STUDIES ON THE PATHOGENESIS OF HEPATITIS DELTA VIRUS

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ERRATA

Designation of Restriction Enzymes: Throughout this thesis, restriction enzymes should be designated with roman numerals, e.g., Hind 111 should read Hind III. Similarly, DNA dependent RNA polymerase should read DNA dependent RNA polymerase II.

Page 45. 1st paragragh, 3rd sentence should read "The DNA concentration was determined by absorption spectrophotometry ..... a final concentration of 1 μg/μl."

Page 74. 3rd paragraph, 1st sentence should read "In one experiment performed over a 12 hr period with Hδ27 cells which were ca. 70% confluent when pulse-labelled, ... to ca. 60% of the untreated cultures."

CONTENTS

ABSTRACT

The primary aim of this study was to investigate the molecular basis of the cytopathic effect of hepatitis delta virus (HDV). HDV is a defective RNA virus that requires a helper function provided by hepatitis B virus (HBV) for its expression and replication. Natural infection with HDV occurs either as a coinfection with HBV, or superinfection of a HBV carrier and may result in fulminant, acute or chronic infection. Acute delta hepatitis results commonly in degenerative changes to the hepatocytes that include shrunken eosinophilic cytoplasm and pyknotic nuclei. The parenchyma is predominantly free of inflammatory cells, even in areas with significant histologic change, consistent with a direct cytopathic effect. In contrast, the histological features associated with chronic HDV infection are suggestive of a necro-inflammatory disease.

The virus-specific antigen, hepatitis delta antigen (HDAg), is expressed as two polypeptides (HDAg-p24 and HDAg-p27) that differ by the addition of 19 amino acids at the carboxy terminus of HDAg-p24 to create HDAg-p27. HDAg-p24 is expressed at high levels in hepatocytes during acute HDV infection, with only small amounts of HDAg-p27 being produced. As the HDV infection progresses to chronicity, the ratio of HDAg-p24:p27 in the liver is decreased. Furthermore, HDAg-p24 expression is necessary for HDV RNA replication, while HDV RNA replication is inhibited by the expression of HDAg-p27.

Thus, a major goal of this study was to investigate the proposal that HDAg-p24 is directly cytotoxic to hepatocytes during the acute phase of HDV infection, and to investigate the role of HDAg-p27 expression in the promotion of persistent infection. An in vitro cell culture model was developed to examine the direct effect of HDAg-p24

expression in stably-transfected eukaryotic cell lines. HDAg-p24 was shown to be directly cytotoxic when expressed under the control of the inducible human metallothionein (MTIIA) promoter in HepG2 and HeLa cells. In these cell lines, HDAg-p24 expression was associated initially with a significantly reduced rate of RNA synthesis followed by reduced rates of DNA synthesis, by a vast increase in cell doubling times, and degenerative cytological changes which resembled those seen in acute-HDV related hepatitis.

Site-directed mutagenesis was used to create HDV cDNA with the capacity to express the large HDAg polypeptide, HDAg-p27. The HDAg-p27 gene was also placed under the control of the MTIIA promoter and expressed in stably-transfected HeLa cells. In contrast to the effects of HDAg-p24 expression, HDAg-p27 expression resulted in a minimal effect on cellular RNA and DNA synthesis, no significant increase in cell doubling times, and normal cytology. Thus, the non-cytotoxic nature of HDAg-p27 and its ability to inhibit HDV RNA replication, are consistent with the proposal that HDAg-p27 expression may promote persistence.

These results were confirmed in a transient transfection system using the Cos7 cell line. In this system, transcription of a mutant form of HDAg mRNA in Cos7 cells that contained a two nucleotide deletion to create a novel stop codon and thus a truncated form of HDAg, was non-cytotoxic. This confirmed that HDAg-p24-related cytoxicity is due to the direct action of HDAg-p24 per se, and is not a result of events leading to the expression of HDAg-p24. Furthermore, it was shown that HDAg-p27 could modulate HDAg-p24 related cytotoxicity when coexpressed in the same cell.

It is possible that the transient interference of HBV replication following superinfection of a HBV carrier is a result of HDAg-p24-induced cytotoxicity during the acute phase of infection. The expression of high levels of HDAg-p24, but not HDAg-p27, was shown to inhibit the multiplication of both poliovirus and adenovirus. Thus, the mechanism of transient interference is not HBV-specific. During acute HDV infection, the high levels of HDAg-p24 expression may destroy HBV-producing cells, and this is reflected in a reduction of replicative markers of HBV in the serum and liver of infected patients.

Since HDAg-p27 is non-cytotoxic and may modulate cytotoxicity due to HDAg-p24, then continued expression of HDAg in chronic HDV infection may induce an immune response which is the cause of the continuing hepatitis. Consequently, the nature of the immune infiltrate in acute- and chronic- phase HDV-infected liver samples was examined by immunofluorescent staining. A dramatic decrease in the ratio of CD4+:CD8+ lymphocytes was observed in the persistently-infected liver samples compared with an acute phase sample. A high proportion of the CD8+ lymphocytes can be expected to represent cytotoxic T-lymphocytes (CTL). In addition, hepatic expression of HLA Class I antigens was detected in the majority of the persistently-infected liver samples. These results provide circumstantial evidence to suggest that HDAg may represent a target for CTL in persistent infection, although this finding will need to be confirmed.

On the basis of this study, a model is proposed for HDV pathogenesis. During the acute phase, HDAg-p24 represents the vast bulk of expressed HDAg, and this has a direct cytotoxic effect. The effect of HDAg-p24 is dose-dependent, so that it is likely that hepatocytes which express low levels of HDAg-p24 survive the cytotoxic action

of HDAg-p24. In addition, HBV replication is transiently suppressed as a result of the cytotoxic action of HDAg-p24 on HBV-producing cells. Thereafter, the ratio of HDAg-p24:p27 in the liver decreases leading to a reduced level of HDV RNA replication, and in turn, persistent infection. During persistent infection HDAg may constitute a target for CTL, and the action of these cells will determine the severity of ongoing hepatitis.

DECLARATION

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

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

Susan Henshall (nee Cole) Date 18/2/94

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ABBREVIATIONS

aa	amino acid
ALT	alanine aminotransferase
Anti-HD	antibody to hepatitis delta antigen
APS	ammonium persulphate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BSA	bovine serum albumin
CPE	cytopathic effect
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
DDW	double-distilled water
DTT	dithiothreitol
E.coli	Escherichia coli
FITC	fluorescein isothiocyanate
gp	glycoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HBS	HEPES-buffered saline; 20mM HEPES (pH 7.4), 150mM NaCl
HLA	human leukocyte antigen
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	isopropylthio-B-D-galactoside
kb	kilobase

kD	kilodalton
MCS	multiple cloning site
MOPS	3-[N-morpholino] propanesulphonic acid. MOPS buffer; 20mM MOPS, 50mM Na acetate, 100mM EDTA, pH 5.5-7.0
mRNA	messenger RNA
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline;150mM NaCl, 6mM K_2HPO_4, 2mM KH_2PO_4, pH 7.2
PVP	polyvinylpyrrolidone
RT	room temperature
SDS	sodium dodecyl sulphate
sol	solution
SSC	standard saline citrate; 150mM NaCl, 15mM Na citrate, pH 7.1
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
TBS	Tris-buffered saline; 25mM Tris-HCl (pH 7.4), 140mM NaCl, 3mM KCl
TCA	trichloroacetic acid
TE-8	10mM Tris-HCl (pH 8.0), 1mM EDTA
TEMED	tetramethylene-diamine
TN	10mM Tris-HCl (pH 7.4), 150mM NaCl
TNT	10mM Tris-HCl (pH 7.4), 150mM NaCl, 0.05% (v/v) Tween-20
tRNA	transfer RNA
ssDNA	salmon sperm DNA
UV	ultraviolet
Xgal	5-bromo-4-chloro-3-indolyl-B-D-galactosidase

CHAPTER ONE

INTRODUCTION

The work presented in this thesis is concerned predominantly with the pathogenesis of hepatitis delta virus (HDV) infection. However, due to the dependent nature of HDV on hepatitis B virus (HBV), I have included a brief overview of the biology of HBV where it is relevant to the subsequent discussion of HDV.

1.1 Hepatitis B Virus

1.1.A The Hepadnavirus Family

The discovery of the 'Australian antigen' in the mid-1960s (Blumberg et al, 1967), and the observation that a 42 nm particle was associated with 'Australian antigen' hepatitis (Dane et al, 1970), led to major advances in the characterisation of HBV within the next two decades. However, HBV infection remains a major health problem, with almost 300 million carriers world-wide. Much interest is now concentrated on global control and prevention of HBV infection.

HBV is the prototype of a small family of DNA viruses known as the Hepadnaviridae. In addition to HBV, four other hepadnaviruses have been identified. These are woodchuck hepatitis virus (WHV; Summers et al, 1978), Beechey ground squirrel hepatitis virus (GSHV; Marion et al, 1980), Pekin duck hepatitis B virus (DHBV: Mason et al, 1980) and heron hepatitis B virus (HHBV; Sprengel et al, 1988). Characteristically, these viruses are species specific and share a common virion

morphology as well as genome size and organisation. Furthermore, all are relatively hepatotrophic and have the ability to cause acute and persistent infection.

1.1.B **HBV Transmission and Carrier State**

The principal modes of spread of HBV are by parenteral, sexual or perinatal transmission. The predominant factor in determining the outcome of HBV infection is the age that the individual is exposed to the virus; thus, persistent infection is most common following perinatal transmission, and 70-90% of neonates born to HBsAg carriers who are HBeAg-positive (see below) develop chronic HBV infection, defined by persistence of serum HBsAg for greater than six months (Beasley et al, 1981; Stevens et al, 1987). In contrast, infection of adults by HBV results commonly in an acute self-limited infection leaving the patient with a long-lasting immunity against recurrent infection. However, approximately 5% of individuals infected as adults develop chronic HBV infection which is associated with a wide spectrum of histological changes in the liver. Other predisposing factors to persistence are (1) gender, with almost twice as many males as females becoming chronically infected (Szmuness et al, 1978); and (2) immunological status, with immunosuppressed patients, such as haemodialysis patients, at much greater risk of chronic infection (Szmuness et al, 1978; Hoofnagle and Alter, 1984).

1.1.C **The HBV -Virion and -Protein Products**

(i) Virus-Associated Particles

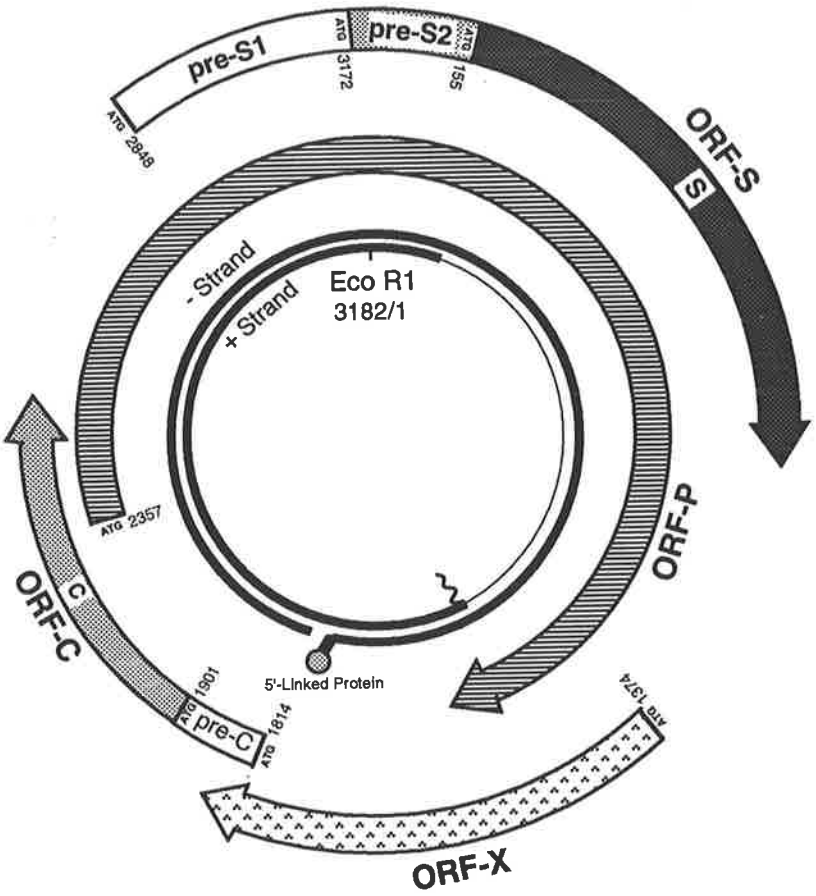
During HBV infection, three distinct forms of virus-associated particles exist in the serum: (1) spherical, and (2) filamentous forms, both 22 nm in diameter, composed

of hepatitis B surface antigen (HBsAg), and (3) the infectious virion, or Dane particle. The former two particles are non-infectious, and the 22 nm spherical particles are present in a 10^3-10^6 fold excess over the complete virion (Bond and Hall, 1972). The hepatitis B virion is a 42 nm particle comprised of a HBsAg envelope which surrounds a 27 nm nucleocapsid which is released from virions by detergent treatment (Almeida et al, 1971). The nucleocapsid or core particle is comprised of a serologically-distinct hepatitis B core antigen (HBcAg) and contains the viral genome, a multifunctional polymerase product (Toh et al, 1983; Radziwill, 1990) and a serine/threonine protein kinase activity (Albin and Robinson, 1980). The HBx gene product was identified recently in hepatitis B virions [Wu et al, 1990; section 1.1.C(iii)], and may be the virion-associated protein kinase.

(ii) Genome Structure

In common with other hepadnaviruses, the HBV genome is one of the smallest described in mammalian DNA viruses. The genome is a circular, partially double-stranded DNA molecule of ca. 3.2 kb (Figure 1.1). The long or (-) strand is full-length but has a nick at a defined site to produce fixed 5' and 3' ends, and has a protein molecule covalently bound at the 5' end. The short or (+) strand has a fixed 5' end which is linked to an oligoribonucleotide (Will et al, 1987), and a variable 3' end creating a gap which varies in length from 20-50% of the (-) strand (Delius et al, 1983). The genome is held in a circular configuration by a ca. 200 bp overlap at the 5' ends of both strands (Sattler and Robinson, 1979). Sequence analysis has identified four overlapping open reading frames (ORF -S, -P, -C and -X), which increase the viral protein coding capacity by approximately 1.5 times. These ORF are

Fig 1.1 The structure and genome organisation of HBV DNA; the positions of the ORF are shown with arrowheads to indicate the direction of transcription and translation. The thin line of the inside circle indicates the position of the single-stranded gap. This figure was adapted from a figure by Dr. T. Macnaughton.



Structure and Genome Organisation of HBV DNA

conserved between the three mammalian hepadnaviruses and are all present on the (-) strand of the viral DNA (Tiollais et al, 1985).

(iii) Protein Products

ORF S is subdivided into the pre-S1, pre-S2 and S domains, delineated by three in-frame initiation codons. The S domain, which spans ca. 678 bp, encodes the major HBsAg polypeptide which is expressed in both glycosylated (gp27) and non-glycosylated forms (p24), and is the predominant component of the 22 nm HBsAg particles. Translation from the AUG codon immediately upstream of the S gene (pre-S2 domain), produces the middle protein (gp33 and gp36). The large HBsAg protein, translated from the first AUG codon (pre-S1 domain), is also detected as a glycosylated (gp42) and a non-glycosylated (p39) polypeptide. Thus, the large and middle forms of HBsAg are co-terminal with the major form, and contain 174 and 55 unique aa respectively, at the amino terminus. The major, middle and large proteins are present in the mature HBV virion in approximately equimolar amounts (Heermann et al, 1984). In contrast, the 22 nm subviral particles are composed predominantly of the major protein with small, but variable amounts of the middle and only trace quantities of the large HBsAg proteins (Heerman et al, 1984).

The HBV C gene (ORF C) encodes two polypeptides, a non-particulate secretory protein (hepatitis B e antigen; HBeAg), and the major capsid polypeptide, HBcAg. Nucleic acid sequence analysis revealed that ORF C contains two in-frame initiation codons and translation of the capsid protein begins from the second initiation site. The ORF C mRNA is transcribed in a way so that two species of mRNA are produced that differ by the addition of ca. 30 nt at the 5' end. The longer species encodes the

HBeAg which is translated from the first AUG codon located 29aa upstream of the HBcAg initiation site. This pre-core region contains a signal sequence of 19aa at the amino terminus that targets the nascent precore protein to the endoplasmic reticulum (ER) to produce the pre-core protein derivative p22 (Ou et al, 1986). Following cleavage of the pre-core signal sequence by a signal peptidase, the polypeptide is processed at the carboxy terminus, and the protein (p15-18) is secreted as mature HBeAg (McLachlan et al, 1987; Standring et al, 1988). Similarly, the 5' end of the shorter RNA is positioned between the two in-frame AUG codons so that the second AUG is the first translation initiation site to be recognised by the ribosomes, thus ensuring efficient translation of the core protein (Will et al, 1987). The HBV core protein is a highly basic, phosphoprotein of ca. 21 kD that is assembled into the virus nucleocapsid structure along with the pregenomic RNA and the viral polymerase, as the first step of DNA synthesis and virion assembly, in the cytoplasm of infected hepatocytes.

ORF P overlaps the entire ORF S region, the 3' end of ORF C and the 5' end of ORF X. The putative polyprotein precursor of ORF P is approximately 90 kD, and is believed to encode the viral DNA-dependent DNA polymerase, the protein covalently bound to the 5' end of the L strand of the genome (Bartenschlager and Schaller, 1988), the reverse transcriptase, and a RNase H activity; the existence of these latter two functions were based initially on nucleic acid sequence homology with several retroviral reverse transcriptases (Toh et al, 1983) and E.coli RNase H (Khudyakov and Makhov, 1989). Recently, activity gel analysis has identified a major (70 kD) and minor (90 kD) reverse transcriptase activity in HBV virions (Bavand et al, 1989).

The fourth and smallest HBV ORF, ORF X, is conserved among the mammalian hepadnaviruses and encodes a nucleoprotein (Siddiqui et al, 1987) which functions as a transcriptional transactivator of HBV- and other heterologous-regulatory sequences (Siddiqui et al, 1989; Colgrove et al, 1989). It has been proposed that the X-protein acts as a viral transactivator which alters normal host cell gene expression leading to tumorigenesis, and expression of the HBx gene in transgenic mice was shown to induce liver cancer in these mice (Kim et al, 1991). Recently, a 16.5 kD phosphoprotein was identified in HBV virions that showed a protein kinase activity similar to that of the HBV X-protein synthesised in E.coli. (Wu et al, 1990). The ability of the X-protein to function as a transactivator was abolished by the addition of a known protein kinase inhibitor, suggesting that the X-protein may be related to the protein kinase activity detected previously in virions (Albin and Robinson, 1980). It was proposed that the X gene product activates transcription by phosphorylating factors involved in the regulation of transcription (Wu et al, 1990). The role of the X gene product in HBV replication is still unclear, although GSHV variants with mutations in the X gene failed to replicate in vivo suggesting that the X-protein may be required for hepadnavirus replication (Ganem and Varmus, 1987).

1.1.D **Pathogenesis**

Liver samples from HBV carriers show a spectrum of histological diagnoses ranging from normal liver to severe hepatitis. Thus, 'healthy' carriers show little or no inflammation of the liver, despite ongoing virus replication or expression of HBsAg. Patients with chronic liver disease are generally divided into two categories depending on the degree of lymphocytic infiltration of the liver. Chronic persistent hepatitis (CPH) is characterised by lymphocytic inflammation restricted mainly to the portal tracts but

leaving the parenchymal limiting plate intact, whereas, in chronic active hepatitis (CAH) inflammation is extended to the periportal tracts and parenchyma, with erosion of the limiting plate by piecemeal necrosis, which may ultimately progress to cirrhosis. Although some controversy remains over the mechanism of cell injury in HBV infection, several lines of evidence support a role for immune-mediated liver damage in HBV-associated hepatitis (Alexander, 1990). In HBV-infected patients with inflammatory lesions of the liver, there is a close histological association between areas of necrosis and the presence of an inflammatory infiltrate (Dienstag, 1984). This is also reflected in clinical findings where the onset of liver injury correlates with the induction of the host immune response (Thomas et al, 1984). It is thought that a cytotoxic T-lymphocyte (CTL) response to both HBcAg and HBeAg expressed on the surface of infected hepatocytes is the principal mechanism involved (Dudley et al, 1972; Pignatelli et al, 1987; Eddleston et al, 1988; Mondelli et al, 1988). Serologically, 'healthy' carriers and patients with CPH are usually anti-HBe positive with a very low level of serum HBV DNA detectable only by PCR (Baker et al, 1991). Patients with CAH most commonly express markers of replicative HBV infection, including serum HBV and circulating HBeAg (Hoofnagle and Alter, 1984).

HBeAg in acute phase patients and short term carriers is a good predictor of HBV replication and correlates closely with HBV DNA in the serum. In chronic carriers, seroconversion from the HBeAg carrier state to anti-HBe is often associated with a transient increase in histological activity and raised ALT that is followed by cessation of HBV replication and disappearance of HBV DNA from the serum leading to decreased inflammatory activity in the liver (Thomas et al, 1984). However, a small percentage of chronic carriers with a severe form of hepatitis are anti-HBe positive but continue to express high levels of HBV DNA in the serum and HBcAg in liver. These

patients are infected with HBV variants which have been identified as having a single or two single point mutation(s) within the pre-core region of the genome that results in the formation of a new translational stop codon, and thus, are unable to secrete HBeAg (Carman et al, 1989; Raimondo et al, 1990; Kosaka et al, 1991). The pathogenic role of HBeAg expression is unknown. However, it has been proposed that secreted HBeAg may subvert the immune response. Thus, in patients infected with pre-core mutant viruses, the complete CTL response is directed towards infected hepatocytes resulting in severe liver disease (Bonino et al, 1991a). The efficiency of perinatal transmission of pre-core mutant HBV appears to be reduced greatly, compared with wild-type HBV. The vast majority of HBV-carrier mothers who infect their infants in the perinatal period are HBeAg-positive, and greater than 90% of these infants develop a chronic carrier state. It has been proposed that HBeAg may cross the placenta, and that in utero exposure to HBeAg can induce immunologic tolerance in the neonate that favours the persistence of HBV after perinatal infection (Milich et al, 1990).

1.2 Hepatitis Delta Virus

1.2.A Transmission

The human hepatitis delta agent was first discovered by Rizzetto and coworkers following the detection of a novel antigen, named hepatitis delta antigen (HDAg), in the nuclei of infected hepatocytes in a proportion of HBV carriers (Rizzetto et al, 1977). HDAg was antigenically distinct from HBcAg and HBsAg, and chimpanzee transmission studies determined that it was associated with a defective virus, named HDV, which required the presence of HBV for its expression and replication (Rizzetto

et al, 1980). WHV is capable of providing the function of the helper hepadnavirus in an experimental model system (Ponzetto et al, 1984a).

The routes of infection by HDV are similar to those of HBV, although perinatal transmission appears to be rarely involved, primarily because most HDV-infected HBV carrier mothers are anti-HBe positive and thus are unlikely to transmit HBV (and HDV) to their infants (Ramia and Bahakim, 1988). Horizontal transmission is most important in endemic regions, by mechanisms likely to involve inapparent transfer of body fluids (Popper et al, 1983).

HDV infection is world-wide, although there are distinct regions of high incidence, viz. parts of Southern Europe, the Western Pacific Region and Central Africa, where 20-80% of HBV carriers are also chronically-infected with HDV (Rizzetto et al, 1988). In the Western Amazon Basin, where the frequency of HDV infection among HBV carriers approaches 100%, HDV has been responsible for severe epidemics of fulminant hepatitis, first recognised in an outbreak in the Yucpa Indians of Western Venezuela in 1978-81 (Hadler et al, 1984). Subsequent investigations have shown that superinfection by HDV was responsible for large outbreaks of viral hepatitis in Columbia, originally described as Santa Marta hepatitis in 1931 (Ljunggren et al, 1985), and Labrea Fever in Brazil in 1940 (Andrade et al, 1983). In the United States, Britain and Northern Europe, HDV-associated hepatitis is restricted predominantly to high-risk groups, and intravenous drug users (IVDU) provide the major reservoir of HDV infection in these countries. In the United States, a multicentre survey revealed that 42% of HBsAg-positive IVDU were also anti-HD positive (Ponzetto et al, 1984b), consistent with similar surveys on the prevalence of the HDV carrier rate in IVDU in Europe. In Australia, approximately 5-10% of HBsAg-positive patients are also

positive for markers of HDV infection and are confined almost exclusively to IVDU and migrants from Southern Europe (Williams and Cossart, 1983). Although, the prevalence of HDV generally correlates directly with the prevalence of HBV, little evidence of HDV infection is noted in some regions, for example, Japan, where the incidence of HBV is relatively high (Mitamura, 1991). Similarly, the level of HDV infection in some high risk groups does not always reflect the level of HBV infection, eg., there is a relatively low level of HDV among HBsAg-positive male homosexuals (Weisfuse et al, 1989).

1.2.B **Clinical Patterns of HDV Infection**

The clinical features associated with HDV infection closely resemble those seen in many presentations of HBV infection, although HDV-infected patients often show increased severity of disease and morbidity. Natural infection with HDV can occur as a coinfection with HBV, or superinfection of a HBV carrier. A third pattern of HDV transmission is now recognized, namely latent infection, which results from infection after orthotopic liver transplantation (Negro and Rizzetto, 1991) (see section 1.2.L).

Coinfection with HBV and HDV results in a self-limited acute viral hepatitis which resolves in ca. 95% of cases, although the acute hepatitis can be more severe than that seen after infection with HBV alone (Shattock et al, 1985). The rate of progression to the chronic carrier state is not increased after coinfection; thus, HDV does not promote chronicity (Smedile et al, 1981; Moestrup et al, 1983). In a small proportion of cases, coinfection results in severe acute fulminant hepatitis associated with high mortality (Smedile et al, 1982; Shattock et al, 1985).

Superinfection of a HBV chronic carrier results in chronic HDV infection in 70-90% of patients (Rizzetto et al, 1983), and an accelerated rate of progression to CAH and cirrhosis, compared with chronic HBV infection alone. The rate of fulminant hepatitis is also increased as a result of superinfection of HBV carriers (Popper et al, 1983, Govindarajan et al, 1985). The risk of transition to chronicity appears to be unrelated to the presence of anti-HBe in the serum, consistent with the idea that the establishment of a chronic HDV infection is independent of ongoing HBV replication (Moestrup et al, 1983).

After superinfection of a HBV chronic carrier, a striking suppression of HBV replication coincides with the peak of HDV replication (Rizzetto et al, 1986). In a majority of cases, this interference is transient and the HBsAg titre and serum HBV DNA return to their original levels (Ponzetto et al, 1987). The most common outcome of HDV superinfection of a HBV carrier is seroconversion from HBeAg-positive to HBeAg-negative/anti-HBe positive in >90% of carriers (Rizzetto et al, 1986). Very rarely, superinfection by HDV results in the termination of the carrier state with seroconversion to anti-HBs (Moestrup et al, 1983; De Cock et al, 1985). In the long term, clearance of HBV DNA occurs in a significantly higher proportion of patients with HBV and HDV than in patients with chronic HBV infection alone (Krogsgaard et al, 1985). The exact mechanism of HDV-induced interference is unknown.

1.2.C **Diagnosis of Acute and Chronic HDV Infection**

Coinfection and superinfection can be distinguished by the presence of anti-HBc IgM which indicates acute HBV infection. In both coinfection and superinfection, acute HDV infection is characterised by an incubation period of 3-4 weeks, followed by a

brief period of HDV-viraemia (10-14 days), monitored by the detection of HDAg and HDV RNA in the serum of infected patients (Craig et al, 1986). Chimpanzee studies showed that the first marker of acute HDV infection was the appearance of intrahepatic HDAg (Ponzetto et al, 1988), although a liver biopsy is unlikely to be performed on patients at this stage of infection. The appearance of HDAg in the serum is followed closely by elevated levels of ALT and the appearance of clinical symptoms (Hoofnagle, 1989; Ponzetto et al, 1988). Biphasic illness, characterised by two peaks in ALT levels, is a feature of coinfections (Craig et al, 1986).

Early studies suggested that HDV viraemia was followed by a 'window' period of 2-11 weeks before the appearance of anti-HD in the serum (Aragona et al, 1987). However, more recent studies have described cases where no delay was detected between the disappearance of HDAg in the serum and the detection of IgM anti-HD, and in many patients, these two markers were detectable at the same time (Shattock et al, 1989). In any case, the IgM anti-HD profile is the most reliable serum marker to distinguish acute from chronic infection; acute self-limited HDV hepatitis is characterised by transient, low titre IgM anti-HD, detectable for 2-3 weeks (Rizzetto et al, 1988). This is accompanied by persistent low titre IgG anti-HD which often appears during convalescence. In contrast to acute infection, chronic infection is determined by the presence of persistent high titre IgM and IgG anti-HD (Rizzetto et al, 1988; Hoofnagle, 1989; Shattock et al, 1989) and may be confirmed by the presence of intrahepatic HDAg or by serum HDV RNA.

1.2.D Animal Models of HDV Infection

In common with HBV, the use of experimental models for replication and pathogenesis studies has proved difficult. The dependence on HBV for both entry and release of HDV from host cells (Kuo et al, 1989), initially restricted HDV studies to the chimpanzee model of HBV infection (Rizzetto et al, 1980). A full-length HDV cDNA clone was prepared from HDV isolated from a chimpanzee serum (Wang et al, 1986). This clone is discussed in section 1.2.H below. Although the pattern and outcome of HDV infection in chimpanzees closely resembles that of human HDV infection, extensive use of the chimpanzee model for HDV infection is restricted by the high cost and limited availability of HBV-carrier animals (Ponzetto et al, 1987). HDV was transmitted successfully to chronic WHV-carrier woodchucks in an attempt to provide a more accessible animal model (Ponzetto et al, 1984a). Experimental HDV infection in woodchucks shows biological and pathological features similar to those noted in humans and has the potential to provide a useful animal model. However, the eastern woodchuck is not available in Australia for laboratory use. Attempts to transmit HDV to DHBV-infected Pekin ducks were inconclusive, and only transient expression of serum HDAg and HDV RNA was reported (Forzani et al, 1988; Freiman et al, 1988). Recently, experimental transmission of HDV to the SCID mouse was demonstrated (Netter et al, 1993). Transient HDV RNA replication was detected in the liver, but cell-to-cell spread of the virus was inhibited due to the absence of HBV or WHV. This experimental model has been proposed to be a likely model for the HDV transplant patient (Gowans and Bonino, 1993), and is discussed further in Section 1.2.L.

1.2.E Cell Culture Models of HDV Infection

Initial attempts to demonstrate HDV replication after direct inoculation of continuous hepatoma-derived cell lines with HDV-infected serum proved unsuccessful (Taylor et al, 1987). However, primary woodchuck hepatocyte cultures were able to sustain HDV replication but the release of progeny virions could not be demonstrated, presumably because coinfection with WHV was unsuccessful (Taylor et al, 1987). An alternative approach involved transfection of continuous cell cultures with HDV cDNA. Transfection has become a widely used technique to study certain aspects of viral replication and gene expression as it provides an easily manipulated model system in cell lines which would normally be resistant to infection. Both hepatocyte-like and non-hepatocyte cell lines have been shown to support HDV RNA replication (Glenn et al, 1990; Macnaughton et al, 1990a), and have been used extensively to examine the intracellular events of HDV replication and pathogenesis (see below). Recently, a human hepatoma cell line cotransfected with HDV- and HBV- cDNA resulted in the secretion of mature HDV virions into the culture medium (Wu et al, 1991). In a more recent study, the relative infectivity of HDV particles produced after transfection of HBV- and HDV- cDNA into a hepatoma-derived cell line, was measured by their ability to infect primary chimpanzee hepatocyte cultures (Sureau et al, 1993). The results showed that the large HBsAg protein was essential for the recombinant particle to be infectious. However, this does not exclude a role for the middle or major HBsAg proteins in receptor-binding as it was shown previously that epitopes to both these regions are exposed at the surface of HDV particles (Sureau et al, 1992). Furthermore, successful in vitro infection of primary chimpanzee hepatocytes was only possible with HDV, but not HBV (Sureau et al, 1991). Thus, the results in this model system are consistent with the hypothesis that although the HBsAg proteins may be

involved in the binding of HDV- and HBV- to hepatocytes, it is likely that the mechanism of uptake by these two viruses is different; they may bind to a different receptor or to the same receptor with a different level of efficiency [Sureau et al, 1992; see section 1.2.J(i)].

1.2.F **Extrahepatic Sites of HDV Replication**

Despite clear evidence of infection and replication of HBV and WHV in a number of extrahepatic sites including the spleen and kidney (Negro et al, 1989), there is no evidence for extrahepatic replication of HDV (Negro et al, 1989; Netter et al, 1993). Nevertheless, following transfection of cultured cell lines with HDV cDNA, HDV RNA replication has been described in both hepatocyte-like and non-hepatocyte cell lines (Glenn et al, 1990; see section 1.2.E). Thus, the limiting event for extrahepatic HDV infection and replication in vivo appears to be virus entry, and supports the above hypothesis that HDV and HBV may use different mechanisms of virus entry to the cell.

1.2.G **Structure of HDV**

Electron microscopy of HDV prepared from human serum showed that the virus was a 36 nm particle (Bonino et al, 1981) with an envelope comprised of HBsAg (or WHsAg). In experimental HDV infection in chimpanzees, circulating HDV particles had a HBV-derived envelope composed of approximately 95% p24/gp27, 5% gp33/gp36 and 1% p39/gp42 polypeptides (Bonino et al, 1986). This protein composition is similar to that of the 22 nm HBsAg particles rather than that of mature HBV virions (Bonino et al, 1986). Recent studies which examined the virion structure suggest that the HDAg and genomic HDV RNA form a stable ribonucleoprotein (RNP) complex

within the HDV particles that was detected by electron microscopy as a roughly spherical core-like structure with a diameter of 19 nm (Ryu et al, 1993).

1.2.H **HDV RNA**

Molecular cloning and sequencing of different HDV-isolates showed that the HDV genome is a single-stranded, covalently closed circular RNA molecule of ca. 1.7 kb, that may be considered as negative sense (Wang et al, 1986; Kos et al, 1986; Makino et al, 1987; Kuo et al, 1988a; Chao et al, 1990a; Chao et al, 1991a). However, because of initial uncertainty over the genome coding potential (Wang et al, 1986; Makino et al, 1987), virus RNA is referred to as genomic sense RNA, and complementary RNA as antigenomic sense. The HDV RNA sequence has a G+C content of 60%, and shows extensive regions of intramolecular complementarity predicted by computer analysis (Wang et al, 1986), so that the genome is able to form a stable rod-like structure resembling that of viroids and virusoids of plants. A model was proposed for the structure of the HDV RNA based on the ability of HDV RNA to form an unbranched rod structure (Figure 1.2; Branch et al, 1989); this model divides the genome into a viroid-like region, spanning a highly conserved domain of ca. 295 bases including the putative self-cleavage sites (see below), and a protein-coding region encoding the HDAg, that has a unique poly(A^+) signal located directly downstream of the HDAg gene. These predicted rod-like structures have been detected by electron microscopy of HDV RNA preparations examined under non-denaturing conditions (Kos et al, 1986). A second similarity between HDV RNA on the one hand, and virusoids and one of the viroids [avocado sunblotch viroid (ASBV)] on the other hand, is the ability for magnesium-dependent self-cleavage and religation to occur in vitro at specific unique sites on both the genomic- (nt 688-689) and

HDV Genome Organisation

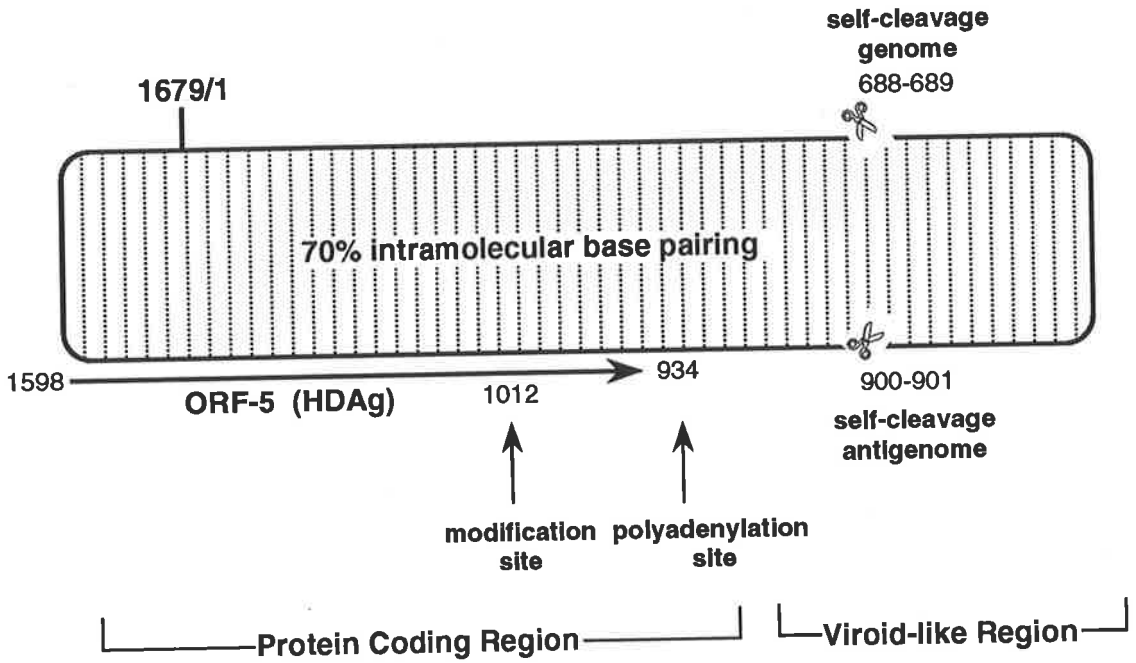


Fig 1.2 Structure and Genome Organisation of HDV RNA according to the model proposed by Branch et al, 1989.

antigenomic- (nt 900-901) strands of HDV RNA (Kuo et al, 1988b; Sharmeen et al, 1988; Sharmeen et al, 1989; Wu and Lai, 1989). The minimum contiguous sequence needed for self-cleavage has been defined as 104 bases on the genomic RNA and 86 bases on the antigenomic RNA. Thus, the two sites are believed to lie opposite each other on the rod structure proposed for HDV RNA (Kuo et al, 1988b). The ability of plant satellite RNAs and ASBV to self-cleave is dependent on a characteristic secondary structure called a 'hammerhead', formed by base-pairing of highly conserved short nucleotide sequences (Hutchins et al, 1986; Forster and Symons, 1987). The self-cleavage domains described for HDV appear to be unrelated to this 'hammerhead' structure, although recent evidence suggests that some structural requirements do exist (Macnaughton et al, 1992). However, there are also significant differences between these plant pathogens and HDV RNA; most importantly, HDV RNA is approximately 5-10 fold larger than viroid RNA, and viroids do not have the capacity to encode proteins.

The first complete HDV RNA sequence to be reported was determined from a cDNA clone prepared after virus from an Italian patient was passaged serially five times in chimpanzees (Wang et al, 1986). In addition, virus from the same HDV-infected patient, submitted to two of the above serial transmissions in chimpanzees, was used to infect WHV carrier woodchucks, and a HDV RNA isolate was then cloned from the liver of one of the infected woodchucks (Kuo et al, 1988a). A comparison of the complete nucleotide sequence of the woodchuck- and chimpanzee- derived clones showed that these two isolates have ca. 98.5% homology (Kuo et al, 1988a). To date, seven additional HDV RNA isolates have been cloned directly from human serum and the complete cDNA sequenced. These isolates came from Southern California (Makino et al, 1987), the island of Nauru in the South Pacific (Chao et al, 1990a),

France (Saldanha et al, 1990), Japan (Imazeki et al, 1991), Taiwan (Chao et al, 1991a), Lebanon (Lee et al, 1992) and Peru (Casey et al, 1993). A comparison of the nucleic acid sequence of these HDV isolates showed that the majority of the HDV isolates were 82-89% identical to one another (Chao et al, 1991a; Casey et al, 1993), however, the Peruvian HDV isolate was only ca. 62% identical to the HDV isolates from other continents (Casey et al, 1993). The clinical significance of this observation is discussed in detail in Chapter 8.4.

Individual sequence analysis of the chimpanzee (Wang et al, 1986) and three different human HDV isolates (Makino et al, 1987; Chao et al, 1990a; Chao et al, 1991a) has identified multiple ORF capable of coding more than 100 bp on both the genomic and antigenomic strands. However, comparison of these four HDV sequences revealed that only two ORF, both on the antigenomic strand, are conserved (Chao et al, 1991a). Only one of these, ORF 5, has been assigned a protein product, namely HDAg (see below; Wang et al, 1986; Weiner et al, 1988). Sequence analysis of the chimpanzee- and woodchuck- derived clones predicted HDAg to be a protein of 195aa (Wang et al, 1986; Kuo et al, 1988a), whereas the sequence from the human-derived cDNA predicted HDAg to be 214aa (Makino et al, 1987). Further analysis of the chimpanzee-derived isolate showed sequence heterogeneity at aa 195 (Wang et al, 1986). The mechanism of HDAg expression from these different RNA species has been resolved more recently (see below).

In addition, a smaller ORF that corresponds to ORF 3 of the Southern Californian isolate, is conserved. However, the relative positions of the start and termination codons of ORF 3 between the four HDV sequences are highly variable so that it is not

clear whether this ORF is a functional coding domain (Makino et al, 1987; Chao et al, 1991a).

1.2.I HDAg Structure, Function and Expression

(i) HDAg Expression

In natural infection, HDAg is detected in the serum and liver by immunoblot analysis as two polypeptides of 24 kD and 27 kD (Bergmann and Gerin, 1986; Macnaughton et al, 1990a) or 27 kD and 29 kD (Bonino et al, 1986; Roggendorf et al, 1987) using antisera from chronic HDV carriers. The differences in the molecular weight most probably reflect different molecular size markers used in independent laboratories. Analysis of recombinant ORF 5-derived polypeptides produced in yeast and bacterial cells demonstrated that ORF 5 encoded both HDAg-p24 and -p27 (Weiner et al, 1988). It was shown in an amber suppressor E.coli mutant that the codon at position 195 was an amber stop codon that was read through to permit synthesis of HDAg-p27 in this system (Weiner et al, 1988). However, in natural infection the synthesis of HDAg-p27 results from an entirely different mechanism, and involves a specific base change in ORF 5 that converts the termination codon at aa 195 from a UAG to a UGG codon (Figure 1.2; Luo et al, 1990). Recent studies demonstrated that the substrate for this mutation is the genomic strand, not the antigenomic strand; the original mutation is a U → C base transition in the genomic RNA that is subsequently reflected in the antigenomic RNA as an A → G base change in ORF 5 (Casey et al, 1992; Zheng et al, 1992). This mutation was shown to occur in nuclear extracts from uninfected cells indicating that the change is not simply the result of a misincorporation during HDV RNA replication but is a form of RNA editing, although the exact nature of the modification is unknown (Zheng et al, 1992).

(ii) HDAg mRNA

An 800 nt linear poly(A^+) transcript has been detected in HDV-infected liver and in cell lines transfected with full-length HDV cDNA, that is likely to be the mRNA for HDAg (Hsieh et al, 1990; Jilbert et al, 1991). This mRNA is antigenomic and cytoplasmic, and is approximately 500 times less abundant (600 copies/cell), than the nuclear genomic RNA (300 000 copies/cell; Chen et al, 1986). The 5' and 3' ends of the transcript have been mapped, placing the start of the transcript at position 1631, 33 bases upstream of the predicted initiation codon for HDAg, and extending 697 nt (Figure 1.3). Site-directed mutagenesis of a poly(A^+) signal sequence, identified 15-20 bases upstream of the poly(A^+) site at position 934, showed that this sequence is required for HDAg synthesis and HDV replication (Hsieh et al, 1990). This poly(A^+) site is the only one in the whole HDV genome. Recent studies which examined the regulation of polyadenylation of antigenomic HDV RNA indicated the presence of cis-acting regulatory elements necessary for optimal polyadenylation immediately downstream of the poly(A^+) signal (nt 923-848), close to the self-cleavage site. However, polyadenylation was not dependent on self-cleavage of the antigenomic HDV RNA (Hsieh and Taylor, 1991).

(iii) Functional Domains of HDAg

Both HDAg-p24 and -p27 have been demonstrated to bind HDV RNA specifically in vitro with equal efficiency (Chang et al, 1988; Lin et al, 1990; Macnaughton et al, 1990b; Chao et al, 1991b; Hwang et al, 1992). Recent studies showed that the RNA-

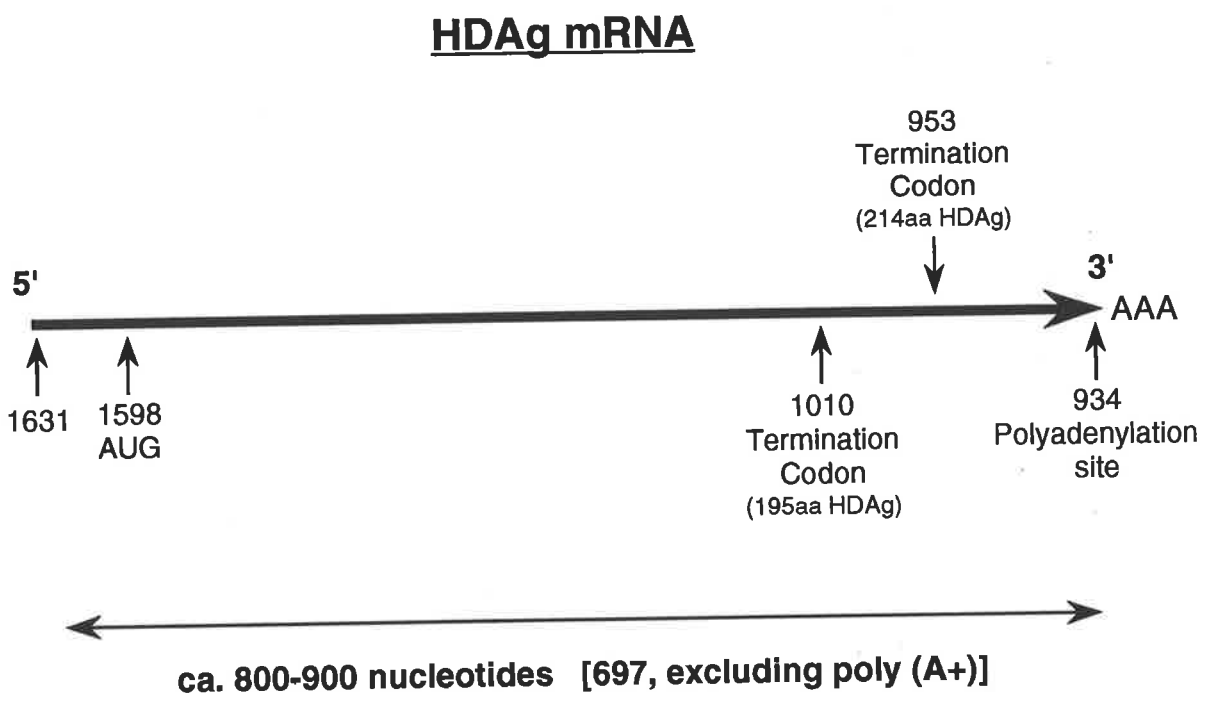


Fig 1.3 Putative HDAg mRNA as described by Hsieh et al, 1990

binding properties of HDAg require two arginine-rich motifs (ARMs; aa97 to aa107 and aa136 to aa146) which are separated by 29aa and constitute the RNA-binding domain (Lee et al, 1993; Figure 1.4). Deletion of the 29aa spacer sequence resulted in a marked inhibition in the ability of HDAg to bind HDV RNA. Thus, it was proposed that the spacer sequence may be required to enable the ARMs in HDAg to bind to different parts of the putative HDV RNA rod-like structure (Chao et al, 1991b; Lee et al, 1993). The ability of HDAg to bind to HDV RNA may play an important role in the replication strategy of the virus. This is discussed in Section 1.2.J(iii).

The N-terminal region of HDAg contains a nuclear localisation signal (NLS) which is composed of two discontinuous stretches of basic aa (aa69 to aa75 and aa85 to aa88; Figure 1.4). The N-terminal domain spanning aa69 to aa75 has been referred to as the core NLS since it displays some nuclear targeting activity in the absence of the downstream domain, although both regions are required for efficient nuclear transport of HDAg (Xia et al, 1992; Lazinski and Taylor, 1993). Deletion of the whole NLS from either HDAg-p24 or HDAg-p27 altered the localisation of HDAg in transfected cells so that a significant amount was seen in the cytoplasm. Nevertheless, HDAg-p24 and HDAg-p27 were still detected in small amounts in the nucleus. Thus, it is possible that a secondary NLS exists and this possibility is consistent with the observation by Chang et al (1992), who showed that sequences within the N-terminal 50 aa of HDAg were capable of directing a β-galactosidase fusion protein to the nucleus at low efficiency. Alternatively, the mutant HDAg with the deleted NLS may enter the nucleus by passive diffusion, as the nuclear pore is thought to be permeable to molecules smaller than 50 kD (Lazinski and Taylor, 1993).

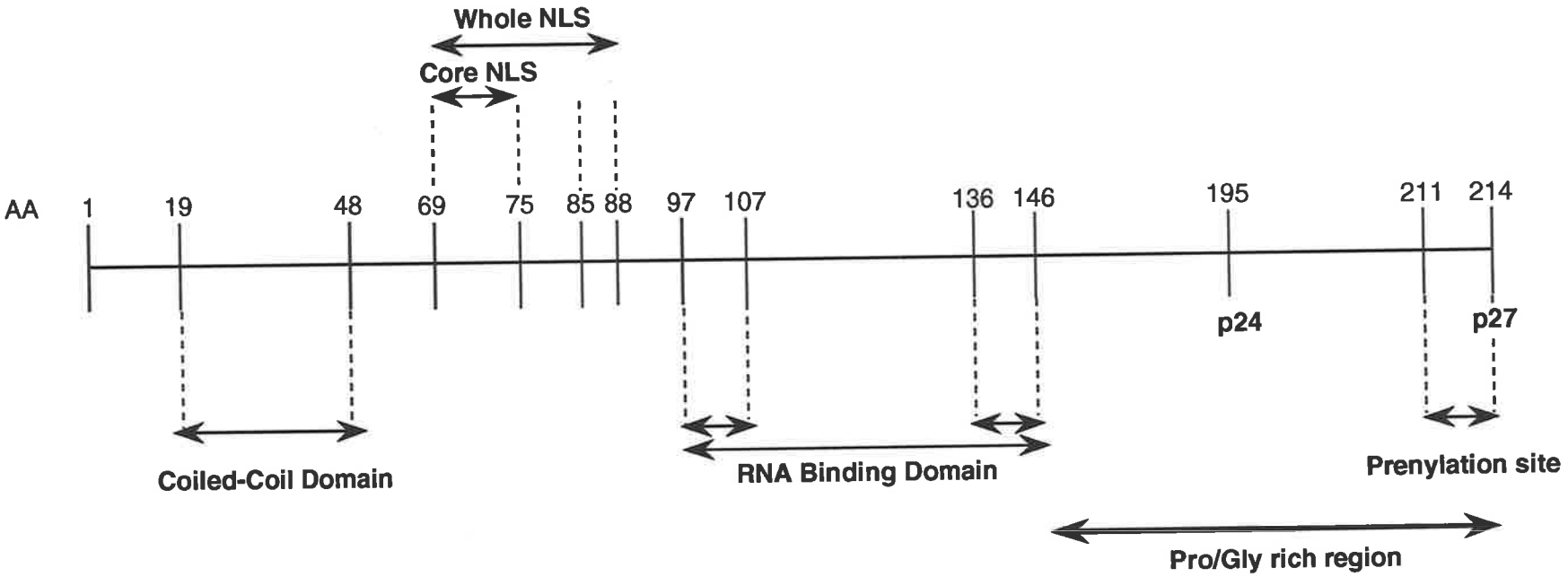


Fig 1.4 Functional Domains of HDAg

Studies which characterised recombinant HDAg-p24 in a cell line transfected with the HDAg gene only, showed that large multimeric aggregates of HDAg were present in crude extracts from cells (Macnaughton et al, 1990b). More recent studies demonstrated that large, multimeric complexes containing multiple copies of HDAg proteins were present in extracts of HDV-infected liver. In addition, HDAg-p24:p27 dimers were also present in these extracts, as well as in circulating virus particles (Wang and Lemon, 1993). In this study, the formation of HDAg dimers was mediated through the N-terminal 81 aa of HDAg. Previous studies identified a putative leucine zipper in this region (spanning aa 30 to aa51) that was believed initially to facilitate dimerisation (Xia et al, 1992). However, more recently, mutational analysis showed that the presence of the leucine residues within the putative leucine zipper motif were not required for HDAg-p24 and HDAg-p27 to form protein complexes in vitro (Chang et al, 1993), and it has been suggested that a coiled-coil region may fulfil this function (Lazinski and Taylor, 1993). This region was located by computer analysis and spans aa21 to aa48 (Lazinski and Taylor, 1993). HDAg-p24 and HDAg-p27 mutants in which a portion of this coiled-coil domain was deleted (aa19 to aa31) were unable to dimerise, consistent with the hypothesis that this region facilitates dimerisation. The formation of oligomeric forms of HDAg was shown to be important in the regulation of HDV RNA replication [see section 1.2.J(iii)].

The C-terminal end of HDAg-p27 is characterised by a region spanning aa146 to aa214 which contains a high proportion of proline and glycine residues (ca. 39%) compared with ca. 12% in the N-terminal region of HDAg (Figure 1.4; Lazinski and Taylor, 1993). This pro/gly rich region is believed to play a role in virus packaging [see section 1.2.J(iv)]. In addition, a prenylation site was identified at the C-terminus of HDAg-p27 spanning aa211 to aa214 (Glenn et al, 1992; Figure 1.4). The C-

terminal aa of HDAg-p27 (Cys-Arg-Pro-Gln-COOH) were shown to be a substrate for prenyltransferases which add 20 carbon moities derived from mevalonic acid to the cysteine residue at aa 211 (Glenn et al, 1992). The resulting hydrophobic modification may result in targeting HDAg-p27 to the ER membrane as part of the process of virus assembly (Glenn et al, 1992), and this is discussed in section 1.2.J(iv) below.

1.2.J **HDV Replication**

(i) Early Events

Although the exact mechanism used by HDV to gain entry into the hepatocyte is unknown, it was proposed that HDV attachment to the cell requires the HBsAg large protein (Sureau et al, 1993; section 1.2.E), despite comprising only 1% of the total HBsAg content of the HDV envelope (Bonino et al, 1986). In addition, it remains possible that the HBsAg middle and major proteins are also involved, although their role in HDV attachment to hepatocytes is yet to be defined (Sureau et al, 1993). The receptor used by HDV and HBV has not been identified on the hepatocyte.

(ii) HDV RNA Replication and Transcription

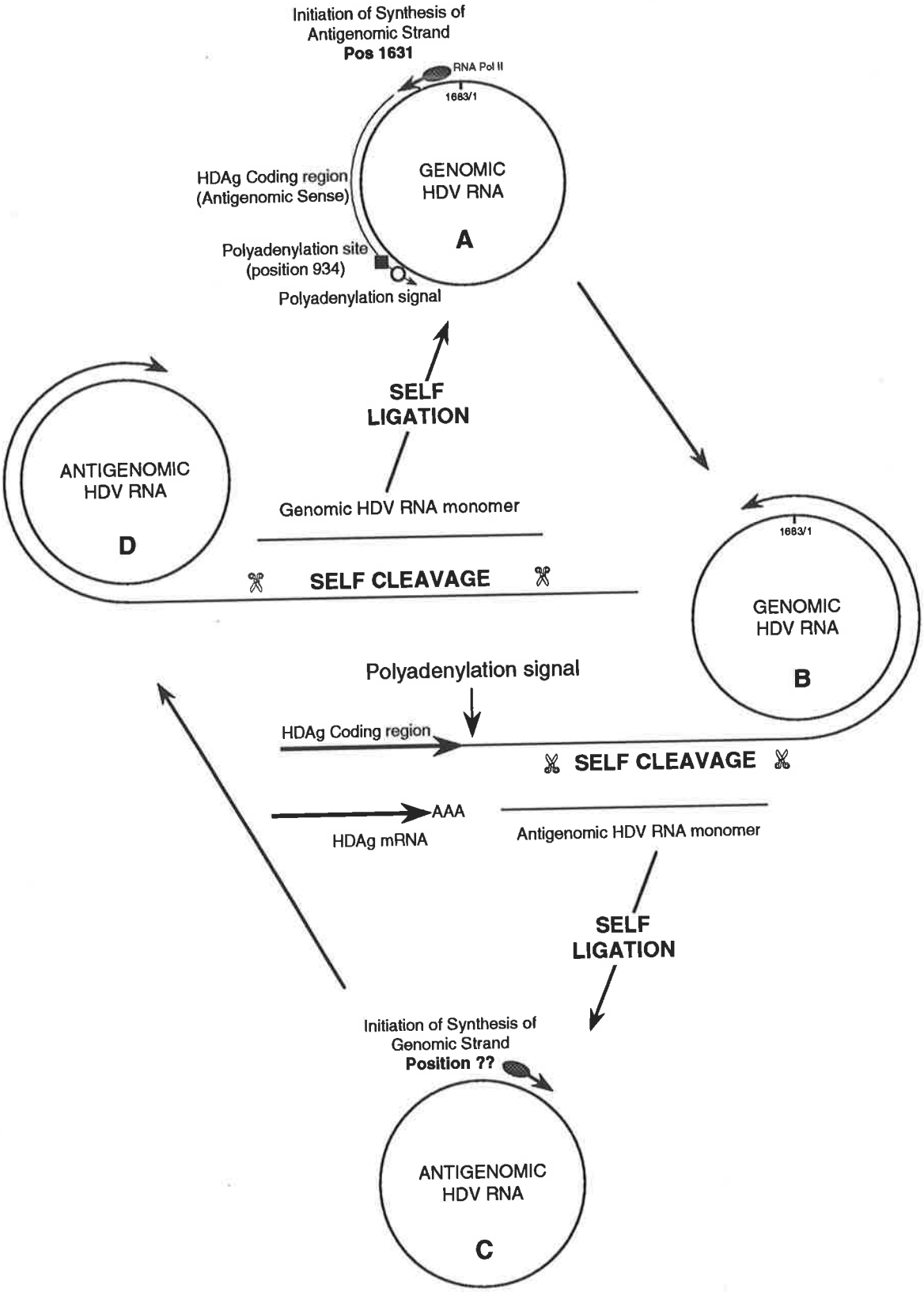
The exact mechanism of HDV RNA replication is uncertain, although it is clear that there is no DNA intermediate (Chen et al, 1986), and thus replication involves RNA-directed RNA synthesis. HDV replicative intermediates were detected in the nucleus of infected hepatocytes by in situ hybridisation suggesting that this is the site of replication (Gowans et al, 1987). Examination of RNA extracted from HDV-infected liver showed that antigenomic HDV RNA was 20-30 times less abundant than genomic RNA. The bulk of antigenomic RNA is base-paired with a small proportion

of the total genomic RNA in a double-stranded complex. Furthermore, multimeric forms of both genomic and antigenomic RNA have been detected, and more than half of the monomeric and multimeric forms are circular. These observations, plus the demonstration that both genomic- and antigenomic- HDV RNA are capable of undergoing autocatalytic self-cleavage and self-ligation reactions in vitro (see section 1.2.H), suggest that HDV RNA replicates via a rolling circle mechanism similar to that of some viroids (Chen et al, 1986). This has recently been confirmed following the observation that the template for HDV RNA-directed RNA transcription (in addition to the input viral RNA) is most likely to be a processed monomeric HDV RNA replicative intermediate produced as a result of in vivo self-cleavage of multimeric antigenomic- and genomic- sense HDV RNA (Macnaughton et al, 1992), consistent with a double-rolling circle model of replication (see below).

In vitro transcription studies have determined that the enzyme responsible for HDV RNA-RNA synthesis is likely to be host cell DNA-dependent-RNA Polymerase II, based on its sensitivity to α-amanitin in a nuclear transcription assay (Macnaughton et al, 1991). The mechanism required to redirect the template specificity of this enzyme is unknown, although the proposed secondary structure of HDV RNA may play a role. In vitro promoter activity was localized to a region of HDV cDNA encompassing a 250 bp sequence upstream of the HDAg gene, although it is unclear if this region represents the HDV RNA promoter counterpart that is recognised by RNA Polymerase II (Macnaughton et al, 1993).

The exact details of the HDV RNA replication strategy are speculative (Figure 1.5). Antigenomic HDV RNA transcription directed by the input virus genomic RNA is believed to initiate at a single transcriptional start site directly upstream of the 5'

Fig 1.5 The proposed mechanism of HDV genome replication (by double rolling circle) including the generation of HDAg mRNA. (A) Transcription of antigenomic sense HDV RNA from input virion RNA initiates immediately upstream of the HDAg gene; (B) Continued transcription from the circular template leads to the production of multimeric length antigenomic HDV RNA, which is subsequently self-cleaved to a ca. 800 nt species then processed into the mRNA species for the production of HDAg, and into monomeric forms; (C) The monomeric forms of the antigenomic RNA then self-ligate into a circular species which serves as the template for transcription of genomic sense HDV RNA; and (D) the production of a multimeric intermediate that self-cleaves and self-ligates to form new monomeric, circular, genomic sense HDV RNA. This figure was adapted from a figure by Dr. T. Macnaughton.



HDV RNA Replication

terminus (position 1631) of the putative HDAg mRNA transcript (Figure 1.5A; Hsieh et al, 1990). It was proposed that, once transcription extends past the polyadenylation signal downstream of the HDAg coding region, the nascent RNA is processed to produce the HDAg mRNA, and the 3' continuing transcript eventually becomes the 1700 bp full-length antigenomic RNA (Figure 1.5B; Hsieh and Taylor, 1991). Transcription continues from the circular genomic template, bypasses the polyadenylation signal, and generates multimeric antigenomic RNA, which self-cleaves to unit-length RNA (Hsieh and Taylor, 1991; Macnaughton et al, 1992). The mechanism by which the HDV transcriptional complex may suppress the polyadenylation signal to generate the multimeric forms of antigenomic RNA is unknown, although HDAg has been proposed to play a role in this process (see below). The antigenomic sense monomeric RNA molecules then circularise, most likely by self-ligation (see 1.2.H), in order to act as the template for nascent genomic strands (Figure 1.5C). Transcription directed by the circular antigenomic template is initiated from an unknown site. Multimeric linear genomic sense RNA is synthesised and processed into nascent virus RNA in a similar manner to that described above (Figure 1.5D).

(iii) The Role of HDAg in HDV replication

Studies to examine the role of HDAg in cell lines transfected with full-length HDV cDNA showed that HDV RNA replication required the presence of HDAg-p24 (Kuo et al, 1989; Macnaughton et al, 1991). It is proposed that a multifunctional role may exist for HDAg in HDV RNA replication; (1) Since HDAg contains a NLS (Xia et al, 1992), HDAg may facilitate transport of HDV RNA to the cell nucleus to permit replication (Glenn et al, 1990; Macnaughton et al, 1991; Xia et al, 1992); (2) the switch from

production of HDAg-p24 to HDAg-p27 may regulate the level of virus RNA replication. Although HDAg-p24 was demonstrated to trans-activate HDV RNA replication (Kuo et al, 1989), HDAg-p27 was shown to be a dominant trans-inhibitor of this process (Chao et al, 1990b; Glenn and White, 1991; Table 1.1). Mutational analysis of the C-terminal end of HDAg suggests that the inhibitory effect of HDAg-p27 is not simply a function of the additional 19 aa, implying that the action of HDAg-p24 may require specific sequence and structural components (Glenn and White, 1991). HDAg is not part of the transcriptional complex per se, since in vitro transcription experiments using nuclear homogenates from HDAg-positive and -negative cell lines indicated that HDV RNA transcription from genomic HDV RNA was independent of HDAg-p24, as comparable levels of HDV-specific transcripts were detected in HDAg-positive and -negative homogenates (Macnaughton et al, 1991). The ability of HDAg-p27 to reduce the level of HDV RNA replication in the presence of a 20-fold excess of HDAg-p24 has led to the proposal that HDAg-p27 interacts with a 'replication complex' containing multiple HDAg-p24 molecules (Chao et al, 1990b; Wang and Lemon, 1993; Lazinski and Taylor, 1993). There are several lines of evidence which support the existence of such a complex that include, (a) the detection of multimeric complexes of HDAg in extracts of HDV-infected livers that were associated with a HDV-specific RNA species (Wang and Lemon, 1993); (b) Ryu et al (1993) have isolated HDV-specific RNP structures from the nuclei of cells undergoing HDV RNA replication that contained both genomic and antigenomic HDV RNA; and (c) recent studies by Lazinski and Taylor (1993), showed that the presence of the coiled-coil domain in the N-terminal region of HDAg is required for the formation of HDAg dimers and for HDAg-p27 to inhibit RNA replication. In addition, although necessary for HDAg-p24 function, the RNA-binding domain could be deleted from HDAg-p27 without compromising its ability to inhibit replication (Lazinski and Taylor, 1993). On

Table 1.1

Properties of HDAg-p24 and HDAg-p27

	HDAg-p24	**HDAg-p27**
Size	195aa (24 kD)	214aa (27 kD)
Localisation	Nuclear	Nuclear
HDV RNA-Binding Activity	Yes	Yes
Dimerisation	Yes	Yes
Trans-Activation of HDV RNA Replication	Yes	No
Trans-Inhibition of HDV RNA Replication	No	Yes
Phosphorylated	Yes	Yes
Glycosylated	No	No
Prenylated	No	Yes
Initiation of Virion Morphogenesis	No	Yes

the basis of this evidence, it was proposed that multimers of HDAg-p24 bind to HDV RNA in a RNP structure to facilitate replication, and that replication is inhibited following a direct HDAg-p24:HDAg-p27 interaction. The mechanism by which this interaction may interfere with the replication process is unclear. It was suggested that the interaction may trigger the assembly of HD virions in the cytoplasm to reduce the nuclear pool of HDV RNA, and as a consequence reduce the level of replication (see below; Lazinski and Taylor, 1993); (3) HDAg may regulate polyadenylation of antigenomic HDV RNA (Hsieh and Taylor, 1991). It was proposed that as nascent antigenomic RNA is transcribed, it is able to fold into a rod-like structure to which HDAg then binds, and the polyadenylation process is suppressed as a result (Hsieh and Taylor, 1991), and finally, (4) the change from expression of HDAg-p24 to HDAg-p27 is likely to initiate HDV virion assembly and release (see below).

(iv) HDV Virion Assembly

The events involved in HDV virion assembly are unknown. HDAg-p27 was shown to be essential for the formation of HDV particles. Conversely, HDAg-p24 alone was insufficient for particle formation (Chang et al, 1991; Ryu et al, 1992; Chen et al, 1992; Table 1.1) and HDAg-p24 is only packaged if HDAg-p27 is also present. However, deletion of the N-terminal coiled-coil region from the HDAg-p27 gene results in the expression of a mutant HDAg-p27 which is unable to facilitate packaging of HDAg-p24 (Lazinski and Taylor, 1993). This suggests that a direct protein-protein interaction between HDAg-p24 and HDAg-p27 via this domain is essential for the assembly of the HD virion (Chen et al, 1992; Lazinski and Taylor, 1993). It is clear that there must be some interaction between the HDAg and/or HDV RNA and the HBsAg during virus assembly, although the packaging signals are not well defined. The presence of the

prenylation site at aa211 to aa214 of HDAg-p27 (see Section 1.2.I(iii); Figure 1.4) was shown to be essential for the formation of HDV particles (Glenn et al, 1992). A HDAg-p27 mutant in which the cysteine residue was converted to a serine was not prenylated nor was it able to form particles with HBsAg. It was proposed that prenylation targets HDAg-p27 to the ER membrane which contains HBsAg (Glenn et al, 1992). In addition, recent studies showed that the presence of the pro/gly rich region at the C-terminal end of HDAg-p27 (that includes the prenylation site) is required for HDAg-p27 to be packaged with the HBsAg (Lazinski and Taylor, 1993). Together, these results and the recent observation by Ryu et al (1993) that a HDV-specific-RNP was detected in the nuclei of cells undergoing HDV replication, have led to the proposal that HDAg-p27 binds to the HDV RNP in the nucleus via a coiled-coil interaction with HDAg-p24 and exports the complex to the ER where the isoprenylated pro/gly rich region of HDAg-p27 interacts with the HBsAg to initiate virion morphogenesis (Lazinski and Taylor, 1993).

1.2.K **HDV Pathogenesis**

The pathogenic nature of HDV was clearly demonstrated in chimpanzee studies in which the duration and magnitude of HDV-associated hepatitis in superinfected HBV carrier chimpanzees was related temporally to HDV replication (Ponzetto et al, 1987). The histological features observed in the acute phase of HDV infection suggested that HDV is directly cytopathic (Popper et al, 1983).

Acute delta hepatitis commonly results in degenerative changes in the parenchyma characterised by hepatocytes with shrunken eosinophilic cytoplasm and pyknotic nuclei, sometimes progressing to acidophilic bodies (Popper et al, 1983; Verme et al,

1986). The parenchyma is predominantly free of inflammatory cells, even in areas with significant histologic change, consistent with a direct cytopathic effect. In contrast, portal tracts are expanded and densely infiltrated by mononuclear cells (Rizzetto et al, 1983; Verme et al, 1986). In acute HDV hepatitis, a close association exists between areas of necrosis and HDAg-positive cells, with HDAg found predominantly in the nucleus, but also in the cytoplasm in some cases (Rizzetto et al, 1983). In some outbreaks of severe acute HDV-associated hepatitis, small-droplet steatosis in the hepatocytes which undergo focal necrosis is a feature (Popper et al, 1983). However, this does not appear to be a widespread feature, and is thought to be the result of additional genetic and environmental factors unique to specific populations (Govindarajan et al, 1985). The molecular basis of the proposed cytopathic effect of HDV is unknown, although a number of mechanisms have been suggested, that include (a) one of the virus-coded proteins may be directly cytotoxic (Macnaughton et al, 1990a); (b) competion for host cell enzymes during HDV RNA replication may cause a cytopathic effect (Gowans et al, 1987); and (c) antigenomic HDV RNA may anneal with specific sequences in host 7SL RNA, to inhibit the formation of a functional signal recognition particle (SRP) which plays a crucial role in the translation of host secretory proteins (Negro et al, 1989). This may occur by simple base-pairing (Negro et al, 1989) or alternatively, by 7SL RNA cleavage as a result of interaction with HDV RNA in the vicinity of the self-cleavage site (Young and Hicke, 1990).

In contrast, in patients with chronic HDV-induced hepatitis, the distribution of the inflammatory infiltrate in the liver correlates with HDV RNA and HDAg expression (Negro et al, 1988a), but not necessarily with areas of hepatocyte degeneration. This histologic presentation more closely resembles that seen in HBV-associated hepatitis

(section 1.1.D) and suggests that in chronic HDV-associated hepatitis, liver injury is likely to be the result of the host immune response to ongoing HDV (or HBV) infection, rather than a direct cytopathic effect of HDV. The mechanism by which the virulence of HDV might be attenuated during the course of infection is unknown. It is clear that 'healthy' HDV carriers exist with essentially normal liver histology (Hadziyannis et al, 1991), and it is possible that the variation in the host immune response between individuals may be a major contributing factor to the outcome of chronic HDV-related liver disease. This point is discussed in detail in Chapter 8.4.

1.2.L **The Transplant Model of HDV Infection**

The evaluation of patients who require orthotopic liver transplantation as a result of end-stage HBV- or HDV- related liver disease, has identified a unique model of HDV infection, namely, latent HDV infection. Studies of post-transplant patients have shown that although there is a high rate of HDV reinfection (ca. 75%) in these patients, reinfection may occur in the absence of detectable HBV replication (Rizzetto et al, 1987; Ottobrelli et al, 1991; Davies et al, 1992). In these patients there is no evidence of liver disease, despite the presence of HDAg and HDV RNA in a variable, but small number of hepatocytes. Clinical disease only occurred in these patients upon reinfection of the graft with HBV some time later, whereupon HDV spread to a significantly greater number of hepatocytes (Ottobrelli et al, 1991; Davies et al, 1992). In contrast, patients who were simultaneously reinfected by both HBV and HDV experienced a recrudescence of liver disease soon after reactivation (Ottobrelli et al, 1991). On the basis of these results, it is unclear if HDV is non-pathogenic per se, or if the absence of liver disease simply reflects the small number of hepatocytes reinfected initially with HDV, since the release of infectious HDV particles is inhibited

in the absence of HBV infection (Mason and Taylor, 1991). A likely model for the transplant patient has recently been developed in the SCID mouse (Netter et al, 1993). In this experimental model, as in the transplant patients, HDV infection was restricted to the number of hepatocytes infected initially, since cell-to-cell spread is inhibited due to the lack of HBsAg or WHsAg. The results of HDV infection studies in this model showed a marked decrease in the level of HDV replicative intermediates and HDAg-positive hepatocytes ten days post-inoculation, that was not attributable to host immune clearance since the rate of this decrease was the same in normal mice and the SCID mice which lack T- and B- lymphocytes (Netter et al, 1993). Thus, it was proposed that HDV infection was the direct cause of cell death, consistent with the above hypothesis in the transplant patients that the lack of a detectable cytotoxic effect was due to the small number of hepatocytes reinfected initially with HDV.

1.3 General Viral Pathogenesis

Viral pathogenesis can be defined as the mechanism by which viruses produce disease in the host (Tyler and Fields, 1990). The 'virulence' of the infecting virus is determined by a combination of both host and viral factors, which influence the outcome of the virus-host cell interaction. At the cellular level, it is possible to identify specific viral proteins or viral genes which are important for the induction of disease, although, in many cases the molecular mechanism of viral pathogenicity remains unknown.

In the event that virus infection results in virus-induced CPE or cell injury, this may be due to (1) secondary effects resulting from virus-adaptation of the host cell machinery to permit virus replication, or (2) the direct cytotoxic effect of viral gene

products per se. Poliovirus is a good example of the first mechanism; the specificity of the host cell translational machinery is changed to permit the efficient translation of poliovirus mRNA while host mRNA translation is inhibited. In this example, the mechanism by which host translation is down-regulated is believed to be the result of cleavage of one of the components (p220) of the cap-binding protein (CBP) complex which is involved in translation of capped mRNA (Etchison et al, 1982). Unlike host cell mRNA, poliovirus mRNA is not capped and thus does not require a functional CBP complex for continuous translation of viral mRNA. The poliovirus 2A protease is believed to be involved indirectly in the cleavage of p220 and the inactivation of the CBP complex (Bernstein et al, 1985; Lloyd et al, 1986).

There are relatively few examples of viral proteins that are known to be cytotoxic to the host. Studies which examined the cytopathogenesis of Parvoviruses provided direct evidence of the cytotoxic nature of Parvovirus non-structural (NS) proteins. A stably-transfected cell line was developed in which the expression of the NS transcription unit was regulated by the addition of dexamethasone to the culture medium. Clones of neoplastic human cells that were induced to express NS proteins showed a direct CPE and eventual cell death (Caillet-Fauquet et al, 1990).

Using a similar system, Friedman et al (1989) examined the cytotoxic properties of the herpes simplex virus type 1 (HSV-1) glycoprotein gC1. gC1 was expressed in stably-transfected L cells under the control of the inducible Mouse Mammary Tumour Virus (MMTV) promoter, and was shown to induce in the short term, bizarre cell shapes including syncytial formation and in the long term, cell death. It is difficult to predict the role of gC1 in HSV-1 pathogenesis in vivo as it is only one of a number of HSV-1 genes which have been implicated in HSV-1 induced cytopathology (Tyler and Fields,

1990). Furthermore, in each of the examples presented above, the mechanism of cytotoxicity is unknown.

Studies which examined transformation caused by adenoviruses resulted in the identification of cytotoxic adenoviral proteins, and also a virus-encoded protein with the ability to overcome this cytotoxicity. The adenovirus early region 1 (E1) encodes two distinct transcription units, designated E1A and E1B, which function in concert to regulate expression of other adenovirus early genes in productively infected cells and in the transformation of primary rodent cells (White and Stillman, 1987). However, transfection of the E1A gene only into primary rodent cells resulted in acute cytotoxicity that was characterised by host cell chromosomal DNA fragmentation and cytolysis, analogous to cells undergoing apoptosis (White et al, 1991). In contrast, coexpression of the E1B-19 kD protein with the E1A proteins was sufficient to maintain host cell viability during infection by preventing the induction of cell death caused by the E1A proteins (White et al, 1991; White et al, 1992). The mechanism by which this cytotoxicity is either induced or suppressed is not known.

1.4 Aims of Thesis

The general aim of this thesis was to investigate the molecular basis of the cytopathic effect of HDV, and to develop a model for HDV pathogenicity based on the results of these studies. Since it is proposed that HDV is directly cytopathic, and because HDV is a relatively simple virus, it was considered that the molecular mechanism for this cytotoxicity could be elucidated. During the period of this thesis (1989-1993) several mechanisms were suggested to account for HDV-induced cytotoxicity. Thus, the

major goal of this project was to examine the pathogenic role of HDAg in HDV-associated hepatitis.

The specific aims of the project were (a) to develop in vitro cell culture models to examine the direct effect of the expression of HDAg-p24 and HDAg-p27 in transfected eukaryotic cell lines, (b) to examine the effect of coexpression of HDAg-p24 and -p27 on cytotoxicity, and (c) to examine the mechanism of HDV interference of HBV replication in this model system.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Eukaryotic Cell Culture

2.1.A Routine Maintenance

All routine cell culture work was performed using Dulbecco's Modified Eagles Medium (DMEM; Flow or Gibco) containing 20mM HEPES and supplemented with 5-10% non-inactivated foetal calf serum (FCS; Flow, Gibco, CSL), 100 units/ml penicillin (CSL) and 40 µg/ml gentamycin (DBL). All continuous cell lines (Table 2.1) were maintained in 75 cm^2 culture flasks (Costar, Corning, Nunc, Falcon). The medium was changed twice per week and the cells subcultured every 5-7 days. For long term storage, cells were stored in DMEM containing 20% FCS and 10% dimethlysulphoxide (DMSO; Univar) under liquid nitrogen. All cell lines were shown to be mycoplasma free by routine testing performed in the Division of Clinical Microbiology, IMVS.

2.1.B Coverslip Cultures

Coverslip cultures were used for immunofluorescence assays, some cytological analyses and ^{3}H-thymidine and ^{3}H-uridine incorporation assays. 13 mm diameter glass coverslips (Lomb) were soaked overnight in chromic acid, washed for 4 hr in running tap water, rinsed in DDW and stored under methanol. Prior to use, the coverslips were rinsed in fresh methanol, flame sterilised, placed in the 16 mm wells of a 24 well multiplate (Costar, Nunc) and seeded with trypsinised cells at varying cell densities.

TABLE 2.1

Eukaryotic Cell Lines Used Throughout This Study

CELL LINE	ORIGIN	REFERENCE
Hep G2	Hepatoblastoma	Knowles et al (1980)
Hela	Cervical carcinoma	ATCC CCL 2.1
Cos7	Monkey kidney	Gluzman (1981)
H1δ8	HH1 transfected with HDV cDNA	Macnaughton et al (1991)
H1δ9	HH1 transfected with HDV cDNA	Macnaughton et al (1991)

2.2 Prokaryotic Cell Culture

2.2.A Growth Media and Bacterial Strains

Nutrient broth (2YT broth) containing tryptone (16 g/litre), yeast extract (10 g/litre) and NaCl (10 g/litre; pH 7.4) was the general growth media used for all E.coli cultures. For large scale preparation of recombinant vectors and plasmids, Super Broth (Appendix 1) was substituted for 2YT broth. All nutrient agar plates were prepared using 2YT broth containing 2% agar (Difco). All recombinant plasmids and vectors used in this study carried an ampicillin resistance gene, and selection of transformed cells was performed in liquid broth and solid media containing 50 µg/ml ampicillin (Sigma). Bacterial cultures were incubated at 37°C. The recA- E.coli strain DH-5α, used throughout this study was obtained from Professor Paul Manning (University of Adelaide).

2.2.B Transformation Procedure

A single colony picked from an overnight culture of DH-5α, was transferred to 20 ml of 2YT broth and incubated, with shaking, at 37°C until the culture reached an OD of 0.6 at 550 nm. The cells were chilled on ice for 10 min, pelleted at 4^0C in a bench centrifuge (2 500 g, 5 min) and resuspended in 10 ml of cold 100mM $CaCl_2$ in DDW. After incubation on ice for 30 min the cells were repelleted and finally resuspended in 2.0 ml of cold 100mM $CaCl_2$. The cells were stored on ice for at least 60 min prior to use. Competent DH-5α cells (0.2 ml) were mixed gently with up to 50 ng DNA (1-10 µl ligation mix) and left on ice for a further 30 min. The cell/DNA mix was heat-shocked at 42^0C for 90 sec, 800 µl of 2YT broth was added, then incubated at 37^0C for 40 min with shaking. The culture was plated directly onto selection plates or after

concentration by centrifugation. Transformants containing plasmids that utilise the Lac Z colour selection system were plated onto 2YT agar which contained 2% Xgal (Boehringer) and 100mM IPTG (Boehringer) [see section 2.3.A(iii)].

2.3 Molecular Cloning Techniques

2.3.A Plasmid Vectors

(i) pSV2neo (Figure 2.1)

pSV2neo is a eukaryotic expression vector (Southern and Berg, 1982), that does not contain its own promoter sequences. It was generously provided by Alan Robbins, Dept. of Biochemistry, University of Adelaide. It comprises a pBR322 origin of replication and ampicillin- and neomycin (neo)- resistance genes. The product of the neo gene inactivates the cytotoxic aminoglycoside antibiotic G418. Hence, transfection leading to stable integration will confer G418 resistance which allows selection of transfected eukaryotic cells. Foreign DNA, inserted into the Eco R1 or Bam H1 restriction enzymes sites, must include its own promoter sequences and polyadenylation signals for efficient gene expression.

(ii) pSVL (Figure 2.2)

pSVL is a eukaryotic high efficiency expression vector (Pharmacia) that includes a pBR322 origin of replication and an ampicillin resistance gene. Genes inserted into the MCS are expressed from the first AUG codon under the control of the efficient SV40 late promoter. pSVL may be used for transient expression of any foreign DNA, and is not designed to integrate into the cellular chromosome.

Fig 2.1 The major features of the eukaryotic expression vector pSV2neo.

Fig 2.2 The major features of pSVL, showing the location of the MCS relative to the SV40 late promoter.

Fig 2.1 pSV2neo

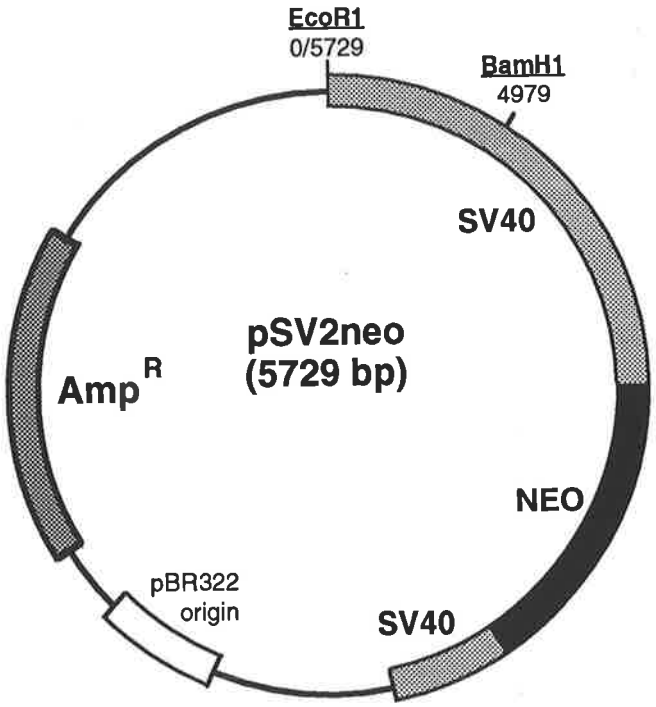
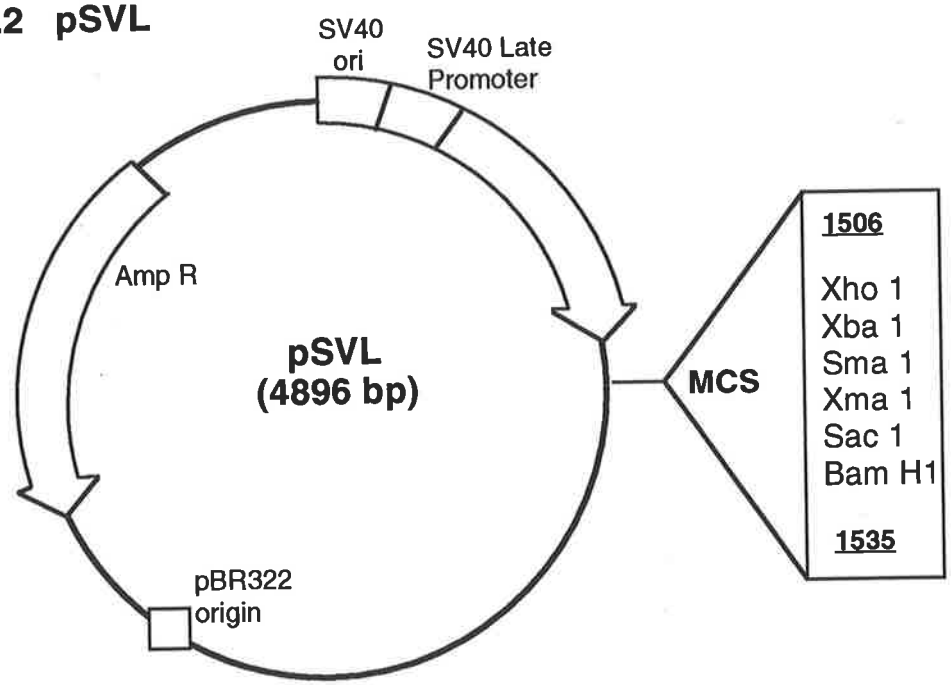


Fig 2.2 pSVL



(iii) pBLUESCRIPT KS+ (Figure 2.3)

The phagemid pBluescript KS+ (Stratagene) is derived from pUC19 and contains the Col E1 origin of replication and an ampicillin resistance gene. The MCS present within the Lac Z gene contains 21 unique restriction sites flanked by T3- and T7- RNA polymerase promoters used for the generation of strand-specific RNA probes. In addition, the MCS contains the M13 forward and reverse primer binding sites to allow sequencing of cloned foreign DNA in both directions.

Insertion of any DNA species into the MCS disrupts the Lac Z gene such that subsequent transformation into an E.coli strain containing the Lac Z M15 deletion (including DH-5α) results in a lack of a functional Lac Z gene product. Thus, when these transformants are plated on agar containing Xgal (Boehringer) and IPTG (Boehringer), they appear white, while transformants containing no foreign insert appear blue.

(iv) pTMδXbaA (Figure 2.4)

pTMδXbaA (Macnaughton, Ph.D. thesis) contains a full-length copy of HDV cDNA derived from a chimpanzee HDV isolate (Wang et al, 1986), inserted into the Xba 1 site of the vector pGEM-3 (Promega). pTMδXbaA contains a pBR322 origin of replication, an ampicillin resistance gene and SP6- and T7- RNA polymerase promoters which flank the HDV cDNA insert, and thus can be utilised to generate strand-specific riboprobes to detect genomic- and antigenomic- sense HDV RNA. This plasmid was the primary source of HDV cDNA used in subsequent cloning

Fig 2.3 The major features of the phagemid pBluescript KS+, showing the location of the MCS relative to the T3- and T7- RNA polymerase promoters.

Fig 2.4 The recombinant plasmid pTMδXbaA, (Macnaughton, PhD thesis) contains a full-length copy of HDV cDNA inserted into the Xba 1 site of pGEM-3 (Promega).

Fig 2.5 The major features of pUCMT, showing the location of the MT11A promoter relative to the unique Eco R1 site.

Fig 2.3 pBluescript KS+

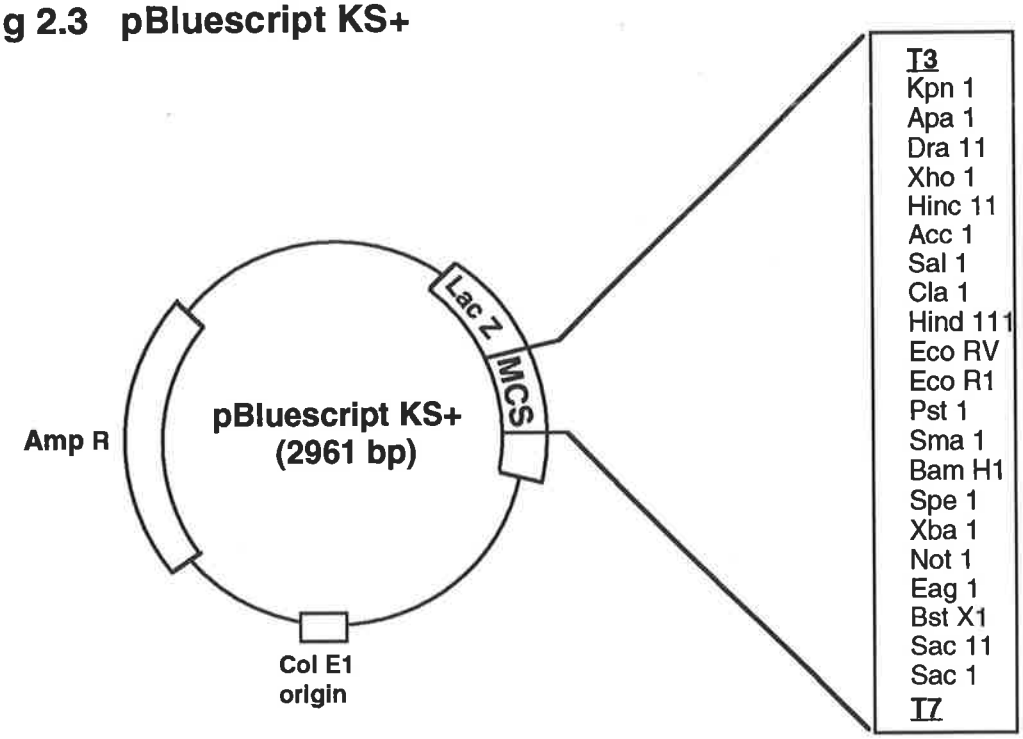


Fig 2.4 pTMδXbaA

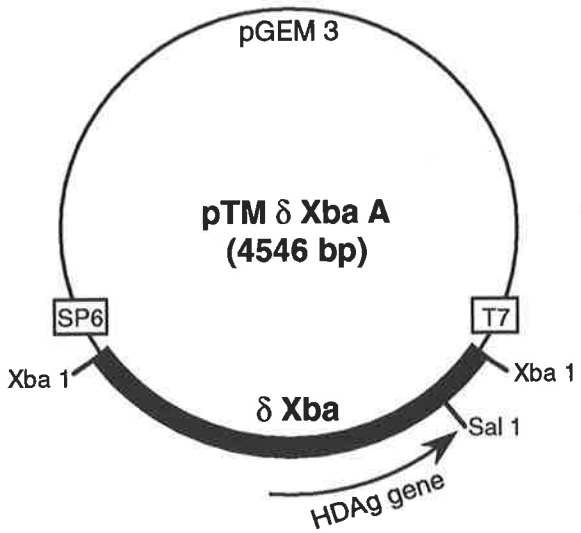
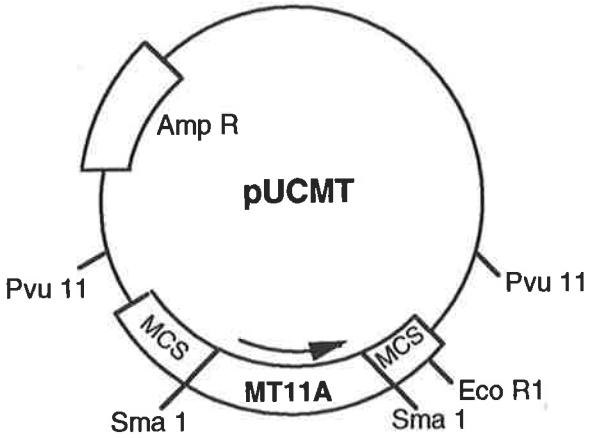


Fig 2.5 pUCMT



experiments (described in Chapter 3.2), as well as the template for PCR-based experiments [see section 2.3.G(ii)].

(v) pUCMT (Figure 2.5)

pUCMT is a pUC18-derived vector comprising a pBR322 origin of replication and an ampicillin resistance gene. The inducible human metallothionein (MTIIA) promoter sequence (Karin and Richards, 1982) is inserted into the Sma 1 site in the MCS to ensure that expression of any foreign DNA inserted into the unique Eco R1 site is controlled by heavy metal ions. pUCMT was a gift from Alan Robbins, Dept. of Biochemistry, University of Adelaide.

2.3.B **Extraction and Precipitation of DNA**

Phenol was prepared for use in extraction of DNA and RNA (section 2.6) as described in Sambrook et al (1989); crystallised phenol (BDH) was melted at 68°C then 8-hydroxyquinoline added to a final concentration of 0.1%. The molten phenol was then washed once in 1M Tris-HCl (pH 8.0) followed by repeated washes in 0.1M Tris-HCl (pH 8.0) until the pH value of the aqueous phase was ≥ pH 7.6. Phenol was stored under 0.1M Tris-HCl (pH 8.0) at 4°C.

Crude DNA preparations were extracted by the addition of an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), vortexed, and then centrifuged at ca. 10 000 g for 3 min. The aqueous phase, which contained the DNA, was transferred to a clean tube and the DNA precipitated by the addition of 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. The DNA was recovered by

centrifugation at ca. 15 000 g for 30 min, and the pellet washed 1-3 times in 70% ethanol before freeze-drying. The process of extraction and precipitation of DNA described above, will be referred to as ethanol precipitation in this thesis.

2.3.C **DNA Modifying Enzymes**

(i) Restriction Enzymes

Restriction enzymes (RE) were purchased from several manufacturers (Pharmacia, Boehringer, New England Biolabs, Amersham). All digestions were performed at 37°C for 90-180 min with the enzyme: DNA ratio appropriate for the particular enzyme used. All RE digests utilised either the single buffer system described by Sambrook et al (1989), (Appendix 2) or the specific RE buffer supplied by the enzyme manufacturer.

(ii) Blunt-ending

5' overhangs created by RE digestion were end-filled immediately after completion of the digestion step in the same reaction tube by the addition of 0.5mM of each dNTP and 0.05 units/ml of the Klenow fragment of DNA polymerase I (Boehringer). The reaction was incubated at 37°C for 15 min, heated to 70°C for 10 min to inactivate the enzyme and the DNA was ethanol precipitated.

The procedure to end-fill 3' overhangs was similar, although in this case T4 DNA polymerase (Promega) was used in a reaction mixture containing 70mM Tris-HCl (pH 7.4), 5mM $MgCl_2$, 5mM DTT, 0.1mM of each dNTP, 1.0 µg/µl DNA and 0.5 units/µl enzyme. The T4 DNA polymerase was inactivated at 75°C for 10 min prior to ethanol precipitation of the DNA.

(iii) Alkaline Phosphatase

In order to prevent self-ligation of vector DNA following RE digestion, 5' terminal phosphates were removed in a reaction mixture containing 50mM Tris-HCl (pH 9.0), 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 0.1-0.2 ng DNA/µl and 0.1 units of alkaline phosphatase (Pharmacia)/µg of DNA. The reaction was incubated at 37°C for 30 min, followed by digestion with 50 µg/ml proteinase K (Boehringer), 0.5% SDS at 56°C for 30 min to inactivate the alkaline phosphatase, and the DNA then ethanol precipitated prior to use in ligation reactions.

(iv) Polynucleotide Kinase

Terminal 5' phosphates were added to DNA in a reaction mixture containing 10mM Tris-acetate (pH 7.5), 10mM magnesium acetate, 50mM potassium acetate, 1mM ATP and 1 unit of polynucleotide kinase (Pharmacia)/µg of DNA. The reaction was incubated at 37°C for 1 hr, then at 65°C for 5 min to inactivate the enzyme, and the DNA then ethanol precipitated.

(v) Ligation

Ligation of vector and insert DNA was performed using bacteriophage T4 DNA ligase, in a buffer containing 20mM Tris-HCl (pH 7.6), 5mM $MgCl_2$, 5mM DTT and 0.05 mg/ml BSA (Boehringer). Cohesive end ligation mixtures were incubated at RT for 2-6 hr with final DNA concentrations of 2.5-5 ng/µl, and vector: insert molar ratios of 1:1 to 1:3. Blunt-end ligations were incubated at RT for 16-24 hr with a final DNA concentration

of 5-10 ng/µl, and vector: insert ratios of 1:3 to 1:10. T4 DNA ligase (Boehringer) was added to 1 unit/0.1-0.2 µg DNA for all ligation reactions.

2.3.D **Agarose Gel Electrophoresis**

For routine analysis, DNA was separated in horizontal submerged agarose gels (1-2%) using a BRL model H3 electrophoresis system and a Biorad model 3000 *xi* power supply. Agarose (Biorad) was dissolved in TAE buffer by heating, cooled to ca. 50°C and poured into gel-forming trays with a 1-2 mm thick comb and allowed to set. The DNA samples were mixed with one sixth volume of 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), loaded onto the gel and electrophoresed at 50-200V until the dye front neared the end of the gel. The gel was then stained at RT for 15 min in DDW containing 20 ng/ml of ethidium bromide, washed in DDW, the DNA visualised using a UV transilluminator and photographed using Polaroid type 667 film. DNA fragment sizes were determined by comparison with Pst 1-digested λ DNA (Appendix 3).

Two methods were used for the purification of specific DNA fragments from agarose gels.

(i) Electroelution

Electrophoresis was performed as described above except that preparative grade agarose (BRL) was used. The band containing the desired DNA fragment was exised and the gel slice was inserted into a 2.5 cm diameter dialysis bag (Selby Scientific, Adelaide) containing 2.0-3.0 ml of 0.2xTBE. The DNA was electroeluted for 30 min at 200V, then the current was reversed for 1 min. The eluate was removed from the

dialysis bag, extracted with butanol to ca. 300 µl, the DNA ethanol precipitated and redissolved in 50-100 µl of TE-8.

(ii) Recovery Through Glass Wool

Electrophoresis was performed as described above (2.3.D) except that the desired fragment of DNA was separated in a 1% low melting point agarose gel. The gel slice was packed into a 0.5 ml Eppendorf tube with a small hole in the bottom, that contained a 1-2 mm depth of siliconised glass wool. This tube was placed inside a 1.5 ml Eppendorf tube and centrifuged at 6 500 rpm for 5 min. The eluate was collected in the 1.5 ml tube, the DNA ethanol precipitated and redissolved in 20 µl of TE-8.

2.3.E **Plasmid Amplification**

(i) Small Scale Plasmid Amplification

Transformants were screened by RE analysis of minipreparation plasmid DNA. Antibiotic-resistant single colonies were inoculated into 3 ml of 2YT broth containing 50 µg/ml ampicillin and incubated overnight with shaking at 37°C. 1.5 ml of this culture was transferred to an Eppendorf tube and centrifuged at ca. 10 000 g for 10 min. The supernatant was removed, the pellet resuspended in 100 µl of lysis sol A [50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA and 5 mg/ml lysozyme (Boehringer)] and incubated for 5 min at RT, then 200 µl of lysis sol B (0.2M NaOH, 1% SDS) was added and held on ice for 5 min. Finally 150 µl of 3M sodium acetate (pH 5.2) was added, vortexed, held on ice for 5 min and then centrifuged at 10 000 g for 10 min. The supernatant was transferred to a clean Eppendorf tube and the

DNA was ethanol precipitated and then redissolved in 100 µl of TE-8 containing 300 µg/ml of RNase A (Boehringer). 5 µl of this DNA was analysed by RE mapping to select recombinant plasmids.

(ii) Large Scale Plasmid Amplification

A single colony of DH-5α cells containing the recombinant plasmid, isolated on an ampicillin selection plate, was inoculated into 25 ml of 2YT containing 50 µg/ml ampicillin and incubated overnight at 37°C with shaking. This culture was then transferred to 500 ml of Superbroth containing 50 µg/ml ampicillin and incubated at 37°C with shaking for a further 6 hr, and finally chloramphenicol (Parke Davis) was added to a final concentration of 150 µg/ml and incubation continued overnight. The cells were pelleted (7 000 g, 10 min, 4°C), resuspended in 100 ml STET, transferred to a 500 ml volumetric flask, 50 mg lysozyme added and the mixture swirled on a boiling water bath for 5 min. The mixture was then transferred to a 250 ml polypropylene centrifuge tube, cooled on ice and centrifuged for 15 min at 20 000 g at 4°C. The supernatant was transferred to a clean 250 ml centrifuge tube and an equal volume of isopropanol (cooled to -20°C) was added. The precipitate was collected by centrifugation (20 000 g, 30 min, -10°C), and the pellet redissolved in 17 ml of TE-8. $CsCl_2$ was added to a final concentration of 1.05 g/ml and 0.7 ml ethidium bromide (10 mg/ml) added. The solution was clarified by centrifugation (16 500 g, 35 min, 20°C), the supernatant transferred to two Beckman 13.5 ml heat seal tubes, and then centrifuged in a Beckman L8 Ultracentrifuge in an 80 Ti rotor (49 700 rpm, 20 hr, 20°C) to separate plasmid and chromosomal DNA.

The tubes were examined under long wave U.V. light and the lower band corresponding to the plasmid DNA was collected using a 2 ml syringe fitted with a 19G needle. The ethidium bromide was removed by extraction with water-saturated butanol, the DNA was ethanol precipitated, and redissolved in 2.0 ml of TE-8. Finally, the DNA was ethanol precipitated again and redissolved in 200 µl of TE-8. The DNA concentration was determined by adsorption spectrophotometry at 260 nm (where 1 OD unit at A^{260} = 50 µg/ml) and adjusted to a final concentration of 1 µg/µl. The DNA was stored at -20°C in small aliquots.

2.3.F **Transfection**

Stable transfection of plasmid DNA into eukaryotic cells was performed using either the polybrene technique (Kawai and Nishizawa, 1984) or using Lipofectin (BRL). In both cases, Geneticin (G418; Gibco) selection was used at a predetermined optimum concentration. Transient transfection of plasmid DNA into eukaryotic cells was performed using DOTAP (Boehringer).

(i) Adherent Cell Technique

This method was used for HepG2 cells and is based on the methods described by Kawai and Nishizawa (1984) and Morgan et al (1986). A subconfluent (40-50%) 25 cm^2 flask of HepG2 cells, seeded the previous day ($1x10^6$ cells/flask), was washed briefly with 10 ml of DMEM containing 5% FCS and 5 mg/ml polybrene. This medium was then removed and replaced with the transfection mix consisting of 1 ml DMEM containing 5% FCS, 5 mg/ml polybrene and 10 µg plasmid DNA, and the cells were incubated at 37°C for ca. 6 hr with occasional swirling of the flask. The cells were

then shocked with 30% DMSO in DMEM at RT for 4 min, washed once in DMEM, then DMEM + 5% FCS was added and the cells were incubated for 48 hr at 37°C in 5% CO_2. The cells were then trypsinised and resuspended in DMEM containing 5% FCS and 350 µg/ml G418 then seeded into a 48 well plate. The medium was changed every 2-3 days, the plates examined daily, and G418-resistant colonies were detected after 2-3 weeks. Stable single-cell clones were isolated and then amplified under G418 selection for a further 3 months until a liquid nitrogen stock was established.

(ii) Suspension Cell Technique

This method was used for HeLa cells; a subconfluent (~70%) 75 cm^2 flask of HeLa cells was trypsinised to the point where the cells just detached from the surface (over trypsinisation decreased transfection efficiency), and the cells resuspended in 10 ml DMEM + 5% FCS. A cell count was performed and 5 x 10^5 cells were transferred to a 10 ml centrifuge tube and pelleted at 800 g for 5 min in a bench centrifuge. The cell pellet was resuspended in 300 µl of DMEM + 5% FCS + 25 mg/ml polybrene containing 5-10 µg of plasmid DNA, and then incubated at 37°C for 100 min. The cells were gently resuspended at 20 min intervals. 1.5 ml of DMEM + polybrene only was then added and incubated for a further 20 min at 37°C. The cells were transferred to a 25 cm^2 flask and incubated at 37°C in 5% CO_2 until the cells adhered (4-5 hr). The cells were shocked with DMSO as described above; 48 hr after seeding, the cells were trypsinised and reseeded into a 96 well plate in DMEM + 5% FCS and 400 µg/ml G418. Single-cell clones were detected and isolated as described above.

(iii) Lipofectin Transfection Technique

The method was used for HeLa and HepG2 cells and is based on the method described by the manufacturer; a subconfluent (60-70%) 25 cm^2 flask of HepG2 or HeLa cells, seeded the previous day at 1.0 x 10^6 cells and 2 x 10^6 cells/flask respectively, were washed twice in serum-free DMEM, and then 3 ml of DMEM +1% FCS was added. The transfection mix was then prepared [50 µg of Lipofectin (BRL) and 10 µg of plasmid DNA in a final volume of 100 µl] and incubated at RT for 15 min. The mixture was then added dropwise to the cells with swirling and the cells then incubated for 5 hr at 37°C in 5% CO_2. 7 ml of DMEM + 5% FCS was then added and the cells were incubated overnight at 37°C in 5% CO_2. The medium was then removed and replaced with fresh DMEM + 5% FCS only. 48 hr after addition of the plasmid DNA, the cells were trypsinised, resuspended in medium containing G418 and reseeded into 96 well plates. Single-cell clones were isolated as described above.

(iv) Transient Transfection

Transient transfection of plasmid DNA into Cos7 cells using DOTAP (Boehringer) was performed essentially as described by the manufacturer; a subconfluent (60-80%) 60 mm tissue culture dish (Costar) of Cos7 cells was seeded two days prior to transfection. The cells were washed twice in DMEM + 1% FCS and then 3 ml of DMEM + 1% FCS was added. 5 µg of DOTAP was diluted into 100 µl of HBS in a polystyrene tube. In a separate polystyrene tube, 5 µg of plasmid DNA was diluted into 100 µl of HBS. The solutions were mixed and incubated at RT for 5-10 min, added to the cells dropwise, and incubated overnight at 37°C in 5% CO_2. The medium was replaced with fresh DMEM + 5% FCS and then incubated for a further 1-7 days.

Transient transfection of plasmid DNA into Cos7 cells seeded onto 13 mm coverslips was performed as described above, with the exception that the volume of the transfection reagents was reduced; the final transfection mix/13 mm coverslip culture contained 1 µg of DOTAP and 1 µg of plasmid DNA in a final volume of 20 µl of HBS. This solution was added dropwise to one well of a 24 well tissue culture dish which contained 0.5 ml of DMEM + 1% FCS.

2.3.G **Site-Directed Mutagenesis Using PCR**

(i) Production and Purification of Oligonucleotides

Oligonucleotide primers for PCR were synthesised using an Applied Biosystems DNA synthesiser model 380A. The primers were removed from the column by passing 2 ml of concentrated ammonia solution through the column 6-10 times every 20 min for 4 hr. The ammonia solution containing the oligonucleotide was then heated overnight at 55°C, cooled to RT and dried under vacuum. The DNA pellet was redissolved in 500 µl of TE-8 and the DNA concentration was determined by adsorption spectrophotometry at 260 nm (where 1 OD unit at A^{260} = 30 mg/ml) and adjusted to a final concentration of 1 mg/ml.

(ii) Amplification of HDV cDNA

Site-directed mutagenesis of the HDAg gene was performed using a two-step PCR protocol based on the method of Higuchi (1990). This protocol is described fully in Chapter 4.2. The PCR conditions were as follows; the reactions were performed in a 50 µl volume containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2µM of each primer (Figure 2.6), 150µM of each dNTP, 1.5U Taq polymerase (Perkin Elmer

Fig 2.6 Oligonucleotide primers for PCR

Two internal complementary oligonucleotide primers (primer ω and υ) which contained the same desired base change, were used in conjunction with two external wild-type primers (primer α and β) in a two-step PCR to introduce a single base mutation into HDV cDNA at nt 1012.

FIG 2.6 Oligonucleotide Primers for PCR

PRIMER α

Antisense primer, 27 bp (map position 303-329)

5' TGTTCGCTGAAGGGGTCCTCTGGAGGT 3'

PRIMER β

Sense primer, 30 bp (map position 905-934)

5' AGGAAAGAAGGACGCGAGACGCAAACCTGT 3'

MUTANT PRIMER ω

Sense primer, 30 bp (map position 997-1026)

5' GGGAAGAGTATATCC**C**ATGGAAATCCCCGG 3'

MUTANT PRIMER ν

Antisense primer, 30 bp (map position 997-1026)

5' CCGGGGATTTCCAT**G**GGATATACTCTTCCC 3'

Cetus) and 10 ng of plasmid DNA, in a DNA Thermal Cycler (Perkin Elmer Cetus). The first cycle contained a denaturation step of 94°C for 7 min, and this was followed by 40 cycles [denaturation (94°C for 1.5 min), annealing (55°C for 2 min) and extension (72°C for 3 min)], and a final extension period of 72°C for 5 min. 10 µl of each reaction was analysed by agarose gel electrophoresis.

2.4 Viral Antigen Detection

2.4.A Immunofluorescence

(i) Sample Preparation

(a) Coverslip Cultures

Both direct and indirect staining were used to detect HDAg. Coverslip cultures of cells were rinsed in PBS, air dried, fixed in acetone at 4°C for 7 min, air dried again and rehydrated in PBS for at least 15 min prior to staining.

(b) Tissue Sections

Frozen tissue samples were sectioned onto uncoated slides and air dried for 30 min. The sections were fixed in acetone at 4°C for 30 sec. This fixation time was determined to be the optimum time for the detection of CD4 and CD8 T-lymphocyte markers and HLA Class I antigens in frozen liver samples by indirect immunofluorescence, and did not affect the ability to detect HDAg in double staining experiments (see Chapter 6.2). The sections were then air dried again and rehydrated in PBS for at least 15 min prior to staining.

(ii) Staining Protocol

HDAg -p24 and -p27 were detected using either pooled human serum from HDV chronic carriers, or with a rabbit polyclonal HDAg-specific antiserum (a gift from M.M.C. Lai, USC). All labelled secondary antibodies were purchased from commercial suppliers (Wellcome, Silenus, Boehringer). A direct human anti-HD FITC conjugate was prepared in the laboratory (T. Macnaughton). The optimum titres of all antibodies were determined by titration using positive- and negative- tissue samples, or cultured cells, and the highest dilution which gave the optimal signal to noise ratio was chosen for use.

The samples were incubated with the primary antibody or the direct conjugate for 60-120 min at 37°C in a humid chamber, washed twice in PBS for 10 min and for indirect staining incubated for a further 30 min at 37°C with the secondary antibody and washed again for 2 x 10 min in PBS. After staining, the samples were mounted in 90% glycerol/PBS containing 50mM Tris-HCl (pH 8.5), examined with a model BH-2 UV microscope (Olympus) and photographed using a PM-10ADS camera system and PM-CBSP exposure control unit (Olympus).

The specificity of all immunofluorescence staining was verified by the inclusion of negative control cell cultures or tissue sections and/or by substitution of primary antibodies with normal serum from the same species.

Unlabelled and FITC-conjugated antibodies were absorbed against a normal human liver homogenate to reduce non-specific reactions; a liver sample taken at autopsy from a patient negative for markers of HBV and HDV was frozen in liquid nitrogen,

pulverised, passed through a sieve and collected in a beaker containing ice-cold PBS. The homogenate was washed with cold PBS containing 0.2% sodium azide until the supernatant was clear, and then stored at -20°C in small aliquots. For use, the thawed homogenate was washed twice in PBS containing 0.08% sodium azide, and an equal volume of packed homogenate mixed with the antibody at 2x the expected working dilution. The mixture was incubated at 37°C for 1 hr and then 4°C overnight, and then centrifuged (10 000 g, 4°C, 10 min) until all the liver homogenate was removed. The supernatant containing the absorbed antibody was filter sterilised and then stored at -20°C in small aliquots.

2.4.B **Immunoblotting**

(i) Preparation of Iodinated-Protein A

A Sepharose-G25 column was prepared by soaking 5 g of superfine Sephadex-G25 (Pharmacia) in 75 ml DDW and 0.02% sodium azide at RT overnight. This mixture was loaded into a plastic 10 ml pipette plugged with siliconised glass wool and the column was washed with RIP buffer (TBS + 0.5% BSA). 20 µg of Protein A (Sigma) was conjugated to 200 µCi of ^{125}Iodine (Du Pont, NEN) as follows; sol A (0.168M Na_2HP0_4, 0.032M NaH_2P04, pH 7.5) was used as a diluent for sodium metabisulphite (2.5 mg/ml in 25% (v/v) sol A) and chloramine T (2.5 mg/ml in 25% (v/v) sol A). The final reaction was performed in a 70 µl volume mix that contained 20 µg of Protein A (Sigma), 50mM Na_2HP0_4 (pH 7.5), 10mM NaH_2P0_4 (pH 7.5), 200 µCi ^{125}Iodine and 0.71 mg/ml chloramine T. The reaction was incubated at RT for 1 min, followed by the addition of sodium metabisulphite to 0.55 mg/ml to stop the reaction. The reaction mix was then diluted to 190 µl with RIP buffer and the mixture was applied to the column. 0.5 ml fractions were collected by gravity; the fractions that contained the

^{125}Iodine were identified by sampling 2 µl of each fraction and counting in a gamma counter (Packard Instrument Company). The fractions which contained the iodinated-Protein A (fractions 5-7) were separated from those containing free ^{125}Iodine only (fractions 10-12) and those fractions which constituted the first peak were pooled. The iodinated-Protein A was stored at 4°C and could be used for immunoblotting for at least two months after the time of preparation.

(ii) SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970) using a Protean II PAGE System (Biorad). A 15% separating gel and a 5% stacking gel were prepared from a stock solution of 40% acrylamide: bisacrylamide (19:1) (Accugel-40, National Diagnostics). The separating gel contained a final concentration of 0.375M Tris-HCl (pH 8.8), 0.1% SDS, 0.034% APS and 0.0625% TEMED. A final volume of 50 ml of the separating gel solution was poured into the gel former, the surface overlaid with butanol to prevent oxidisation, and polymerised for 1 hr at RT. The surface of the separating gel was washed before the stacking gel was layered on top. 10 ml of the stacking gel in 0.125M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% TEMED and 0.0525% APS was layered over the separating gel and allowed to polymerise as before after the sample comb was inserted.

The samples were mixed with loading buffer [50mM Tris-HCl (pH 6.8), 12.5% (v/v) glycerol, 2.5% SDS, 0.0025% bromophenol blue, 2.5% 2-mercaptoethanol], boiled for 5 min, quenched on ice and loaded onto the gel. The samples were stored at -70°C if required, and then re-boiled prior to loading onto the gel. Low molecular weight dye-linked protein standards (Biorad) were used routinely to monitor running

conditions. In experiments described in Chapters 3.6 and 4.4, the level of HDAg present in zinc-induced cells (see section 2.5.A), was compared with the level in acute-phase HDV-infected chimpanzee hepatocytes. The cells and a sliver of tissue cut from a snap-frozen wedge biopsy were homogenised in equal volumes of 2x loading buffer without bromophenol blue (see above), and each sample was divided into two. One half was adjusted to 0.5M NaOH and incubated at 4°C overnight. These samples were then extracted twice with phenol, and the DNA was then ethanol precipitated as described in section 2.3.B. The DNA concentration in each sample was determined by adsorption spectrophotometry at 260 nm (where 1 OD unit at A^{260} = 50 μg/ml). Based on the assumption that each cell contained 5 pg of DNA, the cell concentration in the original sample was calculated. An aliquot corresponding to $5x10^5$ or $1x10^6$ cells from the second half of each sample was boiled for 5 min, quenched on ice and loaded onto the gel as above.

The running buffer contained 25mM Tris-HCl, 192mM glycine and 0.1% SDS, and electrophoresis was performed at 1W overnight. The protein was transferred from the gel to Immobilon membrane (Millipore) at 40V for 4 hr or 25V overnight, in a buffer comprised of 25mM Tris-HCl, 192mM glycine and 20% (v/v) methanol. After transfer, non-specific binding sites were blocked by incubation of the membrane in TN buffer containing 3% BSA at RT for 4 hr. The membrane was then incubated in TNT + 1% BSA containing a 1/1000 dilution of a human anti-HD serum at 4°C overnight, washed 4 x 8 min in TNT, then incubated for 4 hr at RT with 10^5 cpm/ml of ^{125}I-labelled Protein A in TNT + 1% BSA. The membrane was washed as above, dried, wrapped in plastic film and exposed at -70°C to X-Omat RP or AR X-ray film (Kodak) in cassettes with intensifying screens (Kronex; Dupont, USA). Following exposure, the film was processed in an Ilfospeed 2240 (Ilford) X-ray processor. If required, the level of bound

^{125}I-Protein A for each band identified on the autoradiograph was quantitated by excising the relevant region of the membrane and counting in a gamma counter (Packard Instrument Company).

2.5 The Effect of HDAg Expression in Transfected Cell Lines

2.5.A Induction of HDAg by Zinc

Induction of HDAg expression in stably-transfected cell lines containing the HDAg gene controlled by the human MTIIA promoter, was performed by the addition of zinc sulphate to the culture medium. The test and control cell lines were incubated in DMEM + 5% FCS containing varying concentrations of zinc sulphate described in Chapter 3.3.A.

2.5.B Nucleic Acid Synthesis

Coverslip cultures of transfected and control cell lines were seeded at a density which ensured that the cells were 70% confluent after overnight incubation. The cells were then incubated overnight in varying concentrations of zinc sulphate, pulsed for 4 hr in medium containing 2 μCi/ml ^{3}H-uridine or ^{3}H-thymidine (Amersham) then fixed for 10 min at RT in Carnoys fluid (3:1, methanol: glacial acetic acid). The coverslips were washed twice for 5 min in 10% TCA, rinsed in water, dehydrated in ethanol, air dried, and counted in a scintillation counter.

2.5.C Cell Doubling Time and Cytological Analysis

The medium in duplicate overnight cultures in 25 cm^2 flasks (Costar) was replaced with normal medium or medium supplemented with zinc sulphate, and both flasks re-incubated overnight. The cells were then trypsinised and reseeded at a constant density in fresh normal medium or zinc-supplememted medium in 6 well culture plates. At the same time, 13 mm coverslips were also seeded for cytological analysis. The cell doubling time was determined by counting trypsinised cells using trypan blue exclusion 4 days after seeding. The coverslip cultures used for cytological analysis were fixed in 96% ethanol for 30 min, stained by haemotoxylin and eosin and the cells examined microscopically by Dr. Pauline De La M. Hall for cytological changes.

2.6 Total RNA Extraction from Cultured Cells

Total RNA was extracted from cultured cells by a method adapted from that described previously by Chromczynski and Sacchi (1987). A 60 mm cell culture dish (Costar) containing ca. 10^7 cells was washed twice in PBS, drained for 60 sec, 0.6 ml GIT buffer added [4M guanadinium isothiocyanate (ultrapure; IBI), 25mM sodium citrate (pH 7.0), 0.5% sarkosyl, 0.1M 2-mercaptoethanol], and incubated at RT for 2 min. The contents of the culture dish were transferred to a 1.5 ml Eppendorf tube, and the following reagants were added in the order listed with mixing between each addition; 75 µl 2M sodium acetate (pH 4.6), 0.75 ml phenol and 0.15 ml of chloroform: isoamylalcohol (49:1). The final mix was centrifuged at 10 000 g for 3 min at 4°C. RNA was precipitated from the supernatant with one volume of isopropanol, and recovered by centrifugation at 10 000 g for 15 min at RT. The pellet was washed three times in 80% ethanol, freeze-dried and redissolved in DDW. The concentration

of RNA was determined by adsorption spectrophotometry at 260 nm (where 1 OD unit at A^{260} = 40 µg/ml RNA).

2.7 Northern Blot Analysis

2.7.A RNA Probes

RNA probes (riboprobes) were prepared from template HDV cDNA in the recombinant plasmid pTMδSalA (Macnaughton et al, 1990a). Transcription from the T7 RNA polymerase promoter of pTMδSalA generates a riboprobe that detects antigenomic sense HDV RNA. Riboprobe reactions were performed by the recommended protocol using commercial kits (Promega) and ^{32}P-UTP (3000 Ci/mM; Bresatec, SA).

2.7.B Sample Preparation

10 µg of total RNA, extracted from cultured cells as described above, was added to 20 µl of loading buffer {0.72 ml formamide (ultrapure, USB), 0.16 ml 10xMOPS buffer, 0.26 ml formaldehyde sol [37% (w/v)], 0.18 ml DDW, 0.1 ml glycerol [80% (v/v)], 0.08 ml bromophenol blue}. Immediately prior to electrophoresis, the RNA sample was denatured by heating at 95°C for 2 min, quenched on ice and loaded onto the gel.

RNA was analysed in horizontal submerged agarose gels; agarose (ultrapure, IBI) was dissolved in 1xMOPS buffer by heating, cooled to 50°C and then 5.4 ml of formaldehyde sol [37% (w/v)] was added. The mixture was poured into a gel forming tray (BRL) with a 1-2 mm thick comb and allowed to set. The running buffer contained 1xMOPS buffer and electophoresis was performed at 80-100V for 3-4 hr.

2.7.C Transfer of RNA to Nitrocellulose

The nitrocellulose membrane (Hybond-C extra, Amersham) was wet in 2xSSC and then 20xSSC, prior to transfer of the RNA to the membrane overnight via the standard capillary diffusion method in 20xSSC (Sambrook et al, 1989). The membrane was then rinsed in 2xSSC, air dried and baked in a vacuum oven at 80°C for 2 hr prior to hybridisation.

2.7.D Hybridisation

Prehybridisation and hybridisation reactions were performed for 4 hr and 16-20 hr respectively, at 50°C in 50% formamide, 4xSSC, 50mM Na_2HPO_4, 50mM NaH_2PO_4, 50mM Tris-HCl (pH 7.1), 8xDenhardts sol (0.16% Ficoll, 0.16% PVP), 500 µg/ml ssDNA (Boehringer), 50 µg/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 (5-10 x 10^6 cpm/ml)] for 5 min. This was then quenched on ice, the other ingredients added and the mixture sealed in polythene bags containing the nitrocellulose membrane. After hybridisation, the membrane was washed in 2xSSC, 0.1% SDS at RT for 4 x 5 min, then 2xSSC, 0.1% SDS at 80°C for 2 x 20 min, and finally 0.1xSSC, 0.1% SDS for 2 x 20 min at 80°C.

The washed membranes were wrapped in plastic film and exposed at -70°C to X-Omat RP or AR X-ray film then developed as described in section 2.4.B(ii) above.

CHAPTER THREE

HDAg-p24 ASSOCIATED CYTOTOXICITY

3.1 Introduction

The primary aim of the work in this chapter was to investigate the role of HDAg-p24 expression in HDV-related cytotoxicity. Previous studies by Macnaughton et al (1990b) showed that in a stably-transfected eukaryotic cell line, in which the gene for HDAg-p24 only was expressed, a proportion of cells showed evidence of cytotoxicity, leading to the hypothesis that HDAg-p24 is cytotoxic per se. Furthermore, in a stably-transfected cell line capable of sustaining HDV RNA-directed RNA synthesis, serial passage resulted in a loss of cytotoxicity which was coincident with a decrease in the ratio of HDAg-p24:p27 expression (Macnaughton et al, 1990a). In addition, the ratio of p27:p24 is much higher (ca. 1:1) in chronically-infected liver than acute phase-liver (ca. 1:20), and it is possible that severe liver damage associated with acute HDV hepatitis may be the result of the direct cytotoxic action of HDAg-p24. This is consistent with the results of histological studies of severe acute HDV infection in which large numbers of HDAg-positive hepatocyte nuclei were present at the time of onset of severe hepatic disease (Popper et al, 1983).

This chapter examines the effects of the expression of HDAg-p24 in continuous cell lines derived from HeLa and HepG2 cells. In these cell lines the expression of HDAg-p24 is induced and controlled by the human metallothionein promoter (MTIIA) (Karin and Richards, 1982), and thus HDAg-p24 is only synthesised in these cells after the addition of zinc to the culture medium. Consequently, these cells can be used to distinguish effects which result merely from random integration of the recombinant

plasmid into the cellular chromosome from effects resulting from gene expression, and provide an in vitro model that is easily manipulated.

3.2 Plasmid Construction

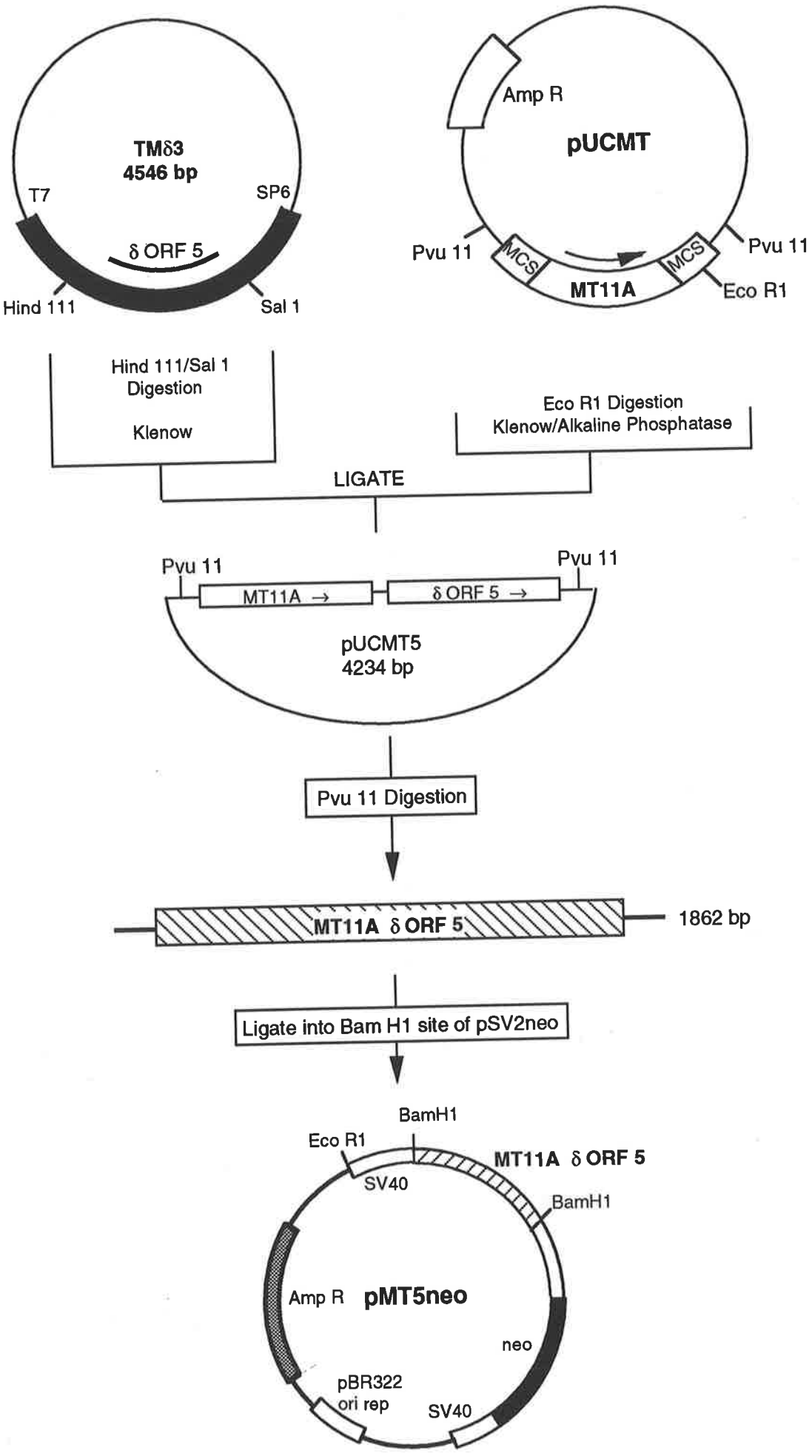
The source of HDV cDNA used to produce the inducible HDAg-p24 expression plasmid pMT5neo, was pTMδXbaA (Chapter 2.3.A (iv)), which contains a full-length copy of HDV cDNA in vector pGEM-3. A 717 bp fragment encoding HDV ORF 5 (map position 962-1679; Wang et al, 1986) including 81 bp upstream and 48 bp downstream of the HDAg-p24 gene was excised by Hind 111/Sal 1 double digestion from pTMδXbaA. This fragment was subsequently end-filled using the Klenow fragment of DNA polymerase 1, and inserted into the unique Eco R1 site of pUCMT (Chapter 2.3.A (v)), thus placing the MTIIA promoter immediately upstream of the HDAg-p24 gene. The 1862 bp fragment encompassing the MTIIA and ORF 5 cDNA was then excised by Pvu 11 digestion and religated into the unique Bam H1 site of the eukaryotic expression vector pSV2neo (Chapter 2.3.A (i)) to produce the final construct pMT5neo (Figure 3.1). This vector contains a neomycin-resistant gene for selection by Geneticin (G418).

3.3 Zinc-Induced HDAg Expression in Transfected Cell Lines

3.3.A Titration of Zinc Sulphate

The concentration of zinc sulphate chosen to induce the expression of HDAg-p24 in Hela- and HepG2- derived transfected cell lines (see 3.3.B) was determined from a titration study using untransfected parent Hela and HepG2 cells. The parent cell lines were grown overnight in normal or zinc-supplemented medium as described in Chapter

Fig 3.1 Strategy for construction of the recombinant plasmid pMT5neo which expresses HDAg-p24 under the control of the MTIIA promoter.


TMδ3
4546 bp
T7
SP6
δ ORF 5
Hind 111
Sal 1
Amp R
pUCMT
Pvu 11
MCS
MT11A
MCS
Pvu 11
Eco R1
Hind 111/Sal 1
Digestion
Klenow
Eco R1 Digestion
Klenow/Alkaline Phosphatase
LIGATE
Pvu 11
Pvu 11
MT11A →
δ ORF 5 →
pUCMT5
4234 bp
Pvu 11 Digestion
MT11A δ ORF 5
1862 bp
Ligate into Bam H1 site of pSV2neo
BamH1
Eco R1
MT11A δ ORF 5
SV40
BamH1
Amp R
pMT5neo
neo
pBR322
ori rep
SV40

2.5.A, using a range of zinc sulphate concentrations from 50μM to 150μM. The results of this study (Table 3.1) showed that increasing the zinc sulphate concentration to 125μM or above resulted in cell death, judged by gross cytology, in both parent cell lines and that this effect was also time-related. Consequently, all future experiments which required the induction of HDAg in HeLa- and HepG2- derived transfected lines, were performed in culture medium containing a maximum concentration of 100μM zinc sulphate. As an additional control, the corresponding parent cell line was grown in medium supplemented with the highest concentration of zinc sulphate used in individual experiments.

3.3.B **Immunofluorescence**

Transfection of pMT5neo into HeLa and HepG2 cells produced a number of neomycin-resistant single-cell clones which were partially expanded and examined by immunofluorescence for constitutive expression of HDAg-p24. Two of these clones derived from HeLa and HepG2 cells showed a low level of constitutive expression in only a small percentage of cells (<5%; Figure 3.2), whereas after culture in medium containing 100μM zinc sulphate for 7 hr, 100% of cells in both clones showed high levels of HDAg-p24 expression in the nucleus. These two clones were subsequently amplified to produce the stable cell lines δMT5A and δMTG25 respectively. The pattern of antigen expression observed in these cell lines after zinc induction is consistent with other reports describing the localisation of recombinant HDAg-p24 in transfected cells (Macnaughton et al, 1990b; Xia et al, 1992).

Table 3.1

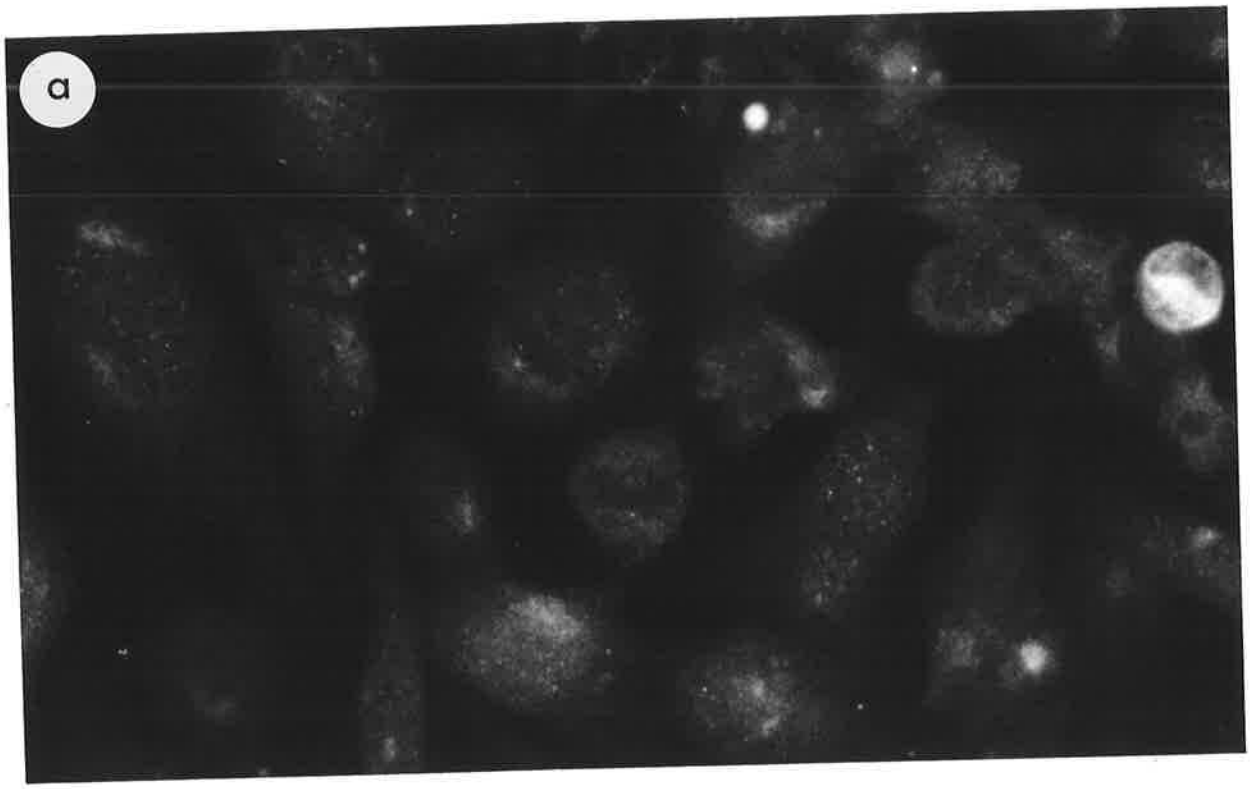
Titration of Zinc Sulphate in HeLa and HepG2 cells

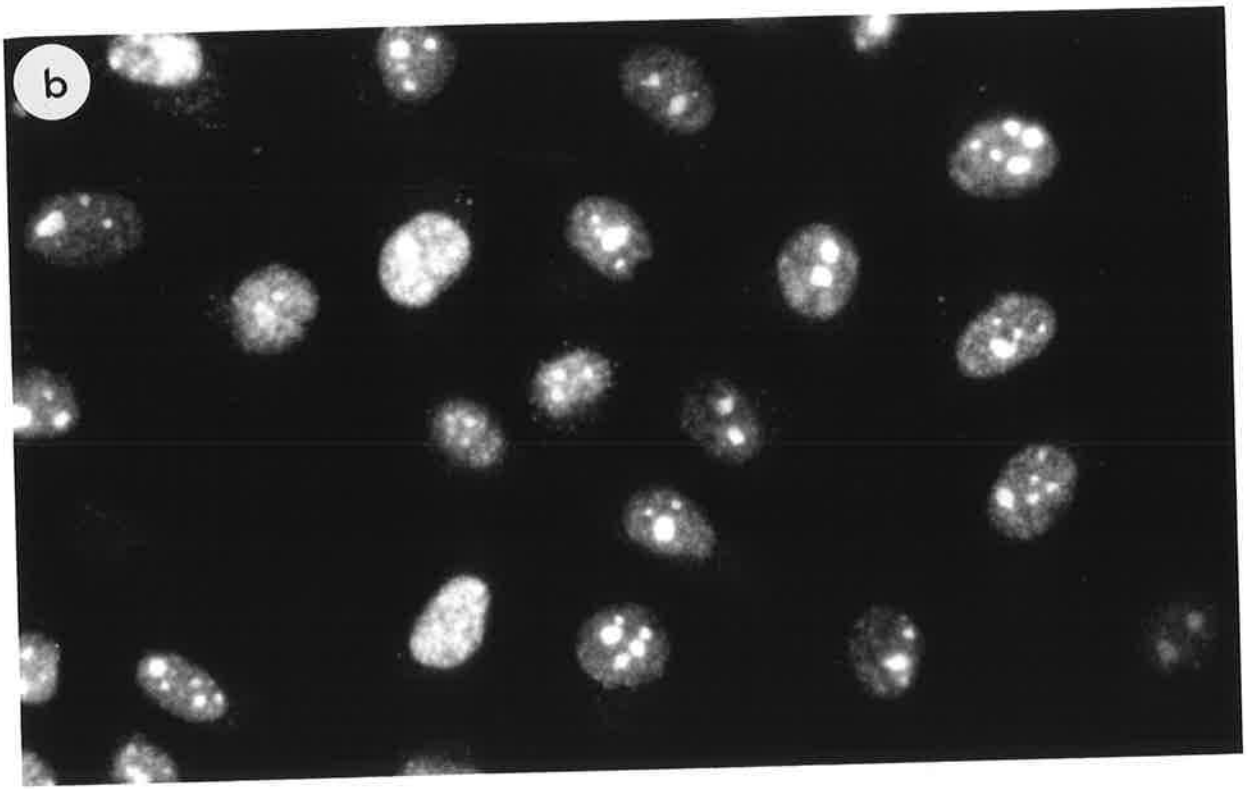
Time (days)	Concentration of zinc sulphate (μM)				
	50	75	100	125	150
2	-	-	-	-	+
9	-	-	-	+	+
14	-	+	+	++	+++*

+++ Cell monolayer destroyed
++ 10-30% of the cell monolayer intact
+ 30-80% of the cell monolayer intact
- Cell monolayer intact

* A detailed cytological analysis following staining with haematoxylin and eosin, is described in Chapter 4.5.B

Fig 3.2 The detection of HDAg in a continuous cell line. δMT5A cells were examined for HDAg by immunofluorescence (A) without zinc induction, or (B) after induction with 100μM zinc sulphate for 7 hr (magnification x 2000).


a


b

3.3.C Immunoblotting

The expression of HDAg in the δMT5A and δMTG25 cell lines was confirmed by immunoblot analysis. The bulk of HDAg in the δMT5A and δMTG25 cells was detected as a polypeptide of 24 kD with a constant proportion of lower molecular weight polypeptides (15 to 21 kD; Figure 3.3A) also present that were considered to be breakdown products which were detected previously (Macnaughton et al, 1990b). In both cases, immunoblot analysis of the parent HeLa and HepG2 cells failed to detect any specific polypeptides. The level of HDAg-p24 in the δMT5A and δMTG25 cells after overnight zinc induction was directly related to the concentration of zinc sulphate in the culture medium. In the δMT5A cell line, the level of induction was quantitated by analysis of the immunoblot in a scanning densitometer (Table 3.2). The results showed that there was a ca. 8.5-fold increase in the level of HDAg-p24 expression in δMT5A cells incubated in medium containing 100μM zinc, compared with the level of expression in uninduced cells.

3.4 The Effect of HDAg-p24 Expression on Cellular Nucleic Acid Synthesis

As the expression of HDAg-p24 was directly related to the concentration of zinc, a range of levels of HDAg-p24 expression was induced in δMT5A and δMTG25 cells by the addition of a range of levels of zinc sulphate to the culture medium. The cells were grown overnight in normal or zinc-supplemented medium as described in Chapter 2.5, and the rate of nucleic acid synthesis was measured by pulse labelling with ^{3}H-uridine or ^{3}H-thymidine for 4 hr. As controls, HeLa and HepG2 cells were also examined. Levels of HDAg-p24 that resulted from induction by ≤ 75μM zinc sulphate were well tolerated, but levels of antigen above this were not. In both the δMT5A and

Fig 3.3 Effect of HDAg-p24 expression on cellular RNA synthesis. (A) immunoblot analysis to detect HDAg-p24 in δMT5A cells after overnight induction with a range of zinc concentrations. (B) HeLa cells and the HeLa-derived cells δMT5A, were incubated overnight in medium containing different concentrations of zinc then pulse-labelled with ^{3}H-uridine for 4 hr and harvested immediately as described in the text. A similar analysis of HepG2 cells and the HepG2-derived δMTG25 cells is also shown.

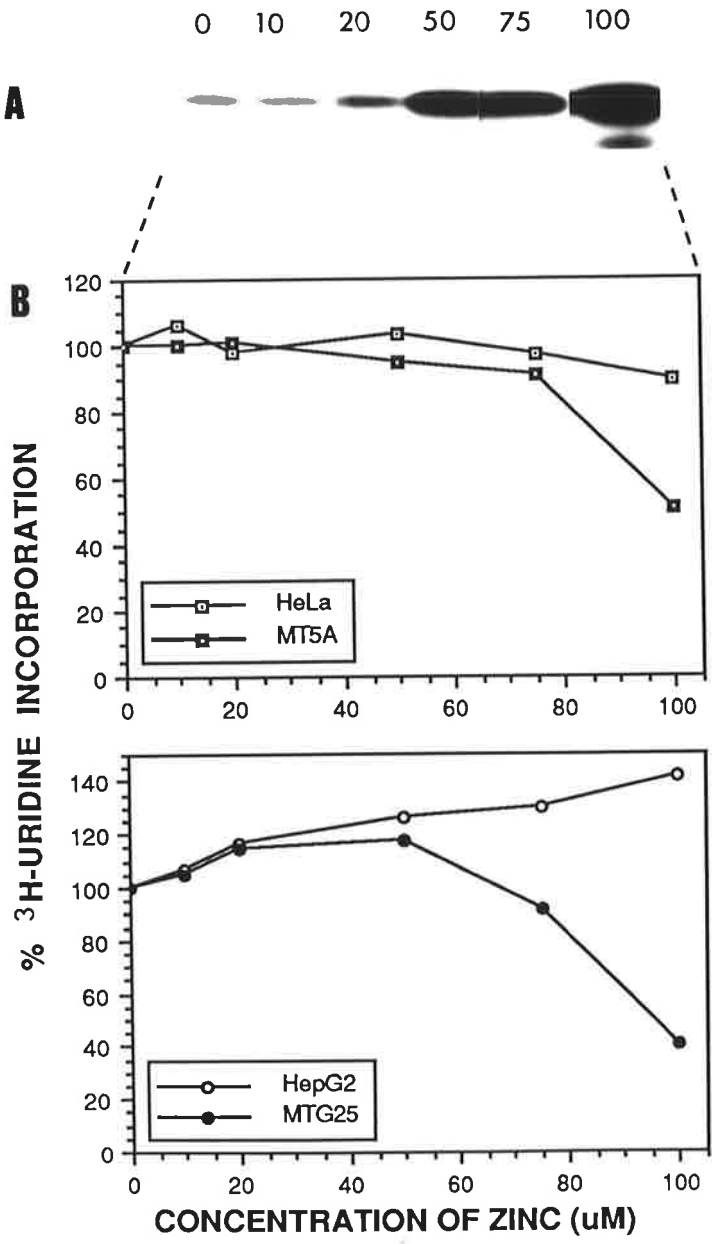

0
10
20
50
75
100
A
B
120
100
80
60
40
20
0
0
20
40
60
80
100
HeLa
MT5A
140
120
100
80
60
40
20
0
HepG2
MTG25
% 3H-URIDINE INCORPORATION
CONCENTRATION OF ZINC (uM)

Table 3.2

Quantitation of the Level of HDAg-p24 Expression
Induced in the δMT5A Cell Line

Concentration of Zinc Sulphate (μM)	Height* (AU)
0	0.206
10	0.154
20	0.306
50	0.987
75	0.989
100	1.733

* This represents the height in arbitrary units (AU) of the peak of HDAg-p24 expression in δMT5A cells at each concentration of zinc, determined by analysis of the immunoblot in Figure 3.3A in a scanning densitometer.

δMTG25 cells, levels of HDAg resulting from induction by 100μM zinc reduced RNA synthesis by 50% - 60%. In the δMT5A cells, the difference between the levels of HDAg induced at 75μM and 100μM was ca. 1.7-fold (Table 3.2). δMT5A and δMTG25 cells that were not treated with zinc, and HeLa and HepG2 cells that were treated with similar levels of zinc used to induce HDAg-p24 in the former cells, showed no reduction in RNA synthesis (Figure 3.3B).

In contrast to the above results, HDAg-p24 expression showed no effect on cellular DNA synthesis; no significant difference was detected in the rate of ^{3}H-thymidine incorporation after a 4 hr pulse in both test and control cells that were previously incubated overnight in varying concentrations of zinc sulphate (Figure 3.4). Because these cultures were approximately 60% confluent when pulse-labelled, it was assumed that S-phase synthesis constituted the bulk of the ^{3}H-thymidine incorporation. Thus, after HDAg-p24 was induced in the cultures for 16 hr, a distinct decrease occurred in the rate of RNA but not DNA synthesis.

3.5 Cell Doubling Times and Cytological Analysis

Measurements of the cell doubling times were performed 5 days after zinc induction using identical six-well culture plates; the doubling times for δMT5A and δMTG25 cells were measured after culture in normal medium or in medium supplemented with 100μM zinc sulphate. The results of these experiments (Table 3.3) showed that the doubling times were 28hr and 57hr respectively, in normal medium. In contrast, in the zinc-supplemented medium, δMT5A and δMTG25 cell numbers decreased over the observation period, suggesting that cell death rather than division occurred. Consequently, in this experiment the cell doubling time was determined as infinity.

Fig 3.4 Effect of HDAg-p24 expression on cellular DNA synthesis. HeLa cells and the HeLa-derived cells δMT5A were incubated overnight in medium containing different concentrations of zinc then pulse-labelled with ^{3}H-thymidine for 4 hr and harvested immediately as described in the text.

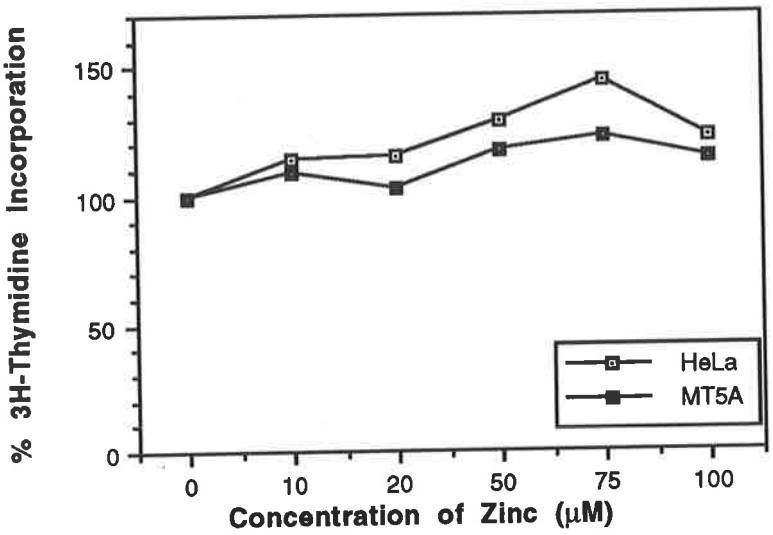

% 3H-Thymidine Incorporation
150
100
50
0
0
10
20
50
75
100
Concentration of Zinc (μM)
HeLa
MT5A

Table 3.3

Cell culture doubling times and mitotic indices of cell lines before and after zinc induction

	Culture doubling times (hr)		Mitotic index	
	No zinc	100μM zinc	No zinc	100μM zinc
δMT5A	28	- *	5.2	0.4
δMTG25	57	- *	2.0	0.2

* Since the cell numbers in the culture decreased, these cultures are assumed to have a doubling time of infinity.

Thus, a major long term effect of the induction of HDAg-p24 expression was cell death in both HeLa- and HepG2- derived cell lines. The mitotic index for the δMT5A and δMTG25 cells was reduced from 5.3 to 0.4 and 2.0 to 0.2 respectively (Table 3.3) after zinc induction, consistent with the vast increase in cell doubling times described above.

Cytological analysis of the zinc-induced and uninduced (control) cells reflected the above results. Zinc induction of the δMT5A (Fig 3.5) and δMTG25 (Fig 3.6) cells resulted in a number of degenerative changes. The first of these was a marked reduction in the number of cells compared with the control cells which had undergone approximately three cell-doubling times described above (Table 3.3). Notable features in the zinc-induced cells were numerous cells with shrunken eosinophilic cytoplasm and pyknotic nuclei, some of which showed evidence of karyolysis. Some cells had lost their nuclei and were seen as round, deeply eosinophilic bodies. The dead cells closely resembled apoptotic bodies seen in both acute and chronic viral hepatitis (Kerr et al, 1972), including HDV-related hepatitis (Popper et al, 1983; Verme et al, 1983). In contrast, cytological analysis of uninduced δMT5A and δMTG25 cells showed none of the degenerative cytological changes observed in the δMT5A and δMTG25 cells following HDAg-p24 expression. In addition, zinc induction of the parent HeLa and HepG2 cells showed none of these changes. These results are consistent with the observed difference in the doubling times of these cell lines in zinc-supplemented medium as described above.

Fig 3.5 Cytological analysis of HeLa cells cultured for 4 days in (a) normal medium or (b) medium supplemented with 100μM zinc, (magnification x 2000), and the HeLa-derived δMT5A cells cultured for the same time period in (c) normal medium, or (d) medium supplemented with 100μM zinc (magnification x 500).

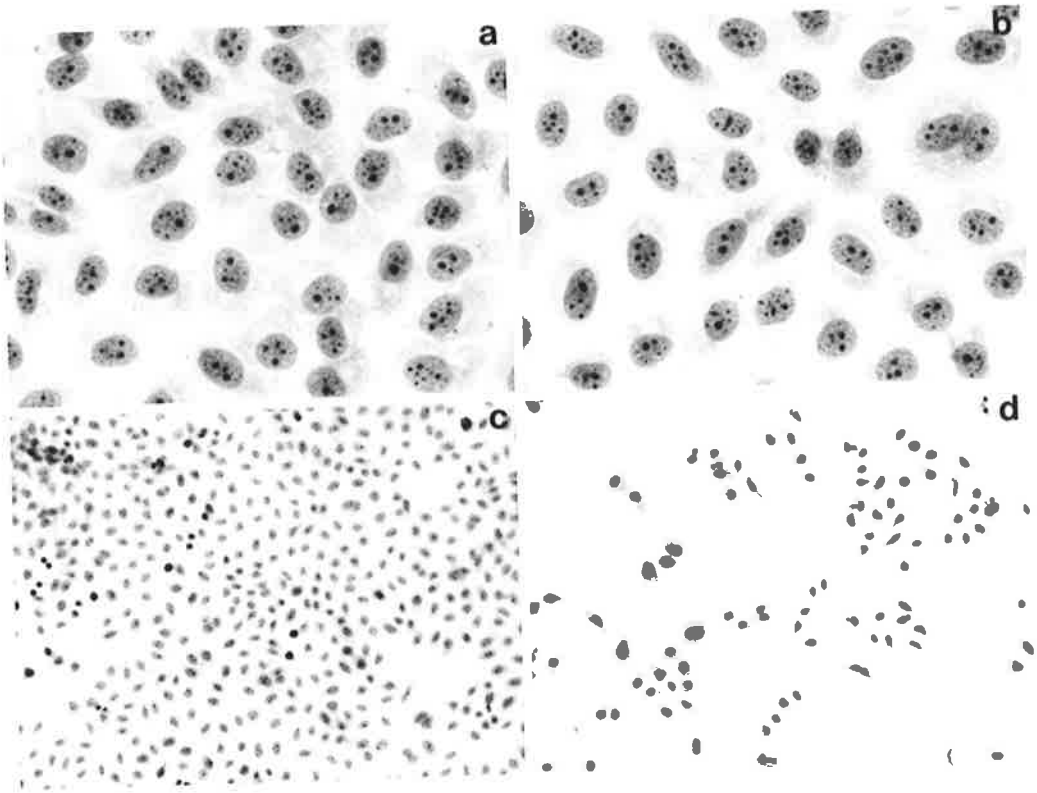
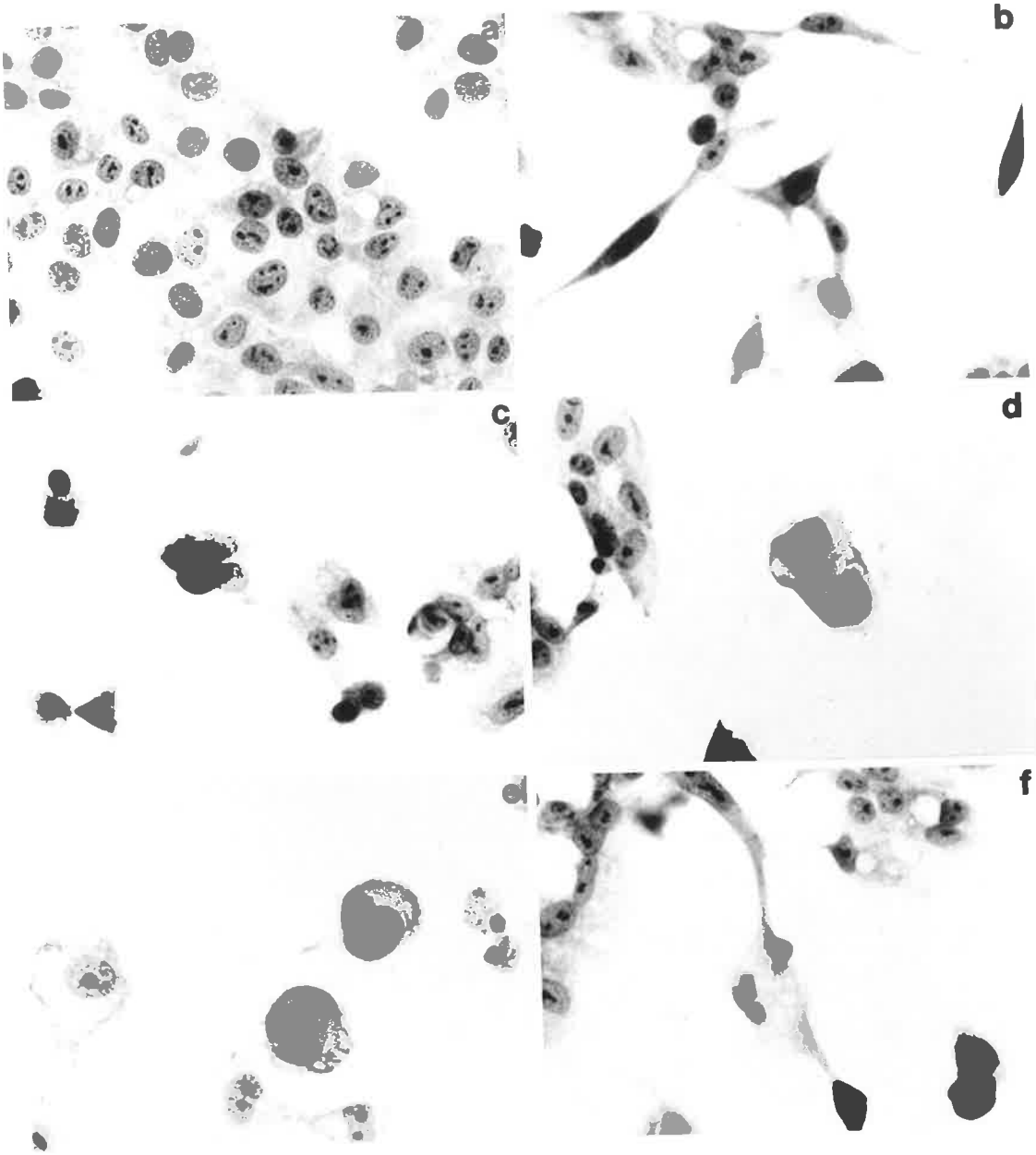

a
b
c
d

Fig 3.6 Cytological analysis of HepG2-derived δMTG25 cells cultured for 4 days in (a) normal medium or (b)-(f) medium supplememted with 100μM zinc (magnification x 2000).


a
b
c
d
e
f

3.6 Comparison of HDAg-p24 Levels in Zinc-Induced Cells and in Acute-Phase Chimpanzee Hepatocytes

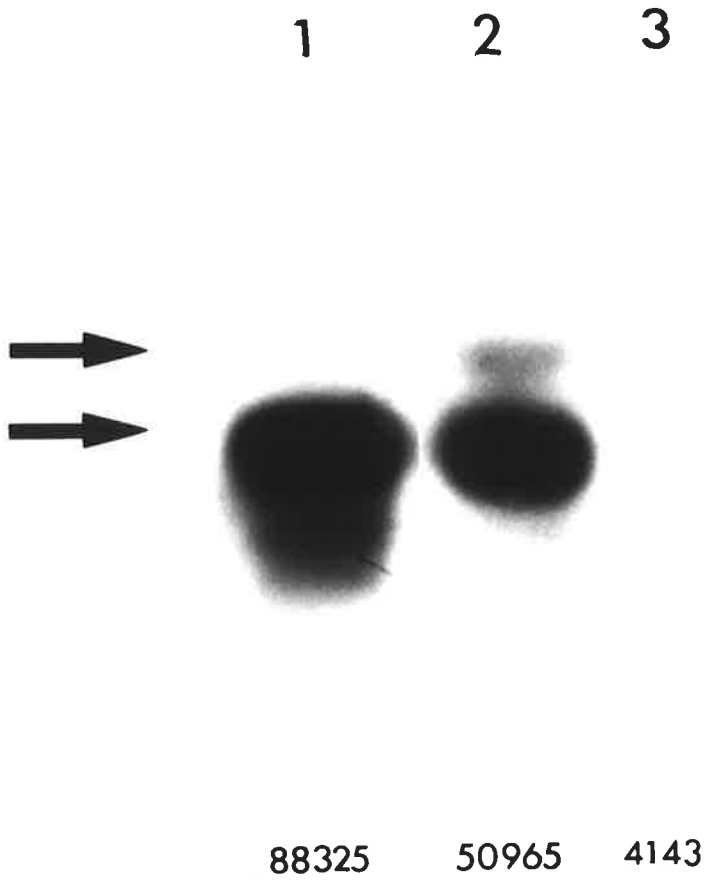
To prove that HDAg-p24-related cytotoxicity was not simply attributed to overexpression, the level of HDAg-p24 present in δMT5A cells after overnight induction was compared with the level of HDAg-p24 present in the hepatocytes of a liver sample from a chimpanzee with acute HDV-related hepatitis. Protein samples were prepared as described in Chapter 2.4.B and adjusted to contain equal concentrations of cells based on the DNA concentration in parallel samples. The level of HDAg was compared by counting bound ^{125}I following polyacrylamide gel electrophoresis and immunoblotting as described in Chapter 2.4.B. In this experiment, it was assumed that δMT5A cells and chimpanzee hepatocytes each contain 5pg of DNA.

The number of ^{125}I counts bound to the cell-derived HDAg was 88 325 cpm compared with the chimpanzee-derived HDAg of 50 965 cpm (Figure 3.7). Thus, because only 50% of chimpanzee hepatocytes were positive for HDAg by immunoperoxidase (data not shown) and because mesenchymal cells comprise a proportion of total liver cells, the level of HDAg in individual chimpanzee hepatocytes was actually slightly higher than the level in δMT5A cells. Thus, cytotoxicity was not simply the result of overexpression of HDAg-p24 in the δMT5A cells.

3.7 Discussion

Since HDV-associated hepatitis is related temporally to the expression and replication of the virus, and because there is a noteable absence of a lymphocytic infiltrate during acute HDV infection, it was suggested that the initial lesion may be the result of a

Fig 3.7 Immunoblot analysis to determine the levels of HDAg-p24 in δMT5A cells and in HDV-infected acute phase chimpanzee hepatocytes. Denatured protein from the equivalent of 10^6 cells was subjected to SDS-PAGE, transferred to nitrocellulose and analysed by immunoblot. After localisation by autoradiography, the HDAg-specific bands were excised and bound ^{125}I counted. *Lane 1* δMT5A cells; *Lane 2* chimp liver homogenate; *Lane 3* negative control liver homogenate. The level of ^{125}I counts bound is shown below the respective lanes. The upper and lower arrows correspond to 27 kD and 24 kD respectively.


1
2
3
88325
50965
4143

direct cytopathic effect and not immune-mediated (Popper et al, 1983; Verme et al, 1983). In this chapter, I have examined the role of HDAg-p24 in HDV-induced cytotoxicity in an in vitro cell culture model that is likely to reflect HDV-induced disease in natural infection.

Two cell lines were developed in which the expression of HDAg-p24 was controlled by the human MTIIA promoter, and consequently the level of HDAg-p24 expression in each cell line was directly proportional to the zinc sulphate concentration added to the culture medium. HDAg-p24 expression was associated initially with a significant decrease in the rate of RNA synthesis and eventually with reduced rates of cell division and degenerative cytological changes which were not observed in uninduced δMT5A and δMTG25 cells. Similarly, the parent HeLa and HepG2 cells were unaffected by the addition of 100μM zinc sulphate. Although overexpression of viral proteins may produce significant pathological changes (Chisari et al, 1989), HDAg-p24-associated cytotoxicity in this model system developed as a result of HDAg-p24 expression at physiological levels.

These results appear to conflict with those of Chen et al (1990) who showed that HDV replication in a continuous cell line was not cytopathic. However, other studies from this laboratory have consistently noted HDV-related cytotoxicity (Macnaughton et al, 1990a; Macnaughton et al, 1990b; Macnaughton et al, 1991) and differences may be related to different strains of HDV. It is also clear that cytotoxicity is dose related, as suggested by the data shown in Figure 3.3A. These results suggest that there is a threshold level below which HDAg-p24 is well tolerated, whereas antigen levels above this are not. This point is relevant to and is discussed further in Chapter 4.4.

It is possible that an event leading to expression of HDAg-p24 and not HDAg-p24 per se caused this cytotoxicity. However, base-pairing between HDV RNA and 7SL RNA cannot account for these results because the HDV cDNA used to produce these cell lines does not contain the sequence responsible for this proposed mechanism (Negro et al, 1989; Young and Hicke, 1990). The mechanism of HDAg-p24-induced cytotoxicity in this system remains unclear. One possible mechanism involves the triggering of apoptosis in cells which express high levels of HDAg-p24. Apoptosis is the term given to describe the programmed pattern of cell death that has been shown to be triggered in cells in response to a range of physiological and pathological stimuli (Kerr et al, 1972). Apoptosis is characterised histologically by the formation of small, roughly spherical, condensed cytoplasmic fragments which may contain pyknotic remnants of nuclei (Wyllie et al, 1980). These membrane-bound particles are referred to as apoptotic bodies. Once formed in tissues, these particles are dispersed rapidly in the intercellular spaces and phagocytosed by resident mononuclear cells eg., Kupffer cells, although there is often no associated inflammation, or they are taken up by adjacent viable cells where they are rapidly degraded by lysosomal enzymes (Wyllie et al, 1980). In the model system described in this Chapter, expression of HDAg-p24 was associated with the appearance of small, round membrane-bound apoptotic-like bodies which contained condensed eosinophilic cytoplasm, sometimes with compacted basophilic nuclear fragments, similar to those seen in acute HDV-related hepatitis (Kerr et al, 1972). Consequently, it is possible that HDAg-related cell death is due to apoptosis. This is consistent with the lack of an immune infiltrate during acute HDV-hepatitis. However, a role for apoptosis in HDAg-p24-related cytotoxicity will need to be confirmed by electron microscopy of induced δMT5A and δMTG25 cells to identify characteristic ultrastructural changes which are associated with the induction of apoptosis (Wyllie et al, 1980). Viral cytotoxicity due to several

lytic viral infections has been shown to be associated with apoptosis (White and Stillman, 1987; Levine et al, 1993; Chapter 8.2).

The results presented in this chapter are consistent with the proposal that HDAg-p24 has a direct cytotoxic effect which may contribute significantly to the extensive hepatocyte injury associated with acute HDV infection. However, these results appear to be inconsistent with the ability of HDV to cause persistent infection.

CHAPTER 4

THE DIFFERENTIAL ROLES OF HDAg IN HDV PATHOGENESIS

4.1 Introduction

The work presented in the previous chapter describes the development of an in vitro cell culture model to examine the direct effect of expression of HDAg-p24 in transfected eukaryotic cell lines. The results showed that HDAg-p24 expression resulted in dose-related degenerative cytological changes. These results are consistent with the proposal that HDV-induced liver injury in acute infection may be the direct result of HDAg-p24 expression. However, the pathogenetic significance of increased levels of HDAg-p27 expression during chronic HDV infection remains unclear (Chapter 3.1).

The specific aim of the experiments described in this chapter was to address the following issues;

(1) to express HDAg-p27 under the control of the MTIIA promoter in a stably-transfected cell line;

(2) to examine the temporal expression of HDAg-p27 and -p24 in two stably-transfected cell lines, and relate this to the appearance of any degenerative changes; and

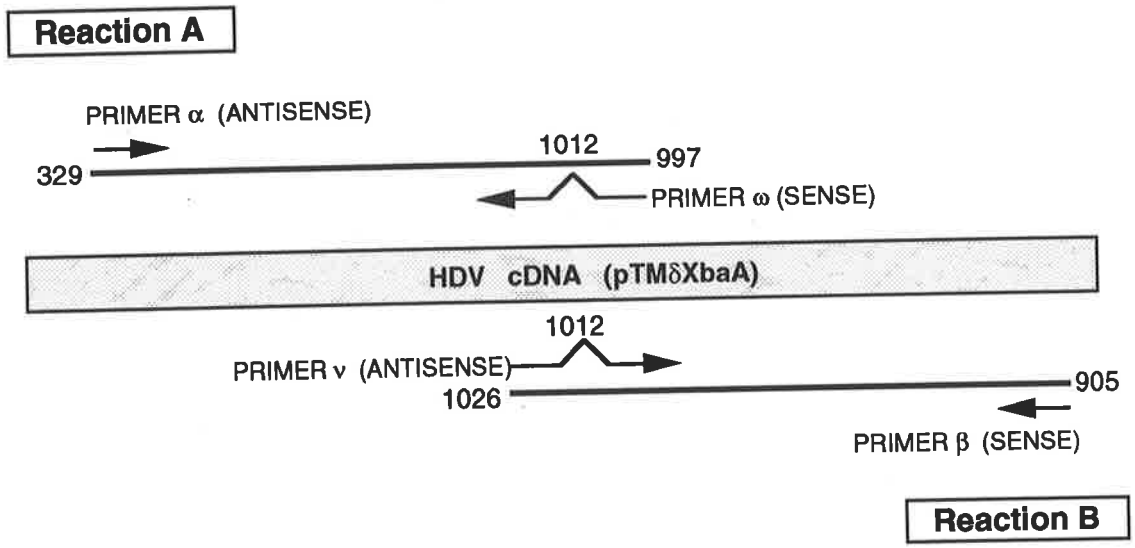
(3) to examine the effect of cell confluency on the effects of HDAg expression.

4.2 Construction of pMT27

In vitro expression studies in E.coli showed that ORF 5 encodes both HDAg-p24 and HDAg-p27 (Weiner et al, 1988). The presence of a stop codon or tryptophan codon dictates which polypeptide is expressed (Chapter 1.2.I; Luo et al, 1990). In order to construct a recombinant plasmid which is capable of expressing HDAg-p27, it was necessary to introduce a single-base mutation into HDV cDNA at nt position 1012 to convert the in-frame stop codon (UAG) to a tryptophan codon (UGG). Site-directed mutagenesis to create this point mutation was performed using a two-step PCR protocol as follows; HDV cDNA in the recombinant plasmid pTMδXbaA (Figure 2.4), was targeted by two sets of specific oligonucleotide primers (Figure 2.6), in such a way that resulted in PCR amplification of two cDNA fragments which overlap in sequence and contain a single base mutation at nt 1012 in ORF 5 (Figure 4.1). In the primary PCR, reaction A used a wild-type, antisense primer (primer α) with a mutant, sense primer (primer ω); reaction B used a wild-type, sense primer (primer β) with a mutant, antisense primer (primer υ) which is complementary to primer ω. The PCR conditions were as described in Chapter 2.3.G. The products of each primary PCR (Figure 4.2) were diluted to ensure that the molar ratio of DNA generated in reactions A and B was 1:1. An equal volume of the diluted products were then mixed together and serial 10-fold dilutions were made. 10 μl of each serial dilution was used as the template for a secondary PCR (reaction C) under the same conditions as above, and using the two wild-type primers (primer α and β). The final product was identified as the 1103 bp DNA band that comigrated on an agarose gel with the PCR product from a single reaction with primers α and β using pTMδXbaA as the template (Figure 4.2).

Fig 4.1 Site-directed mutagenesis of HDV ORF 5 by PCR. Site-directed mutagenesis was performed using a two-step PCR protocol using pTMδXbaA as the HDV cDNA template. Two primary PCR were performed; reaction A used an external wild-type antisense primer (primer α) with an internal mutant sense primer (primer ω); reaction B used an external wild-type sense primer (primer β) with an internal mutant antisense primer (primer υ) which is complementary to primer ω. The PCR products generated in reactions A and B were denatured and reannealed in the same reaction mix (reaction C), and this cDNA was amplified by PCR using the original two external wild-type primers (α and β).

(1) Primary PCR



(2) Secondary PCR

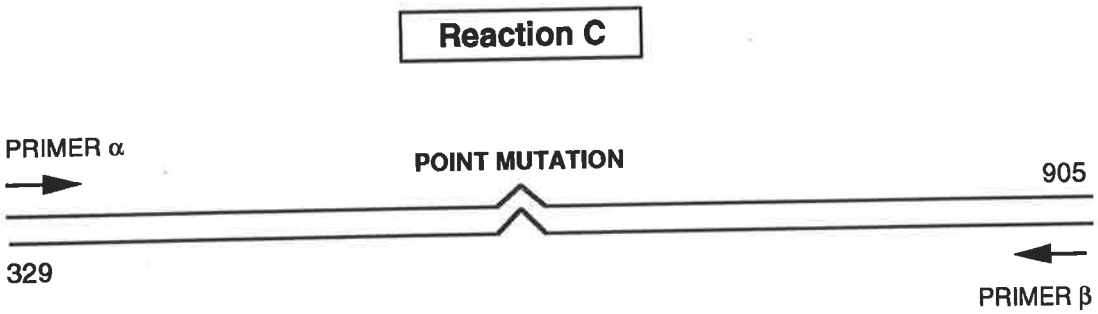
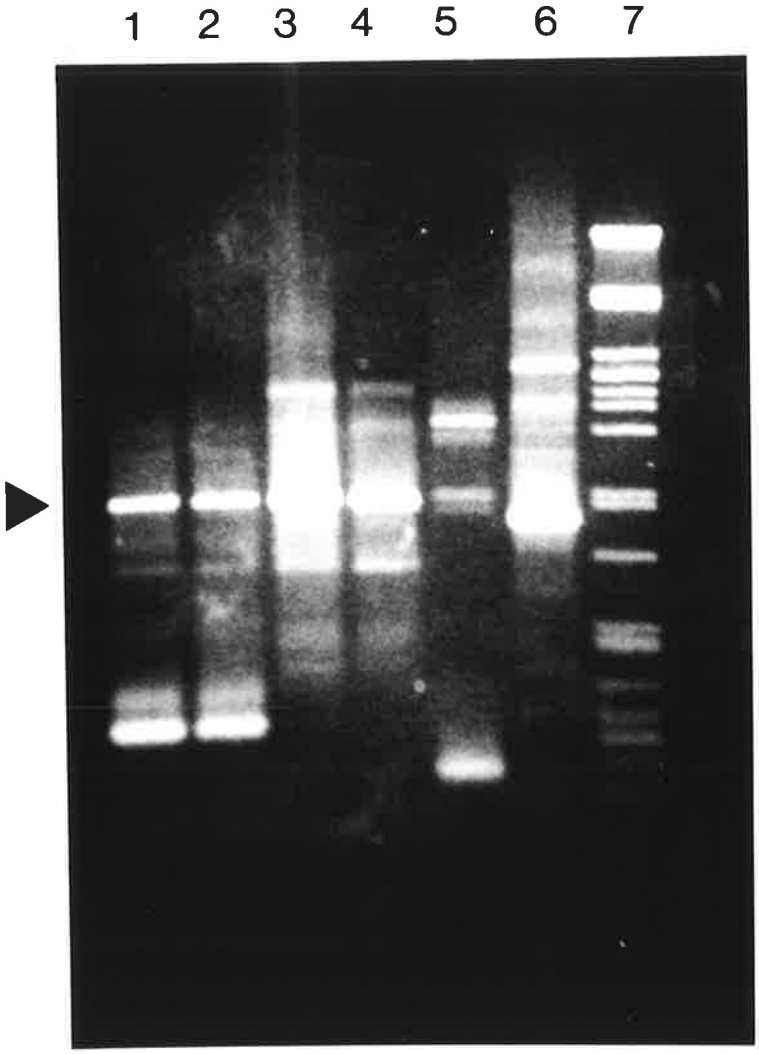


Fig 4.2 Analysis of the products of a secondary PCR by gel electrophoresis. The PCR product of reaction C (Figure 4.1; *Lanes 1 and 2*) was identified as the DNA band that comigrated with the product from a primary PCR with primers α and β using pTMδXbaA as the template (*Lanes 3 and 4*). The arrow indicates the position on the gel of the desired product. *Lanes 4 and 5* show the PCR products of reaction A and reaction B respectively, as described in the text. *Lane 7* shows the DNA size markers (see Appendix 3).


1
2
3
4
5
6
7

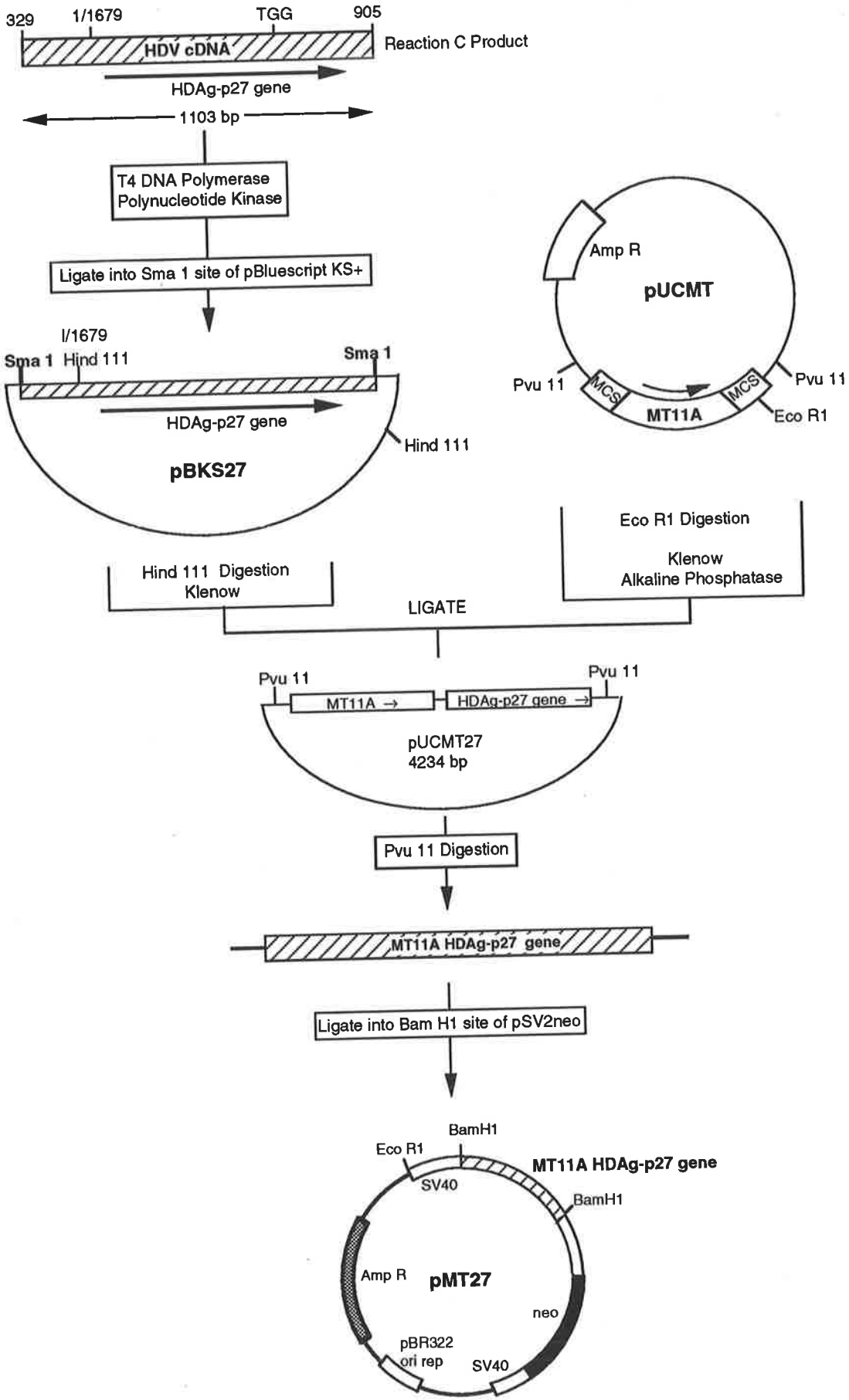
The final product (map position 905-0-329; Wang et al, 1986), was end-filled with T4 DNA Polymerase and subsequently treated with polynucleotide kinase to restore the terminal 5' phosphates, and inserted into the unique Sma 1 site of pBluescript KS+ to create the plasmid pBKS27 (Figure 4.3). At this stage, the presence of the correct point mutation at nt 1012 was confirmed by double stranded DNA sequencing of the cloned PCR product using an Applied Biosystems Model 373 A Automatic DNA Sequencer as described in the manufacturer's instructions.

An 800 bp fragment that encoded the HDAg-p27 gene (map position 953-1598) plus 81 bp upstream of the HDAg-p27 gene (map position 1599-1679), and 48 bp downstream of the gene (map position 905-954), was then excised from pBKS27 by Hind III digestion; the fragment also included 26 bp of the pBluescript KS+ vector sequence downstream of the HDV coding sequence. It was pure coincidence that the RE sites used in this construction and the construction of pMT5neo (Chapter 3.2), generated 48 nt downstream of the genes for HDAg-p27 and -p24 respectively, and thus these 48 nt are not identical. This 800 bp fragment from pBKS27 was subsequently end-filled using the Klenow fragment of DNA polymerase 1, and inserted into the unique Eco R1 site of pUCMT, thus placing the MTIIA promoter immediately upstream of the HDAg-p27 gene. The 1862 bp fragment encompassing the MTIIA promoter and ORF 5 cDNA was then excised by Pvu 11 digestion and religated into the unique Bam H1 site of pSV2neo to produce the final construct pMT27 (Figure 4.3).

4.3 Zinc-induced HDAg Expression in Transfected Cell lines

Transfection of pMT27 into HeLa cells resulted in one expanded clone (Hδ27)

Fig 4.3 Strategy for construction of the recombinant plasmid pMT27 which expresses HDAg-p27 under the control of the MTIIA promoter.


329
1/1679
TGG
905
HDV cDNA
Reaction C Product
HDAg-p27 gene
1103 bp
T4 DNA Polymerase
Polynucleotide Kinase
Ligate into Sma 1 site of pBluescript KS+
I/1679
Sma 1
Hind 111
Sma 1
HDAg-p27 gene
Hind 111
pBKS27
Amp R
pUCMT
Pvu 11
MCS
MT11A
MCS
Pvu 11
Eco R1
Hind 111 Digestion
Klenow
Eco R1 Digestion
Klenow
Alkaline Phosphatase
LIGATE
Pvu 11
MT11A →
HDAg-p27 gene →
Pvu 11
pUCMT27
4234 bp
Pvu 11 Digestion
MT11A HDAg-p27 gene
Ligate into Bam H1 site of pSV2neo
BamH1
Eco R1
MT11A HDAg-p27 gene
SV40
BamH1
Amp R
pMT27
neo
pBR322
ori rep
SV40

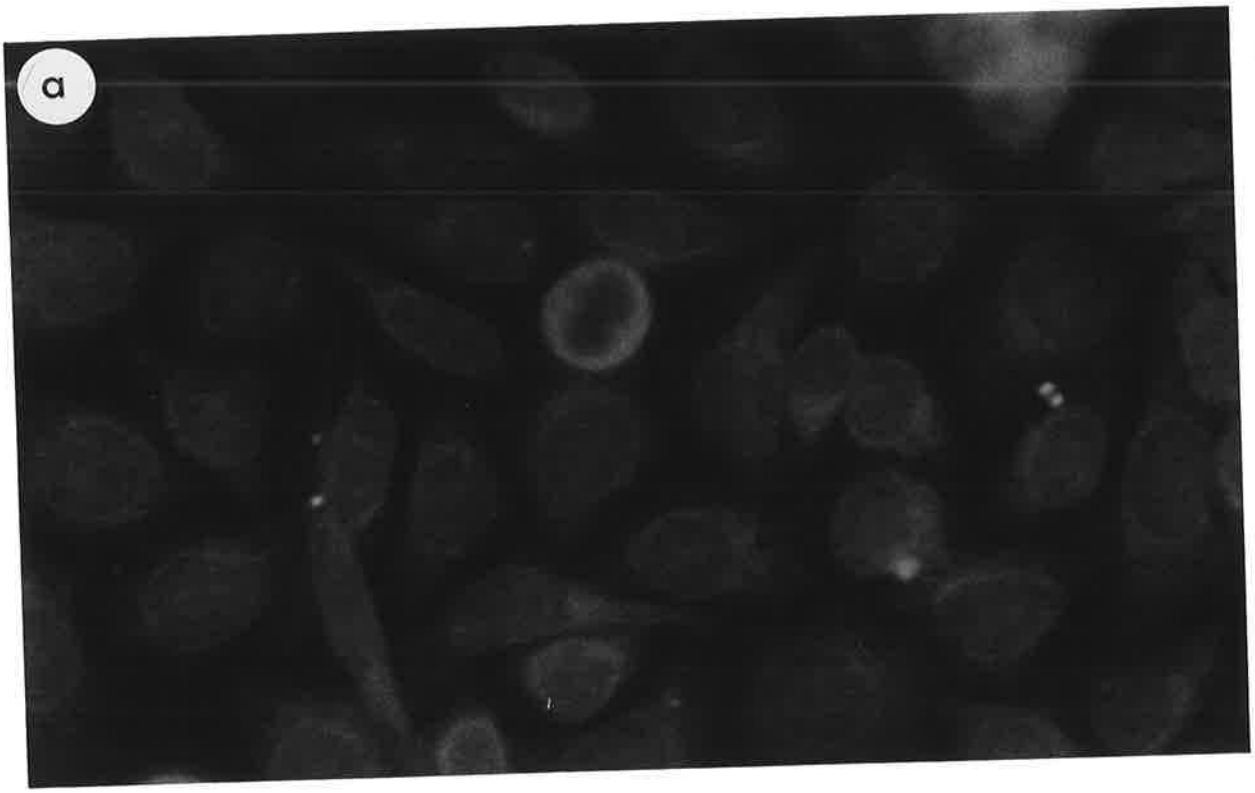
which showed a low constitutive level of HDAg-p27 expression in less than 5% of cells. As described in Chapter 3.3.A, all experiments which required the induction of HDAg in HeLa-derived transfected cell lines were performed in culture medium that contained a maximum concentration of 100μM zinc sulphate. After induction in culture medium containing 100μM zinc sulphate, 100% of cells showed a high level of nuclear staining (Figure 4.4). No difference was noted in the localisation pattern of HDAg-p27 in the Hδ27 cells compared with that of HDAg-p24 in both the δMT5A and δMTG25 cells (Chapter 3.3.B). HDAg-p27 was localised exclusively to the nucleus in transfected eukaryotic cells; the pattern was predominantly nucleolar with some nucleoplasmic staining. These observations are consistent with the pattern of HDAg distribution in a transient transfection system described previously (Xia et al, 1992).

The expression of HDAg-p27 in the Hδ27 cell line was confirmed by immunoblot analysis. A single HDAg-specific polypeptide of 27 kD was detected after overnight zinc induction, and the level of induction was directly related to the concentration of zinc sulphate in the culture medium (Figure 4.5).

4.4 Temporal Expression of HDAg-p27 and HDAg-p24

In the experiment described below, the temporal expression of HDAg-p27 and HDAg-p24 was examined in two stably-transfected cell lines after zinc induction in order to relate HDAg expression to any reduction in cellular nucleic acid synthesis (section 4.5.A), or the appearance of any degenerative cytological changes (section 4.5.B) which resembled those described in the zinc-induced MT5A cells in Chapter 3. The HeLa-derived δMT5A cell line, described in Chapter 3.3, was not used in

Fig 4.4 The detection of HDAg-p27 in a continuous cell line. Hδ27 cells were examined for HDAg by immunofluorescence (a) without zinc induction, or (b) after induction with 100μM zinc sulphate for 7 hr (magnification x 2000).


a

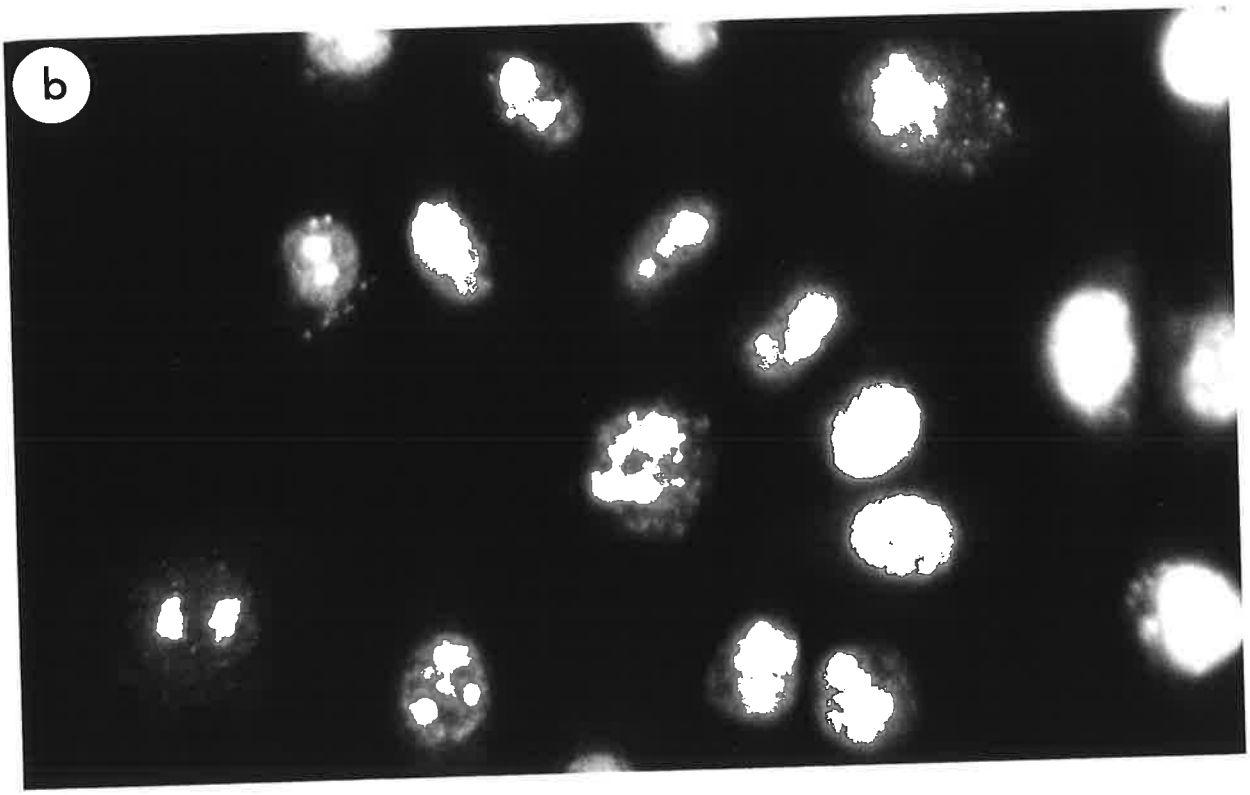
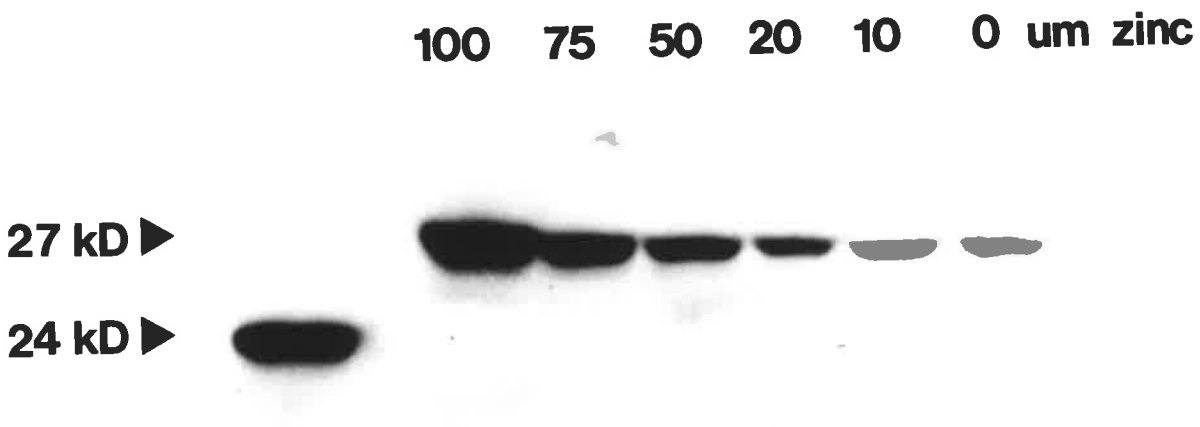

b

Fig 4.5 Immunoblot analysis to detect HDAg in Hδ27 cells showing that the level of HDAg-p27 expression after overnight zinc induction was directly related to the concentration of zinc sulphate in the culture medium.


100 75 50 20 10 0 um zinc
27 kD
24 kD

the experiments described below due to a diminished level of HDAg-p24 expression after zinc induction. A possible explanation is the loss of the integrated HDV cDNA insert from the chromosomal DNA, although this was not confirmed by Southern blot analysis. The Hδ5 cell line is a stably-transfected HeLa-derived line amplified from a single-cell clone transfected with the plasmid pMT5neo (Figure 3.1) as described in Chapter 2.3.F(iii), and this was considered to be a suitable alternative. Upon initial screening of this cell line by immunoblot analysis for HDAg expression, the constitutive level of HDAg-p24 expression prior to zinc induction was similar to that seen in the δMT5A cell line (Chapter 3.3.C). However, in later experiments, immunoblot analysis detected a higher level of constitutive HDAg-p24 expression than expected. Nevertheless, this cell line was considered to be a suitable control cell line for HDAg-p24-induced cytotoxicity and the reasons for this are described in detail below.

The time course for the appearance of HDAg was examined by inducing Hδ27 cells and Hδ5 cells with 100μM zinc sulphate for up to 12 hr. Protein samples were prepared at each time point, as described in Chapter 2.4.B, and adjusted to contain 10^6 cells per sample, based as before, on the DNA concentration in each sample (see Chapter 3.6). A control sample, equivalent to $5x10^5$ cells, was prepared from the hepatocytes of the same chimpanzee described in Chapter 3.6. The level of HDAg in each sample was compared by counting bound ^{125}I following SDS-PAGE and immunoblotting as described in Chapter 2.4.B.

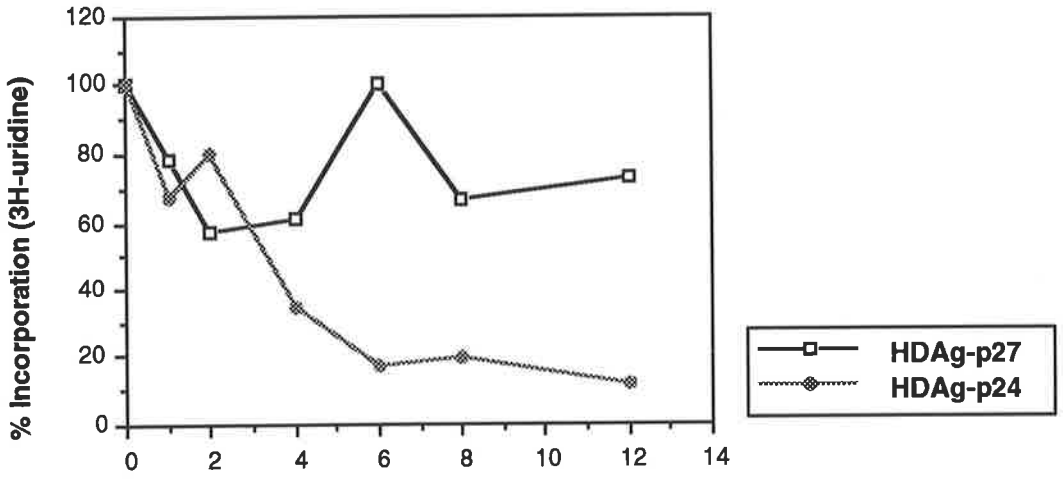
The result of this experiment is shown in Figure 4.6A. The level of HDAg-p27 expression was examined up to 12 hr after zinc induction. An increase in the level of HDAg-p27 expression was detected 4 hr after the addition of zinc sulphate, and

Fig 4.6 The effect of HDAg-p24 and HDAg-p27 expression on cellular nucleic synthesis. (A) the level of ^{125}I counts bound to the nitrocellulose filter following immunoblot analysis to quantitate HDAg at various times post-induction in the Hδ5 and Hδ27 cells. (B)-(C) in the same experiment, 70% confluent cultures of Hδ27 and Hδ5 cells were grown in medium supplemented with 100μM zinc sulphate for up to 12 hr and the rate of RNA synthesis (B), and DNA synthesis (C) was measured at various times post-induction.

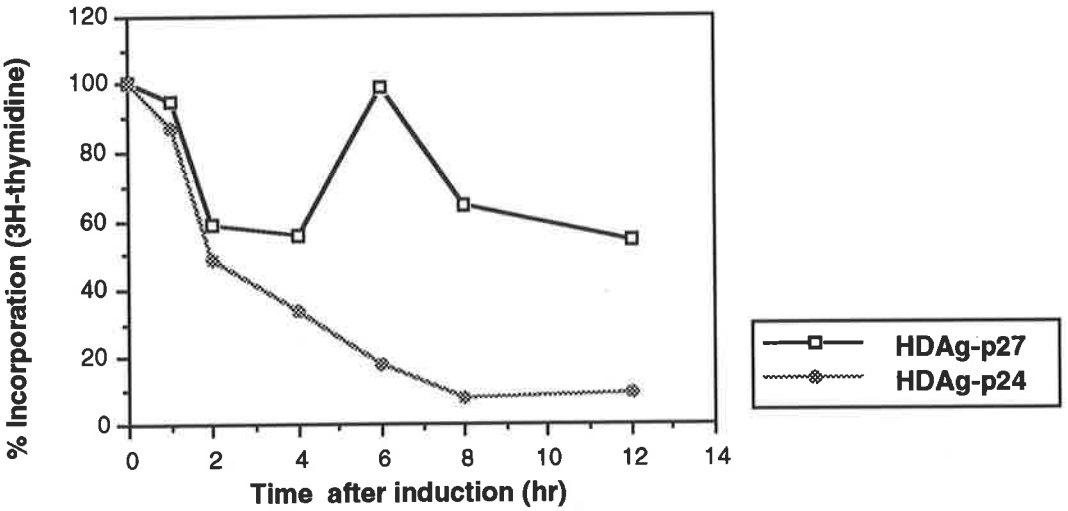
A

Time after induction (hr)	0	4	8	12
HDAg-p27	46	758	872	1676
HDAg-p24	1069	1661	1101	nd

B



C



the highest level was detected 12 hr after zinc induction when the level of bound ^{125}I corresponded to 1676 cpm, i.e. a ca. 40-fold increase in the level of HDAg-p27 expression. Earlier time points were assessed for cellular nucleic acid synthesis (see 4.5.A below), but not HDAg expression. The level of HDAg-p24 expression in Hδ5 cells prior to the addition of zinc sulphate corresponded to 1069 cpm, and 4 hr after induction the number of ^{125}I counts bound to the Hδ5 cell-derived HDAg increased to a maximum of 1661 cpm. At 8 hr after induction, the level of HDAg-p24 detected in the Hδ5 cells was reduced to 1101 cpm (Figure 4.6A). This reduction was due to a loss of viable cells in the culture (see Section 4.5.B). Although the level of HDAg-p24 expressed at 4 hr post-induction represented an increase in the level of expression of only ca. 1.6-fold, this was subsequently shown to be sufficient to induce cytotoxic changes (see below). This finding is consistent with (1) the previous observation that HDAg-p24-related cytotoxicity is dose-related (Chapter 3.4), and (2) the proposal of a threshold level of HDAg-p24 expression in cells, above which HDAg-p24 is not tolerated by the cell. In the δMT5A cells described in Chapter 3, the increase in the level of HDAg-p24 expression induced by 75μM zinc that was non-cytotoxic, compared to the level of HDAg-p24 expression induced by 100μM zinc that was cytotoxic, was ca. 1.7-fold (Table 3.2; Figure 3.3A).

In the above experiment, the level of ^{125}I counts bound to the chimpanzee hepatocyte-derived HDAg was ca. $7.0x10^3/5x10^5$ cells. In the same sample analysed previously (Chapter 3.6), the level of ^{125}I bound was ca. 3.5-fold greater ($2.5x10^4/5x10^5$ cells). This reduction in the level of bound counts may have been the result of (1) long-term storage of the chimpanzee liver sample between experiments, (2) the increased time between the preparation and use of the ^{125}I

labelled Protein A used in this experiment (ca. 6 wk), compared to that used previously (<1wk), and (3) differences is the transfer efficiency of protein to the nitrocellulose. Since it was considered likely that the HDAg in the chimpanzee liver sample would be stable, the latter two factors were most likely to contribute to this change. This experiment showed that the level of HDAg-p27 and HDAg-p24 expression was similar in the first 12 hr after the addition of zinc sulphate to the culture medium.

4.5 The Differential Effects of HDAg-p24 and HDAg-p27 expression

4.5.A The Effect of HDAg Expression on Nucleic Acid Synthesis

The Hδ27 and Hδ5 cell lines were used to examine the differential effects of HDAg-p27 and HDAg-p24 expression respectively, on cellular nucleic acid synthesis in the same experiment as described above. At various times post-induction, the rate of nucleic acid synthesis was measured by pulse-labelling with ^{3}H-uridine or ^{3}H-thymidine for 3 hr as described in Chapter 2.5.B. The level of ^{3}H-uridine or ^{3}H-thymidine incorporation in the uninduced cultures at each time point was used as the standard in these experiments. The rate of nucleic acid synthesis in the parent HeLa cell line was not measured, although the effect of zinc sulphate on the gross cytology was judged at the final time point in each experiment (see section 4.5.B).

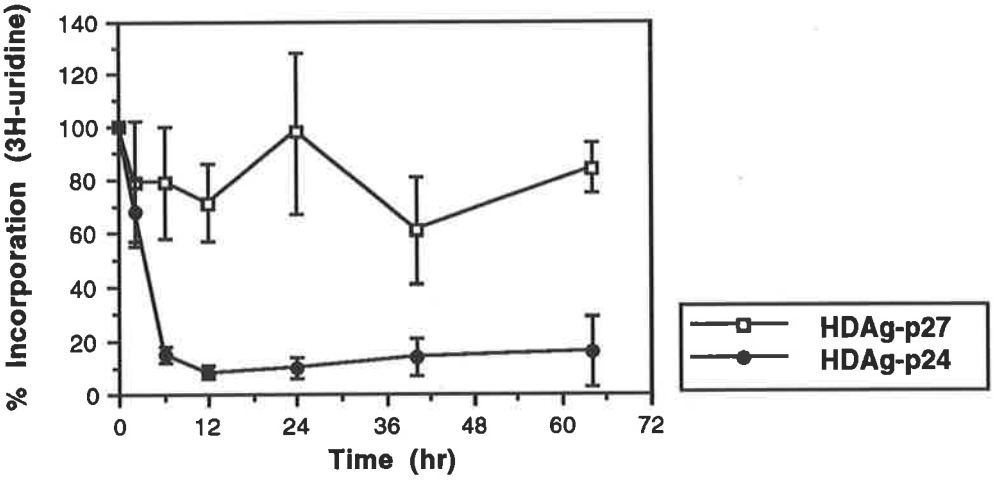
In one experiment performed over a 12 hr period with Hδ27 cells which were ca. 70% when pulse-labelled, the increase in the level of HDAg-p27 expression detected at 4 hr after zinc induction, coincided with a reduction in the rate of ^{3}H-uridine and ^{3}H-thymidine incorporation to ca. 60% of the untreated cultures. This was followed by a transient fluctuation in both RNA and DNA synthesis. However,

these cells appeared to stabilise so that 12 hr post-induction, the rate of nucleic acid synthesis was ca. 60-70% of the control (uninduced) cells (Figure 4.6B,C). In contrast, a marked reduction in cellular nucleic acid synthesis was closely related to HDAg-p24 expression (Figure 4.6B,C). A significant reduction in the level of both ^{3}H-uridine and ^{3}H-thymidine incorporation was detected 4 hr after zinc induction. At this time, the levels of cellular nucleic acid synthesis were reduced to <40% of the untreated cells. At 6 hr after zinc induction, the levels of RNA and DNA synthesis were reduced further to 17.5% and 18.1% of the untreated cells respectively. After this, the level of nucleic acid synthesis appeared to stabilise at ca. 10% of the level of incorporation in the uninduced cultures. In a similar experiment described in Chapter 3.4, zinc sulphate induction of δMT5A and δMTG25 cells for 16 hr resulted in reduced rates of RNA synthesis (50-60%), but not DNA synthesis. This point is discussed in section 4.6.

The above experiment was repeated three times over a three day, rather than a 12 hr period and the combined results are shown in Figures 4.7 and 4.8. In these experiments the cells were pulse-labelled at 70% and 100% confluency. In the zinc-treated Hδ27 cells, although cellular nucleic acid synthesis was reduced initially to approximately 70%, cells which expressed HDAg-p27 recovered, so that the final levels were close to the levels prior to zinc induction. The cause of the fluctuations in nucleic acid synthesis in HDAg-p27-positive cells was unclear but the fluctuations were reduced considerably in the 100% confluent cultures (Figure 4.8). Nevertheless, these results contrast markedly with the results in the HDAg-p24-positive cells. The effect of HDAg-p24 expression was remarkably consistent; within 6 hr post-induction, the synthesis of cellular DNA and RNA was reduced to ca. 10-20% of the rate of nucleic acid synthesis in the uninduced cells. Cytological

Fig 4.7 The effect of HDAg-p24 and HDAg-p27 expression on cellular nucleic acid synthesis. 70% confluent cultures of Hδ27 and Hδ5 cells were grown in medium supplemented with 100μM zinc for three days, and the rate of (A) RNA synthesis, and (B) DNA synthesis was measured at various times post-induction. The error bars were derived from the standard deviation calculated from the average of the results from three experiments.

A


% Incorporation (3H-uridine)
140
120
100
80
60
40
20
0
0
12
24
36
48
60
72
Time (hr)
HDAg-p27
HDAg-p24

B

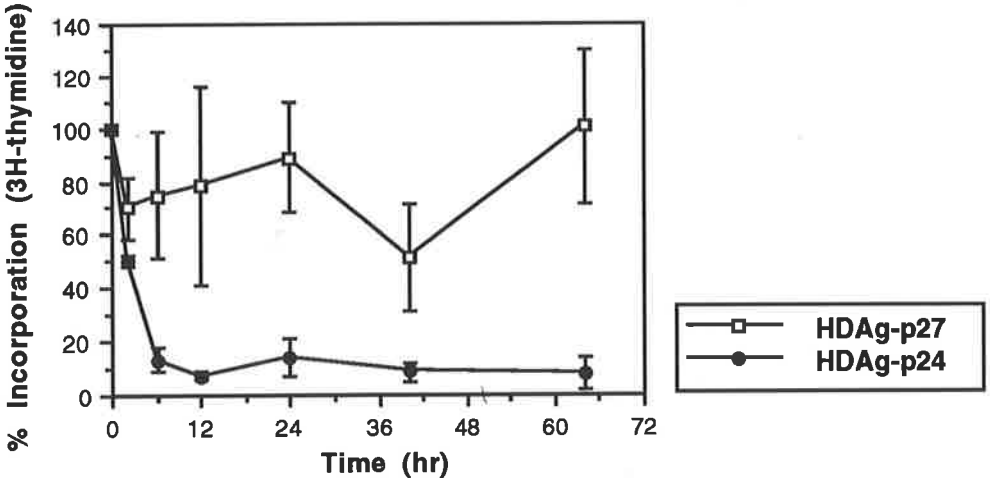
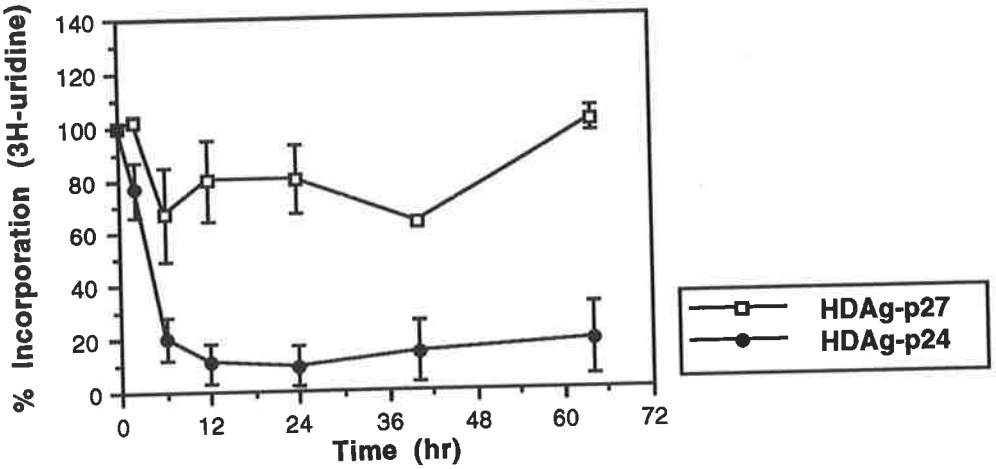
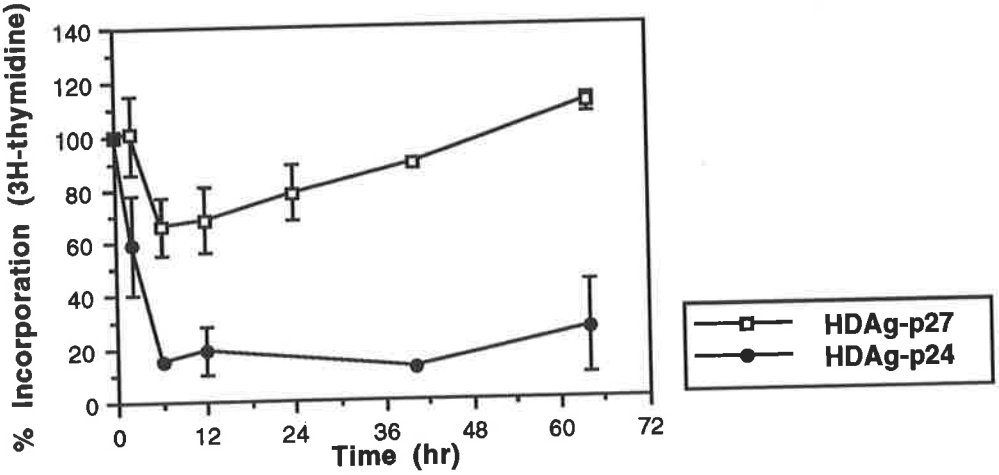

% Incorporation (3H-thymidine)
140
120
100
80
60
40
20
0
0
12
24
36
48
60
72
Time (hr)
HDAg-p27
HDAg-p24

Fig 4.8 The effect of HDAg-p24 and HDAg-p27 expression on cellular nucleic acid synthesis. 100% confluent cultures of Hδ27 and Hδ5 cells were grown in medium supplemented with 100μM zinc for three days, and the rate of (A) RNA synthesis, and (B) DNA synthesis was measured at various times post-induction. The error bars were derived from the standard deviation calculated from the average of the results from three experiments.

A


% Incorporation (3H-uridine)
140
120
100
80
60
40
20
0
0
12
24
36
48
60
72
Time (hr)
HDAg-p27
HDAg-p24

B


% Incorporation (3H-thymidine)
140
120
100
80
60
40
20
0
0
12
24
36
48
60
72
Time (hr)
HDAg-p27
HDAg-p24

examination of each culture confirmed the marked difference between the effect of HDAg-p24 and -p27 (see below). Cellular nucleic acid synthesis was not significantly different in cells that were pulse-labelled at 70% confluency (Figure 4.7) or 100% confluency (Figure 4.8).

4.5.B **Cytological Analysis**

Cytological analysis following zinc induction in the Hδ27 and the Hδ5 cell lines was consistent with the above results. At all time points during the experiments described above, zinc-induced and uninduced Hδ27 cells showed none of the degenerative cytological changes that were associated with HDAg-p24 expression described in Chapter 3 (Figure 4.9). In contrast, in the Hδ5 cell line, there was a marked reduction in the number of cells in the culture ca. 2 hr after the increase in HDAg-p24 expression was detected. A predominant feature of the zinc-induced cultures was the presence of cells with shrunken eosinophilic cytoplasm and pyknotic nuclei, and apoptotic-like bodies similar to those described previously (Chapter 3.5). None of these degenerative cytological changes were observed in the uninduced Hδ5 cells.

Since there was a high constitutive level of HDAg-p24 expression in this cell line, the cytological changes observed in the zinc-induced Hδ5 cells were compared with the cytology of HeLa cells treated with 100μM and 150μM zinc sulphate, since treatment of HeLa cells with 150μM zinc was shown previously to induce cell death (see Chapter 3.3 and Figure 4.10). As expected, cytological analysis of HeLa cells treated with 100μM zinc sulphate did not display any degenerative cytological changes whereas treatment of HeLa cells with 150μM zinc sulphate resulted in cell

Fig 4.9 Cytological analysis of (a) uninduced Hδ5 cells, (b) Hδ5 cells ca. 6 hr post-induction, (c) uninduced Hδ27 cells, and (d) Hδ27 cells ca. 12 hr post-induction (magnification x 2000).

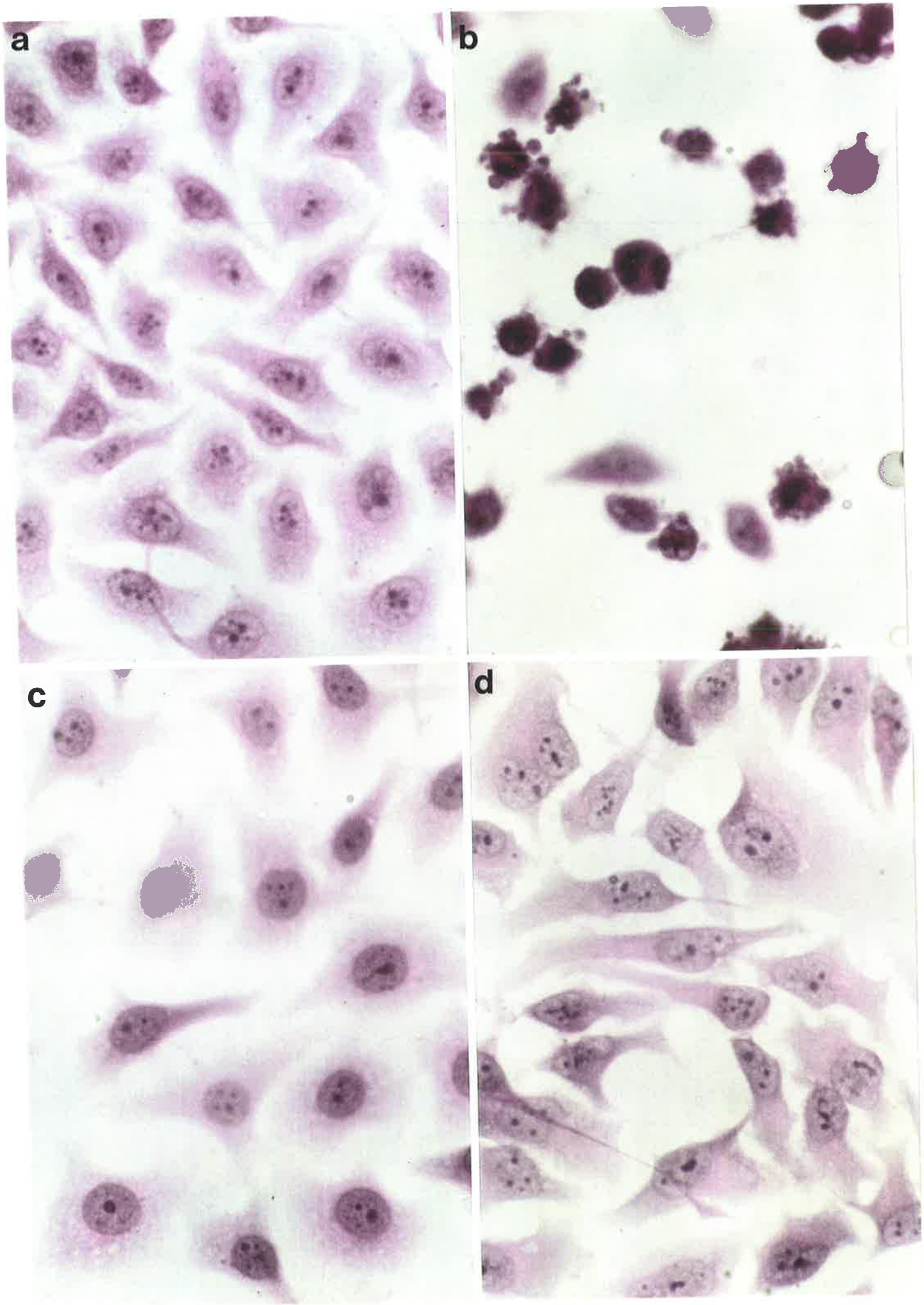
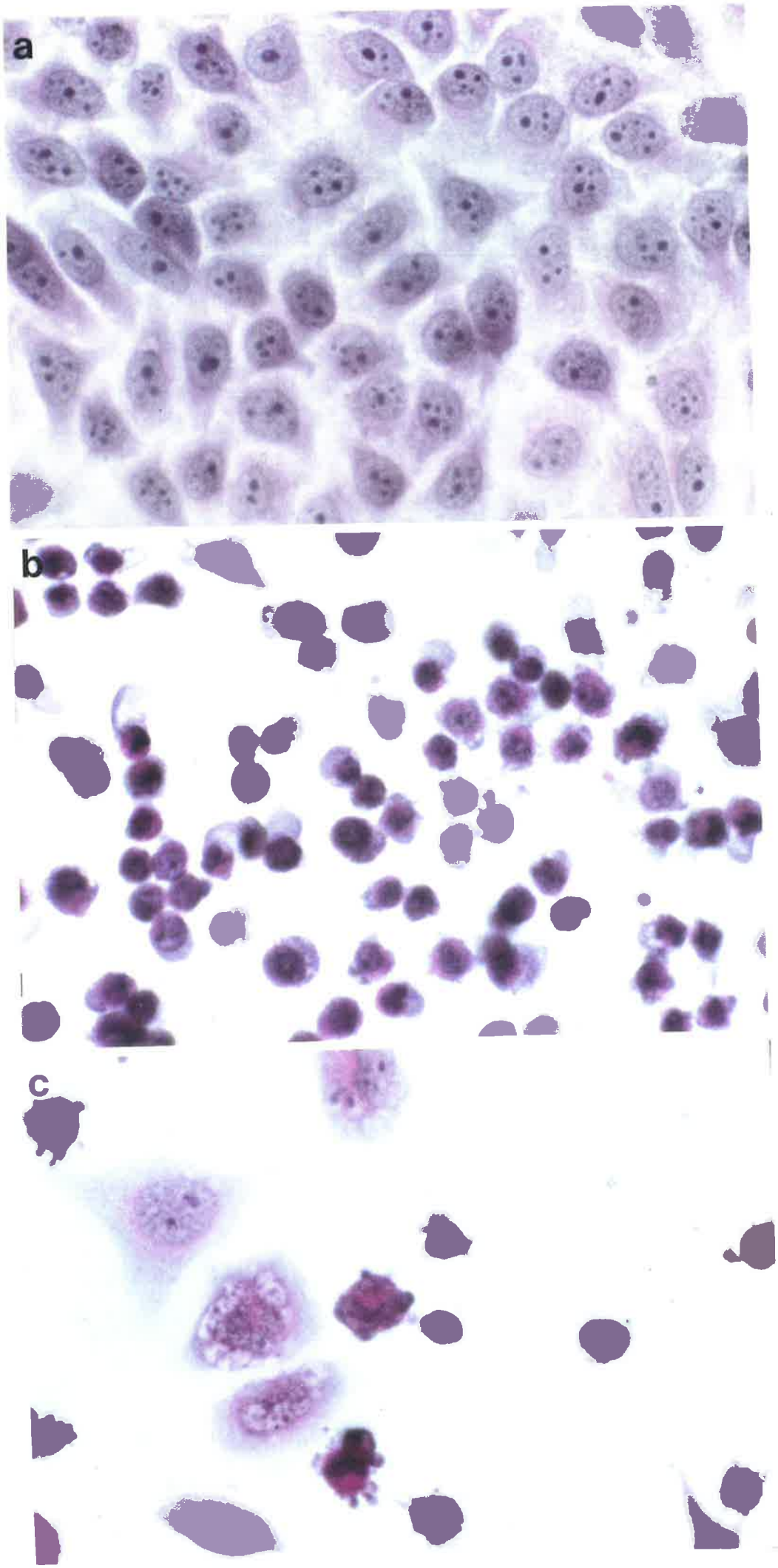

a
b
c
d

Fig 4.10 Cytological analysis of (a) HeLa cells treated with 100μM zinc, (b) HeLa cells treated with 150μM zinc, and (c) Hδ5 cells treated with 100μM zinc (the true eosinophilic nature of the apoptotic-like bodies in this culture are not apparent due to difficulties with the photographic reproduction) (magnification x 2000).


a
b
c

necrosis that was often associated with highly condensed nuclei, but there was no evidence of cells that resembled apoptotic bodies. Furthermore, there was no evidence of a marked reduction in the cell number in the zinc-treated (150μM) HeLa culture that is a feature of HDAg-p24-induced cytotoxicity. Thus, cytotoxicity was not simply the result of high concentrations of zinc sulphate since the gross cytology observed in the zinc-induced Hδ5 cells differed significantly to that observed in HeLa cells treated with a lethal concentration of zinc sulphate.

4.6 Discussion

In this chapter, the effect of HDAg-p27 on cellular nucleic acid synthesis and cytology was examined in an in vitro cell culture system similar to that described in Chapter 3, and compared with HDAg-p24 expression in another cell line (Hδ5) similar to, but distinct from those (δMT5A and δMTG25) examined in Chapter 3. A HeLa-derived cell line was developed in which the expression of HDAg-p27 was controlled by the MTIIA promoter, and consequently, the level of HDAg-p27 expression was directly proportional to the zinc sulphate concentration added to the culture medium. HDAg-p27 expression resulted initially in some reduction in the rate of cellular nucleic acid synthesis, but in the long term, the effect of HDAg-p27 expression on cellular RNA and DNA synthesis was minimal and no cytological degeneration was apparent. In contrast, in the Hδ5 cell line, HDAg-p24 expression induced characteristic cytological changes including eosinophilic cytoplasm and pyknotic nuclei that were similar to those observed in the δMT5A and δMTG25 after zinc induction. HeLa cells treated with the same concentration of zinc sulphate showed no degenerative cytological changes. In addition, cytological analysis of HeLa cells treated with a lethal concentration of zinc sulphate displayed cytotoxic

changes which were quite distinct from the characteristic features of HDAg-induced cytotoxicity. This confirms that HDAg-p24-induced cytotoxicity is not simply the result of exposure to high concentrations of zinc sulphate. In addition, the non-cytotoxic nature of HDAg-p27 in HeLa cells suggests that HDAg-p24-induced cytotoxicity is unlikely to be the result of expression of a foreign protein in the nucleus.

The effects of HDAg-p24 or -p27 expression were independent of the cell confluency level prior to induction of antigen expression in these cell lines. However, HDAg-24-induced cytotoxicity may be more significant in the confluent cultures, as this situation resembles more closely the state of quiescent hepatocytes in natural HDV infection.

Cellular -RNA and -DNA synthesis were both inhibited in the Hδ5 cells 4 hr after the addition of zinc to the culture medium and this was followed by the appearance of degenerative cytological changes to the culture 2 hr later. In contrast, in the δMT5A cell line described in Chapter 3, HDAg-p24 expression was associated with a reduction in RNA, but not DNA synthesis, ca. 16 hr after zinc induction, and subsequently, cytological degeneration was detected 4 days later. These differences may simply be attributable to the use of two distinct transfected cell lines. It is well known that cloned cell lines may vary as a result of 'drift' from the original parent cell line (Huschtscha and Holliday, 1983). Nevertheless, the degenerative cytological changes in the zinc-treated δMT5A cell line were also observed in the zinc-induced Hδ5 cells and were the direct result of HDAg-p24 expression.

Despite some variation between different cell lines, the use of transfected cell lines has proven to be an effective model with which to examine the direct effects of inducible expression of viral proteins, not only in the work presented in this chapter and Chapter 3, but also for other viruses. Studies which examined the cytotoxic properties of the parvovirus non-structural transcription unit and the HSV-1 glycoprotein gC1 both placed the viral gene downstream of the inducible MMTV promoter followed by production of a stably-transfected cell line in which the expression of the viral protein was controlled by the addition of dexamethasone to the culture medium (Friedman et al, 1989; Caillet-Fauquet et al, 1990). In each example, expression of the viral protein was associated with cytotoxicity.

The finding that HDAg-p27 is non-cytotoxic in this model system, together with the previous observation that HDAg-p27 inhibits HDV RNA replication (Chao et al, 1990), are consistent with the proposal that increased expression of HDAg-p27 may promote HDV persistence.

CHAPTER FIVE

HDAg-p24-RELATED CYTOTOXICITY RESULTS FROM PROTEIN EXPRESSION AND IS MODULATED BY HDAg-p27

5.1 Introduction

The work presented in the previous chapters of this thesis described the direct effect of HDAg-p24 and HDAg-p27 expression in stably-transfected hepatoma- and non-hepatoma- cell lines. In these cell lines, HDAg-p24 expression was associated with significantly reduced rates of nucleic acid synthesis, and eventually with reduced rates of cell division and degenerative cytological changes which resembled those seen in acute HDV-related hepatitis. In contrast, HDAg-p27 expression had a minimal effect on cellular nucleic acid synthesis and did not induce any degenerative cytological changes. On the basis of these results, it was proposed that the direct cytotoxic action of HDAg-p24 may contribute to the extensive liver cell injury associated with acute HDV infection, whereas HDAg-p27 expression may promote the establishment of a persistent infection, consistent with its non-cytotoxic nature and its ability to inhibit HDV RNA replication.

The aim of the experiments in this chapter was to address the following unresolved issues;

(i) to confirm the possibility that HDAg-p24-related cytotoxicity is due to the direct action of HDAg-p24 per se, and is not a result of events leading to the expression of HDAg-p24; and

(ii) to investigate the hypothesis that HDAg-p27 can modulate HDAg-p24-related cytotoxicity when coexpressed in the same cell.

A transient transfection system was used in this study in preference to continuous cell lines developed after stable transfection with inducible expression vectors. Transient transfection provides a fast and simple alternative and is designed specifically for short-term experiments. Thus, the need for expensive antibiotic selection is avoided. It is possible to achieve results consistent with those obtained using stably-transfected cell lines due to the high transfection efficiency that can be achieved using current commercially-available transfection reagents. In the study of HDV, transient transfection has been used successfully by others to examine various components of the HDV RNA replication cycle (Kuo et al, 1989).

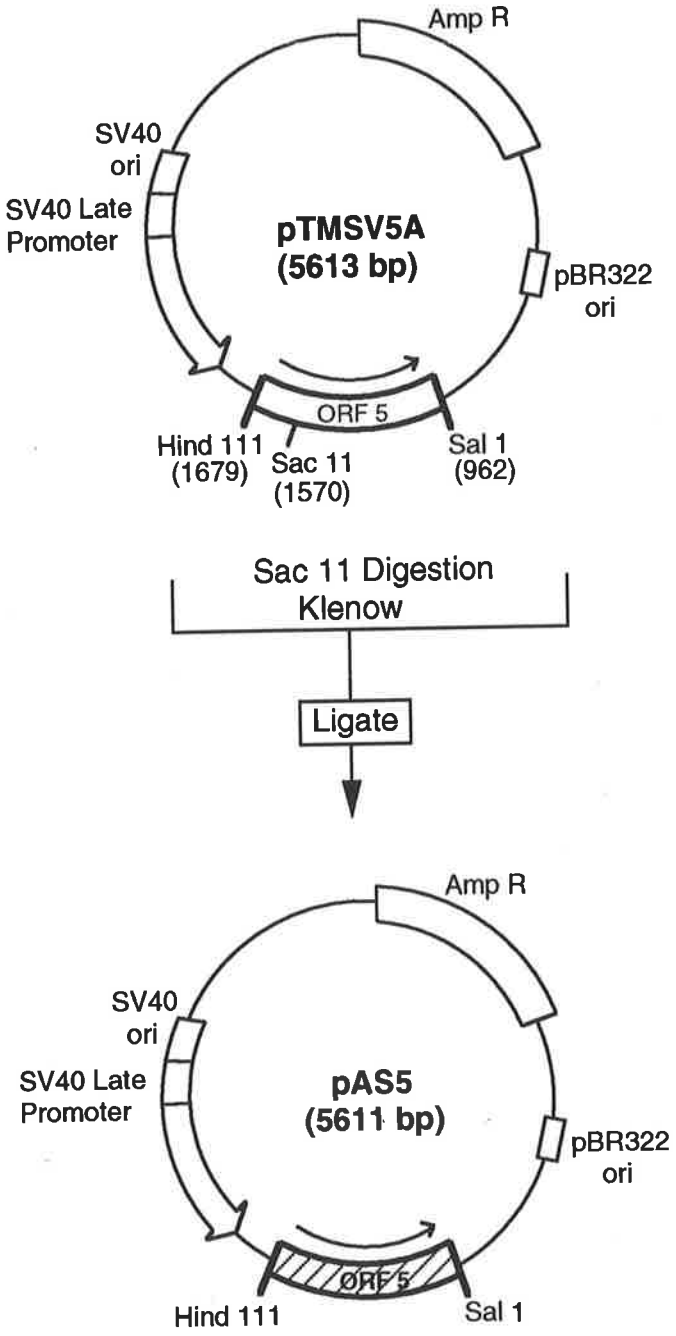
5.2 Construction of HDAg Expression Plasmids

To examine the direct effects of HDAg expression in a transient transfection system, it was necessary to construct recombinant plasmids which contained the HDAg-p24 or HDAg-27 gene inserted into pSVL, a eukaryotic expression vector which is designed specifically for high level transient expression of foreign proteins (Figure 2.2).

5.2.A Construction of pSVL-Derived Plasmids containing a HDAg-p24 -Wild Type or -Mutant Gene

The plasmid pTMSV5A, was a gift from Dr. T. Macnaughton and contains the HDAg-p24 gene inserted into the MCS of pSVL (Figure 5.1). To examine the hypothesis that HDAg-p24 is cytotoxic per se, a pSVL-derived plasmid pAS5 (Figure 5.1), was

Fig 5.1 The pSVL-derived recombinant plasmids pTMSV5A and pAS5. pTMSV5A contains the HDAg-p24 gene under the control of the SV40 promoter. pAS5 was constructed from pTMSV5A and contains a mutated HDAg-p24 gene (shaded) to prevent expression of HDAg-p24. The strategy for the mutagenesis is described in the text and Figure 5.2.


Amp R
SV40
ori
SV40 Late
Promoter
pTMSV5A
(5613 bp)
pBR322
ori
ORF 5
Hind 111
(1679)
Sac 11
(1570)
Sal 1
(962)
Sac 11 Digestion
Klenow
Ligate
Amp R
SV40
ori
SV40 Late
Promoter
pAS5
(5611 bp)
pBR322
ori
ORF 5
Hind 111
Sal 1

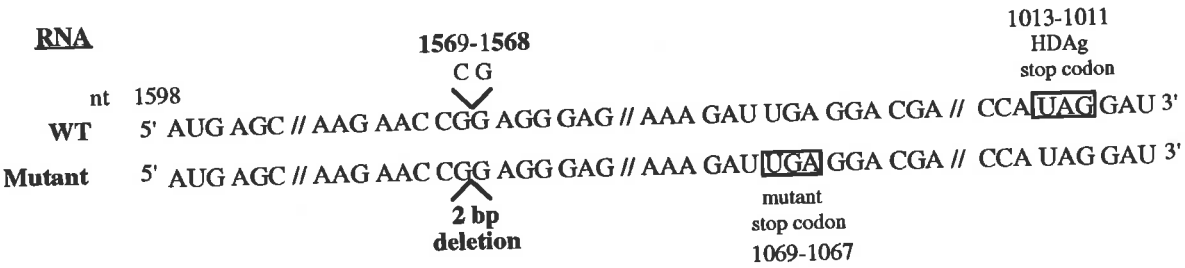
constructed that contains a mutant HDAg-p24 gene which cannot express the antigen. A frame-shift mutation was introduced into the HDAg gene so that, although the mutant mRNA was essentially the same as wild-type, HDAg was not synthesised. The plasmid was produced from pTMSV5A as follows; pTMSV5A was linearised by digestion with Sac 11 (map position 1570; Wang et al, 1986), blunt-ended with the Klenow fragment of DNA polymerase 1 and subsequently religated to produce a Sac 11-resistant construct, pAS5 (Figure 5.1). In this experiment, RE digestion with Sac 11 created 3' overhangs which were then removed by the 3' → 5' exonuclease activity of the Klenow fragment of DNA polymerase 1. Thus, upon religation of the plasmid DNA, a 2 bp deletion was created at nt 1570 of the HDV cDNA. As a result of this deletion, a frame-shift mutation was introduced at aa10 and a nascent stop codon (UGA) at aa44 (Figure 5.2). The presence of this frame-shift mutation was confirmed by double-stranded DNA sequencing of pAS5 using an Applied Biosystems Model 373A Automatic DNA Sequencer as described in the manufacturer's instructions. Thus, translation from the first AUG codon of the HDV mRNA from this construct is predicted to produce a protein of 44 aa, composed of the first 10 N-terminal aa of HDAg and 34 aa of random sequence (Figure 5.2).

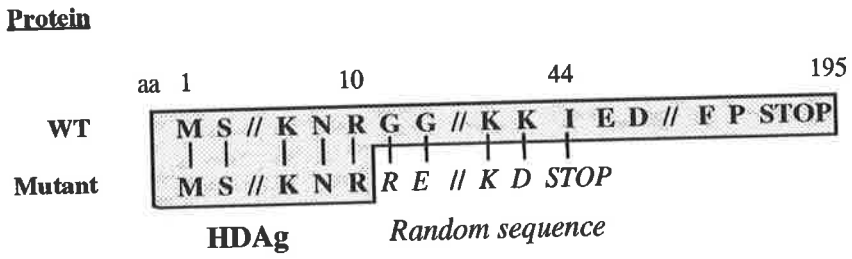
5.2.B **Construction of pSV27**

The HDAg-p27 gene was inserted into the pSVL transient expression vector as follows; an 800 bp fragment which spanned the HDAg-p27 gene, was excised from pBKS27 (described in Chapter 4.2) by Hind III digestion, and included 26 bp of the pBluescript KS+ vector sequence upstream of the HDV coding sequence. This 800 bp fragment was end-filled using the Klenow fragment of DNA polymerase 1 and

Fig 5.2 RNA and aa sequence analysis of the N-terminal region of the wild-type HDAg-p24 gene in pTMSV5A and the mutant HDAg-p24 gene in pAS5. pAS5 contains a frame-shift mutation at aa10 in the HDAg-p24 gene and a nascent stop codon at aa44. Thus, translation from the first AUG codon of the HDV mRNA from this construct is predicted to produce a protein of 44 aa, composed of the first 10 N-terminal aa of the wild-type HDAg and 34 aa of random sequence. Thus, although HDAg-p24 is not synthesised from this construct, the mutant mRNA is essentially the same as wild-type.

Sequence Analysis of the HDAg gene in pTMSV5A and pAS5





inserted into the unique Sma 1 site of pSVL to produce the final construct pSV27 (Figure 5.3).

5.3 HDAg Expression Studies in a Transient Transfection Assay

5.3.A Transient Transfection

The host cell line for the transient transfection assays was the monkey kidney cell line, Cos7 (Gluzman, 1981), which is transformed with the SV40 large T-antigen and allows high level expression from pSVL-constructs. The cells were grown in normal medium as described in Chapter 2.1, for 2 days, or until they were 60-80% confluent, and then transfected using the commercial transfection reagent DOTAP, essentially according to the manufacturer's instructions (see Chapter 2.3.F).

5.3.B HDAg Detection

(i) Immunofluorescence

At day 2 post-transfection, the Cos7 cells were examined by immunofluorescence for expression of HDAg. In each experiment, the transfection efficiency was estimated by the number of HDAg-positive cells. The intracellular localisation of HDAg-p24 and HDAg-p27 expression in Cos7 cells transfected with pTMSV5A or pSV27 respectively, was nuclear (Figure 5.4), and was consistent with the localisation of HDAg-p24 and HDAg-p27 in the stably-transfected cell lines described previously (Chapter 4.3). This pattern of HDAg expression was not altered in cells cotransfected with pTMSV5A and pSV27. Immunofluorescence to detect HDAg expression in Cos7 cells transfected with pAS5 or pSVL was negative.

Fig 5.3 Strategy for the construction of the pSVL-derived recombinant plasmid pSV27, which contains the HDAg-p27 gene under the control of the SV40 promoter.

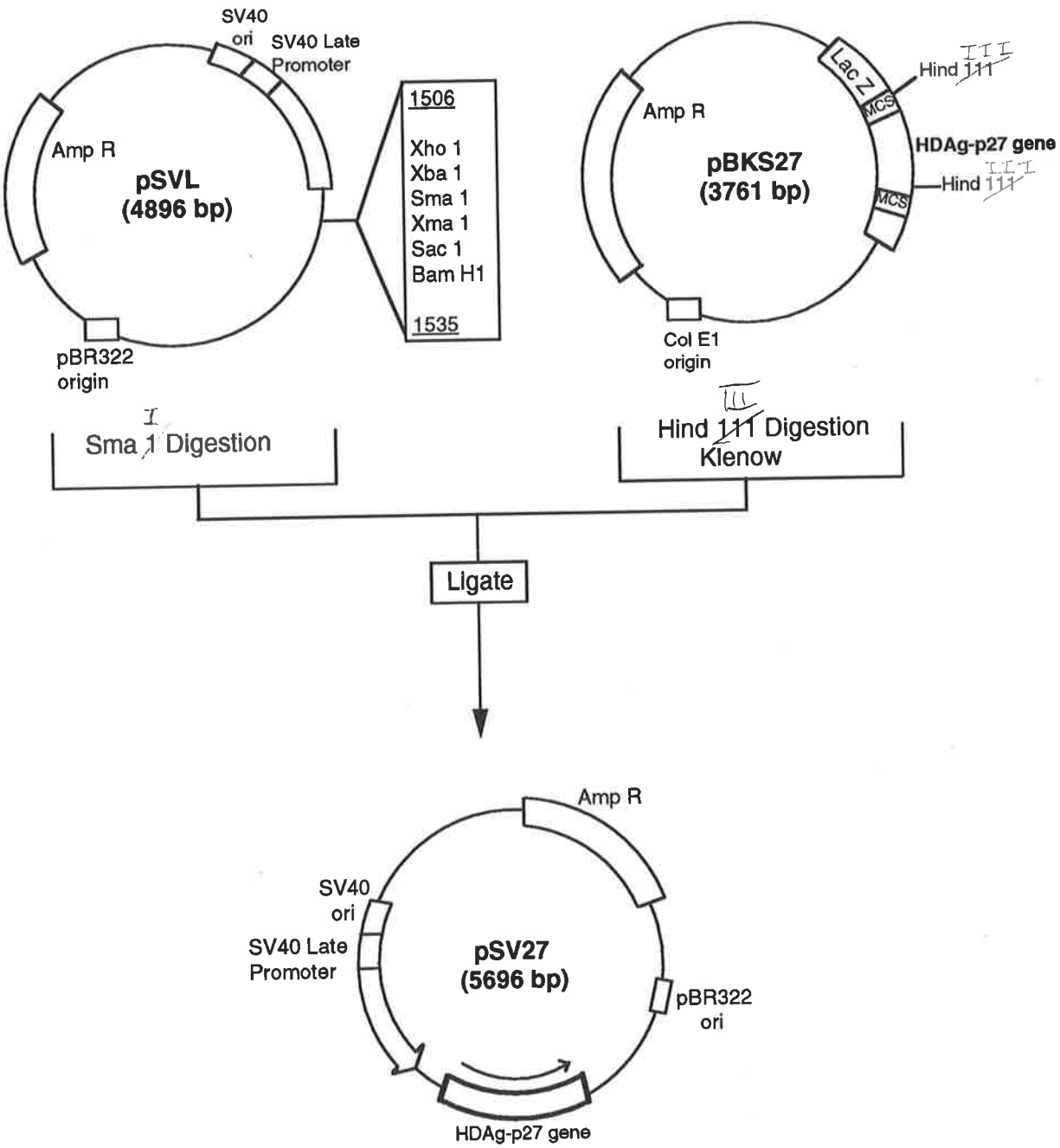
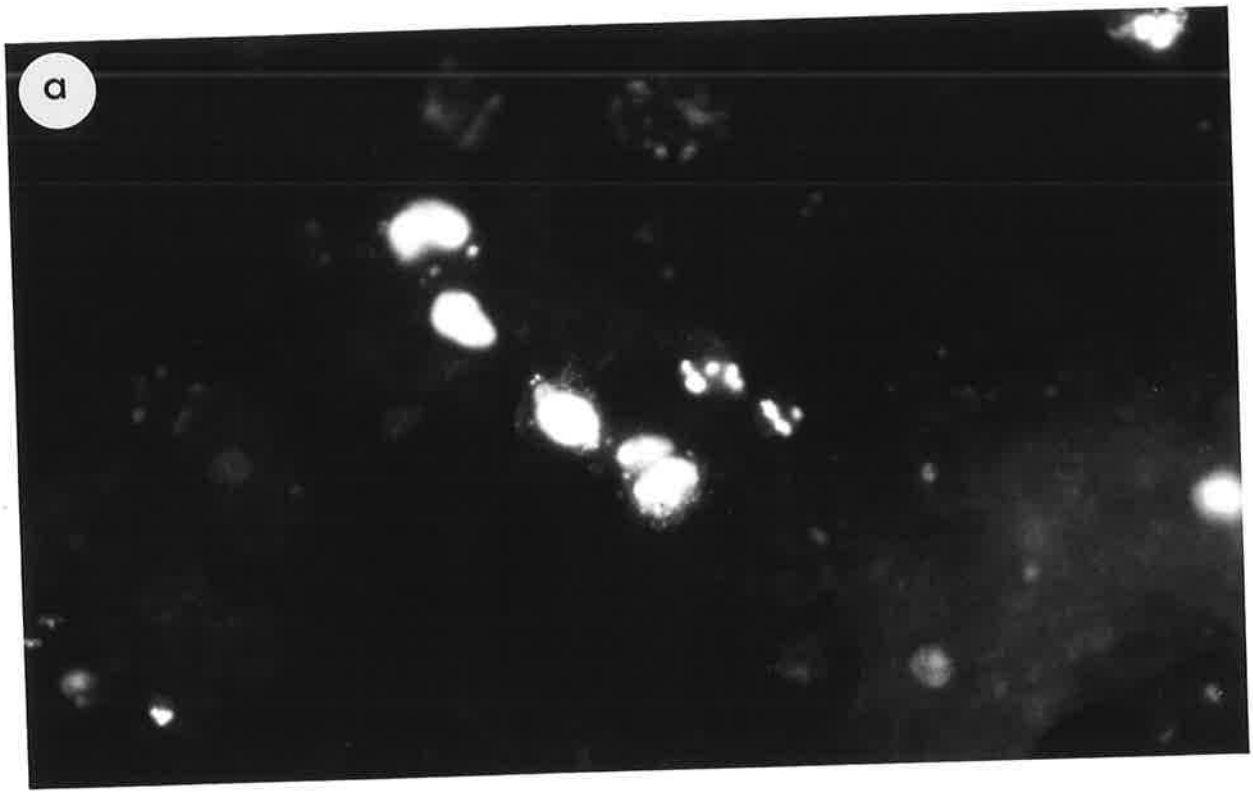
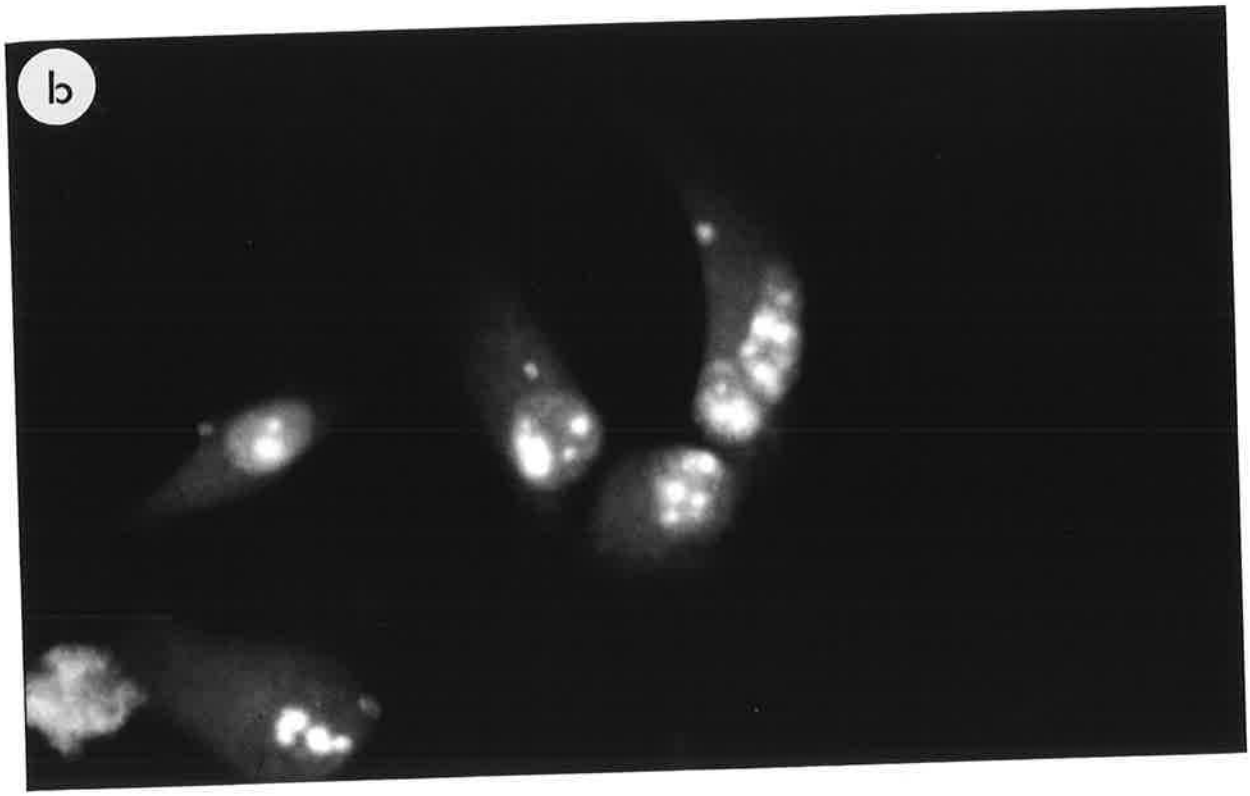

SV40
ori
SV40 Late
Promoter
Amp R
pSVL
(4896 bp)
pBR322
origin
1506
Xho 1
Xba 1
Sma 1
Xma 1
Sac 1
Bam H1
1535
Lac Z
MCS
Hind III
Amp R
pBKS27
(3761 bp)
HDAg-p27 gene
Hind III
MCS
Col E1
origin
Sma I Digestion
Hind III Digestion
Klenow
Ligate
Amp R
SV40
ori
SV40 Late
Promoter
pSV27
(5696 bp)
pBR322
ori
HDAg-p27 gene

Fig 5.4 The detection of HDAg in Cos7 cells by immunofluorescence, at day 2 after transient transfection with (A) pTMSV5A and (B) pSV27 (magnification x 2000).


a


b

(ii) Immunoblotting

The expression of HDAg in Cos7 cells after transfection was confirmed by immunoblot analysis at Day 5 post-transfection (Figure 5.5). In Cos7 cells transfected with pTMSV5A, HDAg was detected as a single polypeptide of 24 kD. Similarly, the bulk of HDAg in Cos7 cells after transfection with pSV27 was detected as a polypeptide of 27 kD but a small proportion was detected at ca. 26 kD that was considered to be a breakdown product which has been detected previously (Sureau et al, 1993). In Cos7 cells cotransfected with pTMSV5A and pSV27, the ratio of HDAg-p24:HDAg-p27 expression reflected the DNA molar ratio of pTMSV5A and pSV27 (1:1 and 10:1) in the transfection mix. Immunoblot analysis of Cos7 cells transfected with pAS5 or pSVL failed to detect any HDAg-specific polypeptides (Figure 5.5). This experiment confirmed the gene cloning work described above.

5.3.C **The Effect on Cell Synthesis**

(i) The Effect on Cellular Nucleic Acid Synthesis

Since HDAg-p24 expression, but not HDAg-p27, was shown previously to induce a marked reduction in cellular nucleic acid synthesis in stably-transfected cells, the level of ^{3}H-uridine incorporation was measured as a marker of cytotoxicity after transient transfection of Cos7 cells with the pSVL constructs described above. Coverslip cultures of Cos7 cells were transfected, then at various times after transfection, pulse-labelled with ^{3}H-uridine for 4 hr (Chapter 2.5.B) and harvested. In these transient transfection assays, the level of ^{3}H-uridine incorporation that resulted from transfection with the expression vector pSVL was used as the standard.

Fig 5.5 Immunoblot analysis to detect HDAg in Cos7 cells after transient transfection with; *Lane 1* pTMSV5A; *Lane 2* pAS5; *Lane 3* pSV27; *Lane 4* pTMSV5A:pSV27 (1:1) and *Lane 5* pTMSV5A:pSV27 (10:1). The upper and lower arrows correspond to 27 kD and 24 kD respectively.

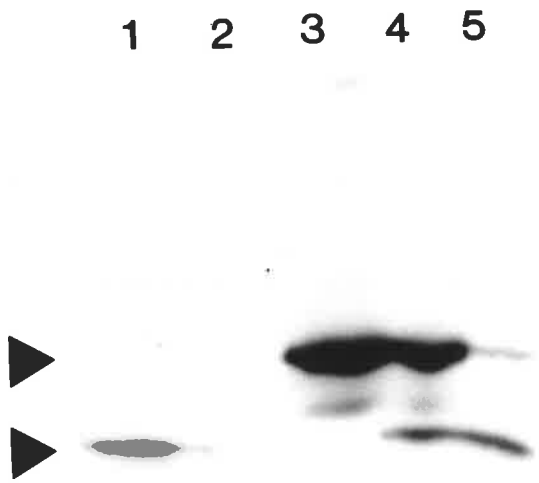
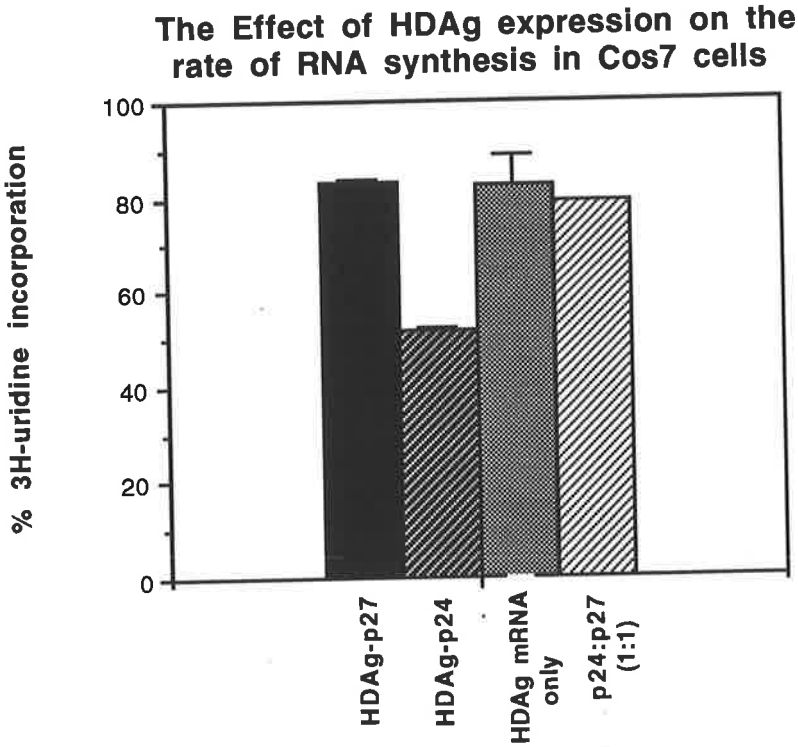

1
2
3
4
5

Figure 5.6 shows the combined results of two experiments at day 5 post-transfection. HDAg-p24 expression in the transient assay had a significant effect on the rate of RNA synthesis (ca. 42% reduction), and while this was not as dramatic as shown previously in the δMT5A, δMTG25, or Hδ5 cell lines, this is most likely due to the use of a transient system in which the transfection efficiency in these two experiments was ca. 35-40%. In contrast, the expression of HDAg-p27 had a minimal effect on cell RNA synthesis. Thus, the effects of HDAg-p24 and HDAg-p27 expression on cellular RNA synthesis in the transient system were consistent with those demonstrated previously in the stably-tranfected cell lines (Chapter 3.4 and 4.5). Furthermore, coexpression of HDAg-p24 and HDAg-p27 resulted in levels of ^{3}H-uridine incorporation similar to those demonstrated when HDAg-p27 only was expressed. Thus, the reduction in cellular RNA synthesis induced by transient HDAg-p24 expression was prevented by cotransfection of pTMSV5A with the HDAg-p27 expression vector, pSV27, in a 1:1 molar ratio (Figure 5.6). In later experiments, attempts to determine the effect of reducing the molar ratio of HDAg-p24:p27 to 10:1 were unsuccessful and this experiment was subsequently performed using a modified experimental approach which is described in Section 5.3.C(iii).

In addition, transfection of the construct pAS5, which contains a chain-termination mutant of the HDAg-p24 gene, was shown to have no significant effect on ^{3}H-uridine incorporation (Figure 5.6). Thus, since transcription of HDAg mRNA was not cytotoxic, this implies that HDAg-p24 is cytotoxic <u>per se</u>. However, this interpretation is only possible if the level of HDV RNA transcribed in the Cos7 cells from plasmids pTMSV5A and pAS5 is similar, and this is examined in section 5.3.D below.

Fig 5.6 Effect of HDAg expression on cellular RNA synthesis in Cos7 cells. Cos7 cells were transfected with the pSVL-constructs described in the text, and then pulse-labelled with ^{3}H-uridine for 4 hr at day 5 post-transfection and harvested as described in Chapter 2.5.B. The error bars were derived from the standard deviation calculated from the average of the results from two experiments.


The Effect of HDAg expression on the rate of RNA synthesis in Cos7 cells
% 3H-uridine incorporation
100
80
60
40
20
0
HDAg-p27
HDAg-p24
HDAg mRNA only
p24:p27 (1:1)

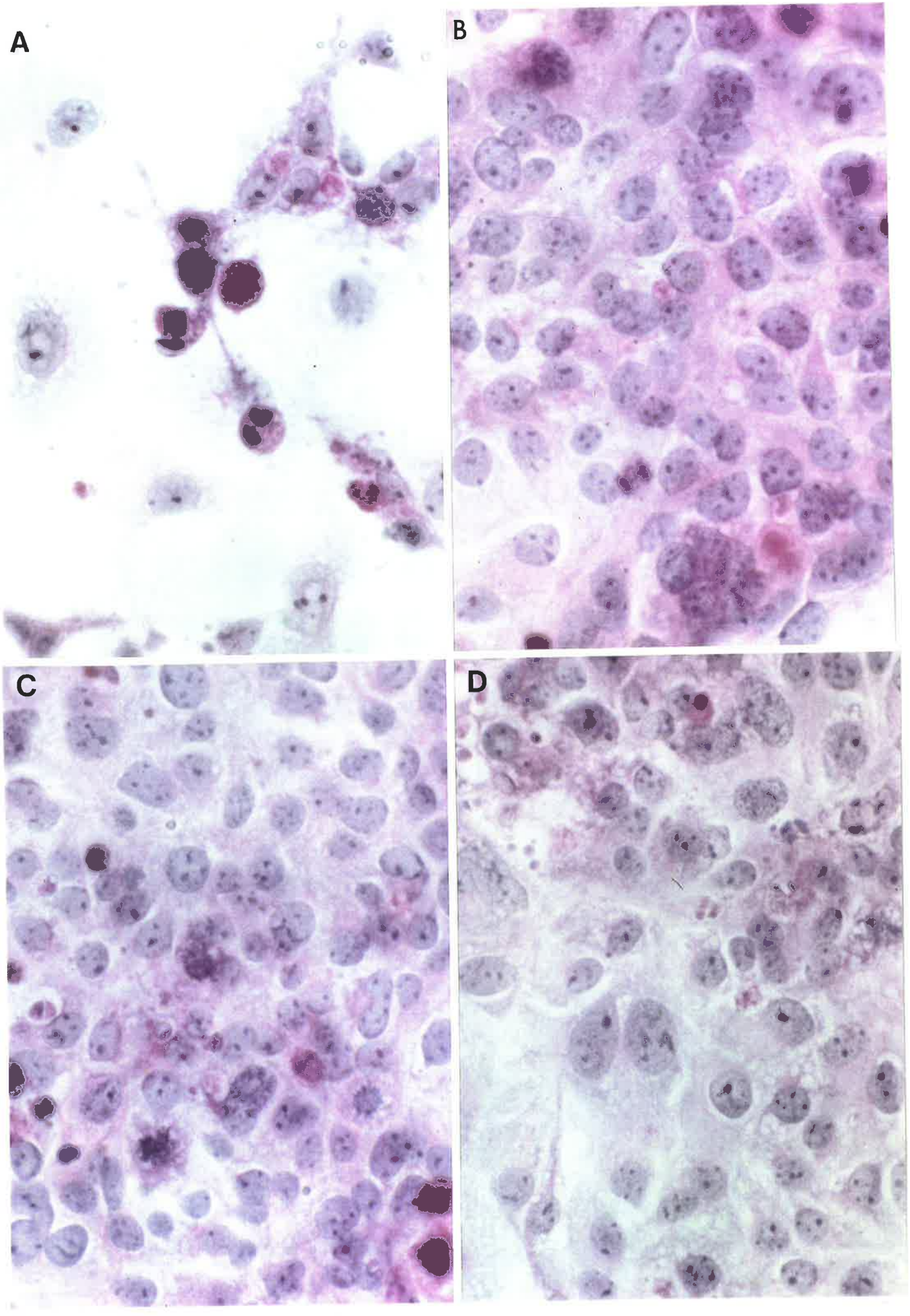
(ii) Cytological analysis

The effect of HDAg expression on cell synthesis was determined not only by the effect on cellular RNA synthesis but was also assessed by the effect on the gross cytology of the transfected Cos7 cells. Cytological analysis was performed on day 5 post-transfection as described in Chapter 2.5.C. The appearance of degenerative cytological changes as a result of transient expression of HDAg-p24 coincided with the reduction in RNA synthesis detected in both experiments (Figure 5.6). The cytological changes included cells with pyknotic nuclei and condensed cytoplasm that resembled apoptotic bodies, similar to those seen in zinc-treated δMT5A, δMTG25 and Hδ5 cells (Figure 5.7). In contrast, a cytotoxic effect was not observed in Cos7 cells that expressed HDAg-p27 nor in Cos7 cells in which HDAg mRNA was transcribed from the plasmid pAS5 without subsequent HDAg-p24 expression (see 5.3.D below; Figure 5.7). In addition, this experiment confirmed in cytological terms the above observation that expression of HDAg-p27 is sufficient to modulate cytotoxicity associated with expression of HDAg-p24 only (Figure 5.7).

(iii) The Effect of Coexpression of HDAg-p24 and HDAg-p27 in a Molar Ratio of 10:1

In the experiments described above, the level of RNA synthesis was measured at day 5 post-transfection. To examine the effect of coexpression of HDAg-p24 and HDAg-p27 in a molar ratio of 10:1, an experiment was performed in which the level of ^{3}H-uridine incorporation was measured daily for four days (Figure 5.8). In this experiment the transfection efficiency was ca. 25-30%, and the greatest reduction in the rate of RNA synthesis due to HDAg-p24 expression occurred two days post-transfection when

Fig 5.7 Cytological analysis of Cos7 cells after transfection with (a) pTMSV5A, (b) pSV27, (c) pAS5, and (d) pTMSV5A and pSV27 in a molar ratio of 1:1 (magnification x 2000).


A
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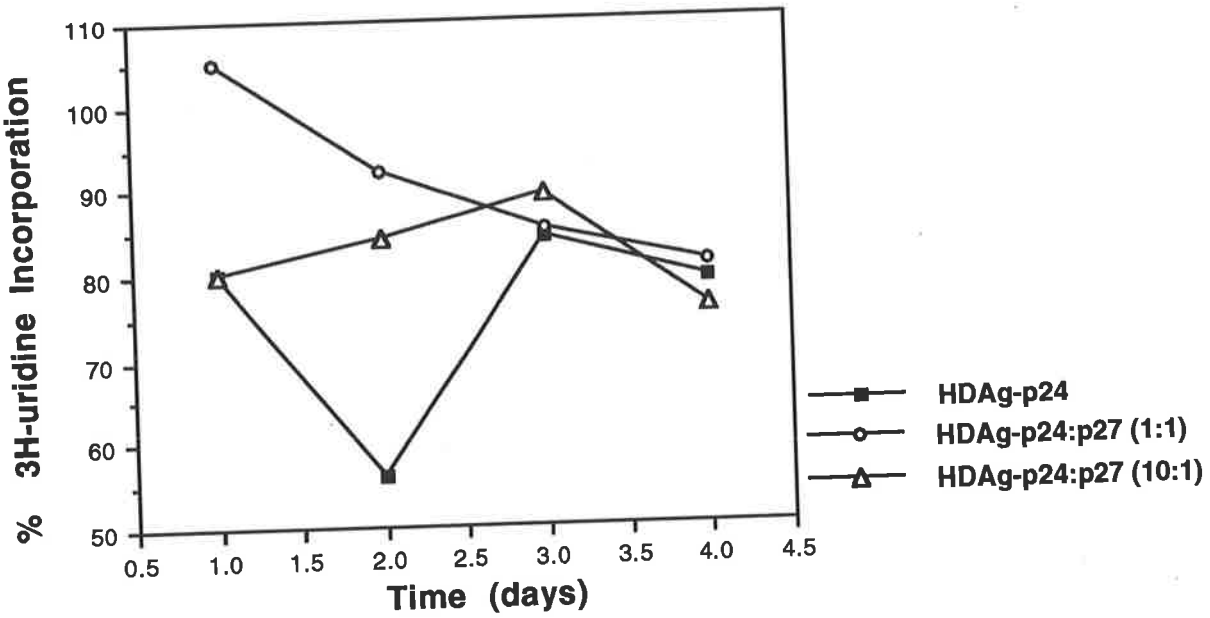
D

Fig 5.8 Effect of coexpression of HDAg-p24 and HDAg-p27 on cellular RNA synthesis. Cos7 cells were transfected with pTMSV5A and pSV27 in a molar ratio of 1:1 and 10:1 respectively and then pulse-labelled with ^{3}H-uridine for 4 hr daily for four days post-transfection and harvested as described in the text.

The effect of coexpression of HDAg-p24 and HDAg-p27 on the rate of RNA synthesis in Cos 7 cells



the level of ^{3}H-uridine incorporation was reduced by ca. 45%. Subsequently, there was an increase in the level of ^{3}H-uridine incorporation from 55% to 82% between day 2 and 3. This was believed to be a consequence of using a transient assay system in which only a varying proportion of cells were HDAg-positive, and consequently the increase in the level of RNA synthesis between day 2 and 3 probably reflected RNA synthesis in untransfected (dividing) cells. In this experiment, there was a 15% lower transfection efficiency compared with the previous experiments described in 5.3.C(i) in which a reduction in RNA synthesis of ca. 42% was still detectable at day 5 post-transfection. It is proposed that, in assays where fewer cells were HDAg-positive (the transfection efficiency was reduced), an overall reduction in RNA synthesis cannot be sustained due to the growth rate of the untransfected cells which constitute a much higher proportion of the culture (in this case ca. 70%). In contrast, in experiments with a high transfection efficiency in which the number of HDAg-positive cells was higher, the extent of HDAg-p24-induced cell death was greater, so that the reduction in the overall rate of RNA synthesis was sustained for a longer period. This suggests that in the transfection experiments monitored at day 5 (Figure 5.6), a reduced level of cellular RNA synthesis should have been detectable much earlier than day 5; in fact, at day 1 post-transfection in one of the two experiments described in Figure 5.6, the rate of cellular RNA synthesis in Cos7 cells that expressed HDAg-p24 was reduced by ca. 30% compared to the level of RNA synthesis as a result of HDAg-p27 expression. Other factors that may contribute to variations between experiments are (1) the confluency of Cos7 cells at the time of transfection, and (2) the growth rate of these cells after seeding.

In the experiment described in Figure 5.8, cotransfection of pTMSV5A and pSV27 in a 10:1 molar ratio was sufficient to prevent a reduction in cellular RNA synthesis that

was associated with transient expression of HDAg-p24 only. This experiment was only performed once due to technical difficulties and time limitations.

5.3.D HDV RNA Detection

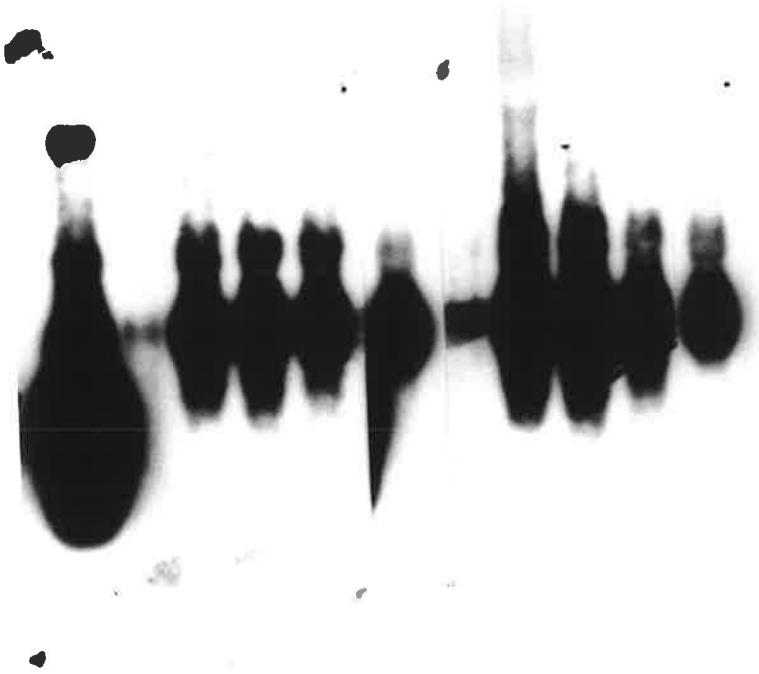
The construct pAS5, was designed to examine the effect of HDV RNA transcription from the HDAg-p24 gene in Cos7 cells in order to confirm that HDAg-p24-associated cytotoxicity was the direct result of HDAg-p24 per se. The results of the experiments described above are consistent with this proposal. However, it was necessary to demonstrate that the level of HDV RNA transcription in cells transfected with pAS5 was similar to that in cells transfected with pTMSV5A. In an independent experiment, HDAg mRNA levels were measured by Northern blot analysis (Chapter 2.7) on days 1-5 post-transfection. The results showed that the levels of mRNA were similar at each time point (Figure 5.9). Although there was no direct measure of the transfection efficiency of Cos7 cells transfected with pAS5, detection of HDAg by immunofluorescence performed at the same time on several coverslip cultures of Cos7 cells transfected with pTMSV5A showed that the transfection efficiency was ca. 35% in all the coverslip cultures examined. Thus, it was assumed that the results described above reflected the levels of mRNA in a similar number of transfected cells.

5.4 Discussion

In a transient transfection system, the expression of HDAg-24 in Cos7 cells was associated with a significant decrease in the rate of RNA synthesis and resulted in the same degenerative cytological changes observed in δMT5A, δMTG25 and Hδ5 cell lines after zinc induction (see Chapter 3.5 and 4.5). Expression of HDAg-p27 after

Fig 5.9 Northern blot analysis to detect antigenomic HDV RNA. *Lane 1* HDV RNA marker (a gift from M. Beard); HDV RNA extracted from Cos7 cells transfected with pAS5 (*Lanes 2-6*) and pTMSV5A (*Lanes 7-11*) on days 1-5 post-transfection.

1 2 3 4 5 6 7 8 9 10 11



transient transfection of the HDAg-p27 gene into Cos7 cells had a minimal effect on RNA synthesis and did not induce any cytological degeneration that is consistent with the cytological analysis of zinc-treated Hδ27 cells (Chapter 4.5). These experiments were technically difficult due to the variable nature of the transfection efficiency, and consequently, the effect on the total culture may not reflect the effect on transfected cells. Despite this, the results of the transient transfection study presented here were consistent with those demonstrated previously in stably-transfected cell lines. Furthermore, the cytological features associated with transient HDAg-p24 expression resembled those seen in acute HDV-hepatitis.

In this system, levels of mRNA transcribed from a mutated HDAg-p24 gene that were similar to levels transcribed from a wild-type HDAg-p24 gene, had little effect on cellular RNA synthesis in Cos7 cells. Thus, since HDAg mRNA synthesis is not cytotoxic, this implies that HDAg-p24-associated cytotoxicity is the direct result of HDAg-p24 per se. Coexpression of HDAg-p27 prevented the reduction in nucleic acid synthesis that was associated with HDAg-p24 expression alone. Even in the presence of low levels of HDAg-p27 expression, the cytotoxic effect of HDAg-p24 was inhibited. Thus, the results suggest that HDAg-p27 can modulate HDAg-p24-related cytotoxicity although the mechanism is unclear. This is discussed further in Chapter 8.

Since HDAg-p27 is non-cytotoxic, and since expression of HDAg-27 modulates cytotoxicity associated with HDAg-p24 expression, it is proposed that the main function of HDAg-p27 in HDV infection is to specifically overcome HDAg-p24-induced cytotoxicity and enhance cell survival and promote HDV persistence.

CHAPTER 6

A ROLE FOR THE HOST IMMUNE RESPONSE IN CHRONIC HDV INFECTION

6.1 Introduction

The previous chapters presented data consistent with the hypothesis that HDAg-p27 may promote HDV persistence and modulate cytotoxicity associated with expression of HDAg-p24. However, any model for HDV-related pathogenesis must account for the hepatitis which is often present in patients with persistent HDV infection. It is possible that continued expression of HDAg in chronic HDV infection may induce an immune response which is the cause of this continuing hepatitis. The histological presentation during persistent HDV infection supports this proposal. In one study of chronic HDV infection, Kojima et al (1986) reported that HDAg-positive hepatocytes showed a similar distribution to infiltrating cells and lymphocytic periempolesis of HDAg-positive cells was a common feature. In addition, Negro et al (1988a) showed that in patients who recovered from HDV-related hepatitis, the liver biopsy preceding remission showed the highest degree of inflammation and number of HDAg-positive hepatocytes, while low levels of HDAg-positive hepatocytes and inflammation were associated with virus persistence (Negro et al, 1988a). On the basis of these observations, Negro et al (1988a) proposed that the host immune response to HDV infection may depend in part on the recognition of HDAg on infected hepatocytes so that the number of HDAg-positive cells may determine the efficiency of the inflammatory reaction which eliminates HDV.

The principal mechanism of specific immunity against established viral infections is CTL. The best defined virus-specific CTL are CD8+ cells which recognise processed fragments of endogenously synthesised viral antigens presented on the cell membrane in association with HLA Class I molecules. As a result, target cell injury is a direct consequence of the host immune response to the virus infection.

Consequently, the aim of the experiments in this chapter was to investigate the hypothesis that a CTL response may be responsible for the continuing hepatitis in chronic HDV infection. In a previous study, the relationship of cytotoxic CD8+ T-lymphocytes to CD4+ T-lymphocytes, a high proportion of which can be expected to be helper/inducer cells, was examined in liver samples from patients with HDV-related CAH (Chu and Liaw, 1989). This study demonstrated that CD8+ T-lymphocytes were predominant in areas of piecemeal necrosis and intralobular necrosis, whereas CD4+ lymphocytes were predominant in portal tracts. Furthermore, the ratio of CD8+:CD4+ cells in areas of necrosis and in portal tracts, was indistinguishable from that demonstrated in liver samples from patients with HBV-related CAH for which there is clear evidence of a role for a CD8+ CTL response (Chu and Liaw, 1989; see section 6.3). However, HLA Class I expression on the hepatocyte membrane in chronic HDV infection has not been examined previously. Thus, it was necessary to address the following issues:

(1) to characterise the composition of the immune infiltrate in acute and chronic HDV infection;

(2) to examine the relationship between HDAg-positive cells and CD8+ lymphocytes; and

(3) to confirm that HDAg-positive hepatocytes express HLA Class I antigen.

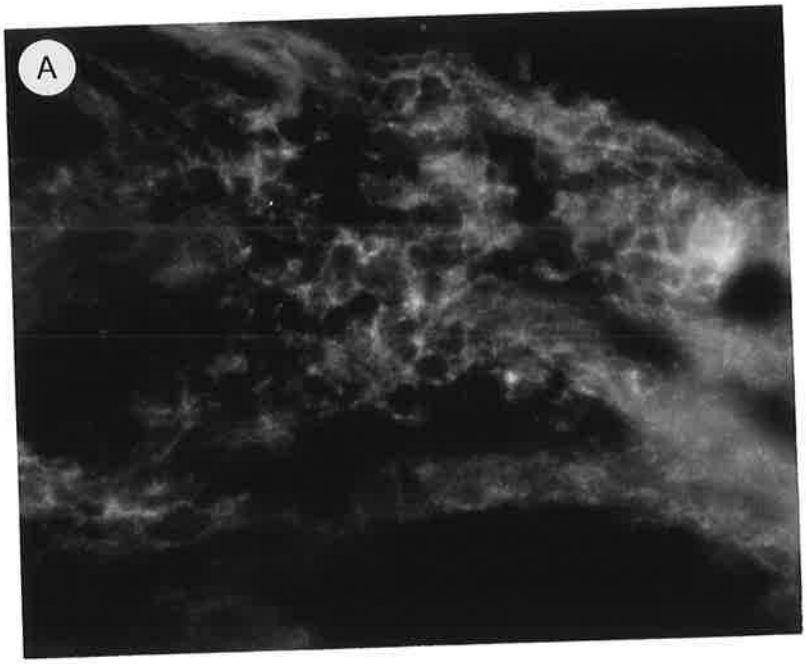
6.2 Immunofluorescence

6.2.A Characterisation of the Lymphocytic Infiltrate in HDV-Infected Liver Samples

The nature of the immune infiltrate present in HDV-infected livers was examined by indirect immunofluorescence for the expression of CD4 and CD8 T-lymphocyte markers, performed as described in Chapter 2.4.A. A sample of chimpanzee liver taken during acute HDV hepatitis and seven samples of human liver, one taken at autopsy and the remainder by biopsy, from chronic HDV carriers were examined. In the chimpanzee sample, large numbers of CD4+ lymphocytes were detected within the hepatic lobules (Figure 6.1A; Table 6.1), but very few CD8+ cells were detected. In contrast, large numbers of CD8+ lymphocytes were detected in the hepatic lobules in all human liver biopsy samples (Figure 6.1C), except those from the patient with end-stage cirrhosis. Biopsy specimen #4 and the autopsy sample which showed evidence of active cirrhosis, contained equal numbers of CD4+ and CD8+ cells in the hepatic lobules (Table 6.1). In the other five human biopsy samples, there was no evidence of CD4+ lymphocytic infiltration into the hepatic lobule, although large numbers of CD4+ cells were detected in the portal tracts.

The relationship between HDAg-positive hepatocytes and CD8+ lymphocytes was then examined by double staining experiments (Table 6.1). Clear evidence of CD8+ lymphocyte/HDAg-positive hepatocyte periempolesis was found in 4/7 persistently-infected human liver samples (Figure 6.1B,C). The histological diagnosis of these samples (# 2,3,4,7) was either CAH with moderate intralobular activity or active cirrhosis. Of the three samples with no evidence of CD8+ lymphocytes adjacent to HDAg-positive hepatocytes, one was a biopsy taken from

Fig 6.1 The detection of CD4+ and CD8+ T-lymphocytes, and HDAg-positive hepatocytes by indirect immunofluoresence. (A) CD4+ lymphocytes detected in a liver biopsy from a chimpanzee with acute HDV hepatitis; (B) HDAg-positive hepatocytes detected in a liver biopsy sample from a patient with chronic HDV hepatitis (biopsy #3), and (C) CD8+ T-lymphocytes detected in the same area of biopsy #3.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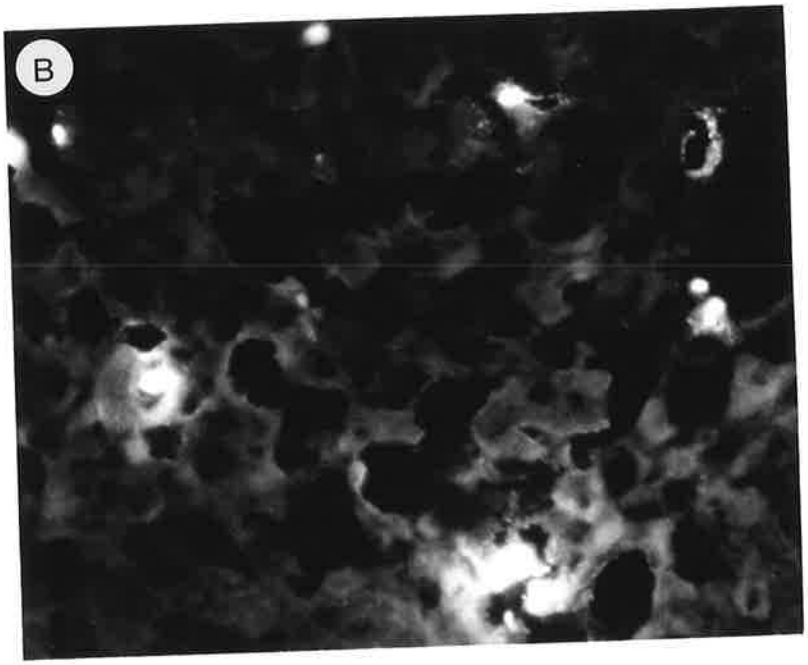
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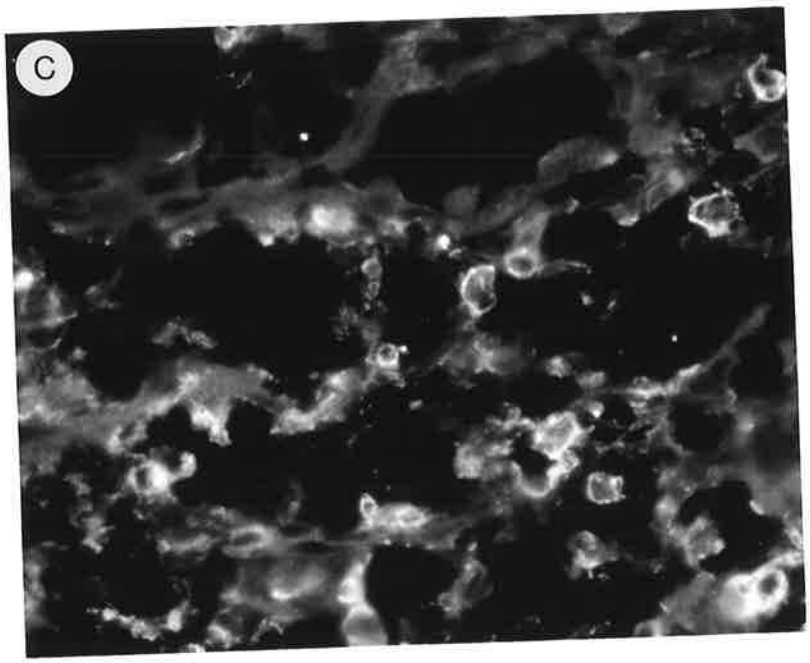

C

TABLE 6.1

Sample	Intrahepatic HDAg*	$CD4^+$ lymphocytes** (hepatic lobule)	$CD8^+$ lymphocytes*** (hepatic lobule)	$CD8^+$ lymphocytes adjacent to HDAg-positive hepatocytes	HDAg-positive/HLA Class I positive-hepatocytes	Histology
Chimpanzee	+++	+++	+	-	ND	Acute
Biopsy # 1	+	-	+++	-	+	CAH (mild)
# 2	+	-	+++	+	-	Active cirrhosis
# 3	+	-	+++	+	+	CAH (moderate)
# 4	+	+++	+++	+	+	Active cirrhosis
# 5	+++	-	-	-	-	End-stage cirrhosis
# 6	+	ND	+++	-	-	CAH (mild)
Autopsy # 7	+++	+++	+++	+	+	CAH and cirrhosis

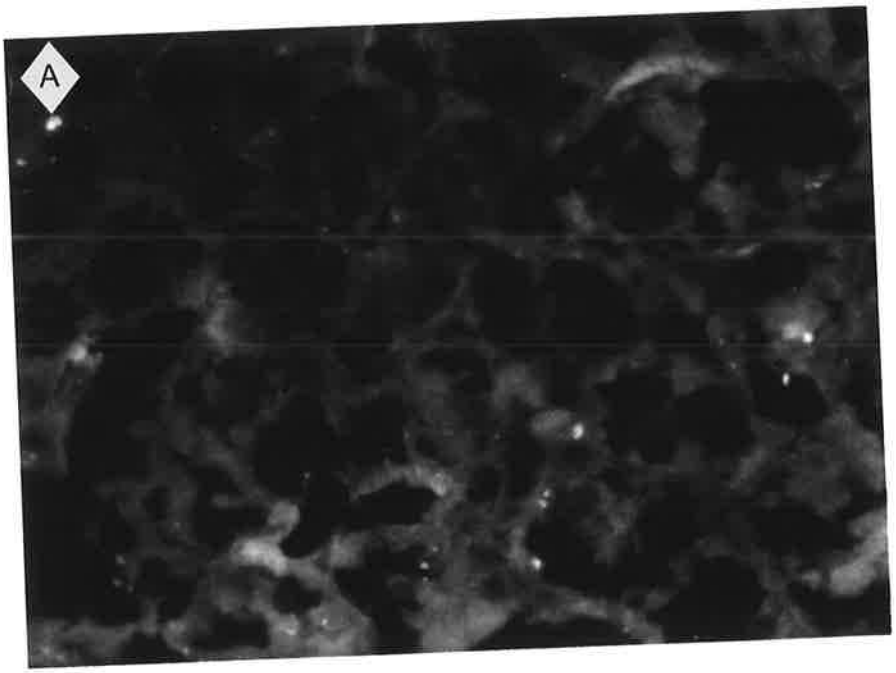
* score indicates the number of HDAg-positive hepatocytes
** score indicates the number of CD4+ lymphocytes
*** score indicates the number of CD8+ lymphocytes
ND not determined

a patient with end-stage cirrhosis and two came from patients with CAH with mild lobular activity. Thus, lymphocytic periempolesis appeared to correlate directly with increased histological activity and inversely, with reduced histological activity as in the patient with end-stage cirrhosis, a stage of disease that may not be expected to contain a lymphocytic infiltrate. There was no evidence of lymphocytic periempolesis in the acute-phase chimpanzee liver biopsy sample.

6.2.B Detection of HLA Class I Antigen in HDV-Infected Liver Samples

Most commonly, viral peptides are presented on the cell surface in association with HLA Class I molecules which are recognised by HLA Class I-restricted cytotoxic CD8+ lymphocytes. Normally, human hepatocytes express little or no HLA Class I antigens (Franco et al, 1988). Thus, a CD8+ CTL response to HDV infection will depend on the induction of HLA Class I antigen on HDAg-positive hepatocytes. To examine if HLA Class I antigen was expressed in the samples described above, frozen sections were used as the substrate in an indirect immunofluorescence or double antigen staining assay (Section 2.4.A). HLA Class I-positive/HDAg-positive hepatocytes were detected in 3/4 liver samples (# 3,4,7) in which evidence of CD8+ lymphocyte periempolesis was demonstrated (Table 6.1; Figure 6.2A-C); the exception was liver biopsy #2 (Table 6.1). In biopsy sample #1 from a patient with mild CAH, 50% of hepatocytes displayed HLA Class I antigen (1-2% of which were HDAg-positive), despite a failure to detect HDAg-positive hepatocytes adjacent to CD8+ lymphocytes. In contrast, HLA Class I expression was not detected in two chronic HDV-infected human liver samples which showed no evidence of periempolesis, viz. patients #5 and #6 with end-stage cirrhosis and mild CAH respectively. By way of comparison, HLA Class I expression was detected in a

Fig 6.2 The detection of HLA Class I antigen and HDAg-positive hepatocytes by indirect immunofluorescence. (A) negative control tissue section (biopsy #3); (B) HLA Class I antigen expression in a liver biopsy sample from a patient with chronic HDV hepatitis (biopsy #3); and (C) HDAg-positive hepatocytes detected in the same area of biopsy #3.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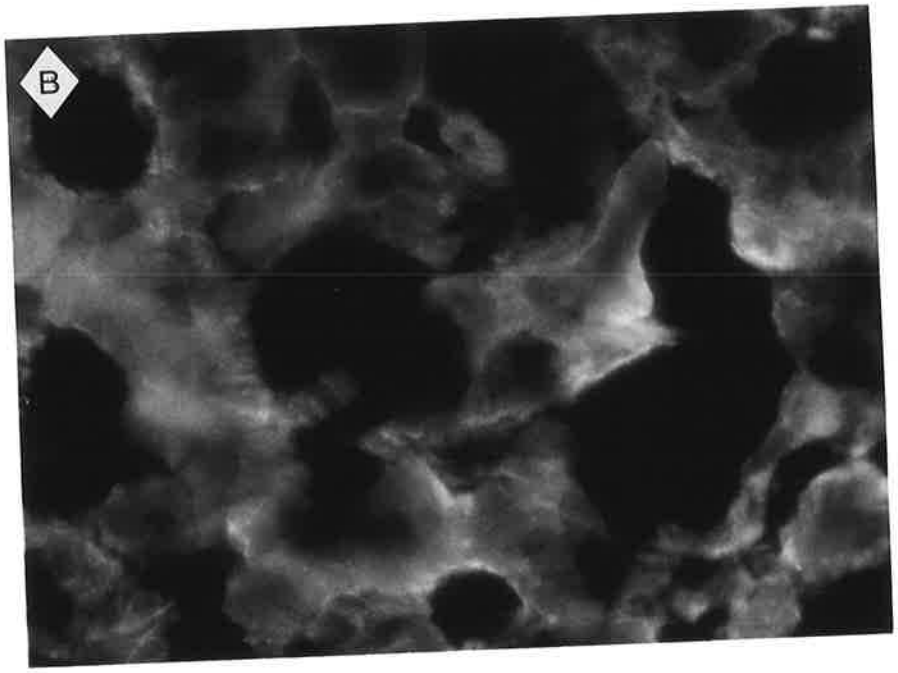
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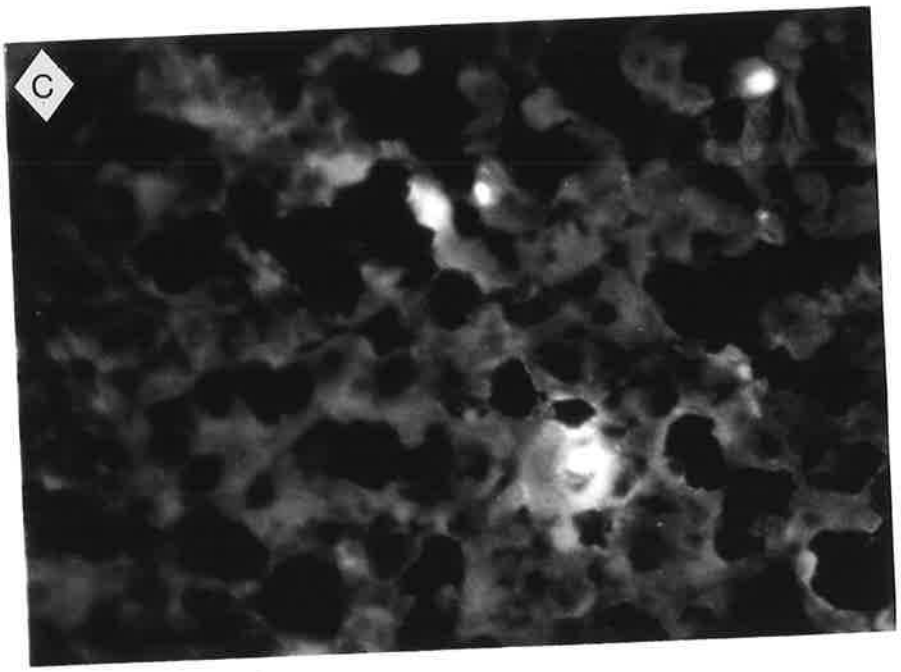
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liver biopsy sample from a patient with CAH who was a HBsAg/HBcAg-positive/HDAg-negative chronic carrier, whereas HLA Class I expression was not detected in a HDAg/HBsAg-negative liver sample. Consideration was given to the examination of HLA Class I expression in a liver sample from a patient that was only HBsAg-positive, but it was considered that a negative result, although expected, may simply reflect undetectable levels of HLA Class I expression rather than a true negative. In fact, even HBcAg-positive/HDAg-negative samples are not always positive for HLA Class I expression (Chu et al, 1987; section 6.3 below).

6.3 Discussion

Due to the unavailability of acute-phase human liver biopsy samples because liver biopsy is rarely performed during this stage of disease, this study was restricted to a single acute-phase liver biopsy sample taken from a chimpanzee with HDV-hepatitis and one autopsy- and six biopsy- samples from patients with chronic HDV hepatitis. These samples were quite unselected and thus may be taken to be representative. The reagents used in this study were designed to detect human CD4 and CD8 T-lymphocyte markers but also detected CD4+ and CD8+ T-lymphocytes in the chimpanzee sample. Although few CD8+ cells were detected in the chimpanzee sample, positive cells stained as intensely as CD8+ cells in the human liver biopsy samples.

The results of this study provide evidence that the ratio of CD4:CD8 lymphocytes in the liver alters dramatically between acute and persistent HDV infection. In an acute-phase biopsy sample, the bulk of the lymphocytes stained positive for CD4

suggesting that these cells were likely to be helper/inducer cells. Furthermore, these cells were localised to the hepatic lobules. In contrast, in persistently-infected human liver samples, CD4+ cells were restricted mainly to the portal tracts and the bulk of lymphocytes were shown to be CD8+, although in two cases, equal numbers of CD4+ and CD8+ lymphocytes were detected. CD8+ lymphocytes were found consistently in the hepatic lobules of chronically-infected samples. This is consistent with the results of a previous study by Chu and Liaw (1989). Although CTL are not restricted to CD8+ cells, a high proportion of these infiltrating lymphocytes can be expected to be CTL. Clear evidence for CD8+ lymphocyte periempolesis was demonstrated in 4/6 patients with CAH or active cirrhosis. However, the lack of a detectable association between HDAg-positive hepatocytes and CD8+ cells by immunofluorescence does not exclude a CTL-mediated response, as degenerate or necrotic liver cells may not stain positive for HDAg. Nevertheless, the results of these studies provide further circumstantial evidence for a role of CD8+ T-lymphocytes in the pathogenesis of chronic HDV infection.

A CD8+ CTL response to HDV infection will depend on the induction of HLA Class I antigen on HDAg-positive hepatocytes since normal hepatocytes express little or no HLA Class I antigen (Franco et al, 1988). A literature search suggested that this report represents the first study in which hepatic expression of HLA Class I antigens in chronic HDV infection has been determined. The inability to detect HLA Class I antigen in two patients with active liver disease is inconsistent with hepatocyte injury as a result of a CTL response, but a low level of HLA Class I expression in these samples cannot be excluded. Factors which may influence the level of HLA Class I antigen expression in HDV-infected hepatocytes are discussed in Chapter 8.4. A previous study which examined HLA Class I

expression during chronic HBV infection showed that HLA Class I antigens were undetected by indirect immunofluorescence in ca. 10% of patients, despite clear evidence for a role for a CD8+ CTL response in the clearance of HBV-infected hepatocytes (see below; Chu et al, 1987).

The data presented in this chapter is consistent with previous histological studies of chronic HDV-related hepatitis (Kojima et al, 1986; Negro et al, 1988a). In addition, recent studies support a role for a cell-mediated immune response in HDV superinfection of chronic WHV-infected woodchucks (Karayiannis et al, 1993). Woodchucks inoculated with purified baculovirus-derived HDAg-p24 or infected with recombinant vaccinia virus which expressed HDAg-p24, failed to develop a humoral response, although on challenge with HDV the immunised animals developed a significantly lower level of viraemia compared to the control (unimmunised) animals. The failure to detect an anti-HD response in animals infected with the recombinant vaccinia virus may be the result of an acute rather than a persistent infection, analogous to the transient, low titre anti-HD response associated with acute self-limited HDV infection (Chapter 1.2.C). Nevertheless, these studies are consistent with a CTL response. Furthermore, computer analysis of the aa sequence of HDAg located a T-cell epitope of HDAg-p24 and HDAg-p27 spanning aa151 to aa154 (Gly-Val-Ile-Pro), and an additional T-cell epitope at the C-terminus of HDAg-p27 spanning aa197 to aa201 (Asp-Ile-Leu-Phe; Protean II-Full Sequence Editor; Proteus Molecular Design Limited).

Conclusive evidence that CTL are involved in the clearance of HDV-infected cells during chronic infection should be sought in a similar manner to that described previously for HBV. Peripheral blood mononuclear cells from patients with chronic

HBV-hepatitis that were incubated with autologous hepatocytes isolated from liver biopsy specimens were shown to be capable of T-cell mediated, HLA-restricted hepatocyte killing (Eddleston et al, 1982). T-cell mediated hepatocyte necrosis was inhibited by anti-HBc, anti-HBe and antibody to HLA Class I proteins, but not HLA class II proteins, suggesting that CD8+ T-lymphocyte cytotoxicity is responsible for hepatocyte injury in chronic HBV infection (Eddleston et al, 1982). In persistent HDV infection, it was proposed previously that a T-cell mediated response may be directed against HBV- but not HDV-encoded antigens (Chu and Liaw, 1989). However, this is unlikely since the major T-cell target antigen in HBV infection was shown to be HBcAg/HBeAg, and the majority of HDV-infected patients are HBeAg-negative/anti-HBe positive (Chu et al, 1990). It is acknowledged that the examination of the phenotypic expression of CD8 and HLA cell markers is not sufficient to demonstrate conclusively the function of HDAg as a target for a HLA Class I-restricted CTL response. Ongoing collaborative projects are designed to clarify this point.

CHAPTER 7

HDAg-ASSOCIATED INTERFERENCE

7.1 Introduction

Experimental and clinical studies have demonstrated that HDV superinfection of a chronic HBV carrier results commonly in a transient interference in HBV replication that coincides with the peak of HDV replication (Rizzetto et al, 1986). Transient suppression of HBV replication has also been well documented following hepatitis A virus (HAV) and non-A/non-B hepatitis (NANB) virus superinfection of HBV chronic carriers, although the degree of inhibition is considerably less (ca. 10-fold reduction in HBV replication) (Rizzetto et al, 1986). In these two latter examples, the mechanism of interference is not thought to be virus-specific and may be mediated by interferon induced by the superinfecting virus (Rizzetto et al, 1986). In contrast, HDV superinfection of HBV chronic carriers results in seroconversion from HBeAg-positive to HBeAg-negative/anti-HBe positive in >90% of carriers (Rizzetto et al, 1986; Chapter 1.2.B), and in rare cases, HBsAg-positivity may be terminated with seroconversion to anti-HBs (Rizzetto et al, 1986). Although interferon induction may contribute to this effect, the marked inhibition of HBV replication suggests that other factors are involved. A number of potential mechanisms exist to explain interference of HBV replication by HDV, which include (1) competition for host cell RNA polymerase II during HBV- and HDV- replication (Gowans et al, 1987); (2) the proposal by Khudyakov and Makhov (1990), that a truncated form of HDAg may be produced as the result of a frame-shift mutation in the HDAg gene, and that this mutant HDAg may compete with the 5' terminal protein on the long

strand of the HBV DNA genome for HBV RNA, thereby inhibiting HBV replication; and (3) HDAg-p24-related cytotoxicity in the acute phase of HDV infection results in the destruction of HBV-producing cells. In this chapter, the third hypothesis is examined.

As in vitro culture of HBV requires primary human hepatocyte cultures which were unavailable, the effect of HDAg expression on HBV replication could not be measured directly. An alternative approach was to measure the inhibitory effect of HDAg-p24 and HDAg-p27 expression on the production of viruses which are readily cultured in vitro such as poliovirus and adenovirus. Consequently, the potential for HDAg expression to cause interference was examined in this way.

7.2 Experimental Design

To determine the direct effect of increasing levels of HDAg-p24 and HDAg-p27 on virus production, the δMT5A or Hδ5, and Hδ27 cell lines were incubated with various concentrations of zinc sulphate prior to infection with poliovirus type 3 or adenovirus type 5. These were obtained from the Diagnostic Services Laboratory in the Division of Medical Virology, IMVS.

The cells were cultured overnight in 6-well culture trays in normal medium, followed by overnight incubation in medium supplemented with 0-100μM zinc sulphate. The parent HeLa cell line was included as a control for the direct effect of zinc sulphate on virus production. Duplicate wells were inoculated with a dilution of the virus stock that was shown previously to produce a confluent CPE three days post-infection. The cells were incubated for a further 2 hr, and then fresh medium

supplemented with zinc sulphate was added to the cells as before. The medium was changed again after overnight incubation. The infected cultures were incubated for an additional 64 hr (in the presence of zinc sulphate), then the cell supernatants and monolayers were harvested, and any intracellular virus present in the cell suspension was released by 3 cycles of freezing and thawing. The debris was removed by filter sterilisation.

The level of virus produced in the HDAg-positive cells was assayed by titration of the clarified virus preparation in the parent HeLa cells. 10-fold serial dilutions were prepared (10^{-2}-10^{-9}), and used to inoculate HeLa cells. Each sample was titrated in duplicate. 2 hr post-inoculation, fresh medium was added and then 3 days post-infection the CPE was determined on the basis of the percentage of the monolayer remaining.

7.3 The Effect of HDAg-p24 and HDAg-p27 Expression on the Replication of Polio- and Adeno- Virus

Secreted and cell-associated virus produced in the HDAg-positive cell lines were titrated, but preliminary experiments showed similar levels of virus in both. Consequently, only the results of virus contained in the culture supernatant are presented. The effect of HDAg-p24 and HDAg-p27 expression on poliovirus type 3 was examined in δMT5A and Hδ27 cells respectively, and the results of these experiments are presented in Table 7.1. The production of poliovirus in the parent HeLa cells and in HeLa cells pretreated with 100μM zinc sulphate was similar. In contrast, poliovirus production was reduced even by low concentrations of HDAg-p24, whereas HDAg-p27 expression had no effect. At the highest level of HDAg-

Table 7.1

HDAg-related Interference of Poliovirus type 3 Replication

Cell Line	Concentration of Zinc (μM)	$TCID_{50}$ *
HeLa	0	$10^{4.5}$
	100	$10^{4.5}$
δMT5A	0	$10^{4.5}$
	10	$10^{3.5}$
	20	$10^{3.5}$
	50	$10^{3.5}$
	75	$10^{2.5}$
	100	$10^{1.5}$
Hδ27	0	$10^{4.5}$
	10	$10^{4.5}$
	20	$10^{5.5}$
	50	$10^{4.5}$
	75	$10^{4.5}$
	100	$10^{5.5}$

* The $TCID_{50}$ was calculated according to the Karber formula (Karber, 1931):

$\text{Log } TCID_{50} = L - d(S - 0.5)$

L = negative log of the lowest virus dilution
d = difference between log virus dilution steps
S = sum of proportion of "positive"; in cell culture monolayers, a unit response in which more than two thirds is destroyed by CPE was scored as positive.

p24 expression, poliovirus production was reduced by at least 10^3-fold.

The effect of HDAg-p24 and HDAg-p27 on adenovirus type 5 production was examined in the Hδ5 and Hδ27 cells respectively, and the results are shown in Table 7.2. The results showed that 100μM zinc sulphate reduced the level of adenovirus production in the parent HeLa cells by 10-fold. Similarly, the 10-fold reduction in adenovirus production observed in cells that expressed HDAg-p27 is likely to be an effect of the zinc sulphate, and thus HDAg-p27 expression had little or no effect on virus production. In contrast, at the highest level of HDAg-p24 expression, the level of adenovirus production was reduced by at least 10^3-fold when the effect of the zinc sulphate on virus production in the parent HeLa cells was considered.

Thus, the results showed that both adeno- and polio- virus production were inhibited by levels of HDAg-p24 induced by 100μM zinc sulphate. In addition, poliovirus production was inhibited at least 10-fold by levels of HDAg-p24 expression induced with 10μM - 75μM zinc sulphate (Table 7.1). In contrast, low levels of HDAg-p24 did not inhibit adenovirus production; the 10-fold reduction in adenovirus production in cells induced with 75μM zinc is likely to be an effect of the zinc sulphate (see above).

7.4 Discussion

The experimental system of choice in which to study HBV interference by HDV, involves the use of stable cell lines cotransfected with HBV- and HDV- cDNA. Nevertheless, this study provides preliminary data on which future work with these

Table 7.2

HDAg-related Interference of Adenovirus type 5 Replication

Cell Line	Concentration of Zinc (μM)	$TCID_{50}$ *
HeLa	0	$10^{6.5}$
	100	$10^{5.5}$
Hδ5	0	$10^{5.5}$
	25	$10^{5.5}$
	50	$10^{5.5}$
	75	$10^{4.5}$
	100	$10^{1.5}$
Hδ27	0	$10^{6.5}$
	25	$10^{6.5}$
	50	$10^{6.5}$
	75	$10^{5.5}$
	100	$10^{5.5}$

* The $TCID_{50}$ was calculated according to the Karber formula (Karber, 1931) in a similar manner as described in Table 7.1.

cell lines can be based. The results of these studies showed that adeno- and polio- virus production was reduced significantly by levels of HDAg-p24 shown previously to be cytotoxic (Chapters 3 and 4; see below). Furthermore, poliovirus production, but not adenovirus production, was inhibited in the presence of non-cytotoxic levels of HDAg-p24. The difference in response to HDAg-p24 expression may be attributed to the use of the δMT5A and Hδ5 cell lines in which the polio- and adeno- viruses were propagated respectively. Since overnight induction of Hδ5 cells with 100μM zinc sulphate was shown previously to result in cell death (Chapter 4.5), it was not surprising that inoculation of this culture with adenovirus resulted in a marked inhibition in virus production. Thus, the results suggest that adenovirus replication and synthesis are resistant to non-cytotoxic levels of HDAg-p24. In contrast, as reported in Chapter 3.4-3.5, overnight induction of δMT5A cells with 100μM zinc resulted in a marked inhibition of cellular RNA synthesis but did not result in degenerative cytological changes which were evident only after 4-5 days in culture with zinc sulphate. Therefore, the progressive reduction in the virus $TCID_{50}$ following infection with poliovirus was not simply the result of a lack of cells receptive to virus infection, but suggests that increasing, but nevertheless, non-cytotoxic levels of HDAg-p24 had a direct inhibitory effect on virus production.

In the experiments described in this Chapter, HeLa cells were inoculated with polio- or adeno- virus ca. 16 hr after zinc induction. In retrospect, to reflect HDV superinfection more closely, an alternative approach would have been to pre-inoculate HeLa cells with virus prior to induction of HDAg expression. Despite this technical deficiency, it is significant that HDAg-p27, a non-cytotoxic protein had little or no effect on polio- or adeno- virus synthesis, whereas HDAg-p24 a

cytotoxic protein, interfered with virus production by (1) destroying cells that are normally permissive to infection, and by (2) an inhibitory effect on cell biosynthesis in response to non-cytotoxic levels of HDAg-p24, that caused a reduction of poliovirus but not adenovirus production.

The work presented in this chapter suggests that two interference mechanisms suppress HBV replication as a result of HDV superinfection;

(1) destruction of HBV-producing cells; and

(2) impairment of hepatocyte biosynthesis.

In addition, two mechanisms which were suggested previously i.e.,

(3) the host immune response; and

(4) specific mechanisms of interference, brings the potential number of interference mechanisms to four, and these are discussed below.

(1) destruction of HBV-producing cells; high levels of HDAg-p24 expression during acute HDV infection may destroy HBV-producing cells. This proposal is supported by the above results and by a previous finding that in HDV superinfection of HBV carrier chimpanzees, increased levels of ALT were preceded by a significant increase in the level of intrahepatic HDAg (Negro et al, 1988b); (2) impairment of hepatocyte biosynthesis; non-cytotoxic levels of HDAg-p24 may cause physiological changes in hepatocytes that may reduce the capacity of these cells to support HBV replication; (3) a host response to HDV infection; in a previous study in woodchucks, the levels of WHV RNA and DNA were analysed in the liver and extrahepatic tissues taken from WHV-carrier woodchucks during acute- and chronic- HDV infection (Negro et al, 1989). The levels of WHV DNA replicative intermediates in the spleen were suppressed following superinfection with HDV,

and since HDV RNA or HDAg was not detected in any of the extraheptic sites examined, it was suggested that HDV may encode or induce a soluble factor or cytokine as a result of HDV hepatic infection that acts systemically to interfere with WHV DNA replication (Negro et al, 1989). This is similar to the mechanism proposed previously for suppression of HBV replication following HAV and NANB virus superinfection of HBV carriers (Rizzetto et al, 1986); and (4) specific mechanisms; these may operate through a direct competition for enzymes or factors necessary for HBV- and HDV- replication (see section 7.1 and Chapter 8.5).

The observation that HDAg-p27 had no inhibitory effect on the synthesis of poliovirus or adenovirus is consistent with the transient interference of HBV replication. It is possible that the increase in the levels of HDAg-p27 expression during HDV infection and the subsequent modulation of cytotoxicity due to HDAg-p24 would permit an increase in HBV replication to levels similar to those prior to HDV superinfection.

CHAPTER 8

CONCLUDING REMARKS

8.1 Introduction

The experiments described in this thesis examined the pathogenic role of HDV gene expression, in an attempt to determine the molecular basis of HDV-induced liver injury in natural infection. On the basis of the observations from this study, I propose a model for HDV pathogenesis that is discussed in detail below.

8.2 Cytotoxic Properties of HDAg

Although the pathogenic nature of HDV is well established, the histological features associated with acute- and chronic- HDV infection are remarkably different (Chapter 1.2.K), and this has led to considerable conjecture regarding the mechanism of HDV-induced liver damage. The characteristic histopathology associated with acute HDV infection suggests a direct cytopathic effect, whereas the histologic presentation in chronic HDV infection is more suggestive of a host immune response to ongoing HDV (or HBV) infection. It is possible that both hypotheses are correct, and the observations presented in this thesis suggest that the degree of virus-induced cytotoxicity is related to the level and ratio of HDAg-p24 and HDAg-p27 expression in infected hepatocytes. These factors differ with the stage of disease, and the significance of HDAg-p24 and HDAg-p27 expression in HDV pathogenesis is discussed more fully below.

An in vitro cell culture model was developed to examine the role of HDAg in HDV-related cytotoxicity that is likely to reflect HDV-induced disease in natural infection. HDAg-p24 was shown to be cytotoxic in three different stable eukaryotic cell lines derived from HeLa and HepG2 cells, in which the expression of HDAg-p24 was controlled by the human MTIIA promoter. In these cell lines, HDAg-p24 expression was associated with a marked reduction in cellular nucleic acid synthesis and degenerative cytological changes which resembled histological features associated with acute HDV-hepatitis in vivo (Table 8.1). In contrast, HDAg-p27 expression had a minimal effect on cellular nucleic acid synthesis and failed to induce any of the degenerative cytological changes associated with HDAg-p24 expression (Table 8.1). These properties can now be added to the other known characteristics of HDAg-p27 (see Table 1.1). It is possible that the trans-inhibitory function and the non-cytotoxic nature of HDAg-p27 are linked and are part of the overall strategy of HDV to establish a persistent infection. The role of HDAg-p27 in HDV pathogenesis is discussed further in section 8.3.

These studies provide formal proof of previous observations which were consistent with HDV-related cytotoxicity. Macnaughton et al (1990b) showed a progressive loss of cells from cell lines transfected with the HDAg-p24 gene that expressed high levels of HDAg-p24. In addition, in a cell line which supported HDV RNA replication, serial passage resulted in increased levels of HDAg-p27 expression that was coincident with a loss of cytotoxicity (Macnaughton et al, 1990a).

Nevertheless, a major finding of this thesis i.e., HDAg-p24-related cytotoxicity, is not accepted universally and several workers dispute a role for HDAg expression in HDV pathogenesis; Sureau et al (1991) found that HDV infection of primary

Table 8.1

Cytotoxic Properties of HDAg-p24 and HDAg-p27

	HDAg-p24	HDAg-p27
Inhibits Cellular RNA Synthesis	Yes	No
Inhibits Cellular DNA Synthesis	Yes	No
Eosinophilic Cytoplasm	Yes	No
Pyknotic Nuclei	Yes	No
Apoptotic Bodies	Yes	No
Karyolysis	Yes	No

chimpanzee hepatocytes was maintained for at least 42 days without any obvious cytotoxicity. In addition, Negro et al (1993) reported that only a minor proportion (<5%) of HDV-infected hepatocytes displayed features associated with direct cytotoxicity i.e. condensed eosinophilic cytoplasm with a pyknotic nucleus. Furthermore, clinical studies of HDV carriers who underwent orthotopic liver transplantation for HDV-related cirrhosis showed that HDV reinfection of the graft may occur in the absence of HBV infection and without concomitant liver disease (Chapter 1.2.L). This has been interpreted as evidence for HDV being non-cytopathic (Ottobrelli et al, 1991), but may simply reflect a limited number of HDV-infected hepatocytes in the graft. In these patients, the level of serum ALT remained normal and there were no obvious histological changes in the liver until reinfection of the graft with HBV some time later, whereupon the number of HDV-infected hepatocytes increased markedly and coincided with the onset of clinical disease (Ottobrelli et al, 1991).

However, recent studies of HDV infection in the SCID mouse support a role for HDAg-related cytotoxicity; Netter et al (1993) showed that there was a progressive loss of HDAg-positive hepatocytes following the peak of HDV replication. Since this was not attributed to immune clearance by T- and B- lymphocytes, it was proposed that the loss of HDAg-positive cells was a direct result of HDAg-related cytotoxicity (Netter et al, 1993). HDV-infected mouse hepatocytes were shown to be HDAg-positive by immunostaining, although the ratio of HDAg-p24:p27 was not demonstrated in these cells. Thus, any potential influence exerted by HDAg-p27 could not be estimated, and as work described in this thesis suggests that expression of HDAg-p27 may modulate HDAg-p24-associated cytotoxicity it is possible that a lack of HDAg-p27 in the mouse hepatocytes may have contributed

to the cytotoxicity. Although unlikely, it is possible that mouse hepatocytes lack the capacity to edit HDV RNA, required for HDAg-p27 expression. The ratio of HDAg-p27:p24 in HDV-infected hepatocytes after HDV reinfection has not been examined in HDV transplant patients, and the SCID mouse may represent a model system in which to study the effect of HDAg expression in transplant patients following HDV reinfection.

A major limitation of the model system described in this study is that the effect of HDAg expression was examined in the absence of HDV RNA replication. Nevertheless, this study represents the first reported attempt to determine the direct effect of HDAg-p24 and HDAg-p27 expression using an in vitro cell culture system which is similar to those described previously to examine the cytotoxic properties of several other viral proteins (Chapter 3). Furthermore, the results of this study complement those of previous studies by Macnaughton et al (1990a,b), and closely reflect the histopathology associated with HDV infection (Rizzetto et al, 1983; Verme et al, 1986; see Chapter 1.2.K). In addition, this work has demonstrated clearly that HDAg-p24-related cytotoxicity is dose-related, and it was proposed that cells exhibit a threshold level above which HDAg-p24 is not well-tolerated. It is likely that this threshold will vary between different cell lines in vitro and naturally-infected hepatocytes of different individuals in vivo, and this may provide an explanation for the results of studies by Sureau et al (1991) and Negro et al (1993) described above. It is clear also that HDAg-p24-related cytotoxicity is strongly influenced by HDAg-p27 expression, and this is discussed further in section 8.3.

The mechanism of HDAg-related cytotoxicity may be multifactorial, however, two

possible mechanisms are more likely than others;

(1) HDAg-p24 may induce apoptosis; recent studies have demonstrated that cytotoxicity due to infection with adenovirus (White and Stillman, 1987), Epstein-Barr virus (EBV; Henderson et al, 1991) or Sindbis virus (Levine et al, 1993) is associated with apoptosis, and the adenovirus proteins which are associated with the induction of apoptosis have been identified, namely the adenovirus early proteins (E1A; see section 8.4; White et al, 1991). However, in each of these examples the exact mechanism by which apoptosis is induced is unknown, although, unlike simple cell necrosis, a specific set of cellular components are involved (Williams, 1991). Therefore, the potential exists for apoptosis to be blocked, and several studies support this hypothesis as a mechanism to sustain cell survival and promote viral persistence (see below).

(2) HDAg-p24 may reduce cell synthesis in a manner similar to that proposed for adenovirus E1A, which has been reported to repress cellular transcription as well as trans-activate adenovirus transcription (Berk, 1986), in addition to its postulated role in apoptosis (see above). In previous studies, adenovirus E1A was shown to reduce transcription of several cellular genes by repression of cellular enhancer elements (Berk, 1986). Although the exact mechanism of repression is unknown, the interaction of E1A with a cellular protein of ca. 300 kD (p300; Harlow et al, 1986) present in host RNA polymerase II transcription complexes, was shown to result in the release of p300 from these complexes (Harlow et al, 1986), and subsequent studies by Stein et al (1990), demonstrated that the interaction of E1A with p300 correlated closely with a reduction in enhancer-stimulated host transcription.

Recently, it was proposed that the E1A-mediated release of p300 from cellular RNA polymerase II complexes inhibits signal transfer from the enhancer element to the basal transcription machinery, thereby decreasing the level of transcription from the cellular promoter (Akusjarvi, 1993).

An interaction of HDAg-p24 with p300 could be beneficial to HDV replication, since it is possible that HDAg-p24-mediated release of p300 from cellular RNA polymerase II complexes may somehow trigger the release of other cellular transcription factors(s) (TF) e.g. TFIID, TFIIID, from these complexes, that may then be used by HDV to trans-activate RNA polymerase II-directed RNA transcription of the HDV genome. This is consistent with previous findings that HDAg-p24 trans-activates HDV RNA replication [Kuo et al, 1989; Chapter 1.2.J(iii)] but is not part of the HDV transcriptional complex per se (Macnaughton et al, 1991).

The putative ability of HDAg-p24 to bind to cellular factors e.g., p300, may be regulated by the ratio of HDAg-p24:HDAg-27 in HDV-infected hepatocytes (see below).

8.3 The Significance of HDAg-p27 Expression

Until now, the pathogenetic significance of HDAg-p27 expression during chronic HDV infection was uncertain. Work presented in this thesis indicated that coexpression of HDAg-p24 and HDAg-p27 failed to produce the cytotoxicity associated with expression of HDAg-p24 alone. The expression of HDAg-p27 was shown to modulate HDAg-p24-cytotoxicity even when the molar ratio of HDAg-

p24:HDAg-p27 was 10:1. Thus, the crucial event which will determine the outcome of infection is the rate of HDAg-p24- to HDAg-p27- gene conversion during HDV RNA replication. However, the factor(s) which influence this is unknown.

It is likely that this modulating effect occurs in vivo and represents another example of a classical method for a cytopathic virus to promote persistent infection (see below). The mechanism involved in the modulation of HDAg-p24-related cytotoxicity by HDAg-p27 is unknown, but four possibilities are proposed:

(1) Expression of HDAg-p27 can prevent hepatocyte apoptosis; in this regard, HDAg-p27 may have a similar effect as Bcl-2 or the adenovirus E1B-19 kD protein.

Transient high-level expression of adenovirus E1A proteins in HeLa cells was sufficient to induce an acute cytotoxic effect, analogous to cells undergoing apoptosis. However, coexpression of the E1B-19 kD protein with the E1A proteins was able to prevent cytotoxicity (White et al, 1991). The mechanism is unclear, but it was proposed that E1B-19 kD is able to specifically block apoptosis caused by the E1A proteins.

A second example of apoptosis suppression has been described recently that involves the Bcl-2 protooncogene; this study showed that overexpression of Bcl-2 in B lymphocytes blocked apoptosis, thereby extending the lifespan of these cells and promoting transformation (Hockenbery et al, 1990).

(2) HDAg-p27 may induce expression of Bcl-2; this may occur in a similar manner to that described for EBV latent membrane protein 1 (LMP 1; Henderson et al, 1991), or by another as-yet unidentified protein functionally analogous to Bcl-2. The mechanism by which LMP 1 induces expression of Bcl-2 is unknown.

The EBV LMP 1 has been shown to induce expression of Bcl-2 in B lymphocytes and block apoptosis (Henderson et al, 1991). This is thought to extend the lifespan of EBV-infected B lymphocytes and contribute to their malignancy. The Bcl-2 oncogene and the E1B-19 kD protein are believed to represent a group of proteins that promote host cell survival by preventing programmed cell death and allowing persistence of otherwise cytolytic virus infections.

(3) Due to a decrease in the ratio of HDAg-p24:HDAg-p27 during chronic infection, HDAg heterodimers fail to interact with cellular factor(s).

In recent studies, HDAg-p24:p27 oligomers were detected in extracts of HDV-infected livers (Wang and Lemon, 1993). It is possible that the HDAg-p27:HDAg-p24 interaction inhibits binding of HDAg-p24 to cellular factor(s) as postulated above in section 8.2.

(4) The viral load in the cell is reduced as a result of virus secretion.

Following a direct HDAg-p24:HDAg-p27 interaction with the putative HDV RNP structure (Ryu et al, 1993), the viral and HDAg-p24 load in the cell is

reduced by initiation of virus assembly and subsequent virus release [Lazinski and Taylor, 1993; see Chapter 1.2.J(iv)].

8.4 The Role of the Host Immune Response

Since HDAg-p27 is non-cytotoxic per se and coexpression of HDAg-p27 and HDAg-p24 prevents the direct cytotoxic effect due to HDAg-p24 expression, any model for HDV pathogenesis must account for the hepatitis which is often associated with chronic HDV infection. Thus, hepatocyte injury during chronic HDV infection may result from the induction of a host immune response to HDV-infected hepatocytes. In this study, evidence was presented for CD8+ lymphocyte periempolesis of HDAg-positive hepatocytes which expressed HLA class 1 antigens. These findings are consistent with the histological studies and with the proposal that liver damage in chronic HDV-related hepatitis may result from the action of virus-specific CTL on HLA-restricted hepatocytes which express HDAg-p24 and/or HDAg-p27. Hepatocyte injury may also result from natural killer (NK) cell-mediated lysis of HDV-infected cells. However, NK cells are unlikely to play a predominant role in immune-mediated lysis of HDV-infected hepatocytes in chronic HDV infection as the peak of NK activation and proliferation generally occurs early in infection prior to the development of a virus-specific T-cell response (Welsh and Vargas-Cortes, 1992). Cytolysis of HDV-infected cells may also be a result of antibody-dependent cell-mediated cytotoxicity (ADCC). Since the principal mediator of ADCC is NK cells, it is unlikely that ADCC plays a major role in immune-mediated clearance of infected cells during chronic HDV infection. However, a role for ADCC mediated by macrophages and/or neutrophils cannot be excluded (Abbas et al, 1991).

It is clear that HDV infection may result in a range of clinical conditions, and while the pathogenic nature of HDV is clear, it is also clear that 'healthy' HDV carriers exist with essentially normal liver histology (Hadziyannis et al, 1991). Variation in the host immune response between individuals to chronic HDV infection may be a major contributing factor in the outcome of chronic liver disease, since the ability of an individual to elicit an efficient CTL response against HDV-infected hepatocytes will vary. Possible differences in the host response to infection may be related to (1) the ability to process HDAg and present HDV-derived peptides via the endogenous pathway (Thomas et al, 1993); and (2) the level of stimulation of HLA Class I expression on hepatocytes, since only very low levels of HLA Class I proteins are expressed constitutively (Franco et al, 1988). Expression of HLA Class I antigens on hepatocytes is stimulated by interferon (IFN) -α and -γ and thus unstimulated hepatocytes may be unable to present adequate levels of HDAg to trigger a response by CTLs (Franco et al, 1988). Double-stranded (ds) RNA is a strong inducer of IFN (Lampson et al, 1967), and it could be expected that ds HDV RNA replicative intermediates would induce IFN synthesis and a subsequent increase in HLA Class I expression. However, antibody to ds RNA that detects viral RNA replicative intermediates in poliovirus-, flavivirus- and HCV- infected cells fails to stain HDV-infected cells (Macnaughton, personal communication) suggesting that ds RNA intermediates in HDV-infected cells may be present at levels which are unable to induce IFN synthesis.

It is clear that the pathogenesis of HDV infection varies worldwide, from mild liver disease in Greek patients (Hadziyannis et al, 1991) to serious liver disease in South American patients (Popper et al, 1983). A recent study compared the nucleic acid sequence of a semiconserved region of HDV cDNA (nt 911 to 1260),

derived from 14 isolates obtained worldwide (Casey et al, 1993). In this study, three South American isolates were shown to be closely related to each other (ca. 94% nt homology), but highly divergent from the other isolates examined (ca. 69% nt homology; Casey et al, 1993). The South American isolates were obtained from individuals in regions with a high prevalence of HDV infection which is associated with serious liver disease (Casey et al, 1993). It was proposed that these HDV isolates represent a South American genotype (HDV genotype III) which may be associated with a more virulent form of HDV-hepatitis (Casey et al, 1993). The majority of HDV isolates examined in the same study were grouped into HDV genotype I (Wang et al, 1986; Makino et al, 1987; Chao et al, 1990a; Imazeki et al, 1990; Saldanha et al, 1990; Chao et al, 1991a; Lee et al, 1992; Casey et al, 1993) and a single isolate from Japan grouped into HDV genotype II (Imazeki et al, 1991). Nucleic acid- and amino acid- sequence analysis of representatives from these three proposed HDV genotypes showed that the least conserved regions were the N-terminus of HDAg-p24 and -p27 and the C-terminus of HDAg-p27, although there was evidence of sequence variation throughout the entire genome (Casey et al, 1993). The exact change(s) in sequence that may increase the virulence of the South American isolates was not identified and this may prove difficult as a single nt change is responsible for the difference between the avirulent Sabin vaccine strain and wild-type poliovirus type 3 (White and Fenner, 1986).

The difference in outcome of HDV infection worldwide may also be influenced by HLA restriction related to specific HLA haplotypes, in particular, the specific HLA haplotype may vary between geographically-divergent populations.

8.5 HBV Interference

HDV superinfection of a chronic HBV carrier results commonly in a transient interference in HBV replication that coincides with the peak of HDV replication (Rizzetto et al, 1986). A number of potential mechanisms exist to explain this interference, and these can be divided into HDV- specific and non-specific mechanisms;

(1) The Specific Mechanisms;

(a) Competition for host cell RNA polymerase II during HBV- and HDV- replication (Gowans et al, 1987);

(b) Competition for host transcription factors required for HBV- and HDV- replication (see section 8.2);

(c) HDAg-p24 may bind to HBV RNA or DNA inhibiting HBV expression and replication. Recent studies which examined hepatitis C virus (HCV) interference of HBV replication in a transfected cell line showed that the HCV core protein suppressed the expression of HBV antigens and replication of the HBV genome (Shih et al, 1993). It was proposed that the HCV core protein may bind to HBV RNA or DNA to suppress transcription, translation or virus packaging of HBV. The exact mechanism of suppression is yet to be determined;

(d) The proposal by Khudyakov and Makhov (1990), that a truncated form of HDAg may be produced as the result of a frame-shift mutation in the HDAg gene, and that this mutant HDAg may compete with the 5' terminal protein

on the long strand of the HBV DNA genome for HBV RNA, thereby inhibiting HBV replication.

(2) The Non-specific Mechanisms;

(a) HDAg-p24-related cytotoxicity in the acute-phase of HDV infection may destroy HBV-producing cells;

The results of this study support the proposal that levels of HDAg-p24 expression that exceed the threshold in individual cells during acute HDV infection, may result in the destruction of HBV-producing cells. This proposal is supported by a previous finding which showed that HDV superinfection of HBV carrier chimpanzees resulted in an increase in the level of ALT that was preceded by a significant increase in the level of intrahepatic HDAg (Negro et al, 1988b). Thus, the transient nature of HDV interference with HBV replication may be explained; it is postulated that a decrease in the ratio of HDAg-p24:HDAg-p27 expression results in modulation of cytotoxicity due to HDAg-p24 by the mechanisms proposed in section 8.3. Therefore, it is possible that this modulating effect of HDAg-p27 allows the level of HBV replication to increase to levels similar to those prior to HDV superinfection.

(b) Loss of hepatocyte "luxury" function;

It was proposed previously that some viral infections may cause physiological changes in specialised cells that may reduce the capacity of

these cells to support non-essential or "luxury" functions e.g., enzyme production, without causing cell death (White and Fenner, 1986). The results of this study suggest that non-cytotoxic levels of HDAg-p24 may result in impairment of hepatocyte biosynthesis that may reduce the capacity of hepatocytes to support HBV replication.

(c) A host response to HDV infection;

On the basis of experimental studies using chronic HDV-infected woodchucks described in Chapter 7.4, Negro et al (1989) proposed that HDV may encode or induce a soluble factor or cytokine which acts systemically to interfere with WHV DNA replication. The induction of lymphokines was proposed previously as a mechanism of HBV suppression by HAV and NANB virus/HCV (Rizzetto et al, 1986; Shih et al, 1993).

8.6 A Model for the Basis of HDV Pathogenicity

Based on all available data, a model for the molecular basis of HDV-pathogenicity is proposed.

(1) During the acute phase of HDV infection, high levels of HDAg-p24 are expressed in hepatocytes coincident with the peak of HDV RNA replication. At this stage in the disease, the severe liver injury is likely to be the direct result of HDAg-p24-induced cytotoxicity. In addition, HBV replication is transiently suppressed as a result of the cytotoxic action of HDAg-p24 on HBV-producing cells.

(2) As a result of increased HDAg-p27 expression, the ratio of HDAg-p24:HDAg-p27 decreases resulting in suppressed levels of HDV RNA replication, modulation of cytotoxicity and persistent HDV infection. During persistent infection, HDAg-p27 and/or HDAg-p24 is processed and presented with HLA Class 1 antigens to constitute a target for CTL, and the action of these cells determines the degree of ongoing hepatitis.

While the observations made in this thesis pertain directly to understanding the primary cause of HDV-induced liver damage, it must be noted that many other factors may influence the severity and outcome of HDV-induced liver disease. These include the status and strain of HBV infection, with the worst outcome observed in patients infected with HBeAg-positive HBV with high levels of HDV replication (Bonino et al, 1991a).

8.7 Future Studies

These studies have provided direct evidence of a role for HDAg expression in HDV pathogenesis. However, it is important to determine the mechanism of HDAg-p24-related cytotoxicity. The two primary mechanisms which have been postulated above (section 8.2) to explain HDAg-p24-associated cytotoxicity may be addressed;

(1) Apoptosis of HDAg-p24-positive cells can be confirmed by electron microscopy to detect the characteristic morphological changes which are associated with this process. Early apoptotic features include chromatin and cytoplasmic condensation, compaction of organelles and loss of microvilli, followed by nuclear fragmentation and the appearance of deeply eosinophilic

apoptotic bodies (Wyllie et al, 1980). In addition, gel electrophoresis of cellular DNA from apoptotic cells reveals a characteristic fragmentation of the DNA into 180-200 bp oligonucleosomal bands (Wyllie et al, 1980).

(2) Further analysis of the multimeric HDAg-p24 aggregates detected previously in extracts of transfected cells which express HDAg-p24 (Macnaughton et al, 1990b) and extracts of HDV-infected liver (Wang and Lemon, 1993) may reveal the presence of cellular transcription factor(s) associated with these complexes.

The potential for HDAg-p27 expression to block both the mechanisms postulated above should also be addressed.

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APPENDIX 1

Bacterial Culture Medium

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	

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

APPENDIX 2

Reaction buffer used for restriction enzyme digestions (Sambrook et al, 1989)

10 x restriction enzyme buffer

1mM potassium glutamate
250mM Tris-acetate (pH 7.5)
100mM magnesium acetate
0.5mg/ml BSA
5mM 2-mercaptoethanol

Optimum dilution of restriction enzyme buffer

Enzyme	Final Concentration
Acc I	1x
Bam HI	1x
Bgl II	1x
Eco RI	1x
Hinc II	1x
Hind III	1x
Kpn II	0.5x
Nco I	1x
Pst I	1x
Pvu II	1x
Sac I	1x
Sac II	0.5x
Sal I	2x
Sma I	1x
Xba I	1x
Xho I	1x

APPENDIX 3

λ Pst DNA STANDARD*
λ DNA (48502 bp) Digested with Pst 1

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

* data from Macnaughton (Ph.D. thesis)

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