THE APPLICATION OF DNA HYBRIDISATION METHODS TO A DETERMINATION OF THE ASSOCIATION OF HEPATITIS B VIRUS WITH CIRRHOSIS AND HEPATOMA

BY

SHAMILA NAIR

Submitted in partial fulfilment
of the
requirements for the degree of
Master of Science (Med.Sc)
in the Department of Virology
University of Natal
1987

Durban 1987

DEDICATED TO MY PARENTS WITH LOVE

AND IN APPRECIATION OF THE SACRIFICES THEY HAVE MADE

TO BRING ME TO THIS POINT IN MY CAREER

ACKNOWLEDGEMENTS

The author offers sincere thanks to the following people:

1 My supervisors: Dr IM Windsor and Professor J van den Ende, for their constructive criticism during the course of this project

2 Particular thanks are due to Dr Windsor of the Department of Virology, for suggesting the project and also for the editing of this manuscript

3 The staff of the Liver Research Unit, University of the Witwatersrand (M Ritchie, L Pitcher and Dr J Dusheiko), especially Dr Dusheiko for the biopsy specimens and his expert assistance on the Southern blot analysis

4 The Council for Scientific and Industrial Research (FRD) for the funding of this project, also the University of Natal for the Graduate Assistance Bursary made available to me

5 Mr AA Chuturgoon of the Department of Biochemistry, University of Natal, Pietermaritzburg, for his constant encouragement and for his advice on the biochemical aspects of this research project

6 The staff of the Department of Virology for help and support

7 I would also like to thank the following people who have made contributions:

The staff of the Microbiology Department at the King Edward V111 Hospital, Mrs S Slogrove (Department of Medicine), and Miss E Rossouw (RIDTE) for friendly assistance

The staff at the Gale Street Police Mortuary and at the King Edward V111 Hospital Mortuary for their co-operation in the collection of specimens

Mr and Mrs S Brimiah for encouragement during the writing up of this thesis

Page

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Autopsy liver material from patients having died of chronic liver disease, cirrhosis, hepatocellular carcinoma (HCC) and causes unrelated to liver diseases was examined by dot blot hybridisation for the presence of HBV DNA. The results indicate that of the patients with chronic liver disease 6/9 were positive for HBV DNA in the liver tissue; of the patients with HCC 3/4 were positive for HBV DNA; of the patients with cirrhosis 4/4 showed the presence of HBV DNA in the liver. Thus by this technique 13/17 (76%) of these patients, all of whom were HBsAg positive, were shown to have HBV DNA present in liver tissue. However, autopsy liver samples were found to be unsuitable for Southern blot hybridisation .

Biopsy liver/tumour tissue was examined for the presence of integrated or non-integrated HBV DNA by Southern blot analysis using the enzymes Eco R1 and Hind 111. 5/5 patients who were both HBsAg and HBeAg positive had extrachromosomal HBV DNA and 2/5 also showed the presence of integrated HBV DNA. 3/4 patients who were HBsAg positive and HBeAg negative had extrachromosomal HBV DNA and all three also had integrated HBV DNA. One control patient was negative for both markers and also for Southern blot hybridisation with the HBV DNA probe.

These results support the hypothesis that HBV is a factor in the development of HCC, and indicate that the dot blot hybridisation method would be suitable for routine evaluation of patients with chronic liver disease or cirrhosis.

CHAPTER 1 GENERAL INTRODUCTION

1.1 HISTORY, CLINICAL EXPRESSION AND EPIDEMIOLOGY

In 1960 Blumberg carried out a study on the polymorphism of human antigens in blood samples collected worldwide from patients who had received a large number of blood transfusions. The studies entailed a search for antibodies against unique antigens which differed from the normal blood group antigens (Blumberg, 1965). A simple Ouchterlony immunodiffusion test revealed a line of precipitation between an antibody present in the blood of an haemophiliac from New York, who had received a number of transfusions, and an antigen present in an Australian aborigine. Thus the antigen was termed Australia antigen or Au. Subsequently, a correlation was shown to exist between the Au antigen and hepatitis, an infection of the liver in humans. This relationship was confirmed in 1967, when a technologist in Blumberg's laboratory developed hepatitis whilst purifying the Au antigen. Thereafter it was revealed that transfusion of blood containing the Au antigen resulted in the

development of hepatitis in the recipient. The Au antigen, also referred to as hepatitis associated antigen (HAA), is now known as hepatitis B surface antigen (HBsAg). The presence of the antigen and its corresponding antibody can be detected by laboratory testing of blood. This is of extreme importance in blood transfusion, as well as other services such as haemodialysis, and exclusion of HBsAg positive individuals has significantly reduced the rate of transmission of the virus now known as hepatitis B virus (Blumberg, 1977).

Serum hepatitis (hepatitis type B), whose causal agent is hepatitis B virus (HBV), is sporadic in occurrence and has a variable incubation period of 2 to 26 weeks. Transmission is predominantly by the parenteral route, but there is now increasing evidence for the spread of HBV by non-parenteral routes such as experimental transmission of HBsAg-positive semen and saliva in non-human primates (Evans, 1982).

Clinical expression following HBV infection is variable and usually asymptomatic, recognisable only by the detection of HBV markers in the serum. Only about 10% of infected adults develop a clinically recognisable hepatitis and this percentage is lower in infants and children (Beasley and Hwang, 1984).

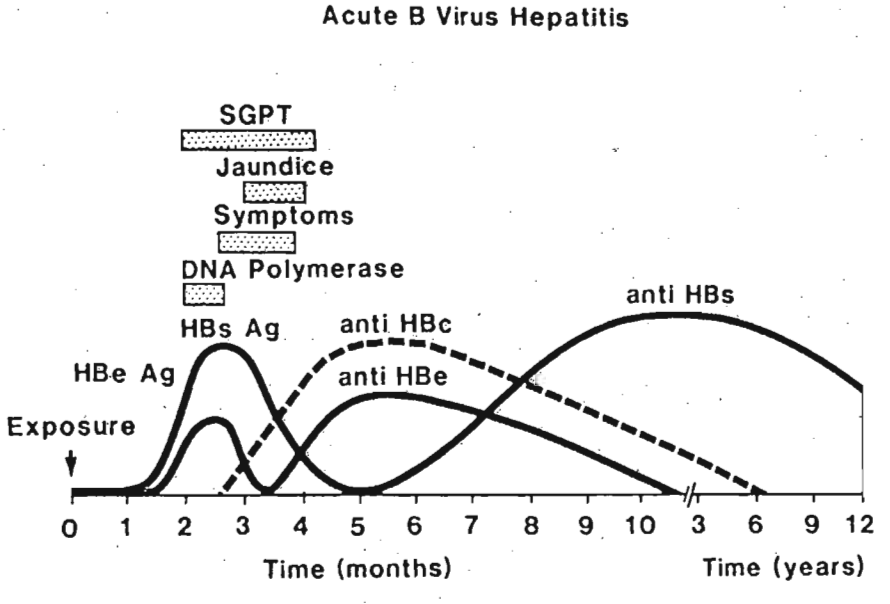


Fig. 1. The serological, clinical and biochemical events associated with the typical course of acute B virus hepatitis (Kew, 1983).

HBV infection is associated with the appearance of HBsAg in the serum from 2 to 8 weeks before biochemical evidence of liver damage or the onset of jaundice (Fig. 1). This antigen persists during the acute phase of infection but is usually cleared from the circulation during convalescence. The appearance of HBsAg is followed by the detection of high levels of the enzyme DNA polymerase which is associated with the core or nucleocapsid of the virus, and also of the 'e' antigen (HBeAg). Antibody secreted against the core (anti-HBc) is found in the serum 2 to 4 weeks after the appearance of HBsAg and antibody to HBeAg (anti-HBe) appears slightly later, its detection coinciding with the loss of detectable amounts of HBeAg. However, antibody to HBsAg (anti-HBs) is often only detectable 4 to 5 months following the detection of HBsAg in the serum (Zuckerman, 1979).

In clinically apparent HBV infections, symptoms range from fatigue and loss of appetite to severe malaise, coma and even death. It has been said that "jaundice is the hallmark of hepatitis", however, only a minority of infected persons ever experience it. Hepatitis B infection is also frequently accompanied by fever, chills and gastrointestinal symptoms, such as nausea and abdominal discomfort (Beasley and Hwang, 1984).

Some individuals do not eliminate the virus after infection with HBV but become chronic carriers. The factors determining the severity of the clinical response as well as the reason for progression to the carrier state and chronic liver disease are poorly understood, but chronic infection, once established, may last for many years (Beasley, 1983). It is believed that the immunologic status, age and sex of the infected individual may influence the likelihood of progression to the carrier state. Certainly HBs antigenaemia is known to be less prolonged in females for reasons that are not understood (Beasley and Hwang, 1984).

The age at which HBV infection occurs is one of the factors determining progression to the carrier state, the probability of becoming a carrier being inversely related to the age at which onset of infection takes place. According to Okada et al., (1976) and Beasley and Hwang, (1984), at least 85% of infected newborns become carriers, whereas less than 10% of infected adults become carriers. The susceptibility of infants to the carrier state may be related to a relative immaturity of their immune system (Hwang et al., 1983; Lee et al., 1983).

There are three types of chronic hepatitis: chronic persistent hepatitis, chronic active hepatitis and chronic

lobular hepatitis. Chronic persistent is usually asymptomatic and has a good prognosis because there is minimal liver damage. Chronic active and chronic lobular hepatitis, however, are severe and have a poor prognosis because of the extensive liver damage associated with these types of hepatitis. Thus it is not surprising that chronic active hepatitis might lead to macronodular cirrhosis and could eventually progress to hepatocellular carcinoma (HCC).

HBV may be involved in the aetiology of human HCC because of the observation that HBV infection often precedes the development of the tumour, the similarity between the geographical distribution of chronic carriers of HBsAg and the incidence of HCC, and the increase in the prevalence of HBV markers in the serum of patients with HCC compared to that seen in the general population (Kew, 1978).

Beasley and Hwang, (1984), also reported that world wide, areas with high HBsAg prevalence have a high incidence of HCC, whereas areas with low HBsAg prevalence have a low incidence of HCC. For instance, both HBsAg carrier states and HCC incidence appear to be universally high among Black South Africans but are very low among American Blacks. In contrast the incidence of HCC among South

African Whites is low, being similar to that seen in Caucasians in Western Europe and the United States. Their prospective study in Taiwan also showed that HBV carriers there have approximately a 200-fold higher risk of developing HCC than have noncarriers, and the lifetime risk of a chronic carrier developing HCC may be as high as 50% in males. They also consider that other environmental factors such as aflatoxins may be involved in the development of HCC in persons who are HBV carriers.

There is also evidence to suggest that increasing severity of cirrhosis is associated with increased risk of HCC (Koshy et al., 1981). HCC is a serious disease, with a poor prognosis. The tumour frequently affects young people. Because epidemiologic studies have established a close association between the occurrence of HCC and chronic HBV infection, the development of an inexpensive HBV vaccine is likely to be the key factor in leading to "ultimate eradication" of HBV infection and hence presumably of much HCC.

1.2 THE VIRUS

The use of recently developed methods for gene cloning and molecular hybridisation techniques has allowed detailed

investigation of the biology of HBV and mechanisms involved in HBV-host cell genome interactions.

HBV is a DNA containing virus with certain unique features; its characterisation has led to the esatablishment of a new group of viruses, the Hepadnaviruses. The virus is a double-shelled, roughly spheroidal particle, either empty or full consisting of a small core and a lipoprotein polypeptide outer envelope (Dane, 1970). Krupp and Chatton, (1982), described spherical and tubular particles of different sizes which were present in the serum of patients with hepatitis B, and which possessed a common antigenicity.

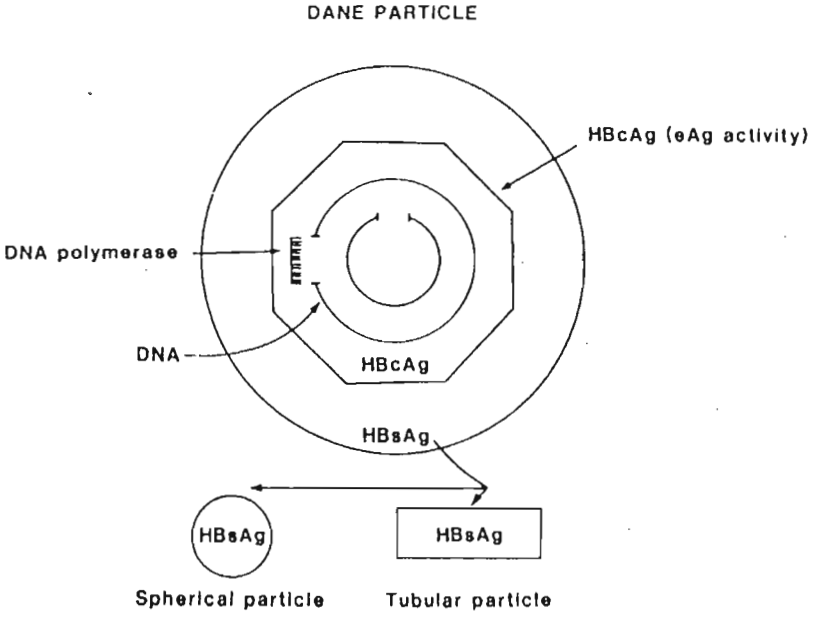


Fig. 2. Diagrammatic representation of HBV particles and their associated antigens (Kew, 1983).

The largest of these spherical forms was first described by Dane, (1970), and is considered to be the complete hepatitis B virion (Fig. 2). The Dane particle is 42nm in diameter and consists of a 27nm core surrounded by an outer coat or envelope composed of HBsAg. Within infected cells the core is found in the nucleus and the double shelled outer coat material is found in the cytoplasm (Krupp and Chatton, 1982). The core, or nucleocapsid, contain double and single stranded DNA and the two enzymes DNA polymerase and a protein kinase. It is thought that the DNA polymerase reaction completes the single stranded DNA to produce a double stranded form, while the protein kinase may assist in the DNA replication. The viral DNA codes for the outer coat material (ie HBsAg) in excessive amounts and material not utilised in coating nucleocapsids leaves the cells as small spherical or tubular particles which circulate in the plasma. A further HBV-associated antigen which is found in the plasma is the 'e' antigen. This is a soluble antigen, circulating alone or complexed with immunoglobulin, whose relationship to the virion was originally obscure but which is now recognised as a signal peptide affecting the excretion of virus (Krupp and Chatton, 1982).

HBV has a small circular DNA genome of 3200 to 3300 base pairs. This genome has a unique structure having an L or long strand which is complete except for a nick of one or a few nucleotides at a fixed point (1800 nucleotides from the Eco R1 site) and an S or short strand which is incomplete, missing from 15 to 50% of its potential sequence complement. The 5`end of the S strand is located at the nucleotide 1560 position but the position of the 3` end varies considerably. The circular configuration of the molecule is maintained by the base pairing of the 5` extremities of both strands which overlap by 250 to 300 nucleotides to form a cohesive end region (Fig. 3) (Sherman and Shafritz, 1984).

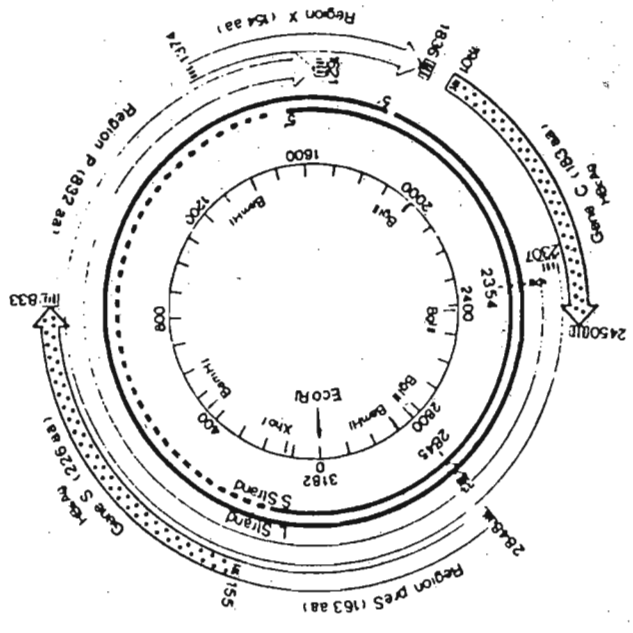


Fig. 3. Restriction map of the genome of HBV showing restriction enzyme sites and the open reading frames and genes on the L strand (Sherman and Shafritz, 1984).

1.3 DIAGNOSTIC METHODS

Insensitive methods, such as the Ouchterlony double diffusion test, were first used to detect HBsAg. However, for present diagnosis of HBV infection highly sensitive radioimmunoassays (RIA) or enzyme immunosorbent assays (ELISA), involving the use of radiolabelled or enzyme - labelled reagents are used to detect a wide range of HBV markers. These markers include HBsAg, Anti-HBs, anti-HBc, HBeAg and anti-HBe. As excessive amounts of HBsAg are produced early in infection during viral replication and the s genes may in some cases be the only regions expressed during persistent infection, detection of HBsAg has provided the most reliable method for diagnosis of acute infection and of the HBV carrier state. Anti-HBs, on the other hand, is a late marker of HBV infection and its presence in high levels in the serum may indicate past infection and immunity. HBcAg may be detected in the nuclei of infected liver cells using a fluorescent antibody technique (Hoofnagle et al., as cited by Zuckerman, 1979). Anti-HBc is present in the serum of chronic carriers, and also in individuals recovering from HBV infection. The detection of HBeAg in patient serum may be taken as a reliable indicator of the presence of

infectious Dane particles in the blood, and there is a significant correlation between HBeAg, infectivity, DNA polymerase activity and the presence of complete virus particles circulating in the blood. Conversely, the presence of anti-HBe in the serum usually signifies reduced or relatively low activity. Virus production, however, has been reported in the presence of anti-HBe in some carriers.

Serum containing very low levels of replicating viral particles may be HBeAg negative but HBV DNA can be detected in serum by molecular hybridisation. The method for HBV DNA detection by molecular hybridisation may therefore be more sensitive for the detection of complete virus particles than the conventional radioimmunoassays for HBeAg.

1.4 STUDIES ON INTEGRATION OF THE VIRAL GENOME

1.4.1 Cloning of the HBV genome

Since HBV cannot be grown in tissue culture and normally infects only man and apes, molecular

studies of the virus and its genome were based on the limited amounts of material obtainable from the plasma of infected individuals.

Burrell and coworkers (1979) inserted fragments of HBV DNA isolated from Dane particles into the plasmid pBR322 and cloned it in E. coli. Some of the transformed cells carrying the hybrid plasmid synthesize antigenic material that reacts specifically with antisera to hepatitis B viral antigens. Cloning of DNA from HBV of three different serotypes in E. coli. via phage derivatives and plasmids has also been reported (Pasek et al., 1979). Valenzuela et al., (1979), examined a full length clone by restriction endonuclease activity analysis, and the nucleotide sequence of an 892 base pair fragment from cloned hepatitis B viral DNA, encoding the surface antigen gene, was determined. The amino acid sequence deduced from this nucleotide code showed the surface antigen to be a protein consisting of 226 amino acids and with a molecular weight of 25 398 daltons. Valenzuela and co-workers also found that the portion of the gene coding for this protein apparently contains no intervening sequences.

The HBV genome has now been studied in great detail and it is now possible to obtain cloned HBV DNA of known nucleic acid sequence which can be used as a probe in diagnostic studies.

1.4.2 HBV DNA integration studies

As described above (1.1) hepatitis is a common and sometimes severe disease with 10-20% of the population in some parts of Africa and Asia being chronic carriers of viral surface antigen.

In 1980, Alexander and co-workers developed a tissue culture cell line (PLC/PRF/5) from the HCC of a Mozambican male with HBs antigenaemia. This cell line produces and secretes HBsAg but no other viral proteins (Chakraborty et al., 1980; Brechot et al., 1980). PLC/PRF/5 therefore contains at least part of the HBV genome in functional form. It was pointed out that the presence of integrated HBV DNA in this HCC cell line as well as in HCC liver material favours the argument for HBV as a cofactor in HCC (Chakraborty et al., 1980; Brechot et al., 1980 and Edman et al., 1980). Subsequently, Shafritz et al.,

(1981), studied HBV DNA in the liver of chronic HBV carriers in the United States. They showed that short term carriers, namely patients who have been positive for HBsAg for longer than six months but less than two years, do not generally have detectable integrated HBV DNA by Southern blot analysis, irrespective of the presence of liver disease. These patients are usually HBeAg positive, DNA polymerase positive and have circulating Dane particles in the peripheral blood. Large amounts of free viral DNA are present in the liver at this stage. In contrast, anti-HBe positive patients may have detectable HBV DNA integration (Brechot et al., 1981). Based on these studies two forms of chronic hepatitis B can be recognised:

a) "Replicative" hepatitis B, in which carriers are HBsAg, HBeAg, HBV DNA positive. These individuals have episomal HBV DNA within hepatocytes. This state represents a relatively early form of the disease.

b) In patients with long-standing hepatitis B, only low levels of HBV replication, or absence of replication predominates. These persons are HBsAg positive, and anti-HBe positive, but

HBeAg and HBV DNA negative, with HBV DNA integrated within the host genome. Presumably their HBsAg is coded from an integrated HBV template.

By analogy with animal tumour viruses, such as adenoviruses and SV40, if HBV has a causal role in HCC, it might be expected to be integrated into the host DNA in tumour cells in chronic HBV carriers.

EXTRA CHROMOSOMAL HBV DNA

(a) Linear (Eco R1)

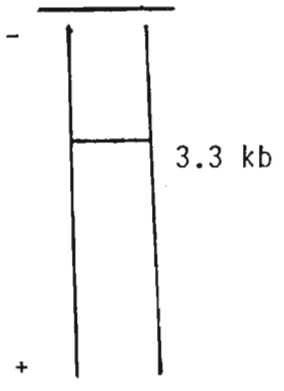


Fig. 4(i). Extrachromosomal HBV DNA as present in the hepatocytes or serum

INTEGRATED HBV DNA

(b) undigested (c) Hind III (d) Eco RI

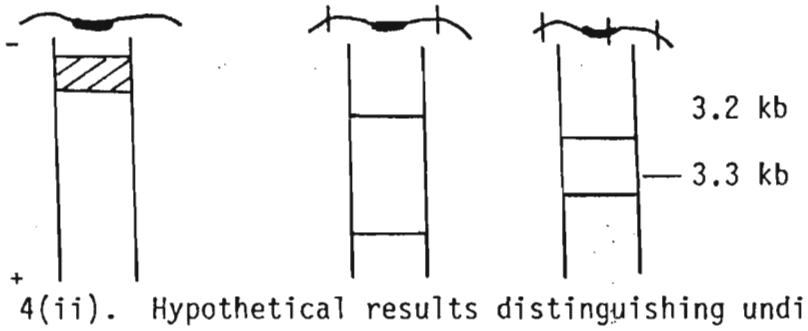


Fig. 4(ii). Hypothetical results distinguishing undigested and digested integrated HBV DNA

Fig. 4. Strategy for restriction enzyme analysis of hepatitis B virus DNA (Adapted from Sherman and Shafritz, 1984).

Different restriction enzymes can be used to distinguish between integrated and non-integrated HBV DNA.

The enzyme Eco R1 cuts once within the HBV genome. Thus, free circular HBV DNA will be cleaved by Eco R1 to yield a linear molecule which migrates like the circular form on gel electrophoresis to form a band at the 3200 to 3300 base pair position (Fig. 4(i)a). However, integrated HBV DNA if untreated, forms a diffuse band at the top of the gel, while an Eco R1 digest will produce high molecular weight molecules consisting of part of the HBV DNA linked to host cell DNA (Fig. 4(ii)). If concatemers (fully replicated HBV genomes remaining linked together) or oligomers (linear tandem repeats of the HBV DNA) are present, 3200 to 3300 base pair length molecules will also be produced.

The enzyme Hind 111, which does not cut the HBV genome, may be used to distinguish between specific and random integration of HBV DNA. The former is defined by the specific banding of molecules larger than the viral genome (ie > 3200 to 3300 base pairs), while the latter will produce only a diffuse smear on gel hybridisation analysis following Hind 111 digestion (Sherman and Shafritz, 1984).

Research has shown that HBV DNA is integrated into the cellular DNA of HCC HBsAg carriers at a very high rate (Hino et al., 1984), and the integration pattern differs from case to case. However, as some similarities in pattern are seen it is possible that there are some preferred sites of viral integration (Koshy et al., 1981).

The molecular basis for the pathogenesis of HCC associated with hepatitis B virus is unknown. Several aspects of hepadnavirus replication resemble the mechanism of retrovirus replication. Like retroviruses, all known HBV coding genes are located on one strand of the virus and are in the same orientation. However, unlike retroviruses, integration does not appear to be necessary for hepadnavirus replication. The cohesive end regions of hepadnaviruses are structurally similar to retroviral long terminal repeats and may serve an analogous function. Murray et al ., (1981) showed that retroviral oncogenes transferred into recipient cells induce neoplastic transformation of those cells. However, transfection of HBV DNA has failed thus far to produce neoplastic transformation and so it may not contain an oncogene as such. Moreover, HBV DNA does not contain sequence homology to any of the known retroviral oncogenes. However, Chakraborty et al., (1981), have identified two strong viral promoters in HBV, one in the cohesive end region and the other in the pre-S region, and it has been

noted that in hepadnavirus integrations the cohesive end region with its strong promoter has been conserved.

Two possibilities outlined by Dejean et al., (1983), may explain the oncogenicity of HBV. The first possibility is that the promoter-containing region becomes integrated adjacent to a cellular proto-oncogene or another transforming gene. However, in those human tumours studied to date, no sequences homologous to any of the known retroviral oncogenes have been found directly adjacent to an integrated HBV gene. An alternative possibility is that during persistent infection, cellular oncogenic sequences could become integrated into the HBV genome creating a mutant, autonomously replicating tumour virus. This mutant virus could then infect other hepatocytes, resulting in enrichment of oncogenic sequences in recipient cells and in their subsequent transformation.

Wen et al., (1983) showed that neoplastic transformation by a DNA tumour virus depends on the presence of certain virus coded antigens, the transformation or T antigens. A protein similar to a T antigen has not been described for HBV, but a nuclear antigen similar to that found in Epstein Barr virus transformed cells has been described.

Sherman and Shafritz, (1984) have pointed out that integration of SV40 and other DNA tumour viruses appears to be a random or semi-random event, occurring at no fixed site in the cellular or viral genome. Rather like SV40, HBV DNA is integrated into host DNA in a complex fashion, with inversions and deletions of viral DNA and with rearrangements of cellular sequences at the site of integration. However, no specific gene expression product associated with or responsible for transformation has so far been identified.

Recently Boender and co-workers (1985) suggested that a number of factors may be responsible for not detecting site specific HBV DNA integration into the human genome by either Southern blot or other methods of analysis. The failure to detect specific integration could be caused by a number of factors such as the presence of a substantial number of copies of HBV in the tumours, the relatively short regions of integrations, rearrangements or deletions occurring during or after the construction of the cell lines, or integration within a highly polymorphic region of the host cell genome.

Thus, molecular studies have resulted in a detailed knowledge of the HBV genome with respect to its integration into the host cell DNA and its relationship to cirrhosis, and also HBV DNA, because of its integration into the human genome and its association with HCC, has often been considered to be oncogenic.

1.5 THE PRESENT STUDY

Windsor and Joseph (1981), reported that of the total number of cases of hepatitis studied in African Black patients in Natal, 11% were due to an HAV infection and 41% to an HBV infection. Also, in 1983, Windsor and Simjee showed that of the 43 Black African HCC patients studied over a period of 14 months, 60% were HBsAg positive and 80% had markers of HBV infection, while of the 65 cirrhotic patients studied, 33% were HBsAg positive and 75% had HBV markers. From Table 1, which depicts the numbers of patients tested for and those found positive for HBsAg at King Edward V111 Hospital, Durban, over a 6 month period in 1986, it can be concluded that HBV infection in Natal is extensive.

TABLE 1. Hospital patients tested for HBV infecton in Natal over a period of 6 months

Month	No of pts	no HBsAg+	%
Jan	690	152	(22)
Feb	819	104	(12)
Mar	1708	188	(11)
Apr	1174	162	(13.7)
May	814	87	(10.0)
Jun	837	126	(15.0)
Mean	6049	819	(13.9)

The majority of the studies on HBV and HCC in Natal have therefore been carried out on Black African patients because of the high prevalence of HBV infection in this population group.

In the present study, both Southern and dot blot hybridisation methods were applied to the detection of HBV DNA in total DNA extracted from liver and tumour specimens obtained from patients with acute and chronic hepatitis B, in the hope of expanding our knowledge of the role of HBV in this group.

CHAPTER 2 INTRODUCTION TO THE METHODOLOGY THEORETICAL CONSIDERATIONS

2.1 GEL ELECTROPHORESIS

Agarose is the most common medium for nucleic acid gel electrophoresis. The three different types available include GTG agarose for high molecular weight DNA and NU Sieve agarose for small DNA fragments. Different concentrations of agarose, ie. from 0.6 to 1.8% can be used to electrophorese DNA or RNA. The voltage can be varied depending upon the size of the DNA, the concentration of agarose used and individual preference. The choice of the different parameters also depends upon the type of research being undertaken.

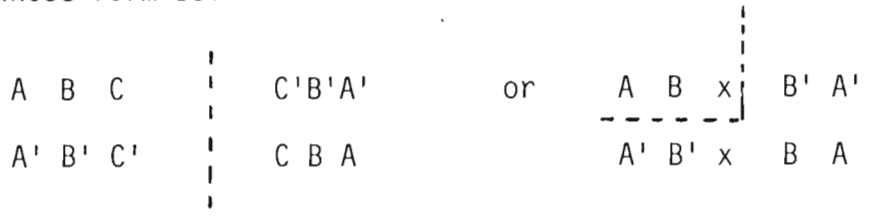
At neutral or alkaline pH, the phosphate groups of DNA give rise to a uniform negative charge per unit length of the DNA molecule. Thus in an electric field, DNA will move towards the anode, with a constant driving force per unit length propelling the molecules, regardless of base composition. Any differences in the rate of movement of different DNA molecules will depend only on their resistances to their movements. If the molecules are in the gel they will have to pass through its pores as they move towards the anode. The longest molecules will have

most difficulty in passing through the pores, and may even be blocked completely, whereas the smallest molecules will be relatively unhindered. Consequently the velocity of movement of a DNA molecule during gel electrophoresis will depend on its size, the smallest molecules moving fastest because the separation depends on chain length rather than molecular weight of the molecules. Thus DNA lengths are referred to as "x number of base pairs" (bp) if double stranded or "x number of nucleotides" (nt) if single stranded. The influence of shape and size rather than molecular weight, is demonstrated by the fact that gel electrophoresis can be used to separate different physical forms of the same DNA from each other, eg covalently closed circular, open circular and linear forms of a plasmid DNA will have different mobilities (Boffey, 1983).

2.2 RESTRICTION ENZYME NUCLEASES

A restriction endonuclease is an enzyme that recognises a specific base sequence in a DNA molecule and makes two cuts at that position, one in each strand, thus generating 3'-OH and 5'-P tremini. Roughly 400 different restriction enzymes have been purified from about 250 different microorganisms and approximately 150 are

commercially available. All but a few of these enzymes recognise sequences having rotational (dyad) symmetry, that is, the recognition site generally has a sequence whose form is:



in which the capital letters represent bases, a prime indicates a complementary base, x is any base, and the dashed line is the axis of symmetry. Sequences having more than 6 bases are known but none have been observed containing fewer than 4 bases (Freifelder, 1983).

The two enzymes used in this study were the Eco R1 enzyme of E. coli which cleaves at the recognition site AAGCTT, TTCGAA and the Hind 111 enzyme of Haemophilus influenzae which cleaves at the recognition site GAATTC. CTTAAG

Restriction enzymes vary greatly in their stability and their ability to tolerate contaminants. The enzymes also show variation of activity depending upon pH, buffer and salt composition. Furthermore, DNA may contain ionic compounds which can reduce the efficiency of the

restriction endonuclease cleavage. Thus these enzymes are sensitive to optimal conditions required for activity.

2.3 SOUTHERN BLOTTING

Gel electrophoresis is a powerful tool for the separation and resolution of complex mixtures of proteins and or nucleic acids. However, the correlation of a band or spot on a gel with particular functional and structural entities is often difficult and gels require careful handling. Further manipulations are usually time consuming and inefficient because the separated samples are located within the gel matrix.

A technique for the transfer of separated macromolecules onto the surface of a membrane was first developed for DNA by Edwin Southern of Edinburgh University (1975) and has since been extended to RNA "Northern blot" and protein "Western blot". These blotting procedures offer a solution to the problems posed in the identification of a molecule after gel electrophoresis, since once transferred, the molecule may be effectively immobilised on the membrane surface. Membranes are relatively easy to handle and thus allow much more efficient analysis of these macromolecules.

The ability to immobilise mlecules at the surface of a membrane has been exploited in a variety of applications other than those involving gel electrophoresis, such as dot blotting and colony/plaque screening. Most of these techniques were originally developed using nitrocellulose (NTC) filter papers or their derivatives. However, nylon based membranes have recently been reported to have advantages in a variety of applications, due to their high physical strength and the avidity with which macromolecules are bound. Thus Hybond-N membrane was used for both Southern and dot blotting (Amersham Research Laboratories (ARL), 1985).

The DNA is carried out of the gel by the flow of solvent and trapped in the Hybond-N paper. It has been found that several factors affect the eficiency of the transfer procedure. For instance, DNA fragments larger than 10Kb in size are transferred very slowly, and should be broken down in the gel before transfer. This can be done either by irradiating the gel with shock wave UV rays before denaturation, or by partial depurination with dilute acid followed by strand cleavage with an alkali. However, the effectiveness of the irradiatioin method has been disputed. Secondly some brands of NTC filter paper seem to retain DNA more effectivley than others. Finally, the time of transfer will depend upon the thickness of the gel, the percentage of agarose in the gel and the sizes

of the fragments to be transferred (Matthew, 1983).

2.4 DOT BLOTTING

A dot blot hybridisation method has been used (Kafatos et al., 1979), for rapidly determining the relative concentrations of nucleic acids in a mixture, as well as the extent of sequence homology between related RNA or DNA species. Multiple samples of DNAs, identical in quantity, are spotted next to each other on a single NTC filter, in dots of uniform diameter. The filter is then hybridised with a radioactive probe, such as an RNA or DNA mixture which may contain the corresponding sequences in unknown proportions. Conditions are chosen to avoid saturation of the filter bound DNA. The extent of hybridisation with each of the DNA dots is evaluated semi-quantitatively after autoradiography, by visual comparison to a standard consisting of a dilution series of radioactive DNA, similarly spotted on an NTC filter in dots of the same diameter. This approach may be used for the evaluation of the relative abundance of nucleic acid sequences in a mixture. Moreover dot hybridisation under progressively more stringent conditions can be used for semi-quantitative evaluation of the extent of similarity between homologous sequences (Kafatos et al., 1979).

2.5 PLASMID ISOLATION

Some bacterial genes are not located in the main chromosomal DNA but in independently replicating molecules of circular, double-stranded DNA called plasmids. Plasmids vary in size depending upon the organism in which they are found, eg pBR322 has 4362 whereas M13 has 7229 bp. Many plasmids carry gene markers which code for antibiotic resistance, antibiotic synthesis, toxin production, nitrogen fixation, production of degradative enzymes and conjugation. So plasmids are obviously of great interest in their own right. However, in the context of this research, plasmids are mainly of interest as vectors for the cloning of DNA. It is possible to obtain large quantities of a particular DNA by inserting it into plasmid (vector) DNA which can then be introduced into a suitable host bacterium in which the plasmid will replicate. Culture of the bacteria will result in the production of more plasmid DNA, which can then be isolated from the cells and the inserted DNA recovered.

As Boffey (1983), pointed out, most of the plasmids used as cloning vectors do not occur in nature, but have been extensively modified so that they have properties useful

for cloning, eg pBR322 contains ampicillin and tetracycline resistance genes alllowing identification of transformants (amp^r, tet^r) or recombinants (amp^s tet^r or amp^r tet^s). These markers are extremely useful, being widely employed for selection and maintenance of the plasmid in bacterial cultures. The plasmid must be replicated in the host cell and if the replication is relaxed (ie not stringently coupled to chromosomal DNA replication) there is the possibility of increasing the number of plasmid copies up to seven thousand per cell by selectively inhibiting protein synthesis and hence chromosomal DNA replication. This results in higher yields of plasmid DNA with respect to chromosomal DNA. The isolation of pure, intact plasmid DNA in high yields is made simpler if the plasmid is small. Small plasmids are relatively resistant to shearing and will therefore remain in their native supercoiled, covalently closed circular (CCC) form, whilst the high molecular weight chromosomal DNA will be broken into large linear fragments. These differences in size and shape can be exploited to separate the two types of DNA from each other. Desirable characteristics of the host bacterium include ease of transformation, of culture and of lysis (Boffey, 1983).

2.6 NICK TRANSLATION

If a single-stranded DNA molecule is placed in contact with a complementary single stranded DNA sequence, the two molecules will associate with one another by hydrogen bonding between the bases on their respective strands. This association, or hybridisation, forms the basis of very powerful techniques for detecting and quantifying specific nucleic acid sequences. Whether the hybridistion is done in solution or using nucleic acid immobilised on filters, a radioactively labelled probe is required. The enzyme, DNA polymerase 1 from E. coli catalyses a reaction which can be used to replace existing unlabelled nucleotides in DNA with radioactive ones. The reaction has been called nick translation and is very widely used in molecular biology (Matthew, 1983).

The initial step in the procedure is to create free 3'-hydroxyl (OH) groups within the unlabelled DNA by means of a nuclease such as pancreatic deoxyribonuclease (DNase) which "nicks" or cuts one strand of the DNA. The DNA polymerase 1 will then catalyse the addition of a nucleotide residue to the 3'-hydroxyl terminus of the nick (Fig. 5). At the same time the 5' to 3' exonuclease

activity of this enzyme will eliminate the nucleotide unit from the 5' -phosphoryl terminus of the nick. The net result of the reaction will be the incorporation of a new nucleotide with a free 3' -OH group at the position where the original nucleotide was excised. The nick will, therefore have been shifted along by one nucleotide unit in a 3' direction. Continued 3' shift, or translation, of the nick will result in the sequential addition of new nucleotides to the DNA while pre-existing nucleotides will be removed.

The DNA thus acts as both primer for the DNA polymerase, because of its free 3' -OH group, as well as template, because the opposite strand of the DNA duplex dictates what type of nucleotide is to be incorporated. If radioactively labelled deoxyribonucleoside triphosphates (dNTPs) are used as substrates, the original unlabelled nucleotides in the DNA will be replaced by labelled ones. By means of this "hot for cold swop" of nucleotides, about 50% of the residues in the DNA can be labelled (Matthew, 1983).

The nick translation reaction has to be carried out slowly and at fairly low temperatures eg incubation for 60 minutes at 15^{0}C. This requires careful monitoring because at higher temperatures 'snap back' DNA (Fig. 6) may be generated and thus affect the efficiency of labelling (Maniatis et al., 1982).

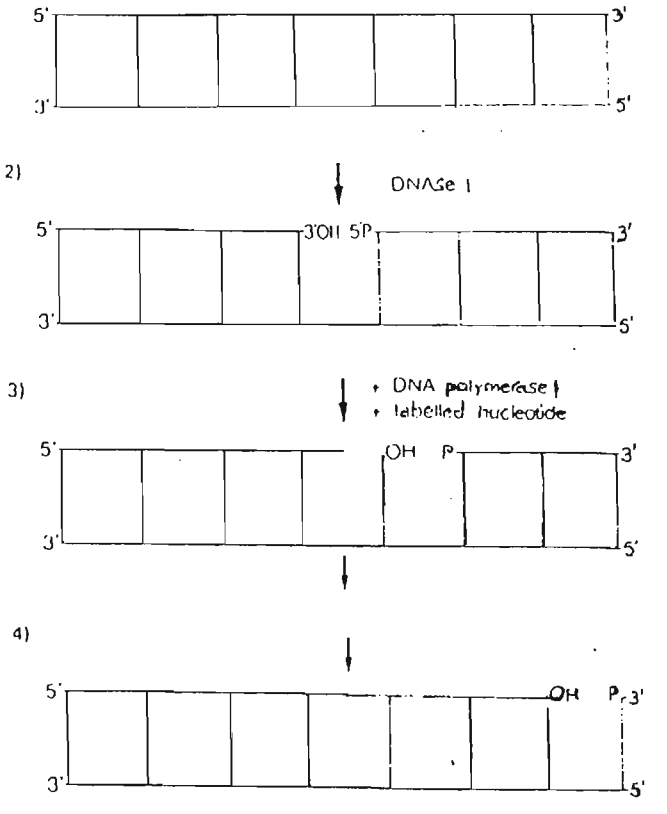


Fig. 5. A schematic representation of the nick translation reaction.

1 Intact double stranded DNA

2 A nick produced by the enzyme DNase 1

3 The first nucleotide has been replaced by a labelled nucleotide and it has been moved along in a 5' to 3' direction

4 The nick has been translated two more positions along the DNA (Matthew, 1983).

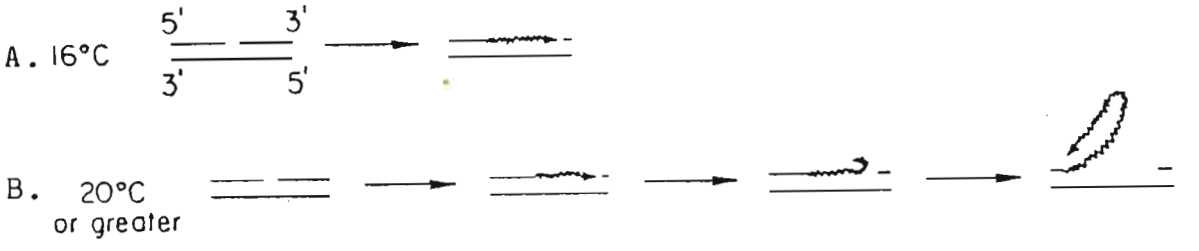


Fig. 6 A. Nick translation reaction at optimal temperature

B. Formation of "snapback" DNA at higher temperatures (Adapted from Maniatis et al., 1982).

2.7 PROPERTIES OF LABELLED DNA

The most important property of the DNA probe is its specific activity. The higher the specific activity, the more sensitive and accurate the detection and quantification of specific sequences will be. In order to make a probe of high specific activity, sufficient labelled nucleotide must be added to the reaction mixture to replace all the corresponding nucleotides in the DNA. For instance, if 4000 picomoles (pmol) of DNA are to be labelled, then 1000 pmol of dCTP will be required to replace all the "cold" dCTP, whereas 100 pmol could replace only 10% of it. In practice, an incorporation of 30-50% is usually obtained. The specific activity of the probe will obviously also depend on the specific activity of the nucleotide(s) used in the nick translation. Adequate detection of unique sequences by Southern blotting for example, requires a probe with an activity of approximately 10^8 cpm/ug.

The amount of DNase added to the reaction is also an important parameter. If the DNase concentration is high many nicks will be generated and the DNA polymerase can begin translating at many sites. The reaction rate will therefore be high, and a probe of high specific activity will be generated in the standard incubation time,

although the single stranded length of the labelled probe, and hence its subsequent rate of hybridisation to complementary sequences, will be reduced at such high DNase concentrations. Thus, for most applications only sufficient DNase is added to produce a probe with a single stranded length of about 400 nucleotides. Another important requirement for a probe is that it should be labelled throughout at uniform specific activity. If, for example, a section of the probe is poorly labelled, a restriction fragment corresponding to that part of the probe might not be detected on a Southern blot. The stability of a nick translated probe on storage is dependent on the type of isotopic label used. A ^{32}P labelled probe with a specific activity of 10^8 cpm/ug is stable for 7-8 days, after which its single stranded length declines rapidly. ^{32}P labelled probes have a half life of 14 days, during which time the specific activity decreases accordingly. However, ^{3}H probes may be stable for 6 to 9 months. Thus the amount of probe used in hybridisation (in ul) will depend upon its activity with respect to the half life of the isotope label.

DNA can also be labelled <u>in vivo</u>. For example, labelled viral DNA can be extracted from virus-infected cells which have been grown in the presence of ^{32}P. However,

nick translation has several advantages in that labelled DNA can be produced in the absence of a tissue culture system, the DNA can be labelled to a much higher specific activity and DNA can be purified when convenient, stored and then labelled rapidly when required.

2.8 HYBRIDISATION

Considerable effort has been devoted by such workers as Meinkoth and Wahl (1985) to determine the parameters affecting hybridisation kinetics. Although much of this work was applied to the annealing of two complementary strands in solution, the general principles apply to mixed phase reactions, and to a large extent, to reactions involving self-complementary probes such as those generated by nick translation.

Many of the factors which affect hybridsation rates also affect hybrid stability and can be expressed in these terms. The melting temperature (Tm) of a hybrid is affected by ionic strength, pH, probe length and base composition of the probe as well as by the concentration of helix destabilizing agents such as formamide (Meinkoth and Wahl, 1985).

For nick translated probes or single stranded probes longer than 100 nucleotides, melting temperature (Tm) decreases by $1^{o}C$ for every 1% of mis-matched base pairs (Boffey, 1983). This might suggest that filter hybridisation should be carried out under the most stringent conditions possible, that is, as close to the Tm of the expected hybrid as is compatible with hybrid stabiity and reasonable reaction rate. However, a common approach is to carry out hybridisation under conditions of relatively low stringency which allows a high rate of hybridisation and follows this with treatment to remove poorly matched probes. Most workers use temperatures of between 65 and $72^{o}C$ in the absence of formamide, or 37 to $45^{o}C$ in the presence of 50% formamide, followed by a series of post hybridisation washes of increasing stringency, that is, at higher temperatures or, more commonly, lower ionic strengths. The use of SDS to assist in the removal of non-specifically bound probes is also commonplace. Using this approach, it is possible to detect related sequences and to gain some estimate of the extent of sequence relatedness (or degree of mis-matching) if filters are autographed following each wash (ARL, 1985).

The above assumes first order kinetics for the hybridisation reaction, since the concentration of the probe should be in excess over that of the target sequences and the rate of hybridisation will then be proportional to probe concentration. However, in filter hybridisation, background problems dictate that relatively low concentrations of radioactively labelled probe be used in order to obtain high signal-to-noise ratios. The generally accepted view is that 10ng of probe/ml of hybridisation mixture is optimal when 10% dextran sulphate is present, with an increase to 50-100 ng/ml in the absence of dextran sulphate (ARL, 1985). These concentrations will usually be high enough to give first order kinetics for the reaction. The rate of hybridisation is increased by the use of dextran sulphate or other polymers. This effect may be due to promotion of network formation by overlapping probe sequences. The increase in rate seen with dextran sulphate is more pronounced with increasing probe length and is not seen at all with oligonucleotide probes. It should however, be noted that the use of dextran polymers may also lead to increased backgrounds and it is important that hybridisation times should be optimised for signal-to-noise ratio (Meinkoth and Wahl, 1985).

CHAPTER 3 MATERIALS AND METHODS

3.1 Specimen Collection and History

3.1.1 Tissue specimens for dot blot hybridisation

Autopsy blood specimens in addition to autopsy liver material were collected from patients who died of chronic hepatitis or HCC as well as from causes unrelated to liver diseases. The latter were used as controls. Autopsy material was collected 2-3 days following death of the patient. The liver specimens were stored at $-70^{o}C$ and the blood was analysed for HBV markers.

Liver autopsy samples were collected as follows: nine samples from patients with chronic liver disease (9/9 were HBsAg positive and 3/9 HBeAg positive), four samples from patients with cirrhosis (4/4 were HBsAg positive and 4/4 HBeAg negative), four samples from patients with HCC (4/4 were HBsAg positive and 1/4 HBeAg positive) and ten samples from patients having died of unrelated causes. These ten patients had no HBV markers.

3.1.2 Tissue specimens for Southern blotting

Tumour tissue specimens obtained as soon after death as possible from patients dying of HCC, or after resection of HCC, were collected by the Liver Research Unit, University of the Witwatersrand. Resected specimens were frozen in liquid nitrogen and stored at - 70^{o}C. Of the 10 biopsy specimens collected: 9/10 were HBsAg positive, 4/9 being HBeAg negative and 5/9 HBeAg positive while 1/10 was negative for HBV markers. Liver as well as tumour tissue was collected from 2 patients who were both HBsAg positive and one of whom was HBeAg positive.

3.2 EXTRACTION OF TOTAL CELLULAR DNA FROM LIVER or TUMOUR TISSUE

Approximately 0.5g tissue was sliced on ice in a petri dish with a sterile scalpel blade. It was then homogenised under liquid nitrogen to a powder form and placed in a siliconised eppendorf tube containing 450ul of lysing solution (2% SDS, 0.05 M EDTA, 0.2 M NaCl, 0.1 M Tris-HCl, pH 8.2). To this mixture 5ul of proteinase K (10mg/ml in 0.01 M Tris-HCl, pH 7.5) to a final concentration of 100ug/ml was added and the tissue incubated overnight at 37^{o}C. This was followed by phenol/chloroform extraction and back extraction with 24:1 chloroform : isoamyl alcohol. The aqueous phase was dialysed against a 1xTE

buffer overnight and treated with DNase free RNase. The phenol/back extraction was repeated and phenol was then removed by an ether extraction. DNA was concentrated and purified by passing it through an Elutip-d (Schleicher Schuell) column. The eluted DNA was ethanol precipitated using ammonium acetate at -70^{o}C to yield highly purified DNA.

3.2.1 Quantification of the extracted DNA

The concentration of DNA in the sample was calculated following determination of the optical density (OD) ratio at 260/280nm using an ultra violet U/V spectrophotometer, Beckman model 24. OD 260/280 ratios of 1.6 or greater were acceptable.

3.3 DOT BLOTTING

Dot blotting was applied to autopsy material as described by Kafatos et al., (1979), using Biorad dot blot apparatus, and Hybond-N membranes. The apparatus was made up of a perspex platform, covered by a layer of thin rubber sheeting, over which a baselayer of Whatmann 3MM filter paper was placed followed by the Hybond-N membrane, both papers being moistened in a solution of ammonium acetate and sodium hydroxide (2 M NH_4OAc, 0.04 M NaOH). Diluted DNA samples were loaded into the wells and washed through with standard sodium citrate buffer (3 M

NaCl, 0.3 M NaCi).

For dot blots, three liver samples were used from patients seropositive for both HBsAg and HBeAg, 14 from patients HBsAg positive but HBeAg negative, and two from patients seronegative for both markers (Table 2). Serum samples were also tested from one patient seropositive for both markers, and from nine HBsAg positive but HBeAg negative patients. HBsAg positive and negative control sera were also tested.

A series of dilutions to 100pg DNA for each of the test samples and controls was tested.

TABLE 2. Autopsy test samples used for dot blot hybridisation

Pt No	Diagnosis	HBV markers in blood			Sample origin	
		HBsAg	HBeAg	anti-e	liver	serum
1	chronic	+		+	+	
2	liver	+	+		+	+
3	disease	+			+	
4		+	+		+	
5	"	+		+	+	
6	"	+	+		+	
7	"	+			+	
8	"	+			+	
9	"	+		+	+	
10	cirrhosis	+		+	+	
11	"	+		+	+	
12	"	+		+	+	
13	"	+		+	+	
14	HCC	+		+	+	
15	"	+	+		+	
16	"	+			+	
17	"	+		+	+	
21	normal	-	-		+	
26	"	-	-		+	+
Pos control		+				+
Neg control		-				+

3.4 RESTRICTION ENZYME DIGESTION OF EXTRACTED BIOPSY LIVER DNA

Restriction enzyme digestion of extracted DNA was carried out prior to loading of samples onto the agarose gels. The enzymes used were Eco R1 and Hind 111 (Amersham). The amount of enzyme required to digest DNA depended upon the concentration of the enzyme in question. An example of a digest for a 20ul loading sample is given below.

Example:

If enzyme Eco R1 is supplied at 3000 units/500ul, then 1 ul is equivalent to 6 units and 1 unit cuts 1 ug of DNA, thus 2 ul of enzyme is equivalent to 12 units which can be used to digest 12 ug or less DNA.

In certain cases it was necessary to increase the amount of enzyme used to 5 units/ug of DNA to ensure complete digestion when incubated overnight. The completeness of digestion was monitored by adding 10ul aliquots of the digestion reaction mix to λ DNA (2 ug of λ DNA). The digested DNA was electrophoresed together with a molecular weight marker 111 (Boehringer Mannheim).

3.5 GEL ELECTROPHORESIS

A perspex horizontal gel apparatus was used (Bethesda Research Laboratories). DNA was electrophoresed in 1% agarose in tris-borate running buffer (89 mM tris-borate, 2.5 mM EDTA, pH 8.3 diluted 1:10 for use). The wells were loaded according to their size with a 1:6 dilution of

tracking dye (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol in water).

A Biorad powerpak (Type 500/200) was used to electrophorese the samples at a constant voltage of 30 V overnight. Ethidium bromide (10 mg/ml) was used as a staining dye at a concentration of 0.1 ul/ml agarose. The electrophoresed gel was examined under UV light to visualise the DNA bands and excess gel was trimmed off. The gel was placed under a plastic cover at $4^{o}C$ until required for Southern blotting.

3.6 SOUTHERN BLOTTING (Table 3)

The electrophoresed gel was transferred to a container for sequential washings in: 0.25 M HCl (2x 20 min), distilled water (10 min), denaturing solution 0.5 M NaOH in 1 M NaCl (2x 20 min), distilled water (5 min) and finally neutralised in 0.5 M Tris in 3 M NaCl pH 7.4 (30 min). DNA was subsequently transferred to the Hybond-N nylon membrane by the technique of Southern (Fig. 7) and the filter baked in a vacuum oven at $80^{o}C$ for 2 hr.

TABLE 3. Biopsy liver DNA used for Southern blot hybridisation

Pt.	HBV markers		Sample origin	
	HBsAg	HBeAg	Tumour	Liver
MJ	+	-	+	
FM	+	—	+	
PS	+	—	+	
RM	+	—		+
RM	+	—	+	
Sc	-	-		+
BN	+	+	+	
MD	+	+	+	
TV	+	+	+	
JM	+	+		+
JM	+	+	+	
McD	+	+	+	

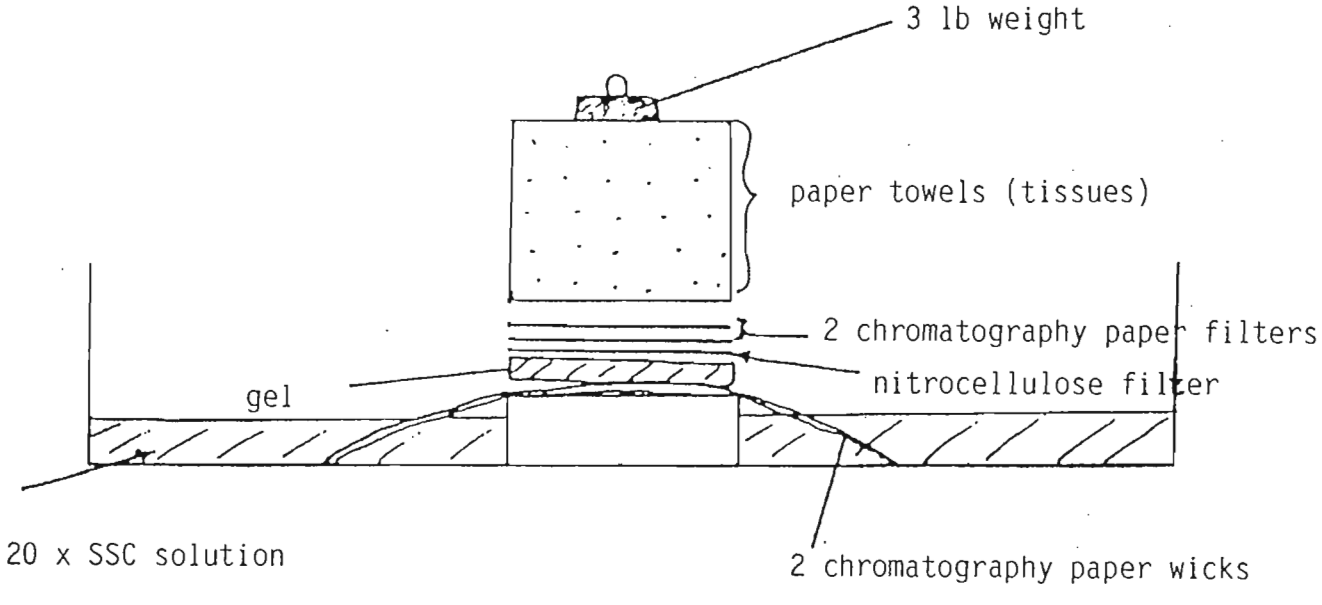


Fig. 7. Illustration of Southern transfer.

3.7 PREPARATION AND EXTRACTION OF THE HBV DNA PROBE

3.7.1 Preparation of the probe

The entire 3.3Kb HBV genome, cloned into the plasmid pBR325 by Anne Moriaty of Georgetown University, Washington DC and grown in transformed E. coli HB101 was obtained.

The HBV genome has been inserted into the chloramphenicol resistance site in pBR325. Tetracycline and ampicillin resistant colonies of E. coli HB101 containing the HBV DNA plasmid were replated twice onto nutrient agar plates in order to obtain pure colony growth. Either tetracycline, ampicillin or chloramphenicol was added to the warm nutrient agar before pouring of the plates. Single colonies from the original "tetracycline growth colony" plate, the original "ampicillin growth colony" plate and the original "chloramphenicol growth colony" plate were used to inoculate plates containing these respective antibiotic containing media. The plates were then incubated at $37^{o}C$ overnight.

As expected, colonies were found only on the ampicillin and tetracycline containing plates. One colony from each plate was used to inoculate 10 ml

nutrient broth, which was then incubated overnight at 37^{o}C with vigorous shaking.

3.7.2 Maxiprep extraction of the probe

The 10 ml overnight culture from above (3.7.1) was used to inoculate 1L of nutrient broth in a 2.5 L Erlenmeyer flask. This was then incubated with vigorous shaking at 37^{o}C for 3.5 hr until late log phase of growth was reached. Following this incubation, chloramphenicol was added at a concentration of 34 mg/ml (0.17g chloramphenicol was dissolved in 5 ml methanol) to inhibit chromosomal DNA synthesis. This culture mixture was further incubated at 37^{o}C for 21 hr with vigorous shaking. Each 1L volume was then decanted into 3 x 500 ml bottles and spun at 7 000 rpm for 20 min at 4^{o}C in a Beckman-J21 centrifuge using a JA-21 rotor. The pellets obtained were resuspended in 150 ml TE buffer and spun at 10 000 rpm for 20 min at 4^{o}C. The pellet was resuspended in 20 ml of 25% sucrose in 0.05 M Tris HCl, pH 8.0, 1 ml lysozyme (stock 10 mg/ml in 10 mM Tris-HCl, ph 7.5) was added and the bottle was left on ice for 30 min, 5 ml of Triton X-100 in Tris-HCl buffer (1 ml of Triton X-100 10%, 12.5 ml 0.05 M EDTA pH 8.5, 5 ml 1 M Tris-HCl pH 8.0, 81.5 ml sterile distilled water) was added

and incubated on ice for a further 10 min. After the addition of 0.85 ml of a solution of 10 mg/ml RNase, the mixture was spun at 20 000 rpm for 30 min at 4°C. The supernatant was poured into clean polyallomer tubes, 0.95 g/ml cesium chloride (CsCl) was added and the contents of the tube mixed well. Ethidium bromide was added to a final concentration of 1 mg/ml and the mixture spun at 45 000 rpm for 36 hr at 4°C in a Beckman L8-55 ultracentrifuge using a 50Ti rotor. The lower band of plasmid DNA visualised by UV light was sucked off into a sterile tube using a sterile 2 ml syringe with an 18g needle. Ethidium bromide was extracted from the DNA solution with equal volumes of isoamyl alcohol. This aqueous phase was measured into a sterile tube and 2 volumes of sterile distilled water (in order to dilute the CsCl), plus 2 volumes of ethanol were added and the contents of the tube mixed well. The tubes were covered with parafilm and left at -20°C overnight. The precipitated DNA was pelleted at 3 000 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol in TE buffer and re-spun at 3 000 rpm for 25 min at 4°C. The final pellet was resuspended in TE buffer. The DNA concentration was calculated and the sample stored at -20°C. Purified HBV DNA was obtained by Eco R1 digestion, agarose electrophoresis and gel extraction.

3.8 RADIOISOTOPE LABELLING OF THE PROBE BY NICK TRANSLATION

Purified HBV DNA was nick translated with ^{32}P dCTP to a specific activity of 1 x10^8. The DNA sample to be labelled was dissolved either in distilled water or in TE buffer to a final concentration of 200 ug/ml, to dilute impurities which might interfere in the labelling reaction. Appropriate volumes of DNA solution, nucleotide/buffer solution, radioactively labelled ^{32}P dCTP, water and enzyme solution were added sequentially to a 1.5 ml microfuge tube which was placed in an ice bath. The tube was capped and the contents mixed gently. This tube was then placed in a constant temperature bath carefully controlled at 15°C, to avoid the generation of "snap back" DNA which may be formed by DNA polymerase copying the newly synthesised strand at higher temperatures (Fig. 6).

The progress of the reaction was monitored to determine the exact reaction time giving the highest specific activity. To monitor counts as well as to measure the percentage incorporation of the label, 2 ul of the reaction mix was added to 100 ul of carrier DNA (stock solution of 50 ug/ml). Of this, 10 ul was spotted onto a GF/C filter paper for total counts. To the remaining 90 ul reaction mix, 2-4 ml of 10% cold TCA was added, the tube placed in an ice bath for 10 min and the precipitated DNA collected by vacuum filtration on a GF/C filter disc. The

filters were counted for 1 min using the 3H channel of the Beckman LS-250 scintillation counter without scintillation fluid.

$$\% \text{ incorporation} = \frac{\text{ppt counts} \times 100}{\text{total counts} \times 9}$$

The labelled probe was passed through a column of Sephadex G 50 in order to separate labelled from unlabelled nucleotides.

Pure vector pBR325 was also labelled and used as a negative control to probe the dot blots.

3.9 HYBRIDISATION OF PROBE TO SOUTHERN AND DOT BLOTS

A modification of Maniatis's hybridisation method was applied to dot blot hybridisation of autopsy liver DNA.

The Southern blots of biopsy liver DNA (Table 3) were hybridised as follows:

The blot was labelled, wet in 2 x SSC and placed in a plastic bag. To the 5 ml prehybridisation solution (10 ml 1 M Hepes pH 7.0, 20 ml 100 x Denhardts, 0.2 ml poly A (10 mg/ml, 30 ml 20 x SSC, 2 x stock diluted 1:1 with deionised formamide), 5 ml of deionised formamide and 100 ul of herring sperm DNA (5 mg/ml) was added. This premix was prewarmed to 42^{o}C and added to the blot which was prehybridised for 6 hr at 42^{o}C. The final hybridisation solution was prepared by adding 2.5 ml of

the prehybridisation solution (to which 20 g of dextran sulphate in 30 ml of water was added), to 2.5 ml of deionised formamide and 100 ul of herring sperm DNA. The premix was removed and the warmed hybridisation mix with the denatured labelled probe was added to the blot which was allowed to hybridise for 24 hr at 42^{o}C. The filters were removed, rinsed twice in 2 x SSC for two periods of 30 min each. Prewarmed 0.1 x SSC, 0.1% SDS was then added and the filters were shaken at 55^{o}C for two periods of 30 min each. The blots were finally rinsed in cold 0.1 x SSC, 0.1% SDS for two periods of 30 min each. The blots were then taped onto a filter paper, the position of the gel slots marked with labelled "blue ink", the blots sealed in plastic wrap and exposed to a 3M X-ray plate at -70^{o}C overnight.

CHAPTER 4 RESULTS

HBV DNA has been strongly implicated as having a causal role in HCC, the presence of HBV DNA in cirrhosis being reported for the first time by Koshy et al., in 1981. It is therefore to be expected that valuable information regarding the pathogenesis of acute and chronic liver disease following HBV infection could be obtained from studies on the state of HBV DNA in the infected liver cells.

4.1 DOT BLOT HYBRIDISATION

Of the 17 HBsAg positive autopsy patients, 13 (76%) showed the presence of HBV DNA in the liver (6/9 with chronic liver disease, 4/4 with cirrhosis, and 3/4 with HCC).

Visual examination of the dot blots indicates that patients positive for both HBsAg and HBeAg show the strongest hybridisation signals (Figs. 8 and 9). Thus one could surmise that there is a greater concentration of HBV DNA in the liver of these patients. It is therefore possible that, for these patients, the positive hybridisation on the dot blots was due to replicative HBV or episomal HBV, suggesting a high infectivity and active liver disease.

All but one of the remaining ten HBV DNA hybridisation positive patients have HBsAg values one degree of magnitude greater than the values seen in the HBsAg positive patients for whom no HBV DNA integration could be detected (Table 4).

The RIA values effectively divide the patients into three main categories. The first contains patients with extremely high values for HBsAg who were additionally HBeAg positive and who were all strongly positive for HBV DNA hybridisation; the second category includes HBeAg negative patients with a lower level of HBsAg positivity but also positive for HBV DNA hybridisation; the third category consists of patients negative for both HBsAg and HBeAg and with no evidence of HBV DNA association. In this study there therefore appears to be an inverse relationship between HBsAg status and the presence of HBV DNA in the liver as determined by dot blot hybridisation with the labelled probe.

All of the control patients with normal livers were negative for HBsAg, HBeAg and HBcAb. They could thus be assumed to have had no exposure to HBV. For most of these patients dot blot hybridisation was negative even when a 12 ug DNA sample was used. However some reactivity, which was attributed to non-specific binding of the probe, was

observed in one case (Fig. 9), and all positive results were therefore taken from the second row where 1 ug DNA samples were loaded.

As it had been found necessary to use entire pBR325 HBV DNA as the probe in these experiments a further control consisting of ^{32}P pBR325 was introduced (Maniatis et al., 1982; Steyn, 1986). However, attempts to hybridise the labelled plasmid to samples of DNA showing positive hybridisation with ^{32}P pBR325 HBV DNA were unsuccessful and it was therefore concluded that HBV specific hybridisation had been obtained when using the entire ^{32}P pBR325 HBV DNA probe.

TABLE 4. HBsAg and HBeAg status related to the detection of HBV DNA in autopsy liver on dot blot hybridisation with the labelled probe

Pt No.	RIA values		HBV DNA
	sAg	eAg	hybridisation
2	11300	3000	+
3	11186	3689	+
6	10067	3988	+
15	9758	4312	+
1	8613	-	+
5	7435	-	+
10	7428	-	+
12	7400	-	+
9	7326	-	+
11	7100	-	+
13	6549	-	+
17	6416	-	+
14	452	-	+
4	399	-	-
7	307	-	-
16	286	-	-
8	203	-	-

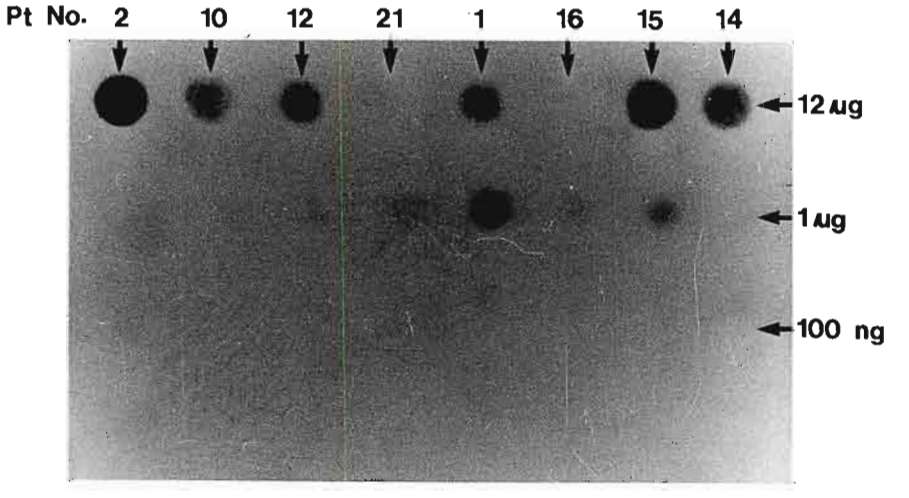


Fig. 8. Dot blot hybridisation of autopsy liver DNA.

HBsAg positive:
Pts 1,2 : hepatitis liver DNA
Pts 10,12 : cirrhotic DNA
Pts 14,15,16: HCC DNA

HbsAg negative:
Pt 21 : normal liver DNA.

Patients 2 and 15 were additionally HBeAg positive.

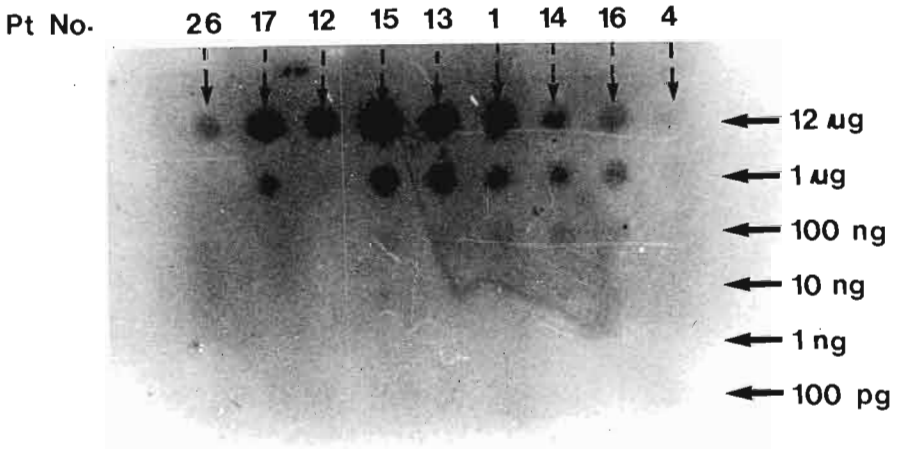


Fig. 9. Dot blot hybridisation of autopsy liver DNA.

HBsAg positive:
Pt 1,4 : hepatitis DNA
Pt 13,12 : cirrhotic DNA
Pt 14,15,16 : HCC DNA

HBsAg negative
Pt 26 : normal liver DNA.

Patient 15 was additionally HBeAg positive. Patient 26 shows non-specific binding at 12 ug DNA but is negative for hybridisation at 1 ug DNA.

4.2 GEL ELECTROPHORESIS

Gel electrophoresis of autopsy liver DNA produced inconsistant banding patterns and was thus unsuccessful. According to Dr. J. Dusheiko (pers comm), this was due to the long time interval between death and post mortem examination. This time interval should be as minimal as possible (1/2 to 1 hr) to avoid loss of DNA as well as degradation.

Completeness of digestion of cellular DNA by the enzymes Eco R1 and Hind 111 was monitored by the addition of λ DNA to the samples. Electrophoresed gels of digested DNA were examined for the different banding patterns produced with the restriction enzymes (Fig. 10).

Gel electrophoresis of undigested and digested biopsy liver DNA produced smears extending from approximately 21 Kbp to below 0.125 Kbp (Fig. 11), which is apparently typical of liver DNA (Dr. J. Dusheiko pers. comm.). Molecular weight marker 111 (Boehringer Mannheim) was electrophoresed together with the liver DNA so as to determine the size of any fragments observed on hybridisation.

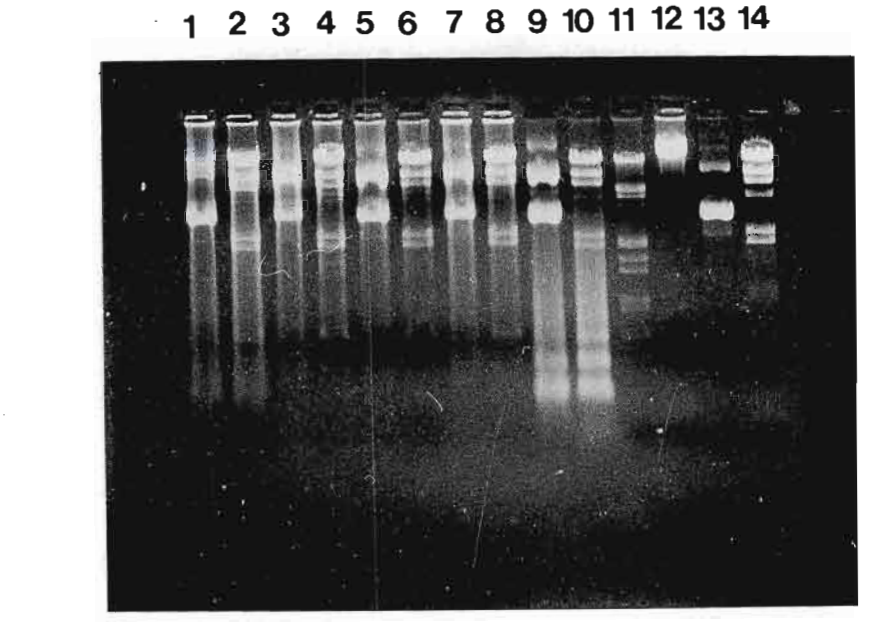


Fig. 10. Gel electrophoresis of Eco R1 and Hind 111 digested DNA.

Lanes 1,3,5,7,9 : Eco R1 digested biopsy liver DNA + λ DNA

Lanes 2,4,6,8,10 : Hind 111 digested biopsy liver DNA + λDNA

Lane 11 : molecular weight marker 111

Lane 12 : undigested λ DNA

Lane 13 : Eco R1 digested λ DNA

Lane 14 : Hind 111 digested λ DNA.

The patterns obtained with the Eco R1 and Hind 111 digested λ DNA indicates that complete digestion has taken place. Thus the conditions used for enzyme digestion of the biopsy liver/tumour DNA were optimal.

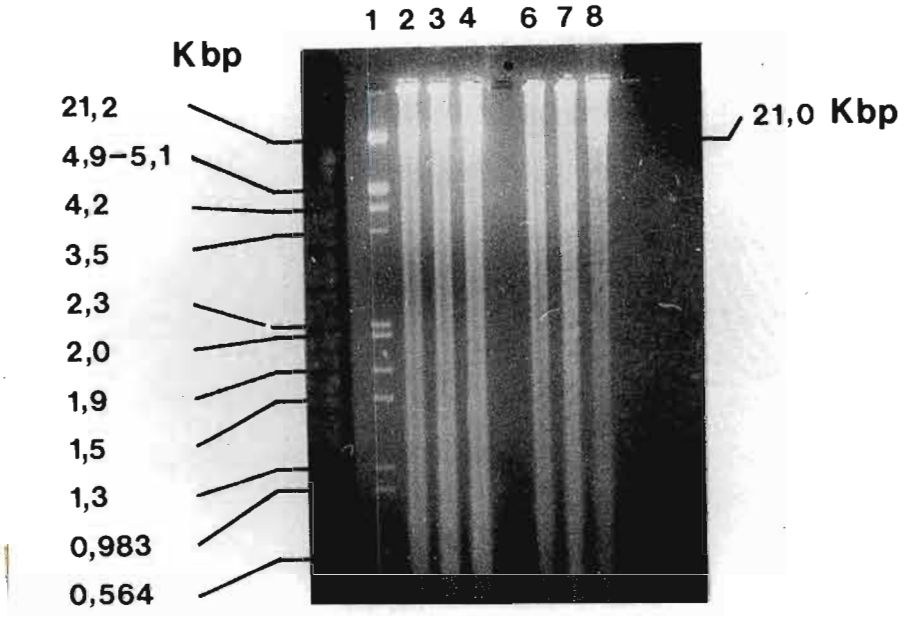


Fig. 11. Gel electrophoresis of biopsy liver DNA.

Lane 1 : molecular weight marker 111

Lanes 2,3,4 : Pt BN Eco R1, Hind 111 and undigested DNA

Lanes 6,7,8 : Pt TV Eco R1, Hind 111 and undigested DNA

In the above example the smears are indicative of total cellular DNA. Bands are visible in the region of 21 Kbp, which continue as smears down the respective lanes to below 0.564 Kbp. The conditions for digestion were as for the samples in Fig. 10.

4.3 HBV DNA Integration (Southern blotting)

There appears to be no simple correlation between HBV markers in the serum and HBV DNA in the tumour. HBV DNA integration into tumour tissue was originally observed only in patients who were HBsAg positive (Shafritz and Kew, 1981). However, Lugassy et al., (1987), have reported the presence of HBV DNA sequences in liver samples of patients who were HBsAg negative and also in patients who had no HBV markers at all. The HBV DNA sequences included free monomeric and integrated forms similar to the restriction patterns seen in HBsAg positive patients.

The various forms of HBV DNA in liver biopsies were examined using the enzymes Hind 111 and Eco R1, which cleave HBV DNA at zero and one site respectively. The restriction patterns obtained suggest the presence of replicative HBV DNA intermediates with the presence of single stranded HBV DNA and thus ongoing HBV replication.

If HBV DNA is integrated into cellular DNA, both undigested and Hind 111 digested material will produce a diffuse hybridisation smear in the high molecular weight region, as Hind 111 digestion will produce bands greater than HBV genome size ($>$3.3 Kb). However, if HBV DNA is not integrated, undigested and digested total cellular DNA will have the same banding pattern and hybridisation will occur in the low molecular weight region of the gel

at or below the position of 3.3 Kbp. Following Eco R1 digestion, sequences greater than genome size (> 3.3Kb) showing a hybridisation signal, must have been derived from integrated HBV DNA (Shafritz et al., 1981)

Hybridisation signals at the 3.2Kb position are indicative of cloned monomeric HBV DNA. Smears of DNA located in the low molecular weight region reflect the presence of single-stranded (0.5 - 1.5 Kb) and partially double stranded replicative intermediates (2 - 2.8 Kb) (Lugassy et al ., 1987).

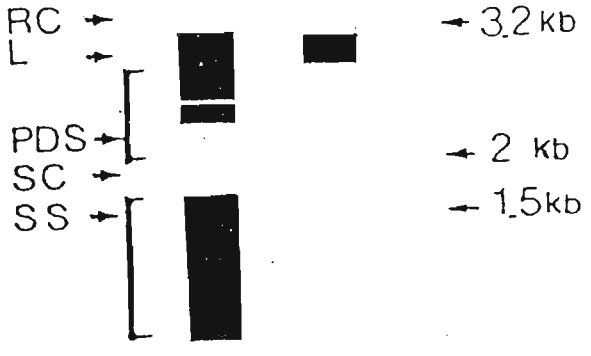


Fig. 12. The various forms of HBV DNA (and their respective sizes) which can be present in the liver following HBV infection: RC - relaxed circular form, L - linear, PDS - partially double stranded, SC - super-coiled and SS - single- stranded HBV DNA. Below 3.2 Kb and above 2 Kb the linear and partially double stranded forms of the virus are present (Lugassy et al., 1987).

Failure to detect HBV DNA integration by Southern blot hybridisation does not necessarily indicate that no integration has occurred. Boender et al., (1985), have suggested that failure to detect specific integration on Southern blots could be due to the presence of insertions or deletions occurring during or after the integration into the host cell genome. Also Bowyer et al., (1987) have shown that the HBV DNA copy number is crucial in Southern blot analysis. They found that samples with a low HBV DNA copy number failed to show HBV DNA hybridisation on blots.

4.3.1 HBV DNA status in patients who were HBsAg and HBeAg positive.

The results of the Southern blots indicated the presence of HBV DNA in the liver of 5/5 (100%) HCC patients with this marker profile. Two HCC patients (BN and MD) showed integrated as well as free replicative forms of HBV DNA in the tumur tissue (eg Pt MD, Fig. 13). This could be due to some cells having integrated the HBV DNA whereas others still have the replicative forms present. Another possibility is that there is a transition from HBeAg positivity to the anti-HBe status which could mean that the patient is no more infectious and is moving towards a chronic carrier state with a percentage of HBV DNA being integrated into the cellular DNA.

A further two patients (McD, Fig. 14 and TV, Fig. 15) showed only replicative intermediates in tumour tissue. These replicative intermediates appear to include relaxed circular, partially double stranded, supercoiled and single stranded forms of the virus ie freely replicating episomal HBV DNA.

The last patient (JM) showed extrachromosomal HBV DNA in the liver (Fig. 16), however, for the same patient there was no detectable HBV DNA in the tumour.

Two of the patients with integrated HBV DNA (eg Pt MD Fig. 13), showed a specific banding pattern on Hind 111 digestion suggesting HBV DNA integration into specific sites on the cellular genome, and supporting the results of Shafritz et al., 1981).

Fig. 13.

A comparison of the enzyme digests with the undigested material indicates integration of HBV DNA as hybridisation signals continue to appear in the high molecular weight region (21 Kbp). An examination of the Hind 111 lane shows at least two hybridisation bands greater than HBV genome size, suggesting integration into specific sites on the host cell genome.

There is also a diffuse hybridisation smear down the lane, indicating a range of forms of HBV DNA, with a strong band of supercoiled DNA at the 2 Kbp position. Thus the tumour cells of this patient contain both integrated and/or free HBV DNA.

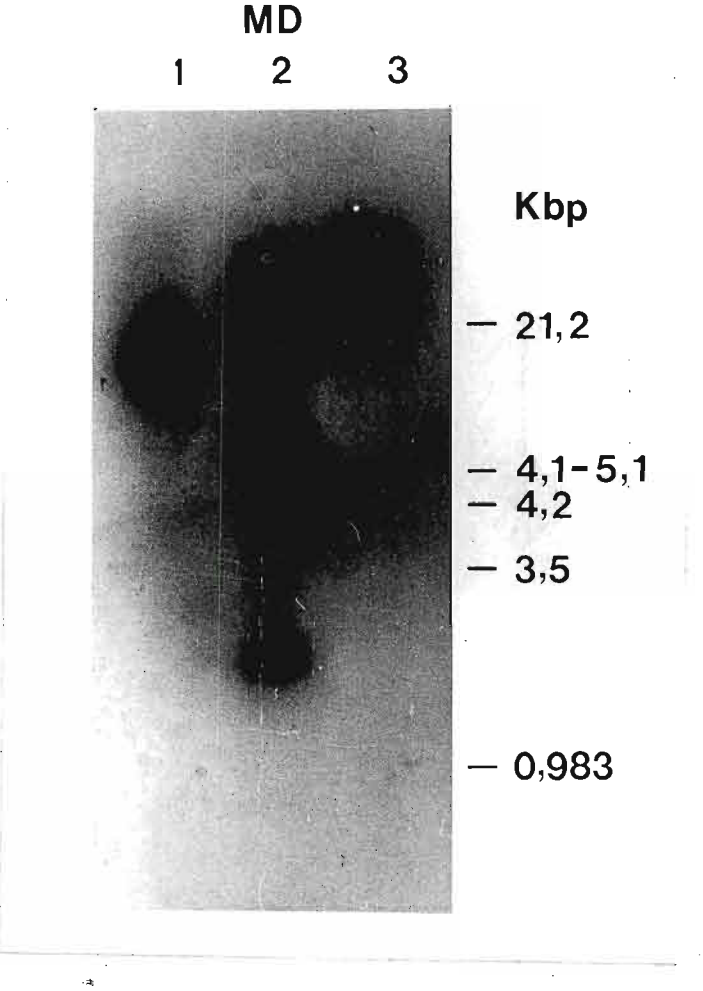


Fig. 13.

Patient MD - Southern blot analysis of HBV DNA in tumour tissue.

Lane 1 : Eco R1 digest

Lane 2 : Hind 111 digest

Lane 3 : undigested tumour DNA

Fig. 14.

There appears to be no integration of HBV DNA into host cell chromosomal DNA as seen by the absence of a hybridisation signal in the high molecular weight region of the blot. However, free replicative intermediates of HBV DNA are clearly present in the region of 3.2 Kbp to below 1.5 Kbp. These intermediates include relaxed circular, partially double stranded, supercoiled and single stranded forms. In addition a strong band is seen at the 5.1 Kbp position. However, as this band occurs in all three lanes it is not considered equivalent to the 5.1 Kbp band in the Hind 111 lane of Fig. 13. Rather it might reflect the presence of HBV DNA covalently linked to cellular DNA, or may be due to multimeric or free dimeric HBV DNA. According to Lugassy et al., (1987), these viral forms are associated with free HBV DNA.

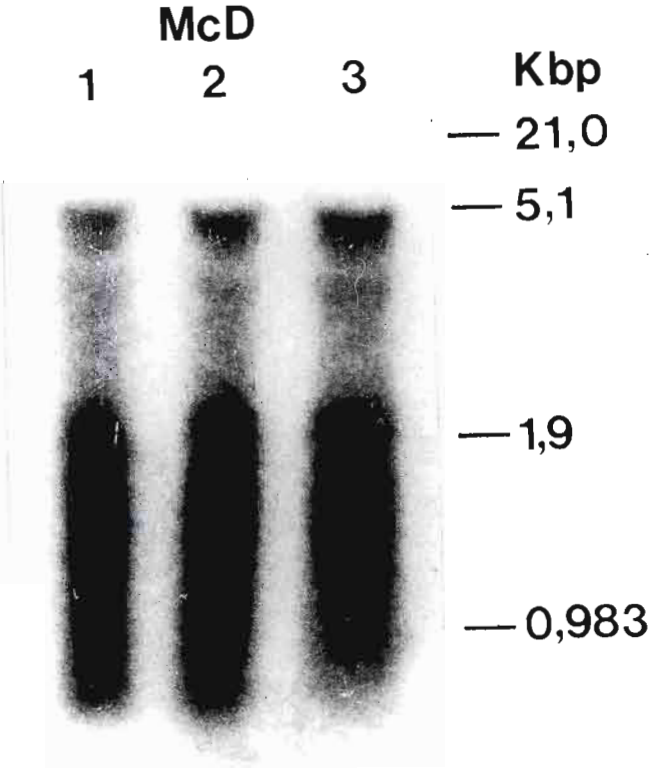


Fig. 14.

Patient McD - Southern blot analysis of HBV DNA in tumour tissue.

Lane 1 : Eco R1 digested tumour DNA

Lane 2 : Hind 111 digest

Lane 3 : undigested tumour DNA

Fig. 15

There is no high molecular weight hybridisation signal in any lane, indicating an absence of HBV DNA integration. In each of the lanes a diffuse hybridisation pattern appears as a smear extending from 3.5 Kbp to below 0.983 Kbp. However, HBV DNA sequences are present at the same position as double stranded full length HBV DNA (3.2 Kbp) and it is possible that single stranded (1.5 Kbp) and partially double stranded sequences are present in the lower base pair region.

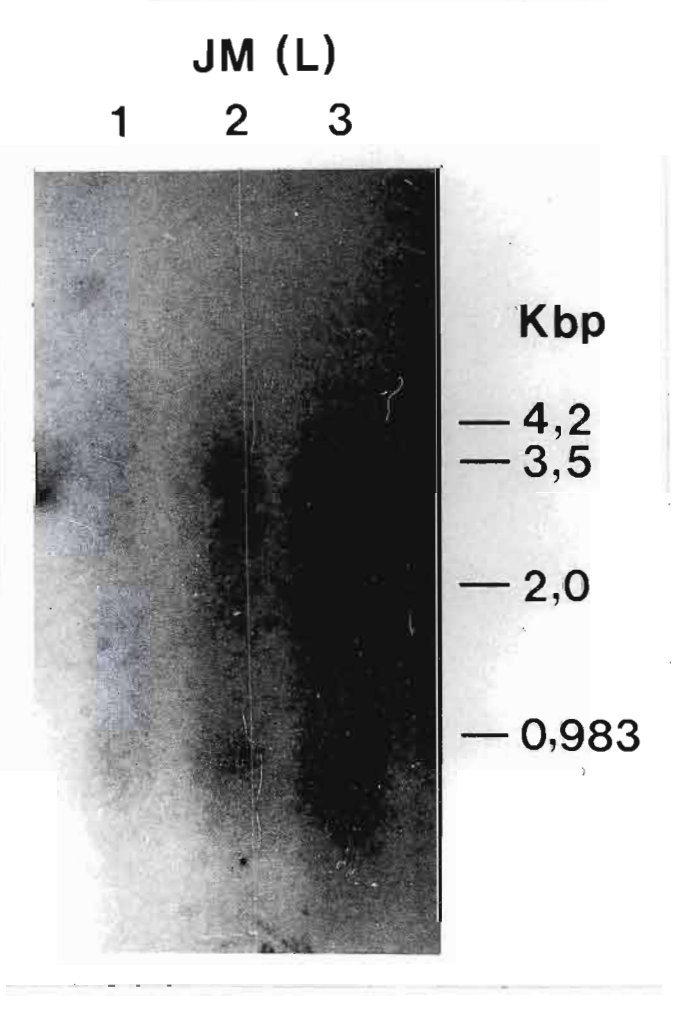


Fig. 15.

Patient JM(L) - Southern blot analysis of HBV DNA in liver tissue.

Lane 1 : Eco R1 digest

Lane 2 : Hind 111 digest

Lane 3 : undigested liver DNA

Fig. 16.

There is no hybridisation in the high molecular weight region indicating the absence of HBV DNA integration. In each lane a diffuse hybridisation pattern is seen as a smear extending from 3.5 Kbp to below 0.983 Kbp, indicating free extrachromosomal HBV DNA in the form of relaxed circular HBV DNA at the 3.2 Kbp position and linear forms of the virus below this position followed by the partially double stranded forms. There is also evidence of supercoiled forms at 2 Kbp and single stranded HBV DNA at 1.5 Kbp positions.

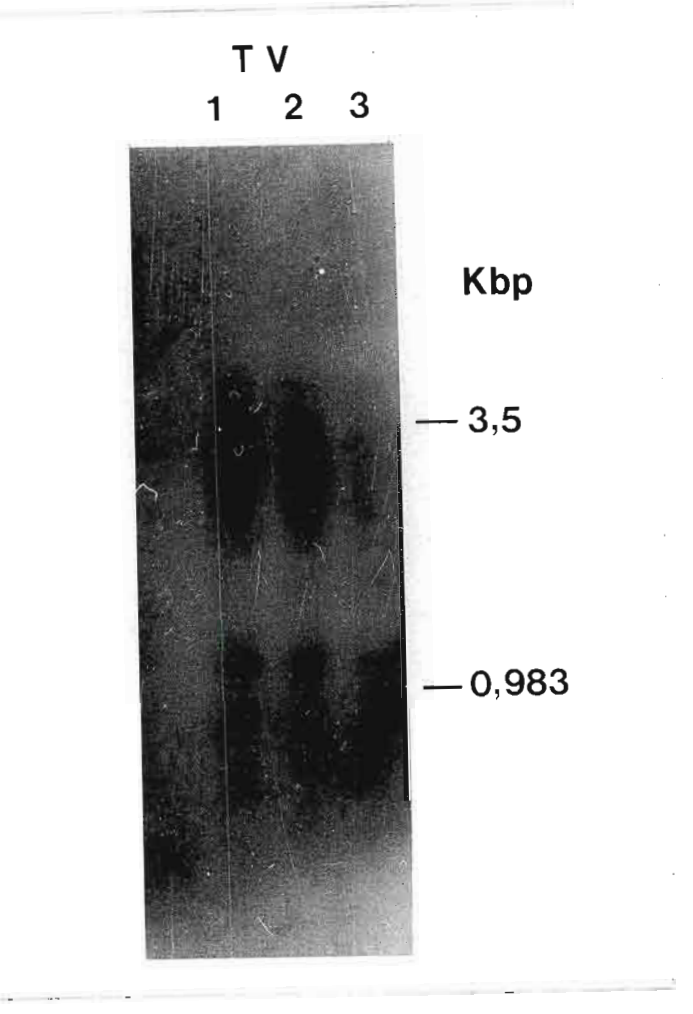


Fig. 16.

Patient TV - Southern blot analysis of HBV DNA in tumour tissue.

Lane 1 : Eco R1 digest

Lane 2 : Hind 111 digest

Lane 3 : undigested tumour DNA

4.3.2 HBV DNA status in HBsAg positive, HBeAg negative patients

3/4 (75%) tumour patients who were HBsAg positive and HBeAg negative showed hybridisation signals on the Southern blots which indicated integrated HBV DNA sequences (Pts. FM, MJ and PS, Figs. 17 and 18). All of these patients appeared also to have replicative intermediates (eg Pt FM Fig. 18). This is in contrast to the results of Lugassy et al., (1987), who did not find any patients who had both integrated as well as free viral DNA. This pattern apparently reflects low viral replication. A specific band greater than HBV genome size was again present for the Hind 111 digested material, suggesting specific site integration of HBV DNA into the human genome.

The finding of HBV DNA integration in these patients who were HBsAg positive and HBeAg negative could carry the following implications:

(a) 'e' Ag is totally absent and only s genes are expressed either with the entire HBV genome being integrated or with only s genes integrated

(b) It is possible that 'e'Ag is actually present but in small amounts undetectable by the

conventional radioimmunoassay.

This may be due to integration of the entire HBV genome with both s gene and minimal e gene expression, or to integrated s gene expression together with expression of extrachromosomal s and e genes.

Fig. 17.

Diffuse hybridisation with high molecular weight material greater than HBV genome size (4.21 - 21 Kbp) is seen in the Eco R1 digest, with more distinct regions of high molecular weight hybridisation appearing in the Hind 111 digest and the undigested DNA, indicating the presence of integrated HBV DNA. A diffuse hybridisation smear of extrachromosomal HBV DNA is also seen extending down the lanes.

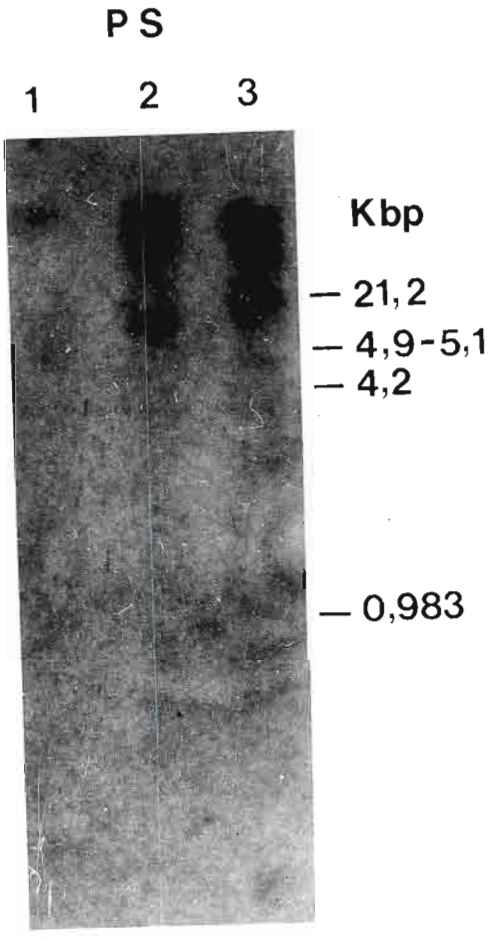


Fig. 17.

Patient PS - Southern blot analysis of HBV DNA in tumour tissue.

Lane 1 : Eco R1 digest

Lane 2 : Hind 111 digest

Lane 3 : undigested tumour DNA

Fig. 18.

The presence of high molecular weight hybridisation signals in all three lanes indicates that integrated HBV DNA was present in the tumour. The Hind 111 digest is similar to that seen in Fig. 17. The Eco R1 digest suggests the presence of concatomers or oligomers of HBV DNA as well as some replicative intermediates smaller than genome size.

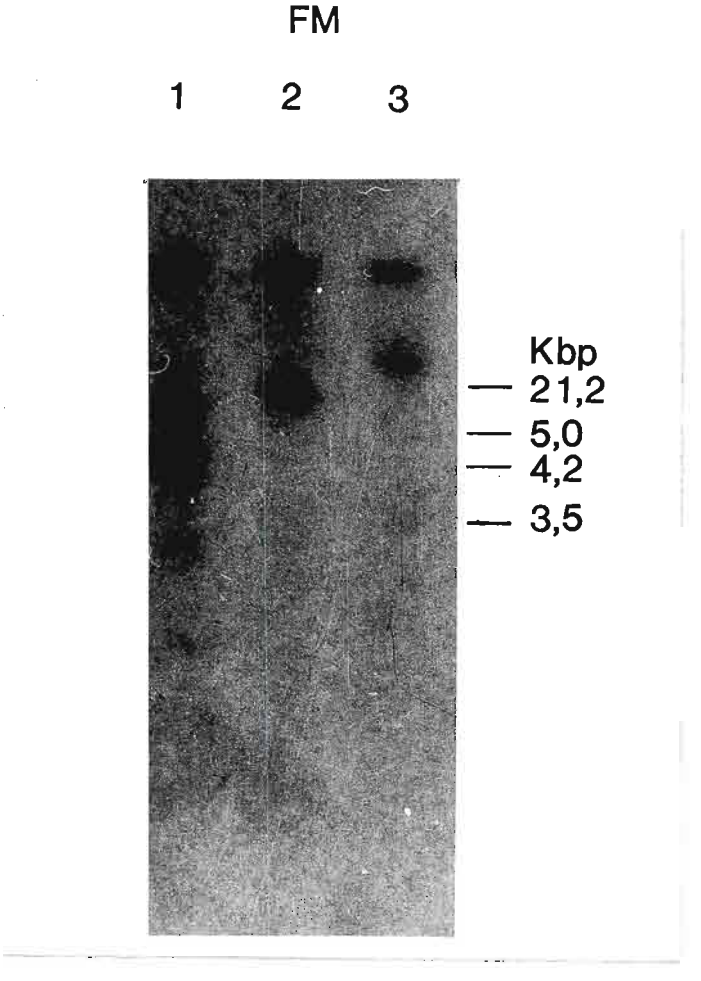


Fig. 18.

Patient FM - Southern blot analysis of HBV DNA in tumour tissue.

Lane 1 : Eco R1 digest

Lane 2 : Hind 111 digest

Lane 3 : undigested tumour DNA

TABLE 5. Summary of HBV DNA status in biopsy liver/tumour specimens

Pt	HBV marker sAg/eAg		HBV DNA status in liver Integrated /	extrachromosomal
BN	+	+	+	+
TV	+	+	-	+
MD	+	+	+	+
McD	+	+	-	+
JM(L)	+	+	-	+
JM(T)	+	+	-	-
Sc	-	-	-	-
PS	+	-	+	+
MJ	+	-	+	+
FM	+	-	+	+
RM(L)	+	-	-	-
RM(T)	+	-	-	-

CHAPTER 5 DISCUSSION

Viral infectivity during the acute and chronic phases of HBV infection is estimated by the detection of HBV markers. However, these provide an indirect estimation of active viral replication. The determination of HBV DNA in the serum of infective carriers is a more direct and quantitative analysis of the presence of Dane particles. In a study by Brechot et al., (1981), HBV DNA was found in 70 - 95% of HBeAg positive sera from HBsAg chronic carriers. Positive HBV DNA in the serum corrrelates closely with replicative HBV infection, and also correlates with the presence of replicative intermediates in liver tissue and thus with active liver disease (Matsuyama et al., 1985).

In the course of this study molecular biology techniques and methods which could be used in the routine determination of the association of HBV with hepatitis, cirrhosis and HCC were utilised. Dot blot and Southern blot methods of hybridisation proved useful in detecting HBV DNA sequences in the diseased liver of patients seropositive for HBsAg, thus supporting an aetiological role for HBV.

HBV DNA in the liver was detectable by dot blot hybridisation in (13/17) 76% of the autopsy patients who were HBsAg

positive. Even although dot blot hybridisation does not distinguish integrated from non-integrated forms of HBV DNA, it is a relatively quick and easy method involving the direct use of the extracted DNA from the tissue, and would thus be readily applicable in the routine diagnostic laboratory.

HBV DNA hybridisation was not detected by Southern blot analysis of autopsy liver material. The same problem was reported by Shafritz et al., (1981), who found that extensive liver fibrosis, as well as DNA degradation due to the long time interval between death and post mortem examination may have led to dilution of HBV sequences and thus negative results on their blots.

However, Southern blot hybridisation proved useful for the analysis of liver biopsy material, distinguishing integrated from non-integrated HBV DNA sequences with the use of the enzymes Eco R1 and Hind 111. Hind 111 digestion has shown that of the tumour biopsy patients 5/9 (56%) had integrated HBV DNA sequences. These results tend to confirm those of Bonino et al., (1981), that HBV DNA in the liver is predominantly integrated in patients with HBV - related hepatocellular carcinoma. However, in this study low molecular weight sequences of HBV DNA (replicative intermediates) were also detected in all 5 tumour patients with integrated sequences.

Bowyer et al., (1987) detected episomal but not integrated HBV DNA in HBeAg positive carriers of HBsAg. In contrast, the results of this study showed that while all five patients with this marker pattern had episomal HBV DNA in the liver or tumour tissue, the hybridisation patterns for two of these patients indicated that integration of HBV DNA had also taken place.

Conversely the three HBsAg - positive HBeAg - positive patients with integrated HBV DNA were simultaneously carrying episomal DNA in the tumour tissue, although it might be expected that viral replication would have ceased in these patients (Bowyer et al., 1987).

Nevertheless, regardless of inferences which might be drawn from the presence of episomal HBV DNA, the detection of integrated HBV DNA in cellular DNA of tumour tissue supports the hypothesis that HBV is a factor in human hepatocellular carcinoma.

With chronic alcohol abuse or cirrhosis the sensitivity of the liver to exposure to agents with oncogenic potential is increased (Beasley and Hwang, 1984), and this could explain possible progression to HCC. The association of HBV with HCC has best been investigated by epidemiologic studies, since studies of HBV gene expression have not revealed the presence

of a transforming gene or an associated oncogene (Murray et al., 1981). In order to prove that integrated HBV DNA may have a causal role in the development of neoplasia, it will first be necessary to demonstrate in tissue culture the expression of integrated HBV genes following HBV infection (Wen et al., 1983), since it is possible that integration of the HBV DNA sequence results in the alteration of adjacent cellular gene expression. However, the majority of experiments relating to HBV DNA integration have used human fibroblast or monkey kidney cells as the recipient cell whereas the most appropriate cells to use would be cultured human hepatocytes (Sherman and Shafritz, 1984).

By the recombinant DNA techniques used in this study one could routinely detect integration of HBV DNA into the human genome. It is possible that detection of integrated HBV DNA could result in treatment leading to the subsequent decrease of HBV DNA copy number and thus result in a probable decrease in percentage integration (Boender et al., 1985). Thus since integrated HBV DNA has been implicated as a cofactor in HCC, a decrease in this integration could probably result in decreased progression to HCC and a longer lifespan for the affected individual.

However, if HBV DNA integration is in some way responsible for the high levels of morbidity and mortality associated with

chronic acute hepatitis, cirrhosis and HCC in many parts of the world the best approach to reduction of such morbidity and mortality would be the prevention of HBV infection in the first place.

Definitive control of HBV infection would require an extensive world-wide programme of vccination. Unfortunately, the currently available hepatitis B vaccine which is produced from human plasma containing HBsAg does not provide the appropriate means as the costs involved in the production of the vaccine are prohibitive for its use in developing countries which have most need for it. However, recombinant DNA technology enables the synthesis of HBsAg by the cultivation of microorganisms and thus could guarantee a continued inexpensive supply of uniform antigens for vaccine use.

The process of gene cloning in genetic research has sparked a new revolution in modern biology, involving the use of living organisms or subcellular components derived from living organisms. Gene cloning has the capacity to make direct alterations to the characteristics of a living organism and also to study these alterations at molecular level. Thus these techniques applied to the study of HBV infection could provide possible answers as to the mechanisms and effects of HBV DNA integration into the human genome, and thereby lead to a better understanding of the pathogenesis and development of HCC, as well as suggesting possible lines of approach for therapeutic intervention at the cellular level or for control of initial HBV infection.

REFERENCES

1 Alexander JJ, Marion PL, Salazar FH, Robinson WS (1980): State of hepatitis B viral DNA in a human hepatoma cell line. J. Virol. 35: 795.

2 Amersham Research Laboratories, Nick Translation, Southern Blotting and Restriction enzyme manuals available with kits (1985 - 1986).

3 Beasley PR, Hwang Lu-Yu (1984): Hepatocellular Carcinoma and Hepatitis B virus. Seminars in Liver Disease 4(2): 113-121.

4 Beasley PR (1983): Hepatitis B virus as the Etiologic agent in Hepatocellular Carcinoma - Epidemiologic Considerations. Hepatology 2(2): 21-26.

5 Blumberg BS, Alter HJ, Visnich S (1965): Hepatitis B virus. J. Am. Med. Assoc. 191: 541-46.

6 Blumberg BS (1977): Australia antigen and the biology of hepatitis B. Science 197: 17-25.

7 Boender PJ, Schalm SW, Heijtink RA (1985): Detection of Integration During Active Replication of Hepatitis B virus in the Liver. J. Med. Virol. 16: 47-54.

8 Bonino F, Hadchouel M, Scotto J, Degos F, Charnay P, Trepo C, Tiollais P, (1981): Detection of hepatitis B virus in liver and serum: A direct appraisal of the chronic carrier state. Lancet ii: 765-768.

9 Boffey SA (1983): Gel electrophoresis of DNA. In : Walker GM, Gaastra W (eds). "Techniques in Molecular Biology". Leaper and Gard Ltd. Bristol.

10 Bowyer SM, Dusheiko GM, Schoub BD, Kew MC (1987): Expression of the hepatitis B virus genome in chronic hepatitis B carriers and patients with hepatocellular carcinoma. Proc. Nat. Acad. Sci. USA 84: 847-850.

11 Brechot C, Pourcel C, Louise A, Rain B, Tiollais P (1980): Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. Nature 286: 533-535.

12 Brechot C, Hadchouel M, Scotto J, Fonck M, Potet F, Vyas GN, Tiollais P (1981): State of hepatitis B virus DNA in hepatocytes of patients with hepatitis B surface antigen-positive and -negative liver disease. Proc. Nat. Acad. Sci. USA 78: 2906-2910.

13 Burrell CJ, Mackay P, Greenaway PJ, Hofschneider PH, Murray K (1979): Expression in Escherichia coli of hepatitis B virus DNA sequences cloned in plasmid pBR322. Nature 279: 43-47.

14 Chakraborty PR, Ruiz-Opazo N, Shouval D, Shafritz DA (1980): Identification of integrated hepatitis B virus DNA and expression of viral RNA in an HBsAg-producing human hepatocellular carcinoma cell line. Nature 286: 531-533.

15 Chakraborty PR, Ruiz-Opazo N, Shafritz DA (1981): Transcription of human hepatitis B virus core antigen gene sequences in an in vitro Hela cellular extract. Virology 111: 647-652.

16 Dane DS, Cameron CM, Briggs M (1970): Virus like particles in serum of patients with Australia A antigen associated hepatitis. lancet ii: 695-698.

17 Dejean A, Brechot C, Tiollais P, Wain-Holson S (1983): Characterisation of integrated hepatitis B virus DNA cloned from a human hepatoma cell-line PLC/PRF/5. Proc. Nat. Acad. Sci. USA 80: 2505-2509.

18 Dusheiko J (1987). Personal communication.

19 Edman JC, Gray P, Valenzuela P, Raqll LB, Rutter WJ (1980): Integration of hepatitis B virus sequences and their expression in a human hepatoma cell line. Nature 286: 535-538.

20 Evans K (1982): "Viral Infections of Humans". 2nd ed. Plenum Medical. p338-339.

21 Freifelder D (1983): "Molecular Biology" - A comprehensive introduction to prokaryotes and eukaryotes. Van Nostrand Rheinhold Publication, U.K.

22 Hino O, Kitagawa T, Koike K, Kobayashi M, Haraq M, Mori W, Nakashima T, Hattori N, Sugano H (1984): Detection of hepatitis B virus in hepatocellular carcinoma in Japan. Hepatology 4: 90-95.

23 Hwang Ly, Beasley RP, Stevens CE, Szmuness W (1983): Immunogenicity of HBV vaccine response of healthy Chinese children. Vaccine 1: 10-12.

24 Kafatos FC, Jones CW, Efstratiadis A (1979): Determination of nucleic acid sequence homologues and relative concentrations by a dot blot hybridisation procedure. Nucl. Acids Res. 7(6): 1541-1552.

25 Kew MC (1978): A review of viral hepatitis. J. Mod. Med. 4(3): 34-35.

26 Kew MC (1983): Viral hepatitis. S. Afr. J. Cont. Med. Educ. 1(1): 30-38.

27 Koshy R, Maupas P, Muller R, Hofschneider PH (1981): Detection of hepatitis B virus - specific DNA in the genomes of human hepatocellular carcinoma and liver cirrhosis tissues. J. Gen. Virol. 57: 95-102.

28 Krupp MA, Chatton MJ (1982): "Current Medical Diagnosis and Treatment". Lange Medical Publications.

29 Lee CY, Hwang LY, Beasley RP (1983): Immunogenicity of HBV vaccine in healthy Chinese newborns. J. Infect. Dis. 148: 526-529.

30 Lugassy C, Bernuau J, Theirs V, Krosgaard K, Degott C, Wantzin P, Schalm SW, Rueff B, Benhamou JP, Tiollais P, Brechot C (1987): Sequences of Hepatitis B Virus DNA in the Serum and Liver of Patients with Acute Benign and Fulminant Hepatitis. The Journal of Infectious Diseases 155: 64-70.

31 Maniatis T, Fritsch EF, Sambrook J (1982): Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York. p387 - 389; p109 - 112.

32 Matsuyama Y, Omataq M, Yokosuka O, Imazeki F, Ito Y, Okuda K (1985): Discordance of hepatitis B e antigen/antibody and hepatitis B virus deoxyribonucleic acid in serum. Gastroenterology 89: 1104-1108.

33 Matthew GP (1983): Labelling of DNA and gel electrophoresis. In : Walker GM, Gaastra W (eds): "Techniques in Molecular Biology. Leaper and Gard Ltd. Bristol.

34 Meinkoth J, Wahl G (1985): Factors affecting filter hybridisation. Anal Biochem 138: 267-284.

35 Murray MJ, Shilo BZ, Chiazo S (1981): Three different human tumour cell lines contain different oncogenes. Cell 25: 355-361.

36 Okade K, Kamiyama I, Inomata M (1976): e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N. Engl. J. Med. 294: 746-749.

37 Pasek M, Goto T, Gilbert W, Zink B, Schaller ½H, Mackay P, Leadbetter G, Murray K (1979): Hepatitis B virus genes and their expression in E. coli. Nature 282: 575-579.

38 Shafritz DA, Kew MC (1981): Identification of integrated hepatitis B virus DNA sequences in human hepatocellular carcinoma. Hepatology 1: 1-8.

39 Shafritz DA, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC (1981): Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. N. Engl. J. Med. 305: 1067-1073.

40 Shafritz DA, Hadziyannis SJ (1984): HBV DNA in liver and serum, viral antigens and antibodies, viral replication and liver disease activity in patients with persistent hepatitis B virus infection. In : Chisari FV (ed): Advances in hepatitis research. Masson Press New York, pp 80-90.

41 Sherman M, Shafritz DA (1984): Hepatitis B virus and Hepatocellular Carcinoma : Molecular Biology and Mechanistic Considerations. Seminars in Liver Disease 4(2): 89-112.

42 Southern EM (1975): Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

43 Steyn L (1985). Personal communication.

44 Valenzuela P, Gray P, Quiroga M, Zaldivar J, Goodman HM, Rutter WJ, (1979): Nucleotide sequence of the gene coding for the major protein of hepatitis B surface antigen. Nature 280: 815-819.

45 Wen YM, Mitamura K, Mercahant B (1983): Nuclear antigen detected in hepatoma cell lines containing integrated hepatitis B virus DNA. Infect Immun 39: 1361-1367.

46 Windsor IM, Joseph M (1981): Viruses associated with Hepatitis in African Black patients, Natal 1981. Paper given at the 23 rd Annual Congress of the SA Society of Pathologists, Pretoria, 1983.

47 Windsor IM, Simjee AE (1983): Hepatoma and cirrhosis in Black African patients, Natal. Paper presented at the Sixth International Congress of Virology, Sendai, Japan, 1984.

48 Zuckerman AJ (1979): Specific serological diagnosis of viral hepatitis. Br. Med. J. 7: 84-86.