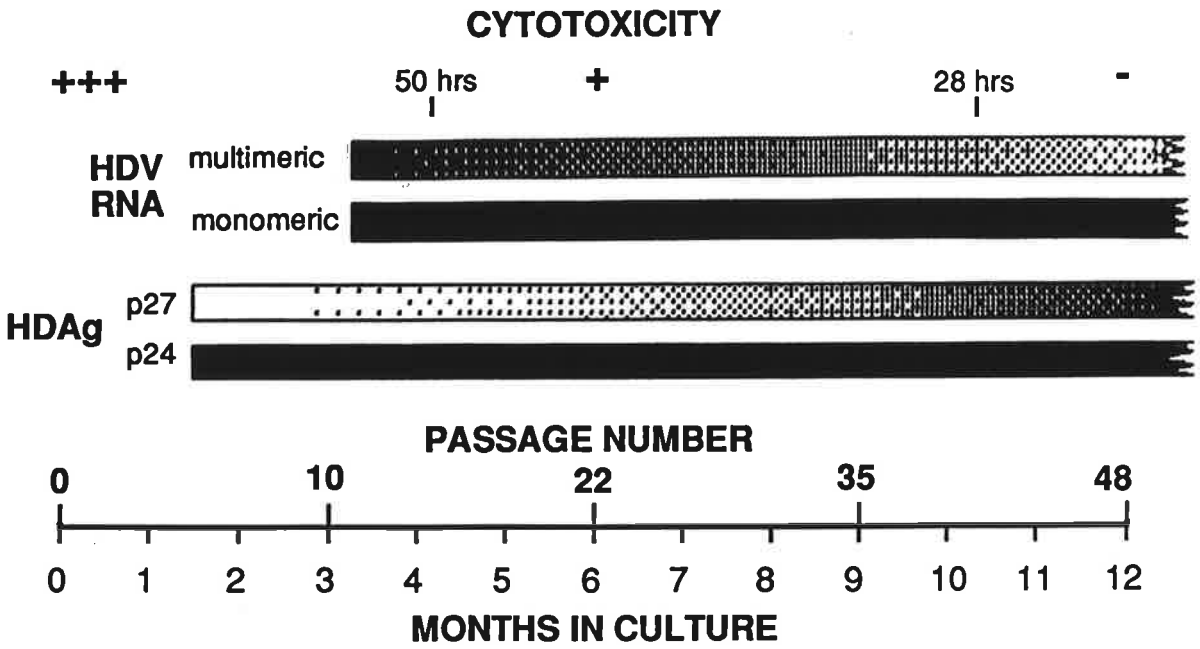
Thus, continous passage of the H1δ8 and H1δ9 cell lines resulted in more rapid cell doubling and a disappearance of spontaneous cell death, that was accompanied by a decrease of multimeric forms of HDV RNA (H1δ9) and the appearance of the larger (p27) HDAg-related polypeptide. The timing of the changes seen in the H1δ9 cell line is shown diagramatically in Fig 7.13.

7.3G(c) HDAg polypetide profiles in HDV-Infected Liver.

The HDAg polypeptide profile of acutely- and chronically- HDV infected liver tissue was compared with that seen in the above cell lines.

A similar association to that noted above in the H1δ8 and H1δ9 cell lines was seen; specifically a ratio of p27: p24 of ca. 1:2 in liver samples from chronic HDV infection (Fig 7.12 lane 10) was noted compared to the ratio of ca. 1:40 seen in acute infection (Fig 7.12 lane 9). Examination of published

Fig 7.13 An outline of the appearance of HDV molecular markers and associated cytotoxicity in the H1δ9 cell line held in continuous culture for 12 months. The indicated times 50 and 28 hours represent cell doubling times.


CYTOTOXICITY
+++
50 hrs
+
28 hrs
-
HDV RNA
multimeric
monomeric
HDAg
p27
p24
PASSAGE NUMBER
0
10
22
35
48
0 1 2 3 4 5 6 7 8 9 10 11 12
MONTHS IN CULTURE

results (Bergmann and Gerin, 1986; Sureau et al, 1989) often show a similar ratio of p27: p24 in acute phase liver samples, but the ratio in serum-derived HDAg is much closer to equimolar during both the acute and chronic stages of infection.

7.3H HBV HELPER FUNCTION.

7.3H(a) HDV Replication.

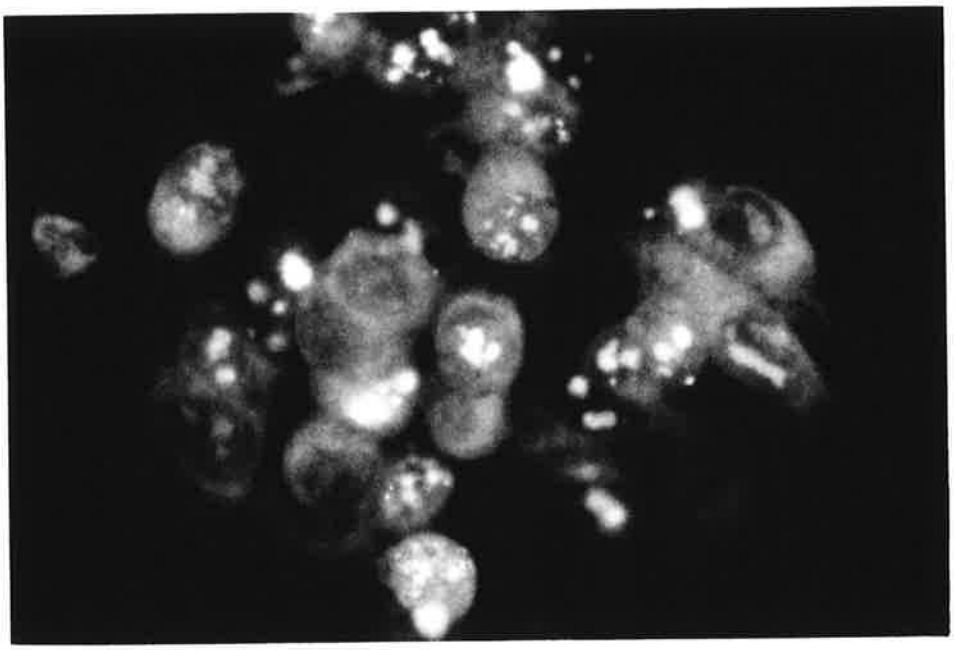
To determine if the HDV RNA replication and HDAg synthesis in the H1δ8 and H1δ9 cell lines depended on HBV-specific functions provided by the parent HH1 cell line, the expression vector pTMδNeo2B (containing dimeric HDV cDNA insert) was transfected into the (HBV DNA negative) HepG2 cell line. As described above, this plasmid was designed to transcribe genomic HDV RNA after transfection. In one of the G418-resistant clones, G2δ6, that was sucessfully expanded HDAg was detected in all cells by immunofluorescence, although the staining intensity was generally lower than that in the H1δ8 and H1δ9 cell lines. In the vast majority of G2δ6 cells the HDAg was only detected in cell nucleoli (Fig 7.14 top), although in ca. 3% of cells, a combined nucleolar and nucleoplasmic HDAg distribution similar to that described above for H1δ8 and H1δ9 was also observed (Fig 7.14 middle). Nucleoplasmic-only staining was not detected in the G2δ6 cell line.

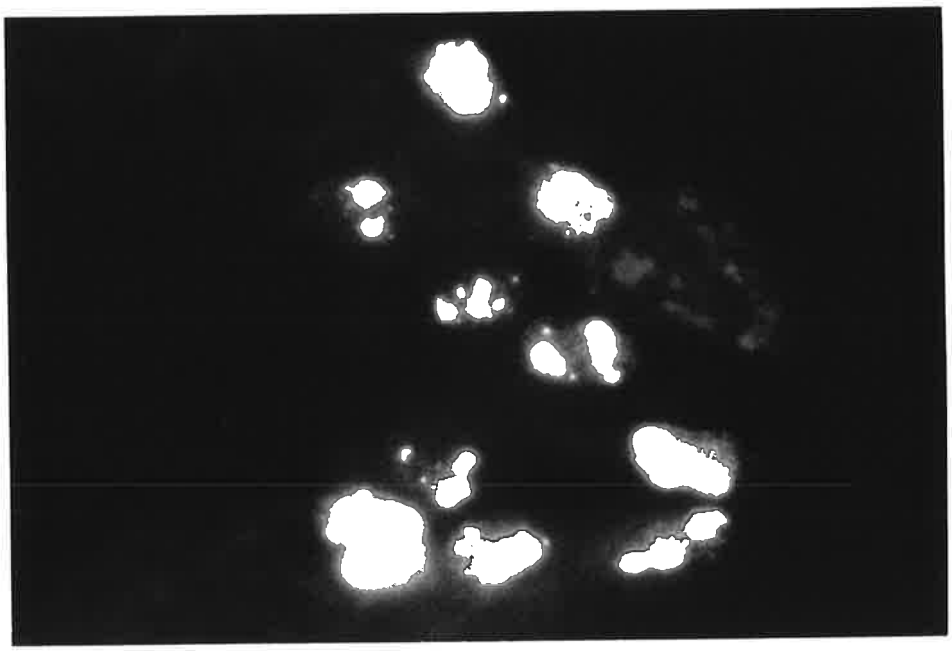
The synthesis of HDAg suggested that HBV-independent HDV RNA replication might be ongoing in the G2δ6 cell line. To investigate this point, total RNA was extracted from the G2δ6 cells and analysed by Northern blot hybridisation. Both genomic and antigenomic HDV RNA (monomeric length only) were detected (Fig 7.15) but at lower levels than that seen above in the H1δ8 and H1δ9 cell lines.

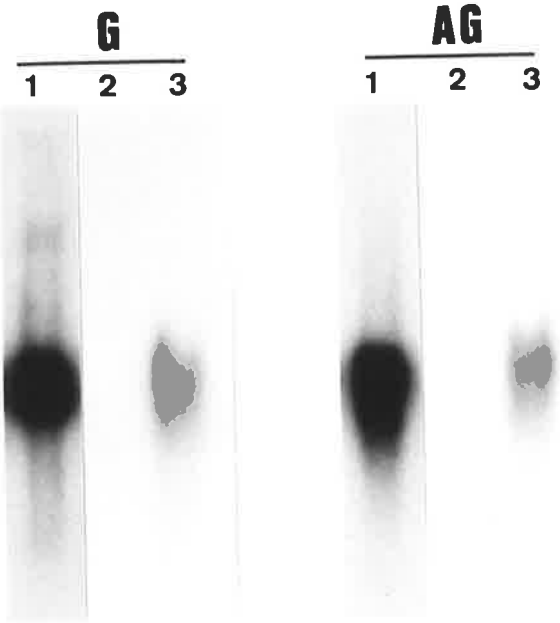
Thus, HDV RNA replication and processing was independent of HBV. However, as the levels of HDV RNA and HDAg were much lower in the G2δ6 cell line in comparison to the H1δ8 and H1δ9 cell lines, it is possible that HBV stimulates HDV replication either directly or indirectly.

Fig 7.14 Patterns of HDAg expression in G2δ6 cells stained with a . FITC-conjugated human anti-HD.
Top nucleolar.
Middle nucleoplasmic and nucleolar.
Magnification x2400

Fig 7.15 (Bottom) Northern blot comparison of RNA extracted from HepG2, G2δ6 and H1δ9 cells after hybridisation with ^{32}P-labelled riboprobes to detect genomic (G) or antigenomic (AG) HDV RNA. Lane 1, 1.0μg of total RNA extracted from H1δ9 cells; lanes 2 and 3, 20.0μg of total RNA extracted from HepG2 and G2δ6 cells respectively.






G
1 2 3
AG
1 2 3

7.3H(b) HDV Encapsidation and Secretion.

Since they are derived from HH1 cells, the H1δ8 and H1δ9 cell lines synthesise all three HBsAg proteins [see Chapter 4.3 D(a)], and therefore contain all the HBV-encoded envelope proteins detected in the HD virion (Bonino et al, 1986). However, Northern blot hybridisation analysis failed to detect encapsidated HDV RNA in the culture fluids even after 1000 fold concentration, or after the cells were grown in calcium-free DMEM, a medium known to stimulate the synthesis and secretion of HBsAg in HH1 cells [see Chapter 4.3 D(a)].

7.4 DISCUSSION.

7.4A HDV RNA REPLICATION IN THE H1δ8 AND H1δ9 CELL LINES.

High levels of monomeric, dimeric and trimeric HDV RNA of both polarities, and two polyadenylated, subgenomic RNAs of antigenomic sense were detected in the H1δ8 and H1δ9 cell lines although the DNA expression plasmid was designed to produce trimeric HDV RNA of antigenomic sense only. Furthermore, as HDV RNA was synthesised in the presence of actinomycin D, it is likely that these cell lines provide a convenient model for HDV RNA replication. Since many of the HDV RNA species were detected as a doublet, suggestive of linear and circular forms, these findings are consistent with a rolling circle mechanism of replication that has been proposed for viroids (Branch and Robertson, 1984) and more recently for HDV (Chen et al, 1986). In spite of this, significant levels of the anticipated HDV dsRNA species were not detected in the H1δ9 cells by immunostaining. This result is consistent with previous observations (Gowans et al, 1987; 1988), and suggest that the dsRNA form is likely to be only a minor component of total HDV RNA.

7.4B THE IDENTITY OF THE HDV RNA REPLICASE.

Replication-competent RNA animal viruses generally use virus coded enzymes for genome replication that may or may not be packaged (White and

Fenner, 1986). However, HDV RNA has insufficient coding capacity for a polymerase (Wang et al, 1986) and a RNA-RNA polymerase activity was not detected in purified virus preparations (Hoyer et al, 1983). It was unclear how HDV overcame this problem. The data presented in this Chapter suggest that the cellular enzyme RNA Polymerase II is a prime candidate for the HDV RNA replicase.

The Role of HDAg.

The above results also confirm that HDAg is required for HDV RNA replication, and it has been shown for the first time that this function is not necessary for RNA transcription itself but is limited to events pre- and/or post-transcription. Since HDV RNA replication did not occur from a defective HDV cDNA containing a mutated HDAg gene but could be complemented in trans by the addition of a wild-type HDAg gene (Kuo et al, 1989), this suggests that HDAg is required for post-transcriptional events. However, in the above experiments and in natural infection, transcription of antigenomic HDV RNA from input genomic HDV RNA is likely to produce the mRNA for HDAg (see Chapter 1, Fig 1.5). Thus, HDAg synthesised de novo could fulfil post-transcriptional requirements at later stages in the cultured cell. It is possible that the only role for HDAg in HDV RNA replication occurs prior to transcription and may be a function of virion-derived antigen in natural infection. This role is likely to be targeting of HDV RNA to the cell nucleus (Glenn et al ,1990).

7.4C HDV-RELATED CYTOTOXICITY.

HDV has been postulated to have a direct cytotoxic effect on infected hepatocytes (Popper et al, 1983; Verme et al, 1983), although this may be limited to the acute infection (Colombo et al, 1983). The data presented in this Chapter support this latter hypothesis and raise the possibility that the changes in the H1δ8 and H1δ9 cells recorded during this study may provide a model for the progression of the cell-virus relationship in vivo. Extended doubling times and spontaneous cell death were features of H1δ8 and H1δ9

cells in early (but not later) passage numbers.

It is possible that this early cytoxicity may have resulted from expression of an HDV product because; (a) the parent HH1 cell line and other hepadnavirus-transfected cell lines held in the laboratory, including the DHBV-positive cell line described in Chapter 5, have never shown similar evidence of cell death; (b) similar cytotoxic changes were seen in HeLa and HepG2 cells transfected with HDV cDNA that expressed HDAg whereas clones that failed to express this antigen showed no cytotoxicity (see Chapter 6). Three of the potential mechanisms that may account for HDV-related cytotoxicity are consistent with the data presented in this chapter. These are; (i) a direct cytotoxic effect of HDAg-p24 (as is also suggested from the data in Chapter 6); (ii) competition for host cell enzymes (Gowans et al, 1987); (iii) binding of HDV RNA to cellular 7SL RNA (Negro et al, 1989b).

The loss of cytotoxicity following extended passage was also of interest. This change was not due to loss of integrated HDV cDNA as the Southern blot hybridisation profile remained unchanged throughout the observation period and reintroduction of G418 into the medium failed to change the characteristics of the H1δ9 cells in later passages. However the loss of cytotoxicity was accompanied by loss of multimeric HDV RNA and by increased appearance of HDAg-p27. It is unknown if these events are connected although it is possible that HDAg-p27 may increase the rate of "self"-cleavage. Published observations show an increased ratio of p27:p24 in liver samples from chronic- compared with acute- infection that are consistent with these results. HDAg-p27 may have a modulating effect on HDV-related hepatocyte injury and is preferentially packaged in virions from acute phase infected livers.

7.4D MECHANISM OF HDAg-p24 AND -p27 SYNTHESIS.

The larger HDAg-related polypeptide (p27) was only expressed in the H1δ8 and H1δ9 cell lines in which HDV RNA replication occurred, despite the fact that the A3 cell line contained the complete gene. This confirms that

occasional translational read-through of the amber stop codon at aa position 195 is not the mechanism for p27 expression, and that an event associated with genome replication is necessary. Recent data (Luo et al, 1990) suggests that the production of this larger polypeptide depends on a mutation in the HDAg gene within the stop codon for the 195 aa form of HDAg that occurs during HDV RNA replication and allows translation to continue to a downstream stop codon thereby producing a HDAg polypeptide of 214 aa. The results of this chapter are consistent with this proposal. The mechanism leading to this mutation is unclear although it may depend on the action of cellular RNA-unwindases (Luo et al, 1990).

7.4E HBV HELPER FUNCTION.

Although the H1δ8 and H1δ9 cells reproduced many of the events of HDV replication in hepatocytes and also synthesised the major-, middle- and large-HBsAg proteins, HDV was never detected in the cell culture fluid even after 1000-fold concentration. This suggests one of two possibilities; (i) that the HDV cDNA clone used in this study is defective, although a closely related HDV cDNA sequence (Kuo et al, 1988) was infectious when inoculated intrahepatically into chimpanzees (Sureau et al, 1989), (ii) that HBV-infected hepatocytes supply another, as yet undetermined, helper function(s) essential to complete the replication cycle.

Thus, although the H1δ8 cells synthesise a number of HBV-specific products, no evidence of HDV morphogenesis has been detected in these cells. It is possible that a HBV product, synthesised as a result of acute replication, is required for packaging and secretion. Experiments to investigate this possibility are currently ongoing.

CHAPTER 8.

CONCLUDING REMARKS.

8.1 INTRODUCTION.

The experiments described in this thesis examined factors affecting hepadnavirus and hepatitis delta virus replication, and viral gene expression *in vitro*, and attempts were made to relate these observations to *in vivo* infections. The significance of these results is discussed more fully below.

8.2 TRANSFECTION.

Transfection has become a powerful technique in the study of virus replication and has been used extensively to examine the mechanisms of hepadnavirus and HDV replication. This technique, which essentially by-passes the early stages of viral infection has, in this and other studies, permitted the *in vitro* synthesis of infectious hepadnaviruses indistinguishable to those produced *in vivo*. Furthermore, following mutagenesis of the viral DNA, transfection has revealed many of the molecular details of the hepadnavirus replication cycle. Since transfection methods played a central role in this thesis, the theory and a selection of the techniques available will be discussed in more detail.

Techniques.

Uptake and expression of exogenous DNA in mammalian cells is very inefficient as both nucleic acid and cell membranes are negatively charged. The transfection methods which have been developed improve this efficiency by overcoming this electrostatic repulsion to permit the two components to be brought into close proximity. The DNA is then internalised, a process that is often facilitated by osmotic shock, then migrates to the nucleus and is finally expressed.

One of the first transfection techniques described, and still the most

widely used, relies on the strong binding affinity that calcium phosphate-DNA precipitates have for cell membranes (Graham and van der Eb, 1973). Since then a number of other transfection techniques and transfection mediators have been developed including protoplast fusion, electroporation, microinoculation, DEAE Dextran and polybrene (reviewed by Spandidos and Wilkie, 1984). Although the technique of choice depends on the application, in this thesis preliminary experiments with the polybrene technique showed the best results and consequently this method was used in all the DNA transfection experiments. Polybrene is a polycation which is thought to bind DNA to cell membranes by forming a positively charged bridge between the two. Finally, a recent development (unfortunately occurring since the DNA transfections described in this thesis were performed) deserves special mention. Although it has been known for some time that DNA can be transferred using synthetic phospholipid vesicles (liposomes), these reagents have become commercially available only recently (lipofectin, BRL; Dotma, Boehringer). These reagents bear a net positive charge that enables them to bind nucleic acids; the complex then fuses with the cell membrane, thereby delivering a proportion of exogeneous DNA into the cytoplasm of the cell. In the future, liposomal reagents are likely to replace all other transfection methods because the method is simple, achieves very high transfection efficiency for both short and long term transfections (up to 90% of cells) and can also be used to transfect RNA, without the cytotoxicity associated with electroporation (previously the most widespread technique used for RNA transfection).

Short Term vs Long Term Transfection.

Transfection methods are generally designed (i) to provide rapid results without the need to select positive cells (short-term transfection) or (ii) to create new stable cell lines with permanently altered genotypes (long-term transfection). The high transfection efficiencies (at least 10% of total cells) required for short term transfection limits the techniques available, viz. DNA

transfer by electroporation, DEAE Dextran or liposomes. On the other hand, although stable transfection is not as dependent on high efficiency, it is necessary for transfected DNA to contain a selectable marker and to become integrated into chromosomal DNA. Since clonal isolation and expansion of stably transfected cells is time consuming (ca. 3 months) this technique is less commonly used than short-term transfection. Nevertheless, as stably-transformed cell lines permit more detailed studies of virus replication and antigen expression, this technique was chosen for all the DNA transfection experiments reported in this thesis. This choice has been vindicated as the analysis of HDAg in A3 cells and the effect of continued HDV RNA replication in the H1δ8 and H1δ9 cells would have been difficult or impossible to perform using short-term transfection.

8.3 HEPADNAVIRUS CULTURE AND GENE EXPRESSION *IN VITRO.*

8.3A INFECTION.

A major aim of this thesis was to cultivate HBV in continuous cell lines and to this end a number of different experimental procedures were evaluated. Some of the resultant protocols incorporated hormonal stimuli, and/or substances promoting gene deregulation or cellular differentiation, while others involved the manipulation of classic variables such as cell type, virus concentration, and adsorption time and temperature. However, despite these attempts, in vitro HBV infection was not achieved. Consequently, factors which regulate HBV protein synthesis were then examined in the hope that this might in itself provide clues to HBV cultivation in vitro. However, although these studies (i) discovered that (low) calcium profoundly affected levels of secreted HBsAg and HBV, (ii) confirmed that 5-azacytidine treatment induced HBcAg synthesis in TK4 B4 cells and (iii) showed that steroid hormones stimulated HBsAg secretion, manipulation of these factors still did not lead to successful cultivation of HBV in cell lines. Nevertheless, a recent preliminary report by Bchini et al (1990), using related techniques, described limited

success in infecting HepG2 cells with HBV in the presence of dexamethasone. Unfortunately it was difficult to compare this report with previous studies since details of reproducibility and the proportion of infected cells were not described. Furthermore it was apparent that infection was dependent on a very high multiplicity of infection, at least 100-fold that required for the infection of primary hepatocytes (Shimizu et al, 1986; Gripon et al, 1988; Rijntjes et al, 1988; Ochiya et al, 1989), and as such it is possible that the mechanism of virus uptake in this report differs to that occurring in vivo. It is possible that these authors reproduced an effect first described by Hirschman et al (1980) who, in an unconfirmed report, described a cytopathic effect and HBcAg synthesis in HeLa cells incubated with very high levels of purified HBV.

Thus reproducible hepadnavirus culture still relies on primary hepatocytes, which are not only difficult to obtain but also tedious to prepare and maintain. This system will have limited applications, and the need to develop a convenient culture system for HBV remains a pressing problem.

8.3B FUTURE PROSPECTS FOR HBV REPLICATION IN CONTINUOUS CELL LINES.

Studies presented here and elsewhere have indicated that two mechanisms responsible for hepadnavirus tissue- and species-specificity may also prevent the culture of HBV in vitro. The first mechanism (extracellular block) is due to the lack of expression of appropriate receptors or the failure to complete the events subsequent to the formation of cccDNA, and appears to be dependent on the state of differentiation of the cells. The second mechanism (intracellular block) is dependent on hepatocyte-specific factors and results in the inability of the host cell to transcribe the 3.5kb "pregenomic" hepadnaviral RNA. Since the latter block can be overcome in the well differentiated human hepatoma cell lines (HepG2, HuH 6 and HuH 7; see Chapters 3-5), these cells are the logical target for cultivation of HBV in vitro. However, before this aim can be fulfiled, a means must be found to regenerate or increase the expression of an active hepadnavirus receptor on these cells.

This might be achieved by reversal of dedifferentiation and the consequential gain of receptor expression. However, this may be accompanied by a change in growth characteristics, in particular the cells may either stop dividing or even die. Thus, an alternative solution may be provided by the identification and cloning of the gene for the hepadnavirus cellular receptor that could be expressed (by transfection) in one of the differentiated hepatoma cell lines and this new cell strain subsequently used for infection with HBV. Precedents for this are well established as this approach has been used to induce the synthesis of poliovirus- (Mendelsohn et al, 1986) and human immunodeficiency virus- (Maddon et al, 1986) receptors that led to the infection of previously-refractile cell lines.

8.4 HEPATITIS DELTA VIRUS.

8.4A CELL SPECIFICITY.

In a similar manner to HBV, in vitro replication of HDV relies on primary hepatocyte cultures (Taylor et al, 1987), but, in contrast to HBV, the replication block in continuous cell lines appears to be restricted to a lack of the receptor, as HDV RNA replication can occur in non-hepatocyte cell lines of both human and animal origin. Since co-expression of HBsAg and HDV RNA has not been tested in these cell lines, it remains possible that another block prevents assembly and/or secretion of mature HDV particles. No data are available on the nature of the HDV receptor nor on the cellular binding protein on the HD virion envelope. Furthermore, either or both may differ from those used during HBV infection since (i) the HBsAg-polypeptide composition of the HDV envelope differs markedly to that of HBV, (ii) HBsAg-enveloped HDV can cross the species barrier and infect woodchucks, (iii) although WHV gains entry to extrahepatic sites in WHV-infected woodchucks, (WHsAg-enveloped) HDV infection was restricted to the liver (Negro et al, 1989a). Fortunately, as for HBV, transfection has provided the means to study many of the molecular details of HDV replication.

8.4B HBV HELPER FUNCTION.

The work presented in this thesis and elsewhere has shown that HDV RNA replication can be initiated from either multimeric HDV cDNA or from full- or multimeric-length HDV RNA transcribed in vitro. Furthermore since this replication could occur in the absence of HBV, it has been suggested that the helper function provided by HBV may be limited to the supply of a HBsAg envelope. Nevertheless in this study, expression of HBsAg in cells which support HDV RNA replication was insufficient for HDV secretion into the culture fluid, suggesting initially that other HBV-specific functions are required or that the HDV cDNA clone used in this study is defective. In situ hybridisation analysis of HDV-infected liver suggested that detectable antigenomic sense HDV RNA was based paired to genomic RNA, whereas the bulk of genomic HDV RNA was present as single stranded RNA (Gowans et al, 1987; 1988). However, since antigenomic HDV RNA was the predominant HDV RNA species detected in the H1δ8 and H1δ9 cell lines and since the ratio of genomic:antigenomic HDV RNA differed markedly to that found in naturally-infected liver, this may account for the lack of secretion. Thus although the mechanism(s) ensuring that genomic rather than antigenomic HDV RNA is packaged is likely to depend primarily on an interaction of HBsAg and/or HDAg with packaging signal(s) unique to genomic sense HDV RNA, it is possible that in the H1δ8 and H1δ9 cell lines, genomic sense HDV RNA may be unavailable for envelopment and secretion.

8.4C PROPERTIES OF HDAg.

Physical Properties.

In this study, expression of the HDAg gene in HepG2 cells produced a polypeptide (p24) with a similar electrophoretic mobility to the smaller of the two HDV-specific polypeptides (p24 and p27) detected in infected sera. This recombinant antigen species was found to have conformationally-dependent epitopes, could self-assemble into a complex with an S value similar to that of 22nm HBsAg particles, and had a density in $CsCl_2$ similar to that of HDAg

extracted from HDV-infected liver.

RNA Binding.

It was shown previously that HDAg p27 had the ability to bind RNA. The study described in this thesis has extended this observation to HDAg p24 and showed that the reaction was specific for HDV RNA. This binding property may play a critical role in the early stages of the HDV replication cycle (see below).

Cytotoxicity.

In contrast to HBV infections, HDV replication itself appears to be directly cytopathic at least in the acute phase. In the work described in this thesis, cytotoxicity was restricted predominantly to early passage number H1δ8 and H1δ9 cells when HDAg p24 was the sole or predominant viral antigen species. In contrast, the moderation of cytotoxicity that occurred in late passage number H1δ8 and H1δ9 cells was associated with a loss of multimeric HDV RNA, appearance of nucleoplasmic HDAg and with the appearance of HDAg p27, thus increasing the proportion of p27:p24. It is possible that HDAg p27 may have a regulatory role in the HDV replication cycle, that by modulating HDAg p24-associated cytotoxicity, may be essential in establishing persistent infection. A similar ratio of p27:p24 has been noted in chronically-infected liver samples. These observations and this hypothesis are consistent with recent data which shows that HDV RNA encoding HDAg p27 is non-infectious (Taylor, 1990) and that HDAg p27 cannot support HDV RNA replication (Glenn, personal communication). Direct detection of HDAg p24 and p27 and HDV RNA in single cells of acute and chronically HDV-infected livers will help answer these questions.

8.4D HBV INTERFERENCE.

To date the mechanism leading to an interference with HBV replication, following superinfection with HDV, is unknown. This effect is not unique to HDV, as interference of HBV replication has also been observed following infection with HAV and HCV (Rizzetto et al, 1986). However, the degree of

interference differs considerably. The latter viruses lead, at most, to a 10-fold reduction in HBV replication by a mechanism that is probably mediated by interferon induction (Rizzetto et al, 1986). In contrast, during the acute phase of HDV superinfection, markers of HBV replication can become negative by conventional assays (Rizzetto et al, 1986). Although this HDV-related interference may in part be interferon-mediated, the profound yet transitory nature of this effect suggests that other factors contribute. It is possible that HDV-associated cytotoxicity which destroys HBV-producing cells provides an additional mechanism. Experiments to resolve this issue are currently underway.

8.4E HDV RNA REPLICATION AND GENE EXPRESSION.

The data presented in this thesis are consistent with the hypothesis that HDV RNA replication occurs by a rolling circle mechanism similar to that proposed for viroids. Moreover it was shown that this reaction is likely to be catalyzed by host-derived RNA polymerase II and thus HDV represents the first animal RNA virus to be identified that relies solely on host-derived enzymes for genome replication. Although a ds RNA species was undetected by immunofluoresence in the H1δ8 and H1δ9 cell lines, as RNA directed RNA replication must produce at least some ds RNA, it is possible that epitopes which occur in circular ds RNA species are not recognised by the antibody used.

Role of HDAg.

Although this and other studies showed that HDAg is essential for HDV RNA replication, this study provides evidence that this requirement is limited to targeting the input HDV RNA molecule to the host cell nucleus. In natural infection this function is likely to be supplied by HDAg packaged in the virion. This property of HDAg may be facilitated by the HDV RNA-specific binding properties demonstrated in this study for HDAg p24. Nevertheless, the possibility that HDAg is also required in subsequent steps of HDV RNA replication such as cleavage and/or ligation in vivo (Taylor 1990) cannot be

excluded at this stage. However, since HDV RNA transcription per se does not require HDAg, de novo antigen could fulfil these latter stages.

Although the mechanism of nuclear targeting in viroid infection is yet to be determined (R. Symons, personal communication), since viroid-coded proteins are unknown, this is likely to be a host cell function and may explain the strict host specificity of these agents. In contrast, this study implies that in HDV infection, nuclear targeting is performed by HDAg. Thus HDV can cross the species barrier to infect humans, chimpanzees and woodchucks, and may ultimately be restricted in its host range by its hepadnavirus envelope.

One of the more intriguing findings of this study was the detection of two antigenomic sense poly(A)$^+$ RNA species in extracts from the H1δ8 and H1δ9 cell lines. While the translated product of the larger species is likely to be HDAg, the product (if any) of the smaller species and its role (if any) in the HDV replication cycle remains to be elucidated.

8.4F REPLICATION STRATEGY OF HDV.

In an attempt to assimilate the data presented in this thesis with data from other studies, the following model is proposed to account for some aspects of the HDV replication cycle.

1. Virus entry into a susceptible HBV-infected hepatocyte is followed by uncoating and transport of the virion RNA to nucleus (facilitated by HDAg).
2. Replication of HDV RNA by a double rolling circle mechanism, during which the mRNA for HDAg is synthesised (see Fig 1.5). Although this step is catalysed by host cell RNA polymerase II, HDAg may be required for post-transcriptional steps such as cleavage and ligation. Since genomic HDV RNA is much more abundant than antigenomic HDV RNA it is likely that transcription from antigenomic to genomic occurs at greater efficiency than the converse leading to a large excess of genomic sense RNA.

3 Initally HDAg-p24 is produced exclusively, but as a result of RNA-RNA replication and a specific point mutation, HDAg-p27 is produced at a later stage. This has the effect of reducing the rate of HDV replication leading to virus persistence.

4. The majority of antigenomic HDV RNA is base paired (in replicative complexes) with genomic HDV RNA. The excess genomic HDV RNA, associates with HDAg, migrates to the cytoplasm, where it is enveloped with HBsAg and is finally exported as complete HDV.

8.5 CONCLUSION.

The last five years have been very productive in the study of both HBV and HDV such that current knowledge of the molecular biology of both of these viruses is now at an advanced level and many of the details of virus-cell and virus-host interactions are well understood. Nevertheless, continued research is still required to uncover many of the more subtle properties of these fascinating viruses.

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APPENDIX 1

Comparison of Cell Culture Media.

COMPONENT	DMEM (mg/L)	CMRL 1969 (mg/L)	MCDB 151 (mg/L)	199 (mg/L)
L-Alanine	-	25.00	8.91	25.00
L-Arginine HCl	84.00	58.00	210.0	70.00
L-Asparagine H_20	-	-	15.01	-
L-Aspartic acid	-	30.00	3.99	35.00
L-Cysteine HCl H_2O	-	100.0	42.04	0.11
L-Cysteine 2HCl	62.60	20.00	-	26.00
L-Glutamic Acid	-	67.00	14.71	75.00
L-Glutamine	584.0	200.0	877.2	100.0
Glycine	30.00	50.00	7.51	50.00
L-Histidine HCl H_2O	42.00	16.20	16.77	21.88
L-Hydroxyproline	-	10.00	-	10.00
L-Isoleucine	104.8	20.00	1.97	20.00
L-Leucine	104.8	60.00	65.58	60.00
L-Lysine HCl	146.5	70.00	18.27	70.00
L-Methionine	30.00	15.00	4.48	15.00
L-Phenylalanine	66.00	25.00	4.96	25.00
L-Proline	-	40.00	34.53	40.00
L-Serine	42.00	25.00	63.06	25.00
L-Threonine	95.20	30.00	11.91	30.00
L-Tryptophan	16.00	10.00	3.06	10.00
L-Tyrosine (Disodium)	103.8	40.00	3.92	57.66
L-Valine	93.36	25.00	35.13	25.00
Ascorbic Acid	-	0.05	-	0.05
Biotin	-	1.00	0.0146	0.01
Calciferol	-	-	-	0.10
Choline Chloride	4.00	2.12	13.96	0.50
Folic Acid	4.00	1.00	0.794	0.01
Inositol	7.00	2.00	18.02	0.05
Menadione	-	-	-	0.016
Niacinamide	4.00	1.00	0.0366	0.025
Nicotinic Acid	-	-	-	0.025
D-Pantothenic Acid	4.00	1.00	0.238	0.01
p-Amino Benzoic Acid	-	0.05	-	-
Putrescine	-	-	0.161	-
Pyridoxal HCl	4.00	1.00	-	0.025
Pyridoxine HCl	-	-	0.0617	0.025
Riboflavin	0.40	1.00	0.0376	0.01
Thiamine HCl	4.00	1.00	0.337	0.01
Vitamin A Acetate	-	-	-	0.14
Vitamin B_{12}	-	-	0.407	-
Calcium Chloride (Anhyd)	**200.0**	**200.0**	**4.40**	**200.0**
Cupric Sulphate	-	-	0.0025	-
Ferric Sulphate $7H_2O$	-	-	0.417	-
Ferric Nitrate $9H_2O$	0.10	-	-	0.72
Magnesium Chloride	-	-	0.122	-
Magnesium Sulphate $7H_2O$	200.0	200.0	148.1	200
Potassium Chloride	400.0	400.0	111.8	400.0
Sodium Acetate	-	-	-	50.00
Sodium Chloride	6400	8000	7559	4500
Sodium Phosphate (Monobasic/Anhydrous)	109.0	60.00	-	125.0
(Dibasic/Anhydrous)	-	180.0	282.9	-
Zinc Sulphate $7H_2O$	-	-	0.863	-

APPENDIX 1 (cont)

Comparison of Cell Culture Media (cont)

COMPONENT	DMEM (mg/L)	CMRL 1969 (mg/L)	MCDB 151 (mg/L)	199 (mg/L)
Adenine HCl	-	-	30.89	-
Adenine Sulphate	-	-	-	10.00
Adenosine Triphosphate	-	-	-	1.00
Adenylic Acid	-	-	-	0.20
Cholesterol	-	-	-	0.20
Deoxyribose	-	-	-	0.50
D-Glucose	**4500**	**1000**	**1081**	**1000**
Glutathione	-	0.05	-	0.05
Guanine HCl	-	-	-	0.30
HEPES	5958	5958	5958	5958
Hypoxanthine	-	-	-	0.30
Phenol Red	15.90	10.00	1.242	21.30
Ribose	-	-	-	0.50
Pyruvic Acid	110.0	-	55.02	-
Thioctic Acid	-	-	0.206	-
Thymidine	-	-	0.0727	-
Thymine	-	-	-	0.30
Uracil	-	-	-	0.30
Xanthine	-	-	-	0.344
Sodium Bicarbonate	3700	3200	1176	2200
pH at 25°C	7.0 ± 0.2	7.2 ± .02	7.5 ± 0.5	6.9 ± 0.3
Osmolarity (mOsm/Kg H_2O)	331 ± 5%	321 ± 5%	325 ± 5%	291 ± 5%

APPENDIX 2

Supplementation of MCDB151 to Prepare LHC-4 Medium.

Calcium Chloride	12mg/litre (Final concentration 16.4mg/L).
Trace Elements	1ml/litre of a mixture containing:

Selenious Acid	$3x10^{-5}$ M
Ammonium Hepta Molybdate	$1x10^{-6}$ M
Ammonium Metavandate	$5x10^{-6}$ M
Nickel Sulphate	$5x10^{-7}$ M
Stannous Chloride	$5x10^{-7}$ M
Sodium Metasilicate	$5x10^{-4}$ M
Manganese Chloride	$1x10^{-6}$ M

Epidermal Growth Factor	5µg/litre (Collaborative Research).
Bovine Insulin	5mg/litre (Sigma).
Human Transferrin	10mg/litre (Sigma).
Hormone Mixture	1ml/litre of following mixture:

Ethanolamine (Unilab)	$5x10^{-4}$ M
Hydrocortisone (Sigma)	$5x10^{-4}$ M
3,3',5-Triiodothyronine (Sigma)	$1x10^{-6}$ M
Phosphoethanolamine (Sigma)	$5x10^{-4}$ M

Bovine Pituitary Protein	35mg/litre of aqueous extract.
DDW	ca. 0.1 volume, untill osmolarity is reduced from 330 to 310 mOsm/Kg H_2O.

APPENDIX 3

Salt solutions used for endothelial cell culture

Hank's Basal Salt Solution

(Ca^{++} and Mg^{++} free)

NaCl	8.00 grams
KCl	0.40 grams
$NaHPO_4.2H_2O$	0.06 grams
KH_2PO_4	0.06 grams
Dextrose	1.00 grams
$NaHCO_3$	0.35 grams
Phenol Red	0.02 grams
DDW	to 1 litre

pH to 7.2, Sterilize by autoclaving.

Earls Salts

NaCl	6.80 grams
KCl	0.40 grams
$CaCl_2$	0.02 grams
$MgSO_4.7H_2O$	0.10 grams
$NaHPO_4.2H_2O$	0.13 grams
Dextrose	1.00 grams
Hepes	8.00 grams
Phenol Red	0.05 grams
DDW	to 1 litre

pH to 7.2, Sterilize by 0.2µm membrane filtration.

APPENDIX 4

BACTERIAL CULTURE MEDIUM

2xYT Medium

Tryptone	16 grams
Yeast Extract	10 grams
NaCl	10 grams
DDW	to 1 litre

pH to 7.4 and sterilize by autoclaving

L Broth

Tryptone	10 grams
Yeast extract	5 grams
NaCl	10 grams
DDW	to 1 litre

pH to 7.4 and sterilize by autoclaving

2YT Agar and L Agar

2YT Agar and L Agar prepared by the addition of 2% powdered Agar (Difco)

Super Broth

Part A

Tryptone	12 grams
Yeast Extract	24 grams
Glycerol	5 mls
DDW	to 900 mls

Part B

KH_2PO_4	11.5 grams
K_2HPO_4	62.5 grams
DDW	to 500 mls

Sterilize both by autoclaving. For use mix 900 mls of Part A with 100 mls of Part B

APPENDIX 5.

Reaction Buffers used for Restriction Enzyme Digestions.

LOW SALT (1OX)

NaCl	0
Tris-HCl (pH 7.5)	100mM
$MgCl_2$	100mM
Dithiothreitol	10mM

Used for: BglII, HpaII, KpnI, MspI, SacI, SacII.

MEDIUM SALT (1OX)

NaCl	500mM
Tris-HCl (pH 7.5)	100mM
$MgCl_2$	100mM
Dithiothreitol	10mM

Used for: AvaI, BamHI, BglI, HindIII, PstI, PvuII.

HIGH SALT (1OX)

NaCl	1000mM
Tris-HCl (pH 7.5)	500mM
$MgCl_2$	100mM
Dithiothreitol	10mM

Used for: EcoRI, NcoI, PvuI, SalI, XbaI, XhoI.

SmaI BUFFER (1OX)

KCl	150mM
Tris-HCl (pH 8.0)	150mM
$MgCl_2$	60mM
2-mercaptoethanol	60mM
Bovine serum albumin	1mg/ml

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SIMULTANEOUS EXPRESSION FROM cDNA OF MARKERS OF HEPATITIS DELTA VIRUS AND HEPADNAVIRUS INFECTION IN CONTINUOUS CELL LINES

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ABSTRACT

Three continuous cell lines were developed that expressed HDV antigen (HDAg) after transfection with hepatitis delta virus (HDV) cDNA. One cell line containing only the gene for HDAg showed nucleolar localisation of HDAg, whereas two cell lines containing a trimeric form of full-length HDV cDNA showed both nucleolar and nucleoplasmic HDAg. The cell lines containing the trimeric HDV cDNA also supported HDV RNA replication and expressed both HDAg-related polypeptides p24 and p27. In an effort to produce infectious HDV particles, one of these cell lines was transiently transfected with duck hepatitis B virus (DHBV) DNA; although DHBV antigens were expressed and simultaneous replications of HDV was occurring, inoculation of the resultant cell culture medium failed to produce HDV infection in DHBV carrier ducks.

INTRODUCTION

Hepatitis delta virus (HDV) is a unique RNA animal virus with a single stranded circular genome reported to be approx 1.7 kb (Kos et al, 1986; Wang et al, 1986). The genome and a HDV- encoded antigen (HDAg) are enveloped by a hepadnavirus surface antigen envelope (e.g. HBsAg) to form the complete virion. Like hepatitis B virus (HBV), the internal components are only detected after detergent disruption, but unlike HBV, HDV has no discrete capsid and the virion structure is still unknown. HBsAg provides the envelope in natural infection (Rizzetto et al, 1983) but in experimental infection, woodchuck hepatitis virus (WHV) can also provide this function (Ponzetto et al, 1984). Thus, HDV is a defective virus which is dependent on hepadnavirus coinfection for expression and replication. However, it is still unknown if the extent of the helper function is restricted to providing an envelope or extends to other functions.

Two reports have suggested limited HDV replication in duck hepatitis B virus (DHBV) carrier ducks (Forzani et al, 1988; Freiman et al, 1988) but other workers including ourselves have been unable to confirm these observations. These results are enigmatic since after transfection with HDV cDNA, HDV RNA replication occurs in a wide range of cell types either with or without hepadnavirus help (Kuo et al, 1989; Macnaughton, unpublished). In direct contrast, hepadnaviruses have a strict requirement for primary hepatocytes in true infection, or for continuous lines of hepatoma cells which support the later stages of virus replication after cDNA transfection.

It was proposed that HDV uses a rolling circle mechanism for genome replication (Chen et al, 1986) which occurs in the nucleus of the infected cell (Gowans et al, 1988), and it has also been reported that HDAg is essential for HDV RNA replication (Kuo et al, 1989). HDAg in liver and serum is composed of two related polypeptides, p24 and p27 and although it is still unclear how these are generated, it appears that p24 is a truncated version of p27 (Weiner et al, 1988). Details of the virus replication strategy are not yet known, and we have therefore developed continuous cell lines in which HDV RNA replication is self-sustaining to allow investigation of the mechanism (Macnaughton et al, 1990b).

In this paper, we compare three continuous cell lines which express HDAg and/or replicate HDV RNA, and

report on our efforts to produce HDV with a DHBsAg envelope by transfection of DHBV DNA into one of these cell lines.

METHODS

Plasmid construction

The plasmid construction has been described in detail (Macnaughton et al, 1990a, 1990b). Briefly, a trimeric form of HDV cDNA or a monomeric copy of the HDAg gene (ORF 5) was inserted into the unique Bam H1 site of the retrovirus expression vector pRSV009 such that antigenomic HDV RNA would result from transcription. The predicted order of the open reading frames (Wang et al, 1986) in antigenomic RNA transcribed from the trimeric form was 5'–ORF 8, ORF 7, and ORF 5–3'. The retrovirus control elements direct the synthesis and processing (capping and polyadenylation) of the mRNA transcript.

Development of continuous cell lines

Continuous lines of HBsAg-positive or -negative human hepatoma cell lines were transfected with the above vectors, which also contain a Neomycin resistance gene. The cells were selected in Geneticin G418-containing media (Macnaughton et al, 1990a, 1990b), single cells were clonally amplified and stable continuous cell lines developed. Northern blot and immunoblotting analyses were performed on different cell passage numbers as described (Macnaughton et al, 1990a, 1990b).

Transient transfection with DHBV DNA

H1δ9 cells (Macnaughton et al, 1990b) at passage #20 and #46 were transfected with DHBV DNA using lipofectin. Briefly, coverslip cultures in 24 well microtitre plates were washed in DMEM+ 2% FCS, then 10µl of a solution containing 1µg of a dimer of DHBV DNA (a gift from John Pugh) in pSV2Neo and 5µg lipofection solution (BRL) was added in 500µl of DMEM and incubated overnight at 37°C. The medium was replaced and both the cells and the medium harvested 3-7 days later.

The cells were fixed in ice cold acetone and stained by immunofluorescence for DHBcAg, DHBsAg and DHBV preS antigens, essentially as described (Macnaughton et al, 1990a). The anti-DHBc and the anti-DHB preS were gifts from Bill Mason and Anna O'Connell. The culture medium was inoculated into DHBV-carrier ducks as described (Qiao et al, 1990).

RESULTS

HDV-associated stable cell lines

Although the HDAg gene was expressed in HeLa and human hepatoma cells after transfection with either trimeric or subgenomic HDV cDNA, HeLa cell clones could not be expanded suggesting that HDAg may be cytotoxic in these cells (Macnaughton et al, 1990a). Properties of the recombinant HDAg (recHDAg) expressed in one of the HepG2- derived cell lines (A3 cells) have been described (Gowans et al, 1990; Macnaughton et al, 1990a). The same expression vector containing a trimeric form of HDV cDNA (Wang et al, 1986; Macnaughton et al, 1990b) was used to transfect HH1 cells, a cell line cloned from the original PLC/PRF/5 human hepatoma cells (Fowler et al, 1983). A few clones were developed, including the H1δ8 and H1δ9 cells described in this report. Details of the H1δ9 cells have been described previously (Macnaughton et al, 1990b). The three cell lines A3, H1δ8, H1δ9 have expressed HDAg stably for more than 12 months.

Expression of HDAg

The bulk of HDAg expressed in the A3 cell line was associated with nucleoli and was comprised of the smaller (p24) of the two HDAg-related polypeptides found in infected serum and liver (Macnaughton et al, 1990a). In contrast, the intranuclear distribution of HDAg in the H1δ8 and H1δ9 cells varied but showed nucleolar expression, nucleoplasmic expression or both. Furthermore, the pattern of HDAg-specific polypeptides present was related to the cell passage number. Initially both cell lines only showed HDAg p24, but in the H1δ8 cells HDAg p27 appeared at passage #11, and in the H1δ9 cells, at passage #22 (Table 1).

Synthesis of HDV RNA

As described above all three cell lines expressed HDAg, although only the A3 cell line was expected to do so, since the expected mRNA transcript in the H1δ8 and H1δ9 cells contained two upstream ORF's. As we have shown that internal initiation using the same mRNA did not occur in an *in vitro* translation system (Jilbert et al, 1990) it was to be expected (see below) that HDV RNA processing took place in the H1δ8 and H1δ9 cells. HDV RNA in the A3 cells was undetected by Northern blot analysis despite the demonstrated production of HDAg, whereas high levels of both genomic and antigenomic HDV RNA were detected in the H1δ8 and H1δ9 cells. In the H1δ8 cells, the vast bulk of this RNA was genome length (1.7 kb) whereas in the H1δ9 cells, dimeric and trimeric forms constituted approximately 10-20% of total HDV RNA (Fig 1). Furthermore, a subgenomic polyA$^+$ antigenomic RNA was also detected in the latter cell line (Jilbert et al, 1990; Macnaughton et al, 1990b). These results suggest that HDV RNA replication was a feature of the H1δ8 and H1δ9 cells (Macnaughton et al, 1990b; Table 1).

DHBV DNA Transfection of H1δ9 cells

Although HDV RNA replication and HDAg expression were features of the H1δ8 and H1δ9 cell lines, secreted HDAg was not detectable in cell culture super-natants. An attempt was made to induce the secretion of complete HDV particles (which may in turn have the ability to superinfect DHBV-carrier ducks) by introducing a DHBV DNA dimer into the H1δ9 cells. Cells of passage #20 and #46 were used. The DHBV DNA construct used was known to be infectious, as a stable cell line containing integrated copies of this DNA continually secretes DHBV which is infectious in DHBV-susceptible ducks (Macnaughton, unpublished).

Three- and seven-days after transfection, the H1δ9 cell coverslips were fixed and stained for DHBV-coded antigens and the supernatants were pooled prior to inoculation into DHBV-carrier ducks. The immunofluorescence results were similar on both days; approximately 15% of cells expressed DHBsAg, 2-3% expressed DHB preS and 5% expressed DHBcAg (Fig 2). These results suggested that widespread expression of the DHBV genome, and possibly active replication of DHBV, was ongoing in a proportion of the H1δ9 cells. Experiments to determine if the supernatant from these cells contained secreted DHBV, HDAg and HDV RNA are currently ongoing.

Inoculation of DHBV carrier ducks

Five ml of pooled supernatant from the above cells was inoculated by the ip route into two- three-week old DHBV-carrier ducks. Serum samples were collected at 3-day intervals for a period of 4 weeks post-inoculation and tested for HDV RNA by Northern blot analysis, using strand specific RNA probes essentially as described (Negro et al, 1989). All the samples were negative, even after amplification by polymerase chain reaction (data not shown). Furthermore, DHBV DNA and DHBsAg levels in the carrier ducks remained unchanged when tested by dot blot and radioimmunoassay respectively (Qiao et al, 1990). These results suggested that HDV superinfection of the DHBV- carrier ducks did not take place.

DISCUSSION

The cell lines described in this paper represent useful models for HDAg expression, HDV RNA replication and pathogenetic mechanisms associated with persistent HDV infection. It was striking that the larger HDAg related polypeptide (p27) was only expressed in the cell lines in which HDV RNA replication occurred, despite the fact that the A3 cell line contained the complete gene. This suggests that occasional translational read-through of the amber stop codon at amino acid position 195 is not the mechanism for p27 expression, and that an event associated with genome replication is necessary. This mechanism has recently been reported (Luo et al, 1990).

We have shown previously that the H1δ9 cells exhibit markers of HDV replication but do not secrete HDV particles (Macnaughton et al, 1990b). In this study, we were unable to transmit HDV to DHBV-carrier ducks by inoculation of supernatants from cell cultures which were undergoing simultaneous replication of HDV and DHBV genome expression. Although neither HBsAg- nor WHsAg- enveloped HDV show as strict species specificity as HBV and WHV respectively, it is possible that HBsAg- or WHsAg-enveloped HDV used in past attempts to superinfect DHBV carrier ducks is not recognised by duck hepatocytes and thus is not internalised. We have therefore explored a different approach to transmit HDV to DHBV-infected ducks, by attempting to produce DHBsAg-enveloped HDV; our lack of success suggests either, that DHBsAg-enveloped HDV particles were not produced by the transfected culture, or that the block in HDV replication in DHBV carrier ducks may occur at a later stage in the virus replication cycle than hepatocyte attachment and uptake.

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FIGURE LEGENDS

Figure 1 Northern blot analysis of RNA extracted from HDV-positive stable cell lines and controls. Tracks 1-5 show genomic RNA and Tracks 6-8 antigenomic RNA. Track 1, HDV RNA purified from serum; Track 2, HH1 cell RNA; Tracks 3 and 6, A3 cell RNA; Tracks 4 and 7, H1δ8 cell RNA; Tracks 5 and 8, H1δ9 cell RNA. The arrows represent monomeric, dimeric and trimeric single-stranded HDV cDNA.

Figure 2 Expression of DHBsAg in H1δ9 cells after transfection with DHBV DNA. Three days after transfection, the cells were fixed in acetone and stained with rabbit anti-DHBsAg. Magnification x2000.

Table 1 - Characteristics of HDV-positive stable cell lines

	Cell line		
	A3	H1δ8	H1δ9
HDV RNA replication	-	+	+
HDAg p24	+	+	+
HDAg p27	-	+*	+*
HDAg Intracellular localisation	N	N, NP	N, NP
Associated cytotoxicity	-	+	+

* only after several passages in culture, see text for details

N - nucleolar, NP - nucleoplasmic

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CHARACTERIZATION OF A SUBGENOMIC HDV-SPECIFIC POLY(A)$^+$ RNA SPECIES ISOLATED FROM HUMAN HEPATOMA CELLS SUPPORTING HDV RNA REPLICATION.

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INTRODUCTION

The hepatitis delta virus (HDV) has been shown to possess a ca. 1700 base single-stranded negative sense RNA genome and encodes at least one viral-specific polypeptide, the hepatitis delta antigen (HDAg). HDAg can be detected in serum and liver as 24 and 27 kDa or 27 and 29 kDa polypeptide species (Bergmann and Gerin, 1986; Bonino *et al.*, 1986). Since no DNA intermediates have been detected in infected liver, HDV replication is considered to proceed via RNA-directed RNA synthesis, both genomic and antigenomic sense HDV RNA species have been detected in total RNA extracted from naturally infected liver tissue (Chen *et al.*, 1986) and from hepatoma cells stably transfected with trimeric HDV cDNA (Gowans *et al.*, 1989a; Macnaughton *et al.*, 1989a).

Expression studies using HDV cDNA (Gowans *et al.*, 1989b; Macnaughton *et al.*, 1989b; Weiner *et al.*, 1988) have identified ORF 5 (nt. 1619-953; Wang *et al.*, 1986) on the antigenomic strand as the coding sequence for HDAg. Thus, *in vivo* synthesis of HDAg requires transcription of antigenomic HDV mRNA and an 800 base subgenomic poly(A)$^+$ RNA species of this polarity isolated from HDV-infected chimpanzee liver (Chen *et al.*, 1986) may represent the HDAg mRNA. Supporting evidence for this includes; (i) the only identified HDV polyadenylation site is located immediately downstream of ORF 5 on the antigenomic strand (Sharmeen *et al.*, 1988); (ii) mutation of this polyadenylation site results in impairment of HDAg

production (Sen-Yung Hsieh, unpublished); and (iii) in cell free extracts HDV RNA can undergo site-specific cleavage downstream of the polyadenylation site (Sharmeen et al., 1988) that may liberate a polyadenylated HDV RNA species. However, the mechanism for synthesis of such a subgenomic HDV RNA species is not known.

This study was designed to examine the strategy for the expression of HDAg, since the specific mRNA has not yet been identified and also since, in a parallel study (Gowans et al., 1989a; Macnaughton et al., 1989a) the expression of HDAg was totally unexpected.

RESULTS

PLC/PRF/5 cells were transfected with trimeric HDV cDNA in the plasmid pRSV009 under control of the Rous Sarcoma Virus LTR (Fig. 1). Single genticin-resistant transfected cells were selected and one clone (H1δ9) expressed high levels of HDAg and both genomic- and antigenomic-HDV RNA (Gowans et al., 1989a; Macnaughton et al., 1989a). As HDV transcripts produced from the input DNA were designed to be of antigenomic polarity with ORF 8 and ORF 7 preceding ORF 5, the expression of HDAg particularly was a surprising result.

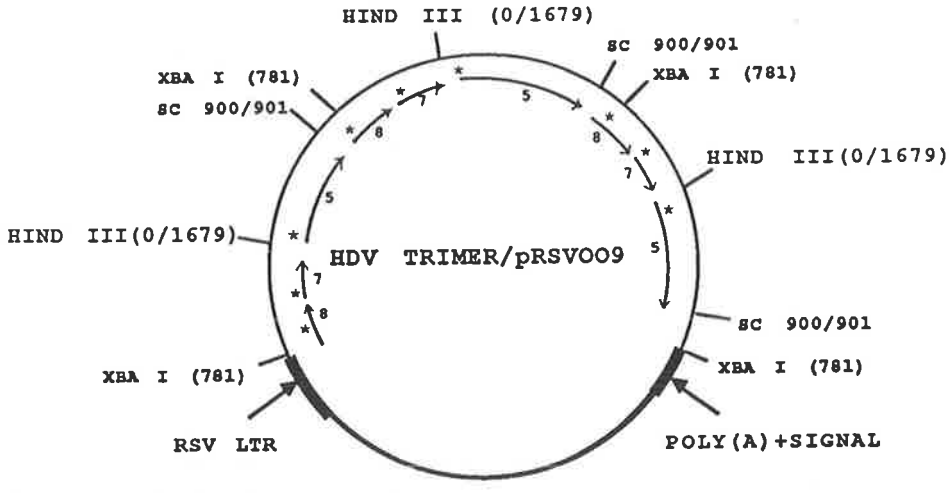

Figure 1. Plasmid pRSV009 containing a trimeric head-to-tail construct of HDV cDNA.

To investigate the mechanism of HDAg expression in general and in the H1δ9 cells, various constructs of HDV cDNA sequences obtained from the Chiron Corporation (Emeryville, CA) were subcloned into pGEM3 (Promega) and genomic- and antigenomic-HDV RNA species were transcribed using either SP6 or T7 RNA polymerase (Promega) for use in in vitro translation experiments (Table 1).

TABLE 1. Plasmid vectors used to transcribe strand-specific HDV RNA for in vitro translation experiments.

Plasmid	RNA Polymerase	Polarity of Transcript	First AUG (nt)[a]
TMδHindXbaA	SP6	+	ORF 5(1598)
TMδXbaHindA	T7	-	ORF 2(786)
TMδHindB	T7	+	ORF 5(1598)
TMδXbaA	SP6	+	ORF 8(731)
TMδXbaB	T7	+	ORF 8(731)

[a] nt = nucleotide position of first AUG in RNA sequence according to Wang et al., 1986.

The transcripts were synthesized with or without the addition of m7GpppG (Biolabs) to produce either capped or uncapped RNA and translated in vitro using a commercially available wheat germ lysate system (Amersham Int) (Fig. 2).

The results of these experiments showed that 24 kDa HDAg was produced from either capped or uncapped HDV RNA only when the HDAg gene (ORF 5) was positioned at the 5' end of the RNA i.e., when the HDAg gene provided the first AUG (nt. 1598) in the mRNA. Thus, within the limits of detection in the experiment, neither polyprotein processing, frame shifting nor internal initiation occurred during in vitro translation of RNA with ORF 8 (nt. 821-507) and ORF 7 (nt. 506-560) upstream of ORF 5.

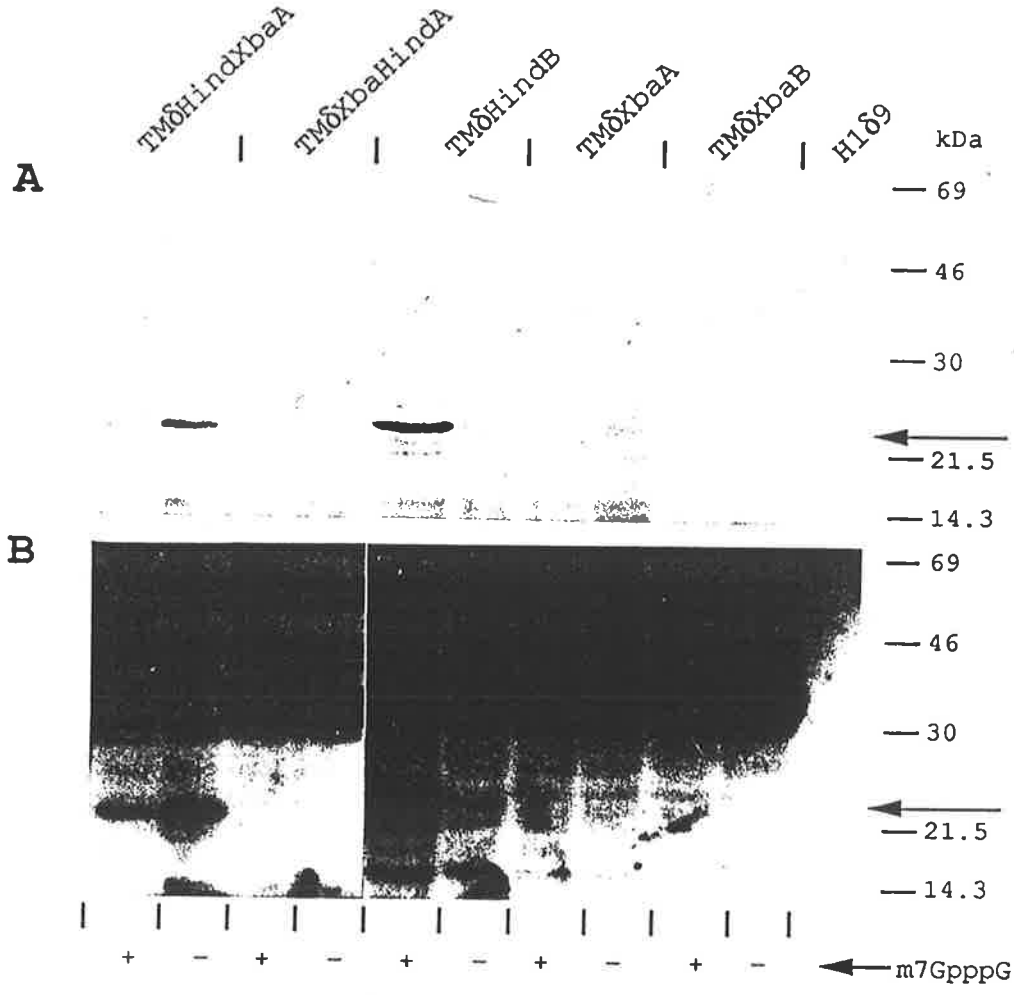

Figure 2. Autoradiographic and immunoblotting analysis of the products of <u>in vitro</u> translation of HDV RNA transcripts, generated from plasmid vectors shown above and described in Table 1. After separation by electrophoresis in 13% polyacrylamide and transfer to Immobilon (Millipore), ^{35}S-labeled protein products were visualized by autoradiography (A); or by immunoblotting (B) to detect HDAg in the same membrane. A lysate of H1δ9 cells was used as an internal 24 kDa HDAg marker (Arrow).

These results suggest that expression of HDAg in the H1δ9 cells was also most likely from mRNA with ORF 5 in the 5′ position, and as these results were at variance with the construction of the input cDNA template, this further suggested that a degree of HDV RNA processing took place within the cells.

Total RNA, poly(A)$^+$ RNA prepared by two passages through oligo(dT) cellulose, and poly(A)$^-$ RNA was then examined by Northern blot hybridization using strand-specific probes. A 900-1000 base antigenomic HDV RNA was detected only in the poly(A)$^+$ sample; 1900 copies were present per cell (Fig. 3, lane f) and represented 0.2% of H1δ9 cell poly(A)$^+$ RNA. No genomic sense HDV-specific poly(A)$^+$ RNA was detected in the H1δ9 cell RNA (Fig. 3, lane c). Poly(A)$^-$ RNA contained both genomic and antigenomic sense HDV RNA in both monomeric and dimeric forms, but contained no subgenomic HDV RNA species (Fig. 3, lanes b,e).

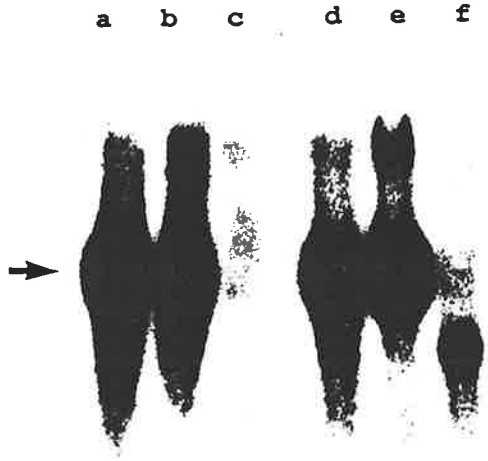

Figure 3. Northern blot hybridization using ^{32}P-labeled RNA to detect H1δ9 cell genomic (lanes a,b,c) and antigenomic (lanes d,e,f) HDV RNA; 25 µg of total cellular RNA (a,d); 25 µg of poly(A)$^-$ RNA (b,e); 5.5 µg of poly(A)$^+$ RNA (c,f). Genomic length HDV RNA is indicated (Arrow).

DISCUSSION

The detection of a subgenomic HDV-specific poly(A)$^+$ RNA species of antigenomic polarity in cells supporting stable HDV replication and HDAg production, supports the hypothesis that such an RNA might function as the template for HDAg production. In addition, the failure to detect HDAg after *in vitro* translation of HDV RNA with ORF 8 and ORF 7 preceding ORF 5, supports the evidence that expression of HDAg requires a specific (monocistronic) mRNA. The mechanism for the production of such an RNA transcript is unknown and experiments are currently

underway to map the 5′ end of this RNA species and to further purify the RNA and confirm its identity by *in vitro* translation.

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CELL CULTURE MODEL SYSTEMS TO STUDY HDV EXPRESSION, REPLICATION AND PATHOGENESIS

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INTRODUCTION

Details of the replication strategy of hepatitis delta virus (HDV) and expression of the virus structural antigen (HDAg) remain unresolved. In natural and experimental infection, replicative intermediates of HDV RNA consistent with a rolling circle mechanism of replication have been detected in RNA extracted from HDV-infected liver (Chen et al, 1986). Replicative intermediates have also been demonstrated in the nuclei of infected hepatocytes indicating that this is the probable intracellular site of HDV RNA replication, and a close association between HDV RNA and HDAg in infected cell nuclei was also noted (Gowans et al, 1987, 1988). A recent report showed that HDAg was essential for HDV RNA replication, as mutation of the HDAg gene resulted in a dramatic reduction in replication which was restored in trans by co-transfection with a wild type HDAg gene (Kuo et al, 1989).

HDV RNA of both genomic and antigenomic polarity has the ability to self-cleave and self-ligate in cell free extracts (Kuo et al, 1988; Sharmeen et al, 1988; Wu et al, 1989a, 1989b), one of several properties that HDV RNA shares with viroids (Branch et al, 1989). These properties of self cleavage and self ligation have the effect of reducing the range of enzymatic functions required for HDV RNA processing, provided that these properties also function in vivo. Thus one of the major unresolved issues in HDV RNA replication is the origin of the HDV RNA replicase.

Studies of the properties of HDAg have been impeded due to a scarcity of material. Although HDAg is usually found as two polypeptides in natural infection, [p24, p27 (Bergmann and Gerin, 1986); p26, p29 (Bonino et al, 1986)], expression of HDAg from cloned sequences usually results in production of the smaller polypeptide only (Weiner et al, 1988). Recombinant HDAg shows an unspecified RNA-binding activity (Chang et al, 1988) and neutralises human anti-HDV antibody activity (Gowans et al, 1989).

RESULTS

We have recently inserted either subgenomic - or genomic-length HDV cDNA into a Rous Sarcoma Virus expression vector which contains a neomycin resistance gene, and after transfection, prepared a number of genticin-resistant stable cell lines. These cell lines now provide models for study of (i) expression and properties of HDAg (ii) HDV RNA replication (iii) persistent infection and pathogenesis.

(i) Expression and Properties of HDAg

Clonal expansion of genticin-resistant cells transfected with the above vector containing the HDAg gene proved difficult. Several clones derived from HepG2 or HeLa cells initially synthesised HDAg but a progressive loss of cells which expressed HDAg was noted and expansion was impossible. These observations suggest that HDAg may have a direct cytotoxic effect. One clone of HepG2 cells that expressed intermediate levels of HDAg was successfully expanded and this cell line (A3) has been stable for 18 months.

Expression of HDAg was independent of the degree of cell confluency and was localised to the nucleolus. HDAg was not secreted. Immunoblotting detected a single polypeptide of 24kDa with a similar electrophoretic mobility to the smaller of the HDAg polypeptides present in HDV-positive sera (Macnaughton et al, 1989a). Storage of recombinant HDAg (rHDAg) produced a second polypeptide with a molecular weight of 20kDa (see Fig 2). Recombinant HDAg from the A3 cells sedimented in a broad peak with a sedimentation coefficient close to that of HBsAg 22 nm

particles; the S value of rHDAg was determined as 50S and that of HBsAg as 58S (Macnaughton et al, 1989a). The density of rHDAg was 1.19 g/cm^3 in cesium chloride (cf HBsAg 1.22 g/cm^3).

Analysis of rHDAg by HPLC in either PBS, 6M urea or 4M guanidine hydrochloride (GuHCl) showed that rHDAg was present in the A3 cells as a polymer with a molecular weight between $5x10^5$-$1x10^6$ Da. The monomeric form of recombinant HDAg was detected in fractions corresponding to 24kDa only when analysed by HPLC in the presence of 4M GuHCl. However, the bulk of rHDAg was immunologically non reactive unless the GuHCl was removed by dialysis prior to RIA to detect HDAg in the fractions (Fig 1). Thus, rHDAg contains conformational-dependent epitopes which are recognised by human anti-HD, and it is likely that native HDAg also contains conformational- dependent epitopes.

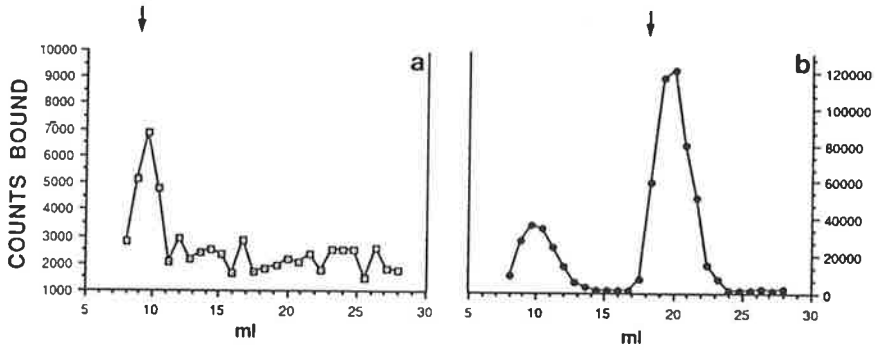

Figure 1. Analysis of recombinant HDAg solubilised and analysed in 4M guanidine hydrochloride (GuHCl) by HPLC. The fractions were tested for HDAg by radioimmunoassay before (a) or after (b) dialysis against PBS to remove the GuHCl. The arrow in (a) shows the position of the void volume ($2x10^6$ Da) and that in (b) shows the position of a marker polypeptide with a molecular weight of 25 kD.

The specificity of binding between rHDAg and HDV RNA was examined by incubating nitrocellulose strips, to which rHDAg was previously bound, with ^{32}P-labelled RNA. Genomic HDV RNA but not human papillomavirus RNA bound strongly to the rHDAg but not to other unrelated protein bands (Fig 2). We have also shown that antigenomic HDV RNA is also bound

to rHDAg with equal efficiency (data not shown). As HDAg has previously been shown to be a phosphoprotein (Chang et al, 1988), it is possible that phosphorylation represents a temporally controlled packaging signal for HDV RNA, as has been described for certain retroviruses (Leader and Katan, 1988).

Figure 2. The specificity of recombinant HDAg for HDV RNA. Recombinant HDAg was separated by SDS-PAGE, and transferred to nitrocellulose which was then cut into strips. Track 1, total protein stained by Coomassie blue; Track 2, Immuno blot using human anti-HD as primary antibody; Tracks 3 and 4, RNA binding assay with ^{32}P-labelled genomic HDV RNA; Tracks 5 and 6, RNA binding assay with ^{32}P-labelled human papillomavirus RNA.

We have also used rHDAg in a diagnostic RIA to detect anti-HD in HBsAg-positive patients and found complete concordance with a conventional assay using liver derived HDAg (Gowans et al, 1989). An indirect immunofluorescence assay to detect anti-HD using the A3 cells as substrate was also developed, and showed similar specificity and increased sensitivity to RIA using rHDAg.

(ii) HDV RNA Replication

HDV-specific RNA was undetectable in the above A3 cells which were transfected with the HDAg gene alone. In contrast, in a stable cell line (H169) derived from PLC/PRF/5 cells transfected with a trimeric form of HDV cDNA that was designed to synthesise antigenomic HDV RNA only, high levels of both genomic and antigenomic HDV RNA were detected (Fig 3). Furthermore, monomeric and trimeric

forms of both RNA species were present in the nucleus of these cells, whereas only monomeric forms were present in the cytoplasm.

Figure 3. Northern blot analysis of RNA extracted from Hlδ9 cells at Pass #20. Tracks (a) and (b) show antigenomic HDV RNA and Tracks (c-f) show genomic HDV RNA. Tracks (a) and (c) contain total cellular RNA while tracks (b) and (d) contain cytoplasmic RNA only. Track (e) contains HDV RNA extracted from the serum of a chronic carrier and Track (f) contains total Hep G2 (control) cell RNA.

As HDAg and HDV were undetectable in the culture supernatant, virion assembly and export were probably not occurring in this cell line. We were also unable to explain the presence of monomeric antigenomic HDV RNA in the cytoplasm of these cells as this does not appear to be mRNA (Jilbert et al, 1989). However, the bulk of the virus RNA was found in the nucleus of the cells by in situ hybridisation, a finding consistent with previous studies (Gowans et al, 1987; 1988).

In vitro transcription of HDV RNA using nuclear run-off assays with the HIδ9 cells showed that HDV RNA transcription was resistant to Actinomycin D indicating

that the input plasmid DNA was not the template. Further experiments demonstrated that HDV RNA transcription was inhibited by 1 ug/ml α-amanatin. A similar result was observed for HBV RNA transcribed from integrated HBV DNA in the same cell line (Fig 4). This result suggests that HDV RNA transcription and replication is performed by RNA polymerase II or by an enzyme with a similar sensitivity to α-amanatin. Although HDAg was previously shown to be essential for HDV RNA replication (Kuo et al, 1989) it is unclear what role this plays in the process.

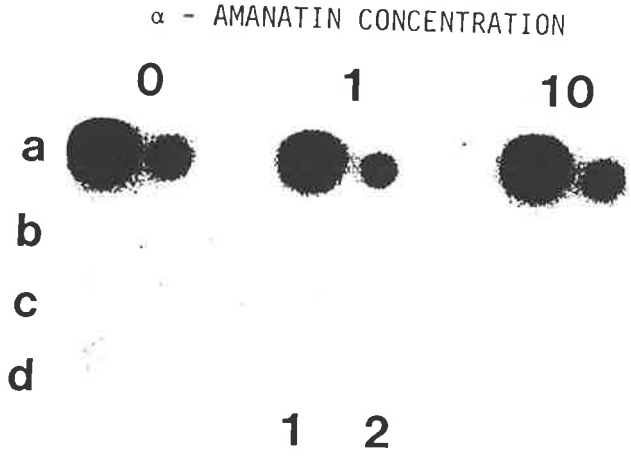

Figure 4. Nuclear transcription run-off analysis from HIδ9 cells. Cell nuclei were added to an in vitro transcription assay containing ^{32}P-UTP with zero (0), 1μg/ml (1) or 10μg/ml (10) α-amanatin respectively. The labelled products were then hybridized to nitrocellulose with bound normal cell DNA (a) or pGEM3 DNA (b) or HDV DNA (c) or HBV cDNA (d). The concentration of bound target was 2μg and 250 ng for cell DNA and 6μg and 1μg for other species in positions 1 and 2 respectively.

In summary, we have demonstrated that the HIδ9 cells support replication of both RNA strands of HDV, and that this process has properties that would be expected if the template were RNA and the enzyme involved were RNA polymerase II or an enzyme with similar sensitivity to α-amanatin. Further studies of this model are likely to

allow characterisation of these steps, and the mechanism of HBV dependency in greater detail.

(iii) HIδ9 Cells as a Model for Virus Persistence and Pathogenesis

The HIδ9 cell line has continued to synthesise high levels of HDV RNA for over one year. Initially, the cell line grew slowly (split ratio 1:3) but in the intervening period the doubling rate has increased (split ratio 1:10). This alteration in growth pattern has been accompanied by a change in the polypeptide profile of HDAg from a single polypeptide (p24) in Pass #8 to two polypeptides (p27, p24) in Pass #35 (Fig 5) and by an increase in the efficiency of HDV RNA processing that results in a much higher proportion of monomeric HDV RNA compared with dimeric and trimeric HDV RNA (Macnaughton et al, 1989b). Thus it is clear that HDV RNA replication can proceed whether expression of HDAg is limited to p24 or includes both species. Furthermore, these results suggest that HDAg (p24) or multimeric HDV RNA may have a direct cellular inhibitory function, a possibility that is consistent with the data in section (i) above.

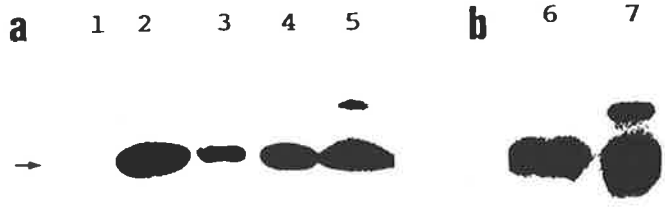

Figure 5. Immunoblot analysis of (a) HDAg expressed in continuous cell lines. Track 1; PLC/PRF/5 control: Track 2; A3 cell line: Tracks 3,4,5; Hlδ9 cell line at Pass #8, #22 and #35 respectively. (b) HDAg extracted from: Track 6; acute phase chimpanzee liver: Track 7; chronically infected human liver. The arrow shows the position of 24kDa.

We then examined naturally infected liver samples in an attempt to correlate an increased relative HDAg p27 content with later stages of infection. In the liver of a chimpanzee with acute HDV infection, the ratio of HDAg

p27:p24 was low (approx 1:40) whereas in a chronically infected human liver, the ratio of p27:p24 was closer to equimolar (1:3). In contrast, although we were unable to examine serum from the same chimpanzee, we and others have shown that in acute and chronic infection, the ratio of p27:p24 is equimolar. This indicates a positive selection for p27 in packaging in the acutely infected liver. It has been suggested previously that HDV may be directly cytopathic in acute infection but not necessarily in chronic infection (Colombo et al, 1983), and it is possible that p27 exerts a modulating effect that may provide a molecular basis for this hypothesis. Further experiments are in progress to further elucidate this relationship.

SUMMARY

Due to the transient nature of HDV replication in natural and experimental hosts and to the inability of the virus to replicate in easily handled cell lines, studies of the replication and expression of HDV have been impeded. These difficulties have been overcome by the development of the cell lines described in this paper.

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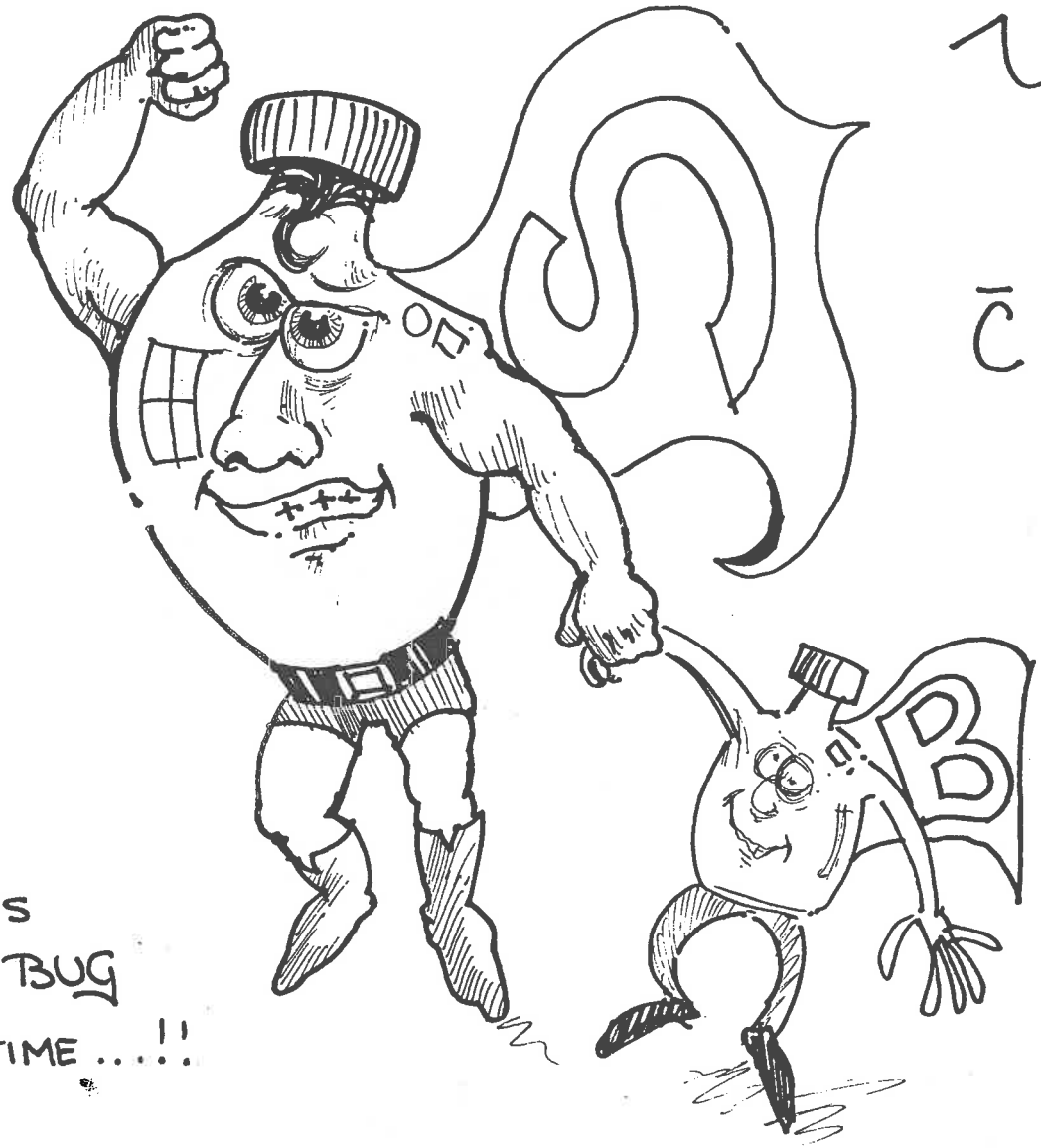

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