LTR ..... long terminal repeat

LMT ..... low melting temperature

LB ..... Luria - Bertani

$MgSO_4$ ..... magnesium sulphate

mRNA ..... messenger ribonucleic acid

MHBs^t ..... truncated middle surface protein

µg ..... micrograms

µl ..... microlitres

ml ..... millilitre

mM ..... millimolar

- ..... minus

M ..... molar

Mw ..... molecular weight

ng ..... nanogram

nm ..... nanometres

ORFs ..... open reading frames

$O.D._x$ ..... optical density at x nm

/ ..... per

% ..... percentage

PBS ..... phosphate buffered saline

pmol ..... picomoles

pfu ..... plaque forming units

+ ..... plus

5' ..... 5 prime

ed. ..... editor

ECL ..... enhanced chemiluminescence

EDTA ..... ethylene diaminotetraacetate

Fl-dUTP ..... fluorescein - 11 - dUTP

FCS ..... foetal calf serum

g ..... grams

g/l ..... grams per litre

g ..... gravitational force unit

G ..... guanosine

HBV ..... Hepatitis B virus

HBeAg ..... Hepatitis B virus e antigen

HBsAg ..... Hepatitis B virus surface antigen

HBxAg ..... Hepatitis B virus x antigen

HCV ..... Hepatitis C virus

HCC ..... Hepatocellular carcinoma

HIV ..... human immunodeficiency virus

IU ..... inhibitory units

IPTG ..... β-D-isopropyl-thiogalactopyranoside

Kbp ..... kilobase pair

λ ..... lambda

LiCl ..... lithium chloride

l ..... litre

pH ..... log [hydrogen ion]

L ..... long

LIST OF ABBREVIATIONS, SYMBOLS AND UNITS

A ..... adenine

AFP ..... α-fetoprotein

et al ..... and others

anti HBc ..... antibody against HBV core antigen

anti HBs ..... antibody against HBV surface antigen

Ag ..... antigen

b.p ..... base pairs

β ..... beta

BSA ..... bovine serum albumin fraction

CO_2 ..... carbon dioxide

CaMV ..... Cauliflower Mosaic Virus

cm^2 ..... centimeters square

ca ..... circa

CP ..... core promotor

C ..... cytidine

°C ..... degrees Celcius

DNA ..... deoxyribonucleic acid

dUTP ..... deoxyuracil triphosphate

DMSO ..... dimethylsulfoxide

DR ..... direct repeat

ds ..... double stranded

DHBV ..... Duck hepatitis B virus

CHAPTER 2

CHAPTER 3

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This dissertation is dedicated to my parents,

Jeremy and Beryl Bailey.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university, nor has it been prepared under the aegis or with the assistance of any other body or organisation or person outside the University of the Witwatersrand, Johannesburg.

Catherine Patricia Bondonno

31 Day of March, 1995

ABSTRACT

Hepatitis B virus (HBV) infection resulting in integration of the viral DNA into host liver cell DNA is associated with the development of hepatocellular carcinoma (HCC). This is indicated by epidemiological trends, molecular studies and studies of animal models infected with viruses closely related to HBV. However, little is known about the mechanism by which the integrated HBV DNA induces HCC despite continuing analysis of the integrated HBV DNA and its surrounding cellular sequences. The Tong/HCC cell line, a cell line established from a HCC of a 59 year old Italian male, has five integrated HBV sequences which to date have not been cloned and sequenced. In this study, DNA from the Tong/HCC cell line was isolated and used to create a genomic DNA library using Lambda gt11. An initial library titre of 1 x 10^5 pfu/ml was obtained. To increase the titre, the library was amplified and showed a final titre of 6.8 x 10^7 pfu/ml with 93% recombinants. The Tong/HCC cell line genomic library was screened for the presence of HBV DNA sequences with a probe consisting of the entire HBV genome (3.2 kb). 15 positive recombinants were detected, 10 of which were isolated and rescreened. Another aspect to this study involved detecting the presence of HBsAg sequences, completely or partially expressed as a fusion protein, using a monoclonal antibody raised against serum HBsAg. This was performed after the third screen. Five percent of the HBV DNA positive recombinants reacted with the antibody. This work was performed so that in future studies the five integrated HBV DNA sequences and the flanking cellular DNA in the Tong/HCC cell line could be sequenced and analysed further.

CONSTRUCTION AND SCREENING OF A DNA LIBRARY TO DETECT INTEGRATED HEPATITIS B VIRUS DNA

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March, 1995

HBV or heterologous viruses. The X gene product also has a protein kinase activity and can phosphorylate serine and threonine residues on both the X - encoded protein as well as other substrates such as histone and casein (Wu et al., 1990).

Another functionally important element that has been identified in the hepatitis B virus genome is two 11 bp direct repeat sequences (5' TTCACCTCTGC 3') designated DR1 and DR2. They are approximately 225 bp apart and play a critical role in viral DNA replication. The 5' end of the L(-) strand lies within DR1 and the 5' end of the S(+) strand is at the 3' boundary of DR2 as seen in Figure 1.1 (Molnar-Kimber et al., 1984; Seeger et al., 1986).

Research has revealed that the HBV genome has 3 RNA polymerase II dependent promoters, namely the presurface gene promoter, SP1, the surface gene promoter, SP11, and the core promoter, CP (Cattaneo et al., 1983; Rall et al., 1983; Laub and Treinin, 1987; Yaginuma and Koike, 1989).

Also present are two tissue specific enhancers (Jameel and Siddiqui, 1986; Shaul et al., 1985; Yee, 1989), a glucocorticoid-responsive regulatory element (Tur-Kaspa, 1986) and the conserved hexanucleotide, TATAAA, which plays a role in the correct polyadenylation and processing of HBV RNA transcripts (Ganem and Varmus, 1987).

1.4. Replication of HBV

The unusual replication strategy of HBV was first determined in DHBV (Summers and Mason, 1982; Mason et al., 1982) and later confirmed for HBV (Blum et al., 1984; Miller et al., 1984; Will et al., 1987). It differs from that of other DNA viruses

the S region alone code for polypeptides of 38 000 daltons, 31 000 daltons and 24 000 daltons respectively.

There are five antigenic specificities found on the HBsAg particles. A group specific determinant (a) is shared by all HBsAg preparations. Two pairs of subtype determinants (dy an l wr) are present and for the most part they are mutually exclusive. Eight HBsAg subtypes have been identified: ayw_1, ayw_2, ayw_3, ayw_4, ayr, adr, adw_2 and adw_4 (Couroucé et al., 1976).

1.3.3. The P gene

This ORF overlaps each of the others and codes information contained within the other three frames of the genome. It specifies a virion-associated DNA polymerase or reverse transcriptase, important in the replication of the virus (Sherker and Marion, 1991). A terminal protein, which is bound to the 5' end of the minus strand, is encoded by the 5' end of the P ORF (Bosch et al., 1988). It functions as a primer for the synthesis of the virus minus strand from the RNA pregenome (Lien et al., 1985; Seeger et al., 1986; Will et al., 1987). Another protein encoded by this region is RNase H (Toh et al., 1983; Bartenschlager et al., 1990; Chang et al., 1990) which degrades all but the most 5' region of the RNA pregenome during replication (Will et al., 1987).

1.3.4. The X gene

The X ORF got its name because its gene product and function were completely unknown for several years. It is now known to encode a transactivating protein which has trans-acting properties that allow it to modulate the level of transcription of several host (Twu and Schloemer, 1987; Twu and Schloemer, 1989) or viral (Spandau and Lee, 1988; Twu and Robinson, 1989) regulatory sequences, either from

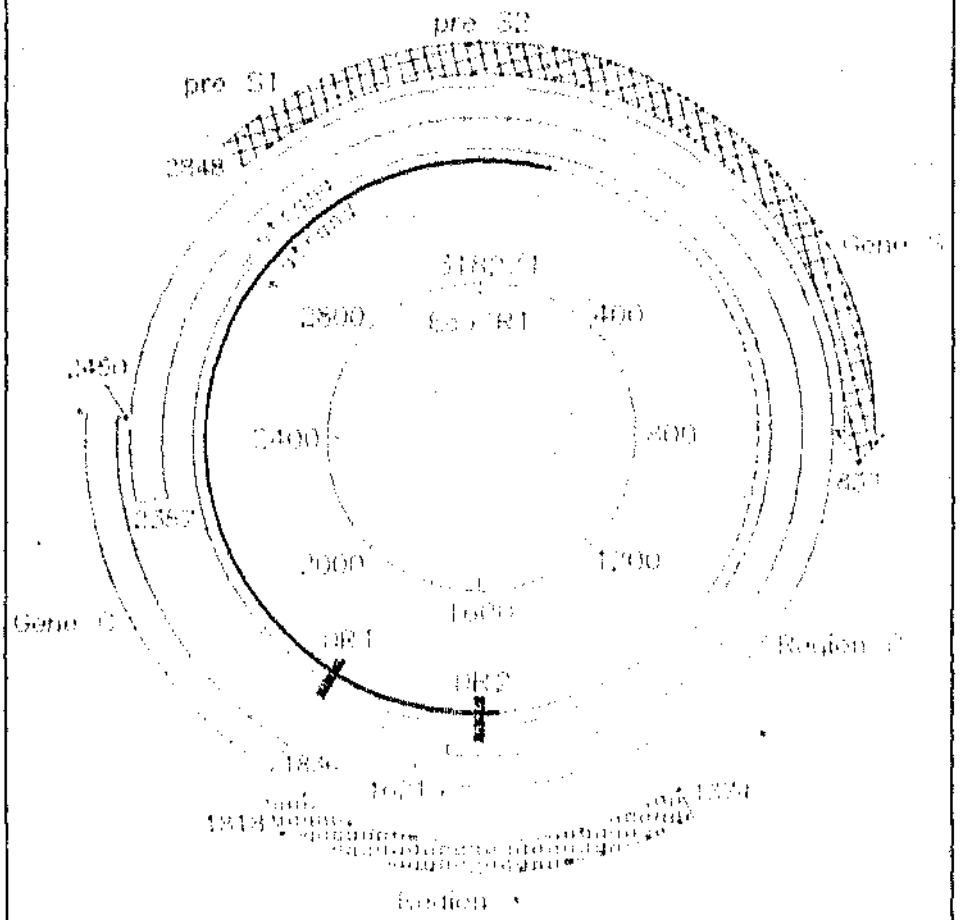

Figure 1.1. Structure and genetic organization of the HBV genome. The broad arrows surrounding the genome represent the four large open reading frames of the (+) strand transcript. The partial restriction map and the numbering of the nucleotides correspond to the adr genome.

1.3. The genetic organization of HBV

HBV has the smallest genome of all known viruses affecting man. It is diagrammatically represented in Figure 1.1. It is a circular, partially double stranded (ds) DNA molecule with a single stranded region of variable length. The long (L) or minus (-) strand has a fixed length of approximately 3 200 bp and is nicked at around 225 bp from the 5' end of the plus strand. The short (S) or plus (+) strand has a variable length ranging from 1700 - 2800 nucleotides in different molecules. The circular structure is maintained by the base-pairing of the 5' ends of the two strands. A protein is covalently attached to the 5' end of the long DNA strand (Gerlich and Robinson, 1980) and is referred to as the terminal protein.

There are four major open reading frames (ORFs) which reside on the long strand, the C, S, P and X ORFs.

1.3.1. The C gene

The C ORF includes a short pre-C sequence and codes for a major viral core or nucleocapsid polypeptide of 22 000 daltons. A truncated form of this C gene product displaying e antigen (eAg) epitopes, and not the core epitopes, is found in sera during active viral replication.

1.3.2. The S gene

The S ORF includes the pre-S1, pre-S2 and S regions. It specifies viral surface antigen reactive polypeptides in the virion envelope and in the incomplete viral forms which are found in the serum and liver of infected individuals. The glycosylated and non-glycosylated forms of three polypeptides are encoded by this region. The entire ORF (pre-S1, pre-S2 and S region), the pre-S2 and S region, and

At first the narrow host range (humans and higher primates) of HBV as well as its inability to be propagated in cultured cells made these viruses difficult to study. The development of workable animal models of HBV infection and the ongoing advances in molecular genetics has provided insight into the molecular structure of this virus, its replication strategy and its oncogenic potential.

1.2. Biology of HBV infection

Exposure of an adult to HBV results in an acute infection lasting up to 20 weeks with subsequent development of protective antibody against the virus (Robinson, 1984). During infection the virus and viral antigens are found in the blood and body fluids with high amounts of viral antigens and nucleic acids in the liver. The most abundant forms of virus particles in the blood at this time are spherical or filamentous and are defective, containing only the viral envelope and no nucleocapsid (Sherker and Marion, 1991). Normally, infection with HBV results in a moderately severe illness with hepatocellular injury and inflammation (Redeker, 1975). However, some individuals experience mild or no liver injury despite extensive hepatic infection. This variation has been attributed to differences in the host immune response to virally infected cells (Dudley et al., 1972).

5-10% of infected individuals do not resolve primary infection but develop persistent, usually life long, hepatic infection (Gianem, 1982). Though a minority, these HBV carriers form a reservoir from which infection is spread to other individuals.

Mortality from HBV infection usually results from chronic rather than acute infection (WHO, 1990). Severe chronic HBV infection frequently leads to premature death from liver failure, but is more importantly associated with HCC.

Other etiological factors of HCC include aflatoxin contamination of foods (Munoz and Bosch, 1987; Lisker-Melman et al., 1989), liver cirrhosis where the association is recognised but not understood (Smuzness, 1978; Blumberg and London, 1981), tobacco smoking (Trichopoulos et al., 1980; Lam et al., 1982; Munoz and Bosch, 1987), excessive alcohol consumption (Tuyns, 1980; Yu, et al., 1983; Hardell et al., 1984), the use of sex hormones (Jick and Herman, 1978), certain parasitic infestations, for example schistosomiasis (Nakashima et al., 1975; Abe et al., 1993), exposure to the radioactive element thorostat (Battifora, 1976), infection with the hepatitis C virus (Saito et al., 1990), the use of pharmacological doses of anabolic steroids as well as various metabolic liver diseases (Lisker-Melman et al., 1989).

Human HCC is a complex and multifactorial disease that is linked to many agents and conditions. Patients with HCC may be exposed simultaneously to multiple etiological factors, depending on their living conditions, which may vary between countries or even a single geographical location.

1.1. The Hepatitis B Virus

The hepatitis B virus was first distinguished from other hepatitis - inducing viruses in 1965 (Blumberg et al., 1965). Originally, it was thought to be unique and not a member of any recognised virus group. However, related viruses have since been discovered in eastern woodchucks (Summers et al., 1978), Beechey ground squirrels (Marion et al., 1980) and Pekin ducks (Mason et al., 1980). These viruses are classified as Hepadnaviridae (Gust et al., 1986) and have similar antigenic, ultrastructural, molecular and biological features. Chronic liver disease and HCC have, however, only been observed in infected woodchucks (Summers et al., 1978; Popper et al., 1981).

Hepatocellular carcinoma (HCC) is the most frequent malignant tumour of the liver. Worldwide, HCC is the seventh most common form of cancer in males and the ninth in females (Parkin et al., 1984). The frequency of HCC varies with respect to geographical location. It is the most recurrent malignant tumour among males in Western, Middle and Eastern Africa and second most recurrent tumour among males in Southern Africa and Southeast Asia. HCC is not as prevalent in China and rare in most parts of America, Europe, Northern Africa and Eastern Asia (Robinson, 1984; Parkin et al., 1984).

Hepatocellular carcinoma is almost always in an advanced stage when first detected and the prognosis is poor as few patients survive beyond a year (Kew, 1987). Surgery alone can prolong survival but only 10% of patients are suitable for this therapy. Chemotherapy gives poor results and liver transplantation has been abandoned owing to the frequent recurrence of the tumour in the transplanted liver (Edwards and Bouchier, 1991).

The occurrence of HCC increases with age, although HCC has been found in a patient as young as eight months (Wu et al., 1987). In most cases the tumour is detected between sixty and eighty years of age (Kew, 1986).

Chronic hepatitis B virus (HBV) infection has emerged as the most important cause of HCC worldwide. Although several vaccines are now available to prevent HBV infection, there are over 250 million chronic carriers of HBV and no effective cure. An estimated 1-2 million deaths due to severe hepatitis, cirrhosis or hepatocellular carcinoma are attributed to HBV infection each year (World Health Organization, 1990).

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INTRODUCTION

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PKC ..... protein kinase C

pre S2/S' ..... truncated HBV middle surface gene

RPN ..... random prime labelling system

RFLP ..... restriction fragment length polymorphism

rpm ..... revolutions per minute

RNAse ..... ribonuclease

RNA ..... ribonucleic acid

S ..... short

SDS ..... sodium dodecyl sulphate

NaOH ..... ydroxide

SP1 ..... pre-surface gene promotor

SP11 ..... surface gene promotor

T ..... thymidine

TBS ..... tris-buffered saline

TBS-T ..... tris-buffered saline with tween 20

TE ..... Tris-EDTA

Tris ..... Tris-methylhydroxyaminomethane

U/µg ..... units per microgram

U ..... uracil

v/v ..... volume to volume

w/v ..... weight to volume

WHV ..... Woodchuck hepatitis virus

X-Gal ..... 5-bromo-4-chloro-3-indolyl- β galactopyranoside

- induced HCC.

1.5.3.2 Chromosomal translocations

Chromosomal translocations could result in crucial genes being brought under control of host regulatory sequences thus altering their expression. Integration of HBV DNA has been associated with the translocation of host chromosomes (Hino et al., 1986; Tokino et al., 1987)) for example, an HBV integration in an HCC was determined to be flanked on one side by sequences from chromosome 17 and on the other side by sequences from chromosome 18 (Hino et al., 1986). No functional alteration in chromosomal expression has yet been attributed to these translocations and limited data is available as most investigators have tended to focus on the regions adjacent to the integrated virus.

1.5.3.3 Chromosomal rearrangements and deletions

In many cancers the development of malignancy has been associated with the deletion or mutation of a tumour suppressor gene (Orkin et al., 1984; Cavance et al., 1985; Hansen et al., 1985; Koufos et al., 1985). Integrations of HBV are connected with micro- and macrodeletions and rearrangements of cellular DNA. Restriction fragment length polymorphism (RFLP) studies of human liver tumours have revealed significant allele losses from regions of chromosomes 4, 5q, 11p, 13q, 16q and 17p. This suggests an important role for the inactivation of tumour suppressor genes that reside on these specific chromosomal regions in the development of HCC. Genes whose altered expression might affect tumour progression invasion and metastasis are also candidates for a role in the transformation process. The presence of mutations in the tumour suppressor gene p53 (located on chromosome 17p) is well documented and recently, a reduction to hemizygosity of the E-cadherin cell adhesion gene (localized on 16q) was discovered in 64% hepatocellular carcinomas

The long latency between HBV infection and the development of HCC as well as the inability of HBV to transform hepatocytes *in vitro* (Sherker and Marion, 1991) cannot be explained by classic mechanisms of oncogenesis as HBV does not contain a directly transforming oncogene (Tiollais et al., 1985; Ganem and Varmus, 1987; Sherker and Marion, 1991) as found in retroviruses.

Proto-oncogenes (cellular oncogenes) are believed to encode tightly regulated proteins whose expression plays an important role in cell growth. Alterations in their expression is believed to be an important cause of many malignancies and may result from a qualitative change in the coding region of the gene or a quantitative change in the expression of an unaltered gene product (Weinberg, 1985; Fausto and Shank, 1987). Mechanisms by which this may occur include insertional mutagenesis, gene amplification, chromosomal translocations, chromosomal rearrangements and deletions as well as the transactivation of important host regulatory genes. Specific chromosomal alterations have been discovered in some HBV-related HCC's that may be relevant to the molecular basis of hepatocarcinogenesis and are summarized below.

1.5.3.1 Insertional mutagenesis

Integrated, often extensively rearranged HBV DNA, could directly activate cellular proto-oncogenes by providing promoters and enhancers that would increase the expression of adjacent genes. Although this mechanism has been shown to occur (Dejean et al., 1986; Moroy et al., 1986; Ochiya et al., 1986; de The et al., 1987; Rogler et al., 1987; Benbrook et al., 1988; Brand et al., 1988; Hsu et al., 1988; Zhou et al., 1988; Wang et al., 1990; Graef et al., 1994), direct insertional activation of cellular oncogenes has not been observed in the vast majority of hepadnavirus - associated tumours studied thus far and hence does not explain the majority of HBV

Recent restriction-fragment-length polymorphism (RFLP) studies of human liver tumours have revealed significant allele losses from regions of chromosome 4 (Bentow et al., 1989; Zhang et al., 1990), 5q (Fujimori et al., 1991), 11p (Wang et al., 1988; Fujimori et al., 1991), 13q (Wang et al., 1988), 16q (Tsuda et al., 1990; Zhang et al., 1990; Fujimori et al., 1991; Slagle et al., 1993) and 17p (Fujimori et al., 1991; Slagle et al., 1991; Scorsone et al., 1992). In addition to deletion, significant rearrangement of cellular flanking sequences and integrated viral sequences has also been observed (Twist et al., 1981; Dejean et al., 1983; Koike et al., 1983; Koshy et al., 1983; Koch et al., 1984a; Koch et al., 1984b; Ou and Rutter, 1985; Ziemer et al., 1985; Mizusawa et al., 1985);

The only aspect of hepadnavirus integration that appears nonrandom is the frequent use of sequences in or near the DR's as recombination sites. The integrated viral DNA in HCC is usually so extensively rearranged that it is difficult to deduce the structure of the virus-cell DNA junction, however 18 out of 40 integrants studied have at least one junction within the short stretch between the beginning of DR1 and the end of DR2 (Shetker and Marion, 1991). A nonspecific mechanism of integration could occur in addition to that involving DR1 and DR2.

1.5.3. HBV as an oncogenic virus

The overwhelming epidemiological evidence linking HBV to the development of hepatocellular carcinoma and the finding of integrated viral sequences in tumour genomes of patients with HCC suggests a role for HBV in oncogenesis. However, a specific viral oncogenic mechanism has not been shown for HBV in the development of most HCC's in spite of extensive research in many laboratories.

and characterised from at least 15 different tumours or cell lines (Dejean et al., 1983; Shaul et al., 1984; Koch et al., 1984a; Mizusawa et al., 1985; Yaginuma et al., 1985; Ziemer et al., 1985; Choo et al., 1986; Hino et al., 1986; Nagaya et al., 1987; Matsumoto et al., 1988; Zhou et al., 1988, to name but a few). The data analyzed so far indicates the following:

There are usually multiple copies of viral DNA integrated into the genome of HCC. As an example, in the Alexander cell line (PLC/PRF/5) HBV DNA is integrated into eight different sites (Edman et al., 1980; Brechot et al., 1980; Marion et al., 1980; Ziemer et al., 1985), and the Tong/HCC cell line has five integrations (Stevens et al., 1987);

The integrated viral DNA is usually not full length (3 200 bp) but subgenomic fragments (Dejean et al., 1983; Shaul et al., 1984; Misuzawa et al., 1985; Ziemer et al., 1985). There are, however, some integrations in HCC's that contain a greater than unit length HBV DNA sequence with an intact copy of the genome (Dejean et al., 1983). No particular region of the HBV genome is conserved in different clones of integrated HBV sequences;

Examination of HBV DNA insertions has not revealed a unique cellular site for viral integration. Very few integrations have been found near or within cellular genes, and there is an apparent preference for integrations within noncoding repetitive cellular DNA sequences (Nagaya et al., 1987; Matsubara and Tokina, 1990). HBV integrations into the human genome have been mapped to at least 12 different chromosomes (Bowcock et al., 1985; Rogler et al., 1987; Dejean et al., 1986; Hino et al., 1986; Nagaya et al., 1987; Blanquet et al., 1988; Zhou et al., 1988), with chromosomes 3,5,6,11,17 and 18 each represented in more than one integration;

1.5.2.1 Studies by Southern blot hybridization

Southern transfer (Southern, 1975) allows the physical mapping of regions complementary to labelled HBV DNA probes. The frequency of HBV DNA integrated in the genome of HCC has been studied by many investigators (Shafritz et al., 1981; Koshy et al., 1981; Brechot et al., 1982; Hino et al., 1984; Imazeki et al., 1986). Integrated viral sequences were found in 75 - 100% of HBsAg-seropositive patients and in a smaller fraction of the animal carriers (Gerin et al., 1982; Marion et al., 1987). Seronegativity does not necessarily preclude HBV integration, as in a significant number of HBsAg-seronegative cases, integrated HBV sequences were also found (Shafritz et al., 1981; Brechot et al., 1982a; Brechot et al., 1982b; Hino et al., 1984; Imazeki et al., 1986;). These results offer evidence that integration plays a part in tumour formation, but the significance of the integrated sequences is unclear. HBV sequences are not indicative of tumorous tissue as HBV DNA has been observed in both non-tumorous parts of livers with HCC and in chronic hepatitis or cirrhosis without HCC.

The pattern of viral integration in individual HCC's is thought to be unique and identical from cell to cell within a tumour which suggests clonal origin of the tumour (Esumi et al., 1986; Blum et al., 1987; Aoki and Robinson, 1989; Chen et al., 1989). More than one clonal tumour may exist within the same liver (Esumi et al., 1986; Chen et al., 1989), however, restriction patterns have varied within individual tumours (Brechot et al., 1980; Shafritz and Kew, 1981; Shafritz et al., 1981; Koshy et al., 1981; Dejean et al., 1983).

1.5.2.2 Cloning and sequencing of integrated viral DNA

The role of HBV DNA integration in liver carcinogenesis still remains uncertain (Chen, 1993). To date more than 40 different HBV integrations have been cloned

infection. Among those who had antibody to HBsAg upon entry into the study, the incidence of HCC was low and not statistically different from those having no markers of current or past HBV infection. This suggests that individuals who recover from acute HBV infection and do not develop a persistent infection have little or no chance of developing HCC.

Another study was done by Sakuma et al. (1982) who followed 202 HBsAg-seropositive and 2928 seronegative employees of the Japan National Railways (98% men). After 5 years, 4 individuals in the seropositive group had developed HCC while there was no cancer development in the seronegative group.

A study of the distribution of HCC and HBsAg carriers in China, however, has revealed a prevalence of HBsAg in the coastal and inland regions, but the prevalence of HCC was significantly higher in the coastal regions when compared to the inland regions (Su, 1982). This raises the possibility that other factors in addition to HBV infection may play a role in the development of HCC in this population.

1.5.2. Molecular studies of HBV-related HCC

In 1980 4 groups demonstrated the presence of HBV DNA integrated into the genome of HCC (Marion et al., 1980; Chakraborty et al., 1980; Brechot et al., 1980; Edman et al., 1980). Subsequently, the structure of integrated DNA in human HCC cell lines, human HCC and HBV-related viruses in animals has been analyzed to try and elucidate a mechanism for cell transformation.

There is little or no correlation between the prevalence of HBV infection in males and females and the development of HCC (Lin et al., 1986). A study on Alaskan native HBV carriers found that while the incidences of chronic active hepatitis and cirrhosis were comparable between males and females, males showed an approximately sixfold increase in incidence of HCC (McMahon et al., 1990). This suggests that being male is an independent risk factor in the development of HCC among HBsAg-positive individuals.

1.5.1.2 Case-control studies

These were performed to determine the relative risk (RR) of developing HCC among HBsAg carriers. In high risk populations such as those of Africa and Southeast Asia the RR ranges from 10-20 (Prince et al., 1975; Kew et al., 1979; Lingao et al., 1981; Lam et al., 1982; Yeh et al., 1985). In the low risk populations of the U.S.A., where the prevalence rates of HBsAg in the control populations are very low, the RR is higher, from 10 to infinity (Yarrish et al., 1980; Austin et al., 1985).

1.5.1.3 Cohort studies

Several large cohort studies have been published comparing the occurrence of HCC among HBV carriers with that of a non carrier control population (Obata et al., 1980; Beasley et al., 1982, Sakuma et al., 1982; Lohiya, et al., 1988; McMahon et al., 1990). All show a markedly increased risk for HCC among HBV carriers.

In the largest of these, Beasley (1982) recruited 22 707 male government employees, of whom 3454 were seropositive and the balance negative for HBsAg. After a follow up period of 6.2 years, 113 men in the positive group and three in the negative group had developed primary liver cancer (mostly HCC). This meant that the risk of developing HCC was 200 fold greater in male individuals with evidence of HBV

1.5.1. Epidemiological evidence

Several lines of epidemiological evidence support an association between persistent HBV infection and the development of HCC.

1.5.1.1. Correlation studies

HBV infection, defined by the presence of hepatitis B surface antigen (HBsAg) in the serum, and death rates from HCC correlates in different regions of the world (Smuzness, 1978; Beasley et al., 1982). HBV infection is lowest in North America and Eastern Europe, while in other regions, such as Southeast Asia and Africa it is at least ten fold higher (Smuzness, 1978; Blumberg and London, 1981). The mean proportions of HCC found on autopsy in the U.S.A. and Europe are 0.39% and 0.45% respectively and those found in Africa and Asia range between 2-8%. HCC constitutes 2.5% of the total cancers in the U.S.A. and Western Europe, whereas in areas of Africa and Southeast Asia it constitutes 20-40% of the total cancers (Smuzness, 1978). Exceptions to this correlation are found with the Greenland Eskimos where there is a high prevalence of HBsAg carriers and a low incidence rate of HCC (Melbye et al., 1984), while in Japan, infection with Hepatitis C Virus (HCV) and HCC have been associated (Saito et al., 1990).

With the inclusion of anti-HBs and anti-HBc (core) as serum markers, HBV infection rates in patients with HCC reached 78-96% in Africa (Lutwick, 1979) and the U.S.A. (Omata et al.,1979). This indicates that the majority of patients with HCC have had a previous infection or have current antigenemia.

HBV has been shown to replicate in extrahepatic tissues. This has been well defined in several animal models such as ducks (Tagawa et al., 1985; Jilbert et al., 1987; Freiman et al., 1988) and woodchucks (Korba, et al., 1988; Ogston, et al., 1989). The first studies documenting the extrahepatic biology of human HBV concentrated on readily obtainable tissue and body fluids such as peripheral blood mononuclear cells (Lamelin and Trepo, 1990; Leung et al., 1994), saliva, semen, urine, faeces and biliary and pancreatic juices (Heathcote et al., 1974; Villarejos et al., 1974; Hoofs et al., 1980; Fagan et al., 1986). Since then HBV nucleic acid sequences and proteins have been detected in pancreas, kidney, skin (Dejean et al., 1984a; Naumova et al., 1985; Yoffe et al., 1990; Mason et al., 1993), lymphoid tissue (Naumova et al., 1985; Laure et al., 1985; Mason et al., 1993), thyroid and adrenal glands, gonads (Naumova et al., 1985), placenta (Laure et al., 1985), spleen, bone marrow, colon, stomach, testes, periadrenal ganglia (Mason et al., 1985) and peripheral blood leucocytes (Leung et al., 1994). The pathological significance of the extrahepatic distribution and biology of HBV is not well understood and the detection of HBV in extrahepatic tissues is seldom accompanied by evidence of tissue damage. Little is known about the mechanisms behind this apparent lack of disease.

1.5. Hepatitis B Virus infection and hepatocellular carcinoma

Association of HCC with viral hepatitis was postulated long before any of the specific viruses causing hepatitis had been identified (Payet et al., 1956; Steiner and Davies, 1957). Only after the discovery of the Australia antigen (HBsAg, Blumberg et al., 1965) was this association confirmed. The evidence for this relationship comes from epidemiological studies, molecular studies of human tissue and HCC cell lines, obtained from human HBV carriers who developed liver cancer, as well as studies of animal models infected with viruses closely related to HBV.

a primer for plus strand DNA synthesis (Lien et al., 1985; Seeger et al., 1986). DNA plus strand synthesis can only proceed until the polymerase reaches the 5' end of the protein-linked DNA minus strand. At this point it is thought that a template switch takes place (Ganem and Varmus, 1987). The last 5 to 8 nucleotides of the 3' end of the DNA plus strand are homologous to the first 5 to 8 nucleotides of the other end of the DNA minus strand which allows the template switch to take place and results in the circular DNA conformation of the HBV genome. The plus strand is then elongated, stabilising the circular intermediate (Will et al., 1987). Complete synthesis of this strand is not necessary for coating of the nucleocapsids and export of virus particles. This explains why infectious particles contain DNA with a single stranded region (Tiollais et al., 1985). Some of the completed nucleocapsids are enveloped and exported while others appear to re-enter the cycle of infection within the same cell (Tuttleman et al., 1986a) thus expanding or replenishing the intracellular transcriptional template CCC DNA.

The HBV replication cycle resembles that of retroviruses and CaMV in having a common reverse transcription step. There are, however, essential differences. Integration of viral DNA into the host genome is not an essential step for HBV as it is in retroviral and CaMV replication (Rogler and Summers, 1984). Minus-strand synthesis of retroviruses and CaMV DNA is primed by a host tRNA while HBV DNA minus-strand synthesis is primed by a protein. The order of the HBV genes C, P and S can be compared to that of the retroviral genes, gag, pol and env, but where retrovirus proteins are translated from mRNA produced by splicing from a single primary transcript, HBV proteins are translated from at least two unspliced primary transcripts (Tiollais et al., 1985). For all three classes of virus, promoters are closely followed by polyadenalation signals that are passed over during the first transcription run.

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in that an RNA copy of the genome is used as an intermediate in replication which includes a reverse transcription step resembling that of retroviruses (Summers and Mason, 1982).

On entering the hepatocyte the plus strand of the virus is extended and the genome becomes fully double stranded. Once in the nucleus it converts into a covalently closed circular (CCC) supercoiled DNA molecule (Tuttleman et al., 1986a; Tuttleman et al., 1986b), the minus strand of which acts as a template for viral RNA transcription by cellular RNA polymerase II (Mason et al., 1983). Three different-sized major mRNA transcripts with common 3' termini encode the viral proteins. The largest of these mRNA species, approximately 3.5 kb in length, consists of a group of mRNAs that are greater than genome length but whose 5' end varies within a range of 130-160 nucleotides (Buscher et al., 1985; Enders et al., 1985; Moroy et al., 1985). This duplication of sequences, a redundancy, arises because the initiation of transcription is close to the start of ORF pre-C, upstream of the only known polyadenylation site in the viral genome. The polyadenylation signals are passed over during the first transcription run but honoured on the second passage. (Ganem and Varmus, 1987). The smallest of the 3.5 kb RNA species is packaged in viral core particles (Enders et al., 1987), along with viral DNA polymerase and terminal protein, and functions as the pregenome from which the minus strand is synthesised by reverse transcription. Initiation of reverse transcription takes place at DR1 close to the 3' end of the pregenome (Will et al., 1987) and the terminal protein acts as a primer (Molnar-Kimber et al., 1984;).

During DNA synthesis the RNA pregenome is degraded by RNAse H, leaving a small fragment of about 20 bp containing the DR1 sequence at the 5' end. This fragment is cleaved off and transposed to DR2 (Will et al., 1987) where it acts as

2.1 Tong/HCC cell culture

2.1.1. Culture conditions of Tong/HCC

Tong/HCC cells were cultured in 25 cm^2 tissue culture flasks (Nunc) for cell maintenance and 75 cm^2 flasks (NUNC) for large scale culture of cells to be used for genomic DNA isolation. The medium used was RPMI 1640 (Gibco) buffered with $NaHCO_3$ (2 g/l) and HEPES buffer (Sigma, U.S.A.; 2g/l) and supplemented with 5% foetal calf serum (FCS, Delta). The FCS was inactivated at 56°C for 1.5 hours. The antibiotics used were Penicillin (100 000 IU/l), Streptomycin (100 mg/l) and Gentamycin (50 mg/l). All these antibiotics were produced by Bio Whittaker (U.S.A.). The cells were incubated at 37°C without CO_2 supplementation.

The Tong/HCC cells were maintained under conditions of retarded growth by using the conditions and media described above, but supplemented with 2% FCS.

2.1.2. Trypsinisation of confluent cells

The cells were routinely split in a ratio of 1:3 every three days. For transfer from small (25 cm^2) flasks to medium (75 cm^2) flasks, the contents of one small flask was trypsinised and transferred to one medium flask, equating to a 1:3 split ratio. Splitting of the cells was performed as follows. The growth medium was replaced with medium containing no FCS to wash the cells. 0.25% bovine pancreatic trypsin and 0.2% EDTA were added to the cells. When the cells began to show a round morphology the trypsinizing solution was poured off. The cells were removed from the side of the flask by hitting it twice. RPMI 1640 supplemented with 10% FCS was added to the cells which were then distributed into three tissue culture flasks.

CHAPTER 2

MATERIALS AND METHODS

1.7. Aim of this study

Despite continuing analysis of integrated HBV DNA and the surrounding cellular sequences, little is known about how the integrated HBV DNA induces hepatocellular carcinoma. The five integrated HBV sequences of the Tong/HCC cell line and it's flanking cellular sequences have not been cloned and sequenced to date. A study of this cell line would contribute to our understanding of how HBV DNA causes HCC. So the aim of this study was to create an expression genomic DNA library of the Tong/HCC cell line in Lambda gt11 and then to screen this library with nucleic acid probes and HBsAg - specific antibodies. This should determine the presence of HBV DNA sequences as well as HBsAg sequences completely or partially expressed as a fusion protein.

To find out if this is indeed the case or whether the integration of HBV DNA is directly involved in the induction of HCC, it is necessary to analyze more and more integrations and their flanking cellular DNA sequences. The five HBV integrations in the Tong/HCC cell line have not to date been cloned and sequenced.

1.6. The Tong/HCC cell line

The Tong/HCC cell line is a continuous adherent cell line established from a hepatocellular carcinoma of a HBsAg-positive 59-year-old Italian male (Stevenson et al., 1987) whose serum was positive for HBsAg, HBeAg, HBV DNA, anti-HBc, and had high α-fetoprotein (AFP) levels. It is of interest in this study as it is a newly established cell line with the following characteristics. The Tong/HCC cell line morphologically resembles other isolated HCC cell lines and has a modal chromosomal number of 64. Both HBsAg and AFP are produced while supernatants from the cell culture medium and lysates of the cell are negative for HBeAg. No free HBV DNA molecules are found in the cells and a stable integration pattern of five distinct HBV DNA bands is observed using restriction enzyme analysis. A translocation on Chromosome 15 has been noted. This cell line has all the characteristics of an HCC cell line but is unable to produce tumours in athymic nude mice (Stevenson et al., 1987).

Table 1.1: Four different integration patterns of HBV DNAs in hepatocytes.

		STRAND POLARITY	
		core→surface→x	x→surface→core
END SPECIFICITY	DR1	I 	II 
	DR2	III 	IV 

A mechanism other than integration could be responsible for the development of HCC. The c-*myc* proto-oncogene is rearranged and aberrantly expressed in three of nine hepatomas in WHV - infected woodchucks, but WHV DNA is not present in or near the vicinity of the infected loci (Moroy et al., 1986). The overproduction of a single structural viral gene which causes severe, prolonged cellular injury and thus a proliferative response would create the opportunity for the accumulation of multiple chromosomal abnormalities or other genetic changes which could be sufficient for the acquisition of the malignant phenotype. Research on transgenic mice that produce large quantities of the HBV large envelope polypeptide and the HBV surface antigen found that they develop severe, prolonged hepatocellular injury followed by inflammation, regenerative hyperplasia, transcriptional deregulation, aneuploidy and eventually HCC. The frequency, severity and age of onset of liver cell injury corresponded to the incidence of HCC (Chisari et al., 1989).

The DNA sequence of the HBV integration site in independent HCC's has been published (Koshy et al., 1983; Dejean et al., 1984; Koch et al., 1984; Mizusawa et al., 1985; Yaginuma et al., 1985; Ziemer et al., 1985; Dejean et al., 1986). As stated above, there is no preferred site as to the target of HBV insertion on the host chromosome. However, with the observation of two virus - cell DNA junctions occurring within a 11 bp directly repeated sequence (DR1 and DR2) in the HBV genome it was thought that HBV integration might occur via a site - specific or site - preferential mechanism (Delius et al., 1983). A growing number of virus junctions have been mapped in or near the DR's (Koshy et al., 1983; Dejean et al., 1984; Rogler et al., 1985; Yaginuma et al., 1985; Chao et al., 1986; Shih et al., 1987; Yaginuma et al., 1987). It is likely, therefore, that conditions favourable for integration include some characteristic feature of the DR - cohesive overlap region. Nagaya et al. (1987) have analyzed 19 integrants, along with data from 13 reported cases, and have shown that one - half of the integrants have at least one virus - cell DNA junction falling within the cohesive end region of the HBV DNA. Shih et al. (1987) identified two hepatoma samples with a single site of integrated HBV which were characterised by cloning and sequencing. Comparison of their results with others led them to conclude that the preferred site of HBV recombination may be localized in an extremely narrow region (ca. 9 bp) within the cohesive overlap (ca. 200 bp).

Shih et al. (1987) examined all the integration patterns of HBV DNA currently available in the literature and divided the patterns into four different groups according to their end specificity and strand polarity.

In a few cases the integration of HBV DNA has been shown to play a direct role in the mutagenesis of cellular controlling genes resulting in the onset of HCC. For example, one cloned HBV integration occurred in a retinoic acid receptor gene (Brand et al., 1988). Another integration was identified with a deletion of cellular DNA at chromosome 11p13 (Rogler et al., 1985). A tumour suppressor gene is found in this area, the loss of which can lead to Wilm's tumour and hepatoblastoma (Koufos et al., 1985). HBV DNA has also been found integrated in the cyclin A gene (Wang et al., 1990). Cyclins are important in the control of cell division, so disruption of the gene by viral insertion might contribute to tumorigenesis. Cloning and sequencing of integrations from woodchuck HCC's has found two instances where woodchuck DNA hepatitis has integrated in the c-myc gene (Hsu et al., 1988).

However, the majority of HBV integrations in HCC's are not associated with known cellular oncogenes or a common cellular DNA sequence. Additionally, the integrated HBV DNA structure varies significantly among different HCC's. In some integrations the viral sequences are colinear with the viral genome while others contain highly rearranged viral genomes (Ogston et al., 1982; Nagaya et al., 1987; Yokosuka et al., 1985; Hino et al., 1989). HBV integrations are also commonly associated with chromosomal abnormalities including large deletions (Hino et al., 1986; Rogler et al., 1985), duplications (Mizusawa et al., 1985; Yaginuma et al., 1985; Tokino et al., 1987) and chromosomal translocations (Hino et al., 1986; Tokino et al., 1987). This, in association with rearranged HBV DNA has led to the theory that post - integration rearrangement is an important mechanism involved in generating mutations in cellular DNA. Such rearrangement may completely eliminate HBV DNA from specific cellular sites although direct evidence for this has not been demonstrated.

wild-type HBV genome (Kekulé et al., 1990) and have only been detected in integrated HBV DNA cloned from HBV-associated HCC's. PreS2/S' genes code for C-terminally truncated middle surface proteins (MHBs') which have been demonstrated to possess transactivator activity (Hildt et al., 1992). These proteins are localised in the endoplasmic reticulum (Meyer et al., 1992; Hildt et al., 1992) and can utilize the transcription factors AP-1, AP-2 and NF-kB for transactivation (Meyer et al., 1992; Natoli et al., 1992; Lauer et al., 1994). It has been hypothesised that an indirect mechanism involving excitation of one or several signal transduction pathways conveys the transactivator effect from the endoplasmic reticulum to the nuclear transcription machinery (Hildt et al., 1992; Lauer et al., 1992; Meyer et al., 1992; Lauer et al., 1994).

The high correlation of HCC with the surface antigen carrier state (Beasley, 1988) and the observation that one third of HBV-associated HCC's harbour at least one transactivating preS2/S' gene (Lauer et al., 1994) supports the notion that chromosomally integrated preS2/S' genes might contribute to HBV-associated liver oncogenesis, however, definite evidence for the presumed oncogenic potential of this transactivator still has to be established.

1.5.4. Theoretical considerations of the mechanism of oncogenesis.

The finding that HCC's arising in HBV carriers contain hepatitis virus integrations has raised much interest in the process of HBV DNA integration and its role in the development of HCC (Summers et al. 1980; Shafritz et al., 1981; Dejean et al., 1983). The integration process cannot be reproduced in cultured cells. Therefore, HBV integrants in HCC's have been analyzed in an attempt to understand their effect on the induction of HCC.

The variety of different X responsive transcription regulatory elements, the failure of the X protein to directly bind any DNA sequence (Wu et al., 1990) and the cell specificity of the X transactivation effect (Seto et al., 1989) suggest that the transactivation mechanism does not involve a direct sequence specific interaction with DNA but is probably mediated through an effect on cellular transcription factors (Twu et al., 1993).

Recently, Kekule et al. (1993) discovered that the HBxAg causes an increase in the endogenous protein kinase C (PKC) activator, sn-1,2 diacylglycerc , with the subsequent activation of the PKC pathway and thus the activation of the transcription factor AP-1 (Jun-Fos) and other PKC-dependent transcription factors (AP-2, NF-kB). The PKC signalling pathway mediates tumour promotion by growth factors, mitogens and phorbol esters and thus overproduction of PKC causes disordered growth in mammalian cells. This mechanism might account for the oncogenic potential of HBx.

The X gene, encoding the transactivator protein HBx, could be oncogenic however, the fact that the X gene appears to be present only in a part of HBV-associated HCC's limits the role of this transactivator in liver carcinogenesis (Choo et al., 1986; Imai et al., 1987; Nagaya et al., 1987; Chen et al., 1988).

1.5.3.4.2. Truncated pre S/S gene transactivator

The second HBV-encoded transactivator is generated after 3' truncation of the HBV middle surface gene (preS2/S, Caselmann et al., 1990; Kekule et al., 1990). The preS/S sequences have essential biological functions including the regulation of virus assembly, the involvement in virus attachment to the hepatocyte and the regulation of the genetic restriction of the immune response (Pontisso et al., 1989). However, in contrast to X, the 3'-truncated preS2/S (preS2/S') wild-type is not active in the

1.5.3.4.1. X gene transactivator

The X-ORF encodes a viral transactivator, the HBx antigen (HBxAg), which in tissue culture cells can function as a transcriptional control element to upregulate the expression of other viral genes (Twu and Schloemer, 1987; Spandau and Lee, 1988; Seto et al, 1990). Thus a possible mechanism for the development of HCC could be that the trans-activating properties of the HBxAg could result in cell transformation by activation of a cellular gene(s), resulting in tumour formation after a long latent period (Twu and Schloemer, 1987). Since the HBV genome has been found to be integrated into the cellular genome within the HBV X gene region (Koshy et al., 1983; Dejean et al., 1984b; Shaul et al., 1984), it is possible that a fusion protein consisting of part of the HBxAg and part of a cellular protein could possess trans-activating activity (Twu and Schloemer, 1987).

The HBxAg has been shown to activate transcription of a number of genes controlled by regulatory sequences. These include, human interferon-β gene (Twu and Schloemer, 1987), human immunodeficiency virus type-1 (HIV-1, Twu and Schloemer, 1987; Seto et al., 1988; Siddiqui et al., 1989), simian virus 40 (Spandau and Lee, 1988; Zahm et al., 1988; Twu and Robinson, 1989; Twu et al., 1989), the c-myc promoter (Koike et al., 1987; Koike et al., 1989), the Rous sarcoma LTR (Zahm et al., 1988), AP-2 binding sequences (Seto et al., 1990), c-jun promoter (Twu et al., 1993; Natoli et al., 1994) and HBV regulatory sequences (Spandau and Lee, 1988; Siddiqui et al., 1989). Genes known to be activated by the HBxAg when they are expressed in cells include interferon-β (Twu and Schloemer, 1987), HIV (Twu and Robinson, 1989), class 1 major histocompatibility complex gene and the c-myc gene (Koike et al., 1987; Koike et al., 1989).

is that in the vast majority of tumours with p53 alterations there are mutations in both alleles (double hit hypothesis). Such double mutation is known to be characteristic of tumour suppressor inactivation (Oliner, 1994).

The wild-type p53 protein has a very short half life and is present in minute amounts in normal cells. The mutant p53 proteins have a longer half life (Oren et al., 1981; Sturzbecher et al., 1987) and are present in high concentrations. The ratio of mutant to wild-type p53 in a cell could be critical in regulating cell division as over expression of the wild-type protein in a cell with mutant p53 protein suppresses transformation (Finlay et al., 1989; Eliyahu et al., 1989), cell growth (Baker et al., 1990; Michalovitz et al., 1990) and the tumorigenic potential of the cells. It is important to note that in HBV-related HCCs, viral integration is an early event (Takada et al., 1990) whereas p53 mutations appear to occur later on during tumour progression (Tanaka et al., 1993). Acquisition of p53 expression may be one of the major steps for transformation of the tumour cells into cells with more aggressive biological behaviour during the course of tumour growth (Hsu et al., 1993).

1.5.3.4 Transactivation

Recently two transactivators, trans-acting proteins which function by increasing transcription from the target promoters, encoded by the HBV genome have been identified. The first one is the X gene product (Twu and Schloemer, 1987; Zahm et al., 1988; Seto et al., 1990) and the second one is a product of a truncated preS/S region (Kekule et al., 1990). Both may play a role in the development of HCC.

studied (Slagle et al., 1993). However, the genes that may serve as potential targets for the selective deletions on chromosomes 4, 5q, 11p and 13q remain to be identified.

The presence of mutant p53 has been reported in many human carcinomas (Cattoretti et al., 1988; van den Berg et al., 1989; Bartek et al., 1990; Iggo et al., 1990; Gutersson et al., 1991; Levine et al., 1991) including HCC. The exact biochemical function of the wildtype p53 protein is unknown but it is thought to transactivate the transcription of a set of genes that may down - regulate cellular growth related genes (Fields and Jang, 1990; Raycroft et al., 1990; Oliner, 1994), inhibit cell cycle progression, cell division and transformation (Finlay et al., 1989; Baker et al., 1990; Oliner, 1994) and thus act as a tumour suppressor gene. In contrast, the mutant forms fail to do so and thus contribute toward oncogenicity (Finlay et al., 1989; Fields and Jang, 1990; Raycroft et al., 1990; Levine et al., 1991; Oliner, 1994).

Mutations in the p53 gene have been found in 18-58% of the HCC's collected from various geographical locations in the world (Bressac et al., 1991; Hsu et al., 1991; Murakami et al., 1991; Beutow et al., 1992; Oda et al., 1992; Scorsone et al., 1992; Sheu et al., 1992; Lai et al., 1993; Oliner, 1994). The majority of p53 mutations identified are missense changes in one of four "hotspots" located within exons 5 to 8 of the gene (Hollstein et al., 1991; Levine et al., 1991; Vogelstein and Kinzler, 1992). Other mutations identified are microdeletions and base insertions (Murakami et al., 1991; Oda et al., 1992). Codon 249 is a mutational hotspot for HCC's from southern Africa and China which is thought to be related to high aflatoxin exposure (Bressac et al., 1991; Hsu et al., 1991; Scorsone et al., 1992). An important finding

2.4.1 Labelling of DNA probe

For each screen 300 ng HBV DNA was labelled with fluorescein-11-dUTP (Fl-dUTP) using the random primed hexamer labelling process according to the Amersham enhanced chemiluminescence (ECL) protocol.

300 ng HBV probe was diluted to 20 µl in sterile distilled water (15 ng/µl). The DNA was denatured by boiling for 5 minutes followed by snap cooling on ice. To a microcentrifuge tube in an ice bath the following reagents were added: 14 µl sterile distilled water, 10 µl nucleotide mix, 5 µl random nonamer primers, 20 µl denatured DNA, 1 µl (4 units) DNA polymerase I "Klenow" fragment. The total volume being 50 µl. The reaction mixture was incubated in a water bath at 37C for 1 hour. The reaction was stopped by the addition of 2 µl 0.5M EDTA. The labelled probes were stored at -20C in the dark.

2.4.1.2. Plating of packaged phage on LB plates

A 10^{-2} dilution of the amplified Tong/HCC genomic library was prepared using phage buffer. 100 µl of the diluted phage was added to 100 µl of E.coli Y1090 cells prepared as described in section 5.1. The phage were allowed to adsorb to the bacterial cells by incubating for 30 minutes at 37C. 3 ml of TB top agar at a temperature of 45C was added to the mixture, vortexed gently and immediatly poured onto an LB plate (188 cm^2 for first screen, Pyrex) prewarmed to 37C. The plates had been placed at room temperature overnight to remove any excess moisture. Ten plates were prepared at a time and were incubated at 37C overnight. Care was taken that the plates grew to confluence without the plaques touching each other. The following morning the plates were removed from the incubator and placed at 4C for 1.5 hours to harden the agar.

10 µg of pBR322 plasmid DNA containing insert HBV was digested with 20 units BamHI at 37°C for 3 hours. The entire reaction mixture was electrophoresed through a 0.8% low melting temperature agarose gel (SeaRate-LMT, Amersham).

The 1850 and 1350 bp fragments were isolated from the gel slices using an improved phenol-based method developed by Favre (1992). The two DNA fragments were cut out of the gel and placed in a microcentrifuge tube. Any buffer that came with the slice was removed. The volume of the gel section was estimated from its weight. 1 volume TE buffer was added and the mixture placed in a water bath at 65°C for 5-10 minutes. The solution was pipetted up and down to make sure that the agarose was fully melted.

Immediately afterwards, 1 volume of Tris-buffered phenol (at room temperature) was added. The solution was mixed thoroughly by inversion and centrifuged for 3 minutes at 12 000 rpm (at room temperature). The extraction was repeated and the aqueous phase moved to a new tube containing 0.1 volume of 4 M LiCl and mixed. A white precipitate appeared immediately. The tube was placed on ice for 2 minutes and then spun as before. The supernatant was transferred to a new tube leaving a transparent pellet. 1 µl of a 20 mg/ml glycogen solution was added as a carrier molecule to improve the final yield of DNA. 2.5 volumes of cold ethanol were added, the sample mixed by inversion and left at -70°C for 30 minutes. After centrifugation as before the pellet was washed with 1 ml of cold 70% ethanol, dried under vacuum and resuspended in 20 µl of TE.

The plasmids were isolated using the Plasmid Maxi preparation kit (Qiagen) which is designed to obtain quantities of up to 500 µg plasmid DNA from 150-500 ml cultures of E.coli.

Residual protein was removed from the DNA solution by the addition of an equal volume of Tris-equilibrated (1M) phenol-chloroform (Anatech, pH 7.4) followed by centrifugation for 5 minutes at 12 000 g in a microfuge at 4C. Any remaining phenol in the aqueous phase was removed by the addition of an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged as before. The aqueous phase, containing DNA, was retained and stored at -20C.

Plasmid DNA was quantified by electrophoretic comparison to known quantities (0.1, 0.3 and 0.5 µg/µl) of linear Lambda phage DNA (Boehringer Mannheim, Germany) run through a 0.8% agarose gel.

2.4.1.1.2 Isolation of probe from vector

The HBV subtype adw genome has a BamHI restriction site at position 1850 relative to the EcoR1 site. The first T of the sequence 5'GAATTC' in the (+) strand corresponding to the unique EcoR1 site that exists in most genomes is used as reference origin of the physical map. Nucleotide numbering is from 5'->3' in the (+) strand. When the EcoR1 site does not exist the base occupying the same position is taken as position 1. As no two viruses sequenced to date have the same length, no standardised numbering system has been proposed (Wain-Hobson and Tiollais, 1984). Restriction of the probe generates two fragments of approximately 1850 bp and 1350 bp in length.

2.4. Screening of the amplified Tong/HCC cell line genomic library

2.4.1. Screening for integrated HBV DNA

The Tong/HCC cell line genomic library was screened for the presence of HBV DNA using HBV DNA labelled with the Amersham enhanced chemiluminescence (ECL) random prime labelling system (RPN 3040).

2.4.1.1. Preparation of labelled DNA probe

The probe, consisting of the entire HBV genome (3.2 kb) which had been inserted into the BamH1 site of pBR322 and subsequently used to infect E.coli strain HB101 cells, was kindly supplied by Mr J. Dewa, of the University of the Witwatersrand.

2.4.1.1.1 Large scale isolation of plasmids containing HBV genomes

The transformed E.coli HB101 cells from glycerol frozen stocks stored at -70 C were grown up overnight at 37 C on LB plates supplemented with ampicillin (100 µg/ml). A single colony was picked off and inoculated into 10 ml of LB medium (ampicillin, 50 µg/ml). This culture was incubated with shaking at 37 C overnight. The following morning a secondary culture was prepared by inoculating 1 ml of the overnight culture into 500 ml of LB medium to which had been added ampicillin (50 µg/ml) and incubated at 37 C until the OD_{600} had reached 0.6 (approximately 2.5 hours).

The bacterial cells were harvested by centrifugation at 3 800 g for 15 minutes at 4 C and washed by resuspending the pellet in 100 ml ice cold STE buffer. The cells were collected by centrifugation as before.

expresses endogenous β-galactosidase which does not allow for the colour selection of recombinants or IPTG induction of expression.

The following morning the number of plaques were counted, the percentage recombinants and the titre of the library determined. All formulas used are shown in Appendix B.

2.3. Amplification of Tong/HCC genomic library

200 µl of the Tong/HCC genomic library was made up to 20 ml with phage buffer (a 10^2 dilution). This was divided into 400 x 50 µl aliquots and added to 400 x 200 µl of freshly prepared E.coli strain Y1090 cells (80 ml in total) prepared as described previously. The phage were allowed to adsorb to the bacterial cells by incubating at 37C for 30 minutes. 3 ml of TB top agar (at 45C) was added to the adsorbed phage, poured onto LB plates prewarmed to 37C and incubated at 37C overnight. The next morning 3 ml of phage buffer was added to each plate containing the plaque forming units (pfu). The plates were incubated at room temperature for 1 hour. The TB top agar containing the pfus was scraped off using a glass spreader and collected in a flask. The solution was agitated for 2 hours at room temperature and then centrifuged at 3 000 rpm for 15 minutes at 4C to remove the top agar. The supernatant was collected and the centrifugation repeated twice more. 0.3% chloroform was added to the collected supernatant. The titre of the library was determined as described in section 5.2 of this chapter. Stocks of the library were prepared by adding dimethylsulfoxide (DMSO) to a final concentration of 7% and storing at -70C.

2.2.5. Titration of packaged phage on LB plates

2.2.5.1 Preparation of bacterial culture for infection

E.coli strain Y1090 (r) was used as host for the packaged ligated DNA and E.coli strain LE392 for the positive control. E.coli strain Y1090 contains the plasmid pMC9 (Amp', Tet'). To maintain pMC9 it was necessary to initially streak strain Y1090 on LB plates supplemented with 100 µg/ml ampicillin and 15 µg/ml tetracycline. All subsequent growth occurred in the absence of ampicillin and tetracycline.

The bacterial culture was prepared by inoculating 3 ml of Luria-Bertani (LB) medium, supplemented with 0.2% maltose and 10 mM $MgSO_4$, with a single colony from a freshly streaked plate. The culture was incubated with shaking at 37C overnight. A secondary culture was prepared by inoculating 500 µl of the overnight culture into 50 ml of LB medium supplemented as before. This culture was incubated with shaking for 2.5 hours at 37C or until the OD_{600} had reached 0.6-0.8.

2.2.5.2 Titration of the packaged phage

A tenfold serial dilution of the packaging reaction ranging from 10^{-1} to 10^{-10} was prepared using phage buffer. 100 µl of the diluted phage was added to 100 µl of freshly prepared bacterial cells. The phage were allowed to adsorb to the bacterial cells by incubating at 37C for 30 minutes. 3 ml of TB top agar at a temperature of 45C was added to the mixture, vortexed gently and immediatly poured onto an LB plate prewarmed to 37C. The plates were incubated overnight at 37C.

The top agar contained IPTG (0.7 ml of a 20 mg/ml stock solution) and X-gal (0.7 ml of a 50 mg/ml stock solution prepared in dimethylformamide) for colour selection. This was not included for the positive control as E.coli strain LE392

The success of the ligations and the optimal ratio of Lambda gt11 arms to DNA insert was determined by running an aliquot of each reaction through a 0.8% agarose gel, to view band intensiy, and a 0.2% agarose gel poured on a 1% base, to distinguish any molecular weight differences.

2.2.3.2 Ligation of Lambda gt11 arms and EcoR1 digested Tong/HCC DNA

Five reactions, each containing 1 µg of insert DNA, 1 µg of Lambda gt11 arms (molar ratio of 1:1), 3 U of T4 DNA ligase and 10x ligase buffer made up to a total volume of 10 µl, were incubated at 14C for 18 hours. An aliquot of each reaction was electrophoresed through a 0.8% agarose gel to observe if ligation had occurred.

2.2.4. In vitro packaging of ligated DNA

The ligated DNA was packaged using Promega's Packagene *in vitro* packaging system (Cat. no. T3260). All five ligation reactions were used. A positive control packaging reaction was performed concurrently using Lambda *cI857 Sam7* DNA that was provided with the packaging system.

Six packaging extracts were thawed out on ice. To each extract one of the above ligation reactions including the positive control reaction was added, mixed in by gently tapping the bottom of the tube and incubated at 22C for 3 hours.

After packaging of the ligated DNA, all 5 reactions were pooled and made up to a total of 2,5 ml with phage buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$). 1 ml of chloroform was added and the mixture stored at 4C. The packaged positive control was made up to a total of 500 µl with phage buffer. 25 µl of chloroform was added and stored at 4C.

2.2.3. Ligation of Lambda gt11 arms to Tong/HCC genomic DNA

2.2.3.1 Determination of optimal ligation conditions

A series of test ligations was performed to determine the ratio of Lambda gt11 arms to insert DNA that would form optimal ligation conditions. The molar concentration of the Lambda gt11 arms was kept constant while the amount of insert DNA was adjusted. Lambda phage gt11 arms were bought as predigested, dephosphorylated, purified EcoR1 arms (Promega, cat. no. T3260).

The reactions were set up as described in Table 3.1 and incubated for 18 hours at 14°C.

Concurrent with the optimisation ligations a positive control ligation was set up using the pSP64 Vector (2 990bp) predigested with EcoR1 (Promega). The reaction, containing 1 µg Lambda gt11 EcoR1 arms, 0.4 µg positive control insert, 3 U T4 DNA ligase and 10x ligase buffer made up to a toal volume of 10 µl, was incubated at 14°C for 18 hours.

Table 2.3.1: Optimization of Lambda gt11 arms:DNA insert ligation

	A	B	C	D
Lambda gt11 arms (0.8 µg/µl; 1 µg = 0.035 pmol)	2µl	2µl	2µl	2µl
Insert DNA (10 ng/µl; 10 ng = 0.0025 pmol)	-	3µl	2µl	1µl
ligase 10x buffer	1µl	1µl	1µl	1µl
nuclease free H2O	6µl	3µl	4µl	5µl
T4 DNA ligase (3 Weiss units/µl)	1µl	1µl	1µl	1µl

6µg of Tong/HCC genomic DNA was digested with 20 units of EcoRI (Boehringer Mannheim, Germany) at 37C. 5 µl aliquots were taken after 5, 10, 15, 20, 25 and 30 minutes of digestion. 1 µl of 0.1 M EDTA (pH 8.0) was immediatly added to the sample which was subsequently snap frozen at -70C until all the samples had been taken. Uncut DNA was included as a control. All aliquots were heated at 68C for 10 minutes and run on a 0.4% agarose gel poured on a 1% base to check the extent of digestion.

2.2.2.2 Large-scale preparation of partially digested genomic DNA

Using the optimised conditions determined in step 2.2.2.1 a large-scale reaction was carried out.

150 µg of Tong/HCC genomic DNA was digested with 250 U of EcoRI at 37C for 20 minutes. The reaction mixture was snap frozen at -70C and an aliquot removed for electrophoresis through a 0.4% agarose gel poured on a 1% base, to check the degree of digestion. Once it was ascertained that the majority of the fragments were in the size range of 5-7 kbp the reaction mixture was removed from -70C, 5 µl of 0.5 M EDTA (pH 8.0) added and heated at 68C for 10 minutes to inactivate the enzyme.

The EcoR1 restricted DNA was precipitated by adding 0.07 volume 3 M sodium acetate (pH 6.0), 2 volumes ice-cold ethanol (Merck) and stored at -70C overnight. After centrifugation at 12 000g for 15 minutes in a microfuge the pellet was washed with 70% ethanol. Following a repeat centrifugation the precipitated DNA was dissolved in distilled water at a concentration of 0.25 µg/µl and stored at -20C.

hours. The dialysis tubing used (Mw cutoff = 12 000) was prepared as per Sambrook et al. (1989). Allowance was made for the volume of the sample to increase 1.5-2.0 fold.

After dialysis, sodium acetate (pH 6.5) was added to a final concentration of 0.3 M and was followed by the addition of 0.8 volumes of isopropanol (Merck). The tube was inverted gently end over end for 10 minutes. The precipitate of DNA was spooled out with a glass pasteur pipette with a heat sealed end and placed in 1 ml of TE (pH 8.0). The DNA was dissolved by leaving the tube rotating overnight on a wheel (20 rpm) at 4°C.

Genomic DNA was quantified by electrophoretic comparison to known quantities (0.1, 0.3 and 0.5 µg/µl) of linear Lambda phage DNA (Boehringer Mannheim, Germany) run through a 0.8% agarose gel. To check if the genomic DNA had a high-molecular-mass a sample was run through a 0.2% agarose gel poured on a 1% support at 4°C. Purity of the preparation was determined by spectrophotometric analysis at OD_{260} and OD_{280}. An OD_{260}/OD_{280} reading between 1.8 and 2.0 indicated the DNA preparation was free from contaminating protein, phenol or isopropanol. This method yielded genomic DNA with a concentration of 0.2-0.3 µg/µl. DNA was stored at 4°C.

2.2.2. Partial digestion of high-molecular-mass genomic DNA

2.2.2.1 Optimization of restriction endonuclease digestion

In order to establish the time period required to generate fragments with an average size of 5-7 kbp the following small scale reaction was performed.

The monolayers were washed twice with ice-cold Tris-buffered saline (TBS). A rubber policeman was used to scrape the cells into 0.5 ml of TBS which was subsequently stored on ice. The flasks were washed with 1 ml of TBS which was combined with the previous washing. The cells were recovered by centrifugation at 1 500*g* for 10 minutes at 4°C in a Beckman bench top centrifuge and resuspended in 10 volumes of ice-cold TBS. After a repeat centrifugation the cells were resuspended in 1 ml TE (pH 8.0) at a concentration of 5 x 10^7 cells/ml. The cell suspension was transfered to a 50 ml Erlenmeyer flask and 10ml of extraction buffer (10 mM Tris-Cl, pH 8.0, 0.1 M EDTA, pH 8.0, 20 ul/ml pancreatic RNAse A, 0.5% SDS) added followed by incubation for 1 hour at 37°C.

Proteinase K (Boehringer Mannheim, Germany) was added to a final concentration of 100 µg/ml and gently mixed into the viscous solution using a glass rod. The suspension of lysed cells was placed in a water bath for 3 hours at 50°C and stirred regularly.

The solution was cooled to room temperature and poured into a centrifuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the two phases mixed by inverting the tube gently and repeatedly for 10 minutes. The phases were separated by centrifugation at 5 000*g* for 15 minutes at room temperature. A wide bore pipette was used to move the viscous aqueous phase to another centrifuge tube and the phenol:chloroform:isoamyl alcohol extraction repeated twice more.

After the third extraction the aqueous phase was dialysed 4 times against 4 liters of 50 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0) at 4°C over a total period of 48

2.1.3. Preparation of stock cultures

Viable stocks of Tong/HCC cells were stored at -70°C in NUNC cryotubes at a concentration of ca. 1.2x 10^6 cells/ml. Freezing of the cells was performed as follows. The cells were trypsinized (0.25% bovine pancreatic trypsin, 0.2% EDTA) and washed by resuspending in RPMI 1640 without FCS followed by centrifugation at 800 r.p.m. The washing was repeated and the pellet resuspended in a freezing mixture consisting of 90% FCS and 10% DMSO. The cryotubes were insulated with cotton wool and tin foil and placed directly into -70°C without any intermediate cooling steps. This method resulted in a 80% minimum viability on thawing and reestablishment in standard conditions.

2.2 Construction of a Tong/HCC cell line genomic library

A Tong/HCC cell line genomic library was created using lambda phage gt11. All solutions used are described in Appendix A.

2.2.1. Isolation of Tong/HCC genomic DNA

High-molecular-mass genomic DNA was isolated according to the modified method of Blin and Stafford (1976) in Sambrook et al. (1989) from the Tong/HCC cell line developed previously by Stevenson et al. (1987). The Tong/HCC cells were grown in monolayer culture in 75 cm^2 Nunc Tissue culture flasks. Ca. 1 x 10^7 cells were used for each extraction.

3.1.2.2 Large-scale partial genomic DNA digestion

Bulk digestion of genomic DNA using the pre-established time period of 20 minutes (Figure 3.2, lane 6) yielded fragments of DNA in the size range of 5 to 7 kb (Figure 3.3).

3.1.3. Ligation of Lambda gt11 arms to Tong/HCC genomic DNA

3.1.3.1 Determination of optimal ligation conditions

To determine the optimal conditions for the ligation of Tong/HCC genomic DNA, the molar concentration of the Lambda gt11 arms was kept constant while the amount of insert DNA was adjusted. The molar ratio of Lambda DNA (43 kb) to genomic DNA (5-7 kb) varied between 1:2.8 and 1:0.9. An aliquot of each reaction was run through a 0.8% agarose gel, to view band intensity (Figure 3.4), and a 0.2% agarose gel, to distinguish any molecular weight differences (Figure 3.5).

All the ligation reactions performed were successful as determined by the lack of insert DNA below the ligated DNA in each lane. A molar ratio of Lambda gt11 arms to genomic DNA of 1:1.8 (lane 4, Figure 3.4) gave the band with the brightest intensity and thus was determined to be the optimal ratio for all future ligation reactions. No difference in molecular weight was observed between the ligated Lambda gt11 arms (lane 2, figure 3.5) and the ligated Lambda gt11 arms with insert genomic DNA (lanes 3-5, Figure 3.5). With a DNA insert size of 5-7 kb a difference in molecular weight could only be observed using a technique such as pulse-field electrophoresis, however the lack of digested Tong/HCC DNA below the Lambda DNA indicated that the ligations were successful.

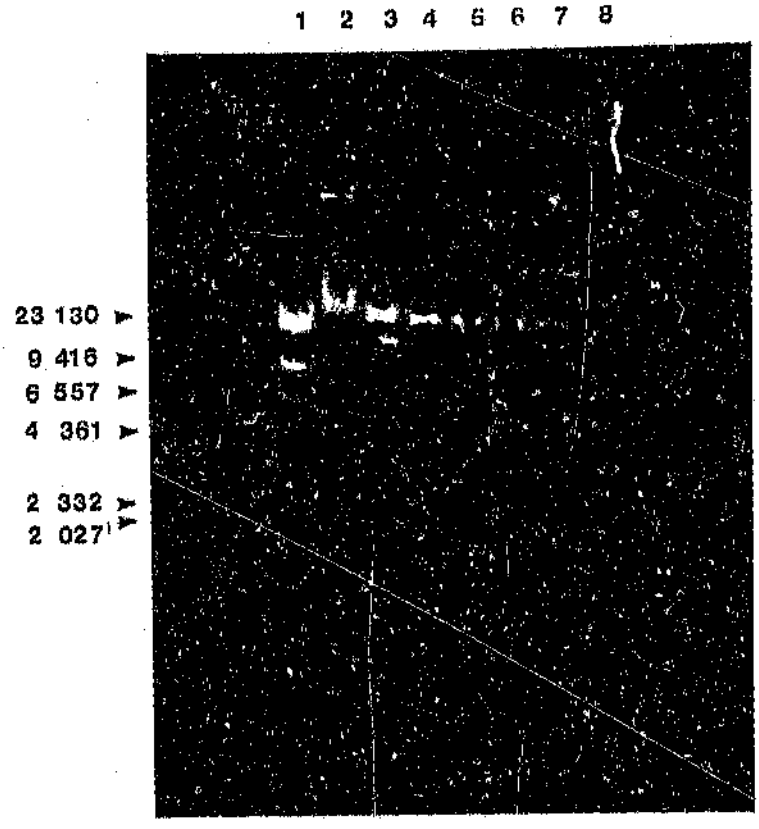

Figure 3.3. Time dependent optimization of the partial *Eco*RI digestion of high-molecular-mass Tong/HCC DNA. Lane 1, λ DNA restricted with *Hind*III; lane 2, uncut Tong/HCC DNA; lanes 3-8, Tong/HCC DNA digested for 5, 10, 15, 20, 25 and 30 minutes with *Eco*RI.

3.1.2 Partial digestion of high-molecular-mass genomic DNA

3.1.2.1 Optimization of restriction endonuclease digestion

Optimal conditions for the preparation of fragments of genomic DNA in the size range of 5 to 7 kb was determined by time dependent partial *Eco*RI digestions ranging from 5 to 30 minutes (Figure 3.2). Uncut Tong/HCC DNA was incubated simultaneously for 30 minutes to confirm the absence of other enzymes in the DNA preparation. A digestion time of 20 minutes yielded the maximum number of fragments in the required size range (Figure 3.2, lane 6). All amounts were scaled up proportionally for the large scale digestion of Tong/HCC DNA.

Originally, the optimal conditions for the preparation of fragments of genomic DNA in the size range of 5 to 7 kb was determined by *Eco*RI enzyme concentration dependent partial digestions as recommended by Promega (Promega Protocols and Applications Guide) for creating a Lambda gt11 library. The *Eco*RI enzyme concentrations ranged from 3.3 U/µg to 0.0116 U/µg. Total DNA digestion was observed for an *Eco*RI enzyme concentration of 3.3 U/µg and no digestion was observed for all subsequent *Eco*RI enzyme concentrations (data not shown). This method is ineffective in determining the optimal conditions for the partial digestion of Tong/HCC DNA and should only be used when there are unknown inhibitors in the preparation or if it is not possible to determine DNA concentration (Frischauf, 1987).

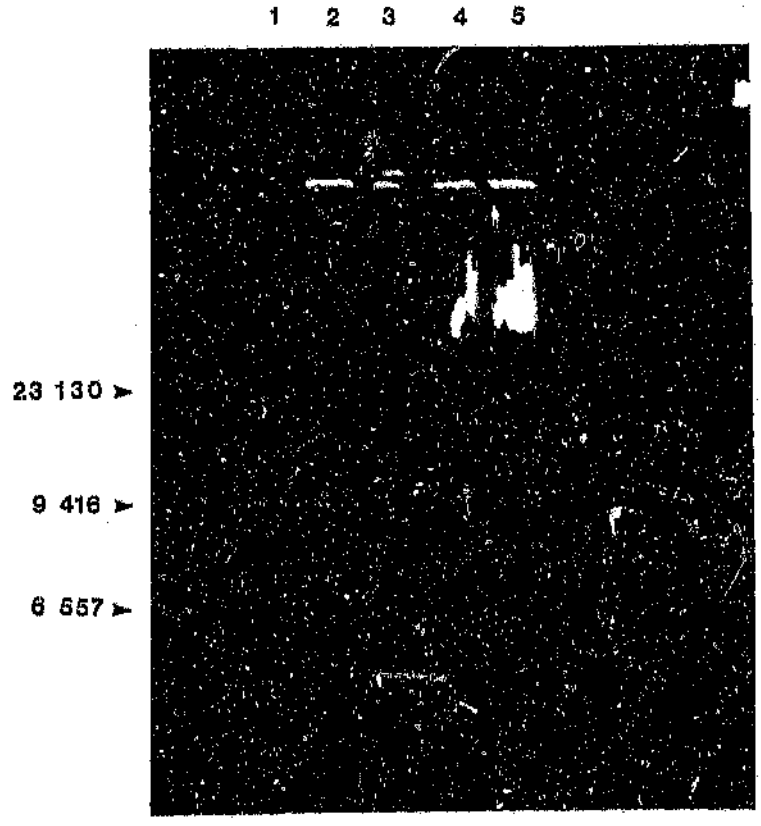

Figure 3.2. High-molecular-mass Tong HCC genomic DNA run on a 0.2% agarose gel with uncut lambda DNA. Lane 1, λ DNA restricted with *Hind*III; lane 2, 0.3 μg Tong-HCC DNA; lanes 3-5, 0.1, 0.3 and 0.5 μg uncut λ DNA.

A number of genomic DNA isolation methods were attempted but the modified method of Blin and Stafford (1976) in Sambrook et al (1989) consistently yielded a large amount of DNA with a high molecular weight.

Eight 75cm^2 tissue culture flasks of Tong/HCC cells grown to confluence yielded approximately 5 x 10^7 cells from which 250 - 300 µg of genomic DNA could be extracted. This compared favourably with the expected yield for this method of 200 µg (Sambrook et al., 1989). The OD_{260}/OD_{280} readings were between 1.8 and 2.0 indicating that the DNA preparation was free from contaminating protein, phenol or isopropanol.

The isolated Tong/HCC genomic DNA did not migrate faster than uncut Lambda DNA, which has a molecular mass of 48.5 kb, when run on a 0.2% agarose gel (Figure 3.1). Most of the DNA loaded onto the gel remained in the wells being of too high a mass to penetrate the low percentage agarose.

Ethanol precipitation yielded DNA of a low molecular weight (< 50 kb) as opposed to the expected 100 - 150 kb reported by Sambrook et al., (1987). DNA in the size range of 100 - 150 kb could only be obtained with dialysis followed by isopropanol precipitation after the phenol:chloroform:isoamyl alcohol extraction.

3.1 Construction of a Tong/HCC cell line genomic library

3.1.1. Isolation of Tong/HCC genomic DNA

Before isolation of the genomic DNA it was ascertained that the Tong/HCC cell line had the correct morphology, namely an epithelial pattern with multinuclei and granules in the cytoplasm (figure 3.1). The presence of multi-nucleated giant cells was not unusual. The Tong/HCC cell line had a doubling time of approximately 48 hours.

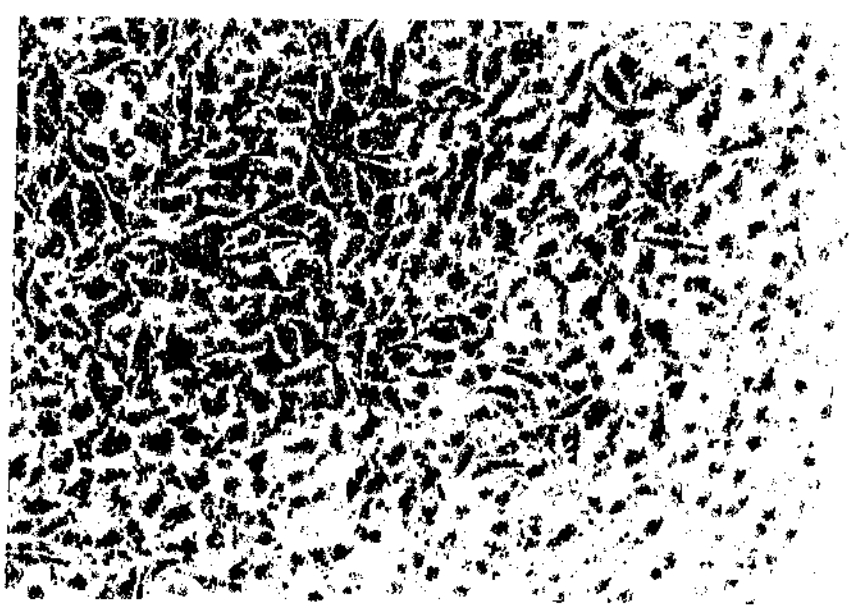

Figure 3.1: Tong/HCC cell line. Photographed at 100% confluency, phase contrast, 200x magnification.

CHAPTER 3

RESULTS

2.4.2.2 Filter screening with antibodies

The non-specific binding sites on the filters were blocked by soaking the filters in 5% blocking agent (Amersham) in TBS-T at 4C with gentle agitation (60 rpm) overnight. The following morning the filters were washed for 2 x 15 minutes followed by 2 x 5 minutes in TBS-T. Mouse anti-HBsAg monoclonal antibody (kindly supplied by Mr C.C. Chen of the University of the Witwatersrand) was diluted in TBS-T and the filters were incubated in this solution for 1 hour at room temperature. The filters were washed as before. The HRP labelled antibody was diluted 1:1000 in TBS-T.

The filters were incubated in this solution at room temperature for 1 hour and subsequently washed for 1 x 15 minutes and 4 x 5 minutes in TBS-T.

2.4.2.3 Signal generation and detection

This was performed using the method detailed in section 2.4.1.6.

expelled into a microcentrifuge tube containing 200 µl of phage buffer and incubated for 1 hour at room temperature. 0.3% chloroform was added to each tube which was subsequently stored at 4C.

From these samples an additional 3 screens were made to try and obtain material with 100% purity and homology.

2.4.2. Screening for HBV protein

LB plates that had tested positive for phages with integrated HBV DNA were used for the detection of fusion proteins produced by these phages. This was performed using the Amersham enhanced chemiluminescence (ECL) Western blotting system (RPN 2108).

2.4.2.1 Filter preparation for screening with antibodies

The nitrocellulose filters (Hybond C, Amersham) were saturated with 10 mM IPTG and air dried. The LB plates were overlayed with these filters and incubated at 37C for 3.5 hours. To harden the agar, the plates were placed at 4C for 20 minutes. The plates, filters and agar were asymmetrically labelled for later reorientation. The filters were removed carefully, rinsed in TBS-Tween (TBS-T) buffer to remove any top agar and screened immediately.

The unbound conjugate was removed by washing twice for 10 minutes and twice for 5 minutes in 0.1% v/v Tween-20 in antibody wash buffer (2 ml/cm^2) at room temperature with gentle agitation.

2.4.1.6. Signal generation and detection

The Amersham ECL random prime system uses a detection method comprising two reagents which are mixed immediately before use. Detection reagent 1 decays to hydrogen peroxide, the substrate for peroxidase. Reduction of hydrogen peroxide by peroxidase is coupled to a light producing reaction by detection reagent 2. This contains luminol which, on oxidation, produces blue light. The light output is increased and prolonged by the presence of an enhancer.

Detection reagent 1 was mixed with detection reagent 2 in equal volumes (0.125 ml/cm^2 of membrane). Excess buffer was drained from the washed membranes which were then placed on a piece of Saran wrap. The detection buffer was added directly to the membranes on the side carrying the DNA and incubated for 1 minute at room temperature. Excess detection reagent was removed by gently laying the membranes, DNA side up, on a folded tissue for a few seconds. The membranes were wrapped in Saran wrap and autoradiographed for 10 minutes.

2.4.1.7 Isolation and purification of positive plaques

A clear transparant sheet was placed on top of each film. The positive signals were marked together with the asymmetric filter markings. These were then aligned on the agar plates and the positive plaques identified. Using a borosilicate Pasteur pipette, two positive plaques were picked off each agar plate. The agarose plugs were gently

2.4.1.4. Hybridization

The membranes were prehybridized in buffer (0.2 ml/cm^2) made from hybridization buffer tablets (Amersham, RPM 131) for 1 hour at 60°C with constant gentle agitation (60 rpm). 300 ng of labelled HBV DNA, which had been denatured by boiling for 5 minutes and snap cooling on ice, was added to the prehybridization buffer. 1 ml of the buffer was withdrawn, mixed with the probe and added back to the main solution to avoid placing the probe directly onto the membranes. The membranes were hybridized in this solution overnight at 60°C with gentle agitation as before.

The following morning the membranes were washed in a solution of 1x SSC, 0.1% SDS (2-5 ml/cm^2) at 60°C for 15 minutes with gentle agitation followed by a second wash of 0.5x SSC, 0.1% SDS under the same conditions.

2.4.1.5. Blocking and antibody binding

The membranes, that had been hybridized, were rinsed briefly in antibody wash buffer at room temperature. Subsequently, they were blocked in 0.5 ml/cm^2 of 0.5% blocking agent (Amersham) in antibody wash buffer for 1 hour at room temperature with gentle agitation. The membranes were washed in antibody wash buffer as before.

The anti-fluorescein-HRP conjugate was diluted 1000 fold in a volume of 0.5% w/v BSA in antibody wash buffer equivalent to that used for hybridization. The membranes were incubated in this solution with gentle agitation for 1 hour at room temperature.

2.4.1.3. Plaque blotting

The LB plates were overlayed with Hybond positively charged nylon membrane discs (Amersham) by gently placing the membrane on one edge of the plate and progressively laying it down so as to avoid air bubbles being trapped between the top agar and the membrane. The plates, membranes and agar were all asymmetrically labelled for later reorientation. The membranes were left on the plates for 1 to 10 minutes at room temperature and then removed from the agar surface and allowed to air dry, plaque side up on filter paper (Whatman 3MM).

The DNA was denatured and fixed by placing the membranes, plaque side up, on two sheets of filter paper saturated with denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 7 minutes.

The membranes were then set, plaque side up, on a pad of filter paper soaked in neutralising solution (1.5 M NaCl; 0.5 M Tris-HCl pH 7.2; 1 mM EDTA) for 3 minutes. This was repeated with fresh filter paper soaked in the same solution.

The membranes were subsequently washed in 2 x SSC, transferred to dry filter paper and air dried, plaque side up.

The DNA was fixed to the membranes by placing the membranes on two pieces of filter paper soaked in 0.4 M NaOH for 5 minutes. This short alkali fixation time is required for use with ECL gene detection systems.

The membranes were washed twice in 5 x SSC for 1 minute with gentle agitation and air dried, plaque side up, on filter paper.

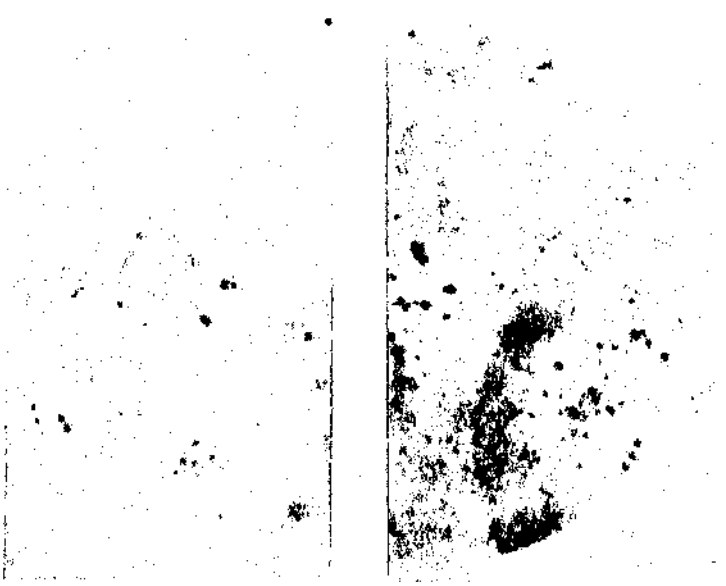

Plate A Plate B

Figure 3.10a: Screen 3 of the Tong-HCC genomic library using the 1850 and 1350 bp fragments of *Bam*H1 digested HBV in pBR322. Plates A and B.

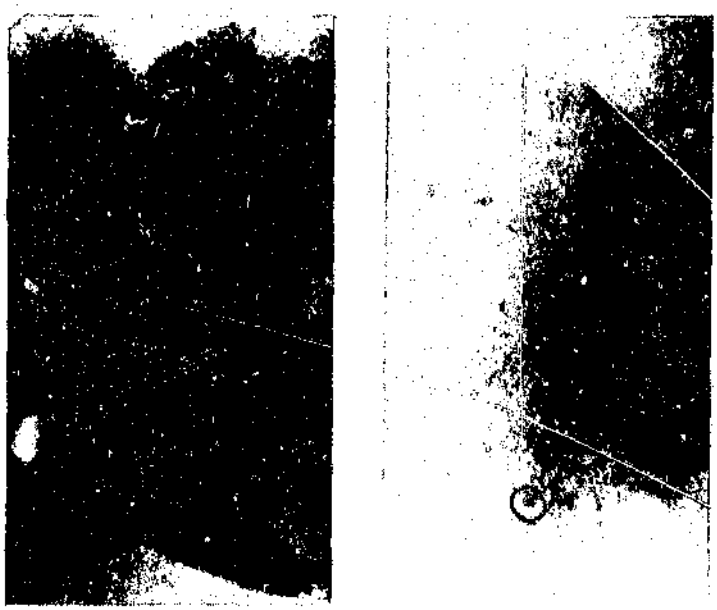

Plate A Plate B

Figure 3.9: Screen 1 of the Tong HCC genomic library with the 1850 and 1350 bp fragments of *Bam*H1 digested HBV in pBR322. Plaques isolated for further screening are circled.

For each screen 300 ng HBV DNA was labelled with fluorescein-11-dUTP using the random primed hexamer labelling process according to the Amersham ECL protocol. This process yielded approximately 750 ng of labelled probe.

3.2.2. Screening for integrated HBV DNA

The Tong/HCC cell line genomic library was screened for the presence of HBV DNA using the Amersham enhanced chemiluminescence (ECL) random prime labelling system (RPN 3040).

Once a screen was performed, the plaques giving positive signals were isolated, the phage grown up and rescreened in order to obtain material with 100% purity and homology. This was done three times.

From the first screen 10 positive plaques were obtained (Figure 3.9). It was very difficult to realign positive hybridization signals with positive plaques on their respective plates. An agar plug containing several plaques was picked so as to be sure of obtaining the phages containing integrated HBV DNA.

The second screen was also successful with positive plaques detected on all ten plates. A single plaque was isolated from each plate for further screening.

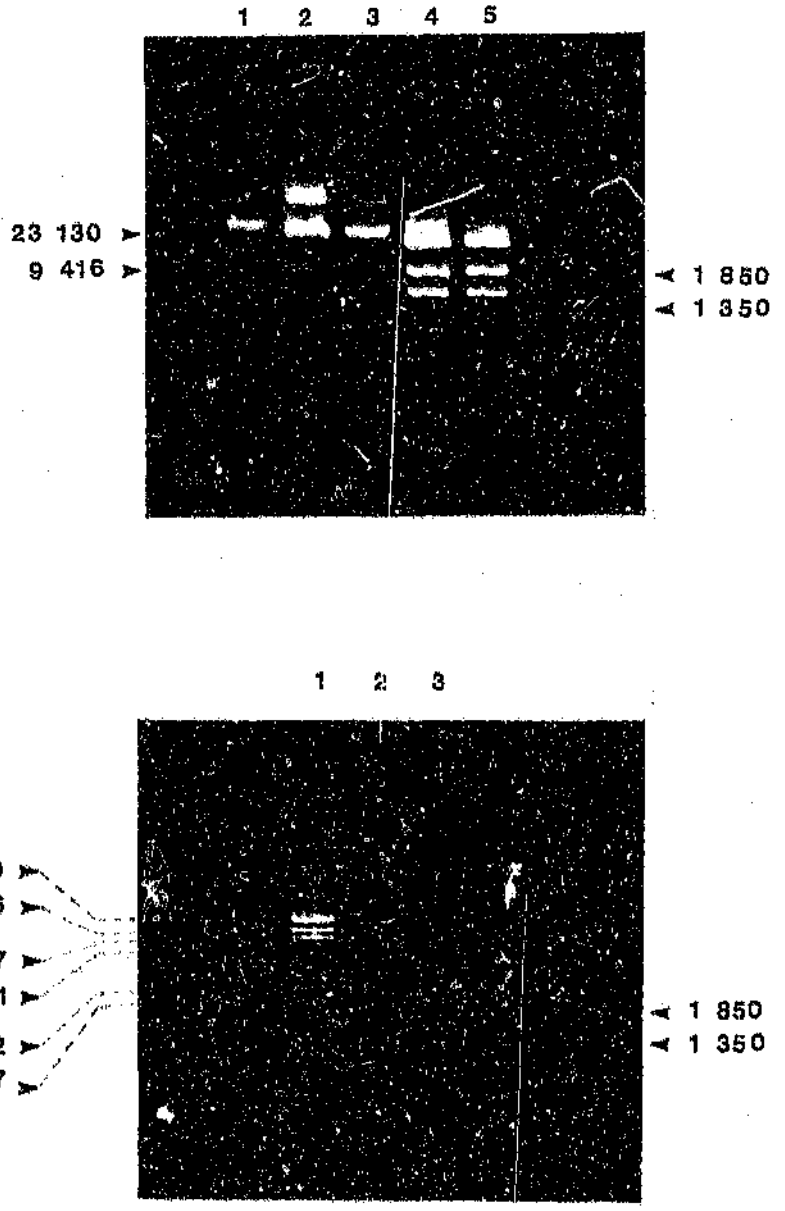

Figure 3.8. a. pHR322 restricted with *Bam*H1 run on a 0.8% agarose gel. Lane 1, λDNA restricted with *Hind*111, lane 2-5, *Bam*H1 restricted pHR322.

b. HBV DNA *Bam*H1 restricted probe inserts isolated from gel a using the phenol based method of Favre (1992). Lane 1, λDNA restricted with *Hind*111, lanes 2 and 3, *Bam*H1 restricted HBV isolated from pHR322.

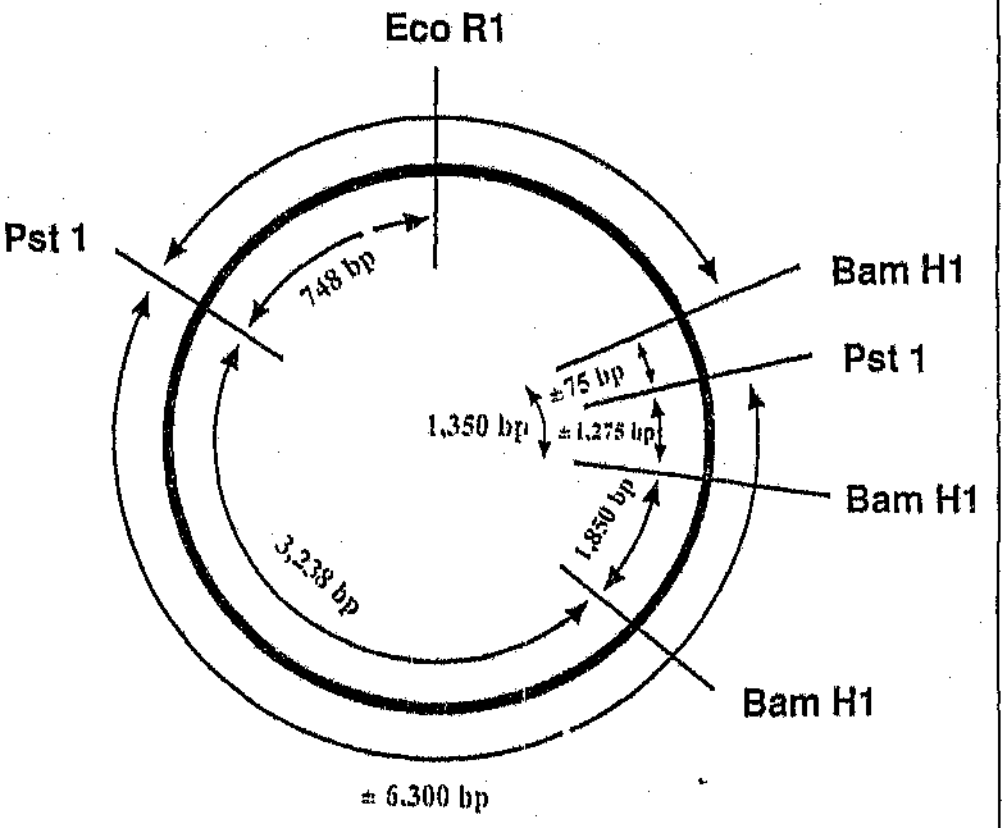

Figure 3.2. Diagrammatic representation of the pBR322 plasmid with ligated insert HBV DNA insert, showing the 1850 and 1350 bp fragments, in the *Bam*H1 restriction site.

Originally, the entire pBR322 plasmid was used to screen the Tong-HCC genomic library. A high background level was obtained and it was decided to continue screening the library with HBV DNA isolated from the probe.

*Bam*H1 restriction of the pBR322 plasmid yielded three fragments of 4363, 1850 and 1350 bp (Figure 3.8a). The 1850 and 1350 bp fragments made up the HBV DNA insert which contains a *Bam*H1 restriction site at position 1350 relative to the *Eco*R1 site (figure 3.7). The restricted pBR322 was run on a 0.8% agarose gel from which the two HBV DNA fragments were isolated. Numerous methods were attempted but the procedure that yielded the highest recovery rate of DNA from the gel (80% figure 3.8b) was the phenol based method developed by Favre (1992) detailed in Materials and Methods.

gives the background level of non-recombinant phage to be 5.25 x 10^3 pfu/µg of arms and is below the typical background levels which range from 1-5 x 10^4 pfu/µg of arms.

3.1.5.3 Titration of the amplified Tong/HCC library

Different recombinant phages may grow at different rates resulting in unequal representation of the recombinants in an amplified library. To try and avoid this the Tong/HCC gene library was amplified in fractions which were then combined for the screening of the library.

The titre of the amplified Tong/HCC library was obtained by preparing a serial dilution in triplicate ranging from 10^{-1} to 10^{-10} and plating. The titre of the amplified library was determined to be 6.8 x 10^7 pfu/ml and yielded 7% non-recombinants and 93% recombinants as determined by a white versus blue colony count.

3.2 Screening of the Tong/HCC cell line genomic library

3.2.1 Preparation of labelled DNA probe

The probe, which consisted of the entire HBV genome, had previously been inserted into the *Bam*H1 site of pBR322 (Figure 3.6) and subsequently used to transform competent E.coli HB101 cells. These cells were used for the large scale isolation of the pBR322 plasmid with the Plasmid Maxi preparation kit of Qiagen.

500 µg of pBR322 was obtained from a 500 ml HB101 cell culture using the above method and corresponded with the expected yield of the Qiagen Plasmid Maxi preparation kit of 500 µg.

The titre was determined by plating a serial dilution of 10^{1} to 10^{7}. A large serial dilution was prepared to ascertain what dilution factors should be used for the titration of the Tong/HCC gene library. The titre of the packaged positive control was 4 x 10^{7} pfu/ml with a packaging efficiency of the arms of 4 x 10^{7} recombinants/µg. This was lower than the expected efficiency of 5 x 10^{7} to 2 x 10^{8} pfu/µg. The lower value obtained could have resulted from the packaged phage being stored at 4°C for two days before the titration allowing a drop in titre.

Background levels of religated arms could not be determined due to the absence of colour selection of the recombinants. They were expected to be low, ranging from 1-5 x 10^{4} pfu/µg of Lambda gt11 arms (Promega Technical Bulletin 006, 1992).

3.1.5.2 Titration of Tong/HCC gene library

The Tong/HCC DNA ligated to Lambda gt11 arms was packaged in E.coli strain Y1090. The Δ(*lac*U169) deletion present in Y1090 prevents the generation of endogenous functional β-galactosidase products and thus allows colour selection of recombinant and non-recombinant phages in the presence of X-Gal.

The titre of the Tong/HCC gene library was determined by preparing a serial dilution in triplicate of 10^{1} to 10^{10} and plating. The titre of the Tong/HCC gene library was 1 x 10^{6} pfu/ml with a packaging efficiency of the arms of 1 x 10^{5} recombinants/µg. Since typical recombinant efficiencies should range from 5-9 x 10^{6} pfu/µg arms it was decided to amplify the library.

Colour selection using IPTG and X-Gal was used when titrating the Tong/HCC library and the number of blue (non-recombinants) and white (recombinants) plaques counted. The library yielded 5% non-recombinants and 95% recombinants. This

3.1.3.2 Ligation of Lambda gt11 *Eco*RI arms and *Eco*RI digested Tong/HCC DNA

Three of the five ligation reactions of Lambda gt11 *Eco*RI arms to *Eco*RI digested Tong/HCC DNA using the conditions optimized before were successful (figure 3.6, lanes 2,3 and 6). No insert DNA was observed below the ligated DNA. 1 µg of Lambda gt11 arms and 3 Weiss units of T4 DNA ligase were added to the two unsuccessful ligation reactions and incubated at 14°C for a further 20 hours. When an aliquot of each reaction was run on a 0.8% agarose gel it was observed that the ligation was succesful (Figure 3.6, lanes 4 and 5).

3.1.4 Packaging of ligated DNA

The ligated DNA was packaged using Promega's Packagene *in vitro* packaging system (Cat. No. T3260). All five ligation reactions were used. A positive control packaging reaction was performed concurrently using Lambda cI857 *sam*7 DNA that was provided with the packaging system.

3.1.5 Titration of packaged phage

All formulas used are found in Appendix B.

3.1.5.1 Titration of positive control

The positive control DNA (λcI857 *sam*7) was packaged in E.coli strain LE392, a host having a *sup*F58 mutation. Colour selection of recombinants or induction of β-galactosidase fusion protein expression, using IPTG and X-Gal, could not be used with LE392 cells which express endogenous β-galactosidase.

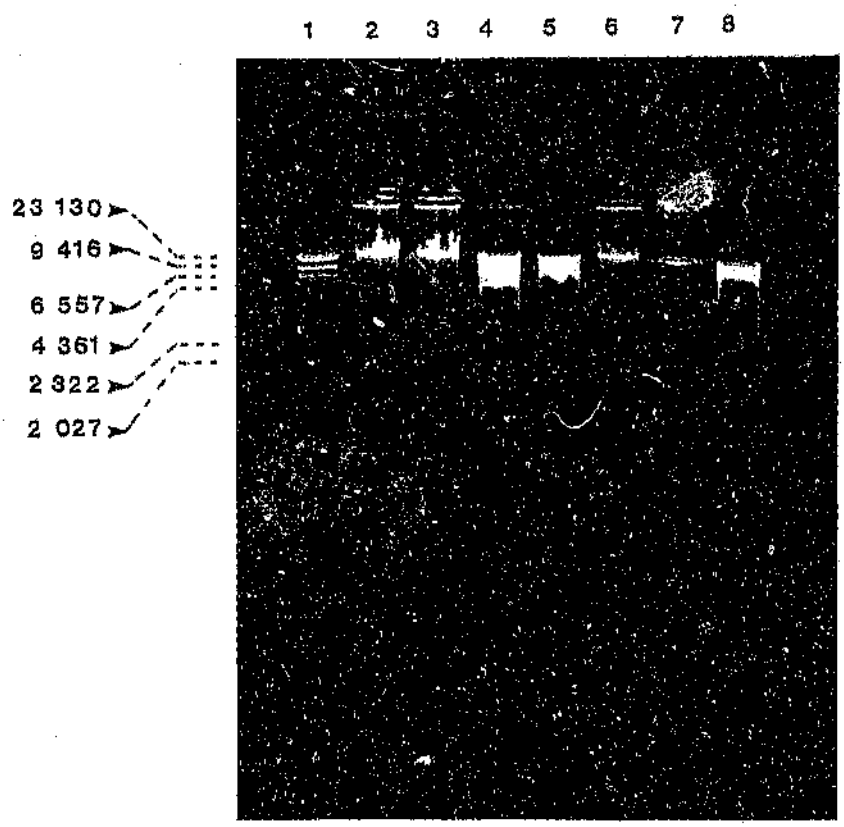

Figure 3.6. Ligation of Lambda gt11 *Eco*RI arms to *Eco*RI digested Tong/HCC DNA. Lane 1, Lambda DNA digested with *Hind*111, Lanes 2-7, successful ligation of Lambda gt11 arms and digested Tong/HCC DNA, lane 8, *Eco*R1 digested Tong/HCC DNA.

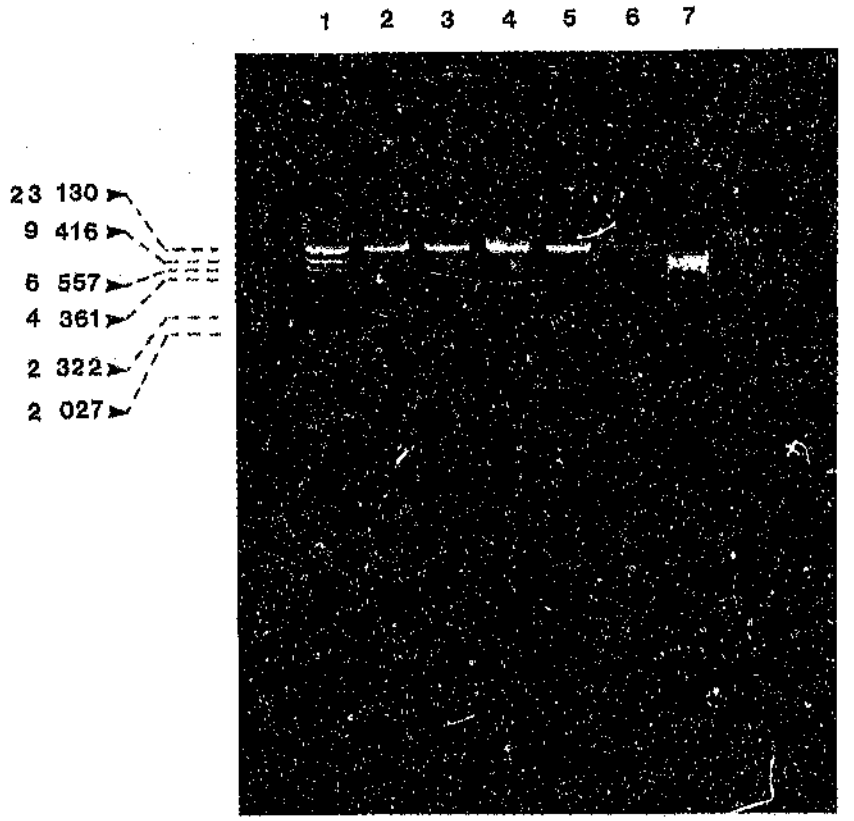

Figure 3.5. Determination of optimal conditions for the ligation of Lambda gt11 arms to genomic Tong/HCC DNA. Aliquots of each reaction run on a 0.8% agarose gel to view band intensity. Lane 1, λ DNA restricted with *Hind*III, lane 2, λ gt11 *Eco*RI arms, lane 3, molar ratio of λ gt11 *Eco*RI arms to insert DNA of 1:2.8, lane 4, molar ratio of 1:1.8, lane 5, molar ratio of 1:0.9, lane 6, positive control ligation, lane 7, *Eco*RI digested Tong/HCC DNA.

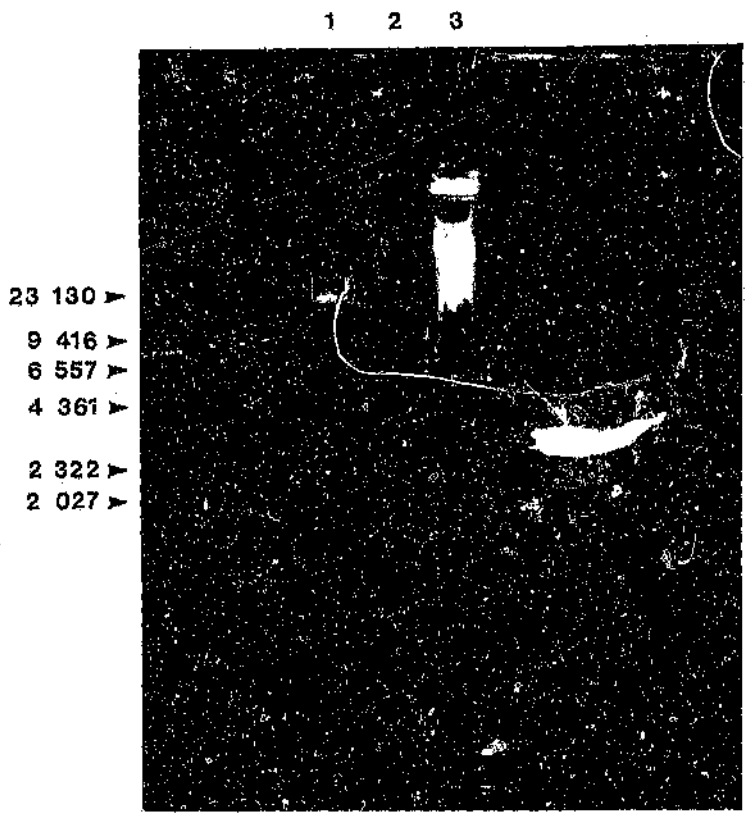

Figure 3.4. Large-scale partial digestion of high-molecular-mass Tong/HCC genomic DNA with *Eco*RI. Lane 1, λ DNA restricted with *Hind*III, lane 2, aliqot of Tong/HCC DNA restricted with *Eco*RI for 20 minutes, lane 3, uncut Tong/HCC DNA showing slight degradation due to age of sample (not sample used for digestion).

rearranged. Although present their epitopes in the fusion protein may be altered and therefore not detected.

If available, clean monospecific antisera is preferable to using monoclonal antibodies for Lambda gt11 screening. An antiserum may react with several epitopes in the fusion polypeptides, whereas the monoclonals can only recognise a single epitope. Greater signal intensities would be expected because of the recognition of multiple epitopes in one cloned sequence.

It is interesting to speculate on the nature of the integrated sequences in the Tong/HCC cell line using the four classes of HBV integrants described in Table 1.1, found in the Introduction.

Looking first at an integrant capable of producing the HBsAg. Since the Tong/HCC cell line is a HBsAg producing cell line at least one of the five integrants falls into this category. If the strand polarity is core → surface → x with the virus - cell junction at DR1, the second virus - cell junction could fall anywhere in the P or X ORF (between 800 and 1820 nt, Figure 1.1), bearing in mind that all five integrations in the Tong/HCC cell line are greater than genome length (3.2 kb). However, this may include rearrangements, translocations and inverted repeats. However, if the virus - cell junction was at DR2 the second virus - cell junction could only fall in the P ORF area (between 800 and 1600 nt) after the S ORF. An enhancer element is located between 966 and 1308 nt (Shaul et al., 1985). It is possible that the second virus - cell junction could place this enhancer next to or upstream from a cellular promoter that regulates cell growth. The X ORF encodes a viral transactivator which can upregulate the expression of other viral genes (Twu and Schloemer, 1987; Spandau and Lee, 1988; Seto et al., 1990). A virus - cell

The Tong/HCC cell line has five HBV integrations and it is a HBsAg producing cell line. The expression of HBsAg in the Tong/HCC cell line is lower than other HBsAg producing cell lines such as PLC/PRF/5 and Hep3B (Aspinall et al.,1986). When the Tong/HCC genomic DNA library was analysed for the presence of S gene sequences that were expressed as part of a fusion protein, only a small minority of the clones (5%) gave a positive signal. There are a number of reasons for the low signal obtained.

It is possible that of the five HBV integrations only one or two are capable of producing HBsAg.

It is also conceivable that these integrants could have been under represented in the amplified library. This is due to the fact that different recombinant phage clones may grow at very different rates resulting in unequal representation of the recombinants in the amplified library.

Since the foreign DNA cloned into Lambda gt11 is expressed as a β-galactosidase fusion protein under transcriptional control of the vector, both entire and incomplete HBsAg sequences will be expressed as part of the fusion protein. Of the recombinants in the Tong/HCC genomic DNA library that gave a positive result when probed with HBV DNA, only a few reacted with the monoclonal antibody raised against the HBsAg. This implies that the majority of integrants do not have HBsAg sequences. However, a disadvantage of using monoclonal antibodies is that they often recognise only a short epitope or an epitope not present in the partly denatured, partly folded antigen expression in E.coli. Therefore the S gene could have been present but not detected by the monoclonal antibody. The same applies for sequences of the S gene that may have been interrupted, partially deleted or

molecules or molecules annealed intermolecularly at their cohesive ends. The titre of the Tong/HCC gene library (1 x 10^7 pfu/ml) and the packaging efficiency of the arms (1 x 10^7 pfu/µg) was lower than expected so the library was amplified which resulted in a titre of 6,8 x 10^7 pfu/ml.

One of the problems with amplifying a library is that different recombinant phages may grow at different rates resulting in unequal representation of the recombinants in the library. This can be avoided if the library is amplified in fractions as was performed in this study.

Several methods are available for detecting recombinants with HBV DNA sequences in the Tong/HCC genomic DNA library. The two methods used in this study, namely nucleic acid probes and monoclonal antibodies, can be used to screen mixtures of recombinants. Selection using only a single approach is rarely proof that a clone has been identified correctly, because even the most stringent criteria for screening may select false positives.

The nucleic acid probe used to screen the Tong/HCC genomic DNA library consisted of the entire HBV genome. It was used simply to determine the presence of recombinant phages containing integrated HBV DNA sequences and therefore not specific according to which HBV sequences it detected. The 15 positive signals detected in the first screen indicated that we had been successful in creating a Tong/HCC genomic DNA library containing a representative number of recombinants with integrated HBV DNA.

There are two important parameters to consider in ligating bacteriophage Lambda arms to segments of foreign DNA. Firstly the ratio of arms to potential inserts and secondly, the concentration of each DNA species in the reaction mixture. Optimum values for both of these parameters can be worked out from theoretical considerations. By necessity, however, such calculations assume that all the DNA molecules in the ligation reaction are perfectly suitable for ligation. Since this is rarely the case it is necessary to carry out trial ligations.

A molar ratio of Lambda gt11 arms to genomic DNA of 1:1.8 gave the band with the brightest intensity and was therefore determined to be the optimal ratio for all future ligation reactions. An excess of vector DNA would theoretically increase the yield of plaques per microgram of insert DNA, however an increase in background due to religated vector would be expected.

Three of the five ligation reactions using the conditions optimized previously were successful. Since one master reaction mixture was made up and subsequently divided up into 5 reaction tubes, it is not understood why two of the ligation reactions did not work. The addition of Lambda gt11 arms and T4 DNA ligase to the unsuccessful reaction mixtures with subsequent incubation resulted in successful ligation.

Packaging *in vitro* refers to the use of a phage - infected E.coli cell extract to supply mixture of viral proteins and precursors required for encapsidating Lambda DNA. Approximately 0.05 - 0.5% of the DNA molecules present in the reaction can be packaged into the infectious virions. The packaging efficiency is determined by titration. If a packaging efficiency of 1×10^6/µg Lambda DNA (containing 2×10^{10} Lambda molecules) is obtained, it means that 1 in 20 Lambda molecules gives rise to a plaque. These yields are, however, only achieved for long concatemeric

The Lambda gt11 vector accepts small DNA fragments of 0-7.2 kb. Since the molecular weight of HBV is approximately 3 200 bp we decided to partially digest the isolated Tong/HCC DNA until the majority of the fragments were in the size range of 5 - 7 kb. In this way we ensured the cloning of fragments of DNA containing the entire genome of HBV as well as those containing deletions and rearrangements of integrated viral sequences and cellular flanking sequences.

At first, the time-dependent optimization reaction samples were taken at 30 minutes, 2 hours, 5 hours, overnight and overnight plus 3 hours. Previous experience with high molecular weight mammalian genomic DNA as well as personal communications (H. Venter and C. St. John Ayre) had shown that the DNA normally has a digestion time period of 24 hours. When it was found that the DNA was digested within 30 minutes it was decided to take the optimization reaction samples at 5 minute intervals ranging from 0 to 25 minutes. This short digestion time indicates that the DNA preparation was of a very good quality, free from salts and other inhibitory substances. This can be attributed to the extensive dialysis against TE carried out on the DNA after isolation (see Materials and Methods). No other DNA degrading enzymes were present in the DNA preparation. This was ascertained by incubating a sample of the DNA at 37C for an hour. When run on a 0.8% agarose gel no digestion of the DNA had occurred.

A digestion period of 20 minutes yielded the maximum number of fragments in the required size range of 5 - 7 kb. This was determined by relating the intensity of flourescence to the mass distribution of the partially digested DNA when the reactions were analysed on a 0.8% agarose gel.

The modified method of Blin and Stafford (1976) is a standardized procedure designed to minimize the number of steps and manipulations. It consistently yielded good quality DNA of a high concentration which migrated slower than uncut Lambda DNA when run on a 0.2% agarose gel. It is suggested in the method that to isolate DNA with a molecular weight of 100 - 150 kb it is sufficient to perform an ethanol extraction in the presence of 10 M ammonium acetate after the third extraction with phenol. A large quantity of DNA was lost during this procedure and the genomic DNA obtained had a low molecular weight. The yield of the isolated DNA was improved when the aqueous phases, after the third extraction with phenol, were dialysed against TE buffer for 48 hours instead of being precipitated with ammonium acetate and ethanol.

The size of the DNA selected for cloning is 7 kb and the initial size of the isolated genomic DNA is greater than the size of uncut Lambda DNA which is 43 kb (Figure 3.1). Therefore the ratio of fragments suitable for cloning is greater than 0.875 (Sambrook et al., 1982).

The DNA fragments to be cloned should be randomly distributed on the DNA to ensure a library with as complete coverage as possible. The DNA to be cloned is therefore fragmented in a random fashion to obtain a representative library with the minimum number of clones. The most frequently used strategy is cutting with restriction enzymes. *Eco* RI, restriction enzyme recognising a 6 bp palindromic sequence, was used because the higher density of sites on the DNA made it less likely that there are long stretches completely without these sites. In addition, the enhancing or inhibiting effects of sequences surrounding a particular site are less important because of the vicinity of many other sites which will not be subject to the same steric or other considerations.

High-molecular-weight DNA isolated for the construction of genomic Lambda libraries should have a minimal size of 100 - 200 kb. The yield of partial digestion products that can be used for cloning is relatively poor unless the initial length of the DNA is at least fourfold greater than the length of the DNA used for cloning. The size of the DNA can be ascertained on 0.2% agarose gels run at low voltage gradient. It is easy to decide by this criterion whether the size of the DNA is greater than the required lower limit. For a better view of the size distribution of the DNA, pulsed-field gradient gel electrophoresis can be used (Shwartz and Cantor, 1984) but in practice DNA preparations that pass the standard gel test, i.e. do not migrate faster than uncut Lambda DNA, are satisfactory.

It is difficult to isolate DNA of this size by methods that involve extraction with organic solvents and mechanical shearing as a lot of DNA is lost during these procedures. For this reason a number of methods were attempted to ascertain which one yielded the largest amount of DNA with the highest molecular weight.

The genomic DNA isolation method of Matthew (1984) was performed. Although a high concentration of DNA was obtained, its molecular weight was equal to and lower than uncut Lambda DNA when compared on a 0.8% agarose gel. The reason for this shearing or degredation of the DNA is unknown but could possibly be attributed to an extensive phenol:chloroform extraction.

A simple salting out method for extracting genomic DNA developed by Miller et al (1988) was attempted. A high DNA concentration with a large molecular weight was obtained. The DNA preparation, however, was found to have too high a concentration of salts which interfered with restriction enzyme digestion and would have hindered the preparation of the library.

CHAPTER 4

DISCUSSION

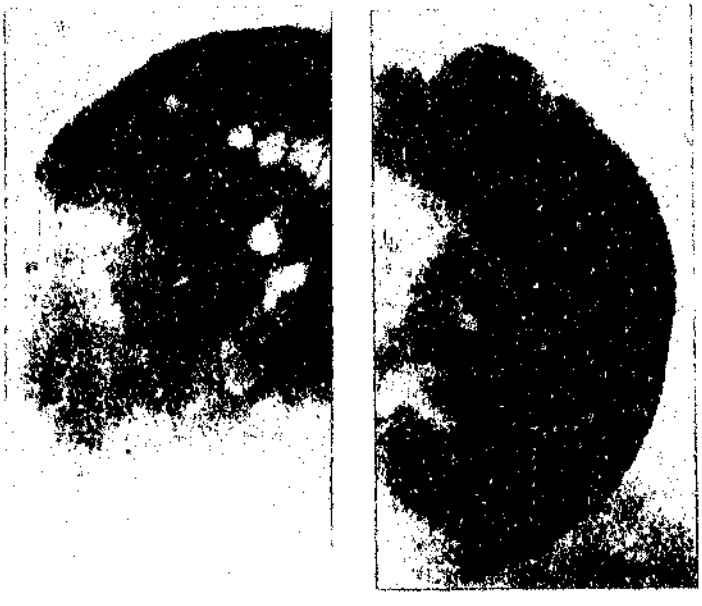

Plate A Plate B

Figure 3.11: Screening of plaques containing integrated HBV DNA from the Tong/HCC genomic library for the presence of fusion protein containing HBsAg sequences.

The outcome of the third screen was a higher percentage of positive plaques (Figures 3.10a, 3.10b and 3.10c). Even though 100% purity and homology had not been obtained it was decided to use these plates for the detection of fusion proteins produced by these phages.

3.2.3. Screening for HBV protein

The Amersham enhanced chemiluminescence (ECL) Western blotting system (RPN2108) and mouse anti-HBsAg monoclonal antibody were used for the detection of fusion proteins produced by the phages that had tested positive for integrated HBV DNA.

A few of the plaques tested positive for HBsAg while the majority of them tested negative (Figure 3.11) indicating that HBsAg sequences are not present in all of the recombinants or is present but not expressed in a form recognisable by the monoclonal antibody raised against the S antigen.

Plate G

Plate H

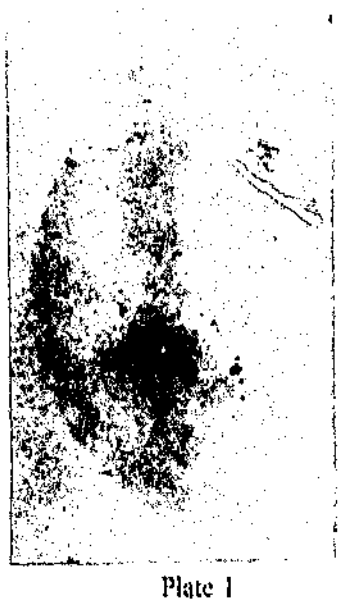

Plate I

Plate J

Figure 3.10c: Screen 3 of the Tong HCC genomic library using the 1850 and 1350 bp fragments of *Bam*HI restricted HBV in pBR322. Plates G, H, I and J.

Plate C Plate D

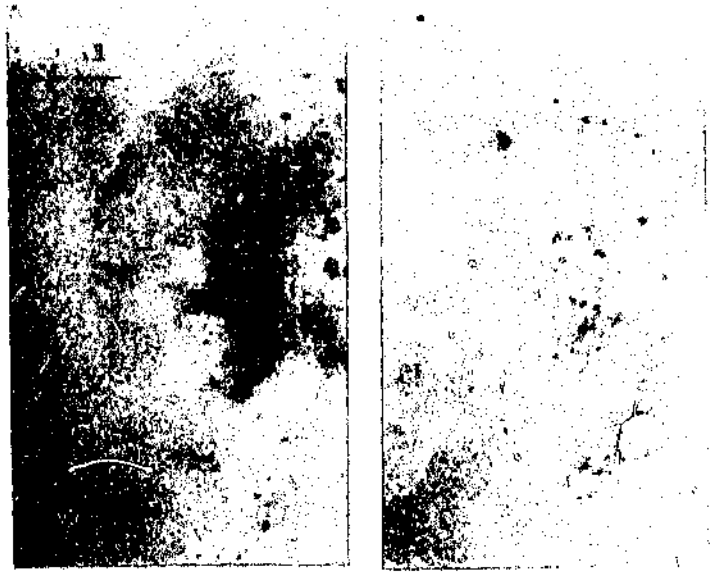

Plate E Plate F

Figure 3.10b: Screen 3 of the Tong/HCC genomic library using the 1850 and 1350 bp fragments of *Bam*H1 digested HBV in pBR322. Plates C, D, E and F.

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To conclude, a Tong/HCC cell line genomic DNA library with a titre of 6,8 x 10^7 pfu/ml was constructed. Screening of this library with HBV DNA revealed the presence of 15 recombinants with integrated HBV DNA sequences. These recombinants were isolated, purified and resereened. Further analysis with a monoclonal antibody raised against serum HBsAg indicated the presence of HBsAg sequences, completely or partially expressed as a fusion protein, in 5% of the positive recombinants. This work will serve as a basis for future studies were the five integrated HBV sequences and the flanking cellular DNA will be sequenced and analysed further.

junction in the X ORF could result in a fusion protein consisting of part of the HBxAg and part of a cellular protein with trans-activating activity (Twu and Schloemer, 1987). A X promoter is found in the upstream region of the X protein coding sequence and overlaps the enhancer element (Treinin and Laub, 1987).

A strand polarity of x → surface → core with the first virus - cell junction at DR1 would result in the second junction falling within nucleotides 1820 and 2850 (incorporating the C ORF and part of the P ORF). A virus - cell junction at DR2 and the same polarity would mean that the second junction lay between nucleotides 1600 and 2800 (C ORF and part of the P and X ORFs). In these regions a number of elements are found that, if positioned next to or upstream from cellular factors regulating cell growth, could alter the normal functioning of the cell. These include a second enhancer that maps to approximately nucleotides 1645 - 1803, a core promoter and part of the X transactivating gene.

Integration in the DR region and interruption of the expression of the S ORF would mean the HBV integrant would be less than genome length (3.2 kb). Matsubara and Tokino (1990) studied 40 clones of published HBV integrants and showed that at least one terminus of the HBV integrant to be located at the S ORF or between the DR regions. An integration point within this region could bring the pre S/S gene transactivator next to cellular DNA. There is a high correlation of HCC with the surface antigen carrier state (Beasley, 1988) as well as an observation that one third of HBV - associated HCC's harbor at least one transactivating preS2/S gene (Lauer et al., 1994). This supports the theory that integrated preS2/S genes might contribute to HBV - associated HCC.

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APPENDIX B

Titration of packaged phage in Tong/HCC gene library

To calculate the titer of the phage:

Formula: $\frac{\text{(number of plaques) x (dilution factor)}}{\text{(volume of extract plated)}} = \text{Pfu/ml}$

To calculate the packaging efficiency of the arms:

Formula: $\frac{\text{(pfu/ml)}}{\text{(concentration of vector DNA in packaging reaction)}} = \text{per microgram recombinants}$

SOLUTION NAME : TB Top Agar

CHEMICAL COMPOUND	CONCENTRATION
Tryptone	1 g/l
NaCl	0.5 g/l
Agar	0.8 g/l

Autoclave. When the solution has cooled add:

$MgSO_4$	1 ml of 1 M solution.

SOLUTION NAME : Tris-buffred Saline (TBS)

CHEMICAL COMPOUND	CONCENTRATION
NaCl	137 mM
Tris	20 mM

Adjusted to pH 7.4 with HCl.

SOLUTION NAME : Tris-buffered Saline-Tween (TBS-T)

CHEMICAL COMPOUND	CONCENTRATION
NaCl	137 mM
Tris	20 mM

Adjusted to pH 7.4 with HCl.

Tween-20	0.1%

SOLUTION NAME: Tris-EDTA

CHEMICAL COMPOUND	CONCENTRATION
Tris-HCl	10 mM (pH 7.5)
EDTA	1 mM

SOLUTION NAME : Phenol:chloroform:isoamyl alcohol

CHEMICAL COMPOUND

Phenol	500 g
Chloroform	500 ml
Isoamyl alcohol	25 ml
TE buffer (pH 8.0)	1 volume

1. Dissolve the phenol in the chloroform and add isoamylalcohol.
2. Add 1 volume TE buffer and stir to emulsify.
3. Let the phases separate.
4. Remove the top phase and repeat TE buffer addition.
5. Let the phases separate.
6. Remove all but 1 cm of the aqueous phase and store in a dark bottle at 4°C.

SOLUTION NAME : Proteinase K solution

Stored as a 20 mg/ml solution in TE buffer at -20°C.

SOLUTION NAME : 20x SSC

CHEMICAL COMPOUND	CONCENTRATION
NaCl	3 M
Sodium citrate	0.3 M

Adjust to pH 7.0 with NaOH or HCl and autoclave.

SOLUTION NAME : STE Buffer

CHEMICAL COMPOUND	CONCENTRATION
NaCl	0.1 M
Tris (pH 8.0)	10 mM
EDTA	1 mM

SOLUTION NAME : Luria-Bertaini (LB) Agar

CHEMICAL COMPOUND	CONCENTRATION
Tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	5 g/ l
Agar	15 g/l

Adjust to pH 7.5 with NaOH and autoclave.

SOLUTION NAME : Luria-Bertaini (LB) Broth

CHEMICAL COMPOUND	CONCENTRATION
Tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	5 g/l

Adjust to pH 7.5 with NaOH and autoclave.

SOLUTION NAME : Neutralizing Solution

CHEMICAL COMPOUND	CONCENTRATION
NaCl	1.5 M
Tris-HCl (pH 7.2)	0.5 M
EDTA	1 mM

SOLUTION NAME : Phage Buffer

CHEMICAL COMPOUND	CONCENTRATION
Tris-HCl (pH 7.4)	20 mM
NaCl	100 mM
$MgSO_4$	10 mM

APPENDIX A

SOLUTIONS USED

SOLUTION NAME : Antibody Wash Buffer

CHEMICAL COMPOUND	CONCENTRATION
Tris-HCl	100 mM
Nacl	150 mM

Adjust to pH 7.5.

SOLUTION NAME : Denaturing Solution

CHEMICAL COMPOUND	CONCENTRATION
NaCl	1.5 M
NaOH	0.5 M

SOLUTION NAME : Extraction Buffer

CHEMICAL COMPOUND	CONCENTRATION
Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	0.1 M
Pancreatic RNAse	20 µl/ml
SDS	0.5%

APPENDICES