EXPRESSION OF THE HEPATITIS B VIRUS GENOME IN CHRONIC HEPATITIS B CARRIERS

Sheila Mary Bowyer

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ABSTRACT

The methylation status of CCGG sites in hepatitis B virus (HBV) DNA was examined to determine whether methylation could be responsible for the selective expression of the HBV surface gene in the progression of chronic hepatitis B infection and hepatocellular carcinoma. Total cellular DNA, determined to have sufficient HBV DNA, was digested with the restriction endonucleases *MspI* and *HpaII*, to determine whether the HBV DNA was methylated, or *HindIII*, to determine whether the HBV DNA was integrated. The cleavage fragments were analysed by Southern blotting and hybridisation to ^{32}P-labelled HBV DNA.

In replicative chronic hepatitis B, hypomethylation of the HBV genome correlated with HBV expression in both virions and infected tissue. Integrated sequences were hypermethylated in the human hepatocellular carcinoma cell line PLC/PRF/5 and in six of the tumour tissues suggesting that methylation plays a role in HBV gene repression. However, since DNA from five other tumours was hypomethylated, the belief that methylation *per se* is an absolute determinant of HBV core gene repression does not hold for human hepatocellular carcinoma tissue.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science (Medicine) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

---------- Simpson ----------

(Name of candidate)

---------- 15th day of December ----------, 1986.

In Memory of my Mother

Helen Winson

1926-1985

PREFACE

There is a high incidence of hepatitis B virus (HBV) infection and a correspondingly high incidence of hepatocellular carcinoma (HCC) in indigenous Africans. HCC develops in young, Black men and women often without evidence of chronic liver disease and progresses rapidly to a fatal outcome. Tumours in Oriental and European patients usually develop on a background of chronic active disease and tumour progression occurs more slowly. Having indirect access to tissue from chronic HBV carriers and patients with HCC permits research into this relatively common and invariably fatal disease. It is hoped that all research efforts in HCC will lead to a lessening of the morbidity from this disease. For these reasons, this is a very important research field in this country.

Chronic hepatitis B carriers may either have predominantly "replicative" or "nonreplicative" hepatitis B virus (HBV) infection. Chronically infected carriers with replicative infection evolve after several years from an early replicative phase to a later, predominantly nonreplicative phase in which HBV expression is diminished. In carriers with replicative infection, HBV DNA in liver exists as several episomal replicative intermediates. Both the core and surface genes are

transcribed from episomal HBV DNA. The majority of long-standing HBV carriers and patients with HBV-related hepatocellular carcinoma have low levels of HBV replication. HBV DNA in liver is predominantly integrated. Transcription of integrated HBV sequences is assumed to be the source of hepatitis B surface antigen in these carriers. The human hepatocellular carcinoma cell line PLC/PRF/5 contains only integrated HBV DNA sequences and expresses only HBsAg.

Methylation of specific cytosine bases has been implicated in gene repression in other viral infections. In particular, two studies have suggested that core antigen expression in the human hepatocellular carcinoma cell line PLC/PRF/5 is regulated by methylation. To determine whether methylation could explain the selective expression of the surface gene in carriers with nonreplicative infection or with HCC, we studied the state of methylation of CCGG sites in HBV DNA in liver tissue obtained from chronic HBV carriers and patients with hepatocellular carcinoma.

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1. The ICI Clinical Research day, Johannesburg, August 1985.
2. The 1st South African/Republic of China Binational Symposium on Molecular Biology and Biotechnology, Pretoria, September 1985.
3. The second biennial Poliomyelitis Research Foundation Conference on Molecular Biology of Viruses, Berg-en-dal, August 1986.
4. Cold Spring Harbor Laboratory Symposium on the Molecular biology of hepatitis B viruses, Long Island, New York, August 1986.

CONTENTS PAGE

CONTENTS PAGE

CONTENTS PAGE

LIST OF ABBREVIATIONS

HBV	Hepatitis B virus
DNA	Deoxyribonucleic acid
DHBV	Duck hepatitis virus
nm	Nanometre
HBsAg	Hepatitis B surface antigen
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
bp	Base-pair
DR	Direct repeat sequence
RNA	Ribonucleic acid
anti-HBc	Antibody to HBcAg
anti-HBs	Antibody to HBsAg
AST	Aspartate transaminase
ALT	Alanine transaminase
DNAP	Positive by DNA polymerase assay
anti-HBe	Antibody to HBeAg
HCC	Hepatocellular carcinoma
Ltk^-	Mouse cells lacking the thymidine kinase gene
E. coli	Escherichia coli
m-RNA	Messenger RNA
5mC	5-methylcytosine
mg	Milligram (10^{-3} g)
ml	Milliliter (10^{-3} l)

μg	Microgram (10^{-6} g)
w/v	Percentage calculated as mgs per 100 ml
$NaDodSO_4$	Sodium dodecyl sulphate
M	Molar
EDTA	Ethylene diamine tetra-acetate
NaCl	Sodium chloride
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris hydrochloride
mM	Millimolar (10^{-3} M)
v/v	Percentage calculated as mls per 100 ml
pg	Picogram (10^{-12} g)
μl	Microliter (10^{-6} l)
$MgCl_2$	Magnesium chloride
BSA	Bovine serum albumin
dNTP	Deoxynucleotide triphosphate
ng	Nanogram (10^{-9} g)
DTT	Dithiothreitol
BRL	Bethseda Research Laboratories
dCTP	Deoxycytidine triphosphate
cpm	Counts per minute
kb (or kbp)	1000 bases (or 1000 base pairs)

LIST OF TABLES PAGE

LIST OF FIGURES PAGE

LIST OF FIGURES

CHAPTER I INTRODUCTION

The hepatitis B-like viruses have been grouped together as a distinct family of viruses, the "hepadna" viruses (for "hepatitis DNA" viruses). They possess several unique characteristics which distinguish them from the other five families of DNA containing viruses (adenovirus, herpesvirus, poxvirus, parvovirus and papovavirus). To date, the well characterised hepadna viruses are the human hepatitis B virus (HBV), duck hepatitis virus (DHBV), woodchuck hepatitis virus and ground squirrel hepatitis virus (Mason *et al.* 1980, Summers *et al.* 1978, Marion *et al.* 1980a).

1. Virion structure and morphology

With the exception of the papovaviruses, the hepadna viruses have the smallest virion of the DNA-containing viruses. The HBV virion is a 42 nanometre (nm) spherical particle, often called a Dane particle. Within the outer envelope of the Dane particle is a 27 nm nucleocapsid containing a circular, partially double-stranded DNA genome. Characteristic of this family of viruses are the abundant non-DNA containing particles also present in serum with the virions. These non-

infectious particles have circular and filamentous forms with, in the case of HBV, a mean diameter of 22 nm (Tiollais et al. 1981). The virion outer coat and the non-DNA containing particles possess a common antigen, hepatitis B surface antigen (HBsAg). The nucleocapsid of the virion expresses the hepatitis B core antigen (HBcAg) and the hepatitis B e antigen (HBeAg, Figure 1). HBeAg is thought to be a breakdown product of HBcAg (Takahashi et al. 1979); transcription from the 'precore' region of the HBV genome is required for the association of HBcAg with the endoplasmic reticulum and the secretion of HBeAg (Ou et al. 1986). Unlike HBcAg, HBeAg is found free in serum, and is a useful and sensitive marker of the presence of ciculating virions.

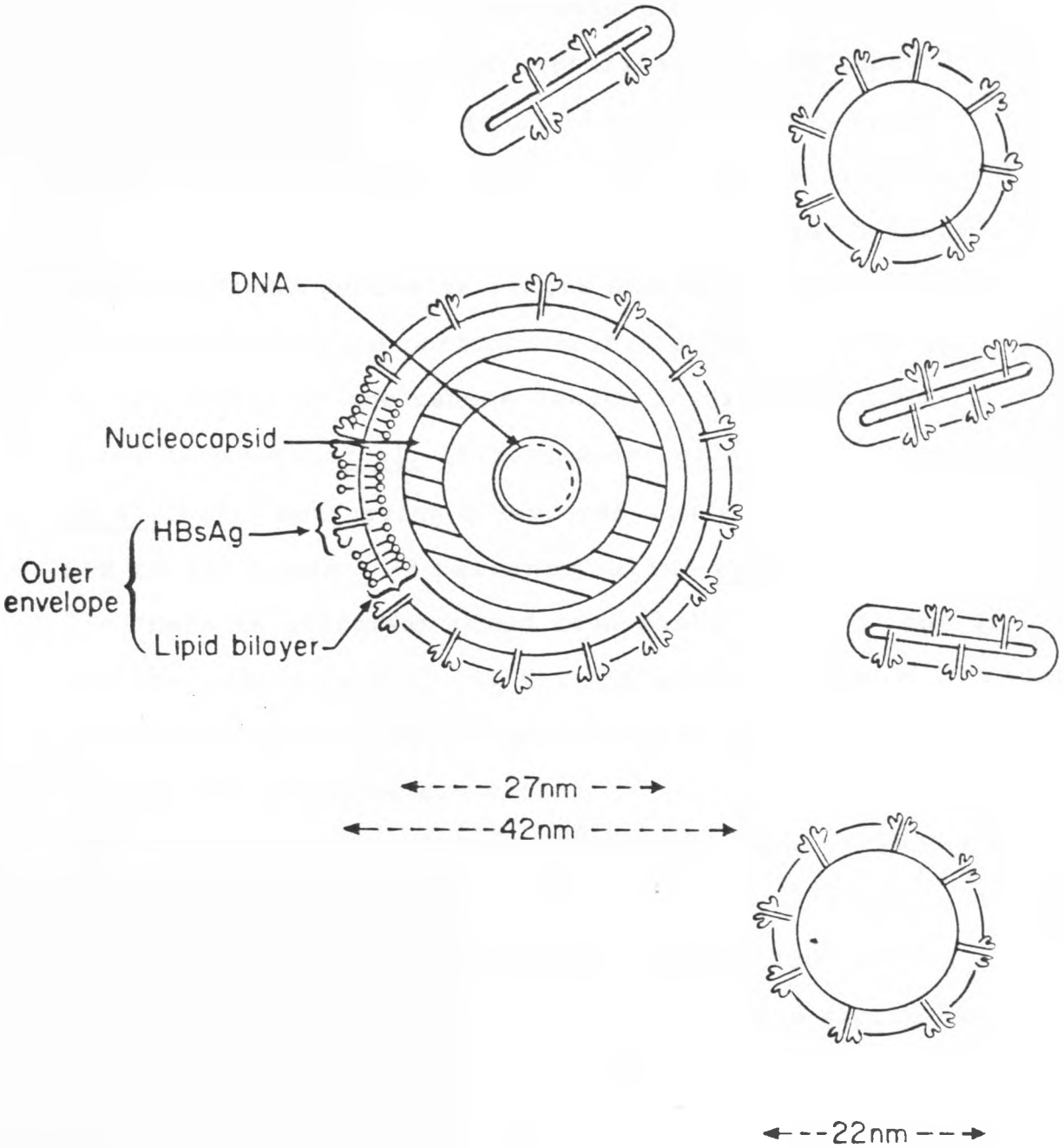

Fig. 1. Model of hepatitis B virus showing virion and non-infectious viral particles, adopted from Tiollais et al. (1981)

2. The DNA molecule

The HBV genome consists of approximately 3200 base-pairs (bps), having the smallest genome of any known animal virus (Robinson *et al.* 1974). Despite its size, the genome is complex. HBV DNA is circular and a large portion of the genome is single stranded. The extent of the single-stranded portion varies, occupying from 15 to 50 percent of the genome length in different molecules (Summers *et al.* 1975, Landers *et al.* 1977, Hruska *et al.* 1977) but having a favoured minimum length of 650 to 700 bases ± 20 percent (Delius *et al.* 1983).

There is a protein bound covalently to the 5' end of the complete strand of the HBV DNA which prevents phosphorylation of the strand (Gerlich *et al.* 1980).

2.1 The cohesive end region

The complete strand of the viral DNA is thus nicked at a unique site (map position 1826) 225 bps from the 5' end of the incomplete strand (Tiollais *et al.* 1985). When HBV DNA is made fully double stranded by the virion DNA polymerase reaction, a nick also remains in the short strand. Thus, HBV is regarded as having a linear genome in a circular conformation. There is an overlap region between the nicked strands and hence HBV DNA is said to have 'cohesive ends' (Sattler *et al.* 1979). This concept is in accord with the finding, from nucleotide

sequence data, that the coding of the virus occurs on one strand (Galibert et al. 1979). The sense direction of the nucleotide sequence was found to be 5' to 3' on the incomplete strand, making it the plus, non-coding strand (Galibert et al. 1982). Since the cohesive ends do not contain any open reading frame, linearisation of the genome should not disrupt any coding regions.
The cohesive end region is bound on both sides by an 11 bp direct repeat (DR) sequence (5'TTCACCTCTGC). One copy of this sequence, termed DR1, starts at nucleotide 1824 while the other, DR2, starts at nucleotide 1590. It is likely that these direct repeats have a biological role since they are conserved in all the hepadna viruses (Tiollais et al. 1985).

2.2 HBV genes

By correlating the amino acid sequences of HBsAg and HBcAg with the nucleotide sequence of HBV DNA, Galibert et al. (1979) were able to localise the genes coding for these two proteins. Two other putative open reading frame regions on the minus strand of the genome were named gene X and gene P (Figure 2).

Translation of the putative P gene would synthesise a protein with a molecular weight of the order of the DNA polymerases (Galibert et al. 1979). The HBV virion contains an endogenous DNA polymerase which functions to complete the single-stranded region of the DNA

molecule by elongation from the 3' end of the short strand (Kaplan et al. 1973). Thus it has been suggested that the DNA polymerase found within the virion is coded by the P region. The putative reverse transcriptase could also be coded by the P region.

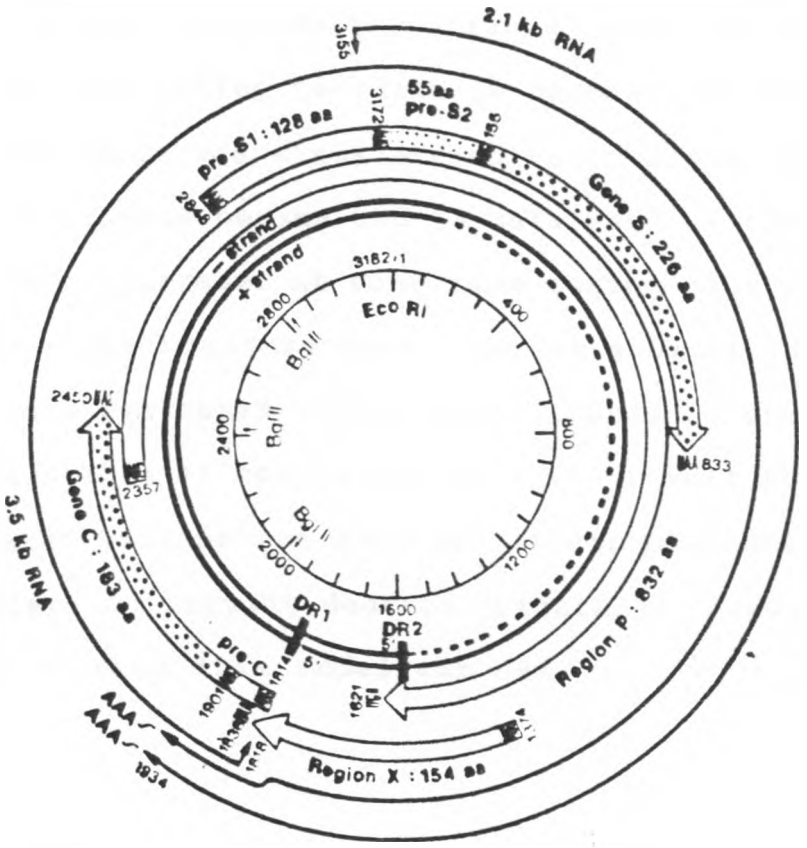

Fig. 2. Physical structure and proposed genetic organisation of the HBV genome

(Tiollais et al. 1985)

3. Replication of HBV

A model for the replication of the DHBV was recently suggested by Summers and Mason (1982a and b). According to their model, once the DHBV virion enters an nepatocyte, the DHBV DNA is made fully double stranded. An RNA intermediate, or 'pre-genome', is then transcribed from the fully double-stranded DNA. A DHBV DNA minus strand is then "reverse transcribed" from the RNA template. The latter template is degraded as the DNA elongates. Once the minus strand is complete, plus strand synthesis begins and is catalysed by the endogenous DNA polymerase. At some time during plus strand synthesis the immature cores receive a packaging signal, hence synthesis of the plus strands is arrested at various stages of completion with the result that when the coated virions are exported, their plus strands are incomplete to varying degrees (Figure 3). Fowler et al. (1984a) confirm this model for HBV.

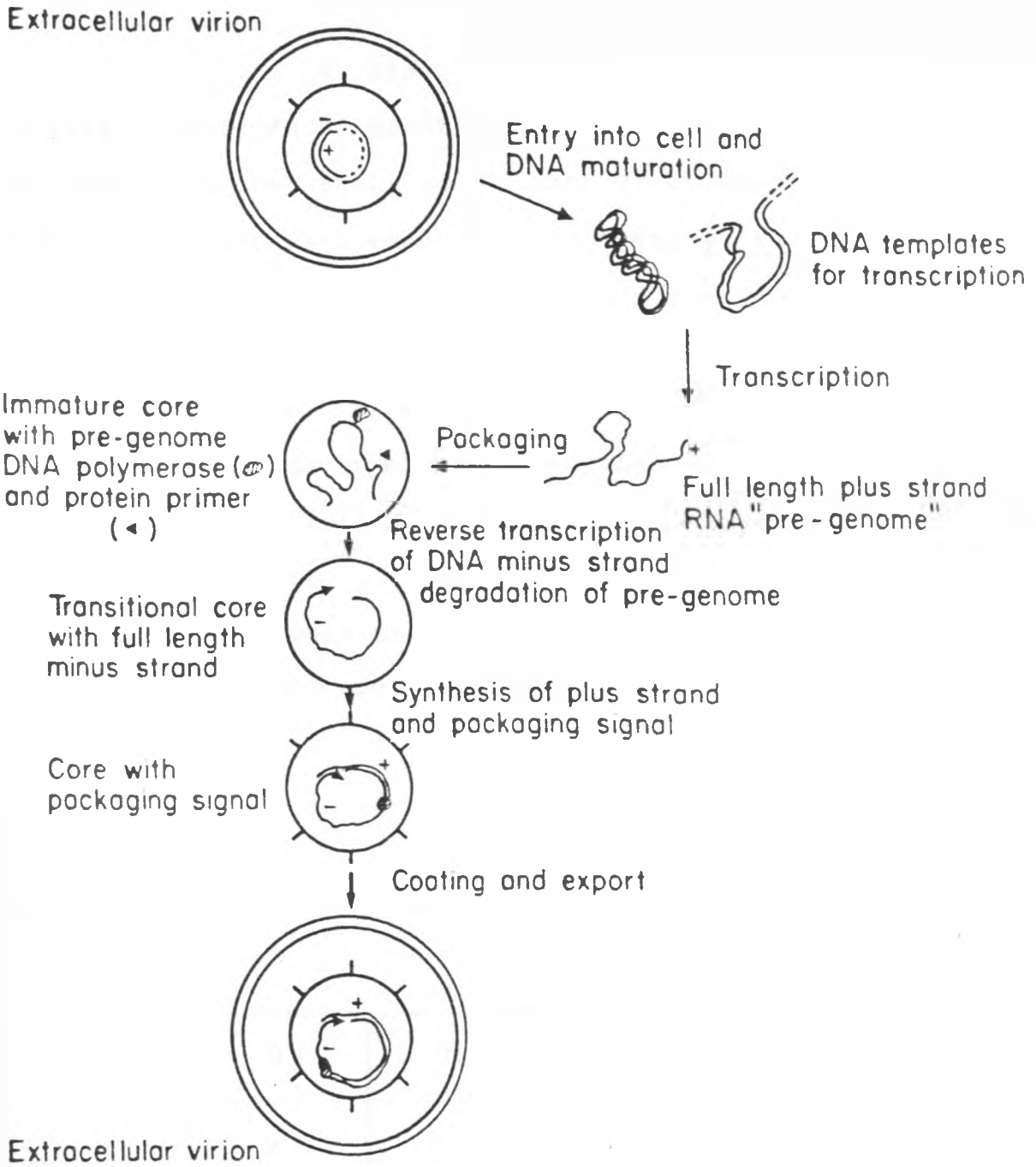

Fig. 3. A general outline for the replication of the genome of HBV-like viruses. (Summers and Mason 1982a)

4. HBsAg subtypes

Different subtypes of HBsAg exist in various geographical regions (Sobeslavsky 1978). Each virus subtype has undergone independent evolution (Fujiyama et al. 1983) but essential regions (and genes) are conserved. The surface antigen has three major antigenic regions: 'a', the group specific antigen, and two mutually exclusive, sub-type specific determinants, 'd'/'y' and 'r'/'w' (Table 1). Thus HBsAg can be classified into four major subtypes: adr (South East Asia), adw (Africa), ayw (Middle East and Southern Europe) and ayr (no prevalence reported) (Szmuness 1978, Fujiyama et al. 1983).

Table 1. Major surface antigen subtypes

a	d	y
w	adw	ayw
r	adr	ayr

Numerous individual amino acid differences exist between the subtypes and indeed, as Siddiqui et al. (1979) have shown, there are even differences between the same subtype. These alterations lead to differences in the restriction maps of the respective genomes. However the main insertions and deletions are in multiples of three and so do not alter the reading frame of the DNA molecule. Adw differs from ayw in having six nucleotides inserted at position 2354 to 2359 and twelve inserted at position 2877 to 2888. In this latter region, position 2856 to 2888, subtype adr has an insertion of 33 bps. In addition, adr differs from ayw by having a 27 bp deletion in position 1791 to 1817 (Ono et al. 1983, Figure 4). The clinical significance of the subtype differences is unknown.

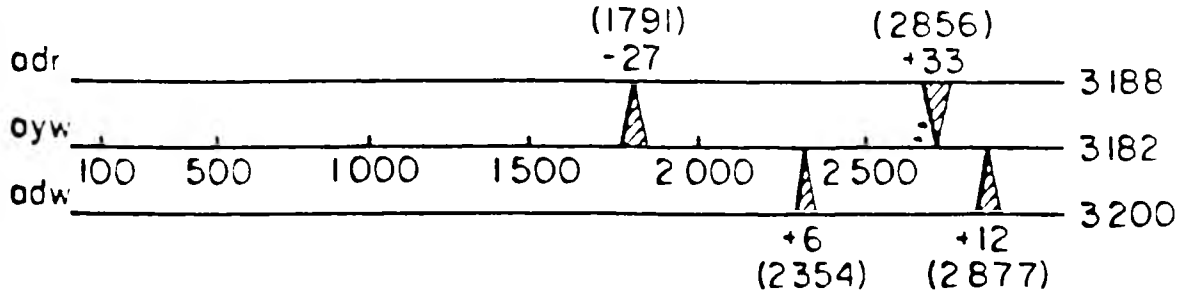

Fig 4. Major differences between surface antigen subtypes

5. Possible outcomes of HBV infection

Most HBV infections progress to an acute attack of type B virus hepatitis (Kew 1979), which may or may not be icteric. The host usually clears the virus within a few months, becomes HBeAg and HBsAg negative and develops antibody to HBcAg (anti-HBc) and HBsAg (anti-HBs). These antibodies impart immunity to reinfection (Fig. 5).

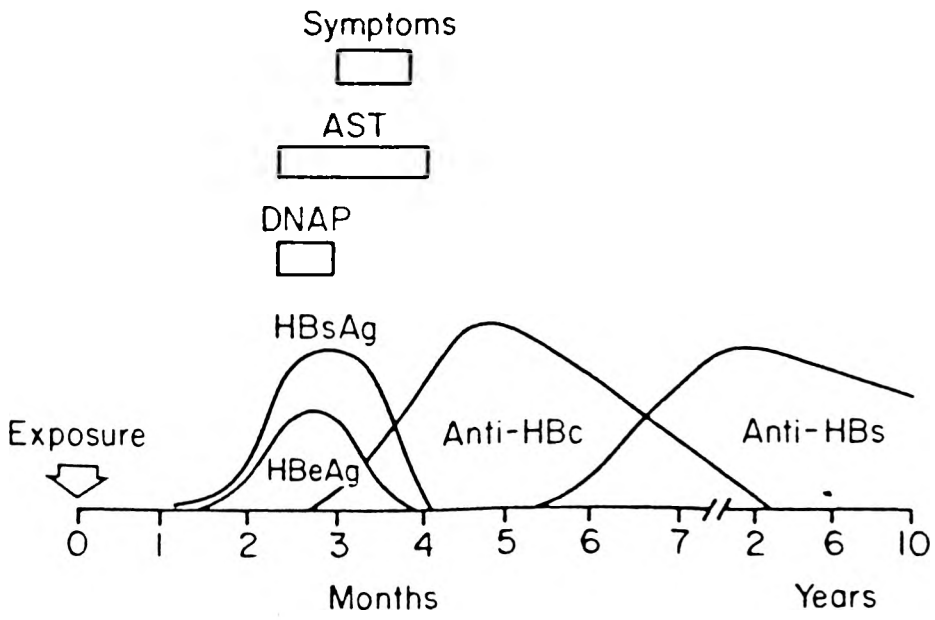

Fig. 5. Acute B-virus hepatitis (Kew 1979)

In a proportion of persons infected with HBV, HBsAg persists for six months or longer and, by definition, the patient is said to be a chronic carrier of the disease. Poorly understood immune defects and the relative youth of the individual at the time of primary infection enhance the probability of persistence. Importantly, although only 5 to 10 percent of infected adults develop chronic hepatitis, 90 percent of neonates infected perinatally become chronic carriers. Other factors suspected of predisposing an individual to the carrier state are sex (there is a higher ratio of male to female carriers), possibly race (Blacks and Orientals have a higher incidence), and genetic factors (? familial disposition, Szmuness 1978).

Two major groups of chronic hepatitis B carriers have been recognised. The first group is characterised by the presence of active viral replication. Within the hepatocytes of a carrier with "replicative" disease, viral DNA is predominantly episomal - or, free within the liver cell nucleus (Brechot et al. 1981). The number of copies of viral DNA is high in these carriers and several intermediate DNA forms, indicating viral replication, are present. The viral DNA transcribes both the core and surface antigen genes and thus both complete viral particles as well as extra, non-infectious particles are released into the serum. These patients are

positive for viral DNA and HBeAg in serum (Hoofnagle et al. 1982). After several years, and sometimes following a period of raised aspartate transaminase (AST) and alanine transaminase (ALT) levels, the disease may evolve to a nonreplicative phase. In "nonreplicative" infection, integrated copies of HBV DNA in host cellular DNA predominate and only HBsAg is expressed. Although these carriers remain HBsAg positive, HBeAg and HBV DNA are not detectable in serum and complete virions are sparse or undetectable. Seroconversion from HBeAg to antibody to HBeAg (anti-HBe) is characteristic of spontaneous transition from replicative to nonreplicative chronic HBV infection and is usually associated with a significant improvement in disease activity and a fall in serum concentrations of AST and ALT (Hoofnagle et al. 1981). Incomplete coat particles, reactive as HBsAg, are present in serum. Presumably HBsAg can be transcribed from the integrated viral sequences as certain human hepatocellular carcinoma cell lines which contain only integrated copies of HBV DNA also express only HBsAg (Figure 6).

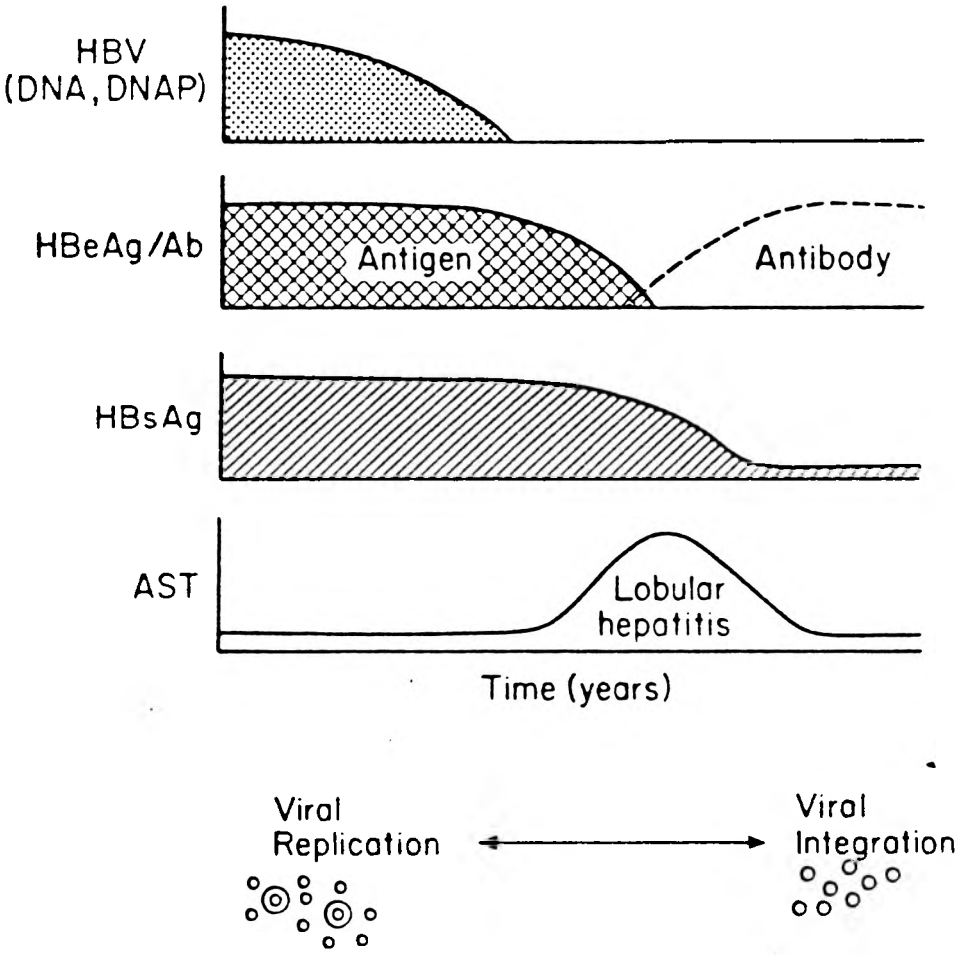

Fig. 6. Virological and serological events during chronic HBV infection (Thomas et al. 1984)

A percentage of carriers (Ø,1 percent of male carriers per year in the Taiwanese study of Beasley et al. 1981) progress further to hepatocellular carcinoma (HCC, Shafritz 1982). Since epidemiologic evidence suggests that it takes ten to thirty years for this progression to take place (Szmuness 1978, Beasley et al. 1981), individuals developing the carrier state early in life may be at greatest risk (Shafritz 1982). Figure 7 shows Shafritz's model of this progression to HCC. Most patients with HCC have predominantly non-replicative infection and are negative for HBeAg and HBV DNA in serum. Since HBV has not yet been propagated in replicative cell culture, most research on the molecular relationship between HBV and HBV DNA in serum is currently confined to the study of human HCC cell lines that contain integrated copies of the HBV genome. The first of these cell lines, PLC/PRF/5, derived from a patient with HBV infection and HCC, has been extensively studied. It has been shown that the PLC/PRF/5 cell line also only expresses HBsAg (Marion et al. 198Øb).

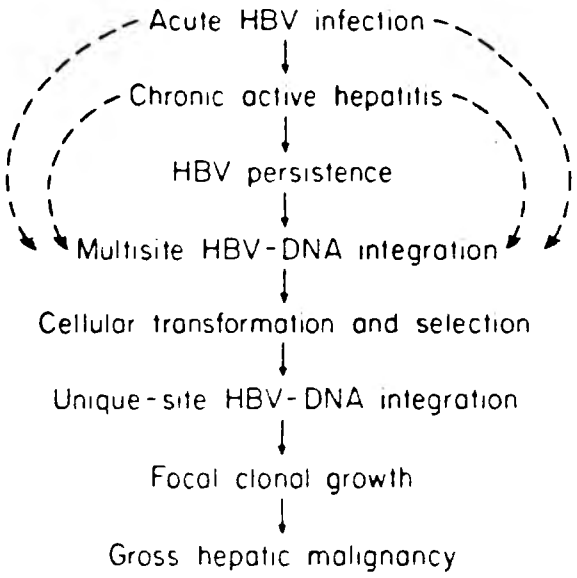

Fig. 7. Model showing potential relationship between integration of HBV-DNA, cellular transformation and development of HCC (Shafritz 1982)

Bearing in mind that there are carriers with levels of viral replication and expression between the two extremes described, and taking into account the sensitivity of Southern blotting for detection of minimal copies of either episomal or integrated HBV DNA, the fact remains that there does appear to be a certain level of HBV gene control during the natural history of the carrier state and in HCC. This dissertation investigates this question of gene control in carriers with 'high' and 'low' levels of HBV replication.

6. Gene control

In chronic carriers who no longer have markers of viral replication, HBsAg expression may persist. The source of HBsAg is assumed to be either selective transcription of episomal HBV DNA or transcription of integrated HBV sequences (Dejean et al. 1982). If the integrated viral DNA can transcribe the surface gene, why is the core gene, and hence HBeAg, not similarly expressed? Either the genetic code has been disrupted or the integrated viral sequences have been modified in vivo. We examined these possibilities in order to plan our strategy for investigating gene expression in HBV infection.

6.1 Genetic code disruption

The position on a DNA strand where transcription begins and ceases (hence defining the protein thus produced) is determined by coding principles. If either the DNA gene sequences or the regulatory sequences of a gene are altered during integration, that particular gene will not be expressed. This can happen in several ways. Both ordered and disordered integrations may occur which could cause critical fragmentations or disruptions of portions of the HBV genome. This did not appear to have taken place in the integrated-HBV sequences cloned by Dejean et al. (1982) from a patient who had died of HCC. They found at least one genome equivalent of HBV DNA had been integrated into the cellular DNA without apparent rearrangement or deletion. Although HBsAg was not detected in poorly differentiated neoplastic liver cells from this patient by immunofluorescent staining, gene S was expressed when the cloned integrated sequences were transfected into mouse LtK- cells (Dejean et al. 1982). Thus, the gene S (and any of its required promotor sequences) was intact but was not expressed in vivo in tumour cells.

Similarly, one of the four PLC/PRF/5 integrated-HBV sequences cloned by Koch et al. (1984) was found to have the putative HBcAg promotor and gene C sequences but, in this case, no expression of HBcAg was detected

after transfection. In these examples the genes appear to have been integrated intact but it is possible that integration could have altered their reading frames and, in this way, changed the genetic code. Chance restoration of the HBsAg reading frame could account for HBsAg expression when the Dejean cloned integrant was transfected into mouse cells and explain the failure of the cloned integrant to express HBsAg *in vivo*. Recombinant HBV genomes, derived by Pasek from circulating virions, expressed HBcAg but not HBsAg in *Escherichia coli* (*E. coli*, Pasek *et al.* 1979). Since the genes for HBcAg and HBsAg are in different translational phases, he reasoned that the situation could be reversed by constructing a new recombinant DNA molecule in the correct reading frame for the HBsAg gene. As predicted, after transfection into *E. coli* HBsAg, but not HBcAg, was expressed (MacKay *et al.* 1981).

If reading frame shifts are responsible for repression *in vivo*, one would expect integrations allowing the correct phase for core expression to occur at least some of the time. Unless, of course, integration is always at the same site of the HBV DNA molecule, which is possible but not yet proven.

Integration of a circular genome, such as HBV DNA, would result in linearisation. If the messenger-RNA (m-RNA) transcript of the core gene is greater than genome

size, it could require covalently closed circular DNA for transcription (Tiollais et al. 1985). Gough and Murray (1982) speculate that the m-RNA of HBcAg does proceed via a precursor that is greater than the length of tne HBV genome. Also, two major classes of poly $(A)^+$ RNAs have been characterised in chimpanzee infected liver (Cattaneo et al. 1983 and 1984). Both classes are unspliced, polyadenylated at a common position and heterozygous at their 5' ends. A 2,1 kb transcript was found to encode the major S protein while a 3,5 kb RNA was found to be the message for the core protein.

Dejean's cloned integrated-HBV segment derived from HCC (Dejean et al. 1982) was 1,2 genome lengths. As more is learnt about the nature of integrated sequences, the importance of m-RNA precursor size in vivo should become clearer.

6.2 DNA modification in vivo

The mode of expression of HBV is complex and could involve many factors, acting either alone or in combination. Even if the genes and their regulatory sequences are not altered during integration, the viral DNA could be modified within the host cell and this too could affect transcription. Evidence is accumulating which suggests that eukaryotic cells control transcription by modifying (or in particular, methylating) specific bases. The only modified base found in

DNA of vertebrates is 5-methylcytosine (5mC, Razin and Riggs 1980). Human cellular DNA contains 3,6 to 4,4 per cent of 5mC (Günthert et al. 1976). The conversion of cytosine to 5mC (Fig. 8) results in the introduction of a methyl group into an exposed position in the major groove of the DNA helix. The binding of proteins such as repressors, histones and hormone receptors to DNA is known to be affected by structural or conformational changes in the major groove of DNA.

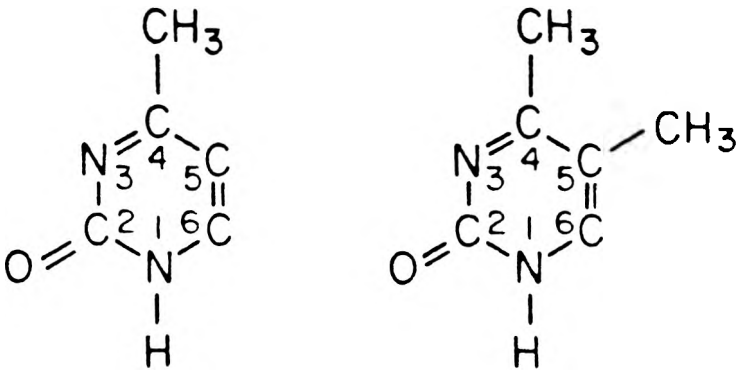

Fig. 8. Structure of cytosine (left) and 5-methylcytosine (right)

The conventional form of DNA is the B form, but Behe and Felsenfield (1981) showed that methylated DNA assumes the Z form at physiological salt concentrations. An alteration from a B to a Z form of DNA may be the mechanism by which methylation suppresses gene

activity. However, it is not certain whether the methylated bases constitute signals in their own right or whether their signal effect is mediated by altering the structure of DNA (Doerfler 1984).

Active chromatin has been found in many cases to be hypomethylated. A large number of publications report an inverse relationship between gene expression and the state of methylation of a gene. Groudine et al. (1981) examined two endogenous retrovirus loci in chick cells, ev-3 (a transcriptionally active gene) and ev-1 (an inactive gene), which differed in that ev-3 was hypomethylated. Exposure to 5'-azacytidine however, which inhibits methylation, resulted in hypomethylation and subsequent transcriptional activation of ev-1. 5'-azacytidine inhibits methylation in two ways:

i. It can be incorporated into replicating DNA and, due to its chemical structure, the cytidine moiety cannot be methylated.

ii. It has the ability to inhibit DNA methyltransferases (Jones et al. 1982).

Sutter and Doerfler (1980) report that adenovirus type 12 DNA integrated into the genome of transformed cells, is extensively methylated at HpaII sites whereas purified virion DNA is not. Furthermore, adenovirus genes integrated in cell lines expressing late viral proteins are undermethylated when compared

to the state of these genes in cell lines that do not synthesise these products.

Desrosiers (1982) found DNA from herpes saimiri virions to be slightly methylated or not at all whereas specific viral genes in virus transformed cell lines were methylated.

7. Methylation in HBV infection

Two studies (Yoakum et al. 1983, Millar and Robinson 1983) have suggested that HBcAg expression is regulated by methylation. Yoakum and co-workers transfected human cells with a plasmid containing a subgenomic fragment of HBV that contained the core antigen gene. After selecting for transformants and establishing these cell lines, no core expression was observed. They then tested the response of one of these cell lines to 5'-azacytidine treatment and reported that, after treatment, greater than 90 percent of cells expressed HBcAg as shown by immunofluorescent assays. They conclude that methylation is responsible for core gene repression.

Millar and Robinson determined the state of methylation of HBV DNA in virions and a virus infected tissue and found that, in both cases, the DNA was hypomethylated. However, HBV DNA extracted from PLC/PRF/5 cells was found to be hypermethylated. The authors conclude

that methylation of critical sites within integrated HBV DNA may account for the differential expression of the viral surface and core genes in the PLC/PRF/5 HCC cell line.

8. Our strategy

These, and many similar experiments which correlate methylation of integrated viral sequences with gene repression in viral infections, led us to investigate the role of methylation in gene control in HBV infection. We examined the state of methylation of HBV DNA in HBV carriers with or without replicative infection, in the PLC/PRF/5 cell line and in patients with HCC. The strategy we used for analysing methylation and gene control was that of specific restriction endonuclease mapping of integrated and episomal HBV DNA using two enzymes which differ in their ability to cleave methylated cytosine. Restriction endonucleases are site specific enzymes which recognise and cleave DNA at specific nucleotide sequences. In particular, both the restriction endonucleases MspI and HpaII cut the sequence CCGG. However, methylation of the internal cytosine base renders the sequence resistant to HpaII endonuclease digestion but not to MspI digestion (Waalwijk et al. 1978). Using the techniques of DNA extraction, restriction endonuclease digestion, agarose gel

electrophoresis and Southern blotting (Figure 9, see Chapter 2 for details), the restriction patterns obtained using these two enzymes were compared. Fewer bands and larger molecular weight fragments obtained after digestion with HpaII, as compared with the pattern after digestion with MspI, indicate an inability to cleave CCGG, and hence methylation of the internal cytosine.

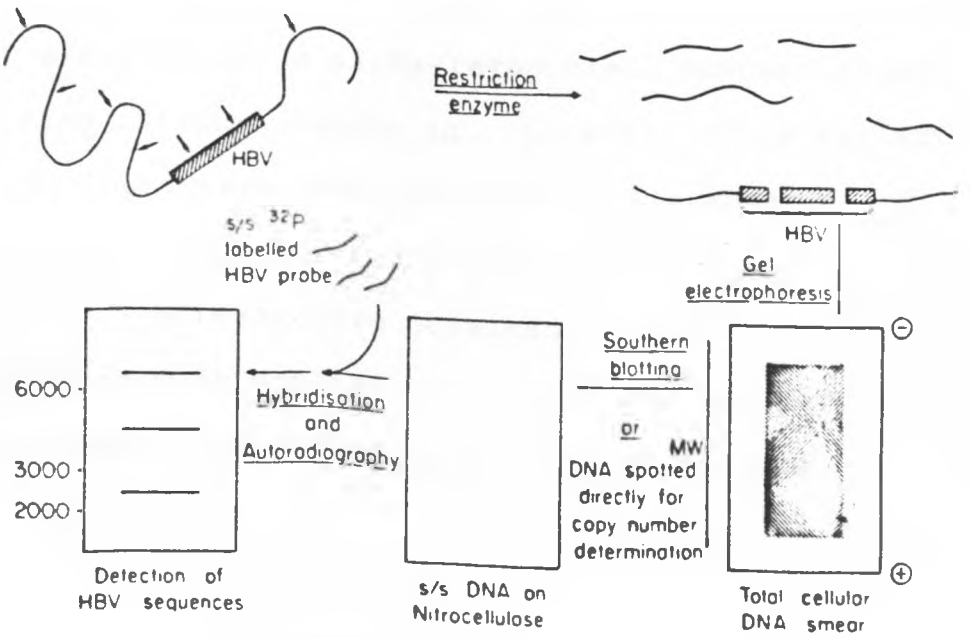

Fig 9 Detection of HBV DNA sequences

CHAPTER II MATERIALS AND METHODS

A total of thirty-nine specimens were analysed (Table 2). Neoplastic and surrounding non-neoplastic tissue from patients with HCC was frozen immediately postmortem or after surgical removal and stored at -70°C. The latter specimens were small hepatocellular carcinomas resected in asymptomatic carriers with nonreplicative HBV infection whose tumours were diagnosed as a result of elevated serum alpha-fetoprotein concentrations during clinical follow up. PLC/PRF/5 cells explanted in nude mouse were obtained from J. Alexander and S. Aspinall (Medunsa, South Africa). Percutaneous liver biopsy specimens were obtained from selected chronic carriers with replicative HBV infection prior to their treatment with recombinant alpha-interferon.

TABLE 2:

MATERIALS

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37 tissue specimens analysed:

35 of the specimens were from HBsAg-positive patients.

a. 29 histologically proven hepatocellular carcinoma (HCC).
   16 adjacent non-neoplastic tissues from these patients with HCC.
b. PLC/PRF/5 HCC cell line.
c. 7 percutaneous liver biopsy samples from patients with chronic hepatitis B.

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2 serum samples:

d. 2 serum samples from patients with chronic hepatitis B.

15/39 of the HBsAg-positive patients were HBeAg positive

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1. Tissue DNA extraction

Grossly visible tumour tissue was carefully separated from non-neoplastic tissue to minimise contamination of tumour and non-neoplastic tissue. Biopsy specimens were chopped directly into a sterile eppendorf tube. Larger tissue samples (approximately 500 milligrams, mg) were placed in 50 milliliter (ml) Falcon tubes half-filled with liquid nitrogen and homogenised under liquid nitrogen to a fine powder. The liquid nitrogen was then allowed to evaporate. Samples were then digested at 37°C overnight with Proteinase K at 100 micrograms (µg)/ml (Boehringer Mannheim) in lysing solution [2 percent weight/volume (w/v) sodium dodecyl sulphate ($NaDodSO_4$), 0,05 Molar (M) ethylene diamine tetra-acetate (EDTA), 0,2 M sodium chloride (NaCl), 0,1 M Tris hydrochloride (Tris-HCl), pH 8,2] followed by three phenol/chloroform extractions. The phenol was equilibrated with T.E. buffer [10 millimolar (mM) Tris in 1 mM EDTA, pH 8] containing 0,1 percent (w/v) 8-hydroxyquinoline and 0,2 percent volume/volume (v/v) mercaptoethanol. RNA was removed by digestion with pancreatic ribonuclease at 100 µg/ml (Boehringer Mannheim) for one hour at 37°C. After further phenol /chloroform extraction, larger samples were purified by dialysis at 4°C against T.E. buffer while smaller

samples were passed through an Elutip-d (Schleicher and Schuell) column according to the maker's instructions. This extraction procedure yielded from 20 µg (biopsy samples) to more than 600 µg of total cellular DNA. HBV virion DNA was extracted from approximately 40 ml of serum using the same protocol as for tissue except that after dialysis DNA was concentrated by ethanol precipitation: For all DNA precipitations one-fifth volume of 10,5 M ammonium acetate and two and a half volumes of ice-cold, 100 percent ethanol were added to the aqueous phase. After centrifugation at 12000 g (where "g" is the acceleration due to gravity), the pellet was then resuspended in T.E. buffer to an estimated concentration of 1000 picograms/microliter (pg/µl). The single-stranded region of the HBV DNA was made double stranded by the DNA polymerase reaction using 2,5 units of DNA polymerase I (Klenow fragment, Boehringer Mannheim) in 50 mM Tris-HCl (pH 7,5), 5 mM Magnesium Chloride ($MgCl_2$) and 50 mM nuclease free bovine serum albumin (BSA) containing the four deoxynucleotide triphosphates (dNTPs, final concentration 20 microMolar) for fifteen to twenty minutes at room temperature. The reaction mixture was then phenol/ chloroform extracted and ethanol precipitated as before except that the pellet was resuspended in one-tenth volume of T.E. buffer to give an estimated DNA

concentration of 10 nanograms (ng)/µl. Reverse transcriptase enzyme was then used to convert any residual single stranded regions to double-stranded DNA according to the protocol of Sattler and Robinson (1979). Each reaction mixture contained 100 ng HBV DNA, 2,5 mM dNTPs, 50 mM Tris-HCl (pH 8,3), 40 mM potassium chloride, 6 mM $MgCl_2$, 0,1 mg/ml BSA and five units of avian myeloblastosis reverse transcriptase (Boehringer Mannheim). The solution was incubated at 37°C for two hours. After further phenol/chloroform extraction, dialysis and ethanol precipitation the pellet was resuspended to an estimated concentration of 1 ng/µl. Twenty-five ng were used subsequently for each digest.

2. Restriction endonuclease analysis

Each DNA sample was digested with the restriction endonucleases HpaII (Boehringer Mannheim), MspI (New England Biolabs), and HindIII (Boehringer Mannheim). The buffers recommended by Maniatis et al. (1982) were used for HpaII [low salt buffer: 10 mM Tris-HCl (pH 7,5), 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT)] and HindIII [medium salt buffer: 50 mM NaCl, 10 mM Tris-HCl (pH 7,5), 10 mM $MgCl_2$ and 1 mM DTT]. The MspI reaction buffer recommended by Bethseda Research Laboratories [BRL, 50 mM Tris-HCl (pH 8,0), 10 mM $MgCl_2$ and 1 mM DTT] was found to give the best results and was thus

used for all MspI digestions. Nuclease free BSA (100 µg/ml) was added to all restriction digests. In a typical digestion protocol three units of restriction endonuclease were added per microgram of DNA and the reaction incubated at 37°C overnight. Completeness of digestion was monitored by adding a microgram of bacteriophage øX174 DNA (New England Nuclear) to a 10 µl aliquot of each incubation mixture and verifying by electrophoresis that the required phage cleavage products were present. After digestion, the DNA was loaded into the slots of a 1 percent agarose gel (BRL electrophoresis grade) and electrophoresed at 30 Volts overnight in 89 mM Tris, 89 mM boric acid and 2,5 mM EDTA (pH 8,3). Two to twenty micrograms of total cellular DNA per gel lane were used, depending on the copy number of HBV DNA per genome present. The DNA was transferred to a nitrocellulose filter by the method of Southern (Figure 10, Southern 1975, Wahl et al. 1979). Prior to Southern blotting, the DNA was not hydrolysed by acid depurination but was immediately denatured by gentle agitataion for one hour at room temperature with 0,5 M sodium hydroxide in 1 M NaCl. It was then neutralised, at room temperature, for a further hour with 0,5 M Tris in 3 M NaCl (pH 7,4). Transfer to nitrocellulose was in 20X SSC (1X SSC is 0,15 M NaCl in 0,015 M sodium citrate).

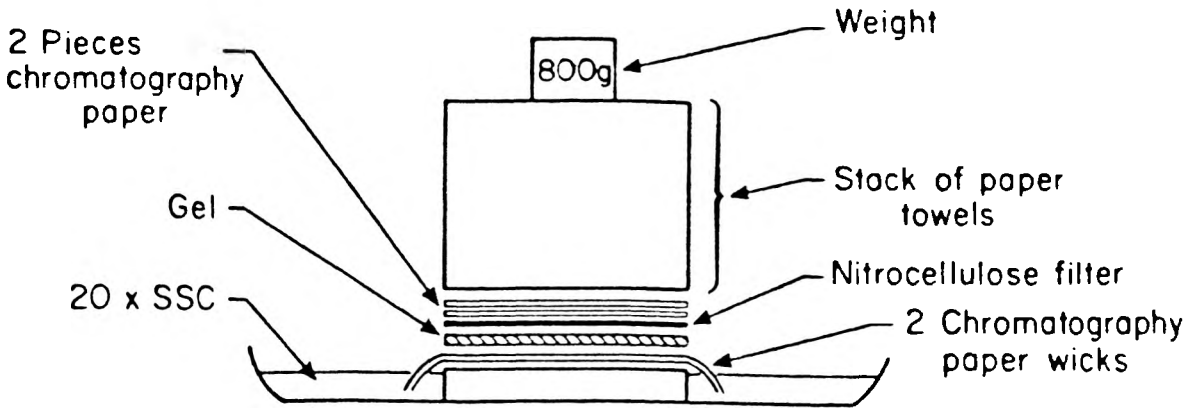

Fig. 10. Method for transfer of DNA from agarose gel to nitrocellulose filter paper

After prehybridisation, the filter was hybridised for twenty-four hours at 37°C to ^{32}P-labelled, nick-translated, pAM12 HBV DNA (Rigby et al. 1977). A typical labelling reaction contained 0,5 µg of HBV DNA in nick-translation buffer [5 µg/ml BSA in 52,5 mM Tris-HCl (pH 7,5) and 5,25 mM $MgCl_2$] containing 20 micromolar of unlabelled deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate. To this was added 10 µl (33 picomoles) of [^{32}P] deoxycytidine triphosphate (dCTP, specific activity 3000 Curies/millimole Amersham, U.K.) and 7 µl of enzyme solution (this contains 3,5 units of DNA polymerase I and 70 pg DNAse I). The final volume of 50 µl

was made up with sterile double-distilled water. [^{32}P] dCTP incorporation was monitored by measuring the specific activity of a 0,5 µl aliquot before and after trichloroacetic acid precipitation. The HBV DNA probes were nick-translated to a specific activity of 1-2 X 10^8 counts per minute (cpm) per microgram of HBV DNA. Hybridisations were performed in 50 percent (v/v) formamide, 5X SSC, 1X Denhardt's solution (0,02 percent (w/v) each of ficoll, polyvinyl pyrollidone and BSA in 3X SSC), 20 mM sodium phosphate buffer (pH 6,5), 10 percent (w/v) sodium dextran sulphate and 100 µg/ml denatured sonicated carrier DNA.

Recombinant HBV probe for these studies, pAM12 (a gift from the laboratory of J. Gerin, Rockville MD USA), contains the complete HBV genome (subtype adw) cloned into pBR325 at the Eco R1 site. Purified HBV probe was separated by electrophoresis and subsequent elctroelution (80 milliamps for one hour) from the gel slice (Sigma Type IV) after release by Eco RI (Boehringer Mannheim) digestion.

After transfer and hybridisation, the nitrocellulose filter was finally washed five times at 65°C with 2X SSC in 0,1 percent $NaDodSO_4$ and three times at 37°C with 0,1X SSC in 0,1 percent $NaDodSO_4$. Filters were then exposed to X-ray film for 2 to 7 days at -70°C using an intensifying screen and the autoradiograms

obtained were then examined.

3. Copy number screening

DNA from carriers with nonreplicative infection and patients with HCC was first screened for the copy number of HBV per haploid genome since the Southern blotting technique has a reported sensitivity of one copy per genome. Copy number was estimated by spotting a sample of total cellular DNA onto nitrocellulose paper in increasing concentrations (1 to 25 µg) together with purified HBV DNA (1 to 100 pg), as a standard. As a control, PLC/PRF/5 DNA was also included on each paper as it has a published copy number of four to six genomes per haploid mammalian cell DNA equivalent (Marion et al. 1980b and Edman et al. 1980). After hybridisation, washing and autoradiography, HBV DNA copy number could be assessed visually. A more accurate assessment was made by cutting out the circles on the filter and measuring the radioactivity of each spot by scintillation counting. From the graph of cpm plotted against pg of HBV DNA standard, the quantity of HBV DNA in picograms of HBV DNA per microgram of total cellular DNA could be extrapolated and from this the copy number was calculated.

CHAPTER III RESULTS

Carriers in the early phase of chronic hepatitis B are HBV DNA and HBeAg positive in serum and have episomal HBV DNA in high copy number in liver cells (Kam et al. 1982). Long-standing carriers are usually HBeAg negative and anti-HBe positive and have predominantly integrated HBV DNA with a lower copy number per cell (Brechot et al. 1981, Di Bisceglie and Hoofnagle, 1986). Early in the study it was realised that samples of DNA from long-standing carriers needed to be tested for HBV DNA copy number prior to analysis by Southern blotting. Thus we initially screened all samples from HBeAg-negative patients and patients with HCC (most of whom are HBeAg-negative) to determine whether Southern blotting would be sufficiently sensitive to analyse methylation. Southern blots were performed whenever the copy number was found to be Ø,2, or greater, so as not to exclude any specimens amenable to further study. The simple dot blot method outlined below proved reliable, as born out by the intensity of the signals obtained in subsequent Southern blots. In our hands, the "cut-off" point for Southern blot analysis was found to be approximately Ø,6 copies per haploid cell genome.

1. Copy number calculation

HBV DNA copy number was assessed visually by examining the autoradiograph obtained after dot blot analysis. Figure 11, a representative autoradiograph, illustrates this procedure. The signals obtained with dilutions of a recombinant HBV-DNA standard (top row; 1, 2, 5, 10, 25, 50, and 100 pg, reading left to right) can be compared to the signals obtained with extracted HBV DNA from the PLC/PRF/5 HCC cell line (bottom row) and six infected liver tissues (1, 2, 5, 10, 15, 20, and 25 µg of total cellular DNA, reading from left to right). HBV DNA copy number was measured by cutting out the circles on the nitrocellulose filter and measuring the radioactivity of each spot by scintillation counting. A standard curve was constructed by plotting the cpm per spot against the picograms of HBV DNA standard spotted. The amount of HBV DNA in picograms per microgram of total cellular DNA in unknown samples was extrapolated from the standard curve and converted to copy number per haploid cell genome by the following calculation: The haploid human genome contains 3×10^6 kbp, thus one human genome would weigh 3,3pg (since 1 kb = $6,6 \times 10^5$ daltons, 1 dalton = $1,67 \times 10^{-24}$g). Similarly, the 3,2 kbp of the HBV genome weigh $3,53 \times 10^{-6}$ pg. Thus, $3,53 \times 10^{-6}$ pg of HBV for every 3,3 pg total cellular DNA is equivalent to 1 copy of HBV DNA per haploid cell genome.

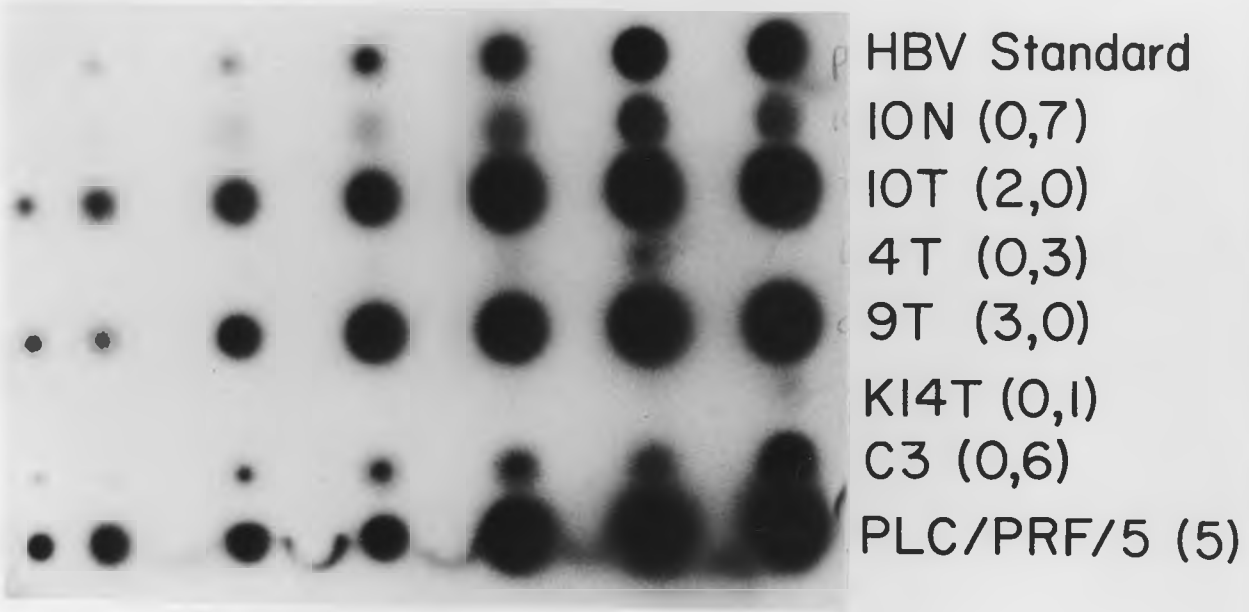

Figure 11:

Dot blot analysis of HBV-specific DNA for copy number determination.

In the example shown above calculated values of copy number of HBV per haploid cell genome are given in brackets. Analysable Southern blots were obtained from specimens 1ØN (Ø,7 copies), 1ØT (2 copies), 9T (3 copies) and C3 (Ø,6 copies). Both 1ØN and C3 gave weak signals as expected.

It was not necessary, for our purposes, to determine copy number of the HBeAg-positive carriers. In only one anti-HBe positive carrier did the HBV DNA copy number permit Southern blot analysis. This preliminary screening for copy number of HBV DNA in HCC revealed that 79 percent (7/9) of the specimens obtained from serologically HBeAg-positive patients and 50 percent (10/20) of the specimens from serologically HBeAg-negative patients had sufficient HBV DNA to permit further analysis. The majority of the corresponding non-neoplastic tissues from patients with HCC had a copy number less than one. For this reason, a comparison of the state of methylation in neoplastic and non-neoplastic tissue from the same patient was possible in only one patient (Table 3).

TABLE 3:

HBV DNA COPY NUMBER IN CHRONIC HBV INFECTION AND HCC

Group	HBeAg status	Number analyzed	Copy number $\geq$ 0.6
Chronic HBV	Negative	3	1
HCC	Positive	9	7
HCC	Negative	20	10
Non-neoplastic	Positive	2	1
Non-neoplastic	Negative	13	1

2. Integration

Total cellular DNA, with sufficient HBV DNA, was digested with HindIII to determine whether the HBV DNA was episomal or integrated. HindIII recognises no site in HBV DNA. Thus by comparing the size of HBV-specific DNA before and after HindIII digestion with the migration position of extrachromosomal, HBV DNA, the integration status of HBV-DNA fragments and molecules can be established (Shafritz 1982). If HBV-DNA sequences are not integrated, the same banding pattern will be produced before and after HindIII digestion and all fragments will be genome size (3,2 kb) or smaller. In contrast, if HBV DNA is integrated at specific sites in the host DNA, the undigested material appears as a high molecular weight smear before digestion with HindIII whereas after digestion, a specific electrophoretic banding pattern is obtained. All bands, both before and after digestion, are greater than 3,2 kb. If integration sites into the host cell DNA are random, the smear remains after digestion but the cleavage products will still all be greater than HBV genome size as the genome itself is not cleaved. The only exception would be if an integrated portion of HBV DNA was detected in a fragment of cellular DNA shorter than 3,2 kb (Figure 12).

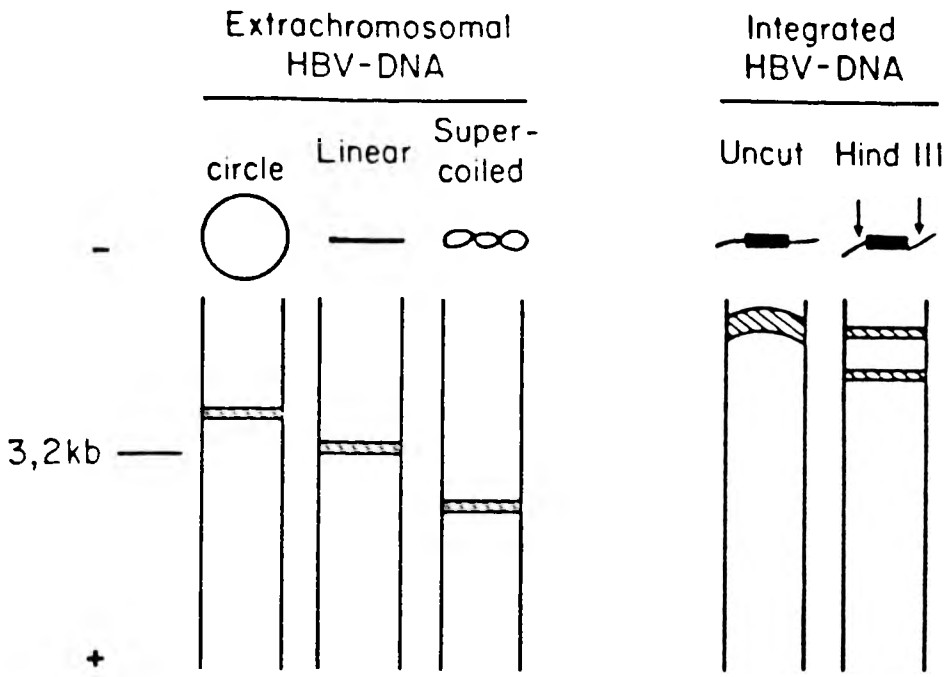

Fig. 12. Strategy for Hind III analysis of HBV-DNA (Shafritz 1982)

2.1 HBV DNA status in replicative infection

Only episomal HBV DNA was detected in Dane particle DNA extracted from the serum of HBeAg-positive chronic carriers. When undigested, the HBV-specific DNA (Figure 13, lane 4), gave a smear which extended from 4,0 to 1,9 kb (heterogenous partially double stranded DNA forms). This smear did not move after digestion with HindIII (lane 1), but bands appeared at 4,0 kb (relaxed circlular HBV DNA) and 3,2 kb (linear HBV DNA).

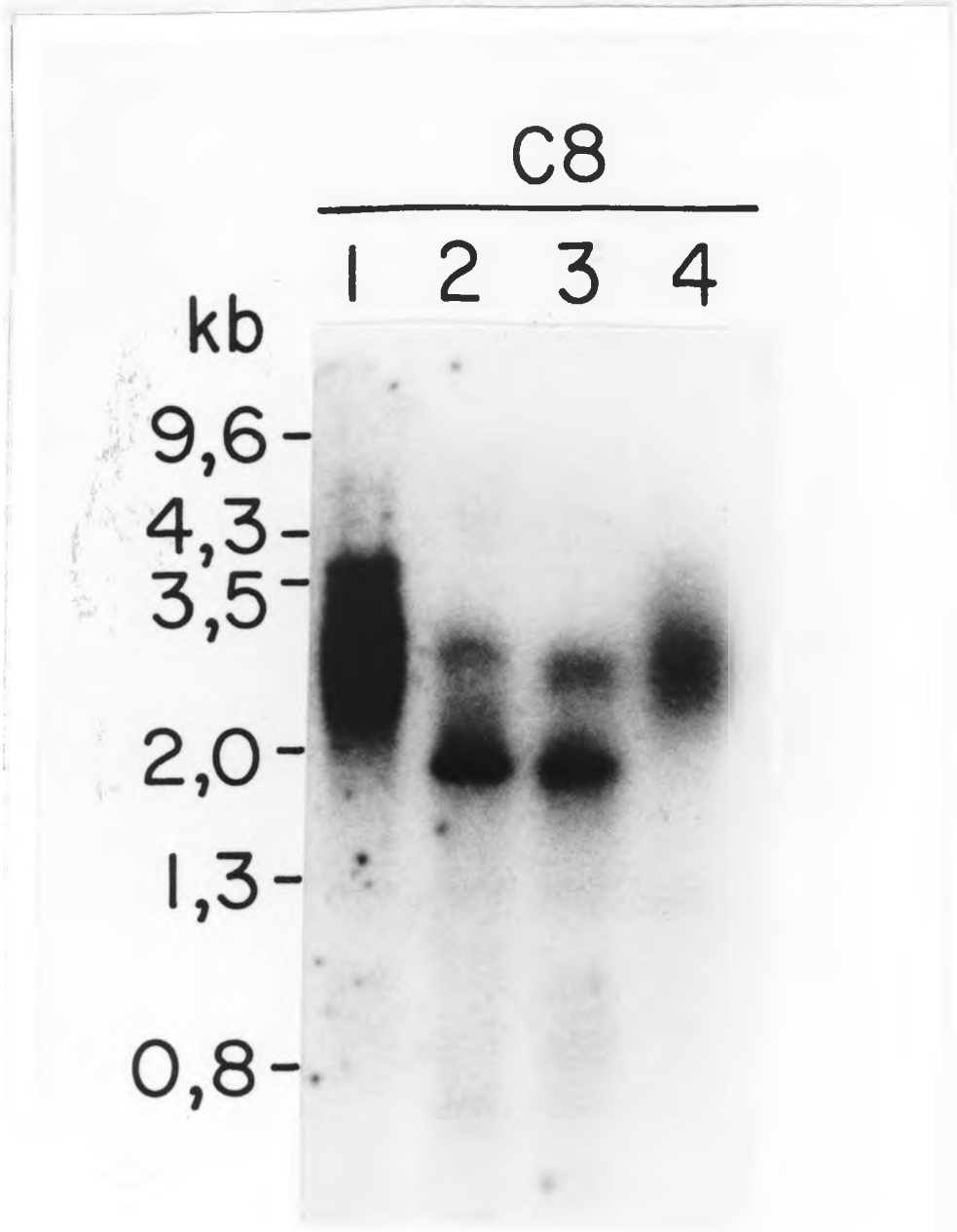

Figure 13:
Patient C8 - Southern blot analysis of HBV DNA in serum. All HBV-specific DNA is genome size and smaller in undigested (lane 4) and HindIII digested (lane 1) DNA, extracted from serum of a replicative carrier, indicating that the HBV DNA is all episomal. The HpaII digest (lane 2) is identical to the MspI digest (lane 3) and this indicates that the HBV-specific DNA is hypomethylated.

The same pattern, indicating predominantly episomal HBV DNA, was found in the liver tissue of the HBeAg-positive patients studied (Table 4). The undigested pattern of the free viral DNA (not shown) showed a 3,2 kb band (this band was at 4,Ø kb in one patient - Figure 14, lane 3) with two smears of DNA which covered the ranges 2,8 to 2,Ø kb (partially double stranded forms) and 1,6 to Ø,4 kb (single minus stranded replicative intermediates).

TABLE 4:

HBV DNA STATUS IN CHRONIC HEPATITIS B AND HCC

Tissue	Number	HBeAg status	Cellular HBV DNA
Chronic hepatitis	4	+	episomal
Chronic hepatitis	1	-	integrated and episomal
HCC	2	+	integrated
HCC	2	+	integrated and episomal
HCC	2	-	episomal
HCC	7	-	integrated

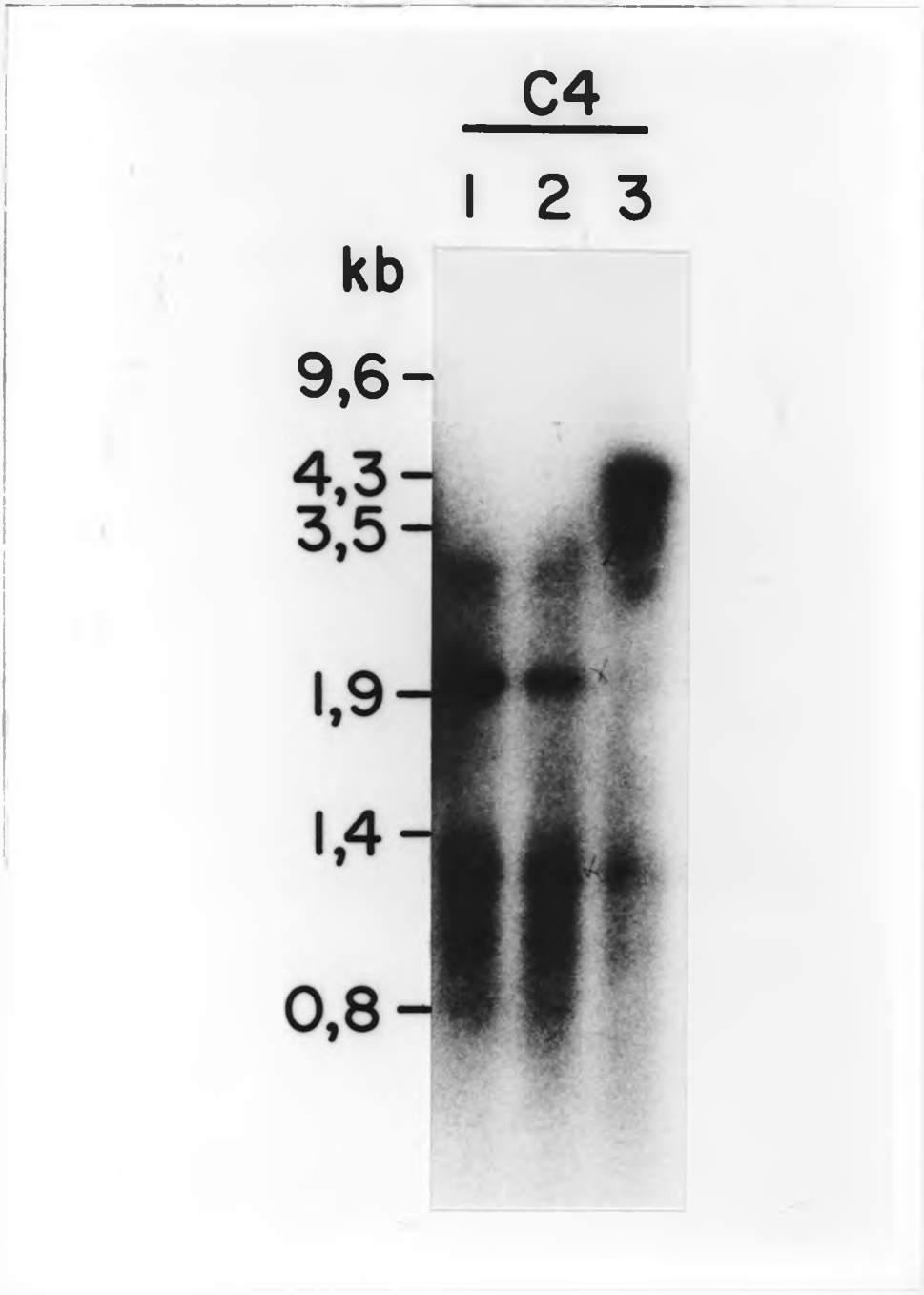

Figure 14:
Patient C4 - Southern blot analysis of infected liver tissue from a serum HBeAg-positive carrier showing undigested free viral DNA (lane 3) which is hypomethylated since lane 1 (HpaII digest) is identical to lane 2 (MspI digest).

2.2 HBV DNA status in non-replicative infection

The autoradiograph of the HindIII digested HBV-specific DNA from the one analysable anti-HBe positive patient (Figure 15, lane 1) shows a smear, greater than HBV genome size extending to a band at 4,Ø kb (relaxed, circular HBV DNA). Thus, both episomal and integrated HBV DNA is present in this carrier.

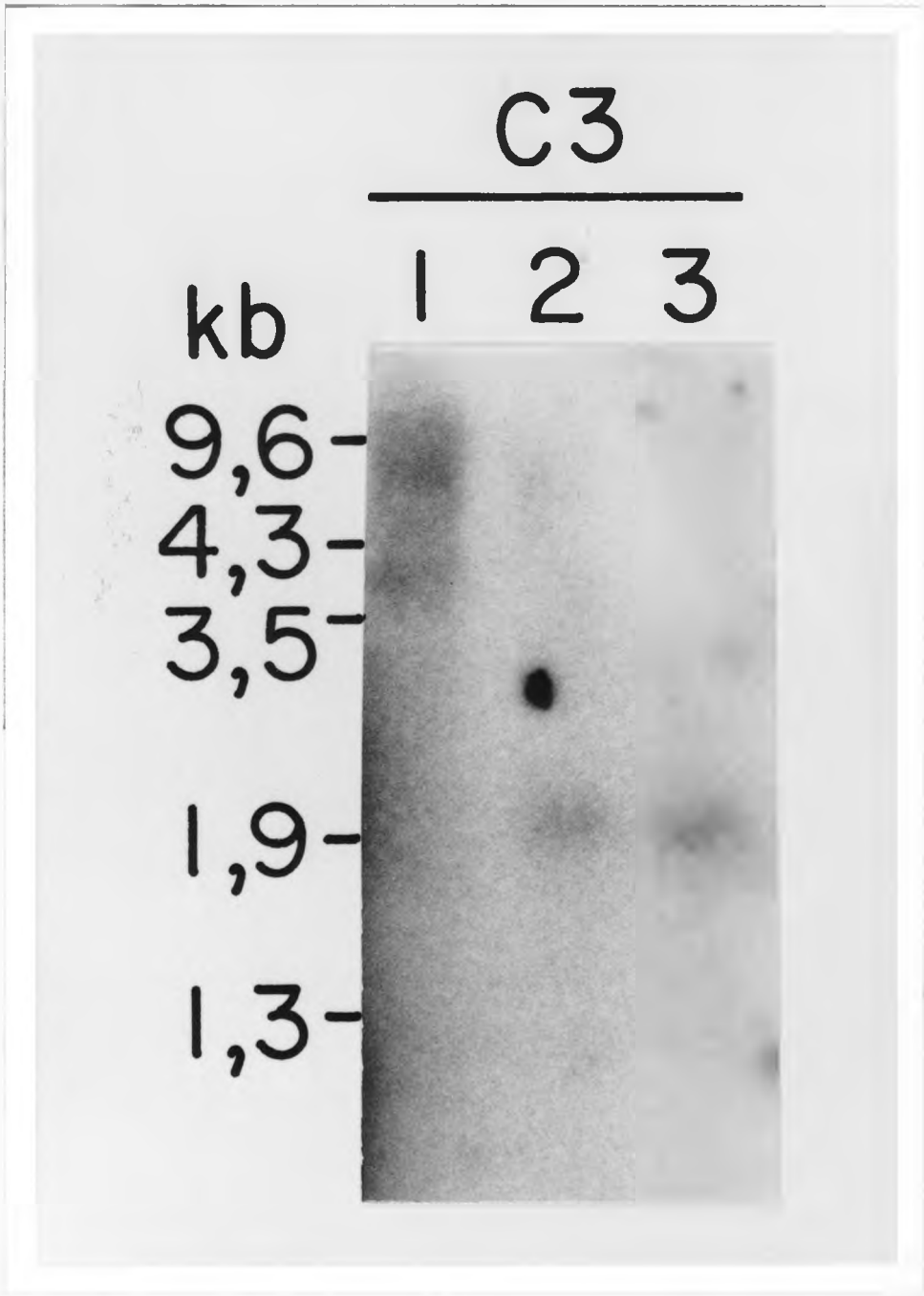

Figure 15:
Patient C3 - Southern blot analysis of integrated and episomal (see HindIII digest, lane 1) HBV-specific DNA from a serum anti-HBe positive carrier. The weak signal in the HpaII (lane 2) and MspI (lane 3) digests make conclusions on methylation status of HBV-specific CCGG sites difficult in this patient.

2.3 HBV DNA status in serum HBeAg-positive HCC

Two of the serologically HBeAg-positive HCC tissues showed both episomal and integrated HBV DNA while in another two samples only integrated HBV DNA was detected (Table 4). In both cases where episomal and integrated species were simultaneously present, integration was specific and low level viral replication was evident. Both high (greater than 9 kb) and low (3,5 - 1,6 kb) molecular weight smears in the undigested DNA (Figure 16, lane 4) are replaced by specific bands after HindIII digestion (lane 1). Some of these bands are greater than genome size (integrated sequences) while others are genome size and smaller (episomal HBV DNA). The HindIII analyses of the two patients where only integrated sequences were present revealed that integration was random in one case but specific in the other. Undigested HBV-specific DNA from the latter (Figure 17, lane 4) appeared on the Southern blot as a high molecular weight smear whereas after HindIII digestion (lane 1) specific bands, larger than genome size, were seen with one distinct band at approximately 2,0 kb. This band is probably from a smaller than genome size integrated fragment.

Three tumours were omitted from integration analysis as poor quality DNA obscured their integration status.

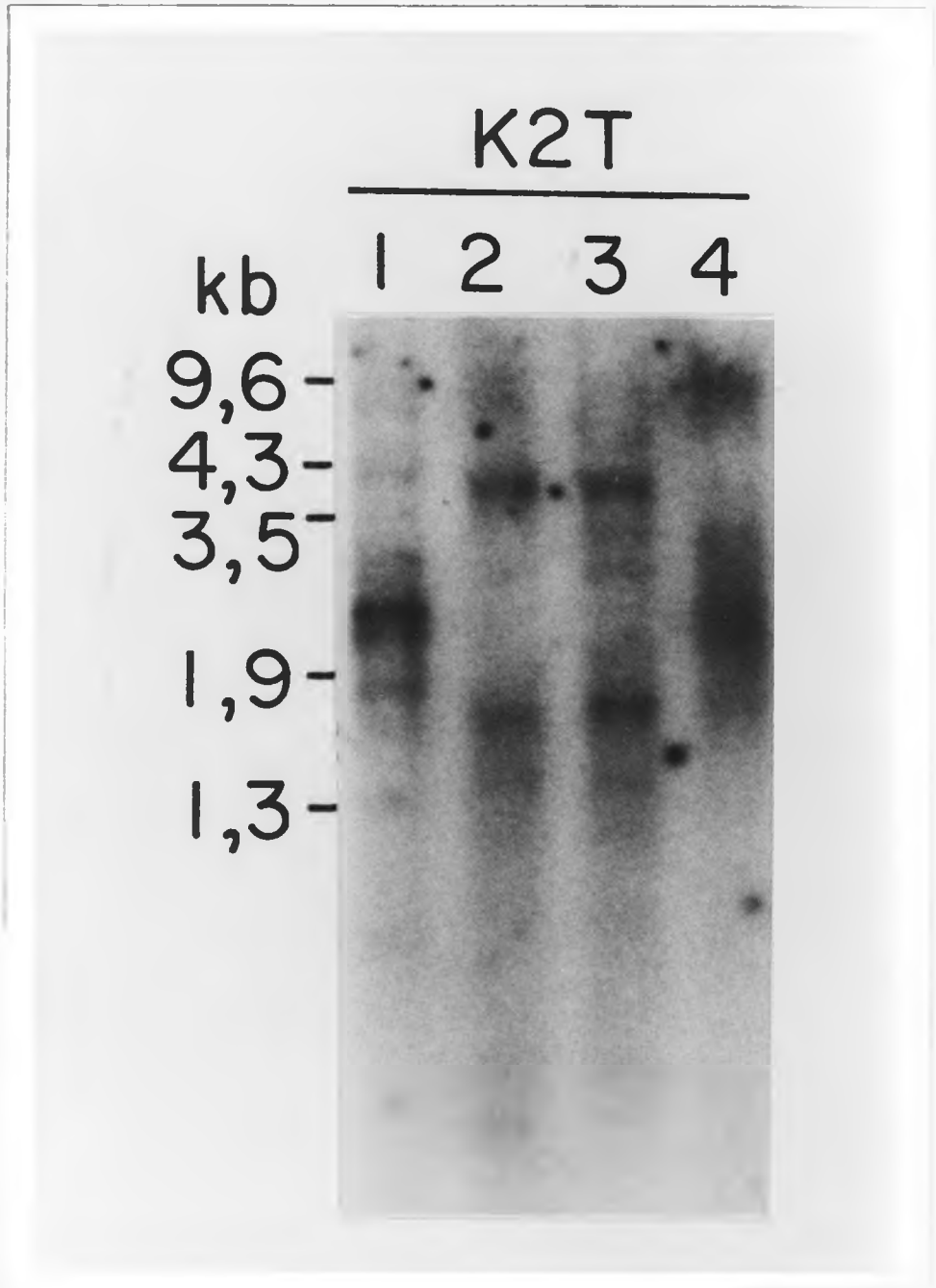

Figure 16:
Patient K2T - detection of both episomal and specifically integrated HBV DNA in a serum HBeAg-positive HCC patient. The appearance of distinct bands in the HindIII digest (lane 1) indicates that integration is specific. DNA extracted from the tissue was also run undigested (lane 4) and digested with HpaII (lane 2) or MspI (lane 3).

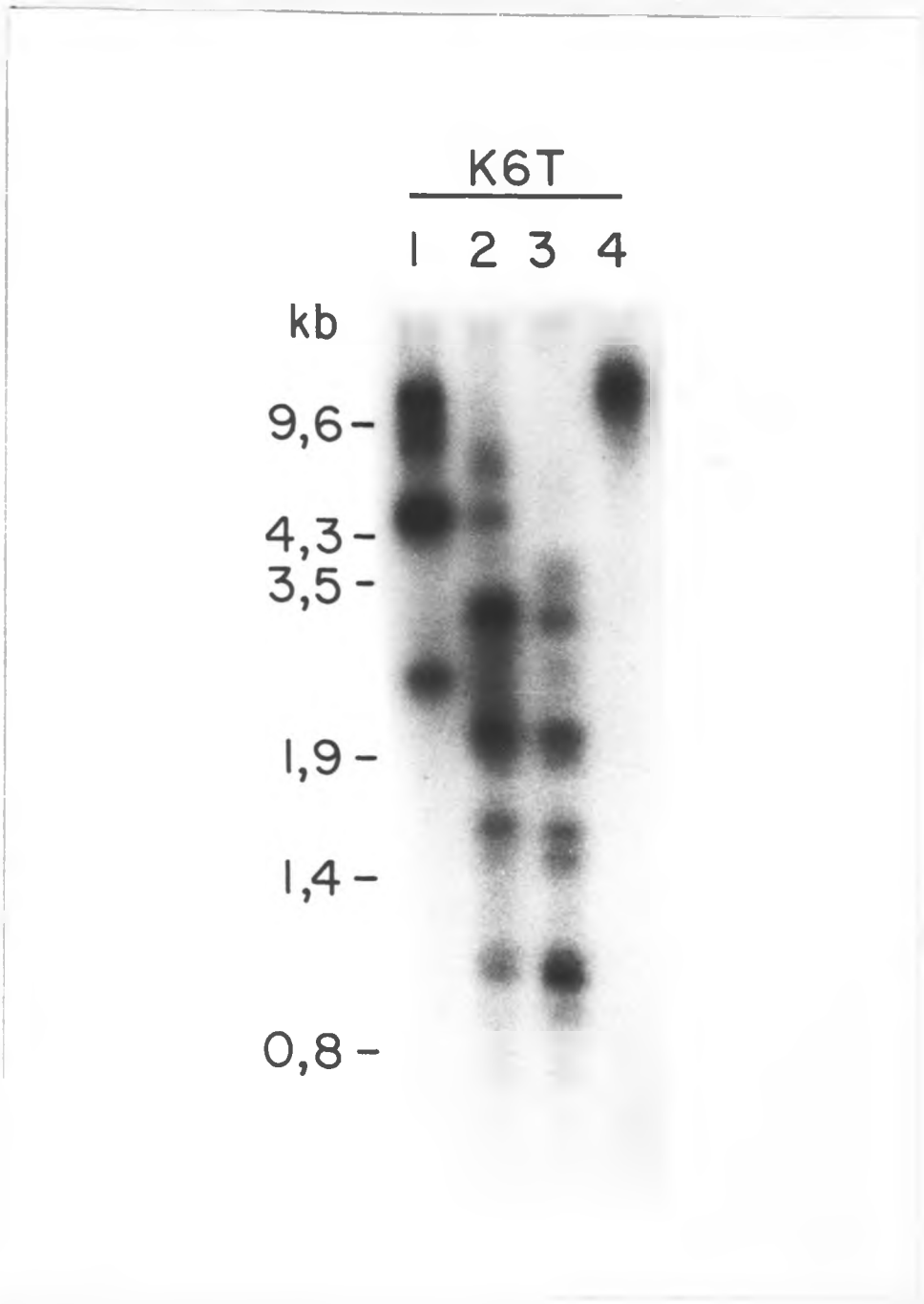

Figure 17:
Patient K6T - specific integration and hypermethylation of HBV-specific DNA in a serum HBeAg-positive HCC patient. Specific integration is evident from a comparison of undigested (lane 4) and HindIII digested (lane 1) HBV-specific DNA. High molecular weight bands present in the HpaII digest (lane 2) but not the MspI digest (lane 3) indicate that the viral DNA is hypermethylated.

2.4 HBV DNA status in serum HBeAg-negative HCC

In two serologically HBeAg-negative HCC tissues only episomal HBV DNA was detected, integrated copies were not visible on the Southern blot (Figure 18). The undigested HBV DNA (lane 4) shows a smear of genome size and smaller. The HindIII digest (lane 1) has the same pattern although the band at 4,Ø kb (relaxed circular HBV DNA) is clearer.

In the remaining seven HBeAg-negative tissues only integrated HBV DNA was detected (Figure 19). Here the pattern was characteristic of randomly integrated HBV DNA in that a heavy smear of HBV-specific DNA, greater than genome size, is seen in both undigested (lane 4) and HindIII digested (lane 1) lanes on the autoradiograph.

One tumour, found to have a copy number of Ø,8, was received too late in the study to be included in further analyses.

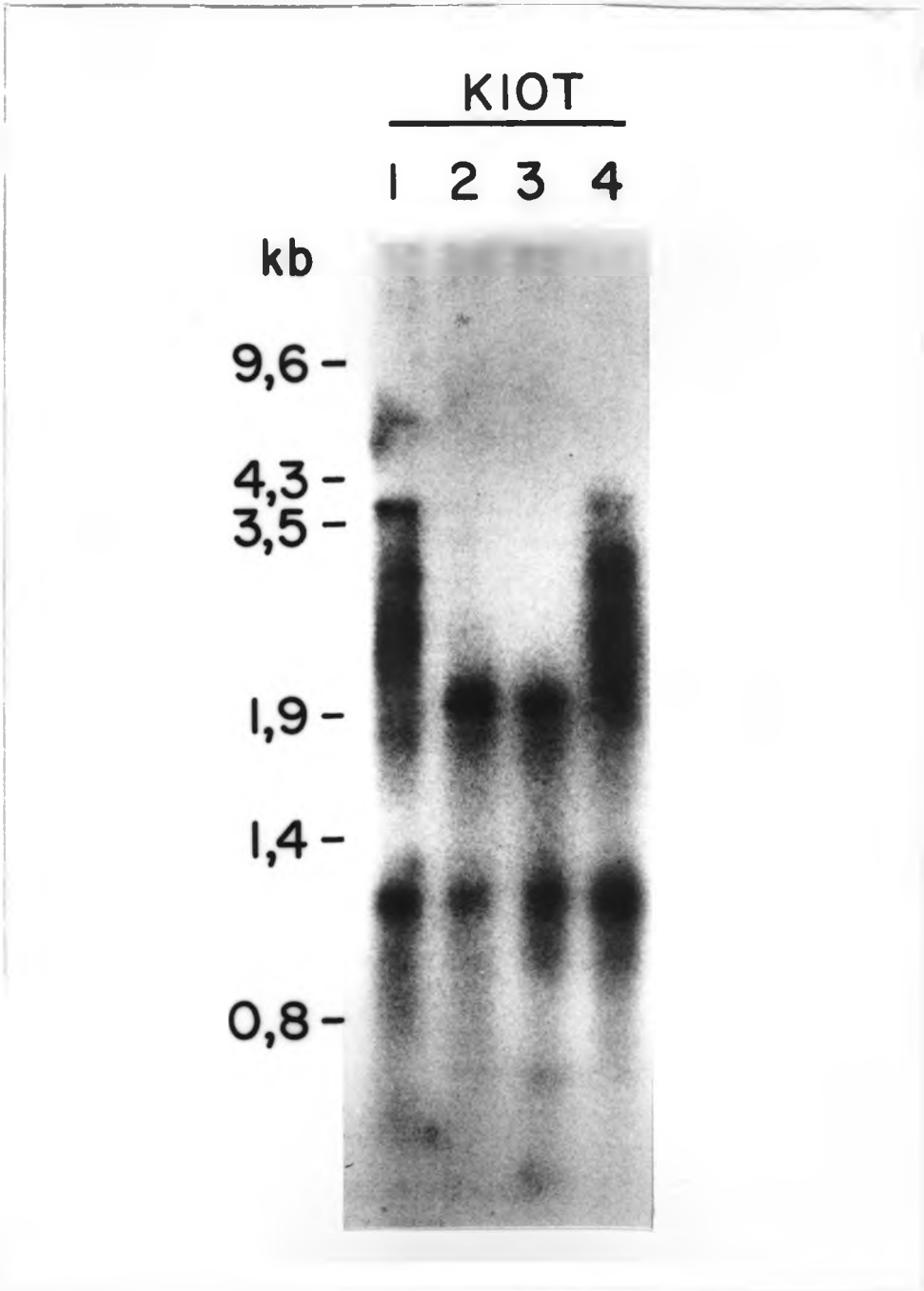

Figure 18:
Patient KlØT - episomal and hypomethylated HBV-specific DNA in a serum HBeAg-negative HCC patient. Only episomal HBV DNA is evident from a comparison of the HindIII digest (lane 1) with the undigested DNA (lane 4). The HBV-specific DNA was hypomethylated as the restriction pattern after the HpaII digestion (lane 2) is identical to that obtained after digestion with MspI (lane 3).

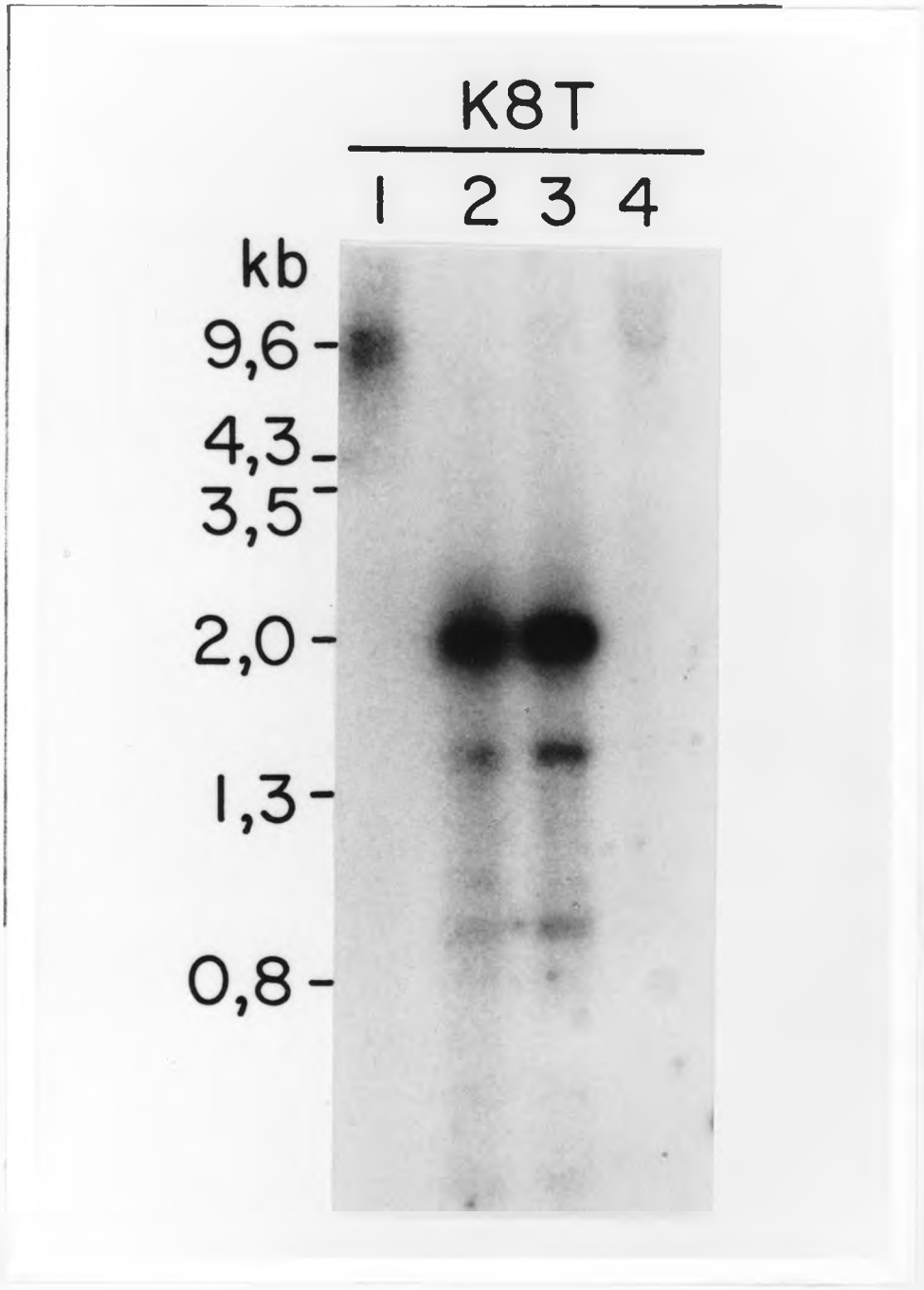

Figure 19:
Patient K8T - random integration (compare undigested HBV specific DNA in lane 4 with the HindIII digest in lane 1) and hypomethylation (compare HpaII digest in lane 2 with MspI digest in lane 3) of HBV-specific DNA in a serum HBeAg-negative HCC patient.

3. Methylation

To determine whether HBV-specific CCGG sites were methylated, the restriction patterns after digestion with HpaII and MspI were compared. Fewer bands and larger molecular weight fragments obtained after digestion with HpaII indicated CCGG site methylation and hence resistance to HpaII digestion (Figure 20).

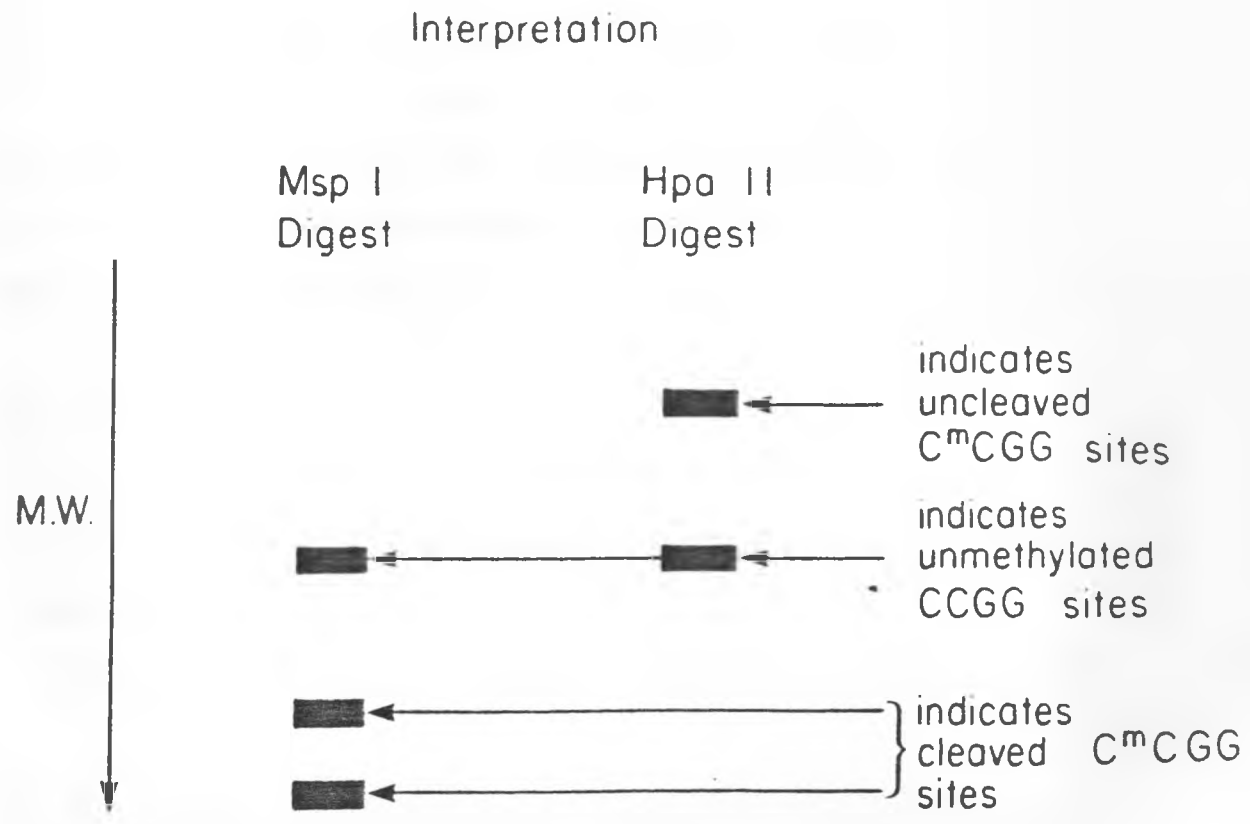

Fig. 20. Interpretation of Msp I/Hpa II restriction maps

3.1 Hypomethylation in replicative infection

The state of methylation of HBV-specific CCGG sites in DNA from HBeAg-positive carriers was examined in both liver tissue and in serum. The Dane particle derived HBV DNA extracted from serum of a replicative carrier was hypomethylated (Figure 13) as the HpaII digest (lane 2) was identical to the MspI digest (lane 3). This identical pattern was also obtained when infected liver tissue from serologically HBeAg-positive carriers was examined (Figures 14 and 21, lanes 1 and 2). Smearing in the lanes of Southern blots from these patients is caused by the presence of HBV replicative intermediates i.e. heterogenous partially double stranded DNA forms and single, minus strand DNA.

TABLE 5:

METHYLATION OF HBV IN CHRONIC HEPATITIS B

	Number	Non-methylated	HBV DNA Status
HBeAg-positive	4	4	4/4 Episomal
HBeAg-negative	1	1	1/1 integrated and episomal

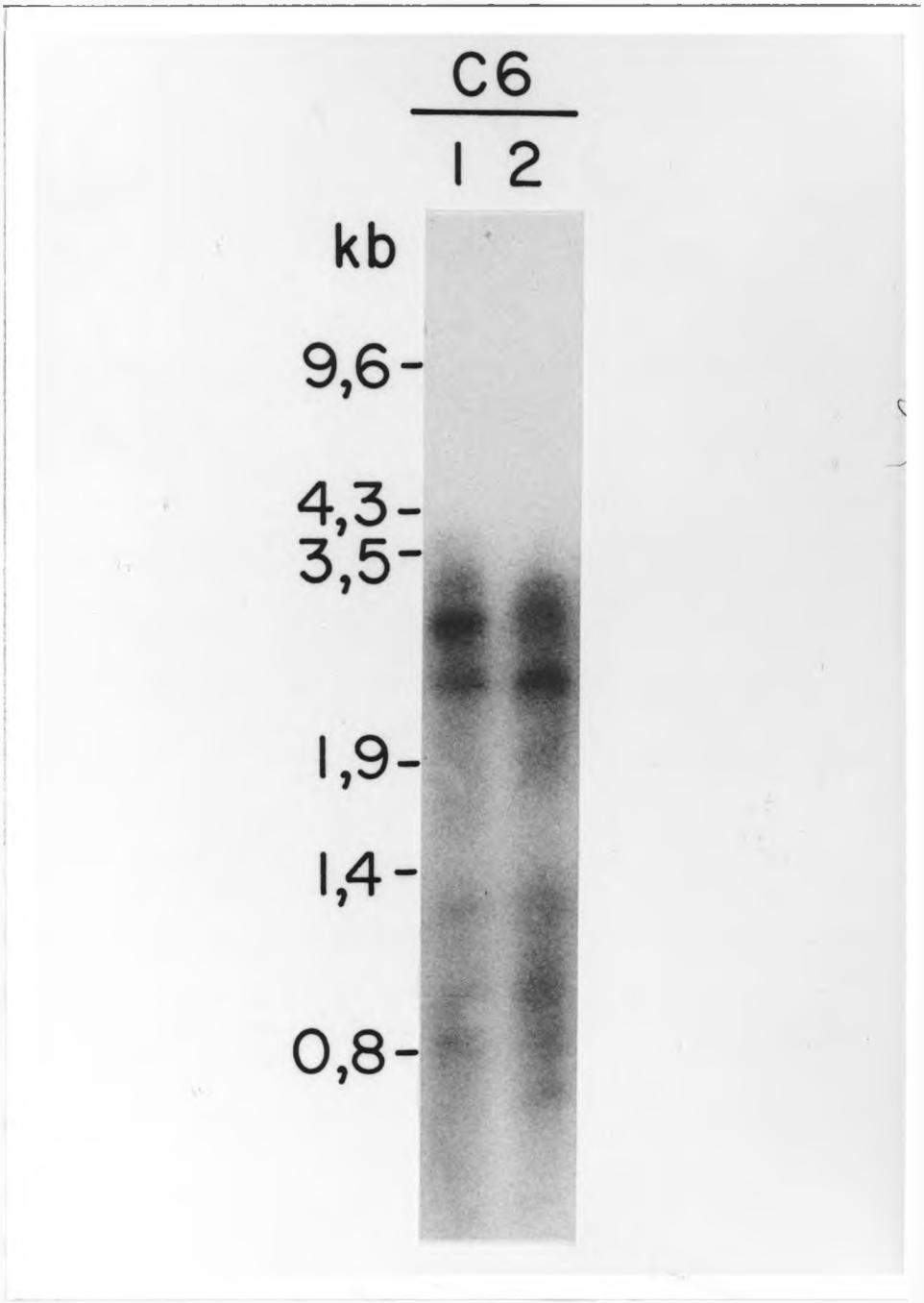

Figure 21:
Patient C6 - Tissue from a serologically HBeAg positive carrier - replicative intermediates obscure the banding pattern.

3.2 Nonreplicative infection

In the long-standing chronic hepatitis B carriers with nonreplicative infection which we examined, viral DNA copy number was frequently low and therefore the role of methylation in influencing HBV gene expression was difficult to ascertain (Table 5). When infected cells are few, the problem of decreased sensitivity inherent in the use of pooled cellular nucleic acid is difficult to overcome in Southern blot analysis. Figure 15 shows the weak HBV-specific signal obtained in the HpaII (lane 2) and MspI (lane 3) digests of DNA from a serologically HBeAg-negative carrier. The MspI and HpaII digests appear identical but the signals are faint. For this reason the DNA from tissue of HBV-related HCC patients was studied in the expectation that clonal amplification of tumour cells infected with HBV DNA would make analysis feasible.

3.3 Hypermethylation in HCC

Hypermethylated HBV DNA was found in the PLC/PRF/5 HCC cell line and in six tumour tissues (Table 6). Three of these tumour tissues were from serologically HBeAg-positive patients. In all three, integrated HBV DNA was detected but only one had traces of episomal HBV DNA. Figure 17 shows the typical hypermethylated pattern with higher molecular weight bands in the HpaII digest (lane 2) which are not present in the MspI digest (lane

3). The other three tumour tissues showing hypermethylation were from serologically HBeAg-negative patients and had only integrated HBV-DNA sequences. Although the signal from patient K9T (copy number 2,5) was weak (Figure 22), the pattern was reproducible. The HpaII digest (lane 2) shows a distinct, high molecular weight band at the 6 kb position which is not present in the MspI digest (lanes 3) whereas the MspI digestion resulted in a series of additional bands at about 1,9 kb. Hypermethylation can also be seen in tumour 1ØT (discussed in section 3.5) and 9T (Figure 23).

TABLE 6:

METHYLATION OF HBV DNA IN HCC

	Number	HBeAg positive	Episomal HBV	Integrated HBV
Methylated	6	3	1	6
Non-methylated	5	1	3	3

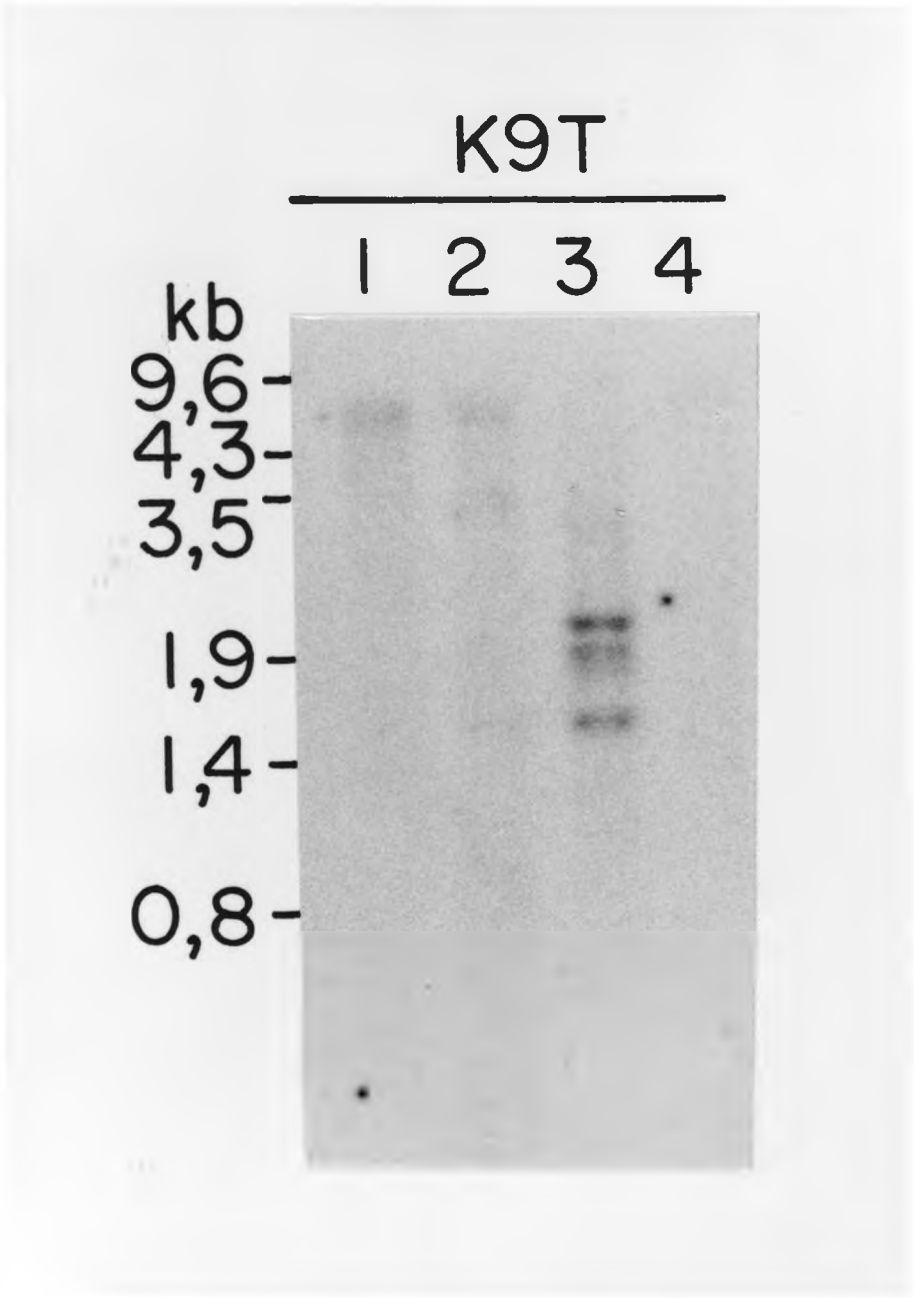

Figure 22:
Patient K9T - integrated and hypermethylated HBV-specific DNA in a serum HBeAg-negative HCC patient. Integration is random (compare undigested sample in lane 4 with the HindIII digest in lane 1). A high molecular weight band is present only in the HpaII digest (lane 2) while several smaller bands, in the region of the 1,9 kb lambda size marker, are present in the MspI digest (lane 3).

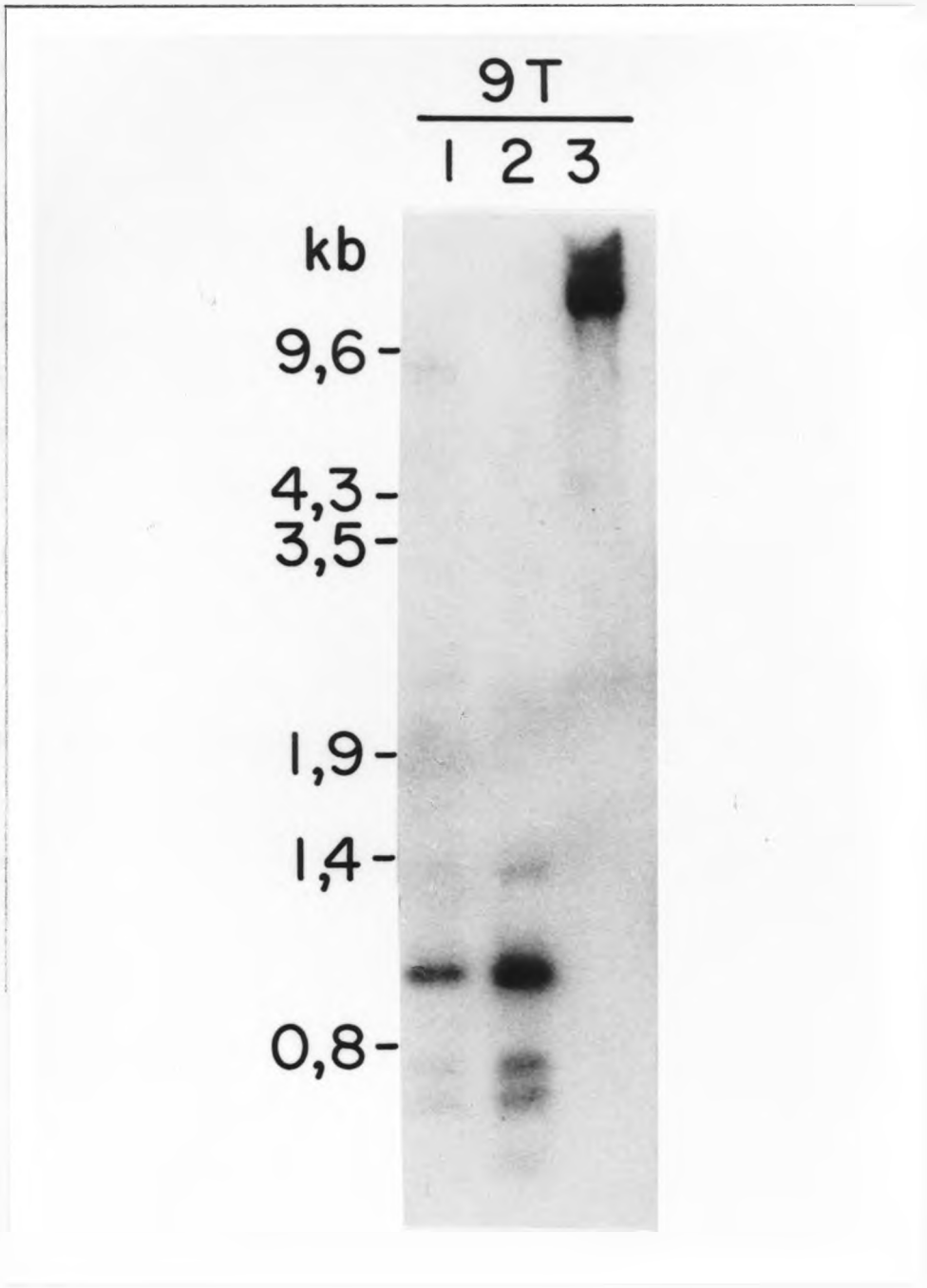

Figure 23:
Patient 9T - hypermethylated HBV-specific DNA. Total cellular DNA was digested with HpaII (lane 1) and MspI (lane 2) or run undigested (lane 3).

The restriction maps obtained with DNA extracted from PLC/PRF/5 nude mice tumour explants were compared to published restriction maps (Miller and Robinson, 1983) for the PLC/PRF/5 cell line. The tumour tissue has intense bands in both MspI (Figure 24, lane 2) and HpaII (lane 1) digests at approximately 8,5 kb and 3,Ø kb but the HpaII digest has additional bands between these common bands. The tumour tissue also shows a smaller band, less than Ø,8 kb, in only the MspI lane (Figure 25). Thus we could confirm methylation of PLC/PRF/5 cells. The first example (Figure 24) shows the additional high molecular weight bands in the HpaII digest more definitely while the second example (Figure 25) shows the smaller band in the MspI digest more clearly.

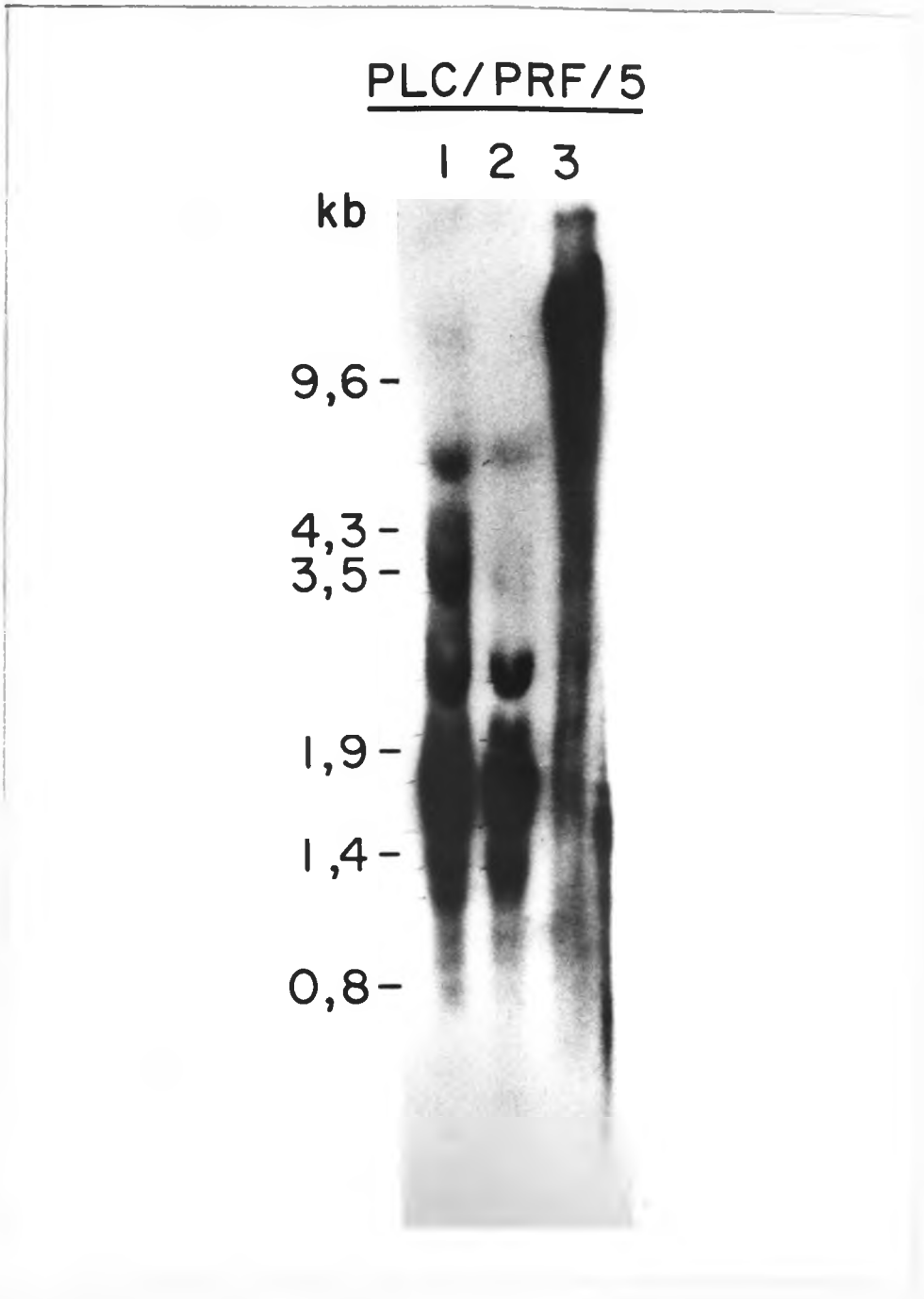

Figure 24:
PLC/PRF/5 - hypermethylated HBV-specific DNA. The tumour tissue has intense bands in both MspI (lane 2) and HpaII (lane 1) digests at approximately 8,5 kb and 3,Ø kb while the HpaII digest has additional bands between these common bands. Lane 3 is undigested HBV-specific DNA from this cell line.

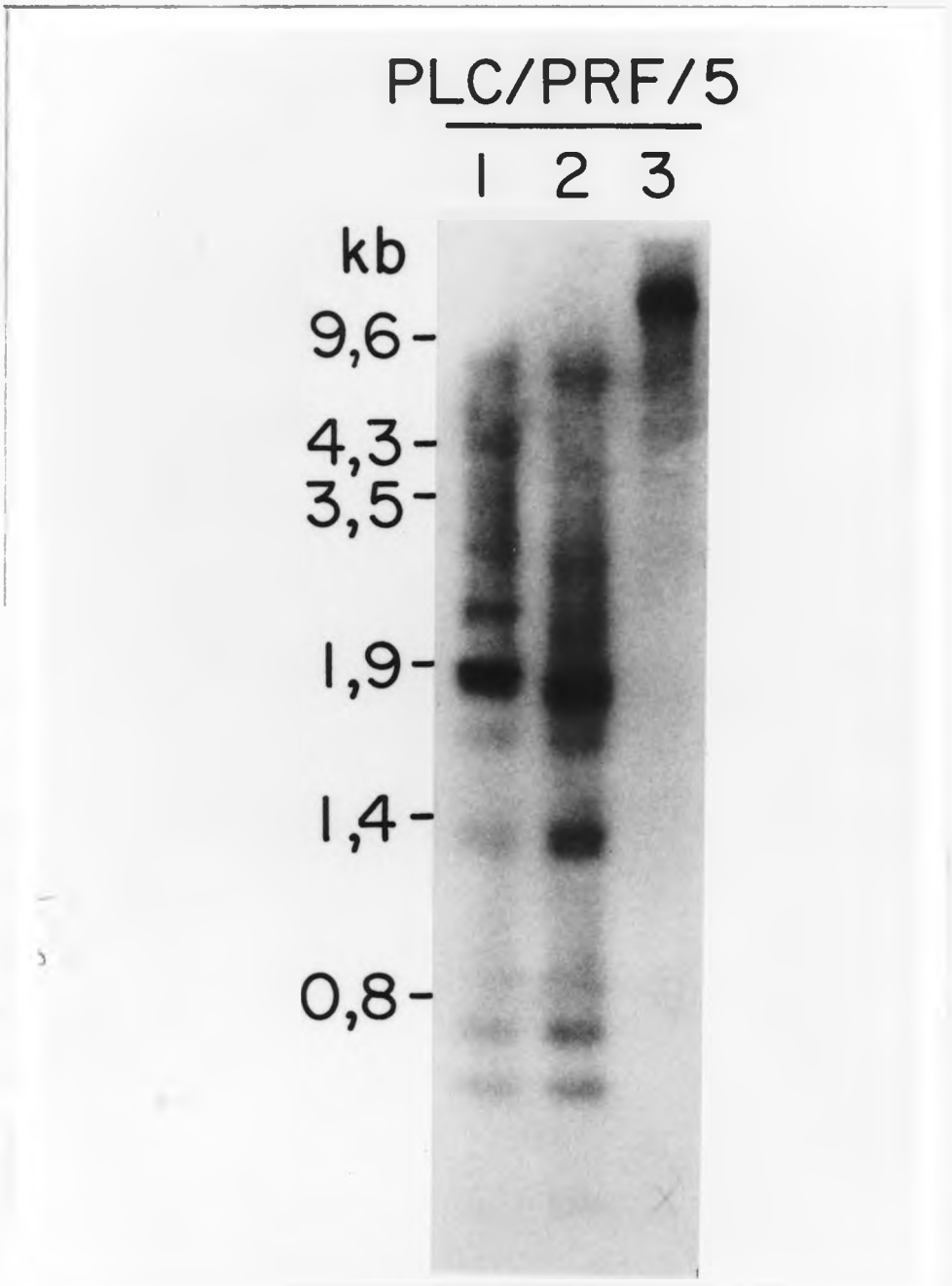

Figure 25:
Southern blot analysis of the PLC/PRF/5 HCC cell line. A low molecular weight band (Ø,8 kb) is present only in the MspI digest (lane 2) while high molecular weight bands are present only in the HpaII digest (lane 1). Lane 3 is undigested HBV-specific DNA.

3.4 Hypomethylation in HCC

Five of the tissues showed a hypomethylated pattern (Table 6). One of these, 1T, was a special instance; although this patient was HBsAg negative in serum, HBV DNA was detectable in tumour tissue (Figure 26). The HBV DNA in this patient was hypomethylated as seen by the identical HpaII (lane 1) and MspI (lane 2) digests.

Three of the tissues with hypomethylated HBV DNA were serologically HBeAg negative. The HindIII digestion pattern of two of these indicated that only episomal HBV DNA was present, no integrated HBV DNA sequences were visible (Figure 18). Thus, since the restriction pattern seen was only that of excess episomal HBV DNA, one might anticipate hypomethylated HBV DNA as was observed (Figure 18). The third of the serologically HBeAg-negative specimens showed only integrated HBV DNA sequences (Figure 19) and these were hypomethylated. The fifth hypomethylated tumour was from an HBeAg-positive patient who had both episomal and integrated HBV DNA (Figure 27).

Two tumours were excluded from the methylation study as their methylation status was unclear.

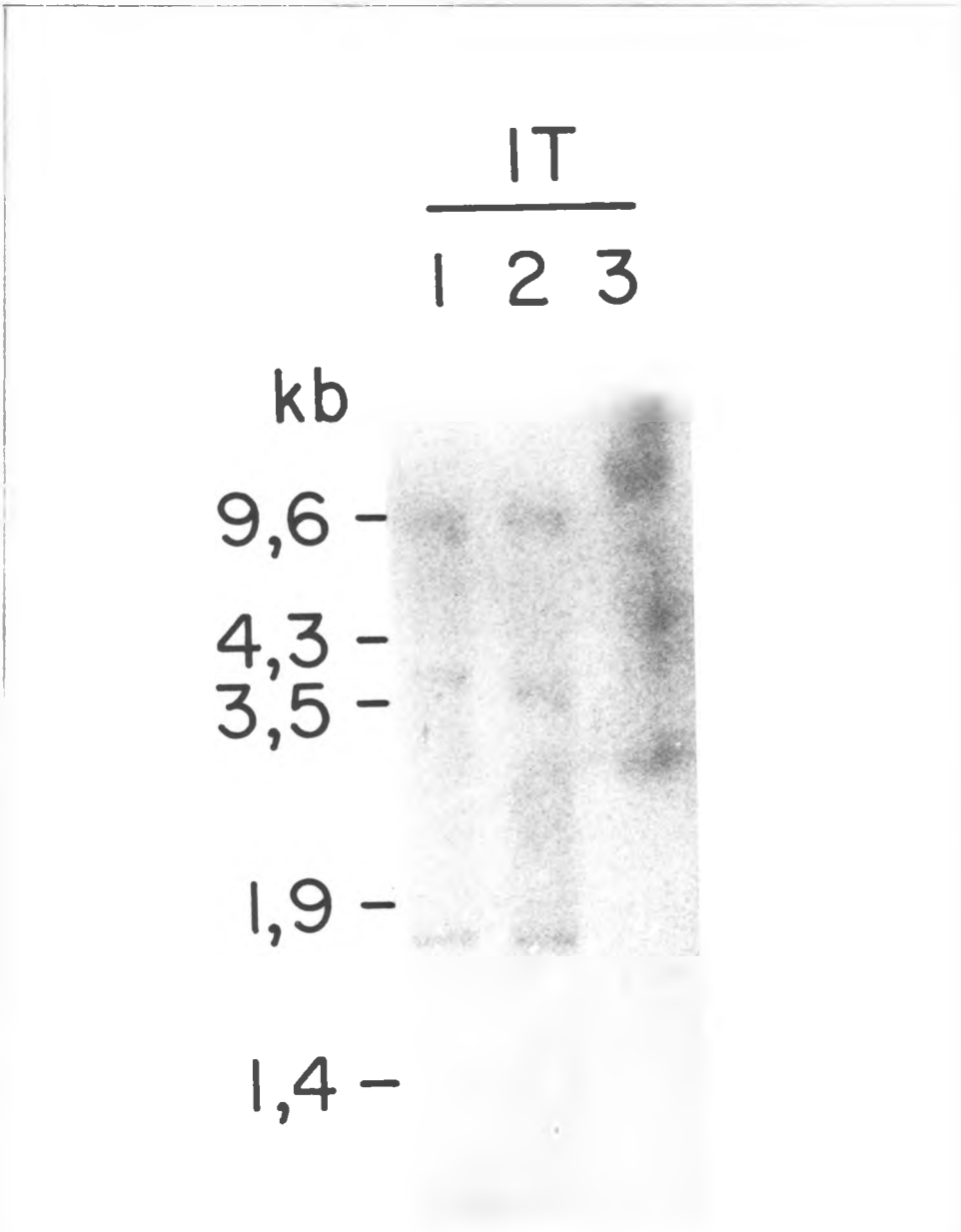

Figure 26:
Patient 1T - hypomethylated HBV-specific DNA in a serum HBsAg-negative HCC patient. Extracted DNA was digested with HpaII (lane 1) and MspI (lane 2) or was not digested (lane 3).

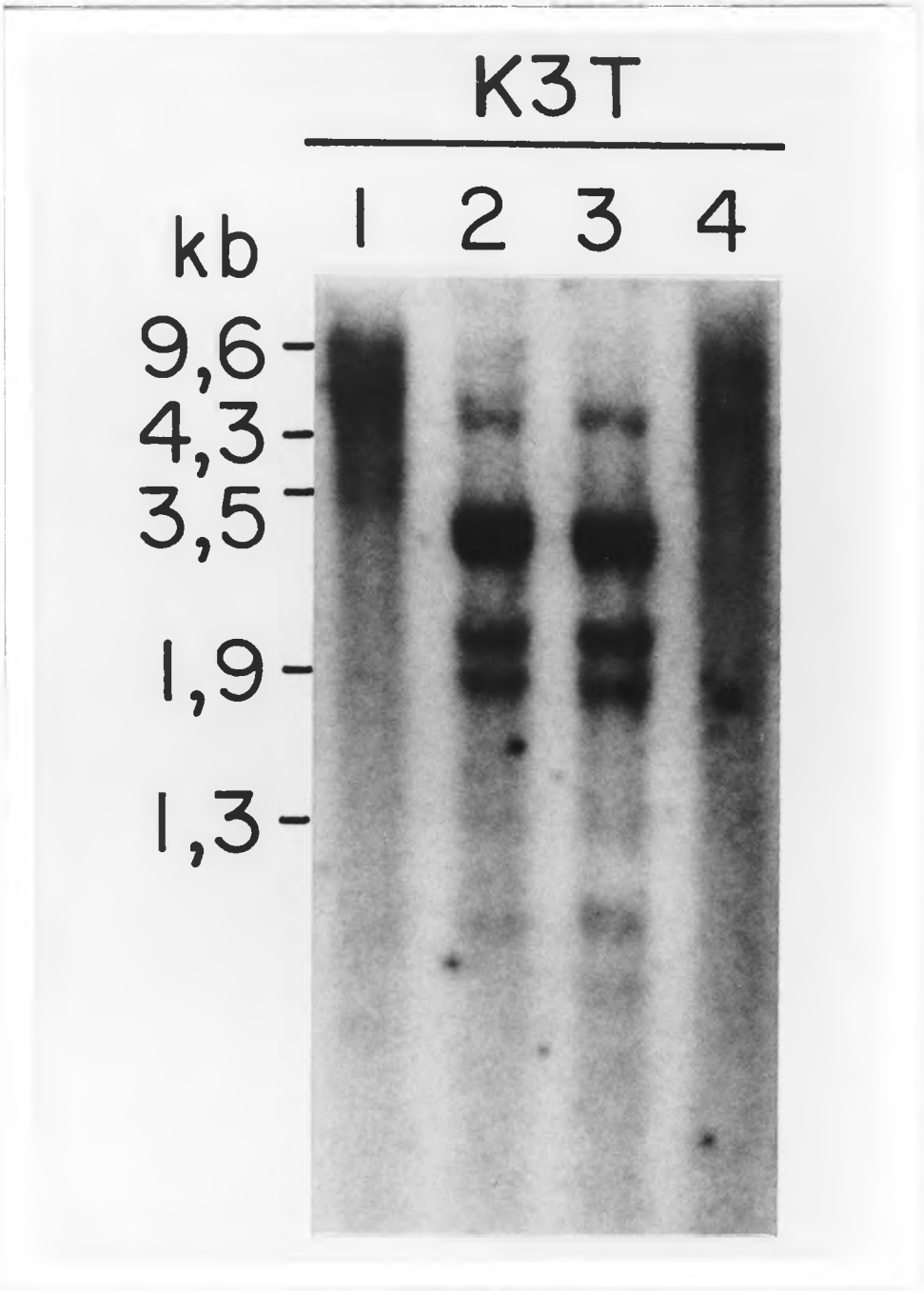

Figure 27:
Patient K3T - episomal, integrated and hypomethylated HBV DNA in a serum HBeAg-positive HCC patient. DNA extracted from this tissue was run undigested (lane 4) or digested with HpaII (lane 2), MspI (lane 3) and HindIII (lane 1).

3.5 Comparison of methylation status in tumour versus adjacent non-neoplastic tissue

Wherever tissue was available, we attempted to compare the state of methylation in tumour tissue with that in adjacent non-neoplastic tissue. However, only one of the 15 adjacent segments of non-neoplastic tissue (1ØN) was assessable. The HpaII (lane 3) and MspI digests (lane 4) were identical in this instance, indicating that the HBV DNA in this non-neoplastic tissue was hypomethylated (Figure 28). Two bands (one at approximately 2,Ø kb and the other at 1,6 kb) were present in both digests. Neither of these bands were present in the digests of the tumour tissue from the same patient. This result was interesting not only because the HBV DNA restriction analysis of the corresponding tumour tissue was totally different, but also because the HBV DNA was hypermethylated in the tumour (Figure 28) Tumour HBV-specific DNA digested with HpaII (lane 1) has high molecular weight bands at 4,Ø kb and approximately 2,5 kb which are not present in the same DNA digested with MspI (lane 2). Also present are four common bands of 1,9 kb and smaller.

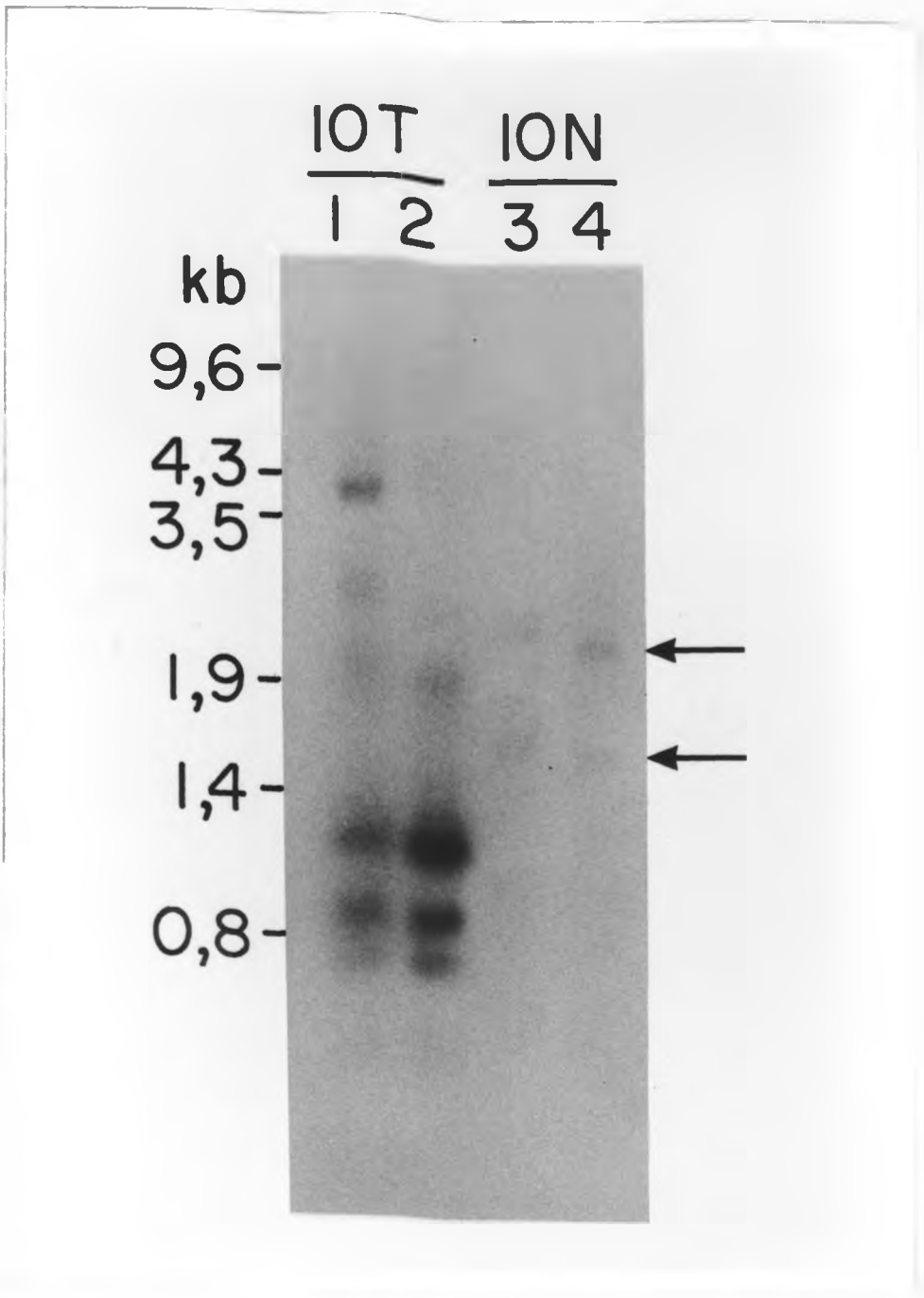

Figure 28:
Patient 1Ø (L and T) - comparison of the state of methylation in tumour tissue versus adjacent non-neoplastic tissue. Southern blot analysis of tumour tissue in this patient (lanes 1 and 2) gave quite different results to those obtained on analysis of the adjacent non-neoplastic tissue (lanes 3 and 4). The HpaII digest from the tumour tissue (lane 1) has heavier bands not present in the MspI digest (lane 2) while the adjacent non-neoplastic tissue shows identical HpaII (lane 3) and MspI (lane 4) digests.

CHAPTER IV DISCUSSION

We observed a low copy number of HBV per cell in HBeAg-negative HBsAg-carriers. These findings are in keeping with the observation that a decline in HBV copy number occurs after seroconversion from HBeAg to anti-HBe. In the tumour tissues studied, although clonal amplification had increased HBV copy number, there was nonetheless a difference between the analysable tumours found in HBeAg-positive (79 percent) versus HBeAg-negative (50 percent) patients. The mean copy number was also higher in HCC patients who were serologically HBeAg-positive (mean value 2) than in serologically HBeAg-negative patients with HCC (mean 0,8).

HindIII restriction digestion analyses confirmed earlier reports (Fowler et al. 1984b) that HBV DNA in infected liver in HBeAg-positive carriers is predominantly episomal. Anti-HBe positive patients have predominantly integrated HBV DNA although episomal HBV DNA can also be detected. Fowler et al. (1986) report that 15 percent of anti-HBe carriers who have detectable serum HBV DNA have episomal HBV DNA in liver tissue. However, they did not find episomal HBV DNA in patients without serum HBV DNA. We were similarly able to detect both

integrated and episomal HBV DNA in tissue in an anti-HBe positive patient. Both episomal and integrated HBV DNA, as well as integration alone, was observed in long-standing carriers with low levels of viral replication who had progressed to HCC. Song et al. (1984) studied HBV viral markers in 106 southern African Blacks with HBV-related HCC and found that HBV replication was present in the minority of these patients and, when occurring, was of low grade activity. Their findings support the hypothesis that HBV DNA is frequently integrated into the human genome of Black patients with HBV-related HCC. Our HindIII restriction digestions confirmed an inverse correlation between serum markers indicative of HBV replication and integrated HBV DNA sequences in infected tissue from patients with chronic hepatitis and HCC. We also confirm that indeed the majority of HBsAg-positive HCC patients possess integrated HBV DNA in tumour tissue. Twelve of the 14 nonreplictive carriers and patients with HCC had detectable integrated HBV DNA sequences. Although episomal HBV DNA was detected in five of these tissues, it was always present in much lower quantities than was found in the specimens from chronic active carriers. In two of the HBeAg-negative patients we found only episomal HBV DNA. Robinson et al. (1984) report that some tumours do not show detectable integrated HBV DNA

sequences by molecular hybridisation techniques with sensitivities down to Ø,Ø1 genome copy per cell. However the presence of very small viral DNA sequences (i.e. less than 1ØØ bps per cell) cannot be excluded and thus, in the two HCC patients in whom we detected episomal HBV DNA alone, it is possible that integrated sequences are present but are below the sensitivity of the Southern blot assay.

Our results indicate that CCGG sites in HBV DNA extracted from HBV virions in serum of HBeAg-positive carriers with replicative infection are hypomethylated. This is in keeping with the findings previously reported by Miller and Robinson (1983). Neither are they methylated in infected tissue from these carriers. Thus in early, replicative, chronic hepatitis B, hypomethylation of the HBV genome correlates with HBV gene expression. This molecular state is not unique to the hepatitis B virus: DNA extracted from virions of simian virus 4Ø (Ketner et al. 1976), herpes saimiri (Desrosiers 1982), adenovirus (Gunthert et al. 1976) and herpes simplex (Sharma et al. 1977) is either slightly methylated or not methylated at all. Herpes simplex is however, transiently methylated during the course of infection (Sharma et al. 1977).

Although the signal was very weak, one carrier with predominantly nonreplicative infection and a copy num-

ber of Ø.6 had hypomethylated HBV DNA on Southern blot analysis; episomal and integrated HBV DNA was present. However, because of the low HBV DNA copy number found in carriers with nonreplicative infection, the possible converse role of methylation in repressing HBV gene expression was difficult to ascertain. The low HBV-DNA copy number in long-standing nonreplicative infection was confirmed when non-neoplastic liver tissue from HCC patients was studied: Only one of the sixteen adjacent non-neoplastic tissues from HCC patients had a copy number greater than one. In only one other case were interpretable Southern blots obtained from both tumour and adjacent non-neoplastic tissue from the same patient. The HBV DNA in this non-neoplastic tissue (1ØL) was integrated but was hypomethylated. This result was interesting because DNA from the corresponding tumour tissue was hypermethylated and the restriction analyses using tumour DNA were totally different than those obtained using DNA from the non-neoplastic tissue. The two tissues, although from the same patient, did not have common bands. There were also more bands in the Southern blot of the tumour tissue suggesting that either there had been rearrangements in the tumour clone or the tumour clone was the result of another integration. In an attempt to overcome the problem of low copy number in nonreplicative infection, we

included HBV-related HCC patients in the study and regarded all HCC tissue (regardless of serum HBeAg status) as being representative of long-standing infection with low level viral replication. This premise is justified since long-standing HBV infection precedes the development of HCC and there is evidence to suggest a measure of gene control in HCC (Dejean et al. 1982). Also, only a minority of patients with HCC are HBeAg- and HBV-DNA positive in serum and, wnere HBeAg is present, it is almost always present in low titer (Kew et al. 1981, Tabor et al. 1977). Methylation of HBV DNA could be demonstrated in HCC but the results were inconsistent.

Integrated HBV DNA sequences were found to be hypermethylated in the PLC/PRF/5 HCC cell line. Methylation of specific viral genes in virus-transformed cell lines has been previously reported for HBV (Millar and Robinson 1983) as well as for herpes saimiri virus (Desrosiers 1982) and adenovirus (Günthert et al. 1976). HBV-DNA sequences were also found to be hypermethylated in six of the tumour tissues studied, suggesting that methylation may play some role in gene repression, but a further five tumours were hypomethylated. Viral expression correlated more closely with the presence of HBV DNA integration than HBV DNA methylation. All six hypermethylated tumours had integrated

HBV DNA. Three were from serum HBeAg-negative patients, but the other three were serum HBeAg-positive. Low titers of HBeAg could be accounted for by coding from small quantities of episomal HBV DNA but this was only detected in one of the three specimens; this could reflect the degree of sensitivity of the methods employed. However, despite several lines of evidence indicating a strong correlation between specific gene activity and hypomethylation, HBeAg expression was detected in only one of the five hypomethylated HCC specimens. Expression of HBeAg in this HCC patient could not be attributed solely to the hypomethylated integrated sequences as episomal sequences were also present. The five hypomethylated HCC specimens included the special case that was serum HBsAg negative. A further two tumours were unusual in that they were HBeAg negative with only episomal HBV DNA detectable; no integrated sequences were visible. Thus, although the HBV DNA was hypomethylated in both of these patients, this would be expected if only the episomal sequences were visualised. The fifth hypomethylated tissue did not express the HBeAg; only integrated HBV-DNA sequences were detected and these were hypomethylated. Since all HCC with integrated HBV DNA did not have hypermethylated HBV DNA, our results suggest that methylation <u>per se</u> is not an absolute determinant of

gene expression in HCC tissue. Thus, other factors, such as gene rearrangements, regulatory gene disruption, reading frame shifts or m-RNA precursor size, must also be considered as possible influences of HBV expression in chronic hepatitis B and HCC.

APPENDIX A

Analysis of tissue and sera from chronic hepatitis B carriers.

Code	HBsAg	HBeAg	Anti-e	Copy No.	HBV Status	Meth. Status	Diagnosis
C1	+	-	+	0,50	NA	NA	CPHB
C2	+	-	NT	0,30	NA	NA	CPHB
C3	+	-	+	0,57	INT. & EPI.	HYPO.	CPHB
C4	+	+	NT	NT	EPI.	HYPO.	CAHB
C5	+	+	NT	NT	EPI.	HYPO.	CAHB
C6	+	+	+	NT	EPI.	HYPO.	CAHB
C7	+	+	NT	NT	EPI.	HYPO.	CAHB
*S1	+	+	-	NT	EPI.	HYPO.	CAHB
*S2	+	+	-	NT	EPI.	HYPO	CAHB

Meth, Methylation; NA, not analysable; NT, not tested; +, positive by RIA; -, negative by RIA; CPHB, chronic persistent hepatitis B; CAHB, chronic active hepatitis B; I, integration; EPI, Episomal; HYPO, Hypomethylated.

* serum samples

APPENDIX B

Analysis of tissue from patients with hepatocellular carcinoma

Code	HBsAg	HBeAg	Anti-e	Copy No.	HBV Status	Meth. Status	Diagnosis
*1L	-	-	-	0,5	NA	NA	N/N
*1T				0,68	INT.	HYPO.	HCC
*2L	+	-	+	0,04	NT	NT	N/N
*2T				0,04	NT	NT	HCC
*3L	+	-	+	0,04	NT	NT	N/N
*3T				0,65	INT.	NA	HCC
*4T	+	-	-	0,27	NA	NA	HCC
*5L	+	-	+	0,04	NT	NT	N/N
*5T				0,04	NT	NT	HCC
*7L	+	-	+	0,04	NT	NT	N/N
*7T				0,04	NT	NT	HCC
*8L	+	-	NT	0,04	NT	NT	N/N
*8T				0,60	INT.	NA	HCC
*9L	+	-	NT	0,04	NT	NT	N/N
*9T				3,00	INT.	HYPER.	HCC
*10L	+	-	NT	0,72	INT.	HYPO.	N/N
*10T				2,00	INT.	HYPER.	HCC
+K8T	+	-	NT	2,98	INT.	HYPO.	HCC
+K9T	+	-	NT	2,50	INT.	HYPER.	HCC
+K10T	+	-	NT	6,95	EPI.	HYPO.	HCC
K11L	+	-	NT	0,19	NA	NA	N/N
+K11T				0,39	NA	NA	HCC
+K12T	+	-	NT	0,13	NT	NT	HCC
+K13T	+	-	NT	0,40	NA	NA	HCC
K14L	+	-	+	0,04	NT	NT	N/N
+K14T				0,30	NA	NA	HCC
K15L	+	-	+	0,10	NT	NT	N/N
+K15T				0,20	NA	NA	HCC
+K16T	+	-	-	2,00	EPI.	HYPO.	HCC
K17L	+	-	NT	0,23	NT	NT	N/N
+K17T				0,80	NT	NT	HCC
K18L	-	-	-	0,04	NT	NT	N/N
+K18T				0,30	NT	NT	HCC
*6L	+	+	-	0,50	NA	NA	N/N
*6T				0,70	INT.	HYPER.	HCC
+K1L	+	+	NT	0,19	NA	NT	N/N
+K1T				1,06	NA	NT	HCC
+K2T	+	+	NT	1,90	EPI. & INT.	HYPER.	HCC
+K3T	+	+	NT	1,57	EPI. & INT.	HYPO.	HCC
+K4T	+	+	NT	0,25	NA	NA	HCC
+K5T	+	+	NT	5,0	NA	NA	HCC
+K6T	+	+	NT	6,00	INT.	HYPER.	HCC
+K7T	+	+	NT	1,20	NA	NA	HCC
K19L	+	+	NT	3,50	NT	NT	N/N
+K19T				0,30	NT	NT	HCC
CL1	+	+	-	5,00	INT.	HYPER.	PLC/PRF/5

NA, not analysable; NT, not tested; N/N, non-neoplastic; +, positive by RIA; -, negative by RIA; INT, Integrated; EPI, Episomal; HYPO, Hypomethylated; HYPER, Hypermethylated.

*HCC tissue from Taiwanese patients

+HCC tissue from African patients

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