

**ONCOGENE EXPRESSION IN HEPATOCELLULAR
CARCINOMA AND DIVIDING CELLS**

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ABSTRACT

An investigation has been made into aspects of the expression of oncogenes in normally dividing cells and in hepatocellular carcinoma (HCC). HCC occurs commonly in Southern Africa, and the aetiology of this tumour is associated with hepatitis B virus (HBV) infection.

c-erbA, *c-myc* and *c-fos* but not *c-Ha-ras* mRNA were elevated in tumours and adjacent hepatic tissue from the same patients when compared to normal liver. Amounts of Fos and Myc protein in the liver tumour specimens were also raised. There was some correlation between the patients' serum α -fetoprotein concentrations, histological features of tumour differentiation, *c-myc* and *c-fos* expression.

Expression of *c-fos* and *c-myc* has been reported to be elevated after stimulation of cells to divide, as occurs during liver regeneration. This was corroborated by the finding that *c-myc*, *c-fos* and *c-jun* mRNA concentrations were increased in cultured 3T6 mouse fibroblasts following treatment with alkaline medium as a mitogenic stimulus. The time course of the expression of these oncogenes was similar to that reported after growth factor stimulation.

The HBV X-gene may be responsible for increased oncogene expression in HCC as a result of its documented *trans* activating properties. This viral gene is unusual in that it has a codon preference which is similar to that of

eukaryotic cell genes. Also HBV may have evolved from a similar ancestral virus to that giving rise to retroviruses. These ideas suggest that the HBV X-gene is a viral oncogene derived from a host homologue.

Low stringency Northern blot hybridisation using a X-gene probe demonstrated a murine transcript in heart and thymus. Attempts to isolate the sequence from mouse heart and thymus cDNA libraries were unsuccessful despite extensive screening with sensitive probes (SP6 polymerase and PCR labelled X-gene fragments). Conserved X-gene sequences were also used for the design of primers in a PCR based method aimed at isolating a mammalian sequence. No significant sequence homology was found between the HBV X-gene and DNA amplified from genomic and cDNA library template sources. The PCR products appeared to have been artefacts of amplification. Failure to detect the homologous gene may have resulted from poor complementarity between the viral and mammalian sequences.

Non-specific amplification is commonly encountered when using PCR. A quick asymmetric re-amplification method based on extension of an internally hybridising X-gene primer was devised to confirm PCR products. The method was specific in that even single base mismatches between the internal primer and template resulted in failure of detectable primer extension.

DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination.

Patrick B. Arbuthnot

Patrick B. Arbuthnot

27th day of October, 1992.

To Pamela

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ABBREVIATIONS

bp base pair

BSA. Bovine Serum Albumin

c-onc. cellular oncogene

EDTA Ethylenediamine Tetraacetic Acid

FCS. Fetal Calf Serum

HBV. Hepatitis B virus

HCC. Hepatocellular Carcinoma

HGF. Hepatocyte Growth Factor

IPTG Isopropyl-β-D-thiogalactopyranoside

kb kilobase

kDa. kilodaltons

MEM. Minimum Essential Medium

ORF. Open Reading Frame

PAGE Polyacrylamide Gel Electrophoresis

PBS. Phosphate Buffered Saline

PCR. Polymerase Chain Reaction

PDGF Platelet derived growth factor

PEG. Polyethylene Glycol

pfu. plaque forming unit

PHA. Phytohaernaglutinin
PMSF Phenyl Methyl Sulphonyl Fluoride
PPP. Platelet Poor Plasma
PVP. Polyvinylpyrrolidone
SDS. Sodium Dodecyl Sulphate
SS-Phenol. . . Salt Saturated Phenol
TCA. Trichloroacetic Acid
TEMED. N,N,N',N'-tetramethylethylenediamine
v-onc. viral oncogene
X-gal. 5-bromo-4-chloro-3-indolyl- β -D-galactoside

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Chapter 1

INTRODUCTION

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1. INTRODUCTION

1.1 HISTORICAL PERSPECTIVE

It has long been known that there is a genetic basis to the cause of cancer. Indeed, it was as early as 1820 that a report appeared in the Edinburgh Medical and Surgical Journal by William Norris which suggested that malignant melanomas occurred more commonly in a particular family (Bishop 1987).

Subsequently, there have been other lines of evidence supporting a genetic basis for the development of cancer:

- 1) generations of cancer cells of a particular malignancy display a similar abnormal phenotype - implying that the characteristics of cancerous cells are passed on from parent to progeny cells;
- 2) many cancers display abnormalities of chromosomal structure; and
- 3) the chemicals which have damaging effects on DNA may also cause cancer.

A large number of vertebrate genes has been shown to have the potential to induce malignant transformation. There are two important classes of

these genes. They are termed oncogenes and tumour suppressor genes (anti-oncogenes). Mutations within these genes are responsible for changes in the control of normal cell division and differentiation which lead to malignant transformation (Bishop, 1991).

1.2 RETROVIRUSES AND ONCOGENES

Perhaps the most fundamental insight into the role of oncogenes in the development of cancer came from studying the transforming type C retroviruses. It was in fact through analysis of these RNA viruses that oncogenes were discovered (Bishop, 1983).

There are essentially two types of retrovirus, viz. acute and slow transforming retroviruses. The acute transforming retroviruses differ from the slow transforming viruses in that members of this type of retrovirus possess so-called viral oncogenes (*v-oncs*). These oncogenes confer the ability to induce malignancy efficiently and with a short latent period. The well described Rous sarcoma virus belongs to this group (Stehelin *et al*, 1976; Bishop, 1983).

There is a number of aspects of retroviral replication which is important in

understanding their acquisition of oncogenes (Varmus, 1982; Bishop, 1983). Shortly after infection of cells the RNA retroviral genome is copied into a linear double stranded DNA molecule through the action of retroviral reverse transcriptase. Synthesis of additional sequences at either end of this DNA, so-called long terminal repeats (LTRs), takes place during this step. The LTRs are derived from a combination of sequences present at the 3' end (U3) the 5' end (U5) or both ends (R) of the retroviral RNA genome and have the structure U3-R-U5. Integration of retroviral DNA into the host genome is a regular process of the retroviral life cycle and is regulated by these LTRs. They are attached directly to the cellular genomic DNA to form a stable provirus structure. The sites at which retroviruses integrate into the host cell DNA appear to be random but transcriptionally active regions may be preferred (Scherdin *et al*, 1990).

Apart from the LTR sequences, there are three important genes within the retroviral genome (Varmus, 1982; Bishop, 1983). They are the: 1) *gag* gene which codes for a protein which is responsible for the packaging of the retroviral RNA in the virus particle; 2) *pol* gene which encodes a polymerase enzyme which has properties of reverse transcription and 3) the *env* gene which encodes the envelope glycoprotein found on the surface of the retrovirus particle.

After integration of the provirus DNA into the host, the steps of viral replication are carried out by the host cell enzymes. Viral RNA is synthesised by cellular RNA polymerase II, and transcripts may serve either as mRNA for viral proteins or as new genomes (Varmus, 1982; Bishop, 1983). Examples of the structure of integrated provirus DNA of acute and slow transforming retroviruses are given in Figure 1.1.

One of the most important concepts concerning oncogenes emerged from the demonstration that viral oncogenes are derived from normal cellular genes (*c-oncs*) which are highly conserved in evolution (Spector *et al*, 1978; Shibuya *et al*, 1980; Shilo and Weinberg, 1981; de Feo-Jones *et al*, 1983). They are acquired by the viruses through a process of anomalous recombination during integration of the provirus into the host genome. This was first demonstrated in a study on the *v-src* oncogene by Bishop and his coworkers (Stehelin *et al*, 1976; Bishop, 1983). Oncogene incorporation into retroviruses usually results in structural differences and altered rates of transcription to account for the transforming properties (Goldfarb and Weinberg, 1981; Swanstrom *et al*, 1983; Takeya and Hanafusa, 1983).

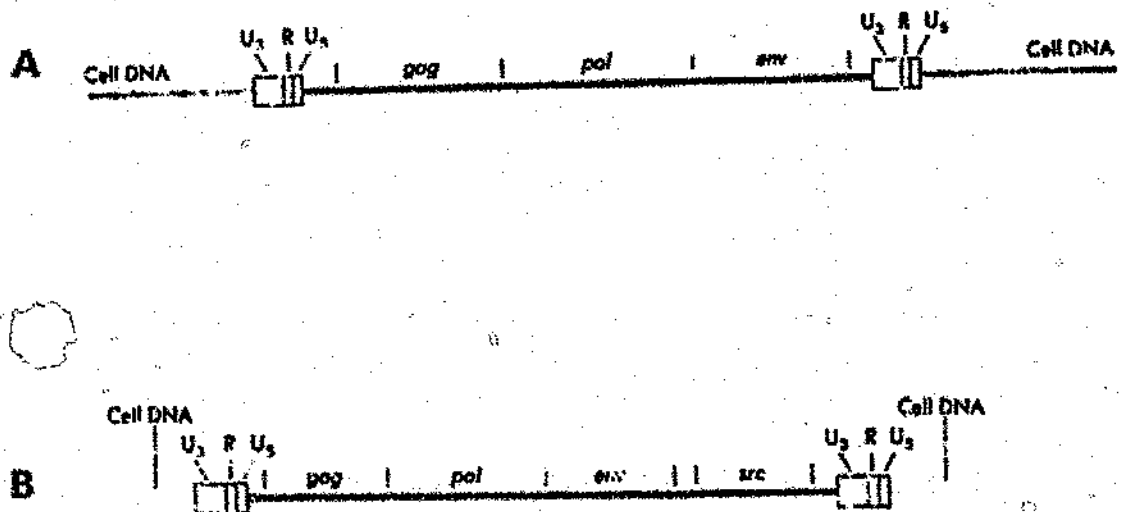


Figure 1.1 Schematic representation of integrated provirus DNA of a slow transforming retrovirus which does not contain an oncogene (A). Integrated provirus DNA of the Rous sarcoma acute transforming retrovirus containing the *src* oncogene is depicted in B. Transcription of the integrated proviral DNA by host cellular RNA polymerase II yields RNA that serves as template for the synthesis of *gag*, *pol*, *env* and *src* retroviral gene products as well as progeny genomes. Adapted from Watson *et al* (1987).

The transforming potential of cellular DNA was confirmed by an important study which demonstrated that transfection of cultured cells with normal DNA fragments is capable of causing transformation (Cooper *et al*, 1980).

Comparison of the sequences of normal cellular oncogenes with those of their mutated derivatives has revealed insights into the mechanisms of activation of oncogenes. Activation may essentially occur through changes in the coding sequences of the gene so that a mutated protein is produced, or else, it may occur as a result of a mutation or amplification that affects the rate and/or timing of transcription of a structurally unaltered oncogene.

Since their initial discovery, the number of known cellular oncogenes has increased dramatically. Table 1.1 shows a classification of these genes according to their cellular locations and known functions (adapted from Morris (1992) and Sleeman (1992)). Cellular oncogenes have been found to control many normal cellular functions which are usually concerned with proliferation and differentiation. These functions include actions as growth factors, protein kinases and nuclear activators of transcription.

Examples				
	Class	Acro- nym	Function	Location
1	Growth factor-like	sis	PDGF	Extracellular
2	Receptor-like protein tyrosine kinase	kit	PDGF receptor-like tyr. kinase	Transmembrane
3	Protein tyrosine kinases not obviously receptors	src	tyr. kinase	Inner cytoplasmic membrane
4	Oncoproteins which have GTPase activity	ras	GTPase	Inner cytomembr.
5	G-proteins	gap	G-protein	Inner cytomembr.
6	Protein serine/threonine kinases	mos	ser/thr kinase	Cytoplasmic
7	Nuclear hormone receptors	erbA	Thyroid hormone receptor	Nucleus
8	Transcription factors	jun	Part of σ complex	Nucleus

Table 1.1: Summarised classification of cellular oncogenes according to the functions and cellular locations of the proteins they encode. Adapted from the reviews by Morris (1992) and Sleeman (1992).

1.3 PROTOONCOGENE EXPRESSION DURING CELL DIVISION

1.3.1 THE CELL CYCLE

The cell cycle can be defined as 'the period between the formation of a daughter cell, by the division of a mother cell, and the subsequent time at which the cell divides to form two more daughter cells' (Mitchison, 1971). This period was originally divided into two phases: interphase and mitosis. Pioneering work done in the 1950s (Howard and Pelc, 1951; 1953) demonstrated that DNA synthesis occurs during the interphase. These workers divided the cell cycle into four stages: 1) G₁ phase (gap 1) which is the interval between mitosis and the onset of DNA synthesis; 2) S phase during which time DNA replication takes place; 3) G₂ phase (gap 2) is the interval between the end of S phase and the commencement of 4) M phase (mitosis) when the chromosomes condense, and cytokinesis is completed.

The phases of the cell cycle have been further characterised by the identification of the so called G₀ phase and 'TD state. Limiting external growth factors and nutrients, or by growth of cells to high density, can influence cells to enter a quiescent (G₀) or terminally differentiated (TD) state (Carpenter and Cohen, 1979; Stiles *et al*, 1979; Barnes and Seto,

1980; Rozengurt, 1983). The term G_0 phase was originally coined by Lajtha (1963) to designate the state of quiescence of cells which they could leave for the G_1 phase upon commencement of division. Subsequent to this original postulate, a considerable body of evidence has implicated G_0 as being a distinct phase of the cell cycle (Torado and Green 1963, Torado *et al.*, 1965; Holley and Kiernan, 1968; Brooks, 1969; Ley and Tobey, 1970; Temin, 1971; Rozengurt, 1986). Stimulation of quiescent cells in the G_0 phase, with for example a growth factor, causes them to progress towards the S phase of the cell cycle and become committed to division (Pardee *et al.*, 1978; Wharton *et al.*, 1982; Baserga, 1985; Prescott, 1987). Transformed cells frequently do not enter a phase of growth arrest (G_0) even when they are deprived of nutrients (Burstin *et al.*, 1974; Cholon and Studzinski, 1974; Pardee and James, 1975, Bartholomew *et al.*, 1976).

The phases of the cell cycle which have been described are depicted diagrammatically in Figure 1.2.

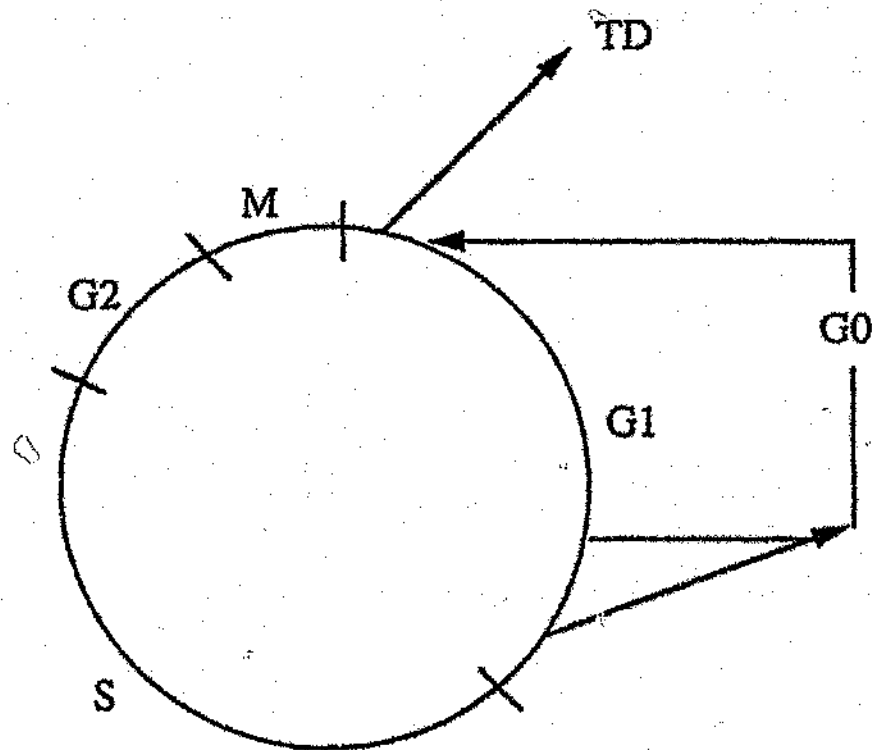


Figure 1.2 Diagrammatic representation of the phases of the mammalian cell cycle (explained in the text). Cells may exit the cell division cycle and enter G_0 , which is a state of quiescence. These quiescent cells may then re-enter the cell cycle at G_1 when active division again commences. Some cells exit the cell cycle after M and terminally differentiate (TD). These cells do not enter the cell cycle again (from Soprano and Cosenza, 1992).

After mitogenic stimulation of a cell, there is a point during the G_1 phase at which it may either commit itself to DNA synthesis and carry on to divide or else it may enter a state of quiescence. This is the so-called restriction or R point of the cell cycle (Pardee, 1974).

In a study on BALB/c3T3 mouse fibroblasts, two additional control points for G_0 to G_1 transition were identified (Pledger *et al.*, 1977; 1978). The experiments were based on a kinetic analysis of the mitogenic effects of the two major components of serum, platelet derived growth factor (PDGF) and platelet poor plasma (PPP). It was shown that PDGF treatment rendered cells competent to respond to further growth factors contained in PPP. Other experiments which entailed defining the timing of the growth factor requirements by cells for transition from G_0 to G_1 , enabled identification of the so-called V and W points. The V point is midway between the G_0 to G_1 transition and requires PPP for the cells to progress to the S phase (progression factors). The W point is similar to the previously described R point, and at this stage, cells are committed to enter the S phase. PPP and PDGF thus appear to be responsible for different activities in the activation of cells to divide. Treatment of BALB/c 3T3 mouse fibroblasts with PDGF results in their attaining a competent state after which they are able to respond to progression factors in PPP and

progress to the S phase.

The normal control of cell division is a complex and diverse process and there are numerous possible inputs for regulation. The role of oncogenes in the cell cycle is discussed here. Other regulatory elements, particularly the cyclins, have recently been reviewed (Hunter and Pines, 1991; Lew and Reed, 1992; O'Farrell, 1992; Soprano and Corenza 1992).

1.3.2 INDUCTION OF NUCLEAR PROTOONCOGENES FOLLOWING MITOGENIC TREATMENT

One of the earliest detectable events following growth factor stimulation of quiescent cells in the G₀ phase involves the induction of immediate early or competence genes (see 4.1.1). Protein synthesis inhibitors do not affect their expression (Lau and Nathans, 1987). They include the nuclear cellular protooncogenes *c-fos*, *c-myc* and *c-jun*.

The *c-myc* transcript is not easily detectable in quiescent cells, and the increase in its concentration is approximately 20 to 40 fold after growth factor stimulation of 3T3 fibroblasts (Denhardt *et al*, 1986). The *c-myc*

gene has been found to be maximally expressed approximately 1 to 2 hours after stimulation of quiescent 3T3 mouse fibroblasts to divide. The concentrations of both the *c-myc* messenger RNA and protein decrease rapidly, but may remain detectable up to 24 hours after stimulation (Morgan and Pledger, 1989; Persson *et al*, 1986). The increase in the transcript concentration is attributable to both an increased transcription rate as well as an increase in the rate of post transcriptional processing of *c-myc* mRNA (Morgan and Pledger, 1989; Persson *et al*, 1986).

The increased concentration of *c-myc* mRNA following mitogenic stimulation is superinduced by the presence of inhibitors of protein synthesis (for example cycloheximide) or a deficiency of amino acids in the medium. The exact mechanism for this is not clear. Possibilities include the inhibition of transcription shutdown and the stabilisation of transcripts (Almendral *et al*, 1988; Olashaw *et al*, 1992).

c-fos mRNA concentration is increased after a mitogenic stimulation of 3T3 mouse fibroblasts prior to the increase in the concentration of *c-myc* mRNA and protein. The peak concentration of *c-fos* mRNA occurs 15 to 30 minutes after stimulation, and the protein concentration reaches a maximum 30 to 60 minutes after treatment with a mitogen. An increased

rate of transcription accounts for most of the increased expression of this gene (Kruller *et al*, 1984). Following maximal expression, the transcript is rapidly degraded, and not usually detectable at 2 hours after stimulation. As with the transcript of *c-myc*, *c-fos* mRNA is superinduced by the presence of cycloheximide in the culture medium (Denhardt *et al*, 1986; Almendral *et al*, 1988). The Fos protein also appears to be able to cause negative feedback on its own expression (Lucibello *et al*, 1989).

The serum responsive element (SRE) is a DNA enhancer sequence which is largely responsible for the regulation of the expression of *c-fos* as well as other immediate early cellular genes by growth factors. This topic has recently been reviewed by Treisman (1992). The functions of SRE sequences appear to be independent of their orientations and locations on genes relative to other promoters and the mRNA cap site. Activity of the SRE is rapidly stimulated following treatment by a wide variety of growth factors, and this is largely responsible for the increase in *c-fos* mRNA concentrations following mitogenic stimulation. The activation of the SRE appears to involve both protein kinase C dependent as well as protein kinase C independent mechanisms, and the activation is linked to signal pathways which involve *ras* (Sassone-Corsi *et al*, 1988).

The kinetics of the expression of the *c-jun* protooncogene family (*c-jun*, *Jun B* and *Jun D*) are similar to those of *c-fos* (Nakabeppu *et al*, 1988). The mRNA of these genes is rapidly induced by mitogens. The peak in *c-jun* messenger RNA concentrations is at 1 hour after stimulation, and there is barely detectable transcript at 3 hours. Also, the concentration of the mRNA is superinduced by the presence of protein synthesis inhibitors in the culture medium (Nakabeppu *et al*, 1988).

1.3.3 FUNCTIONS OF NUCLEAR PROTOONCOGENES DURING CELL DIVISION

Initial experiments to determine the functional role of *c-fos* in the proliferation of cells (Holt *et al*, 1986) were aimed at blocking of endogenous *c-fos* mRNA by the production of *c-fos* antisense transcripts. These antisense sequences produced inhibition of colony formation and proliferation of 3T3 mouse fibroblast cells in culture. In a similar experiment, it was shown (Nishikura and Murray, 1987) that *c-fos* antisense transcripts prevented G_0 to G_1 transition induced by PDGF, but had no effect on the exponential growth of 3T3 fibroblasts. The significance of these apparently conflicting data may be that there is an important role for *c-fos* during the G_0 to G_1 transition of cells, but in

continuously dividing cells, the expression of *c-fos* may need only be residual (Calabretta and Venturelli, 1992).

The protein product of the *c-myc* gene appears to act as a competence factor for BALB/c 3T3 fibroblasts (reviewed by Calabretta and Venturelli, 1992; Bishop, 1992), and the *c-myc* product may function as an intracellular mediator of the mitogenic response induced by PDGF (Armelin *et al*, 1984). BALB/c 3T3 fibroblasts transfected with an exogenous *c-myc* cDNA driven by the murine mammary tumor (MMTV) promoter could be induced to initiate proliferation in the absence of PDGF when the MMTV promoter was active. Subsequently, it was shown (Kaczmarek *et al*, 1985) that microinjection of *c-myc* protein into BALB/c 3T3 cells, cultured in medium that was depleted of PDGF, was capable of initiating DNA synthesis. Also, inhibition of *c-myc* expression in T lymphocytes by exposure to *c-myc* antisense oligodeoxynucleotides resulted in an inhibition of the transition of cells from G1 to S phases of the cell cycle (Helkkila *et al*, 1987).

Other nuclear protooncogenes which appear to be important during the cell division process are *c-myb* and *c-ets*. Expression of *c-myb* is a relatively late event during the cell cycle. Maximal expression of the gene occurs 36

to 40 hours after stimulation of T lymphocytes by phytohaemagglutinin (PHA). This coincides with the entry of these cells into the S-phase of the cell cycle (Torelli *et al.*, 1985; Pauza, 1987). Exposure of the cells to conditions of protein synthesis inhibition decreases the expression of *c-myb* (Reed *et al.*, 1986).

Interestingly, analysis of the expression of two members of the *c-ets* gene family, indicate that the expression of *c-ets-1* is inhibited by growth factor stimulation, but *c-ets-2* expression is induced by signals which cause mitosis in T lymphocytes (Bhat *et al.*, 1990).

1.3.4. DNA BINDING PROPERTIES OF NUCLEAR ONCOGENES

DNA binding by proteins encoded by the nuclear proto-oncogenes *c-myc*, *c-fos* and *c-jun* has been well documented (reviewed by Gutman and Waslyk, 1991; Calabretta and Venturelli, 1992; Curran *et al.*, 1992), and this property is likely to be important in regulating the rate of cell division. It is thought that by acting as transcriptional regulators, *c-myc*, *c-fos* and *c-jun* affect the expression of other genes which are also important in the the control of cell division.

The first reports implicating a link between oncogenes and transcription factors emerged from studies which showed functional and sequence similarity between the *v-jun* oncogene and the DNA binding domain of the yeast transcriptional regulator GCN4 (Vogt *et al.*, 1987; Struhl, 1987). The DNA recognition sequence of GCN4 is similar to the mammalian AP-1 site (Hill *et al.*, 1986; Hope and Struhl, 1987; Lee *et al.*, 1987a,b). This sequence is represented in genes which are responsive to phorbol esters, and has therefore been termed the TPA-responsive element (TRE) (Angel *et al.*, 1987; Lee *et al.*, 1987b). Later TRE was found to be present in genes which are also responsive to a variety of other agents (Curran and Frenza, 1988). Initial studies indicated that Jun was in fact the AP-1 binding

protein (Bohman *et al.*, 1987; Bos *et al.*, 1988). However, it has become evident that AP-1 binding activity comprises a complex mixture of proteins of which Jun is one (Franza *et al.*, 1988; Rauscher *et al.*, 1988). The other two members of the *jun* family, Jun B and Jun D, share sequence similarity with *c-jun* and also bind to the same DNA sequence of seven nucleotides (TGACTCA) (Bohmann *et al.*, 1987; Nakabeppu *et al.*, 1988).

A remarkable feature of the transcriptional activation by Fos and Jun proteins is that they are capable of forming dimers through the interaction of so-called 'leucine zippers' (Landschulz *et al.*, 1988; reviewed by Curran *et al.*, 1992). The leucine zipper region comprises a stretch of 35 amino acids containing 4 to 5 leucine residues separated from each other by 6 amino acids. The leucine residues are located on one side of an amphipathic α -helix region of the protein. These hydrophobic residues are capable of interdigitating with each other and thereby facilitate the formation of protein dimers (Landschulz *et al.*, 1988). These Fos and Jun protein complexes are highly stable (Rauscher *et al.*, 1988a, 1988b; Franza *et al.*, 1988; Lucibello *et al.*, 1988; Chiu *et al.*, 1988; Schönthal *et al.*, 1989; Sassone-Corsi *et al.*, 1988). The heterodimer of Fos and Jun proteins binds to the AP-1 binding site with high affinity, and is a strong transcriptional activator (Nakabeppu *et al.*, 1988; Halazonetis *et al.*, 1988; Kouzarides and

Ziff, 1988; Rauscher *et al*, 1988c). The affinity of the Fos-Jun heterodimer for the AP-1 site is approximately 30 fold higher than is the Jun homodimer. The Jun homodimer is also a less potent transcriptional activator than is the heterodimer (Abel and Maniatis, 1989; Vogt and Bos, 1989; Sellers and Struhl, 1989; Busch and Sassone-Corsi, 1990). The Fos protein is unable to form a homodimer and bind to the AP-1 site (Halazonetis *et al*, 1988).

Myc protein also has a leucine zipper region which is thought to facilitate dimerisation by interaction of the leucine side chains (Blackwell *et al*, 1990). It has however only recently been shown that the protein encoded by the *c-myc* oncogene has sequence specific DNA binding properties (Blackwell *et al*, 1990). The basic/helix-loop-helix/leucine zipper (B/HLH/LZ) motifs within the Myc protein structure are important for this function.

A Myc-associated 'X' factor protein (termed Max) which is thought to be important in the transcriptional activating properties of Myc has recently been isolated. Max appears to be encoded by a highly conserved gene which is expressed in many tissues of a wide variety of vertebrate species. Max interacts with Myc protein in a manner which requires the integrity of the *c-myc* B/HLH/LZ motif (Blackwell *et al*, 1990). *In vitro*, the complex

binds specifically to the hexanucleotide DNA sequence CACGTG (Blackwell *et al.*, 1990; Kerkhoff *et al.*, 1991; Prendergrast and Ziff, 1991). When the Myc:Max complex is formed, it is able to bind to the CACGTG sequence under conditions where neither Myc nor Max are able to bind DNA (Blackwood and Eisenman, 1991; Prendergrast *et al.*, 1991). These DNA binding properties of Myc and Max have recently been reviewed (Blackwood *et al.*, 1991; Pendergrast and Ziff, 1992).

The proteins encoded by the *c-ets1* and *c-ets2* genes have DNA binding activity which resides in the carboxyl terminal of the protein. They bind to a 14 base pair enhancer element of which the sequence ACTTCCT appears to be the essential part (Wasylyk *et al.*, 1990).

The Myb protein binds to a specific core sequence (pyAACG/TG) (Biedenkapp *et al.*, 1988). This protein is unusual in that its DNA binding area is located in the amino terminal portion of the protein (Klempnauer and Sippl, 1987). A possible link between the function of Myc and Myb proteins in cell division activation has recently been reviewed by Bishop (1992).

1.4 TUMOUR SUPPRESSOR GENES

Recent discoveries have confirmed earlier ideas that the transformed phenotype may develop as a result of mutations which occur in cellular genes which negatively regulate the rate of cell division. This negative control is presumably expressed as a signal to the cell to slow down its rate of division and to differentiate (Sager, 1989). These tumour suppressor genes were located at the sites of deletion of parts of chromosomes that have been observed in some rare heritable cancers.

The initially isolated gene of this class was the retinoblastoma (or *RB1*) gene (Friend *et al*, 1986; Dryja *et al*, 1986). Children with a mutation in a *RB1* allele have a 95% chance of developing a retinoblastoma and an increased risk of developing other tumours such as osteosarcomas and fibrosarcomas (Gallie *et al*, 1990). Recently, the products of a number of DNA viruses (for example simian virus 40 (SV40) and papillomaviruses) which are capable of inducing malignancies have been found to produce proteins which bind to the *RB1* gene product, and thereby cause its inactivation. The role of *RB1* gene expression in normal and malignant cell proliferation has recently been reviewed (Hamel *et al*, 1992; Hu *et al*, 1992).

Another tumour suppressor gene, *p53*, has recently been implicated in the cause of a number of different malignancies including hepatocellular carcinoma. Mutations of the *p53* gene are thought to be the commonest genetic abnormality in human malignancies (Levine *et al*, 1991; Quartin *et al*, 1992). The protein of *p53* was originally discovered in extracts from transformed cells with the antiserum from animals infected with SV40 (Linzer and Levine, 1979; Lane and Crawford, 1979). *p53* forms complex with the large T antigen of SV40 which the antiserum recognises. The binding of *p53* by the large T antigen is also important in the transforming properties of SV40.

In humans, the *p53* gene is located on the short arm of chromosome 17 and spans approximately 20 kb of DNA (Benchimol *et al*, 1985). The gene contains 11 exons, the first of which is not coding, and is approximately 10 kb away from the other coding sequences (Oren *et al*, 1983; Zakut-Houri *et al*, 1983). *p53* mRNA is approximately 2.5kb in length, and the protein coded by this transcript is 53 000 daltons (Harlow *et al*, 1985).

The normal role of the *p53* protein in the cell is largely unknown (Levine *et al*, 1991). It is found in the nucleus, and structurally, it comprises three domains - an alpha-helical stretch of 75 amino acids at the amino terminal

end, followed by a hydrophobic region (positions 75-150) and a carboxy terminal end which is an alpha-helical basic region (Pennica *et al.*, 1984). The areas of the protein that are mutated in malignancies are located at certain 'hot spots'. The highest frequency of mutations is found in codons specifying amino acid residues 175, 248 and 273 (Levine *et al.*, 1991). These areas are highly conserved amongst the *p53* genes of different species, suggesting that the regions are functionally very important (Soussi *et al.*, 1987; Levine *et al.*, 1991).

A number of hypotheses has been proposed to explain the mechanism by which *p53* regulates cell division and differentiation. Firstly, *p53* may regulate the assembly and function of the DNA replication-initiation complex which is required to be active prior to the entry of cells into the S-phase of the cell cycle (Braithwaite *et al.*, 1987; Gannon and Lane, 1987). Disruption of normal *p53* function may therefore result in uncontrolled cellular proliferation. Binding of *p53* to a cellular homologue of the SV40 large T antigen appears to be important in this process. Recently, a cellular protein was isolated which is normally closely associated to *p53* (Momand *et al.*, 1992; Oliner *et al.*, 1992). This protein, called MDM2, was purified because of its co-immunoprecipitation with *p53*. It appears to inactivate *p53* through the formation of a protein complex. That is by a similar

mechanism to the association of the SV40 large T antigen of simian virus 40 and p53. The report by Oliner *et al* (1992) also showed that the *MDM2* locus was amplified in 17 out of 47 human sarcomas. This is in keeping with the idea that MDM2 overproduction inactivates p53 by the formation of protein complexes.

A second mechanism of p53 action is that it may function as a transcriptional regulator. The basic C-terminal region has DNA binding properties and may be important in the functioning of the product of this gene as a transcriptional regulator (Kern *et al*, 1991). Interestingly, the mutations within the p53 gene do not appear to affect the binding to MDM2 protein. It is rather by affecting the sequence specific DNA binding of p53 that the tumour suppressor activity is inhibited (Lane, 1992).

A third model for tumour suppressor action of p53 has recently been suggested (Lane, 1992). Normal p53 may monitor the integrity of the genome by functioning as a so-called 'molecular policeman'. If there is DNA damage, then p53 accumulates and suppresses the rate of cell division allowing more time for DNA repair to take place. Cells in which p53 is mutated or in which it is bound to host (eg. MDM2) or viral (eg. SV40 large T antigen) proteins make it incapable of arresting cell growth. As a result,

genetic mutations accumulate at an increased rate and this has the effect of increasing the probability of malignant transformation.

The role of p53 in the process of malignant transformation is by no means completely understood. New insights into the functioning of this protein should provide important concepts for the understanding of the process of carcinogenesis. The p53 mutations found in HCC are discussed in more detail later.

1.5 HEPATOCELLULAR CARCINOMA IN HUMANS

1.5.1 INTRODUCTION

Annually, hepatocellular carcinoma (HCC) affects approximately 250 000 people worldwide. This malignancy occurs commonly in Africa south of the Sahara and in parts of the Far East (Kew, 1986). The highest incidence has been recorded in Southern Africa (Mozambique has an incidence of 103.8 per 100 000 per annum), Taiwan and South East China (Waterhouse *et al*, 1977).

The prognosis of people affected with this malignancy is generally grave, especially amongst African and Chinese patients. The clinical course in African patients is usually fulminant, and survival time may be as short as 11 weeks after the onset of symptoms (Kew and Geddes, 1982). In these patients, it is very rare that the tumours are resectable (1% of Southern African blacks, and 3% amongst Ugandans) and the tumour often does not respond to chemotherapy (Harrison *et al*, 1973; Kew, 1986). The clinical course of patients from lower risk groups is generally more benign. These tumours are often amenable to surgical resection (37% amongst Japanese patients) and may be responsive to chemotherapy (Foster and Burman, 1977).

Africans appear to be unique with respect to the age at which HCC develops. It has been reported that as many as 50% of Mozambican Shangaans with the tumour are less than 30 years of age (Prates, 1961). In other parts of the world, HCC usually develops amongst older patients with a mean age of 55-60 years (Higginson, 1963). The male to female preponderance of patients with HCC is particularly high in African and Oriental patients where the ratio is 4-8 to 1 (Higginson, 1963; Okuda, 1976).

Cirrhosis of the liver is frequently associated with HCC (Kew and Popper, 1984). The frequency of cirrhosis and HCC occurring together is approximately 60% in Southern African blacks (Kew and Geddes, 1982; Patterson *et al*, 1985), and the overall incidence of the association is approximately 80% irrespective of the patients' background (Higginson, 1963).

1.5.2 AETIOLOGY OF HEPATOCELLULAR CARCINOMA

Epidemiological studies of HCC have enabled the identification of a number of different agents which are thought to be important in the development of HCC. Some are listed in Table 1.2, which is a modification of the data presented by Kew (1986).

Serological evidence of infection with hepatitis B virus (HBV) is frequently associated with the development of HCC (Beasley *et al*, 1981). In sub-Saharan Africa and in the Far East, approximately 90% of patients who have HCC are also infected with HBV (Bréchet, 1987). The risk of developing HCC is approximately 100-fold higher in HBV carriers than in their age matched non-carrier counterparts (Beasley *et al*, 1981). In Western countries, alcoholism is frequently associated with the development of HCC, but environmental chemicals, with the possible exception of aflatoxin, are a rare cause of HCC (Popper, 1979).

Most HCC tumours begin as solitary nodules (Tang, 1985). They comprise parenchymal cells which may be well, moderately or poorly differentiated. The tumours generally do not contain Kupffer cells, and the malignant parenchymal cells are arranged in trabeculae two to eight cells thick which

are separated by sinusoids (Peters, 1976).

1.5.2.1 Hepatitis B Virus

The association between HBV infection and HCC is perhaps the strongest ever established between a viral infection and a human neoplasm (Carloni *et al*, 1988). This important link obviously suggests that the virus may be primarily involved in the development of HCC and at least promote the initiation of a premalignant hepatic state (eg. hepatitis or cirrhosis) (Zaman *et al*, 1985).

Hepatitis B virus is a member of the hepadnavirus group of hepatotropic viruses and only infects humans and certain other primates (Blum *et al*, 1989). The hepadnavirus family includes the hepatitis viruses of woodchucks (Summers *et al*, 1978), Pekin duck (Mason *et al*, 1980), heron (Sprengel *et al*, 1988), ground (Marion *et al*, 1980) and tree squirrels (Faltelson *et al*, 1986). These members of the hepadnavirus family have similar genome structures and mechanisms of their replication. They are also capable of inducing malignancies in these animals (reviewed by Rogler *et al*, 1987; Popper, 1988).

Frequent	Occasional
HBV Infection	Smoking
Chronic Aflatoxin ingestion	Haemochromatosis
Cirrhosis	Primary and secondary biliary cirrhosis
	Wilson's disease
	Chronic active hepatitis
	Long term use of oral contraceptive steroids or anabolic androgenic steroids
	Alpha ₁ antitrypsin deficiency

Table 1.2: Factors which are associated with the development of hepatocellular carcinoma (adapted from Kew, 1986).

1.5.2.1.1 Structure of the HBV Genome

The genetic organisation of HBV (Figure 1.3) and the processes involved in viral replication have been the subject of a number of reviews (Michel and Tiollais, 1987; Lieberman *et al*, 1987; Summers, 1987; Blum *et al*, 1989; Schaller and Fischer, 1991; Seeger *et al*, 1991; Ganem, 1991). The virus has a small, circular, partially double stranded DNA genome. The minus (-) or long (L) strand is of a fixed length of 3 200 bases. This strand has a circular structure, but is not covalently closed. The plus (+) or short (S) strand has a variable length, and is usually 50-100% of the length of the - strand. The circular structure of the genomic DNA is maintained by the base pairing between the 3' ends of the two strands. At both sides of this cohesive overlap, there is an 11 base direct repeat of sequence 5' TTCACCTCTGC 3'. These details of the arrangement of the HBV genome are depicted diagrammatically in Figure 1.3.

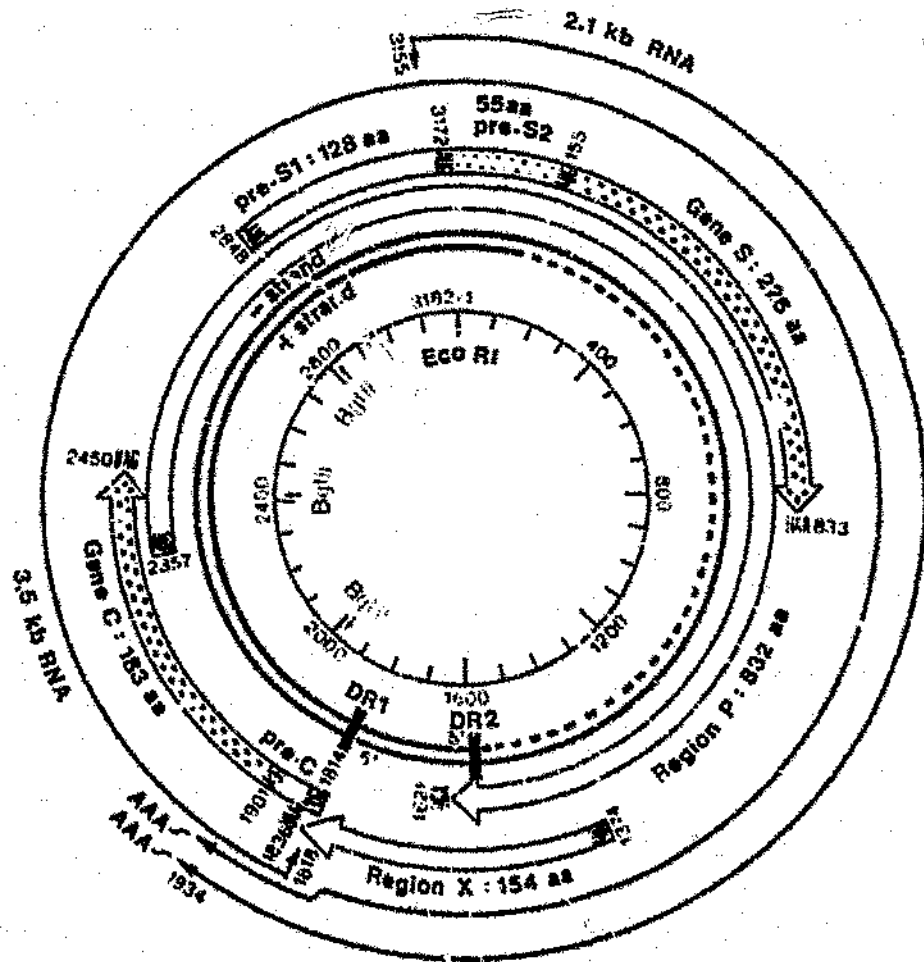


Figure 1.3 The structure and organisation of the HBV genome. The L (-) and S (+) DNA strands comprising the genome are indicated in the centre of the diagram, and the overlapping open reading frames are depicted by the labelled large arrows surrounding the circular DNA. The indicated nucleotide positions are relative to the *EcoRI* digestion site (position 1). The sequences of the L (-) strand to which the 2.1 kb and 3.5 kb RNA transcripts are complementary are shown by the positions of the arrows on the outside of the diagram. DR1 and DR2 are the direct repeats at positions 1824 and 1590 respectively. Adapted from Michel and Tellefsen (1987).

The - strand of HBV contains four open reading frames (ORFs) termed S, C, P and X, and these sequences are conserved amongst the different strains of hepadnaviruses. In summary, the functions of the proteins encoded by these ORFs (reviewed by Michel and Tiollais, 1987; Lieberman *et al*, 1987; Summers, 1988; Blum *et al*, 1989; Schaller and Fischer, 1991; Seeger *et al*, 1991; Ganem, 1991) can be summarised as follows:

1. The S region codes for the three HBsAg proteins of the viral envelope. This sequence is divided into the S gene, pre-S1 and pre-S2 regions. The three surface proteins are the major protein (coded for by S), the middle protein (coded for by the pre-S2/S regions) and the large protein (coded for by pre-S1/pre-S2/S sequences).
2. The C region comprising C and pre-C regions, codes for HBcAg (coded by the C sequence), and a larger HBcAg coded for by pre-C and C sequences. The HBcAg is required for the assembly of viral core particles.
3. The P region codes for DNA polymerase/reverse transcriptase and is important for the replication of the viral genome. This region also codes for a protein which is covalently attached to the 5' end of the DNA minus strand.
4. The region of the HBV genome denoted X encodes the HBxAg. This protein has *trans* activating properties (Twu and Schloemer, 1987;

Wollersheim *et al.*, 1988; Aufiero and Schneider 1990), protein kinase (Wu *et al.*, 1990) and serine protease inhibition properties (Koike, 1992).

There are two major transcripts of the hepatitis B virus. They are unspliced RNA molecules of plus strand polarity which are transcribed from the L (-) strand. The RNA molecules are transcribed from different initiation sites, but they have a common 3' terminus which is polyadenylated (Will *et al.*, 1987; Seeger *et al.*, 1987). These two transcripts were originally characterised in chimpanzee liver specimens which had been infected with HBV (Cataneo *et al.*, 1984). The RNA strands are referred to as 2.1 kb and 3.5 kb RNAs according to their sizes. The 5' end of the 2.1 kb RNA has been mapped to an area about 20 bp upstream of the pre-S2 region, and the 3' end to the beginning of the C gene. The 5' end of the 3.5 kb RNA has been mapped to the pre-C region and the 3' end of this transcript is at the same position as that of the 2.1 kb transcript (Michel and Tiollais, 1987; Lieberman *et al.*, 1987; Summers, 1988; Blum *et al.*, 1989; Schaller and Flecher, 1991; Seeger *et al.*, 1991; Ganem, 1991) (see Figure 1.3).

The 3.5 kb RNA molecule contains the full complement of genetic information of the HBV genome. In addition to the 2.1 kb subgenomic RNA,

two other species of subgenomic RNA have been characterised. They have lengths of 2.4 kb and 0.65 kb (Blum *et al.*, 1989).

The largest genomic RNA serves as template for HBV DNA synthesis and for the coding of proteins not encoded by the smaller subgenomic RNA. That is, the pre-C, C and P gene products (see Figure 1.3). The subgenomic RNA may serve as template for the synthesis of the other proteins.

Important biological characteristics of hepadnavirus infections are the species specificity and the hepatotropism (Blum *et al.*, 1989). The attachment of viral particles to liver cell membranes appears to be a receptor mediated event. A specific receptor on the hepatocyte recognises a peptide sequence which is encoded by the pre-S1 gene (Neurath *et al.*, 1986). After entry of the HBV DNA into the hepatocyte, it may follow one of three different pathways (Blum *et al.*, 1989; Seeger *et al.*, 1991).

1. It may persist in a latent state without replicating actively.
2. It may become integrated into the genome of the host. This aspect of the HBV life cycle appears to be particularly important in the disruption of host cellular genes, and may thus be central to the carcinogenic properties of HBV.
3. The virus may replicate actively.

HBV replication is a complicated process which initially involves the conversion of open circular viral DNA (ocDNA) to covalently closed circular DNA (cccDNA). The - strand of this cccDNA is then used as template for transcription of the 3.5 kb polyadenylated pregenomic RNA. This RNA pregenome is then used as template for the synthesis of the - DNA strand followed by the synthesis of the shorter + DNA strand from the - strand template. This process of HBV replication has been reviewed by Blum *et al* (1989) and Seeger *et al* (1991).

A remarkable feature of the genome of HBV is its compact organisation. It has the smallest genome of any virus known to infect man and uses its genetic materials extremely economically. This is achieved by two important and rare genetic arrangements (Miller *et al*, 1989):

1. The reading frames of the genes coding the HBV proteins are overlapping. This is possible through translation of the reading triplets specifying the amino acids in phase with the first second or third nucleotides of a given gene sequence.
2. All of the gene regulatory DNA elements of HBV reside within the protein coding sequences. That is, although the protein coding capacity of HBV is enhanced by overlapping reading frames, the binding sites for proteins involved in regulating gene expression or

genome replication also reside within open reading frames.

Hybridisation studies on the DNA of HCC specimens have shown that in most cases, HBV DNA is integrated into the genome of the tumours (Bréchet *et al*, 1981; Shafritz and Kew, 1981; Shafritz *et al*, 1981; Koshy *et al*, 1981). Southern blot analysis of the HCC DNA indicates that the sites of HBV integration vary in different tumours, and also, individual tumours often contain multiple viral DNA integrations. The sites of hepadnavirus DNA integration include an area adjacent to a sequence with homology to *erbA* and steroid receptor genes (Dejean *et al*, 1986), a cyclin A gene (Wang *et al*, 1990), and also the *c-myc*, *N-myc-1* and *N-myc-2* genes in woodchuck liver tumours (Moroy *et al*, 1986; Hsu *et al*, 1988; Fourel *et al*, 1990).

It has also been shown that HBV DNA may be duplicated and/or translocated to different cellular locations following the initial insertion (Koch *et al*, 1984; Ziemer *et al*, 1985; Hino *et al*, 1986). HBV DNA is also frequently rearranged as a result of deletions, inversions and/or duplications which occur during integration (Koch *et al*, 1984; Ziemer *et al*, 1985; Hino *et al*, 1986; Dejean *et al*, 1983; Koike *et al*, 1983; Koshy *et al*, 1983; Rogler *et al*, 1985). Chromosomal translocations have also been associated

with HBV integration in HCC (Hino *et al.*, 1986; Meyer *et al.*, 1992).

Another line of evidence implicating HBV and its products in the aetiology of hepatocellular carcinoma comes from the recent data on the product of the HBV X-gene (Kim *et al.*, 1991). Transgenic mice, from germ line cells in which the entire HBx gene under its own regulatory elements has been incorporated, were shown to develop progressive histopathological changes in their livers which ultimately led to hepatocellular carcinoma (Kim *et al.*, 1991).

The HBx protein is a protein kinase (Wu *et al.*, 1990) which has transcriptional activating properties (Wollersheim *et al.*, 1988; Auferio and Schneider, 1990; Twu and Schloemer, 1987). This protein may be linked to the development of HCC through the *trans* activation of viral and host cell gene expression. HBx has been shown to transactivate the protooncogenes *c-fos* and *c-myc* (Balsano *et al.*, 1991).

In addition to the HBx protein, it has also been reported that 3' truncated pre S2/S sequences in integrated HBV DNA encode a transcriptional *trans*-activating property (Kekulé *et al.*, 1991). The product of this sequence was shown to activate *c-myc* expression (Kekulé *et al.*, 1990).

Another important development in understanding the molecular pathology of HBV has been the demonstration of molecular variation in the HBV genome. Patients with chronic hepatitis have been found to be infected with viruses that have different sequences, and some of the regions of the HBV genome from these isolates are poorly conserved (Kaneko and Miller, 1989). Mutant viruses may be responsible for the different clinical courses that HBV infections often take. This may result from altered immunological pressures, caused by the mutations, being placed on the host (Brown *et al*, 1992). HBV mutations may also influence the efficiency with which its DNA is integrated into the host genome by cellular 'Integrase' enzymes. Such an enzyme is topoisomerase I (Wang and Rogler, 1991).

1.5.2.2 Hepatitis C and D Viruses

The hepatitis C virus, originally termed non-A, non-B hepatitis virus has recently been isolated and the genome characterised using a recombinant complementary DNA (cDNA) approach (Choo *et al*, 1989).

The genome of this virus comprises a single plus stranded RNA molecule of approximately 9 400 nucleotides (Kato *et al*, 1990; Houghton *et al*, 1991; Choo *et al*, 1991; Takamizawa *et al*, 1991). The sequence contains an open reading frame which almost spans the entire genome. This ORF may encode a viral protein of 3 011 (Choo *et al*, 1991) or 3 010 (Kato *et al*, 1990; Takamizawa *et al*, 1991) amino acids. A sequence of 324 to 341 nucleotides precedes the large open reading frame, and contains 3 or 4 very small ORFs which may code proteins of up to 28 amino acids in length (Houghton *et al*, 1991).

The genome of HCV does not demonstrate remarkable sequence homology to the other hepatitis viruses. There is however evidence which suggests that HCV is related to human flaviviruses (eg. yellow fever virus) and animal pestiviruses (eg. hog cholera virus) (Miller, 1991).

Numerous data implicate HCV as the major cause of post transfusion hepatitis and community acquired hepatitis around the world (Alter *et al*, 1989; Esteban *et al*, 1989; Kuo *et al*, 1989; van der Poel *et al*, 1989; Choo *et al*, 1990; Miyamura *et al*, 1990). It has also been established that the risk of developing HCC is significantly higher amongst patients who are seropositive for HCV infection (Vargas *et al*, 1989; Saito *et al*, 1990; Colombo *et al*, 1990; Kew *et al*, 1990).

Hepatitis D virus is also an important liver pathogen which has been associated with severe hepatitis and hepatocellular carcinoma. Since this virus requires HBV as a helper virus, it is difficult to distinguish the effects of HBV from those of HDV (Rezetto *et al*, 1980; Smedile *et al*, 1982; Negro *et al*, 1988).

1.5.2.3 Aflatoxin

Aflatoxin is probably one of the most potent hepatocarcinogens which is known to man (Linsell, 1984). It is a mycotoxin produced by the fungus, *Aspergillus flavus oryzae*, which contaminates cereals and other foods when stored in hot and humid conditions. Close correlations between HCC

and aflatoxin exposure have been demonstrated in many parts of the world (Linsell, 1984; Newborne, 1984; van Rensburg *et al.*, 1985).

Ingested aflatoxin is converted by oxidase enzymes, associated with cytochrome P450, to reactive metabolites such as the 2,3 epoxide. This compound is capable of producing chemical modifications of DNA which lead to base substitutions during replication and also breaks in the polynucleotide DNA backbone (Miller and Miller, 1986; Miller and Miller, 1981).

Interest in the role of aflatoxin in causing HCC has been renewed after the demonstration of point mutations occurring at a *p53* 'hot spot' in hepatocellular carcinoma specimens from China and Southern Africa (Hsu *et al.*, 1991; Bressac *et al.*, 1991). Of the hepatomas from the Chinese patients, all had transversion point mutations of G to C or T in the 249th codon (Hsu *et al.*, 1991). Of the 5 tumours derived from Southern African patients analysed by Bressac *et al.* (1991), four had G to T substitutions with three of them occurring at the 249th codon. The base changes described by these two groups are in keeping with the mutations which are induced by carcinogens such as aflatoxin (Harris, 1991).

In another study on p53 mutations in 28 cell lines and hepatocellular carcinoma specimens from Taiwan, a mutation in the p53 gene (homozygous deletion) was found in only one patient (Hosono *et al*, 1991). The reason for this inability to detect mutations may relate to the differential importance of HBV and aflatoxin in causing HCC in the different populations. HBV infection predominates in Taiwan, and aflatoxin may be more important in Southern Africa and China (Carbone, 1991).

The suggestion that a tumour suppressor gene may be involved in hepatocarcinogenesis also came from a different line of evidence (Buetow *et al*, 1989). In this study by Buetow *et al* (1989), screening DNA from HCC specimens against a panel of restriction fragment length polymorphisms (RFLPs), showed a loss of constitutional heterozygosity on chromosome 4. These results were thought to indicate that an anti-oncogene located on chromosome 4 may be partly responsible for causing HCC. This antioncogene is not the same as p53, as this gene is located on chromosome 17 (Benchimol *et al*, 1985).

The demonstration of p53 mutations in HCC and the strong evidence linking HBV infection to this malignancy suggests a complicated molecular basis for hepatocellular carcinogenesis. The relationship between the p53

mutations and HBV infection in HCC could be through the interaction of a virally encoded protein with a mutant p53 to cause abnormal cell division (Harris, 1991).

Chapter 2

MATERIALS AND METHODS

1.6 AIMS

A large body of evidence has recently accumulated which implies that the carcinogenic process involves multiple steps (Bishop, 1991). That is, rather than a single gene being affected, it is likely that loss of a tumour suppressor gene, together with the disruption of a cellular protooncogene may be involved in the progression of many cancers including HCC. This idea is in keeping with the original multistep and/or 'two hit' models (Armitage and Doll, 1954; Knudson, 1971).

In this study on the expression of oncogenes in normal cells and in hepatocellular carcinoma, the aims have been to determine whether:

1. there is evidence of abnormal expression of the oncogenes *c-myc*, *c-fos*, *c-Ha-ras* and *c-erbA* in HCC specimens of Southern African origin.
2. there are alterations in the rate of *c-myc*, *c-fos* and *c-jun* expression following the mitogenic stimulation of cultured cells with alkaline medium.
3. the X-gene of HBV is an oncogene derived from a mammalian host.

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2. MATERIALS AND METHODS

2.1 MATERIALS

The chemicals used were of analytical reagent grade and were obtained from recognised commercial vendors. All radioactive isotopes were purchased from Amersham. The sources of enzymes and other specialised materials are indicated in the relevant sections of the text. Sterile plasticware including tissue culture flasks was supplied by Nunc.

2.1.1 PREPARATION OF TREATED MATERIALS

2.1.1.1 Deionised Formamide

Formamide was deionised by treatment with Bio-Rad or Sigma Chemical Company mixed bed resin. To approximately 100ml of formamide, 2g of resin was added, and the mixture stirred for approximately 4 hours. Thereafter, the solution was filtered and stored at -20°C for up to 6 months before use.

2.1.1.2 SS-Phenol

Salt saturated phenol was prepared according to the procedure outlined by Davis *et al* (1986). To a 500g bottle of phenol, 100ml of 2M Tris, pH 7.4 and 130 ml of water were added, and the phenol carefully heated to 37°C with shaking until it dissolved. The upper aqueous phase was removed and 100ml of 2M Tris, pH7.4, 25 ml m-cresol and 500mg 8-hydroxyquinoline was added. The aqueous and phenol layers were stored together at room temperature. The yellow phenol solution (top layer) was used for nucleic acid extractions.

2.1.1.3 RNase-free Materials

Wherever possible, sterile disposable plasticware was used for experiments involving RNA. All solutions, except those containing Tris, were rendered RNase free by the addition of diethylpyrocarbonate to a concentration of 0.1% and autoclaving. Tris containing solutions were made up in sterile Baxter water and thereafter autoclaved. Pipette tips and glassware were treated by rinsing in chloroform or an aqueous solution of 0.1% diethylpyrocarbonate and then subjected to autoclaving.

2.1.1.4 Siliconising of Glassware

Corex tubes (Corning) and other glassware to be used for work with DNA and RNA were washed thoroughly and then immersed in a solution of 2% dimethyldichlorosilane in chloroform. The materials were then baked for 3 hours at 120°C and after cooling, the procedure repeated. After rinsing and autoclaving, they were ready for further use.

The procedure for siliconising of the glass plates used for sequencing gel electrophoresis was simpler, but the siliconised surface did not last as long. Each time the plates were assembled for electrophoresis, they were wiped with a 2% solution of dimethyldichlorosilane in chloroform and then rinsed with ethanol. It was found that siliconising of both plates resulted in easier handling of the sequencing gels after electrophoresis.

2.1.1.5 Preparation of G-50 Sephadex for Chromatography

Sephadex G-50 was placed in water and incubated overnight (at 5°C) to swell the beads. Thereafter, they were autoclaved to sterilise.

A column was usually prepared using a 2ml Pasteur pipette. The narrow

part of the pipette was plugged with siliconised glass wool before pouring in the Sephadex G-50. The column was washed by passing through several volumes of column wash buffer (10mM Tris, pH 7.4, 0.1mM EDTA, 0.05M NaCl and 0.1% SDS).

2.1.1.6 Preparation of DNase free RNase

A 10 mg/ml solution of RNase A (Boehringer Mannheim) was heated in a boiling water bath for 5 minutes in order to inactivate DNase. Thereafter, the RNase A was stored at -20°C before use.

2.1.1.7 Preparation of LB (Lauria-Bertani) Medium and Agar

10g of bacto-tryptone, 5g of bacto-yeast extract (Difco) and 10g of NaCl were dissolved in 950 ml of water, the pH adjusted to 7.4 and the volume of the solution was finally adjusted to 1 litre before autoclaving to sterilise. LB agar was prepared by making a 1.5% solution of agar in LB medium.

Bottom agar used for the culturing of bacteriophages was prepared by the addition of $MgSO_4$ to LB agar to a concentration of 10mM. Top agar was similar with the exception that the concentration of the agar was 0.8%.

Top and bottom agarose solutions were prepared by substituting agarose for agar at the same concentrations.

Ampicillin stock solutions (50 mg/ml) were sterilised by filtering and then added to the culture medium or agar (on cooling to $\pm 50^{\circ}\text{C}$) if required.

Liquid culture was carried out in glass Erlenmeyer flasks which had been sterilised by autoclaving. Agar plates were prepared in plastic Petri dishes which were supplied sterile (Sterilin, Hounslow, UK).

2.1.1.8 Preparation of Minimum Bacterial Growth Medium (M9-glucose)

The procedure used was essentially that described by Davis *et al* (1986). Initially, a phosphate buffered saline solution was made up as follows: 60g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 30g KH_2PO_4 , 5g NaCl and 10g NH_4Cl were dissolved in 900 ml of water and the pH adjusted to 7.4. 100 ml of a 20% solution (w/v) of glucose was added, 100 ml of this solution was made up to a litre with the addition of water, 1 ml of 1M MgSO_4 (final concentration 1 mM) and 0.1 ml of 1M CaCl_2 (final concentration 0.1 mM). All of the solutions except the glucose solution were autoclaved for sterilisation. The glucose solution was filter sterilised. Minimal agar plates were poured using 1.5%

agar made up in the above minimal medium solution.

2.1.1.9 Preparation of Tissue Culture Media

Eagle's Minimum Essential Medium (MEM) was prepared according to the manufacturer's instructions (Gibco). After appropriate adjustments to the pH, the solutions were sterilised by pressure filtration using Millipore 0.2 μ filters. Foetal calf serum (Gibco) was inactivated at 56°C for 30 minutes before use in media.

2.2 ONCOGENE ANALYSIS IN HEPATOCELLULAR CARCINOMA SPECIMENS

2.2.1 TISSUE SPECIMENS

Hepatocellular carcinoma and adjacent hepatic tissue specimens were removed from eight patients at surgery or soon after death and then immediately frozen in liquid nitrogen. Of the patients, all except one had serological evidence of infection with hepatitis B virus (HBV). Normal liver tissue was obtained at surgery from organ donors who did not have any evidence of liver disease. The malignant or benign nature of the tissue specimens was assessed histologically in the routine anatomical pathology laboratories of the South African Institute for Medical Research.

2.2.2 RNA ANALYSIS

2.2.2.1 Isolation

Total cellular RNA was isolated from the tissues according to the method of Chomczynski and Sacchi (1987). Approximately 50 mg of tissue was homogenised in 1 ml of solution D (4 M guanidinium thiocyanate, 25 mM

sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Sequentially, 0.1 ml of 2 M sodium acetate, pH 4.0, 1 ml of phenol (water saturated), and 0.2 ml of chloroform / isoamyl alcohol mixture (49:1) were added to the homogenate with thorough mixing of the reagents by shaking. The final suspension was incubated on ice for 15 minutes. After centrifugation at 10 000xg for 20 minutes at 4°C, the RNA was present in the upper phase, and the proteins and DNA were at the interphase and in the phenol phase. The aqueous phase was transferred to a new tube, mixed with 1 ml of isopropanol, and then placed at -20°C for at least an hour in order to precipitate the RNA. Sedimentation of the RNA was again by centrifugation (10 000xg at 4°C for 20 minutes), and the RNA pellet was suspended in 0.3 ml of solution D, transferred to a fresh tube, and the RNA reprecipitated with an equal volume of isopropanol. The final RNA pellet was suspended in 50 μ l of 0.5% SDS, and the concentration of this solution determined by absorbance at 260nm using the conversion factor of $1 \text{ OD}_{260\text{nm}} = 40 \mu\text{g RNA/ml}$. The RNA prepared according to this method was generally of high purity - the ratio of absorbance at 260nm to that at 280 nm was consistently ≥ 1.8 .

2.2.2.2 Preparation of Northern and slot blots

20 μ g of total cellular RNA was separated on 1.5% agarose gels (Davis *et al*, 1986) containing 0.66M formaldehyde, 1x MOPS buffer (0.02M MOPS, pH 7.0, 5 mM sodium acetate and 1mM EDTA) and in some cases ethidium bromide to a final concentration of 10 μ g/ml. After electrophoresis, the gels were rinsed for 20 minutes in two changes of 10x SSC buffer (1x SSC = 0.15M NaCl, 0.015M Sodium citrate, pH7.0) and then blotted onto nylon or nitrocellulose membrane (Hybond-NTM or Hybond.CTM, Amersham) using standard procedures (Davis *et al*, 1986). Cross-linking of the RNA onto the nylon membrane was by exposure to short wave UV irradiation for 5 minutes, and fixation to the nitrocellulose was by baking at 80°C for 1 hour.

The slot blot procedure was according to that of Davis *et al* (1986) using a Schleicher and Schuell SRC 072/0 Minifold II apparatus. Serial dilutions of total cellular RNA (5 -0.01 μ g) were applied to the slots, and then affixed to the membranes by UV irradiation. In each of the experiments, an excess of yeast transfer RNA (Sigma Chemical Company) was applied to one of the slots in order to assess non-specific binding of the probe.

2.2.3 DNA ANALYSIS

2.2.3.1 Isolation

Specimens were homogenised in buffer (0.3M sodium acetate, 20 mM Tris-HCl and 1 mM EDTA, pH 7.5), and the cells were lysed by the addition of SDS to 0.5%. The lysate was extracted with an equal volume of SS-phenol/chloroform/ isoamyl alcohol (50:50:1) and the phases were separated by centrifugation. The aqueous phase was removed and re-extracted with SS-phenol and then again with chloroform / isoamyl alcohol (24:1). The aqueous phase was removed and the DNA precipitated by the addition of two volumes of ethanol. After spooling, the DNA was dissolved in TE buffer (10mM Tris-HCl, pH7.6, 1mM EDTA). Recovery of DNA was assessed spectrophotometrically using the conversion factor of $1 \text{ OD}_{260\text{nm}} = 50 \text{ } \mu\text{g/ml}$. DNA prepared according to this method was generally of sufficient purity for Southern blot analysis - the ratio of absorbance at 260nm to that at 280 nm was consistently ≥ 1.6 .

2.2.3.2 Preparation of Southern Blots

15 μ g of DNA was digested with 60 units of restriction enzyme according to the manufacturer's instructions (Boehringer Mannheim) and electrophoresed in a horizontal 0.8% agarose gel containing 1x TAE buffer (0.04M Tris-HCl, pH 7.2, 0.02M sodium acetate and 1mM EDTA) and 10 μ g/ml of ethidium bromide. After electrophoresis, the gels were processed (Davis *et al*, 1986) by incubation twice for 30 minutes in an excess of solution A (1.5M NaCl and 0.5M NaOH) and then twice for 30 minutes in solution B (1M ammonium acetate, 0.02M NaOH). The gel was then blotted onto Hybond-NTM nylon membrane according to standard procedures (Davis *et al*, 1986).

2.2.4 HYBRIDISATION OF SOUTHERN AND NORTHERN BLOTS

The prehybridisation and hybridisation was done essentially according to Wahl *et al* (1979). Briefly, prehybridisation was carried out at 42°C for at least 4 hours in a solution comprising 25 mM KPO₄, 5x SSC, 5x Denhardt's solution (100x Denhardt's solution = 2% BSA, 2% PVP, 2% ficoll), salmon sperm DNA to a concentration of 50 μ g/ml and 50% deionised formamide.

Hybridisation was for 18 hours at 42°C in a solution similar to that of the prehybridisation with the addition of a heat denatured radiolabelled probe (prepared as described in Section 2.2.5). After hybridisation, the Southern blot filters were sequentially washed at 65°C for 30 minutes in the following solutions: 2x SSC; 2xSSC and 0.1% SDS; 0.1x SSC and 0.1% SDS. The Northern blot filters were washed sequentially for 30 minutes at 42 °C each in 5x SSPE; 1x SSPE and 0.1% SDS; 0.1x SSPE and 0.1%SDS (1x SSPE is 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH adjusted to 7.4 with NaOH). Autoradiography was at -70°C using 2 Chronex intensifying screens and Fuji RX medical X-ray film. A Beckman scanning densitometer (model CDS-200) was used to quantitate the intensity of the slots and bands. These scanning data were corrected using a ratio to the intensity of a methylene blue stained image of RNA on the blots or by using an β -actin probe for re-hybridisation to the blots (Davis *et al*, 1986; Sambrook *et al*, 1989). A 0.24 - 9.5kb molecular weight marker ladder (Bethesda Research Laboratories) was used to determine the sizes of the transcripts detected on the Northern blots. The sizes of the restriction fragments detected on the Southern blots was determined using *Hind*III digested phage λ molecular weight markers (Boehringer Mannheim).

2.2.5 PROBE PREPARATION

2.2.5.1 Transformation of Bacteria

All of the oncogene probes used (*c-fos* (van Straaten *et al*, 1983), *c-myc* (Dalla-Favera *et al*, 1982), *c-Ha-ras* (Chang *et al*, 1982) and *c-erbA* (Janssen *et al*, 1982)) had been cloned into derivatives of the plasmid pBR322, and they conferred ampicillin resistance when transfected into the HB101 *E coli* bacterial strain. The procedure used for the transformation was essentially according to that of Davis *et al* (1986) with some minor modifications.

Bacterial HB101 cells from a single colony of a plate culture were grown for several hours at 37°C in 40 ml of LB medium with vigorous shaking until the optical density at 600 nm was 0.3 to 0.5. The cells were then centrifuged (2 500 x g) at room temperature for 5 minutes and the pellet gently resuspended in 20 ml of 50 mM CaCl₂. These cells were then incubated on ice for 30 minutes, recentrifuged at 4°C and then the pellet resuspended in 4 ml of 50 mM CaCl₂. This final suspension of competent cells was incubated overnight at 4°C before use.

In a sterile tube, a solution containing about 0.1 μ g (usually 1-20 μ l) of recombinant plasmid DNA was mixed with 200 μ l of competent cells and incubated on ice for 30 minutes. Thereafter, the cells were 'heat shocked' for 2 minutes in a water bath at 42°C. 1 ml of LB medium containing 50 μ g of ampicillin was added to the tube, and the bacteria allowed to grow at 37°C for 45 minutes.

The transformed cells were then spread onto LB agar plates containing ampicillin (50 μ g/ml), inverted, and grown overnight at 37°C. Plasmid extracts (see below in Section 2.2.5.2) from the colonies were subjected to restriction enzyme mapping before further analysis.

2.2.5.2 Plasmid DNA Preparation

The procedure used was a modification of the technique described by Birnboim and Doly (1979). A bacterial colony selected from a plate of transformed HB101 *E. coli* bacteria was used to inoculate 200ml of LB containing 50 μ g/ml of ampicillin. The broth was cultured overnight at 37°C with vigorous shaking.

The cells were then pelleted by centrifugation at 2 000g for 10 minutes

and the pellet resuspended in 4.5 ml of glucose buffer (25 mM Tris, pH 8.0, 50 mM glucose and 10 mM EDTA). 1.5 ml of a solution containing lysozyme (Boehringer Mannheim) (8mg/ml in glucose buffer) was added and the solution incubated at room temperature for 5 minutes. 12 ml of 0.2 M NaOH with 1% SDS was then added and the solution placed on ice for 5 minutes. Thereafter, 9 ml of ice cold potassium acetate solution (29.4g potassium acetate (3M) and 11.5 ml glacial acetic acid/100 ml) was added to the tube, and it was then centrifuged (12 000g) for 15 minutes at 4°C. After the addition of an equal volume of isopropanol to the supernatant and 15 minutes incubation at -20°C, the DNA was pelleted by centrifugation (12 000g) for 20 minutes at 4°C.

The DNA pellet was resuspended in 0.5 ml of TE buffer (10 mM Tris HCl, pH 7.4, 100µM EDTA). 1/10 volume of 20 x SSC and 25µl DNase free RNase A (Boehringer Mannheim) (10mg/ml) was added to this and then incubated at 37°C for 30 minutes. 50µl of 3 M sodium acetate (pH 7.0) was then added and the solution extracted once with equal volumes of SS-phenol and chloroform and once with an equal volume of chloroform. The aqueous phase was removed, and the DNA recovered after the addition of an equal volume of isopropanol, incubation at -20°C for 20 minutes, and then centrifugation for 20 minutes at 12 000 g. The supernatant was

discarded, the pellet air dried, and the DNA suspended in 50 μ l of TE buffer. Inclusion of the RNase digestion step allowed a reasonably accurate measurement of the DNA concentration by absorbance at 260 nm. Previously, RNA contamination was very significant, and made convenient spectrophotometric assessment of the DNA concentration impossible. The plasmid DNA solution was stored at -20°C until further use.

2.2.5.3 Digestion and Isolation of Cloned Plasmid Inserts

Plasmid DNA was digested using the appropriate restriction endonuclease/s according to the manufacturer's (Boehringer Mannheim) instructions. Generally, 4U of restriction enzyme were used per μ g of plasmid DNA, and the incubations were carried out at 37°C for 2 hours.

Digested plasmid DNA was subjected to 0.8% agarose gel electrophoresis and the DNA bands visualised by staining with ethidium bromide (as described in section 2.2.3.2). The areas of the gel bearing the oncogene insert bands were excised for extraction using the glass milk adsorption technique (Vogelstein and Gillespie, 1979) (GeneClean II[®] Kit, Bio101). This procedure was found to be rapid, reliable and compared favourably with the conventional electroelution technique. Briefly, the procedure was as

follows: Three volumes of 6M NaI stock solution were added to the excised band and this mixture incubated at 55°C for 5 minutes. After this, the agarose had melted. Approximately 1 μ l of glassmilk® solution was added per μ g of DNA estimated to be in the solution and then incubated at room temperature for 5 minutes. The glassmilk®/DNA complex was pelleted by centrifugation for 5 seconds and washed three times with NEW WASH®. The DNA bound to the glassmilk® was eluted into TE buffer by incubation at 60°C for 3 minutes. This DNA was ready for use, and the yield was checked by electrophoresis of a small aliquot and comparison of band intensity with those standard molecular weight marker DNA bands of known amount.

An example of the method of calculation of insert concentration and yield is given by using the data from figure 3.6. The molecular weight marker used was a *Hind*III digest of the bacteriophage lambda. The total number of bases in the genome of this bacteriophage is approximately 48 500bp and the fragments detectable on the gel are 23 130bp, 9 418bp, 6 557bp, 4 361bp, 2 322bp, 2 027bp and 564bp. The smallest 125bp fragment (and sometimes the 564bp fragment) was generally not visible after electrophoresis. The amounts of DNA in each of the bands is therefore the

fraction of the total genome that the band represents multiplied by the total amount of DNA which was loaded onto the gel in the marker lane. This is based on the assumption that digestion is to completion and the restriction fragments which collectively represent the genome of the bacteriophage are present in equimolar amounts.

In the example depicted in figure 3.6, it can be seen that the intensity of the 2 322bp fragment of the marker lane 1 is approximately the same as that of the *c-myc* oncogene fragment (lane 2). The amount of DNA which is present in that band is 96ng. (2 322 divided by 48 500 and multiplied by 2 μ g - 2 μ g being the total amount of bacteriophage marker DNA loaded onto the gel.) A 2 μ l aliquot of the solution containing the *c-myc* fragment was electrophoresed and the concentration per μ l was therefore 48ng/ μ l.

2.2.5.4 Probe Labelling

The multiprime labelling method based on the protocol of Feinberg and Vogelstein (1984) was used for the labelling of the probes. The following components were added to a sterile microfuge tube at the commencement of the reaction:

Heat denatured DNA Template (25 ng)	$\pm 2 \mu\text{l}$
5x Multiprime labelling buffer (250 mM Tris-HCl, pH8.0, 25mM MgCl_2 , 10 mM DTT, 1mM HEPES, 26 A_{260} units/ml random hexadeoxynucleotides)	10 μl
BSA (5mg/ml)	4 μl
Water	27 μl
50 μCi [α - ^{32}P]dCTP (± 3000 Ci/mmol, Amersham)	5 μl
Klenow Enzyme	2 μl

The reaction was allowed to proceed at room temperature for 4 hours. Checking of the incorporation of radiolabel and calculation of the specific activity was according to the instructions of Amersham. Unincorporated ^{32}P -dCTP was removed by glassmilk adsorption (see section 2.2.5.3) or Sephadex G-50 chromatography (Sambrook *et al*, 1989).

2.2.6 WESTERN BLOT ANALYSIS OF ONCOPROTEINS

2.2.6.1 Protein Extraction

Approximately 50 mg of liver tissue was homogenised in a ml of TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet X, 2 mM phenylmethylsulphonyl fluoride (PMSF)). The cell debris was removed by brief centrifugation in a microfuge (10 000xg for 2 minutes). The protein in the supernatant was precipitated by the addition of 5 volumes of ice cold acetone and recovered by centrifugation for 5 minutes (10 000 g). The protein was resuspended in TBS and the concentration determined using the method of Lowry *et al* (1951).

2.2.6.2 Polyacrylamide Gel Electrophoresis of Proteins

100µg of protein was subjected to SDS urea polyacrylamide gel electrophoresis according to the method of Laemmli (1970). In all experiments, two similar gels were run - one was stained with Coomassie blue in order to confirm that equal amounts of protein were loaded on all of the lanes. The gels used for further analysis were electroblotted onto nitrocellulose membranes (Hybond NCTM, Amersham) and the blots probed

with polyclonal rabbit anti-human c-fos and c-myc antibodies (Medac, Hamburg). Detection was performed using ^{125}I -labelled protein A (Amersham) and autoradiography. These procedures were essentially according to the recommendations of Amersham.

The probing procedure was as follows: Initially, non-specific sites on the filters were blocked for 30-60 minutes with blocking solution (3% BSA, 0.1% Tween in TBS buffer) at room temperature. Probing with the primary antibody (diluted 1:250 in blocking solution) was done overnight at 8°C with gentle agitation. Thereafter, the blots were washed twice for 15 minutes in 0.1% Tween in TBS buffer. The bound primary antibodies were detected by incubation in TBS/Tween solution containing 0.3-0.5 $\mu\text{Ci}/\text{blot}$ ^{125}I -labelled protein A for 1 hour. Excess unbound protein A was removed by washing in two changes of ± 250 ml of TBS/Tween solution. The blots were then exposed for autoradiography. The autoradiographs were scanned densitometrically, and the intensities of the signals obtained compared to those in the normal tissue specimens (100%). Rainbow molecular weight markers (Amersham) were used to determine the sizes of the proteins detected by Western blotting.

2.3 CULTURE AND STIMULATION OF 3T6 MOUSE EMBRYO FIBROBLASTS

3T6 mouse embryo fibroblasts (Flow Laboratories) were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 1% fetal calf serum (FCS). For a period of 48 hours prior to experimentation, the FCS concentration was reduced to 0.5%. Subsequently, the confluent monolayers were stimulated by the addition of MEM at pH 9.5 for 5 minutes. Controls were performed in which the cells were subjected to a change of MEM containing 0.5% FCS. After stimulation, the cells were harvested by trypsinisation of the flasks at 2 hourly intervals from 0 to 14 hours.

Trypsinisation was performed as follows: The culture medium was removed from the flasks and the monolayer rinsed once with calcium and magnesium free phosphate buffered saline (PBS) (8g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4 made up to 1 litre with distilled water). Pre-warmed trypsin solution (Difco, 0.2% w/v in calcium and magnesium free PBS) was then added to cover the monolayer of cells, and the flasks were gently agitated to facilitate dissociation of the cells. The contents were then transferred to a sterile tube and the cells collected by centrifugation,

washed with calcium and magnesium free PBS, and stored at -70°C before RNA extraction. Only flasks where the cell viability was assessed as being greater than 85% by trypan blue exclusion (Babior and Cohen, 1981) were used for further RNA analysis.

2.3.1 RNA ANALYSIS

The pelleted cells were subjected to RNA extraction according to the method of Chomczynski and Sacchi (1987) (Section 2.2.2.1). The RNA was then processed by Northern and slot blotting as has been described in previous sections (2.2.2.2-2.2.5.4). The blots were probed with multiprime (Feinberg and Vogelstein, 1984) labelled *c-fos* (van Streaten *et al*, 1983), *c-myc* (Dalla Favera *et al*, 1982) and *c-jun* (Lamph *et al*, 1988) probes. After hybridisation and autoradiography, the signal detected by image analysis was expressed as a ratio to the amount of 18S ribosomal RNA detected on the nitrocellulose blots by staining with methylene blue (Herrin and Schmidt, 1988). The data were then expressed as the difference between the ratios detected after alkaline stimulation and those obtained after replacing with fresh medium in the control cells.

Methylene blue staining was found to work only on nitrocellulose filters and

not on nylon membranes. The staining procedure (Sambrook *et al.*, 1989) was briefly as follows: The filter was initially soaked in 5% acetic acid for 15 minutes at room temperature and then transferred to a solution of 0.5 M sodium acetate (pH 5.2) and 0.4% methylene blue for 10 minutes. Thereafter, the blots were briefly rinsed in water and the stained RNA visualised.

2.3.2 PULSE LABELLING OF RNA

In a second set of experiments, after alkaline stimulation, the cultured cells were pulsed with 10 μ Ci 6-[³H]-uridine (± 40 Ci/mmol) (New England Nuclear) per ml of medium for two hour periods from 0 to 14 hours. Controls were performed in which the cells were not stimulated but pulsed with radioactive uridine. The RNA was extracted as previously described (Chomczynski and Sacchi, 1987) and then subjected to urea denaturing polyacrylamide gel electrophoresis according to the method of Lichter *et al.* (1982). Samples of approximately 50 000 cpm were applied to each lane. Gels processed for fluorography were impregnated with sodium salicylate (Chamberlain, 1979), dried, and then exposed to Fuji RX medical X-ray film for up to 2 weeks at -70°C. Each of the fluorograph lanes was

scanned densitometrically in order to compare the newly synthesized RNA.

2.3.3 SILVER STAINING OF RNA SEPARATED ON DENATURING POLYACRYLAMIDE GELS

The denaturing 5% polyacrylamide gels were stained with silver according to the method of Biedler *et al* (1982).

2.4 ANALYSIS OF MAMMALIAN DNA AND RNA WITH HBV X-GENE PROBES

2.4.1 TISSUE PREPARATION

6 week old mice were sacrificed by cervical dislocation. The following tissues were removed by dissection for further analysis: brain, thymus, heart, liver, spleen and kidney. Hepatocellular carcinoma and adjacent normal hepatic tissue were resected from 3 patients at surgery and then immediately immersed in liquid nitrogen. All of these patients had serological evidence of infection with hepatitis B virus. DNA was also isolated from leucocytes obtained by venesection from uninfected human volunteers.

2.4.2 NORTHERN AND SOUTHERN BLOT ANALYSIS

DNA and RNA were isolated and subjected to Northern and Southern blot hybridisation as described in previous Sections (2.2.2-2.2.5) with the following modifications.

2.4.2.1 Isolation of poly A⁺ mRNA

Polyadenylated RNA species were selected by adsorption to oligo(dT) cellulose according to the method devised by Aviv and Leder (1972) and modified by Davis *et al* (1986).

2.4.2.2 Probe Labelling

Three methods for the labelling of X-gene probes were used:

2.4.2.2.1 Multiprime Labelling

This procedure has already been described in Section 2.2.5.4.

2.4.2.2.2 PCR Labelling

The primers used for the PCR labelling of DNA were synthesised using a Millipore oligonucleotide synthesiser according to the manufacturer's instructions. The synthesis was carried out as a service at the facility of the Biochemistry Department of the University of the Witwatersrand.

The oligonucleotides were 24 base sense (5' CCTTTGTTTACGTCCCGTCGGCGC 3') and antisense (5'

CTACAGCCTCCTAATACAAAGACC 3') strands at the 5' and 3' ends (nucleotides 1420 to 1443 and 1788 to 1765 respectively) of the hepatitis B virus X-gene. The oligonucleotide primers were used at a concentration of 0.25 μ M. 1.0ng of the HBV DNA containing the X-gene fragment provided by Dr W.S. Robinson (Wu *et al*, 1990) was used as template DNA. The PCR buffer contained 50 mM KCl; 10 mM Tris-HCl (pH 8.9) and 1.5 mM MgCl₂. The concentration of dGTP, dATP and dTTP was 50 μ M, while the final concentration of unlabelled dCTP was 10 μ M. 100 μ Cl of ³²P-dCTP ($\pm$ 3000 Ci/mmol, Amersham) was also added to the reaction. Each mixture had a final volume of 100 μ l. PCR was performed using 2.5 units of Taq polymerase (purchased from either Cetus or Promega) for 30 cycles - each cycle being 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. There was a final extension step of 10 minutes at 72°C.

Unincorporated ³²P-dCTP was removed by Sephadex G-50 chromatography (Sambrook *et al*, 1989), and the labelled DNA was denatured by heating in a boiling water bath for 5 minutes prior to use in hybridisation experiments.

2.4.2.2.3 SP6 Probe Labelling

The protocol used was a modification of the procedure devised by Melton *et al* (1984) for RNA synthesis *in vitro*. The standard transcription protocol involved adding the following components to a sterile tube at room temperature:

5x Transcription buffer (200mM Tris-HCl, pH7.5, 30 mM MgCl ₂ , 10 mM spermidine, 50 mM NaCl)	4 μ l
rRNasin® ribonuclease inhibitor (20u/ μ l)	1 μ l
ATP, GTP and UTP mix (2.5 mM each)	4 μ l
CTP 100 μ M	2.4 μ l
Linearized SP64 plasmid template DNA (1 μ g/ μ l in TE buffer)	1 μ l
[α - ³² P]CTP (50 μ Ci) ($\pm$ 3000 Ci/mmol, Amersham)	5 μ l
SP6 RNA polymerase (20 u/ μ l, Promega)	1 μ l
Nuclease free water	to 20 μ l

The reaction was allowed to continue for 60 minutes by incubation at 37°C. The labelled RNA was then purified as follows:

10 units of RNase free DNase I (10u/ μ l, Boehringer Mannheim) was added

to the reaction mixture and then incubated at 37°C for 20 minutes. Thereafter, 40 μ l of RNase free water was added to the mixture, and it was then extracted once with equal volumes of SS-phenol and chloroform and once with chloroform alone. 20 μ l of 5 M ammonium acetate was then added, and the RNA precipitated by the addition of 250 μ l ethanol with incubation at -70°C for 15 minutes. The RNA was recovered by centrifugation at 12 000g for 20 minutes at 4°C. After resuspension in 50 μ l of TE buffer, the percentage incorporation of [α -³²P]CTP was assessed by liquid scintillation counting. This method of probe labelling was highly efficient - percentage incorporations usually ranged from 60-80%.

2.4.2.2 Hybridisation and Post Hybridisation Washes of Blots probed with the X-gene

The conditions of hybridisation and washing were similar to those previously described in Section 2.2.4 with the following modifications to lower the stringency. Hybridisation was carried out in a solution containing 40% formamide (instead of 50%) and 6xSSC; all the other components were similar to those described in section 2.2.4. Washing of the blots after hybridisation was initially in 6x SSC at room temperature for 15 minutes. This was followed by two washes in 2x SSC, 0.1% SDS for 15 minutes.

each. The final wash was in 0.2x SSC, 0.1% SDS for 15 minutes at room temperature. After exposure for autoradiography, if the blots were found to have a high background, they were again washed in 0.2x SSC, 0.1% SDS for 15 to 30 minutes at 42°C. Under these conditions, it was found that stringency was the lowest possible without resulting in non-specific hybridisation - binding of the X gene probes to 18S and 28S ribosomal RNA (used as an indication of non-specific hybridisation) was not detectable.

2.4.3 SP64 HEPATITIS B VIRUS X-GENE CLONE CONSTRUCTION

2.4.3.1 PCR Amplification of the X-gene and Incorporation of Restriction Sites

In order to facilitate the cloning procedure, the HBV X-gene was amplified using PCR and primers which had restriction enzyme recognition sites (*Bam*HI and *Xba*I) at their 5' ends. The sequences of the primers were otherwise similar to those used for the PCR labelling protocol described in Section 2.4.2.1.2. The two primers were 30 nucleotide long sense (5' AGTC**GGATCCC**CTTTGTTTACGTCCCGTCGGCGC 3') and antisense (5' AGTC**TCTAGAG**GGTGCTGGTGCGCAGACCAATTTG 3') sequences located towards the 5' and 3' ends of the hepatitis B virus X-gene respectively (nucleotides 1420 to 1443 and 1816 to 1793). The *Bam*HI and *Xba*I recognition sites are indicated by bold print in the above sequences. In

addition, the primers had a 4 nucleotide 5' terminal redundancy (AGTC), which was required to facilitate later restriction endonuclease digestion. The oligonucleotides were each used at a concentration of 0.25 μ M. 1.0 ng of template DNA (Wu *et al.*, 1990) was used, and the PCR buffer contained 50 mM KCl; 10 mM Tris-HCl (pH8.8) and 1.5 mM $MgCl_2$. The deoxynucleotide triphosphate precursors were included at a concentration of 50 μ M. The volume of the reaction mixture was 100 μ l. PCR was performed using Taq polymerase (Promega) for 30 cycles - each cycle being 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. There was a final extension step at 72°C for 10 minutes. After the reaction was complete, the amplified DNA was purified using the glasspowder adsorption technique (Vogelstein and Gillespie, 1979) (described in Section 2.2.5.3).

2.4.3.2 Restriction Enzyme Digestion

SP64 plasmid DNA was purchased from Boehringer Mannheim. The purified amplified HBV X-gene fragment and the SP64 plasmid DNA were subjected to a double restriction enzyme digestion with *Bam*HI and *Xba*I according to the manufacturer's instructions (Boehringer Mannheim). The recommended concentrations of the buffer components for the use of these enzymes in combination is Tris acetate 33 mmol/l, pH7.9, Mg-acetate 10 mmol/l,

potassium acetate 66mmol/l and dithiothreitol 0.5 mmol/l. The restriction enzymes were used at a concentration of 4 units per μg of DNA digested, and the reactions were allowed to continue for 2 hours at 37°C. After digestion, the plasmid and X-gene DNA was again purified using glass powder adsorption (Vogelstein and Gillaspie, 1979).

2.4.3.3 Ligation of X-gene Fragment and SP64 Plasmid

The SP64 and HBV X-gene fragments were ligated by mixing the following components (Sambrook *et al*, 1989):

HBV X-gene fragment	2 μg
SP64 plasmid DNA	2 μg
10x ligase buffer (Tris-HCl 0.5M, pH 7.4, MgCl ₂ 0.1M, DTT 0.2M, ATP 10mM BSA 50 $\mu\text{g}/\text{ml}$)	2 μl
T4 DNA ligase (4 units, Boehringer)	2 μl
Water	to 20 μl

The reaction mixture was incubated overnight at 15°C.

2.4.3.4 Plasmid DNA Preparation

Transfection of HB101 bacteria was as previously described in section 2.2.5.1. Transformed bacteria were selected by their ability to grow on LB agar plates containing ampicillin. Plasmid DNA preparation was performed according to the method of Birnboim and Doly (1979) with the modifications described in section 2.2.5.2. The plasmid DNA was mapped using the restriction endonucleases *EcoRI*, *BamHI* and *XbaI*. Confirmation of the recombinant SP64 HBV X-gene clone was also by Southern blotting of the digested plasmid DNA. The procedure was similar to that described in Section 2.2.3-2.2.5.

2.4.3.5 Preparation of SP64 HBV X-gene recombinant DNA for Labelling with SP6 RNA Polymerase

The isolated plasmid was subjected to digestion with the restriction endonuclease *EcoRI*. This enzyme digests the recombinant plasmid immediately downstream of the *BamHI* and *XbaI* digestion sites of the polylinker where the X-gene fragment was inserted. A linearised DNA template for 'run off' transcription by SP6 polymerase to produce antisense RNA was thus conveniently created. The promoter of the enzyme was

upstream of the cloned fragment. The digested plasmid was then subjected to 0.8% agarose gel electrophoresis (Section 2.2.3.2), and the linear plasmid isolated from the agarose by glass powder adsorption (Vogelstein and Gillespie, 1979).

The yield of the purified plasmid was assessed after re-electrophoresis on agarose, and comparison of the ethidium bromide stained bands with those of markers of known concentration. The digested plasmid was then ready for production of labelled X-gene transcripts according to the method outlined in section 2.4.2.1.3.



2.5 APPROACHES TO THE ISOLATION OF A MAMMALIAN HOMOLOGUE OF THE X-GENE

2.5.1 cDNA LIBRARY SCREENING

2.5.1.1 Titring and Plating of Phage cDNA Libraries

The cDNA libraries used were purchased from Clontech, Palo Alto. Bacteriophage λ gt10 was the vector used, and the libraries were constructed with reverse transcribed mRNA isolated from mouse heart and thymus. The procedures used for the titring of the libraries was essentially according to standard procedures (Sambrook *et al*, 1989) with few minor modifications described below.

2.5.1.1.1 Preparation of Plating Cells

100 ml of sterile LB medium containing 1% maltose and 10 mM MgSO_4 was inoculated with a fresh colony of *E. coli* λ 77 bacteria (Clontech). The cells were grown at 37°C with vigorous shaking until the optical density at 600 nm was approximately 1.0. The cells were then collected by centrifugation, the supernatant was discarded, and the cell pellet was

resuspended in 50 ml of 10 mM MgSO_4 . The cells could then be stored at 4°C for up to 2 weeks prior to use.

2.5.1.1.2 cDNA Library Titering

A series of 5-10 ten-fold dilutions were made from 1 μl of the cDNA library stocks. The dilutions were made in TMG buffer (10 mM Tris, pH 7.4, 10 mM MgSO_4 , 0.001% gelatin) and were added to sterile tubes. 200 μl of plating cell solution (section 2.5.1.2) was then added to the bacteriophage dilutions, and incubated at 37°C for 15 minutes. 2.5 ml of melted top agar (cooled to 50°C) was then added to each of the tubes. Immediately thereafter, the solution was poured over bottom agar on a 90-mm culture plate. The bottom agar plates had been warmed to 37°C prior to use in order that the top agar would not solidify too rapidly when poured. The top agar was allowed to harden for 20 minutes at room temperature before the plates were inverted and incubated overnight at 37°C. The plaques were counted before they had grown to confluency and the titer in the original stock samples of the libraries could thus be calculated. The units of the titer measurements were plaque forming units (pfu's) per millilitre of stock bacteriophage solution. The titering procedure allowed accurate determination of the amount of sample which would be required for library

plating during screening.

2.5.1.1.3 Plating of cDNA Libraries for Screening

Generally, approximately 5×10^4 pfu's were plated onto a 150 mm LB agar plate. During a primary screen, the total number of plaques analysed was approximately 10^6 and the number of 150 mm plates used was therefore 20.

The plating procedure was as follows: To begin with, 15 ml of plating cells were placed in a sterile tube, and $1-1.5 \times 10^6$ plaque forming units from the titrated stocks were added to the bacteria and mixed. Adsorption of the phage to the bacterial cells was allowed to proceed by incubation at 37°C for 20 minutes. Thereafter, 600 μl aliquots were pipetted into sterile 10ml glass tubes. Six ml of melted sterile LB top agarose at 50°C was added to each tube and then immediately poured over an LB agar plate and swirled for even distribution. The agarose was allowed to harden at room temperature for 20 minutes. Thereafter, the plates were inverted and incubated at 37°C for 10 to 16 hours - that is until plaques were evident, but small enough not to produce confluent lysis over the entire plate. Expansion of the plaques was stopped by cooling the plates to 4°C where they were stored for approximately 1 to 2 hours before the making of lifts. This cooling step also served to harden the top agarose.

2.5.1.1.4 Immobilisation of E. coli phage plaques on Nylon Membranes

The method used was essentially that of Brenton and Cavis (1977) with some modifications (Sambrook *et al*, 1979). The circular nylon membranes were carefully placed onto the plates, and plaque adsorption allowed to proceed for about 10 minutes. During this stage, about six holes were punched asymmetrically around the edges of the filters and their corresponding positions on the plate were marked. The filters were removed from the plates taking care not to dislodge the layer of top agarose. Thereafter, a second set of duplicate filters was processed in the same way.

The filters were sequentially immersed in approximately 250 ml of the following three solutions:

- a) 0.2M NaOH, 1.5 M NaCl
- b) 2x SSC, 0.4 M Tris, pH 7.4
- c) 2x SSC

These solutions were changed after processing approximately 20 filters.

The filters were air dried and then the DNA crosslinked by short wave ultraviolet irradiation for 5 minutes.

2.5.1.1.5 Filter Hybridisation

The hybridisation of the filters was according to the protocols which have already been outlined for Southern blot hybridisation with the modifications described in section 2.4.2.2.

2.5.1.1.6 Secondary Screening

After exposure for autoradiography, the filter positioning holes were marked on the film. A transilluminator was used in order to align the autoradiographs with the original plates from which the plaque lifts were made.

A sterile inverted 'yellow pipette tip' was used to remove a plug of top and bottom agar corresponding to the area on the autoradiograph where there was thought to be a positively hybridising plaque. The plug was then transferred to 500µl of TMG buffer with a drop of chloroform. This suspension of bacteriophages was then ready for titering and rescreening.

2.5.2 PLAQUE PURIFICATION

Three methods were attempted for the process of plaque bacteriophage DNA purification. They were a quick plate lysis method and large scale liquid lysis methods with and without a CsCl gradient centrifugation purification step. It was found that the quicker methods did not always yield the bacteriophage DNA in sufficient quantity or purity to allow convenient analysis of the inserts. The liquid lysis procedure with CsCl centrifugation did however produce large amounts of bacteriophage DNA of good purity.

2.5.2.1 Plate Lysis Method of Bacteriophage DNA Preparation

Agarose plates were prepared as for the screening procedure (section 2.5.1.1.3) except that the plaques were plated at a higher density. This was in order to improve the yield of the bacteriophage DNA. Another modification was that agarose was used for the preparation of the plates. Agar is thought to contain inhibitors of restriction endonucleases which are generally not removed during the extraction procedure (Sambrook *et al*, 1989).

8 ml of TM buffer (50 mM Tris, pH 7.4, 10 mM MgSO₄) per 150 mm plate was added directly to the surface and gently agitated for 1-2 hours at room

temperature.

The bacteriophage diluent was then transferred to a tube, and centrifuged for 20 minutes at 4°C at 2 000xg in order to remove the bacterial debris.

The supernatant was removed then treated with RNase A (1 µg/ml, Boehringer Mannheim) and DNase I (1 µg/ml, Boehringer Mannheim) for 15 minutes at 37°C.

1.6 ml of 5 M NaCl and 1.8 g of solid PEG8000 was then added to this solution and vortexed in order to dissolve the PEG. Thereafter, the solution was incubated on ice for 15 minutes.

The precipitated bacteriophage particles were recovered by centrifugation at 10 000xg for 10 minutes at 4°C. They were resuspended in 0.5 ml of TE buffer and then 5µl 10% SDS and 10µl of 5 M NaCl were added. This solution was then extracted with equal volumes of SS-phenol and chloroform, then an equal volume of chloroform.

The DNA was precipitated with an equal volume of isopropanol, and it was recovered by centrifugation at 10 000 g for 20 minutes. After air-drying of

the pellet, it was resuspended in approximately 50 μ l of TE buffer and stored at 4°C before using.

2.5.2.2 Liquid Lysis Method of Bacteriophage DNA Preparation

c600h// bacteria were cultured overnight at 37°C with vigorous shaking. 250 ml of LB containing 1 mM MgSO_4 was then inoculated with 1 ml of the overnight culture, and incubated at 37°C with vigorous shaking until the optical density at 600 nm reached approximately 0.5. This bacterial suspension was then inoculated with bacteriophage particles (approximately 10^8 pfu) and incubation was continued until lysis occurred (± 12 hours).

5 ml of chloroform was then added to each flask and incubation at 37°C was continued for a further 15 minutes. DNase I and RNase A, each to a final concentration of 1 μ g/ml were then added and the solution incubated at 37°C for 30 minutes.

Solid NaCl to a final concentration of 1M (15g per 250 ml) was added, dissolved, and the solution allowed to stand on ice for 1 hour. The bacterial debris was removed by centrifugation at 10 000 g for 10 minutes.

Solid PEG6000 was added to a concentration of 10%, and after dissolving, the solution was allowed to stand on ice for 1 hour.

The precipitated bacteriophage particles were recovered by centrifugation at 10 000xg for 15 minutes at 4°C. The phage pellet was then resuspended in 10 ml of TE buffer.

Extraction with SS-phenol and chloroform, precipitation with isopropanol and resuspension were as described in the previous section.

2.5.2.3 Liquid Lyase with CsCl centrifugation method of bacteriophage DNA preparation

c600h7 bacteria were cultured overnight at 37°C with vigorous shaking. 250 ml of LB containing 1 mM MgSO₄ was then inoculated with 1 ml of the overnight culture, and incubated at 37°C with shaking until the optical density at 800 nm reached approximately 0.5. This bacterial suspension was then inoculated with bacteriophage particles (approximately 10⁸ pfu) and incubation was continued until lysis occurred (usually overnight).

1 ml of chloroform was added to the bacterial cell suspension and then

shaken for 10 minutes at 37°C. The bacterial debris was removed by centrifugation at 2 000xg for 20 minutes.

The supernatant was decanted and 12 g of solid NaCl and 20 g of solid PEG8000 were added. The solution was mixed to dissolve the NaCl and PEG, then incubated on ice for 1 hour with intermittent shaking.

The bacteriophage particles were precipitated by centrifugation at 4°C for 20 minutes at 10 000xg, then resuspended in 5 ml of buffer C (Tris-HCl pH 7.5, 10 mM MgSO₄).

A discontinuous CsCl gradient was formed by layering 6.5 ml of 3 M CsCl on top of 3.5 ml of 5 M CsCl in a 12 ml polyallomer Beckman SW40Ti rotor tube. The CsCl solutions were made up in buffer C. Approximately 3 ml of the bacteriophage suspension was then layered over the 3.5 M CsCl solution in order to fill the tubes prior to centrifugation.

Centrifugation was at 24 500 rpm for 2 hours (80 000xg) with slow acceleration and deceleration. The bacteriophage particles were visible thereafter as a faint blue band at approximately the position of the interphase between the two layers of the different CsCl solutions. In order

to retrieve the virus particles, the centrifuge tube was punctured with an 18G syringe needle immediately below the band and approximately 1 ml of fluid was drained.

The solution was thereafter dialysed at 4°C for 24 hours against two changes of 2 litres of dialysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 10 mM MgCl₂).

After dialysis, EDTA and SDS were added to final concentrations of 20 mM and 0.5% respectively, and the solution heated to 68°C for 15 minutes. Thereafter, the solution was treated with RNase A (1 µg/ml final concentration) for 1 hour at 37°C.

Extraction with SS-phenol and chloroform, precipitation with isopropanol and resuspension were as described in section 2.5.2.1.

2.5.3 SOUTHERN BLOT ANALYSIS OF BACTERIOPHAGE DNA

Varying amounts (approximately 5 to 20 μg) of the extracted bacteriophage DNA were digested with the restriction endonuclease *EcoRI* according to the manufacturer's instructions (usually 4U/ μg of bacteriophage DNA). The DNA was then subjected to agarose gel electrophoresis with ethidium bromide staining, blotting and probing as previously described (sections 2.4.2.2).

2.5.4 PCR BASED APPROACH TO THE ISOLATION OF HOMOLOGOUS X-GENE SEQUENCES FROM cDNA LIBRARIES

DNA isolated from large scale preparations of bacteriophage particles of putative positive plaques was used as template DNA for amplification. Four primers were used (two sense and two antisense) in four possible combinations. Each of the primers had restriction enzyme recognition sequences at their 5' ends to facilitate cloning for sequencing after PCR amplification.

The sequences of the primers were as follows:

Primer 1 5' AGTCGGATCCCCTTTGTTTACGTCCCGTCGGCGC 3'

Primer 2 5' AGTCGGATCCACGCCCATCAGATCCTGCCCAAGG 3'

These two primers were sense sequences at positions 1420 to 1443 and 1625 to 1648 of the hepatitis B virus genome (primers 1 and 2 respectively). They each also contained a 5' terminal redundancy of 4 nucleotides (5' AGTC 3') as well as restriction digestion sites for the enzyme *Bam*HI.

Primer 3 5' AGTCCTAGACTACAGCCTCCTAATACAAAGACC 3'

Primer 4 5' AGTCCTAGAGGTGCTGGTGCGCAGACCAATTTG 3'

Primers 3 and 4 were antisense sequences at positions 1788 to 1785 and 1816 to 1793 of the HBV genome (primers 3 and 4 respectively). They each contained the 5' terminal redundancy as well as a restriction digestion site for the enzyme *Xba*I.

The reaction mixture for PCR was similar to that which has already been described (section 2.4.3.1). The temperature cycles were however slightly adjusted in that the annealing temperature was changed to 47°C. This was in order to facilitate the hybridisation of primers which were possibly not entirely complementary to the template DNA. The DNA was processed by electrophoresis with or without Southern blotting and then cloned into M13 for sequencing.

2.6 DNA SEQUENCING

2.6.1 M13 CLONING

The procedures used were slight modifications of standard methods (Davis *et al*, 1986; Sambrook *et al*, 1989) and are described below.

2.6.1.1 Ligation of Insert and Vector DNA

M13mp18 and M13mp19 (Yanisch-Perron *et al*, 1985) (purchased from Boehringer Mannheim) were the two filamentous bacteriophage vectors used. Cleavage of the bacteriophage DNA was with the two restriction endonucleases *Bam*HI and *Xba*I according to the recommendations of the manufacturer (Boehringer Mannheim) for the use of these two restriction endonucleases in combination (see section 2.4.3.2). The cleaved DNA was then purified using the glassmilk® adsorption technique (Vogelstein and Gillespie, 1979) described in Section 2.2.5.3. The amplified DNA was also cleaved with the same two restriction endonucleases and purified with glassmilk®.

The products of the digestions were checked by electrophoresis on 0.8%

agarose gels.

The ligation reactions were set up by adding the following solutions to a sterile tube:

10 x Ligation buffer (0.5 M Tris-HCl, pH 7.4, 0.1 M MgCl ₂ , 0.2 M DTT, 10 mM ATP and 50 µg/ml BSA)	1 µl
≈ 0.1 µg cleaved M13 DNA	1 µl
± 0.1 µg cleaved amplified DNA	1 µl
T4 DNA Ligase (2 Weiss units, Boehringer)	1 µl
Water	8 µl

This mixture was then incubated overnight at 15°C.

2.6.1.2 Preparation of Competent *E. coli* cells

A single colony of JM109 *E. coli* was removed from a M9 minimal medium plate and inoculated into 5 ml of LB medium then incubated overnight at 37°C with vigorous shaking. 0.4 ml of this overnight culture was then

used to inoculate 40 ml of LB medium and incubated at 37°C for approximately 2 hours until the optical density at 600 nm was 0.2.

The cells were pelleted by centrifugation (2 000xg for 15 minutes at 4°C) and then resuspended in 20 ml of ice cold 50 mmol CaCl₂. These cells were then allowed to stand on ice for approximately 30 minutes. The cells were then again recovered by centrifugation and resuspended in 4 ml of 50 mmol CaCl₂. These cells were then stored for up to 2 days prior to use.

2.6.1.3 Transfection

The ligated DNA was mixed with 50 mmol/l Tris-HCl, pH7.2 to bring the volume up to 50µl. The DNA was then mixed with 300µl of competent E. coli cells and incubated on ice for 40 minutes. The cells were then subjected to a heat shock by incubation at 42°C for 3 minutes, and then the cells were returned to ice. 200µl of exponentially growing E. coli were then added.

To 1 ml of melted top agar cooled to 50°C, the following were added for each of the transfection experiments:

40µl sterile filtered IPTG solution (100 mmol/l), and

40µl X-gal solution (2% (w/v) in dimethylformamide) (both purchased from Boehringer, Mannheim).

The top agar mix was then added to the transfected cells and then immediately poured onto 90 mm agar plates. The top agar was allowed to harden at room temperature for approximately 15 minutes, then the plates were inverted and incubated overnight at 37°C.

The insert bearing clones were easily detected after the overnight culture by their colourless appearance. Those M13 clones which had not resulted in recombination with the insert gave rise to blue coloured plaques.

2.6.1.4 Preparation of Recombinant M13 Replicative Form (RF) DNA

A 1:100 dilution of an overnight culture of JM109 *E. coli* was made with LB medium. This was then distributed into 10 ml aliquots. Individual colourless plaques were picked and used to inoculate the diluted *E. coli*. The culture was then incubated at 37°C for approximately 6 hours with vigorous shaking.

1.5 ml of the suspension was then transferred to a microfuge tube and

centrifuged for 5 minutes at 10 000xg. The bacterial pellet was recovered and subjected to standard plasmid preparation as was described in section 2.2.5.2. The supernatant was reserved for M13 single stranded (ss) DNA isolation.

The isolated DNA was then digested with various restriction enzymes and subjected to agarose gel electrophoresis with or without Southern blot analysis as was described previously in section 2.2.2-2.2.5.

2.6.1.5. Preparation of Recombinant M13 ssDNA

The supernatant of the recombinant M13 culture (described in section 2.6.1.4) was recentrifuged at 10 000xg for 5 minutes, and the supernatant carefully removed taking care not to disturb any cells which had sedimented. 1/5 volume of a solution of 2.5 M NaCl and 20% (w/v) PEG6000 was added and the mixture then incubated on ice for 15 minutes. The solution was centrifuged for 10 minutes at 10 000xg, and the M13 pellet was resuspended in 100 μ l of TE buffer. This was then subjected to SS-phenol/chloroform extraction and isopropanol precipitation as was previously described (section 2.2.5.2). After suspension of the M13 ssDNA pellet, it was further purified using glassmilk® adsorption (Vogelstein and

Gillespie, 1979) as has been previously described (Section 2.2.5.3). This additional step of ssDNA purification was found to improve the quality of the sequencing reactions.

2.6.2 SEQUENCING OF RECOMBINANT M13 ssDNA

The chain termination method as devised by Sanger *et al* (1977) with a few modifications was used for sequencing. Unless otherwise indicated, all of the reaction components were purchased from Boehringer Mannheim. For the sequencing reactions, the four different nucleotide solutions were made up with the following component concentrations:

Nucleotide solutions	G mixture $\mu\text{mol/l}$	A mixture $\mu\text{mol/l}$	T mixture $\mu\text{mol/l}$	C mixture $\mu\text{mol/l}$
dGTP	5	100	100	100
dTTP	100	100	5	100
dCTP	100	100	100	10
ddGTP	120	-	-	-
ddATP	-	100	-	-
ddTTP	-	-	500	-
ddCTP	-	-	-	100

These solutions were made up in polymerase reaction buffer comprising 7 mM Tris-HCl, pH 7.4, 50 mM NaCl and 7 mM MgCl_2 . No dATP was added to these nucleotide solutions as sufficient of the radioactive form was added later on in the experiment.

The standard sequencing assay was started by annealing the sequencing primer to the ssM13 DNA. The following were added to a tube:

$\pm 0.5 \mu\text{g}$ recombinant single stranded M13 DNA	5 μl
Approximately 0.8 pmol M13 sequencing primer	2 μl
10x Reaction Buffer	1.5 μl
Water	1 μl

After mixing, the solution was heated to 55°C for 10 minutes and then allowed to cool slowly to room temperature. The chain elongation step was commenced by adding the following components to the mixture once it had cooled to room temperature:

16 μCi α -[^{35}S]dATP (≥ 1000 Ci/mmol) (Amersham)	2 μl
1 unit Klenow Enzyme	1 μl

After mixing, the solution was dispensed in $2.5 \mu\text{l}$ aliquots into four labelled microfuge tubes (G, A, T and C). The reactions were started by the addition of $2 \mu\text{l}$ of the appropriate dNTP/ddNTP mixtures to the tubes. The mixtures were then incubated at room temperature for 20 minutes. Thereafter, the reactions were 'chased' by the addition of $2 \mu\text{l}$ of deoxynucleotide solution

(a mixture of dATP, dGTP, dCTP and dTTP each at a concentration of 125 μ M at a pH of 7.5) and further incubated at room temperature for 15 minutes.

The reactions were terminated by the addition of 4 μ l formamide buffer (95% deionised formamide, 1x TBE buffer, 0.01% bromophenol blue and 0.01% xylene cyanol). Thereafter, the DNA was denatured by heating in a boiling water bath for approximately 3 minutes then quick cooled on ice.

2 μ l aliquots were loaded onto a 5% polyacrylamide 8M urea denaturing gel (Davis *et al*, 1986).

The electrophoresis system used was a home made one, and basically conformed to the specifications suggested by Davis *et al* (1986). The proportions of the gel were approximately 20cm x 40cm x 0.4mm. Both of the glass plates were siliconised in order to facilitate separation after the running of the gel.

The samples were run on a preheated gel ($\pm 65^{\circ}\text{C}$) for approximately 2 hours at a constant power of 50 Watts. By this time, the bromophenol blue dye front had reached the end of the gel. A second set of 2 μ l aliquots from

the original reaction mixtures was then loaded and allowed to run for a further 2 hours at the constant power of 50 Watts. In some cases, a third set of aliquots was loaded onto the gel. By making three separate loadings, it was possible to resolve a greater number of nucleotides on the sequencing gel.

After electrophoresis, the gel was soaked in 10% acetic acid for 10 minutes in order to fix the DNA and remove the urea. After transferring to Whatman 3M filter paper, the gel was dried under vacuum for approximately 1 hour at 80°C.

The dried gel was then exposed overnight for autoradiography, and the sequence could thereafter be determined from the X-ray film. Typically, the sequence of approximately 200 nucleotides could be determined from one reaction.

Using both the M13mp18 and M13mp19 vectors enabled sequencing of the inserts in two orientations.

Analysis of the sequencing data was by using the DNA StarTM COMPARE program (registered to Mr J. Burke, Haematology Department, University

of the Witwatersrand). The width of the residues of the unit of comparison (window size) was 30. The setting for the number of adjacent residues of the window that should match was 30% to 50% using all register shifts. The minimum quality - the minimum number of consecutive qualifying windows for homology to be recorded - was one. In all cases, the reverse complement of the sequences were also compared.

2.7 ASYMMETRIC RE-AMPLIFICATION TO CONFIRM PCR PRODUCTS

The oligonucleotides used in the initial PCR amplification were 24 base sense (primer 1) and antisense (primer 2) strands towards the 5' and 3' ends of the hepatitis B virus X-gene. The locations of these primers and their orientation with respect to the HBV genome are indicated in Section 2.4.2.1.2. The oligonucleotide primers were used at a concentration of 0.25 μ M. 1.0 ng of the SP64 clone of the X-gene was used as template DNA. The PCR buffer contained 50 mM KCl; 10 mM Tris-HCl (pH8.8) and 1.5 mM MgCl₂. Each reaction mixture had a volume of 50 μ l and in some cases contained 2.5 μ l of ³²P-dCTP ($\pm$ 3000 Ci/mmol). PCR was performed using Taq polymerase (Promega) for 30 cycles - each cycle being 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. There was a final extension step of 10 minutes at 72°C. After completion, a 1 μ l or 5 μ l aliquot was removed, and this was used as template source for re-amplification. The PCR components for the re-amplification were similar to those of the initial reaction, except that only a single sense 24 base internal primer 3 (HBV nucleotides 1625 to 1648, 5' ACGCCCATCAGATCCTGCCCAAGG 3') was added to a final concentration of 0.25 μ M. Individual reaction mixtures were then subjected to 1, 2, 5, 10 or 15 PCR cycles followed by a 5 minute extension step at 72°C.

Control re-amplification experiments contained:

- a) 0.25 μ M of primer 4 (5' GGTGCTGGTGC~~G~~CAGACCAATTTG 3') which was external to the initially amplified X-gene but present in the SP64 clone (antisense nucleotides 1816 to 1793);
- b) approx. 250ng of non-specifically amplified DNA as template; or
- c) no initially amplified DNA but appropriately diluted primer 2 (0.005 μ M) and the SP64 X-gene clone template DNA (1ng).

A set of five 15 base internal sense primers extending from HBV nucleotides 1624 to 1648 were used in order to assess the effect of a mismatch on the re-amplification step. The nucleotide sequences of these primers were 5' AGATCCTGCCCAAGG 3' (primer 3A, normal sequence), 5' AGATCCT~~C~~CCCAAGG 3'; 5' AGATCCT~~A~~CCCAAGG 3'; 5' AGATCCT~~T~~CCCAAGG 3' (primers 3B to 3D respectively, single mismatches at the 8th base) and 5' AGATCCTGCCCAAG~~C~~ 3' (primer 3E, 3' end transversion).

Aliquots of the products of PCR were subjected to 6% polyacrylamide, as well as native and alkaline denaturing 1.5% agarose gel electrophoresis (Sambrook *et al*, 1989). Staining was with ethidium bromide followed by autoradiography of the dried gels for 15 to 120 minutes if required.

Chapter 3

**ONCOGENE EXPRESSION IN HEPATOCELLULAR
CARCINOMA**

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3.1 INTRODUCTION

The mechanism by which malignancy is induced in hepatocellular carcinoma (HCC) is not well defined. Hepatocarcinogenesis is a multistep process influencing a number of cellular functions and involving the effects of one or a number of different carcinogens. HBV and aflatoxin appear to be the most important liver cancer inducing agents.

Theories concerning the mechanism of malignant induction in HCC can be divided into two categories (modified from Daugherty, 1991):

1. **Specific viral or chemical effects.** These include the activation of oncogenes; inactivation of host tumour suppressor genes, as may result from the effect of aflatoxin on p53 (Bressac *et al*, 1991; Hsu *et al*, 1991); expression of viral oncogenes or viral transcriptional *trans* activating factors, eg. the HBV X protein (Twu and Schloemer, 1987; Wollersheim *et al*, 1988; Aufiero and Schneider 1990). Also included in this group of effects is the mutation of host cellular genes by the process of insertional mutagenesis during integration of hepadnavirus DNA into the host genome. The documented sites of viral DNA integration in human HCC include an area adjacent the cyclin A gene (Wang *et al*, 1990), and a sequence with homology to *c-erbA* and steroid receptor genes (Dejean *et al*, 1986). In woodchuck liver tumours, integration of the hepatitis virus in the *c-*

myc, *N-myc-1* and *N-myc-2* genes has been shown to occur in up to 50% of malignancies (Moroy *et al*, 1986; Hsu *et al*, 1988; Fourel *et al*, 1990).

2. Non-specific mechanisms whereby a state of chronic liver inflammation with rapid cell division is induced, and which in turn leads to the development of a number of mutations and then the transformed phenotype.

Although there have been reports of transforming sequences isolated from HCC (Ochiya *et al*, 1986; Carloni *et al*, 1988), these liver cancer oncogenes have not been demonstrated reproducibly.

Increased expression of the cellular oncogenes *c-myc*, *c-fos* and *c-Ha-ras* is thought to be important for the processes of cell division during rat liver regeneration following partial hepatectomy (Fausto and Shank, 1983; Goyette *et al*, 1983; Makino *et al*, 1985; Bravo *et al*, 1986; Fausto and Shank, 1987; Dean *et al*, 1987). During the early stages of the G1 phase, elevated *c-myc* and *c-fos* expression may confer competence on quiescent liver cells, enabling them to move from G0/G1 to S phase of the cell cycle in the presence of further progression factors (Reed *et al*, 1986; McNurney *et al*, 1987; Owen *et al*, 1987). Increased *c-Ha-ras* expression in hepatocytes occurs at a later stage after hepatectomy. *c-Ha-ras* mRNA concentration is increased two- to threefold during the DNA replication period (S-phase) of regenerating rat liver (Fausto and Shank, 1987). In normal liver tissue, only a small percentage of the cells is dividing (Fausto

and Shank, 1983; Fausto and Shank, 1987), however this fraction may be increased in malignant or inflamed liver cells, and in regenerating nodules of cirrhotic livers. Thus, in these pathological conditions, with an increased number of cells actively dividing, expression of *c-myc*, *c-fos* and *c-Ha-ras* in hepatocytes is likely to be elevated.

Results from investigations of oncogene expression in hepatocellular carcinomas have been conflicting. In studies on three tumour tissue specimens (Zhang *et al*, 1987) and a hepatoma cell line (PCL/PRF/5) (Motte *et al*, 1986), expression of *c-fos*, *c-myc* and *c-Ha-ras* was found to be increased. Similarly, it has been shown in Morris rat hepatomas that *c-Ha-ras* and *c-myc* expression is elevated (Cote and Chiu, 1987). Su *et al* (1986) were, however, unable to show any differences in *c-myc* mRNA concentrations when comparing normal hepatic and tumour tissue from humans.

In an immunohistochemical protein study on Ras p21 and Myc p62 in human hepatocellular carcinoma and non neoplastic liver tissues, most HCC specimens showed enhanced Ras p21 and Myc p62 expression when compared to the normal (Tiniakos *et al*, 1989). In this study by Tiniakos *et al* (1989) the antibody against Ras p21 recognised Harvey-, Kirsten- and N-ras p21.

Altered expression of *c-myc* and the *c-ras* family of protooncogenes has been demonstrated in a number of animal models of chemical

hepatocarcinogenesis (Yaswen *et al*, 1985; Beer *et al*, 1986; Fausto and Shank, 1987; Braun *et al*, 1989). Most of this evidence shows the elevated expression of the *c-myc* oncogene to be a consistent feature during the development of liver cancer in rats, but increased mRNA concentrations of the *c-ras* genes are not always present. Activation of these oncogenes has also been reported to occur specifically in 'oval' cells during hepatocarcinogenesis (Braun *et al*, 1989). The origin of the oval cells in the liver is uncertain, but the evidence suggests that they may be stem cells with the potential to differentiate into normal as well as neoplastic hepatocytes (Tsao and Grisham, 1987).

In this study, in a further attempt to elucidate the role of oncogenes in liver cancer, the expression of *c-fos*, *c-myc*, *c-Ha-ras* and *c-erbA* in human hepatocellular carcinoma specimens of Southern African origin was investigated. Evidence of transcriptional activation, or rearrangement of these oncogenes was sought. Changes in the mRNA and protein concentrations in liver tumour specimens was measured using standard blot probing techniques. Evidence for the presence of oncogene rearrangements (possibly caused by HBV DNA integration) was analysed using Southern blot hybridisation.

3.2 RESULTS

3.2.1 METHODOLOGICAL CONSIDERATIONS

3.2.1.1 RNA Extraction

RNA prepared according to the rapid acid guanidinium-phenol-chloroform (AGPC) method of Chomczynski and Sacchi (1987) was of good quality. This method compared favourably in terms of simplicity of performance with some other methods of RNA extraction - for example, that of Chirgwin *et al* (1979). The purity of the RNA extracted was also good as was reflected by the consistently high A_{260}/A_{280} ratios of ≥ 1.8 . Figure 3.1 (and also Figures 3.12 and 3.13) shows that 18S and 28S ribosomal RNA are present in most abundant quantities.

In the case of extraction of RNA from liver tumour tissue which had been obtained from patients at *post mortem* the extracted RNA was often degraded. Degradation by RNases is thought to be inevitable if the tissue source for the RNA extraction is more than a few hours old. The RNA obtained from fresh surgical specimens was usually intact. RNA which had the appearance of a smear, and in which the 18S and 28S ribosomal RNA

were not at all discernible after electrophoresis, was discarded. This was the case in approximately one of every three tumours studied.

In obtaining *post-mortem* tissue, there were obviously very important practical considerations. It is not always easy to procure the liver tissue immediately after death, and yet the samples were valuable for the use in these experiments. As a result of some degradation, the RNA which was extracted from *post mortem* tissue was often only suitable for slot blot analysis. In these cases the signal

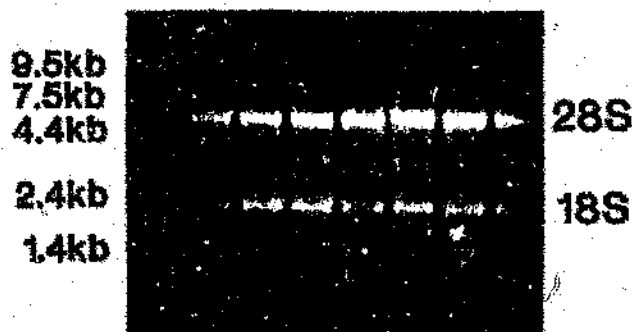


Figure 1 Ethidium bromide stained formaldehyde 1.5% agarose gel of RNA extracted according to the method of Chomczynski and Sacchi (1987). Resolution of the molecular weight markers is depicted in lane 1, and RNA isolated from 7 liver specimens is shown in lanes 2 to 8.

detectable on hybridisation of slot blots could be more easily interpreted than a smear from the Northern blot. These data needed to be interpreted with caution in that the hybridisation signal was lower than the true value. However, in most of the experiments it was found that the oncogene expression in the tumour tissue was considerably higher than that of the normal tissue. This normal tissue was fresh having been obtained at surgery from organ donors, and the RNA intact.

3.2.1.2 Preparation of Oncogene Probe DNA

Transformed HB101 *E.coli* were cultured as was described in Section 2.2.5.2 of Materials and Methods. For large scale preparation of plasmid DNA, the volume of culture used was approximately 250 ml - a volume which generally yielded adequate quantities of plasmid DNA after overnight growth.

The rapid plasmid preparation method of Birnboim and Doly (1979) allowed easy preparation of DNA. With the modifications described in Materials and Methods Section 2.2.5.2, the purity was adequate for most purposes. It was found that it was not necessary to perform more elaborate and time

consuming purification steps such as CsCl gradient centrifugation.

The inclusion of a RNase digestion step resulted in the removal of most of the RNA contaminating the preparation (Figure 3.2). Generally, it was found that DNase activity of the commercial RNase enzyme was effectively removed by incubation in a boiling water bath for 5 minutes (Section 2.1.1.6). The yield of DNA was not affected by the inclusion of this step.

An example of the densitometric scan of the plasmid DNA preparation is also given in Figure 3.2. RNase digestion also allowed more accurate measurement of the DNA concentration by spectrophotometry.

The plasmid preparation method produced DNA of sufficient purity to allow efficient digestion with restriction endonucleases. The multiple bands of the undigested plasmid DNA which represent the circular DNA in various conformations of supercoiling is discernible in Figure 3.2 and lane 2 of Figure 3.3. With this method of plasmid DNA preparation, it was difficult to eliminate all of the bacterial chromosomal DNA. Apart from producing spuriously high spectrophotometric absorbances at 260nm, this recurring problem did not have significant consequences.

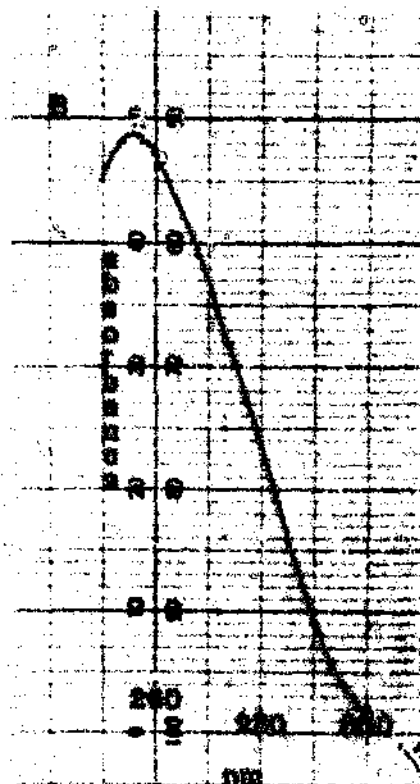
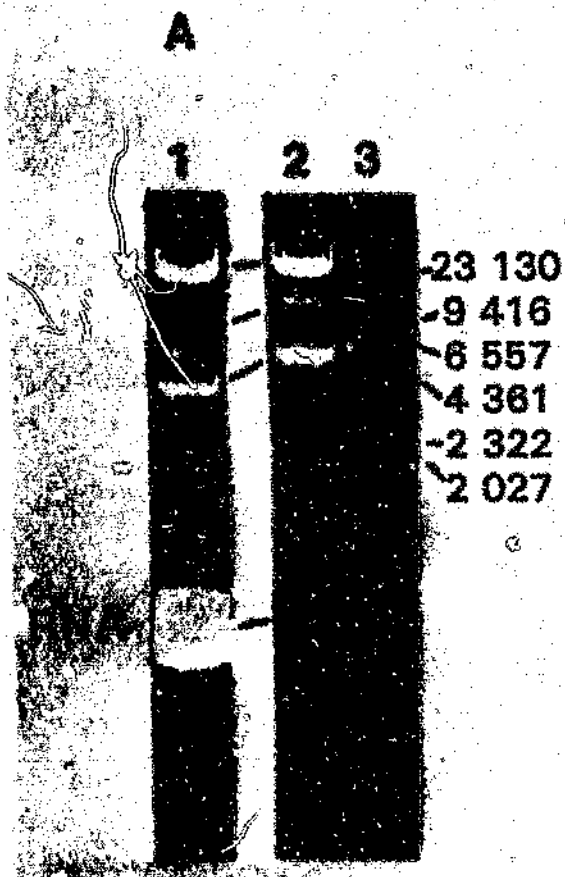


Figure 3.2 Ethidium bromide stained 0.8% agarose gel and spectrophotometric scan of plasmid DNA. **A.** The plasmid preparations were separated by electrophoresis before (lane 1) and after (lane 2) treatment with RNaseA. The molecular weight marker is *Hind*III digested phage λ . **B.** The spectrophotometric scan of this plasmid DNA is given in this panel.

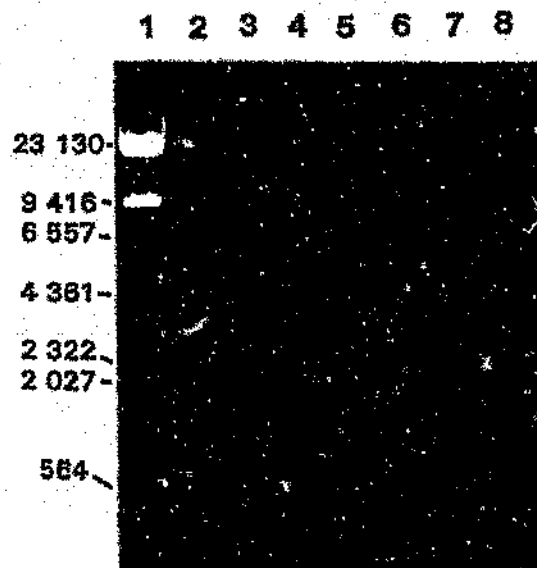


Figure 3.3 *c-erbA* plasmid DNA separated by electrophoresis on an ethidium bromide stained 0.8% agarose gel. Lane 1 represents the molecular weight marker DNA (*Hind*III digested phage λ) and undigested plasmid DNA is depicted in lane 2. Lanes 3 to 8 represent DNA which was prepared as for lane 2, and then subjected to restriction enzyme digestion with *Bam*HI (lane 3), *Bam*HI and *Eco*RI (lane 4), *Eco*RI alone (lane 5), *Eco*RI and *Hind*III (lane 6), *Hind*III alone (lane 7); *Hind*III and *Bam*HI (lane 8).

Restriction enzyme mapping of one plasmid DNA preparation is depicted in Figure 3.3. The plasmid mapped in this case was that which contains the *c-erbA* insert (Janssen *et al*: 1983). The oncogene fragment had been ligated into the *EcoRI* site of pBR323. The restriction endonuclease map of this recombinant plasmid is represented in Figure 3.4.

Examples of purified inserts from the recombinant plasmids are depicted in Figures 3.5 and 3.6. Initially, the plasmid DNA was digested on a large scale and subjected to electrophoresis (Figure 3.5). The insert DNA was isolated by removing the relevant band from the agarose gels and subjecting this to the glass powder adsorption (Section 2.2.5.3) followed by re-electrophoresis (Figure 3.6). Electrophoresis of these purified inserts also confirmed that their sizes were correct.

This probe DNA was of adequate purity for labelling. The calculated percent incorporation of radioactivity (^{32}P -dCTP) was generally in the region of 30-40% - an acceptable figure when using the multiprime labelling method.

The multiprime procedure was preferred to the nick translation protocol as it was more efficient. The incorporation of ^{32}P -dCTP was higher and the amount of

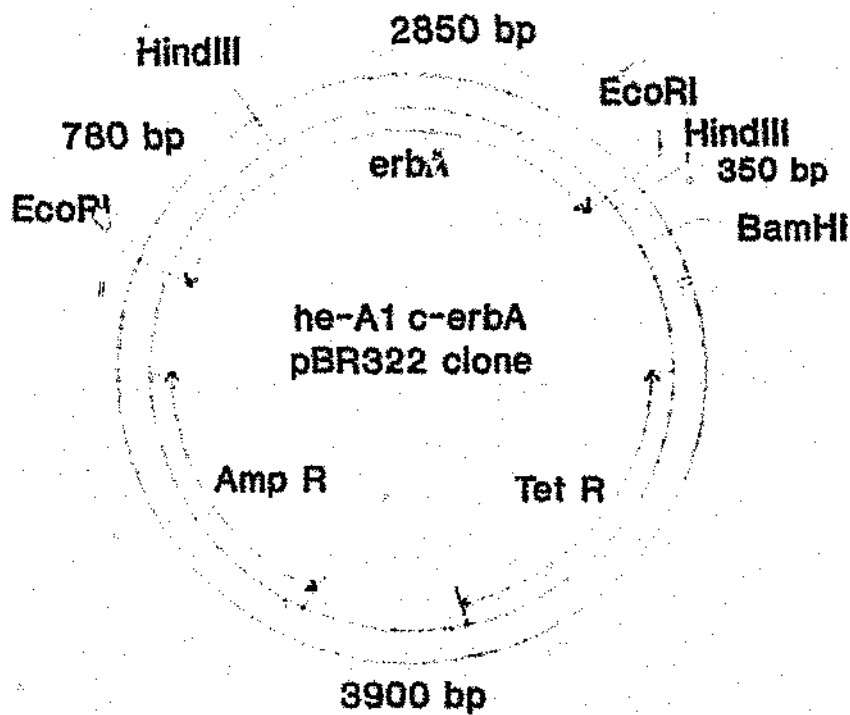


Figure 3.4 Schematic representation of the restriction enzyme map of the *c-erbA pBR322* (*he-A1*) recombinant plasmid. The data in Figure 3.3 confirmed this map.

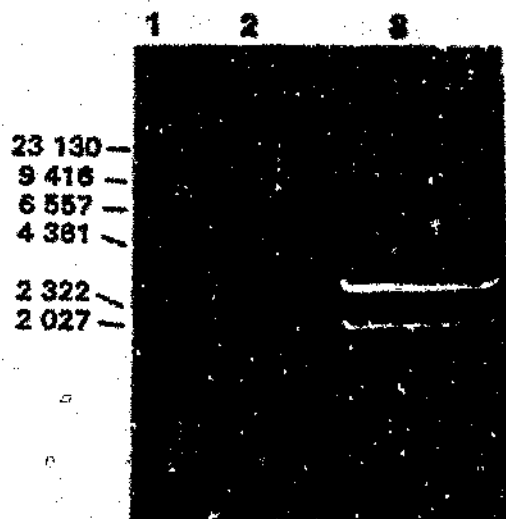


Figure 3.5 Ethidium bromide stained agarose gel used for large scale preparation of probe DNA. The molecular weight markers have been separated in lane 1. The recombinant *c-fos* and actin plasmids were digested with *EcoRI* and *BamHI* respectively. The resolution of the plasmid and insert DNA produced by this digestion is depicted in lane 2 (*c-fos*) and lane 3 (actin).

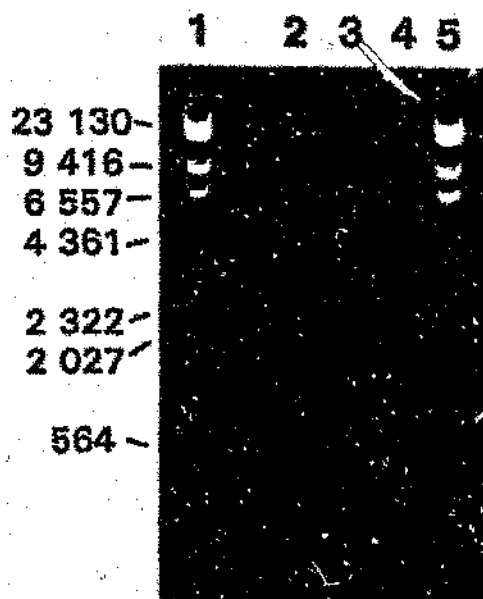


Figure 3.6 Ethidium bromide stained agarose gel of insert DNA, purified after restriction enzyme digestion and electrophoresis followed by glassmilk adsorption purification. Lanes 1 and 5 represent the molecular weight markers (*Hind*III digestion of phage λ), lane 2 the purified c-myc insert, lane 3 the purified c-fos insert and lane 4 the c-jun insert.

probe DNA required for labelling was considerably less.

3.2.2 BLOT ANALYSIS OF RNA, DNA AND PROTEIN ISOLATED FROM LIVER TISSUE SPECIMENS

Figures 3.7 to 3.10 show typical examples of slot blot analysis of similar amounts of RNA isolated from tumour and adjacent tissue from patients with HCC. The blots were probed with the oncogene probes *c-myc*, *c-fos*, *c-erbA* and *c-Ha-ras*. With the exception of *c-Ha-ras*, comparison shows more mRNA transcribed from these oncogenes in the tumour than in the tumour adjacent tissue. Also, there was more *c-myc*, *c-fos* and *c-erbA* mRNA detectable in the tumour adjacent tissue than in the tissue from the two patients without evidence of liver disease (Figure 3.11).

Controls using yeast transfer RNA (see Section 2.2.2.2) showed that under the conditions used, non-specific hybridisation was minimal (Figures 3.7 to 3.10).

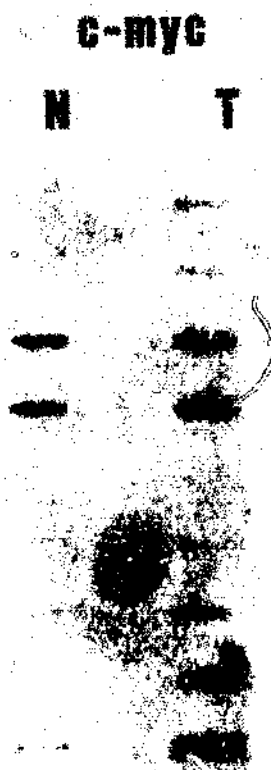


Figure 3.7 Slot blot hybridisation of increasing amounts of RNA (0.5 μ g, 1 μ g, 2.5 μ g and 5 μ g) isolated from tumour (T) and adjacent non-malignant (N) liver tissue from 2 patients with HCC. No signal was detected on the slots where 40 μ g of yeast tRNA was applied (middle of the Figure). The probe used here for the hybridisation was *c-myc*.

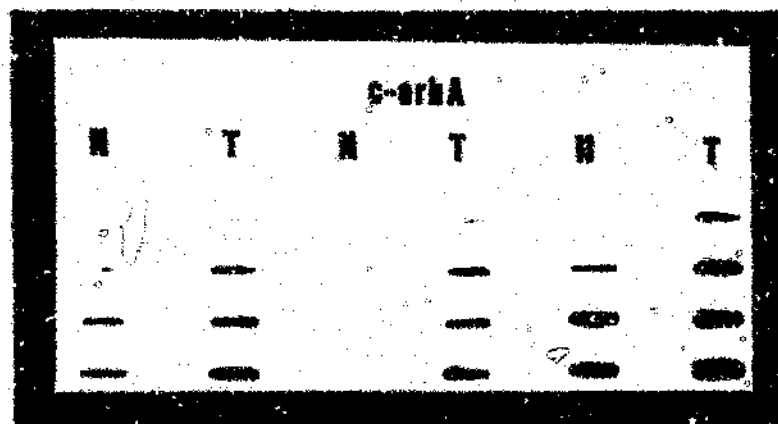


Figure 3.9 Slot blot hybridisation of RNA isolated from tumour (T) and adjacent (N) liver tissue. The probe used here for the hybridisation was *c-erbA*.

c-Ha-ras

N

T



Figure 3.10 Slot blot hybridisation of RNA isolated from tumour (T) and adjacent (N) liver tissue. The probe used here for the hybridisation was c-Ha-ras.

c-myc		c-erbA		c-fos	
N'	N	N'	N	N'	N

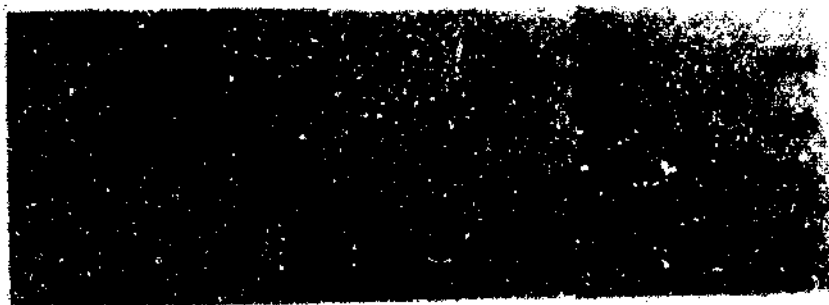


Figure 3.11 Slot blot hybridisation of RNA isolated from normal liver tissue of donor patients who did not have evidence of liver disease (N') and tumour adjacent non-malignant liver tissue (N) of one of the patients with HCC (patient number 1 of Figures 3.12 to 3.14). The blots were hybridised to labelled *c-myc*, *c-erbA* and *c-fos* probes as is indicated in the diagram.

The data represented in these slot blots was corroborated by the Northern blot data obtained from an analysis of intact RNA. This is shown in Figures 3.12 and 3.13. The tissue which was used for these experiments was obtained at surgery, and therefore the quality of the RNA was good -hence the ability to produce clearer Northern blot hybridisation data.

The autoradiographs were processed by densitometric scanning as described in Section 2.2.4 and the results are expressed in Figures 3.14 to 3.17. Here, it can be clearly seen that the oncogenes *c-myc*, *c-fos* and *c-erbA* are expressed at a relatively high level in tumour tissue when compared to tumour adjacent as well as tissue from patients without liver disease.

Another interesting finding was that the tumour with histological features of poor differentiation and which had the highest α -fetoprotein concentration also was found to have the highest level of *c-fos* and *c-myc* mRNA (patient 2 of Table 3.1 and Figures 3.15 and 3.16). Conversely, the tumour with well differentiated histological features and the lowest α -fetoprotein concentration also had the lowest *c-fos* and *c-myc* mRNA in the tumours (patient 1 of Table 3.1 and Figures 3.15 and 3.16).

Patient Number	Degree of Tumour Differentiation	α -fetoprotein concentration (ng/ml)
1 *	Well differentiated	46
2	Poorly differentiated	63 034
3	Well differentiated	622
4	Well differentiated	17 604
5	Moderately differentiated	1 305
6	Well differentiated	122

Table 3.1: Serum α -fetoprotein concentrations and histological assessment of the degree of differentiation of tumours from six patients. * - this patient did not have serological evidence of HBV infection.

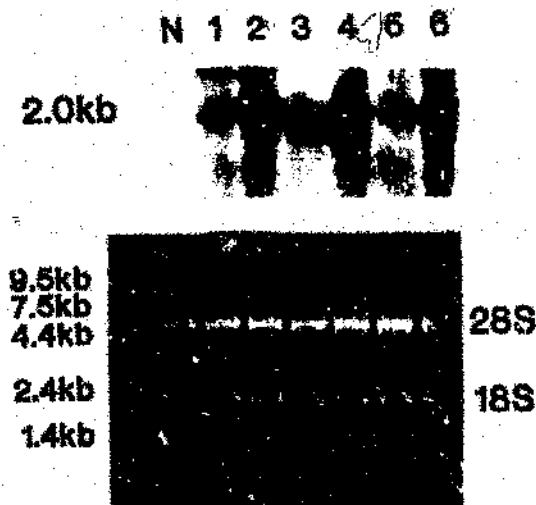


Figure 3.12 Northern blot analysis of RNA extracted from hepatocellular carcinoma specimens (1-6) and normal liver tissue (N) from a patient without evidence of liver disease. The upper panel shows a comparison of the signal obtained after hybridising with a radioactive *c-erbA* probe. The lower panel shows electrophoretic separation (formaldehyde 1.5% agarose gel stained with ethidium bromide) of total cellular RNA and molecular weight RNA prior to blotting.

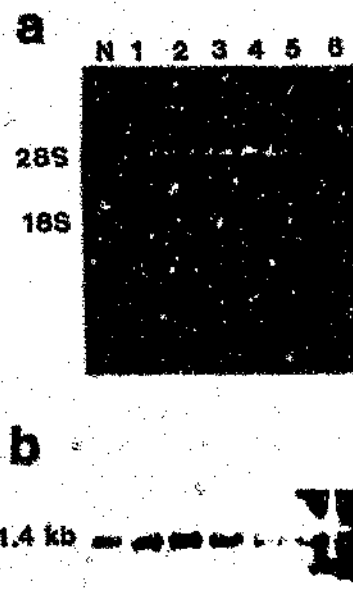


Figure 3.13 Northern blot analysis of RNA extracted from six hepatocellular carcinoma specimens (1-6) and normal tissue (N) from a patient without evidence of liver disease. Resolution of total cellular RNA after agarose gel electrophoresis is depicted in panel a. Samples were processed by hybridisation to a radiolabelled c-Ha-ras probe (panel b).

***c-erbA* mRNA concentrations in hepatocellular carcinoma**

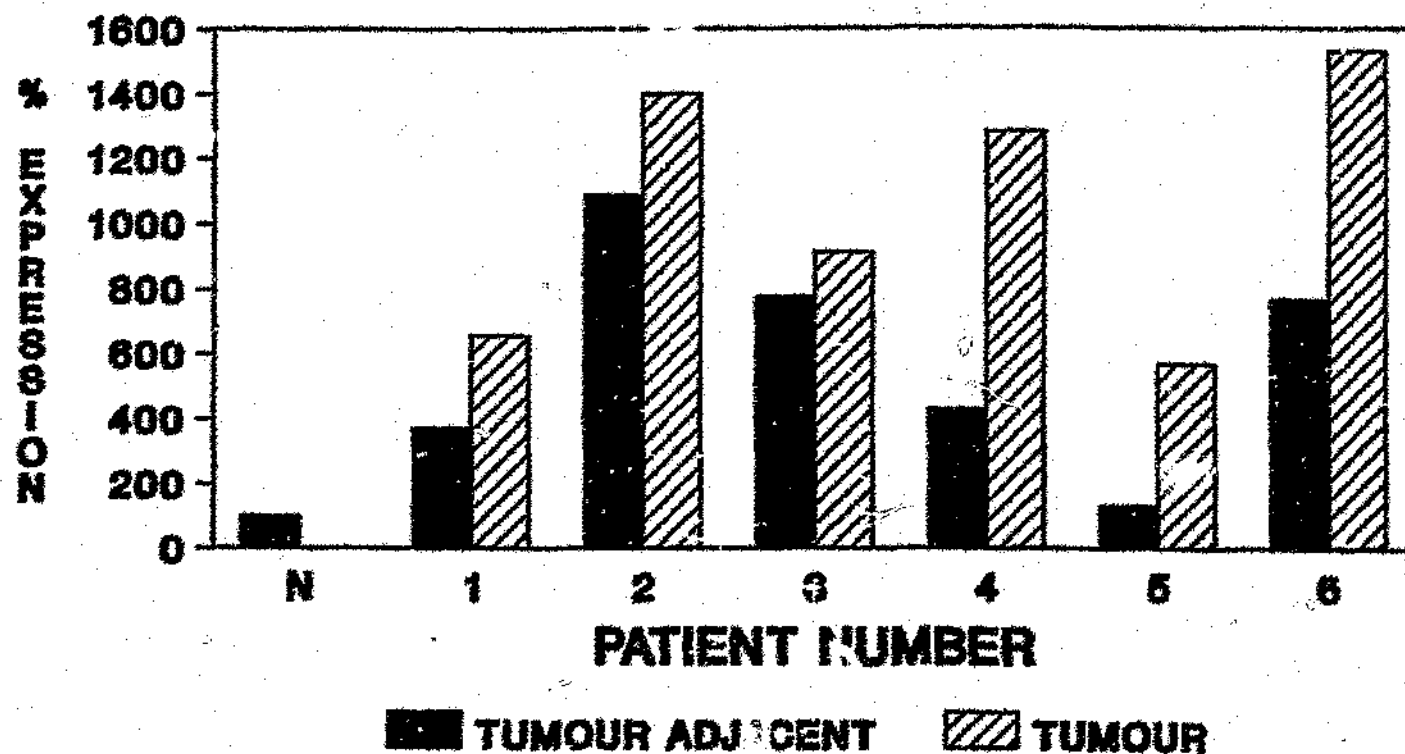


Figure 3.14 A comparison of *c-erbA* mRNA concentrations in normal (N), tumour and adjacent liver tissue of six patients (1-6). Extracted RNA was processed by Northern and/or slot blotting with hybridisation to the radiolabelled *c-erbA* oncogene probe and the intensity of the slots assessed densitometrically. The ratio of the *c-erbA*/actin hybridisation signal in the normal tissue (N) was used as a standard (100%) for the comparison of mRNA expression in tumour and adjacent liver tissue.

***c-myc* mRNA concentrations in hepatocellular carcinoma**

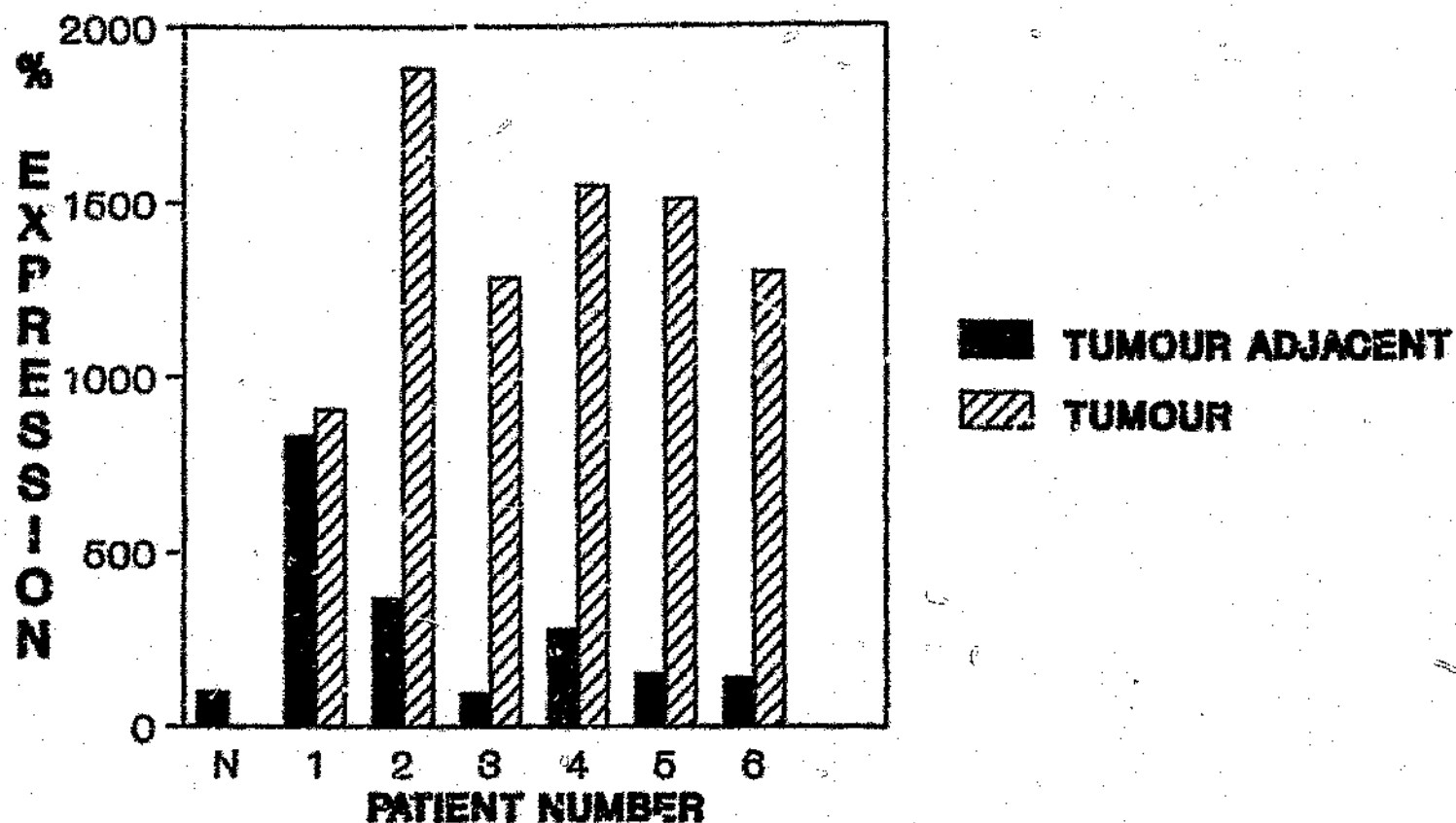


Figure 3.15 A comparison of *c-myc* mRNA concentrations in normal (N), tumour and tumour adjacent liver tissue from the same six patients (1-6) of Figure 3.14. Data were analysed as described in the legend to Figure 3.14.

***c-fos* mRNA concentrations in hepatocellular carcinoma**

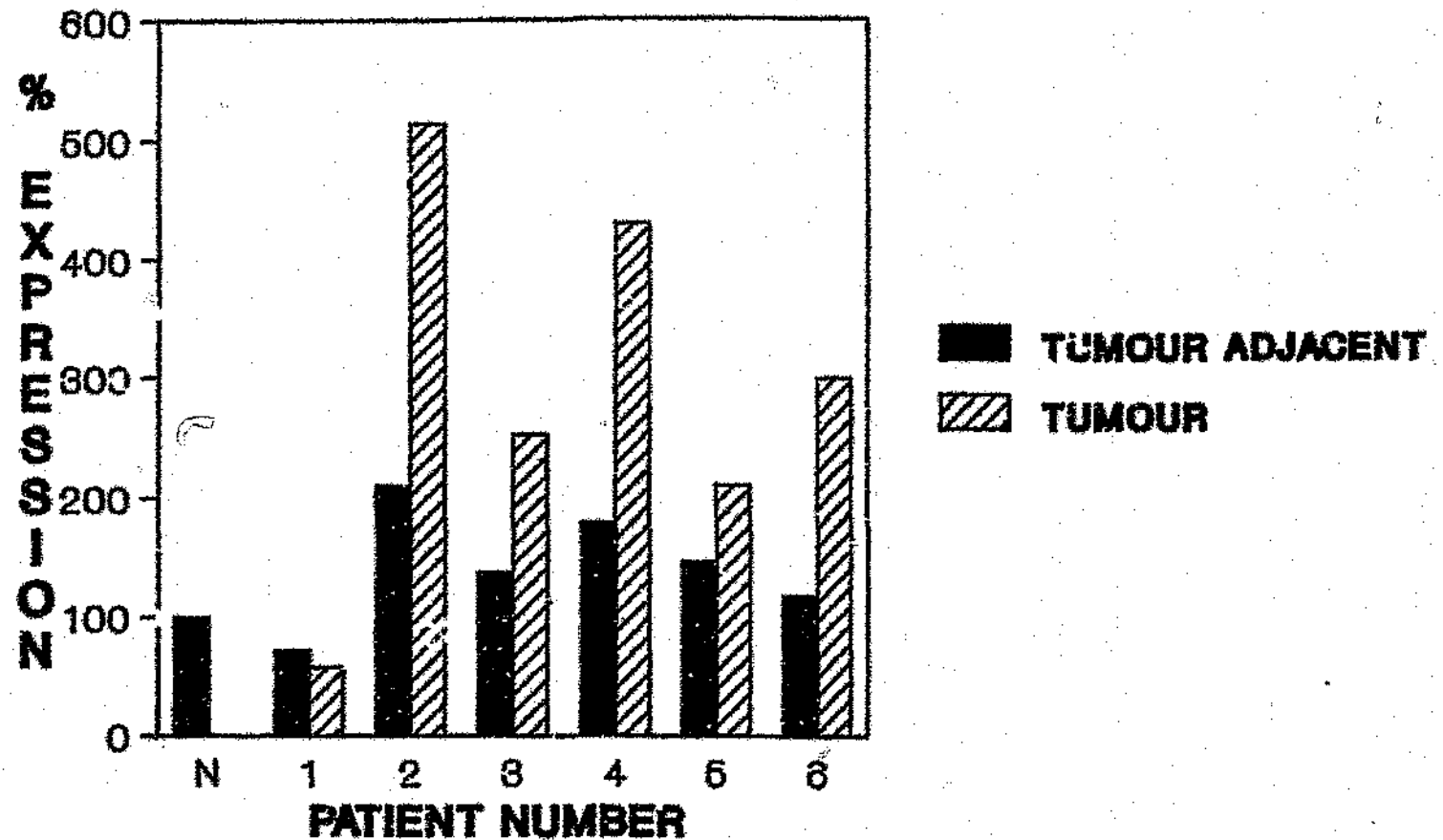
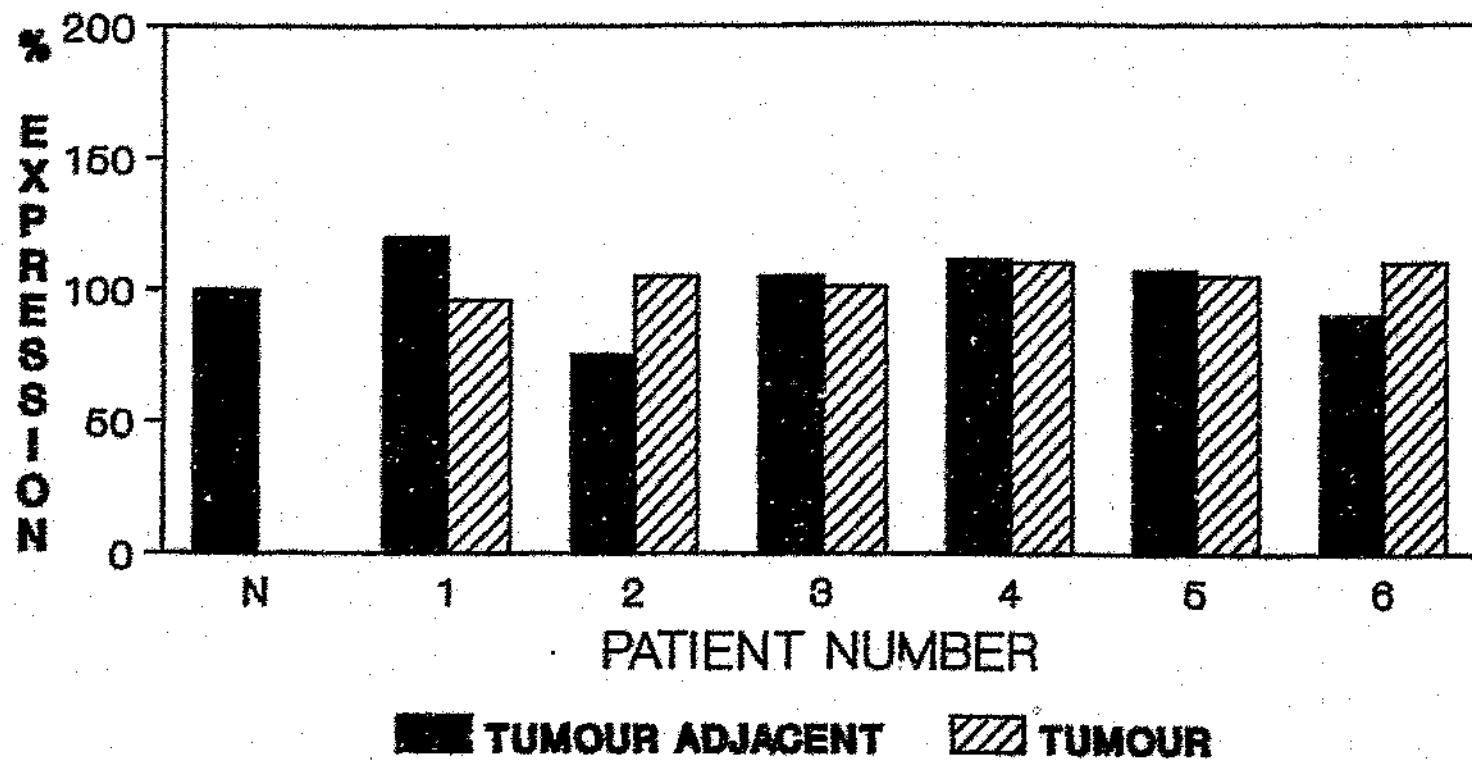


Figure 3.16 A comparison of *c-fos* mRNA concentrations in normal (N), tumour and tumour adjacent liver tissue from the same six patients (1-6) of Figure 3.14. Data were analysed as described in the legend to Figure 3.14.

c-Ha-ras mRNA concentrations in hepatocellular carcinoma



c-Ha-ras mRNA concentrations in hepatocellular carcinoma

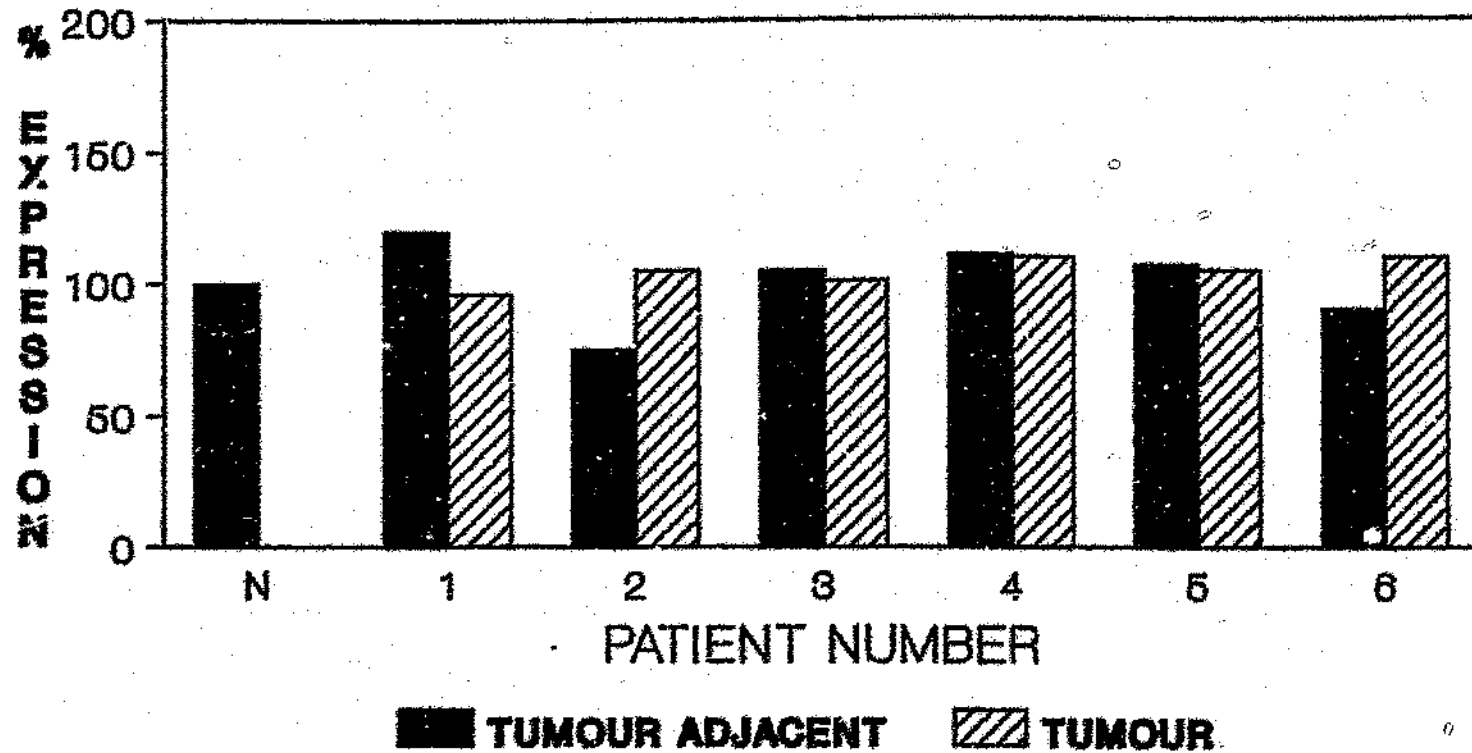


Figure 3.17 A comparison of c-Ha-ras mRNA concentrations in normal (N), tumour and tumour adjacent liver tissue from the same six patients (1-6) of Figure 3.14. Data were analysed as described in the legend to Figure 3.14.

Similar representation of the data obtained when the RNA was probed with the c-Ha-ras probe is given in Figure 3.17. In this case, the intensity of the hybridisation signal was similar regardless of the malignant or benign nature of the tissue which was used as the source for RNA extraction.

Typical examples of Southern blot data from *HindIII* DNA which was probed with the *c-myc*, *c-erbA* and oncogene probes are depicted in Figures 3.18 and 3.19. The essential feature was that the restriction fragments detected were of similar size and amount regardless of their tumour or adjacent normal tissue source. This suggests that there was no rearrangement of these oncogenes which may have resulted from HBV integration. Although an internal hybridisation standard was not used, it appeared that there was no amplification of these oncogenes in the tumours. A consistent feature of liver tumours which arise in association with hepatitis B virus infection is the integration of the DNA of this virus into the genome of the host (Imazeki *et al*, 1987; Shafritz and Kew, 1981; Bréchet *et al*, 1980).

Western blot analysis of proteins extracted from tumour tissues is given in Figure 3.20 and 3.21. Here, it is shown that in hepatocellular carcinoma specimens, the amount of Myc and Fos proteins is elevated.

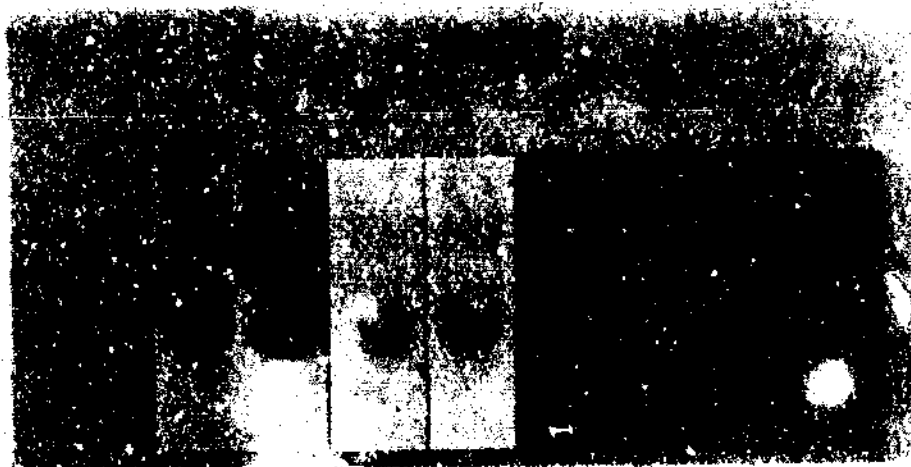


Figure 3.18 Representative Southern blot of DNA extracted from hepatocellular carcinoma (T) and tumour adjacent tissue (N). 10 μ g of DNA was digested with *Hind*III, subjected to electrophoresis and Southern transfer with hybridisation to a radiolabelled c-erbA probe.

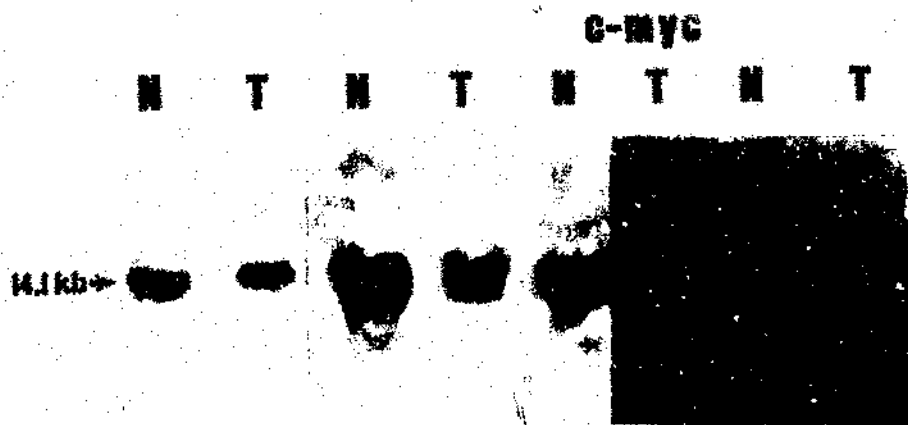


Figure 3.19 Representative Southern blot of DNA extracted from hepatocellular carcinoma (T) and normal tumour adjacent tissue (N). DNA processed as described in the legend to Figure 3.18 was probed with a radiolabelled *c-myc* probe.

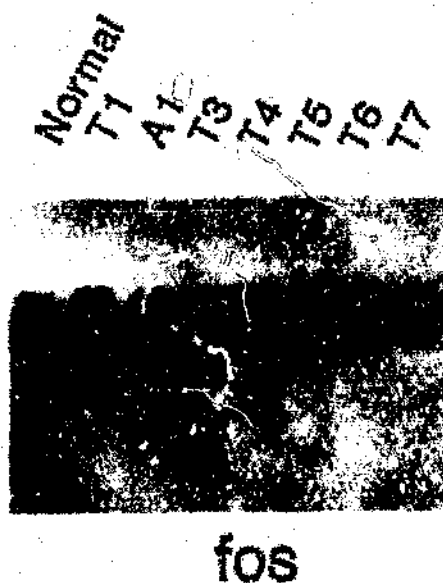


Figure 3.20 Typical Western blot data from normal, tumour adjacent (A) and tumour (T) tissue. The protein samples extracted from the various tissues were probed with a Fos protein antibody. Detection of this bound antibody was by using ^{125}I -labelled protein A and autoradiography.

Normal
T1 A1 T3 T4 T5 T6 T7



myc

Figure 3.21 Typical Western blot data from normal, tumour adjacent (A) and tumour (T) tissue. The protein samples extracted from the various tissues were probed with a Myc protein antibody. Detection of this bound antibody was by using ^{125}I -labelled protein A and autoradiography.



Figure 3.22 Coomassie blue stained polyacrylamide gel of liver protein samples (see legend of Figure 3.20) separated after electrophoresis. This gel represents a duplicate of those used in Figures 3.20 and 3.21 for Western blot analysis.

Figure 3.22 confirmed that the amount of protein from each of the tissue specimens which was loaded onto the polyacrylamide gels was similar. This Figure represents a typical Coomassie blue stained polyacrylamide gel of liver protein samples prior to electroblotting. The gel shown in Figure 3.22 is a duplicate of those which were analysed by Western blotting depicted in Figures 3.20 and 3.21. Quantitation of these Western blot data by densitometric scanning of the autoradiographs is given in Figures 3.23 and 3.24.

Composite graphical comparison of *c-myc* and *c-fos* mRNA and protein concentrations in some of the tumour specimens is represented in Figures 3.25 and 3.26. The extent of the correlation between the amount of messenger RNA and the oncoprotein content can be seen here.

Myc protein concentrations in hepatocellular carcinoma

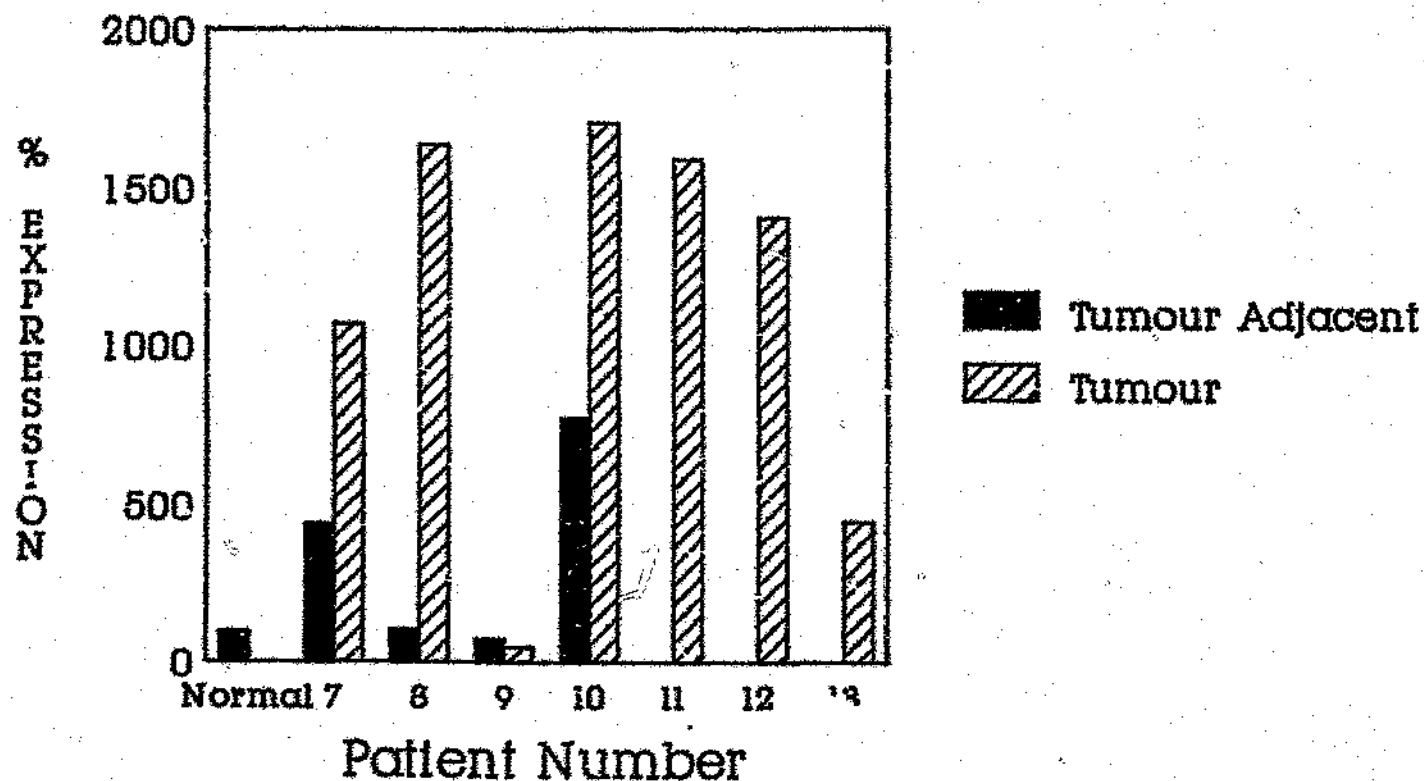


Figure 3.23 Comparison of *c-myc* oncoprotein concentrations in liver tissue from normal donors, tumour adjacent (A) and tumour specimens (T) of patients 7 to 13. Values are expressed relative to the normal (100%). Tumour adjacent tissue was not available from all of the patients studied.

Fos protein concentrations in hepatocellular carcinoma

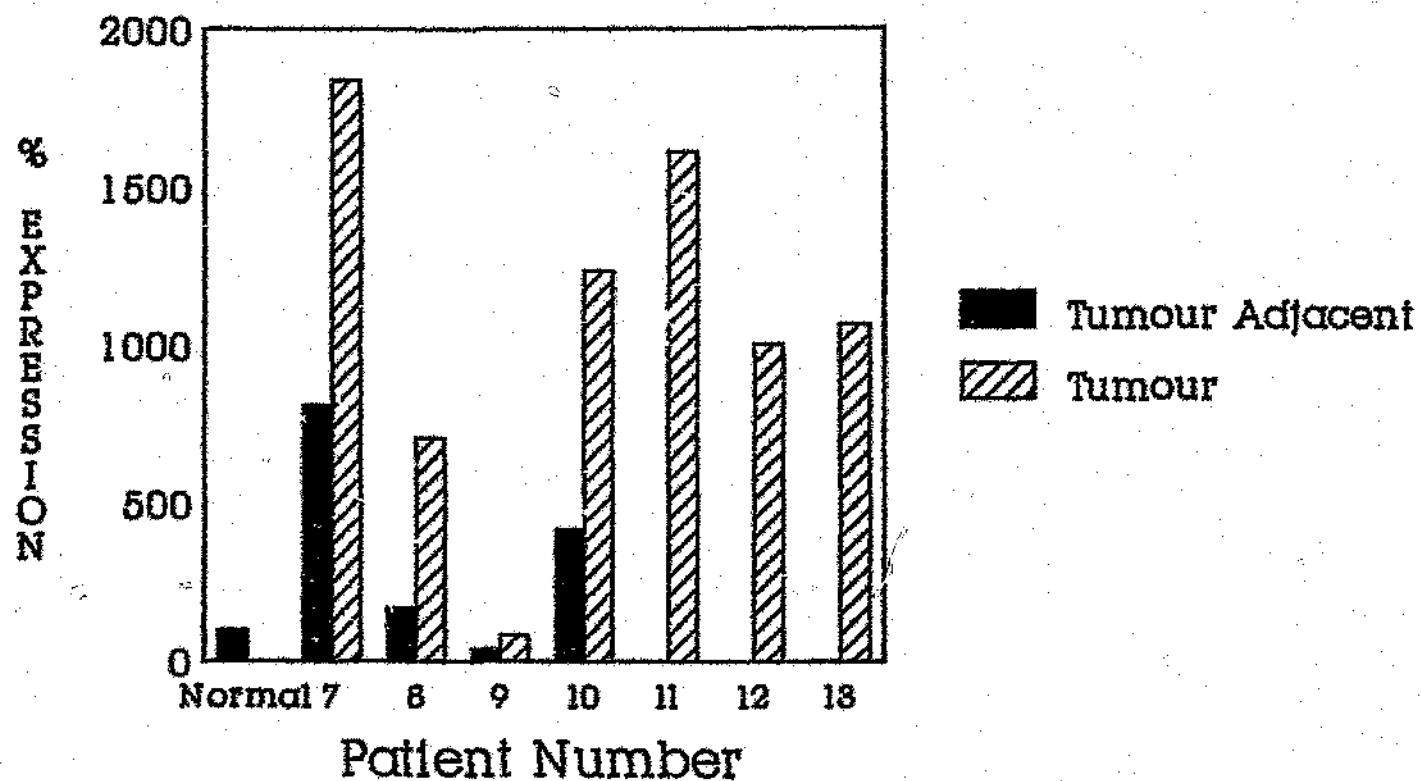


Figure 3.24 Comparison of *c-fos* oncoprotein concentrations in liver tissue from normal donors, tumour adjacent (A) and tumour specimens (T) from patients 7 to 13. Values are expressed relative to the normal (100%). Tumour adjacent tissue was not available from all of the patients studied.

***c-myc* mRNA and protein concentrations in hepatocellular carcinoma**

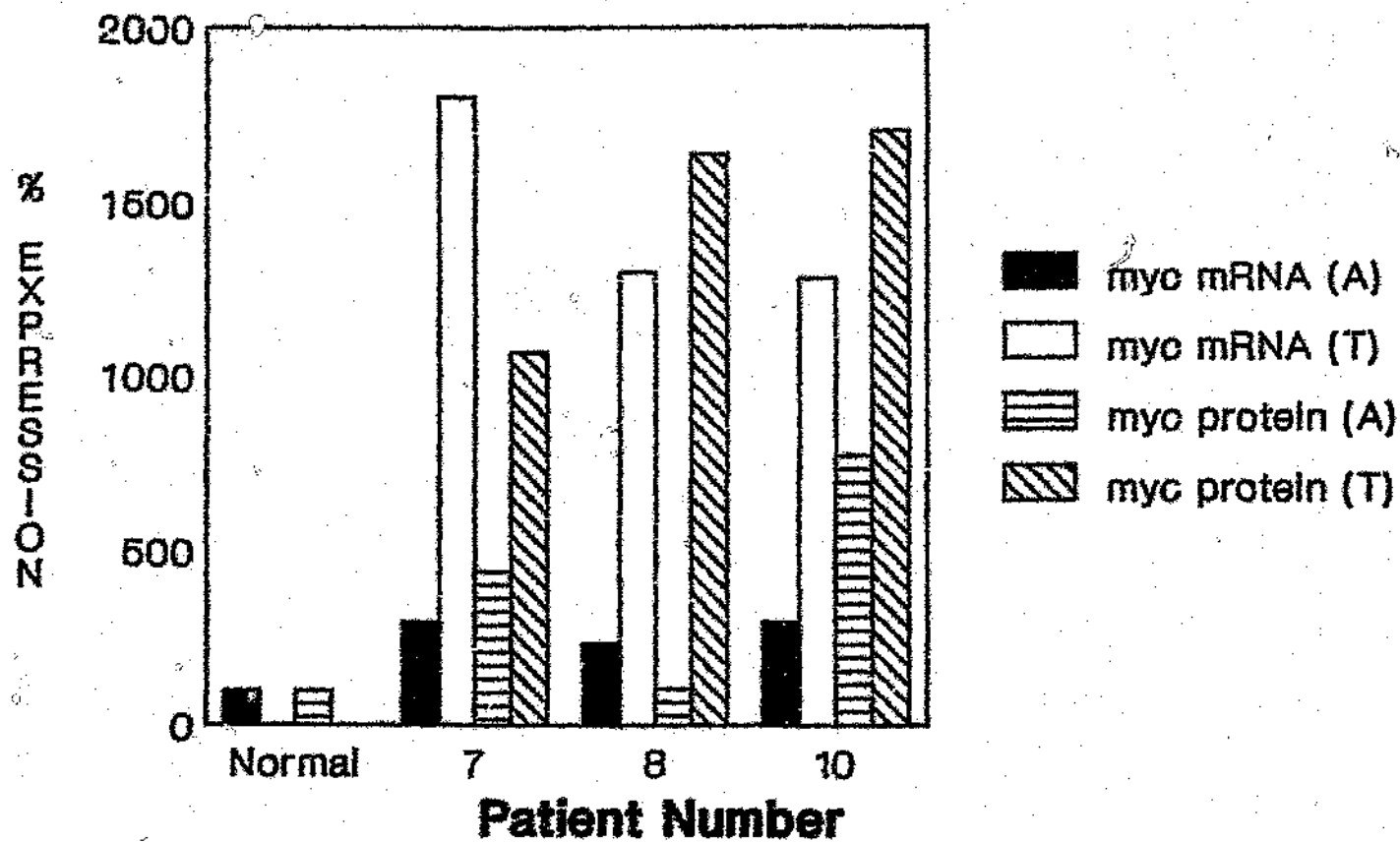


Figure 3.25 Comparison of *c-myc* mRNA and protein concentrations in liver tissue from normal donors, tumour adjacent (A) and tumour (T) tissue from three patients (7 to 10 of Figure 3.23). Values are expressed relative to the normal (100%) and the numbering of the three patients is similar to that given in Figures 3.23 and 3.24.

***c-fos* mRNA and protein concentrations in hepatocellular carcinoma**

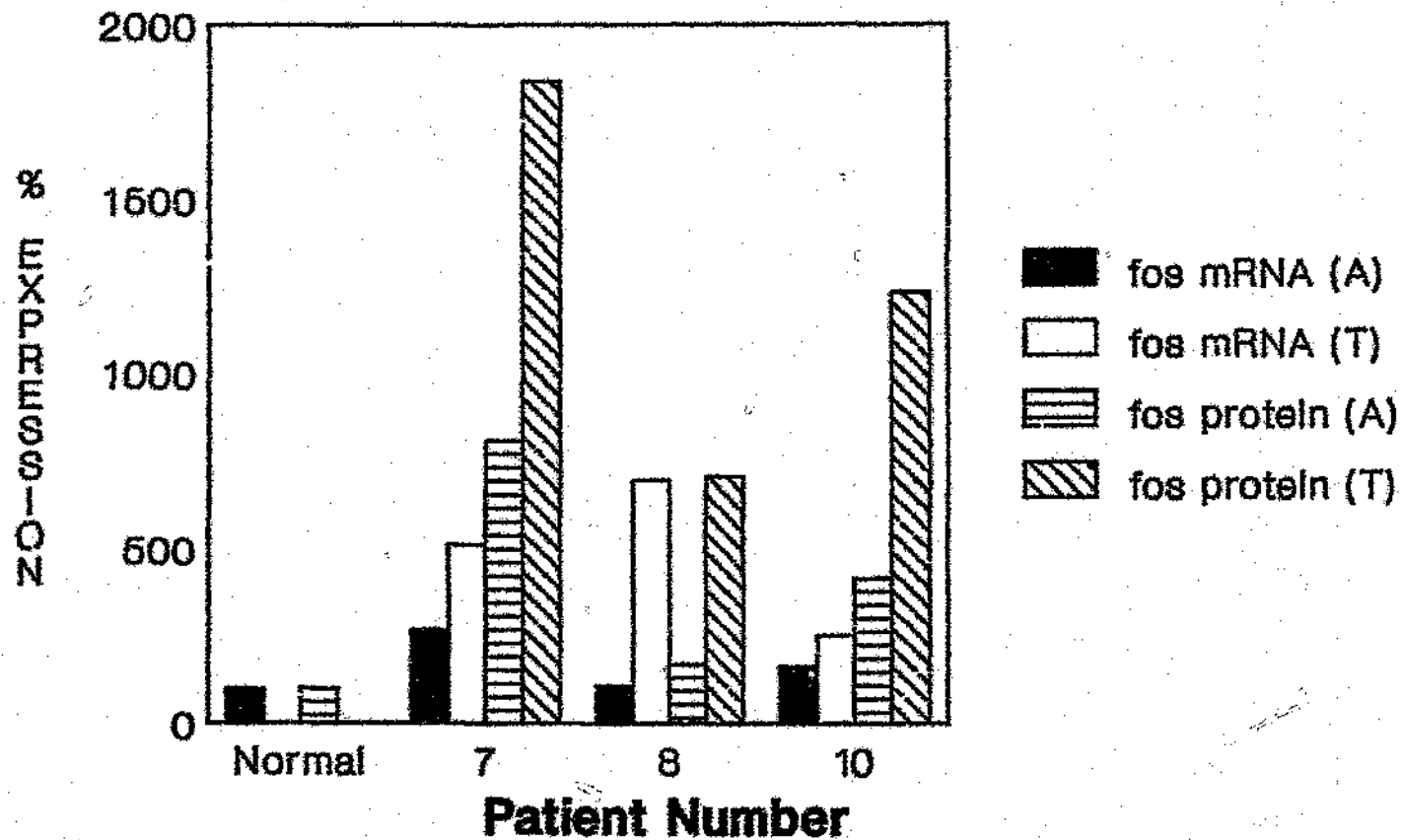


Figure 3.26 Comparison of *c-fos* mRNA and protein concentrations in liver tissue from normal donors, tumour adjacent (A) and tumour (T) tissue from three patients (7 to 10 of Figure 3.24). Values are expressed relative to the normal (100%) and the numbering of the three patients is similar to that given in Figures 3.23 and 3.24.

3.3 DISCUSSION

The finding of elevated oncogene expression in most of the hepatocellular carcinoma specimens studied here supports the idea that their overexpression may be important in the development of this tumour. Despite the abundant evidence for the involvement of HBV in the neoplastic process, no clear mechanism has emerged to explain the process (see Section 1.5). Insertional mutagenesis by integrated HBV DNA has been a popularly invoked theory (Bréchet *et al*, 1981; Shafritz and Kew, 1981; Shafritz *et al*, 1981; Koshy *et al*, 1981) but the sites of HBV integration are not consistent, and HBV DNA does not always integrate at the same site in the host's genome. Although no gross rearrangements of oncogenes were found, HBV integration cannot be definitely excluded as a mechanism for enhancing oncogene expression. (In this study a limited number of restriction endonucleases was used for Southern blot analysis.) In addition, integrated DNA may have long range effects on oncogene transcription.

Elevation of both mRNA and protein from *c-myc* and *c-fos* implies that the activation is at the level of transcription. *c-myc* and *c-fos* may be implicated directly in the process of malignant transformation or alternatively reflect the mitotic status of the tumour cells. *c-myc* and *c-fos* have been recognised as oncogenes which are activated during liver regeneration

(Fausto and Shank, 1983; Fausto and Shark, 1987). Also, in this study, the serum α -fetoprotein concentrations and *c-fos* and *c-myc* expression appeared to correlate with the histological features of tumour differentiation (Table 3.1 and Figures 3.13-14), and the degree of differentiation may correlate with the rate of division of the tumour cells.

A further important point is the fact that the oncoprotein concentrations in the tumour adjacent tissue, determined using the Western blot analysis, were elevated when compared to the totally normal tissue which had been obtained from organ donor patients who had no evidence of liver disease (Figures 3.23 and 3.24). This corroborated the comparison between oncogene mRNA concentrations from tumour, adjacent and normal tissue (Figure 3.11 and Figures 3.14 to 3.16). Elevated oncogene expression in tumour adjacent non-malignant tissue may reflect a pre-neoplastic state (eg cirrhosis or hepatitis) which is part of a multistep hepatocarcinogenic process (Yang *et al.*, 1991). Regenerating liver cells are a feature of hepatitis and cirrhosis of the liver. It is therefore not surprising that elevated *c-myc* and *c-fos* expression was documented in many of the tumour adjacent liver tissue specimens which usually demonstrate features of hepatitis and/or cirrhosis.

Enhanced expression of *c-erbA* in hepatocellular carcinoma may render the cells more sensitive to carcinogenic influences of hormones. *c-erbA* has been shown to have homology to the thyroid hormone receptor (Sap *et al*, 1986; Weinberg *et al*, 1986) and possible hormonal involvement in hepatocarcinogenesis is well documented (Christopherson *et al*, 1975; Porter *et al*, 1987).

The finding of similar tumour and normal *c-Ha-ras* mRNA concentrations suggests that this oncogene may not be involved in the carcinogenic process of the HCC specimens studied here. Small mutations of this gene (such as a point mutation) which do not affect the rate of expression can however not be excluded.

An explanation for the observed increased oncogene expression is that integrated HBV DNA may be producing a transcriptional activator. Possible candidates for trans-activation of the oncogenes are the HBV products of the preS2/S (Kekulé *et al*, 1990) and X-gene (Wollersheim *et al*, 1988) regions. The X-gene is now known to interact with *c-myc* and *c-fos* promoters to increase transcription of these oncogenes (Balsario *et al*, 1991).

3.4 CONCLUSION

It has been proposed that during the development of HCC, there are two important forces which drive the carcinogenic process (Yang *et al*, 1991):

1. the presence of a carcinogenic agent, eg. HBV, and
2. a mitogenic stimulus.

In support of this, it has recently been shown that HCC develops more frequently when associated with increased DNA synthesis (and therefore hepatocyte division) in patients with cirrhosis of the liver (Tarao *et al*, 1992).

A number of hepatocyte mitogens has recently been discovered which may be important in the process of liver regeneration, hepatocarcinogenesis and therefore oncogene activation. They include hepatocyte growth factor (HGF) (Michalopoulos and Zarnegar, 1992), insulin like growth factors (Quin, 1992), transforming growth factors α and β (Castilla *et al*, 1991; Brenner, 1991; Fausto, 1991). An interesting recent finding is that the *c-met* oncogene encodes the cell surface receptor for HGF and is rapidly activated by treatment with HGF (Bottaro *et al* 1991).

Expression of *c-fos* and *c-myc* are elevated during liver regeneration (Fausto

and Shank, 1983; Fausto and Shank, 1987). The finding of increased expression of these oncogenes in the HCC specimens studied here suggests that there may be an elevated rate of hepatocyte proliferation. This idea prompted investigation of the expression of nuclear protooncogenes in cultured cells which are subjected to a mitogenic stimulus. Experimental work to address this is described in Chapter 4.

The possible role of the protein product of the X-gene of the hepatitis B virus as transcriptional activator stimulated interest in this gene. Transcriptional activating properties of the gene had already been demonstrated and have been discussed. The possible eukaryotic origins of the HBV X-gene were investigated, and this is described in chapter 5.

Chapter 4

THE EFFECT OF ALKALINE STIMULATION ON RNA SYNTHESIS IN 3T6 MOUSE FIBROBLASTS

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4. THE EFFECT OF ALKALINE STIMULATION ON RNA SYNTHESIS IN 3T6 MOUSE FIBROBLASTS

4.1 INTRODUCTION

When cells move from a quiescent to an actively dividing state several important controlling events occur. Included is the increased expression of a variety of genes which are thought to play a role in the control of the rate of cell division. Examples of these genes are the nuclear protooncogenes *c-myc*, *c-fos* and *c-jun*. This topic has been discussed in Section 1.3.

An explanation for the overexpression of *c-myc* and *c-fos* in liver tumours (chapter 3) is that there is a higher rate of cell division in the hepatocellular carcinoma specimens. Expression of these oncogenes is increased when cells are stimulated to divide and is a feature of liver cells during regeneration following partial hepatectomy (Fausto and Shank, 1983; Fausto and Shank, 1987). In this chapter, an investigation was made of the effect of alkaline mitogenic treatment of cultured mouse 3T6 fibroblasts on nuclear protooncogene expression.

4.1.1 INTRACELLULAR ALKALINISATION AND THE STIMULATION OF QUIESCENT CELLS TO DIVIDE

The cellular reactions that occur after the addition of a growth factor to cells are complex, and it has therefore been difficult to determine exactly which of the reactions mediate the growth factor response. For example, the tyrosine kinase family of growth factor receptors appear to be important in the transition of cells from a quiescent to actively dividing state. Many of the receptors in this family also stimulate other cellular functions such as migration, changes in shape, production of extracellular matrix (Williams *et al*, 1991). As a result of this complexity of the cellular response to growth factor stimulation, it has been difficult to determine exactly which of the reactions mediate the growth factor response.

One of the earliest effects of growth factors on quiescent cells appears to be the activation of the Na^+/H^+ exchanger (Pouyssegur *et al*, 1982; Moolenaar *et al*, 1982; Pouyssegur, 1987). It has been suggested that the subsequent Na^+ influx and intracellular alkalinisation may be the principal factors involved in the sequence of events initiated by growth factor-target cell interaction (Koch and Leffert, 1979; Rozengurt, 1981; Pouyssegur *et al*, 1982; Moolenaar *et al*, 1983; Rothenberg *et al*, 1983; L'Allemain *et al*,

1984; Muldoon *et al*, 1985; Pouyssegur *et al*, 1985). Moreover, the blocking of this antiporter with amiloride (L'Allemain *et al*, 1984b) inhibits growth factor-induced DNA synthesis. Pouyssegur *et al* (1984) have also demonstrated the inability of mutant mammalian cells which lack the Na^+/H^+ exchange activity, to grow in neutral or acidic pH, whereas at an external alkaline pH they grow at a normal rate.

Furthermore, it has been shown (Bravo and MacDonald-Bravo, 1986) that the progression of cells to the S-phase of the cell cycle is highly sensitive to the external pH. Zetterberg and Larson (1981) have demonstrated that after 5 minutes of treatment with culture medium at a pH of 9.5, 3T3 mouse fibroblasts can be induced to undergo DNA synthesis and mitosis under conditions of starvation. Although not established, the mechanism of the mitogenic stimulation is likely to be by intracellular alkalisation (Zetterberg *et al*, 1982).

Kinetic analysis of 3T3 mouse fibroblasts treated briefly with alkaline medium demonstrates that the progression of cells to the S-phase occurs at a similar time to that after foetal calf serum stimulation. The time taken to enter the S-phase after stimulation is approximately 12 hours (Zetterberg *et al*, 1982).

4.1.2 ANALYSIS OF GENE EXPRESSION IN CULTURED 3T6 MOUSE FIBROBLASTS AFTER ALKALINE TREATMENT

A useful model for the study of gene expression during cell proliferation is that of using cultured 3T3 or 3T6 mouse fibroblasts. These cells can be induced to enter a quiescent state by depriving their medium of foetal calf serum (containing growth factors). They can then be induced to enter the S phase of the cell cycle synchronously by the addition of the appropriate growth factors or a higher concentration of foetal calf serum (Stiles *et al*, 1979). A convenient approach (used here and in many of the previously described experiments) to the understanding of the genetic basis of cell proliferation is to identify the genes whose expression is affected by the exposure to the growth factor stimulus.

An advantage of using the alkaline treatment of cells as a mitogenic stimulus is that cells can be maintained under conditions of relative starvation. The numerous effects of foetal calf serum *per se* can thus be minimised. Also, this experimental model allows assessment of increased pH, as being a trigger required for stimulation of oncogene expression.

Two approaches have been used to characterise some of the transcription

responses of 3T6 mouse embryo fibroblasts to an alkaline mitogenic stimulus. The first was an analysis of total cellular RNA synthesis. Use was made of the pulse labelling technique described in the Methods and Materials Section (2.3.2). The second more specific approach was to use mRNA hybridisation in order to analyse *c-myc*, *c-fos* and *c-jun* expression in 3T6 mouse fibroblasts after treatment with alkaline medium as a mitogenic stimulus

4.2 RESULTS AND DISCUSSION

4.2.1 TOTAL RNA SYNTHESIS FOLLOWING ALKALINE STIMULATION OF 3T6 CELLS

Figure 4.1 is a depiction of the fluorograph which was obtained after pulse labelling to assess changes in the synthesis of total RNA following alkaline stimulation. Visual comparison shows that there are no major changes in the bands which are visible on the autoradiograph, and this was corroborated by densitometric scanning of the autoradiographs.

Figure 4.2 shows a gel similar to that depicted in Figure 4.1 which had been stained with silver and therefore represents changes in total RNA. As with Figure 4.1, there were no obvious differences between the lanes.

A limitation of this set of experiments was the relative insensitivity of detection of the changes which may occur in the expression of specific RNA species. Also, the electrophoretic separation technique used, that of 5% polyacrylamide gel electrophoresis, does not resolve large RNA molecules (approximately larger than 18S ribosomal RNA).

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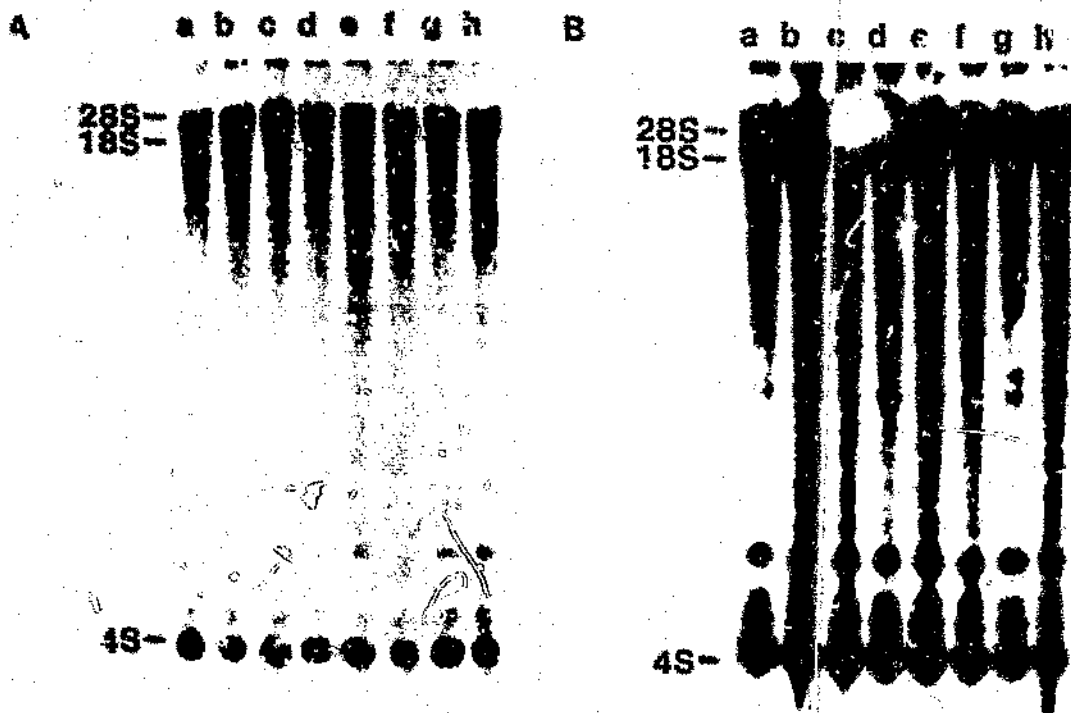


Figure 4.1 Fluorograph of radiolabelled RNA synthesised after alkaline stimulation and separation on 5% polyacrylamide gel electrophoresis. Lanes from left to right (a-h) represent RNA extracted from unstimulated cells (lane a) and at two hour intervals up to 14 hours after alkaline stimulation (lanes b-h). In each case, pulsing with ^3H -uridine was for the final two hours of the incubation after the alkaline treatment. Details of this procedure and the processing of the gels for fluorography is outlined in Section 2.3 of the Materials and Methods. The autoradiograph depicted in A was after 2 days exposure, and that in B was after 3 weeks exposure.

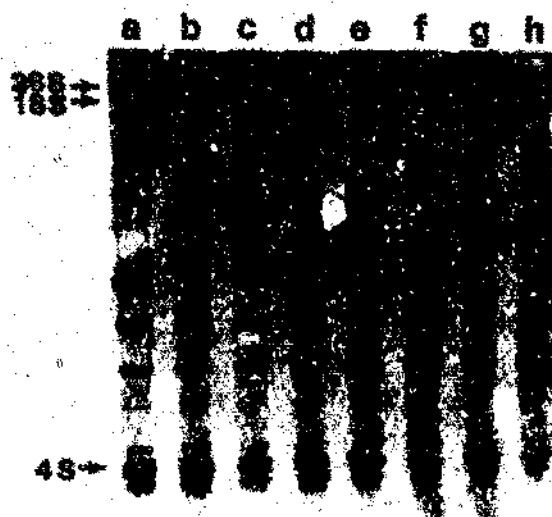


Figure 4.2 Silver stained 5% polyacrylamide gel of 3T6 mouse fibroblast RNA separated by electrophoresis. Lane a represents the control where cells had not been stimulated by alkaline treatment. Lanes b to h represent the separated RNA from 3T6 cells which had been stimulated with alkaline medium and then harvested at 2 hour intervals 2 to 14 hours thereafter (lanes b to h).

4.2.2 ONCOGENE EXPRESSION FOLLOWING ALKALINE STIMULATION

A graphical representation of the time course of the expression of the three oncogenes studied (*c-myc*, *c-fos* and *c-jun*) after alkaline stimulation is given in the Figures 4.3 to 4.5. Oncogene expression (hybridisation ratio) is expressed as a function of the ratio of the intensity of the hybridisation signal (detected by densitometric scanning) to the intensity of the analysed stained image of the 18S ribosomal RNA detected on the nitrocellulose membranes of the Northern blots. The values obtained at each of the time periods represent the difference in ratios obtained for the stimulated and unstimulated cells. These data were obtained from the analysis of RNA extracted from the pooled cells of three independently treated culture flasks. The details of the procedures of analysis have been outlined in Methods Section 2.3.

An important consideration when working with cells in culture, and analysing their response to a stimulus is that the process of changing of culture medium itself may result in the stimulation of various genes such as the ones studied here. The data presented in Figures 4.3 to 4.5 take this into account. The presented data represent the difference between the hybridisation signal detected

***c-myc* mRNA synthesis after alkaline stimulation**

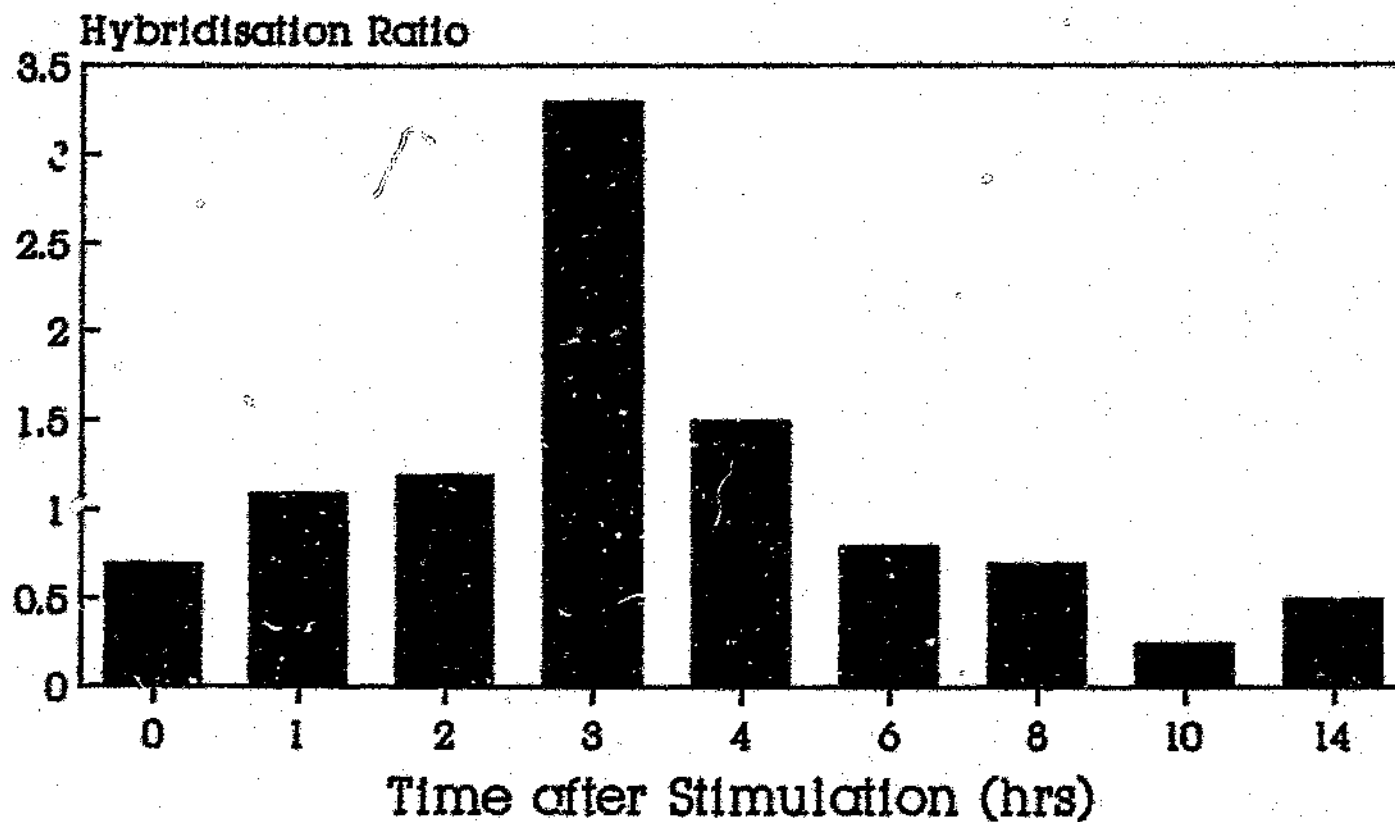


Figure 4.3 Time course of *c-myc* mRNA levels detected after treatment of cultured 3T6 mouse fibroblasts with alkaline medium. RNA extracted from the cultured cells at two hour intervals after alkaline treatment was subjected to Northern blot hybridisation analysis. The signal detected (by densitometric scanning) was expressed as a ratio to the amount of 18S ribosomal RNA detected on the nitrocellulose blots after staining with methylene blue. The data were expressed as the difference between the signal detected after alkaline stimulation and that after changing the culture medium in the control cells.

***c-fos* mRNA synthesis after Alkaline stimulation**

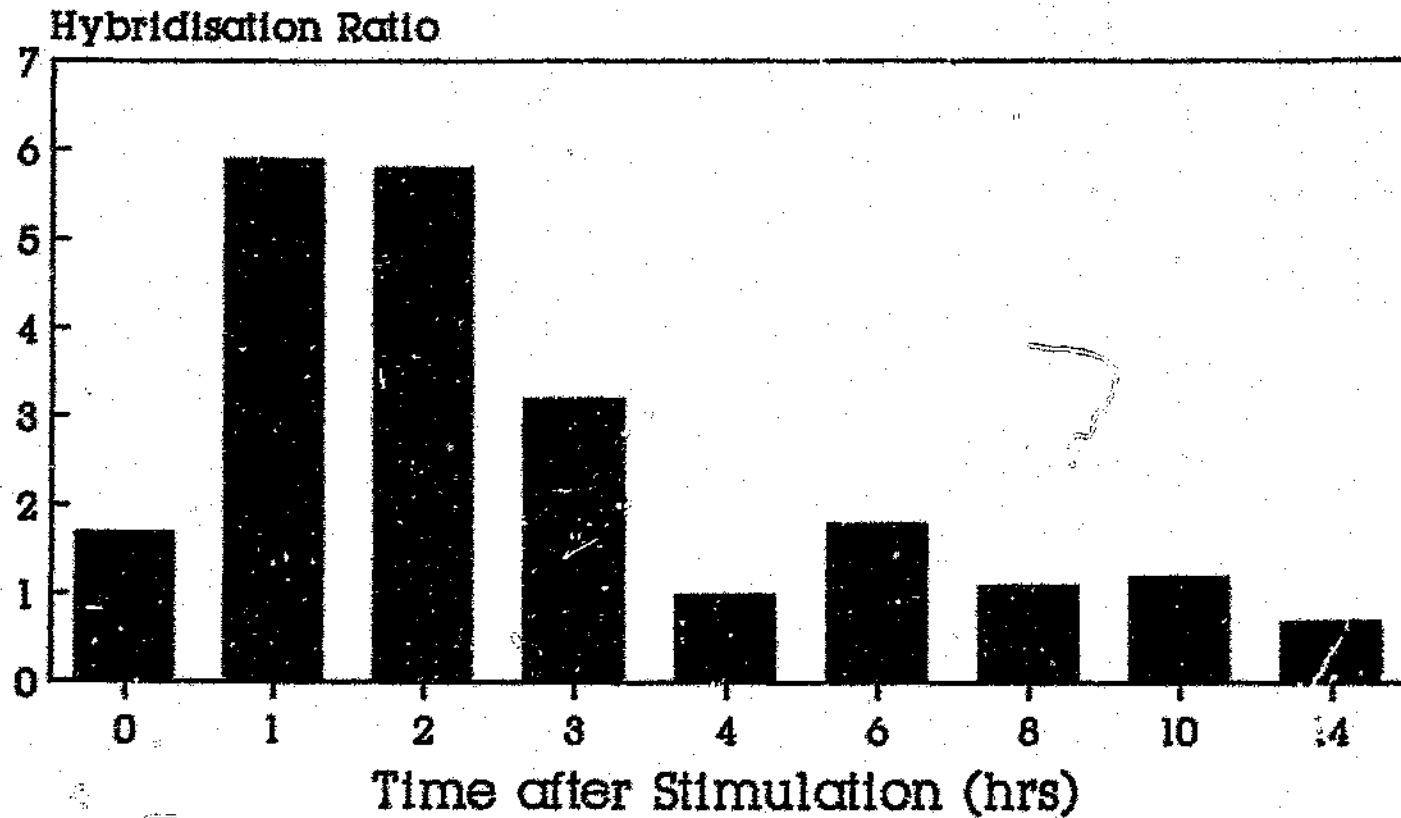


Figure 4.4 Time course of *c-fos* mRNA concentrations detected in 3T6 mouse fibroblasts after treatment of the cells with alkaline medium. The processing of the data was as described in the legend to figure 4.3.

***c-jun* mRNA synthesis after Alkaline stimulation**

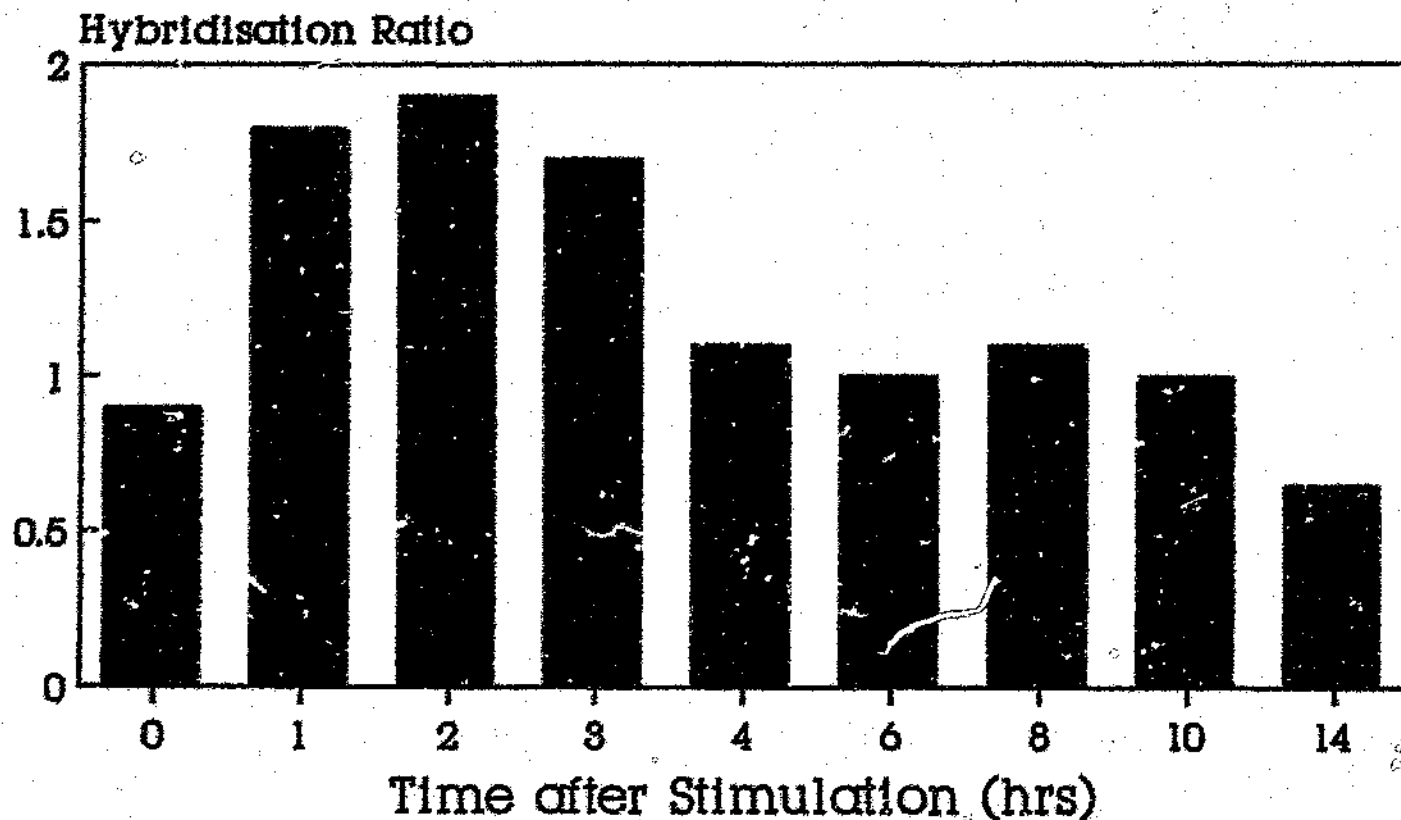


Figure 4.5 Time course of *c-jun* mRNA concentrations detected in 3T6 mouse fibroblasts after treatment of the cells with alkaline medium. The processing of the data was as described in the legend to figure 4.3.

after alkaline stimulation and that obtained after changing the medium in the control cells.

The expression of the three oncogenes was found to be elevated following alkaline treatment. Increased expression of *c-fos* and *c-jun* occurs 1 to 2 hours after the stimulation (Figures 4.4 and 4.5). Elevation of *c-fos* expression is greater than that of *c-jun*. The diagram (Figure 4.4) shows that the increase in *c-fos* expression is approximately 5-fold at its maximum, whereas that of *c-jun* is only 2-3 fold increased. Both *c-fos* and *c-jun* mRNA concentrations reach the baseline (level at time 0 hours) approximately 4 hours after stimulation. This is similar to the time course of *c-fos* and *c-jun* mRNA concentrations after growth factor stimulation (Denhardt *et al*, 1986; Almendral *et al*, 1988; Nakabeppu *et al*, 1988).

The increase in the expression of *c-myc* occurs at a later stage after the alkaline stimulus (Figure 4.3). This diagram shows maximum expression at the time 3-5 hours after stimulation and the baseline level is reached 6 hours after stimulation (Figure 4.3).

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is, the expression of *c-fos* and *c-jun* do appear to parallel each other with respect to the time at which their expression is at a maximum (Nakabeppu *et al*, 1988). Furthermore, the increase in the expression of these two particular oncogenes occurs soon after the growth factor stimulation (Kruljer *et al*, 1984), whereas the expression of *c-myc* occurs at a later stage of the G1 phase of the cell cycle (Morgan and Pledger, 1989; Persson *et al*, 1986).

The previously documented 20 to 40 fold increases in the concentrations of *c-myc* mRNA following treatment with a growth factor (Denhardt *et al*, 1986) were not demonstrated using alkaline treatment. Here, the increase was only approximately 3.5 fold (Figure 4.3). The most important reason for these differences may be that these experimental data were analysed differently (taking into account the effects of changing of culture medium - see page 163) to those of the report by Denhardt *et al* (1986). Also, alkaline treatment is likely to have 'non-mitogenic', and perhaps deleterious, effects on cells which indirectly influence oncogene expression.

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4.3 CONCLUSION

Evidence has been presented here which further implicates *c-myc*, *c-fos* and *c-jun* in the regulation of the cell division process. The expression of these genes has been shown to be increased following mitogenic stimulation with alkaline medium. Growth factors act on a Na^+/H^+ antiporter to increase intracellular pH (pH_i), and this is thought to be important in the mitogenic response (Koch and Leffert, 1979; Rozengurt, 1981; Pouyssegur *et al.*, 1982; Moolenaar *et al.*, 1983; Rothenberg *et al.*, 1983; L'Allemain *et al.*, 1984; Muldoon *et al.*, 1985; Pouyssegur *et al.*, 1985). Mitogenic stimulation of cells by their treatment with alkali is likely to increase pH_i and thereby have the mitogenic effect. The data presented suggest that an increased pH_i *per se* precedes the expression of the cellular oncogenes *c-myc*, *c-fos* and *c-jun*, and intracellular alkalinisation is likely to be an obligatory step in the cell stimulation pathway.

Alkaline treatment of cells may bypass the normally occurring steps in the cascade of events leading from growth factor stimulation to the initiation of DNA synthesis. The induction of cells to enter the S phase of the cell cycle appears to be independent of progression and competence factors. The alkaline mitogenic stimulus can be carried out under conditions of

relative starvation where both PDGF and PPP factors are limited in the culture medium (Zetterberg and Larson, 1981; Zetterberg *et al.*, 1982). Increased expression of *c-jun*, *c-fos* and *c-myc* (competence genes) suggests that in the system used here, alkaline stimulation may at least have effects similar to competence factors.

These data suggest that increased pH_i is the culmination of actions of growth factors which trigger the increase in expression of cellular protooncogenes. By treating cells with alkaline medium, the normally occurring steps in the pathway may be 'short circuited'. It therefore does not appear that increased pH_i is a coincidental permissive effect of the mitogenic treatment of cultured cells.

The mechanism of oncogene activation in hepatocellular carcinoma is uncertain. Their expression may reflect stimulation of liver cancer cells to divide in a similar way to the induction of oncogene expression in stimulated cultured cells. Alternatively, the expression of the nuclear protooncogenes may be a result of transcriptional activation by the product of a gene which may be primarily responsible for the hepatocarcinogenic process. Such a gene is the hepatitis B virus X-gene. This gene has been

shown to have transcriptional activating properties of nuclear protooncogenes (Balsano *et al*, 1991).

Chapter 5

ATTEMPTED ISOLATION OF A MAMMALIAN HOMOLOGUE OF THE HEPATITIS B VIRUS X-GENE

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5. ATTEMPTED ISOLATION OF A MAMMALIAN HOMOLOGUE OF THE HEPATITIS B VIRUS X-GENE

5.1 INTRODUCTION

A general representation of the genetic organisation of the hepatitis B virus genome has been given in the Introduction (Figure 1.3). In addition to the pre S/S (encoding the three envelope proteins), the C (encoding the major core protein, P22c) and the P regions (encoding a DNA polymerase - reverse transcriptase), a fourth open reading frame (ORF), the X-region, codes a protein (HBx) of 154 amino acids with a molecular mass of 17 kDa (Tiollais *et al*, 1985; Chisaka *et al*, 1987; Siddiqui *et al*, 1987; Levrero *et al*, 1990). This gene appears to play an important role in the life cycle of hepatitis B virus. The inability of viruses with mutations in the X-gene to grow in animal hosts (Colgrove *et al*, 1989) and the high degree of conservation of this gene amongst hepadnaviruses suggest this (Lo *et al*, 1988).

The HBx protein has protein kinase activity (Wu *et al*, 1990) and been found to have transcriptional activating properties (Tsu and Schloemer, 1987; Auferio and Schneider 1990; Wollersheim *et al*, 1988). Activity as

a serine protease inhibitor has also recently been documented (Koike, 1992). The proteolytic and protein kinase activities may be important in regulating the interaction between cellular transcription factors and the HBx-responsive DNA elements (Wu *et al*, 1990; Koike, 1992).

There is significant evidence implicating the X-gene in development of hepatocellular carcinoma. Transgenic mice derived from germline cells with the X-gene incorporated progressively develop histopathological changes which ultimately lead to liver malignancy (Kim *et al*, 1991). Transcriptional activation of the cellular oncogenes *c-fos* and *c-myc* by the HBx protein may be important in the development of hepatocellular malignancy (Balsano *et al*, 1991).

An important and striking feature about the genetic organisation of the hepatitis B virus genome is its compact structure. This is achieved by the overlapping ORFs which collectively form the genes of HBV (Miller *et al*, 1989).

There is speculation about the mechanism by which HBV evolved this complex genetic organisation. Computer analysis indicates that the long overlapping surface and polymerase ORFs may have evolved from a common ancestral

genetic element which gave rise to retroviruses and retrovirus-like transposons (Robinson *et al*, 1987).

5.1.1 HEPADNAVIRUS AND RETROVIRUS GENOME HOMOLOGY

The small size of HBV (3.2kb) makes it especially amenable to detailed computer analysis of its sequence and comparison to other known DNA sequences of retroviruses. It has been found that there is a number of conserved sequences of hepadnaviruses which also share homology to corresponding regions of the genomes of retroviruses (Miller and Robinson, 1988; Robinson *et al*, 1987; Miller *et al*, 1989). These similarities are summarised below.

The most highly conserved region amongst hepadnavirus strains is a 111 nucleotide sequence extending from the 3' end of DR-1 into the C-region (see Figure 1.3). Of 11 mammalian hepadnaviruses studied, the degree of homology over the entire sequence is more than 95%, while the degree of conservation amongst the first 50 bases of this sequence is 99% (*ibid*).

This sequence has also been found to have most homology with retroviral sequences. This similarity exists between the first 66 nucleotides of this

sequence and the U-5 sequence within the long terminal repeats (LTRs) of certain retroviruses. Hepadnaviruses however do not contain sequences which are functionally identical to the LTRs of retroviruses. The retroviruses with the highest degree of homology were found to be the type C murine leukaemia/sarcoma viruses and human and simian retrovirus like elements (*ibid*).

Another area of homology between hepadnaviruses and retroviruses is in the core gene. This gene is highly conserved amongst the hepadnavirus strains. Furthermore, it demonstrates homology with the P-30 *gag* protein of the type-C murine leukaemia/sarcoma viruses. The proteins encoded by the hepadnavirus C gene and the retrovirus *gag* genes are functionally similar. The homology between a 98 amino acid sequence of the carboxyl terminals of the core and P-30 *gag* proteins is approximately 41%. This region of homology contains basic amino acids suggesting that these areas of the proteins may be involved in nucleic acid binding (*ibid*).

A third area of homology between the hepadnavirus genes and retrovirus genes is in the predicted amino acid sequences of the polymerase (P) gene of hepadnaviruses and the *pol* gene of retroviruses. There is an approximately 40% degree of homology in a 94 amino acid sequence in the

mid region of these genes (*ibid*).

The areas of significant sequence as well as functional homology between hepadnaviruses and retroviruses suggests a common ancestral origin (Miller and Robinson, 1986; Robinson *et al*, 1987; Miller *et al*, 1989).

5.1.2 CODON PREFERENCE OF HEPADNAVIRUS GENES

It has long been known that the genetic code is degenerate in that a number of different triplet codons can code for a single amino acid. This was revealed by work done in the early 1960s which determined the codons which correspond to particular amino acids (Nirenberg and Matthaei, 1961; Nirenberg and Leder, 1964; Nishimura *et al*, 1965; Crick, 1966). The variation occurs in the third base position of codons and up to four different bases may occur in this position for a particular amino acid.

It has been found that certain organisms appear to have different codon preferences and the codon usage is not random (Ikemura and Ozeki, 1982; Grosjean and Fiers, 1982). That is, the bases which occur in the third position of a codon have preponderances depending on the source of the gene. For example, C and G occur more frequently in the third position of

a codon of a eukaryotic gene than do A and U. In eukaryotic viruses, the converse is true (Wain-Hobson *et al*, 1981).

This bias of specific bases in the third position of codons has significance in attempting to determine the origins of genes which are thought to have arisen through a recombinational event between different organisms.

The core, polymerase and surface genes of the hepadnaviruses have all been found to have a third base preference which is in keeping with viruses of eukaryotic cells (Robinson *et al*, 1987). That is, the bases found at the third position of codons of these genes is more commonly A and U rather than C and G. The codon usage of the X-gene is different. In this gene, G and C are preferred at the third position of the codon. The X-gene codon preference is unlike that of eukaryotic viruses, and is similar to the preference of eukaryotic and phage and prokaryotic cell genes. These data are summarised in Table 5.1 which has been reproduced from the publication by Robinson *et al* (1987).

This codon preference is a particularly interesting finding in that hepadnaviruses appear to be related to retroviruses. Retroviral oncogenes, like the HBV X-gene, have a eukaryotic cell gene codon preference.

Furthermore, retroviral oncogenes are located in positions relative to the other viral genes which are similar to the location of the X-gene of hepadnaviruses (Robinson *et al* 1987). It has been shown that the retroviral oncogenes were derived from their eukaryotic hosts (Stehelin *et al*, 1976; Scolnick *et al*, 1973). That is, the oncogenes were incorporated into the retroviral genome through a recombinational event during the evolution of the virus. The codon preference of the X-gene of HBV suggests that the X-gene has its origins in a eukaryotic genome and was also incorporated into the hepadnavirus genome at some stage during its evolution.

	Eukaryotic cell genes ^a	Eukaryotic virus genes ^a	Phage and prokaryotic cell genes ^a	Hepadnavirus Genes ^b			
				C	P	S	X
$\frac{A+U}{G+C}$	$\frac{1}{50.7}$	$\frac{9.6}{1}$	$\frac{1}{1.2}$	$\frac{17}{1}$	$\frac{2.5}{1}$	$\frac{1.7}{1}$	$\frac{1}{2.5}$

Table 5.1 Third base codon preference of viral and eukaryotic genes expressed as a ratio of A+U to G+C. This table is reproduced from the publication by Robinson *et al* (1987). The data had previously been presented by (a) Wain-Hobson *et al* (1981) and (b) Miller and Robinson (1985) and is based on the analysis of 10 to 16 sequences for each of the ratios which is represented.

5.1.3 EXPERIMENTAL APPROACH TO THE ISOLATION OF AN X-GENE HOMOLOGUE

Initially, the aim of the experiments was to demonstrate the presence of a eukaryotic sequence/s homologous to the hepadnavirus X-gene by Northern and Southern blot hybridisation analysis of RNA and DNA isolated from a murine source.

Thereafter, two experimental approaches were used in an attempt to isolate an X-like sequence:

- 1 Conventional bacteriophage library screening using plaque lift followed by hybridisation to radiolabelled RNA and DNA probes.
- 2 A PCR based approach using primers designed as described in the following section for the amplification of sequences from mammalian genomic and bacteriophage DNA.

5.1.3.1 Design of Hepadnavirus X-gene Primers to be used for PCR amplification of homologous X-like Mammalian Sequences

The rationale behind the design of the PCR primers was based on the idea that areas of X-gene sequence homology amongst different strains of

hepadnaviruses are the most likely to be conserved in the putative mammalian X-gene progenitor.

Based on a computer comparison of X-gene sequences of 14 hepadnavirus strains, two sense and two antisense primers were designed for amplification of putative X-gene homologues (W.S. Robinson, personal communication). A schematic representation of the orientation of the primers on the X-gene is represented in Figure 5.1. Refer to the Materials and Methods Section 2.5.4 for details concerning the sequences of these primers.

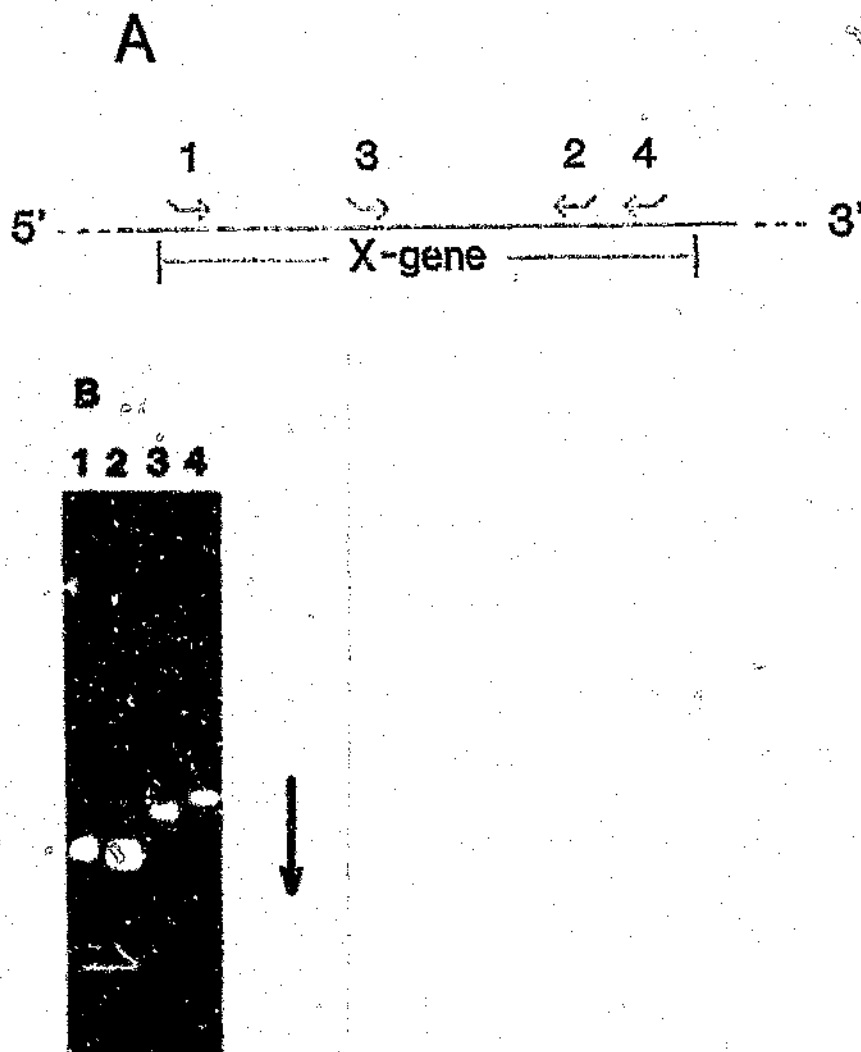


Figure 5.1 A. Schematic representation of the location and orientation of the four X-gene primers used for the amplification of homologous sequences. The primers are represented by the arrows which are pointing to their 3' ends. The linker sequences and the terminal redundancies of the primers are indicated at the 5' ends by the short line which is not parallel to the X-gene.

B. Ethidium bromide stained 0.8% agarose gel confirming the sizes of the fragments amplified using the primers indicated in A from known template. The primer combinations were 3 and 4 (lane B1); 3 and 2 (lane B2); 1 and 2 (lane B3) and 1 and 4 (lane B4). The sizes of the DNA fragments generated are 211bp (lane B1); 183 bp (lane B2); 388 bp (lane B3) and 416 bp (lane B4).

5.2 RESULTS

5.2.1 METHODOLOGICAL CONSIDERATIONS: X-GENE PROBE LABELLING

Three methods of probe labelling were attempted. It was important that the probe be efficiently labelled in order that a mammalian DNA fragment with X-gene homology be identified. Preliminary experiments showed that there was poor complementarity between the derived X-gene fragment and the putative mammalian homologue which was being investigated. This is based on hybridisation experiments which demonstrated that low stringency hybridisation was required in order to detect mammalian sequence/s. This is discussed later. A probe was therefore needed which would efficiently hybridise to the mammalian sequence, and yet not produce an excess of non-specific hybridisation. Spurious binding of the labelled probe would confuse any attempt at identification and isolation of the mammalian homologue of the HBV X-gene fragment.

Initially, the labelling method used was a standard multiprime labelling technique (Feinberg and Vogelstein, 1984). The reaction appeared to be carried out efficiently in that the incorporation of ^{32}P -dCTP into the probe was approximately 40% of the total radioactivity added to the reaction

mixture. However, when this probe was used for hybridisation to Northern blots prepared using RNA extracted from various mouse tissues, absolutely no hybridisation signal was detected. This was despite very low stringency of hybridisation and washing. Hybridisation was carried out at 37°C in a solution containing 40% formamide and the post hybridisation wash was a 15 minute incubation of the blot at room temperature in a 6xSSC solution.

As a first attempt to improve the efficiency of probe labelling, a technique using PCR was used. This method involved a standard PCR amplification reaction where ^{32}P -dCTP was included as one of the precursors. This method generated a highly radioactive probe. The DNA produced after the amplification was of the correct size as assessed by electrophoresis (Figure 5.2).

The PCR labelled X-gene DNA fragment contained almost the entire length of the X-gene (396bp) (see Figure 5.2) and should therefore have a higher T_m than the multiprime labelled probe. The multiprime labelling procedure makes use of randomly generated hexanucleotides which hybridise

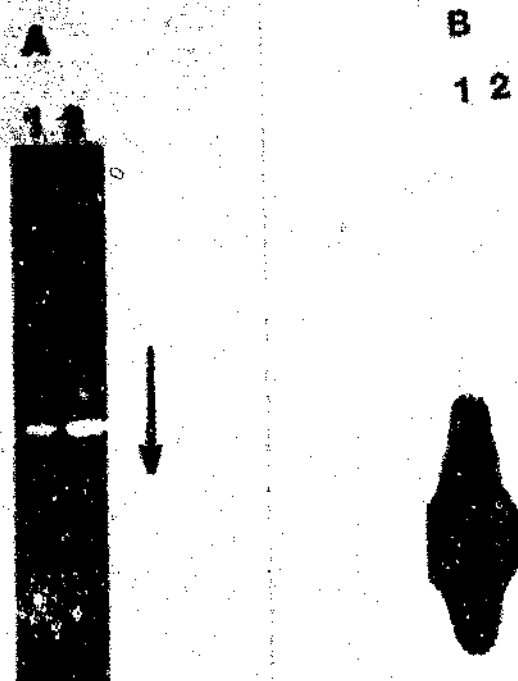


Figure 5.2 A. Ethidium bromide stained X-gene DNA^{in both lanes} prepared using primers 1 and 4 (416 bp; (see Figures 5.1) after separation on a 1.2% agarose gel. ³²P-dCTP was incorporated during amplification of the DNA which is depicted in lane A1. The amount of DNA loaded in each lane was approximately 100 ng, and represented one tenth of the PCR labelling reaction mixture.

B. Autoradiograph (5 minute exposure) of the same gel represented in A.

at a number of sites to the target probe DNA. These are then extended to form longer ligated oligonucleotides which are collectively complementary to the entire probe sequence. The labelled fragments however do not have complementary sequences over the entirety of the template DNA.

The T_m of a hybrid formed between two DNA molecules can be estimated from the following equation (Bolton and McCarthy, 1962):

$$T_m = 81.5^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G+C) - 0.63(\%\text{formamide}) - (600/l)$$

where l is the length of the hybrid in base pairs.

The probe labelled using PCR is longer than the multiprime labelled probes and therefore, the T_m would be higher. The average size of the multiprime probe fragments is not fixed, and is an inverse function of the concentration of the primers (Hogdson and Fisk, 1987). Typically, multiprime probe fragments are up to 50% of the length of the length of the template (Thomas, 1980; Meinkoth and Wahl, 1984). Therefore, under similar conditions of hybridisation, the T_m of the PCR labelled X-gene probe

is approximately 1.5°C higher than the multiprime labelled X-gene probe (600/198 - 600/396).

The number of complementary bases between the X-gene probe and target mammalian DNA is not known. It is therefore not possible to calculate the T_m of the probe hybridising to the target mammalian X-gene homologue using this equation.

The PCR labelled probe appeared to be more efficient than that labelled by the multiprime method. This was assessed by the amount of ^{32}P -dCTP which was incorporated into the labelled product (see Table 5.2). This probe was able to produce a signal when hybridised to Northern blots prepared from mouse tissue RNA. The autoradiograph produced is depicted in Figure 5.3, and the details of the hybridisation conditions and the washing are indicated in the legend. The positive signal is indicated by the arrow. Although at low stringency, the result was produced after a more stringent washing protocol than that using the multiprime labelled probe which produced no signal.

Labeling Method:	Multiprime	PCR	SP6 RNA Polymerase
Probe type	Short multiprimed complementary DNA fragments	Complete length double stranded DNA fragments	Full length single stranded RNA
Approximate % incorporation of ^{32}P -dCTP precursor into probe.	40%	80%	70%
Comparison of Tm hybrids	Lowest Tm. Short labelled DNA strands generated by the extension of random primed hexanucleotides	Higher Tm than multiprime probe - longer probe DNA strands (416 bp) including most of the X-gene sequence.	Highest Tm - almost full-length HBV X-gene single stranded RNA ($\pm$ 400 bases) used as the probe.
Probe stability	Stable	Stable	Subject to RNase degradation

Table 5.2 Comparison of the three methods of X-gene probe labelling which were used for analysis of the putative mammalian homologue of the X-gene - see text (Section 5.2.1) for discussion.

When the PCR labelled probe was used for hybridisation to Southern blots, there was a very faintly visible band on the autoradiograph. DNA of both human and mouse tissue origin gave this signal. The image produced was however of such faint intensity, and not easily distinguishable from the background, that photographic reproduction was not possible.

The third method of probe labelling was SP6 polymerase *in vitro* transcription. The X-gene fragment was cloned into the polylinker site of the SP64 plasmid - that is immediately downstream from the SP6 polymerase promoter.

The protocols used for this cloning experiment have been described in Section 2.4.3 of the Methods Section. The X-gene fragment was amplified using PCR with primers complementary to sequences towards the 5' and 3' ends of the X-gene. The primers were designed so that they contained recognition sites for the restriction endonucleases *Bam*HI and *Xba*I at their 5' ends. The linker sites together with the terminal redundancies were thus incorporated at the ends of the X-gene during the amplification.

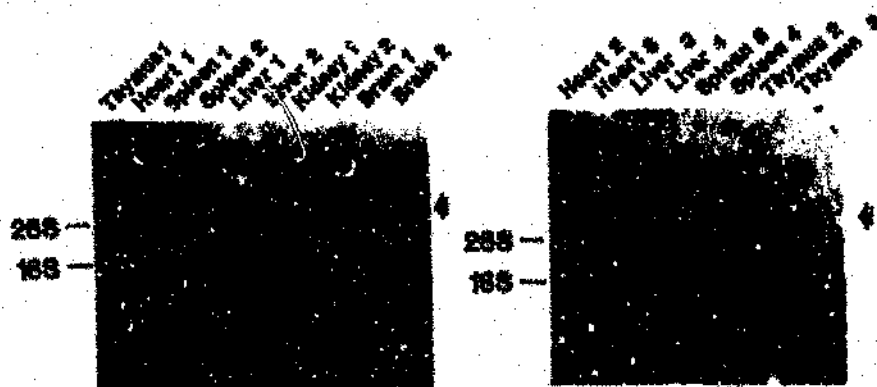


Figure 5.3 Northern blot analysis of RNA extracted from various murine tissues. Total cellular RNA was extracted from the indicated organs then subjected to Northern blotting and hybridisation with the PCR labelled X-gene probe. Hybridisation was carried out in a solution containing 40% formamide at 42°C. The post hybridisation washes were: two of 15 minutes each at room temperature in 6xSSC; followed by two of 15 minutes each at room temperature in 2xSSC and 0.1% SDS; and finally two washes of 15 minutes each at 42°C in 0.2xSSC and 0.1% SDS. Independent but similar hybridisation of two blots is represented in this photograph. The arrows indicate the positions of the putative positively hybridising RNA band.

The X-gene was ligated at the polylinker site of the SP64 plasmid after digestion of both the plasmid and amplified X-gene DNA fragments with *Bam*HI and *Xba*I. Transfection of HB101 *E.coli* bacteria with the recombinant SP64 plasmid conferred ampicillin resistance.

After isolation of a number of bacterial colonies which displayed ampicillin resistance, plasmids were prepared (as described in Section 2.2.5.2) and subjected to restriction enzyme mapping and Southern blot hybridisation with a multiprime labelled X-gene fragment.

Not all of the bacterial colonies which displayed ampicillin resistance were found to have the recombinant SP64 X-gene fragments. This represented about 50% of the colonies which grew on the ampicillin containing plates.

A typical example of a restriction mapping and Southern blot experiment performed in order to confirm the incorporation of the X-gene into the SP64 plasmid is depicted in Figure 5.4. The agarose gels were deliberately overloaded with DNA in order that the small (396 bp) X-gene fragment released by *Bam*HI and *Xba*I digestion be visible after ethidium bromide staining of the gel (Figure 5.4, panel A, lane 2).

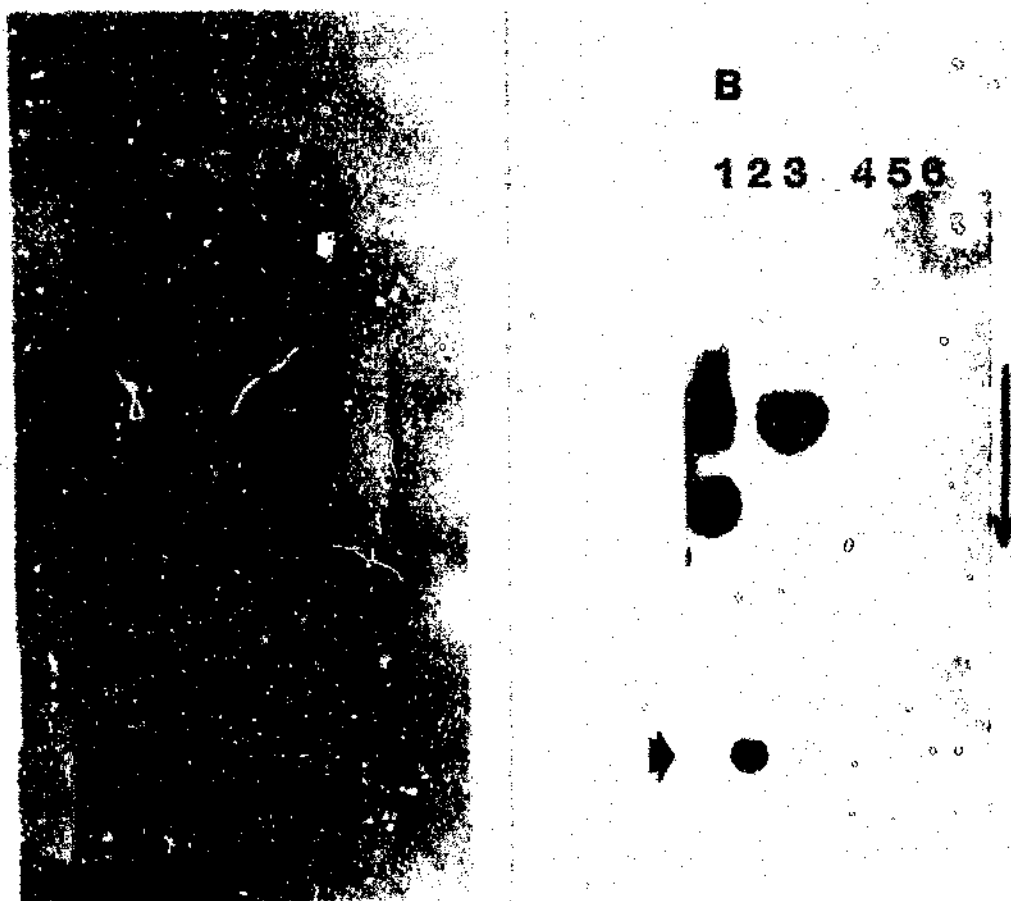


Figure 5.4 Restriction endonuclease mapping and Southern blot hybridisation analysis of recombinant SP64 X-gene plasmid.

A. Ethidium bromide stained 1.2% agarose gel of plasmid DNA which was undigested (lanes A1 and A4), digested with *Bam*HI and *Xba*I (lanes A2 and A5), and *Eco*RI alone (lanes A3 and A6). Lanes A1 to A3 represent an example of a successful plasmid ligation, and lanes A4 to A6 give an example of an unsuccessful recombination.

B. Autoradiograph of the gels indicated in A after Southern blot hybridisation to a multiprime labelled X-gene fragment. The positive hybridisation signal in panel B resulted from the successful incorporation of the 396 bp X-gene fragment (smaller band in lane 2 indicated by the arrow) into the *Bam*HI/*Xba*I digestion site of the SP64 polylinker. The autoradiography data shown in panel B lanes 4 to 6 shows no hybridisation signal and was obtained after similar Southern blot analysis of the SP64 plasmid where X-gene incorporation was unsuccessful.

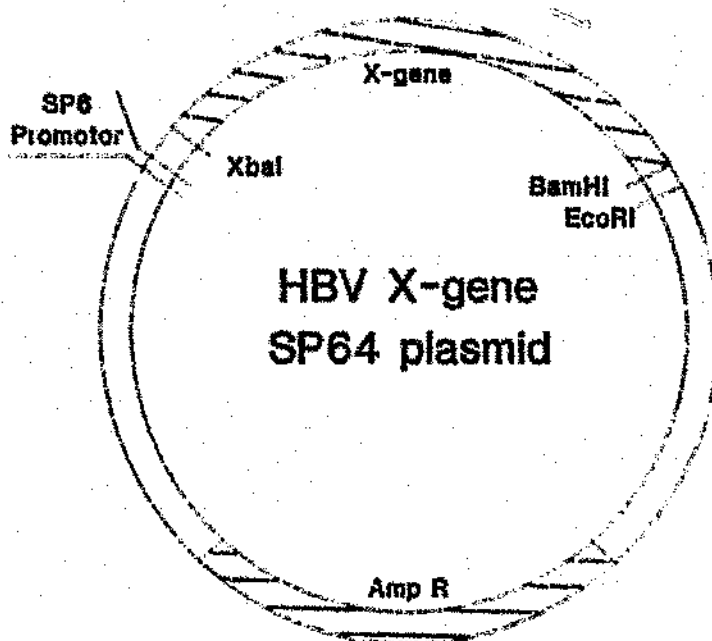


Figure 5.5 Diagrammatic representation of the HBV X-gene recombinant plasmid constructed as described in 2.4.3 of the Materials and Methods Section. The X-gene fragment is represented by the area between the *XbaI* and *BamHI* sites of digestion. The approximate locations of the SP6 RNA polymerase promoter and the ampicillin resistance gene (amp R) are also indicated. The diagram is not drawn to scale as the length of the X-gene fragment is 396 bp and the SP64 plasmid DNA comprises approximately 3000 bp.

Digestion with *EcoRI* has the effect of linearising the plasmid as there is only one site for digestion by this restriction endonuclease. It is located downstream of the site of the X-gene cloning - that is, relative to the SP6 polymerase promoter. The restriction map of the recombinant plasmid is depicted in Figure 5.5. The orientation of the restriction enzyme sites and the primers used to amplify the X-gene DNA resulted in antisense RNA transcript being produced by SP6 polymerase.

The *EcoRI* digested recombinant SP64 plasmid was used as template for the labelling protocol. An example of the electrophoretic separation of the purified linearised plasmid template is given in Figure 5.6. The method of purification was by adsorption to glass powder, and the protocol is outlined in the Methods section 2.2.5.3.

The incorporation of radioactivity after probe labelling was consistently approximately 80% (see Table 5.2). An example of the RNA produced after *in vitro* transcription is depicted in Figure 5.7.

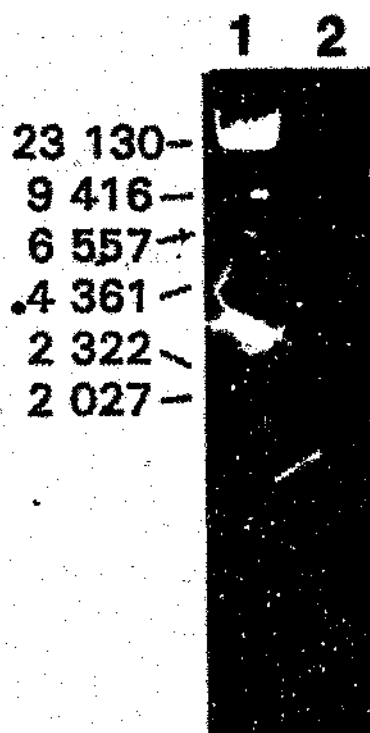


Figure 5.6 Ethidium bromide stained 0.8% agarose gel depicting purified linearised SP64 plasmid. After digestion of the plasmid with *EcoRI* it was purified using glass powder adsorption and then subjected to electrophoresis in order to confirm the purity. Resolution of the molecular weight markers is depicted in lane 1 and the purified linearised plasmid in lane 2. The size of the linear plasmid is 3 401 bp.

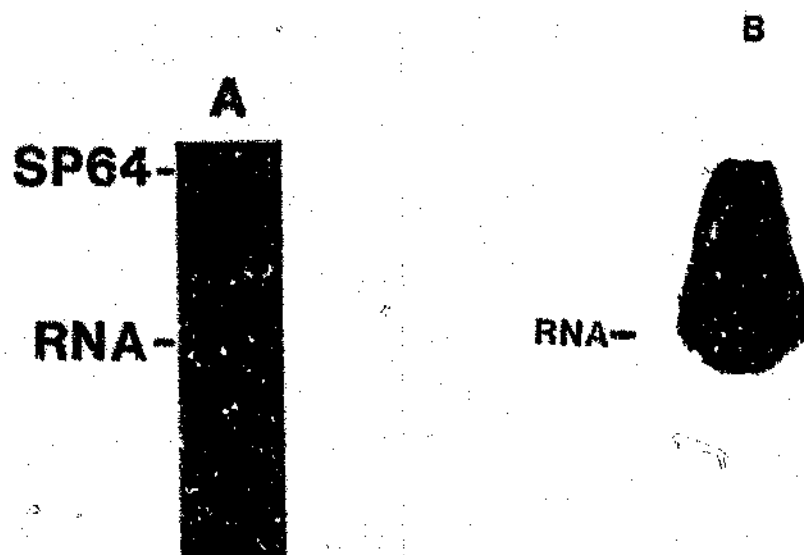


Figure 5.7 5% polyacrylamide gel electrophoretic separation of RNA synthesised *in vitro* using the recombinant SP64 X-gene plasmid as template and SP6 RNA polymerase for transcription. Panel A is an ethidium bromide stained gel of RNA extracted after *in vitro* transcription. The template SP64 DNA and the RNA which is produced are indicated. The size of the RNA is approximately 400 bases. The autoradiograph obtained after a 30 minute exposure of this gel is depicted in panel B.

There are several distinct advantages which this method of probe labelling has over the other two methods used. Firstly, the probe is single stranded and therefore does not hybridise to itself when incubated in a hybridisation solution. During DNA labelling by the symmetric PCR technique, a large amount of double stranded DNA was produced (see Figure 5.2A). When a high concentration of complementary strands of DNA is used in a solution for blot hybridisation, it is likely that many of the strands hybridise to themselves.

The second very important advantage of the use of the SPE polymerase labelled probe is that the melting temperature of a RNA:DNA hybrid is higher than is a DNA:DNA hybrid of the same length. The T_m of a hybrid formed between DNA and RNA can be calculated according to the equation derived by Casey and Davidson (1977):

$$T_m = 79.8^{\circ}\text{C} + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\%G+C) + 11.8(\%G+C)^2 - 0.50(\%\text{formamide}) - (820/l)$$

Comparison of this equation with that for the calculation of a DNA:DNA hybrid (given previously) shows that the T_m of a RNA:DNA hybrid is higher

than that of a DNA:DNA hybrid. For example, the calculated T_m of a hybrid formed between 400 bp DNA molecules comprising 50% G+C in a 40% formamide and 1M Na^+ solution is 79.9°C. That of a RNA:DNA hybrid is 80.8°C, approximately 1°C higher than the DNA:DNA hybrid. Although the number of complementary bases between the X-gene probe and the putative mammalian homologue is not known, the RNA probe would nevertheless have a slightly higher melting temperature than the other probes used - a particularly important property for these experiments. By effectively increasing the melting temperature of the hybrid, the stringency of hybridisation could be increased.

A disadvantage of the use of RNA as a probe is that it is susceptible to degradation by ubiquitous RNases. Careful attention to the elimination of RNase contamination did however reliably produce intact probes.

A comparison of the three methods of probe labelling which have been described is given in Table 5.2.

5.2.2. NORTHERN BLOT ANALYSIS OF RNA EXTRACTED FROM MOUSE TISSUES AND HUMAN HEPATOCELLULAR CARCINOMA

Northern blot analysis of RNA isolated from human hepatocellular carcinoma tissue is depicted in Figure 5.8. The patients investigated both had serological evidence of infection with HBV. There are two bands detectable on the Northern blot, and they represent the characteristic 3.5 kb and 2.1 kb transcripts of HBV (Figure 1.3). They both have RNA sequences complementary to the X-gene. The sequences are detectable in both the tumour and tumour adjacent normal liver tissue. These transcripts have both been described in the Introduction Section 1.5.2.1.

Figure 5.3 shows that when using the PCR labelled probe, a sequence is detectable in mouse RNA which appears to have complementarity to the X-gene. The transcript was large, and this is reflected by its slower electrophoretic mobility than 28S ribosomal RNA. The mice used for these experiments were disease free, and furthermore, HBV is not known to be able to infect mice (Blum *et al*, 1989).

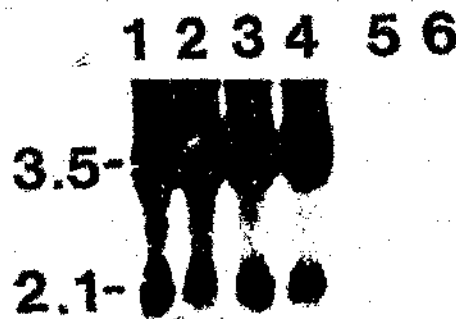


Figure 2.3 Northern blot hybridisation of total cellular RNA isolated from hepatocellular carcinoma (lanes 1 and 3) and adjacent normal tissue (lanes 2 and 4) from two different patients with HBV related liver malignancy. Probing with a multiprime radiolabelled HBV X-gene DNA probe revealed 3.5 kb and 2.1 kb bands. Lanes 5 and 6 represent the result after probing total cellular RNA which was derived from normal liver tissue of donor patients who had no evidence of liver disease. See methods section 2.2.1 for the details concerning the preparation of the tissue specimens.

After a very low stringency wash of 6xSSC at room temperature, the non-specific binding of the probe to 18S and 28S RNA was very high. These bands disappeared when using the conditions described in the legend to Figure 5.3.

The X-gene homologous transcript shown in Figure 5.3 was detectable in heart and thymus tissue isolated from the mice studied. This was however not consistent. For example, it is evident from Figure 5.3 that of the four mice used, the transcript was detectable in the thymus of mouse 1, the heart of mouse 2 and in both tissues of the third mouse. This was despite the loading of similar amounts of RNA onto the gels used for the Northern blot analysis.

This Northern blot data was corroborated when using the SP6 RNA polymerase protocol for the labelling of the X-gene probe on similar blots.

Southern blot analysis performed on DNA isolated from mouse and human sources using a PCR labelled probe showed extremely low levels of hybridisation. A single band was barely discernible and could not be photographically reproduced.

The stronger signal detected by Northern blot hybridisation was thought to relate to the melting temperature of RNA:DNA hybrids being higher than that of DNA:DNA hybrids. The empirically derived equations for the calculation of the melting temperatures of these two hybrids has already been discussed.

5.2.3 cDNA LIBRARY SCREENING

Initially, the PCR labelling protocol and thereafter the SP6 polymerase techniques were used for the probe labelling. The problems of non-specific hybridisation when screening libraries are notorious and this proved to be particularly problematic in these experiments. In order to exclude the non-specific detection of plaques, duplicate filters were prepared from each of the plates. While this is the generally accepted protocol (Sambrook *et al*, 1989), it proved to be extremely difficult in that the detected signals were extremely variable as a result of the apparent lack of complementarity to the HBV X-gene probe.

The bacteriophage vector used in the library construction (Clontech) was λ gt10. This was chosen in preference to a λ gt11 library because under the conditions of phage culture, the number of λ gt10 plaques which contain

inserts is considerably higher than plates which are prepared using λ gt11. The insert bearing λ gt10 bacteriophages are genetically selected (Sambrook *et al.*, 1989) by their lytic rather than lysogenic growth on bacterial lawns.

λ gt11 bacteriophage libraries have the advantage of expressing the cloned insert DNA if in frame with the *lacZ* gene. Antibody screening is therefore possible. Antibodies to the HBx protein were not available for the screening of libraries.

An example of some positively hybridising λ gt10 plaques is given in Figure 5.9. On this particular plate three positive plaques are demonstrated, and this was after a secondary screening of bacteriophage particles isolated from a putative positively hybridising plaque identified on the primary screen.

A



B

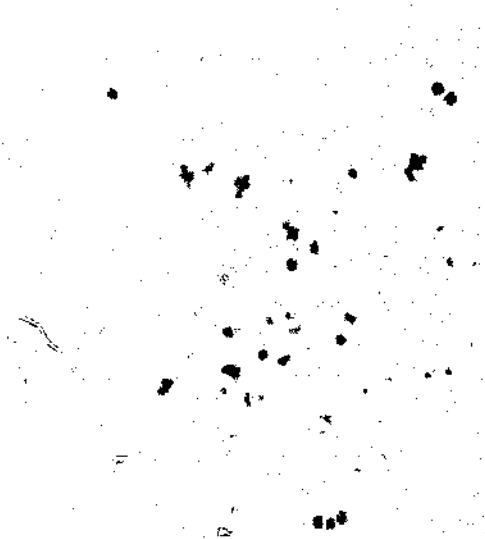


Figure 6.9 An example of an autoradiograph depicting putatively positively hybridising bacteriophage plaques at various stages of washing of the blots during library screening. The positive plaques are indicated by the arrows. This autoradiograph was obtained after hybridisation with the SP6 polymerase generated RNA probe to a 'lift' made from a primary screen. Autoradiography exposure time was for 48 hours. Plate A depicts the autoradiograph after low stringency and that in plate B was after higher stringency washing. Washing of the blot depicted in A was twice for 15 minutes in a solution of 6xSSC at room temperature. The blot in B was further washed as follows: twice for 15 minutes at room temperature in 2xSSC, 0.1% SDS, and once for 20 minutes in a solution of 0.2xSSC, 0.1% SDS at 42°C.

5.2.4 APPROACHES TO THE ISOLATION OF BACTERIOPHAGE DNA FROM POSITIVELY HYBRIDISING PLAQUES

The three methods used for the isolation of bacteriophage DNA have been detailed in Section 2.5.2 of the Methods Chapter. They were two liquid lysate protocols, one using a CsCl gradient purification step and a third method involving a plate lysis technique.

Figure 5.10 shows an example of DNA digested with *EcoRI* after isolation using the plate lysis technique. The remarkable feature, demonstrated here, was that the insert DNA could not be identified reproducibly - that is despite the theoretical presence of inserts in the bacteriophage particles selected for their lytic growth (see Section 5.2.3). The inserts were undetectable in these gels despite overloading with digested DNA.

It has been reported that commercial agar contains inhibitors of restriction enzymes (Sambrook *et al.* 1989), and it is therefore not recommended for use in the plate lysis method of bacteriophage DNA preparation. In these experiments, agarose of electrophoretic grade instead of agar was used for the preparation of culture plates. The endonuclease inhibitors present in agar are not present in

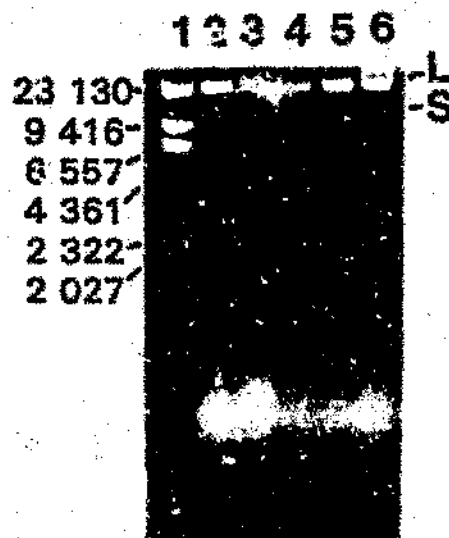


Figure 5.10 Ethidium bromide stained 0.8% agarose gel of DNA isolated using the plate lysis method of bacteriophage DNA purification. The DNA was subjected to *EcoRI* digestion prior to electrophoresis in order to release the insert carrier by each of the clones. The molecular weight markers were separated in lane 1 and the digested bacteriophage DNA isolated from five individual clones is shown in lanes 2 to 6. On this gel, the long (L) and short (S) arms of bacteriophage Agt10 can be easily resolved, but no inserts can be seen. The long arm is approximately 32.5 kb and the short arm 9 kb in size.

good quality agarose (Sambrook *et al*, 1989). Evidence that inhibitors of restriction enzyme digestion were not present in the bacteriophage DNA preparations is that the long and short arms of bacteriophage λ gt10 are visible after digestion (Figure 5.10).

Bacteriophage DNA prepared using the liquid lysate protocol followed by CsCl gradient centrifugation was reproducibly the best method of preparation. Unlike the methods using plate and liquid lysis without CsCl gradient purification, the inserts were readily identified by agarose gel electrophoresis after digestion. This is shown in Figure 5.11.

The reasons for this are not obvious. Possibilities include: 1) The purer bacteriophage DNA allowed easier detection of the insert DNA after restriction endonuclease digestion. That is, contaminating bacterial chromosomal DNA was not present to obscure visualisation of the small amount of insert DNA; 2) Inhibitors of restriction enzymes are effectively removed after the CsCl gradient centrifugation.

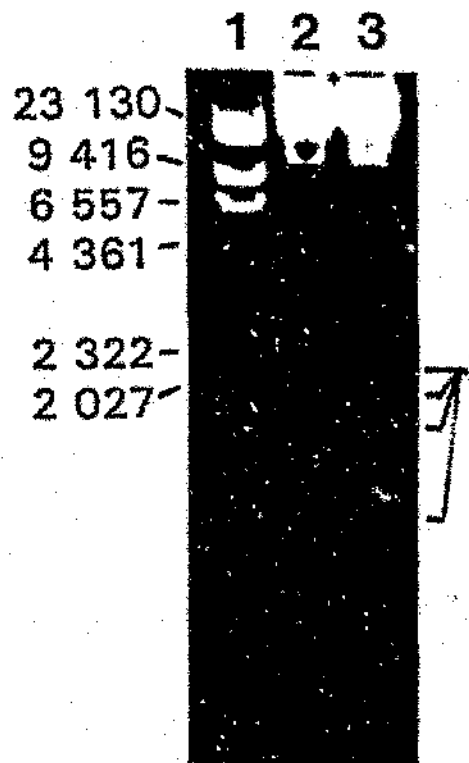


Figure 5.11 Ethidium bromide stained 0.8% agarose gel of DNA isolated using a liquid lysis bacteriophage preparation method followed by CsCl discontinuous gradient centrifugation. Lane 1 represents the separation of the molecular weight markers. Lanes 2 and 3 represent bacteriophage DNA from individual plaques which had been digested with *EcoRI* and then subjected to electrophoresis separation. The numerous inserts which can be detected in these two lanes (approximately ≤ 2100 bp) probably reflect the picking of more than one plaque from the densely plated libraries.

5.2.5 SOUTHERN BLOT ANALYSIS OF RESTRICTION DIGESTED BACTERIOPHAGE DNA.

Southern blot analysis of bacteriophage DNA digested with restriction enzymes produced irreproducible results. It was usually the case that DNA prepared from a positively hybridising plaque did not produce a signal on Southern blot analysis. Also, when Southern blot hybridisation of the plaque DNA was positive, no insert DNA was detectable.

Initially, these results were obtained when DNA was analysed after isolation from plate lysis preparations. It was thought that the reason was that this bacteriophage DNA preparation technique did not yield sufficient DNA of good purity.

When prepared by the CsCl gradient method however, and when an insert was detected, the fragment did not hybridise to the HBV X-gene probe. This was despite numerous attempts to prepare large quantities of λ gt10 DNA which was subjected to electrophoresis with or without Southern blotting. These data suggested that falsely positive hybridising plaques were isolated from the plates during library screening.

This lack of reproducibility strongly suggested that the hybridisation signal being detected was non-specific - an inherent problem with low stringency hybridisation protocols such as were being used here.

In all approximately 100 putatively positive plaques were analysed in this way. In no case was it possible to isolate a definitely positively hybridising plaque which also yielded a hybridising insert of the same size when subjected to Southern blot hybridisation.

5.2.6 PCR BASED APPROACH TO X-GENE HOMOLOGUE ISOLATION

As an alternative to the λ gt10 library screening, a PCR based approach aimed at amplifying an X-gene homologue was used. Four different primers were constructed (2 sense and 2 antisense) which were located at positions on the HBV X-gene which were found to have a high degree of homology amongst the subtypes of the hepadnaviridae (see earlier discussion). The rationale behind the choice of these sequences was that if they were conserved amongst the different species, then the probability of being conserved in the putative mammalian homologue was greater if the X-gene's function had been preserved (see earlier discussion).

A number of different modifications of the amplification protocol were used. The temperatures of annealing during amplification were varied - they ranged from 40°C to 50°C. This was to account for possible lack of complementarity between the primers and template DNA which were being used to direct the amplification step.

The template DNA used was isolated from putative positively hybridising plaques from the cDNA library screening (see 5.2.4 and 5.2.5) as well as genomic human and murine DNA.

After amplification, the products of PCR were analysed by agarose gel electrophoresis. All bands clearly detectable were used for sequencing. These fragments were cloned into the polylinker sites of M13ms18 and M13ms19 filamentous bacteriophages. These two vectors have their polylinkers in opposite orientations and thus allow sequencing from both ends of the same insert. The recombinant bacteriophage plaques were selected by their colourless appearance when grown on X-gal and IPTG containing plates (see Methods Section 2.6.1.3). An example of single stranded M13 DNA prepared for sequencing is depicted in Figure 5.12. An example of a sequencing gel (using the dideoxy chain termination method of Sanger et al, 1973) is depicted in Figure 5.13.

After sequencing of approximately 20 clones, the only areas of significant homology between the amplified fragments and the X-gene sequences were the primer sequences (Figure 5.14). This was determined using the DNA Star™ software package and the criteria for significance indicated in the Methods and Materials Section 5.2: The width of the residues of the unit of comparison (window size) was 30. The setting for the number of adjacent residues of the window that should match was as low as 30% using all register shifts. The minimum quality - the minimum number of consecutive qualifying window homology to be recorded - was one. In all cases, the reverse complement of the sequences were also compared. The Quality value (Q-value), calculated by DNA Star™ using a number of parameters to determine where homology was significant, indicated that there was only homology in the primer sequences.

The most common artefact of the PCR amplification protocol was that of so called 'primer dimer' products of PCR. In this type of amplified DNA sequence, it was found that the DNA comprised one or more tandem repeats of primer sequences sometimes including other non-homologous DNA. An example of this type of artefact is given in Figure 5.14. Here, it

is also shown that comparison of a sequenced amplified product with the primers and consensus X-gene sequence reveals homology only in the primer sequences.

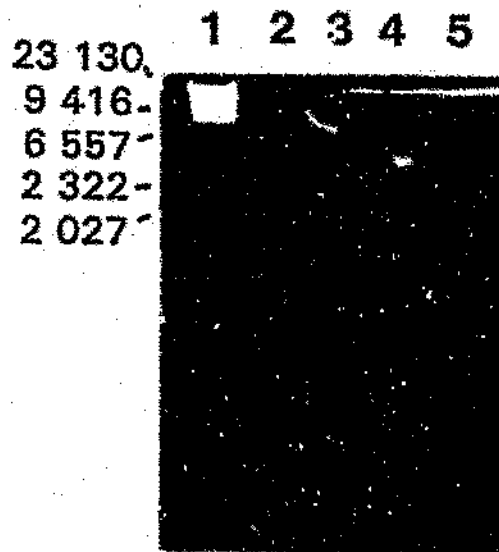


Figure 5.12 Ethidium bromide stained 0.8% agarose gel of single stranded recombinant M13mp18 and M13mp19 DNA. Molecular weight marker DNA is shown in lane 1, and examples of different single stranded M13 DNA clones (indicated by the arrow) which were sequenced is shown in lanes 2 to 5.



Figure 5.13 Example of an autoradiograph from a nucleotide sequencing gel using the dideoxy chain termination method. Depicted here is the data which was derived from sequencing a recombinant M13 vector containing a fragment amplified using the X-gene primers indicated in the text and in Figure 5.1.

```

      +---+---+---+---+---+---+---+---+---+---+
CON 1519 TGCCTTACGCCCCATCAGATCCTGCCCAAGGTCGCTTCACCTCTGCAG 1565
HOM      G   ACGCCCATCAGATCCTGCCCAAGG      T       G
2&4      29 GGATCCACGCCCCATCAGATCCTGCCCAAGGGAATATTTGCAAATTG 75
      +---+---+---+---+---+---+---+---+---+---+

```

```

CON      1781 TTGTATTAGGAGGCTGTAGTTGCAAATTGGTCTGCGCACCAGC 1813
HOM      T  T      GG      TTGCAAATTGGTCTGCGCACCAGC
2&4      47 TCCTGCCCAAGGGAATATTTTCCAAATTGGTCTGCGCACCAGC 89
      +---+---+---+---+---+---+---+---+---+---+

```

Figure 5.14 Comparison of an amplified sequence derived from recombinant bacteriophage template DNA from a putative positive plaque. The primers used were 2 and 4 (see Materials and Methods Section 2.5.4). The numbered HBV X-gene consensus and primer sequences are indicated by 'CON', and the amplified sequence in this case by '2&4'. The areas of sequence homology are indicated as 'HOM'. The primer sequences are indicated in bold print. It can be seen that primer sequence 2, including the *Bam*HI restriction site, occurs at sequence 35 to 48 of 2&4. The complement to primer 4 occurs at sequence 69 to 89 of 2&4. Approximately 250 bp were sequenced from this clone, and the only areas of homology are indicated in this diagram.

5.3 DISCUSSION

A number of different probe labelling procedures was used in an attempt to identify a sequence with putative homology to the HBV X-gene. It was possible to detect a band on Northern blot analysis of mouse heart and thymus RNA. The low stringency conditions of hybridisation which were required to produce a signal suggest an increased probability of non-specific hybridisation giving a spurious signal. The low intensity of the Southern blot hybridisation signal also supported this. Extensive cDNA library screening and re-screening with highly sensitive X-gene probes, together with a PCR based approach, failed to yield a mammalian DNA sequence with homology to the HBV X-gene. Positive plaques found on primary screening may have resulted from non-specific binding of the probe. They were not enriched after secondary and tertiary plating and hybridisation.

Possible modifications to the protocols used here include 1) use of an antibody to screen an expression library/ies constructed using λ gt11 bacteriophage vector. The rationale behind such an approach would be that if a sequence is not detectable using nucleic acid hybridisation, an epitope of the protein may well be recognised by an antibody to the X-gene. In such an experiment, it would also be desirable that the antibody used for the detection be polyclonal rather than monoclonal. The chance of

recognising different epitopes present in the X-like protein would be greater with a polyclonal antibody. 2) Use of degenerate primer sequences in order to probe DNA libraries. The principle behind this method (Sambrook *et al.*, 1989) entails the construction of a number of oligomer probes which have different sequences based on the degeneracy of codons.

5.4 CONCLUSION

The important question being asked in the experiments which have been described in this section is: Does a mammalian gene homologous to the HBV X-gene exist? Unfortunately, it is not possible to answer this conclusively.

The results supporting this idea come from the Northern blot data which suggest that there is a murine RNA sequence with homology to the HBV X-gene in heart and thymus (Figure 5.3). Evidence against the existence of the homologue of the X-gene is that after very extensive screening of murine heart and thymus cDNA libraries and attempts to amplify homologous DNA using PCR, no reproducible results could be obtained.

The experimental data presented here substantiate the idea that if there is

a mammalian progenitor of the hepatitis B virus X-gene, then it has been mutated and/or rearranged to such an extent that the sequence is vastly different from that of the HBV X-gene. This makes it very difficult to isolate such a sequence using viral probes. Rearrangement of sequences when they are integrated into another genome is a well described phenomenon, and it is presumably such rearrangements which could be contributing to the technical difficulties encountered in this attempt to isolate a mammalian homologue of the hepatitis B virus X-gene.

In conclusion, it can be said that there is evidence that the hepatitis B virus does share homology with the retroviruses, and there is also some theoretical evidence for the X-gene having been derived from a mammalian host at some point during its evolution (discussed in Section 5.1). The evidence comes mainly from the data on the HBV X-gene codon preference. The bias of bases in the third position of the X-gene is different to that of other hepadnavirus genes and is not typical of eukaryotic virus genes (Table 5.1). This data on the codon preference is however perhaps not conclusive. The A+U/G+C ratio of 1/2.5 of the X-gene is nowhere near equal to the 1/50.7 ratio of eukaryotic cell genes. (It is perhaps also significant that the X-gene sequence codon preference is similar to that of phage and prokaryotic cell genes - Table 5.1).

The PCR technique is a very powerful method for the amplification of small amounts of DNA. Unfortunately, this property is also its weakness in that non-specifically amplified sequences are easily generated - as was the case in this attempt to isolate a mammalian homologue of the hepatitis B virus X-gene. A methodological approach, using asymmetric reamplification, for the easy detection of non-specifically amplified X-gene sequences is presented in the following Chapter.

Chapter 6

ASYMMETRIC RE-AMPLIFICATION AS A METHOD FOR THE CONFIRMATION OF PCR PRODUCTS

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6. ASYMMETRIC RE-AMPLIFICATION AS A METHOD FOR THE CONFIRMATION OF PCR PRODUCTS

6.1 INTRODUCTION

The polymerase chain reaction is being used increasingly for a wide variety of applications. In most cases, confirmation of the identity of the DNA which has been amplified is required, for example the attempted isolation of a mammalian homologue of the hepatitis B virus X-gene described in the previous chapter. Size determination is not always a reliable method of verification. Non-specific amplification often produces DNA of similar size to that which is expected.

Confirmation protocols have traditionally included a combination of Southern blot hybridisation with an internal probe, restriction mapping and direct sequencing. These methods are often time consuming, expensive and sometimes technically demanding.

Recently, simpler methods have been devised. They include the so called 'hot-blot' oligomer extension method of Parker and Burner (1991). In this procedure, an end labelled oligomer complementary to an internal sequence

is used for liquid hybridisation to an initially amplified DNA sequence. The reaction mixture is then subjected to one cycle of PCR and the products analyzed by electrophoresis and autoradiography. Hybridisation of the nested extended labelled primer, and therefore confirmation of the initially amplified DNA sequence, is indicated by a band at the appropriate position on the autoradiograph. Another method recently developed uses AMV reverse transcriptase for the extension of a labelled internal primer which hybridises to the initially amplified DNA (Kaluz and Reid, 1991). This enzyme extends the labelled internal primer more efficiently than does *Taq* polymerase.

A third protocol which has recently been devised includes three primers in the initial PCR reaction (Kai *et al*, 1991). After the reaction, electrophoretic separation of two amplified fragments of appropriate sizes is confirmation. The advantage of this procedure is that no radioactivity is required.

The simple procedure described here is based on a double amplification protocol. DNA is initially amplified using conventional procedures and a small amount is then subjected to a second asymmetric PCR step where a single internal primer is used to amplify a shorter DNA fragment. The DNA produced from the first PCR step is present in sufficiently high quantities

to allow significant amplification to take place during the second stage even though only one primer is present. Electrophoretic resolution of the extended internal primer DNA with size comparison to a control DNA fragment allows confirmation of the originally amplified DNA. The principle of the technique is outlined in Figure 6.1.

After as few as five cycles of re-amplification, the extended internal primer is detectable by electrophoresis and autoradiography. More cycles of re-amplification allow easier detection by standard ethidium bromide staining.

The method is specific in that it is dependent on precise hybridisation of the internal primer - a single base mismatch may result in lack of primer annealing and therefore extension. The use of only one primer during the second amplification step results in slower linear amplification of the smaller fragment. The logarithmic amplification of artefacts is thus limited.

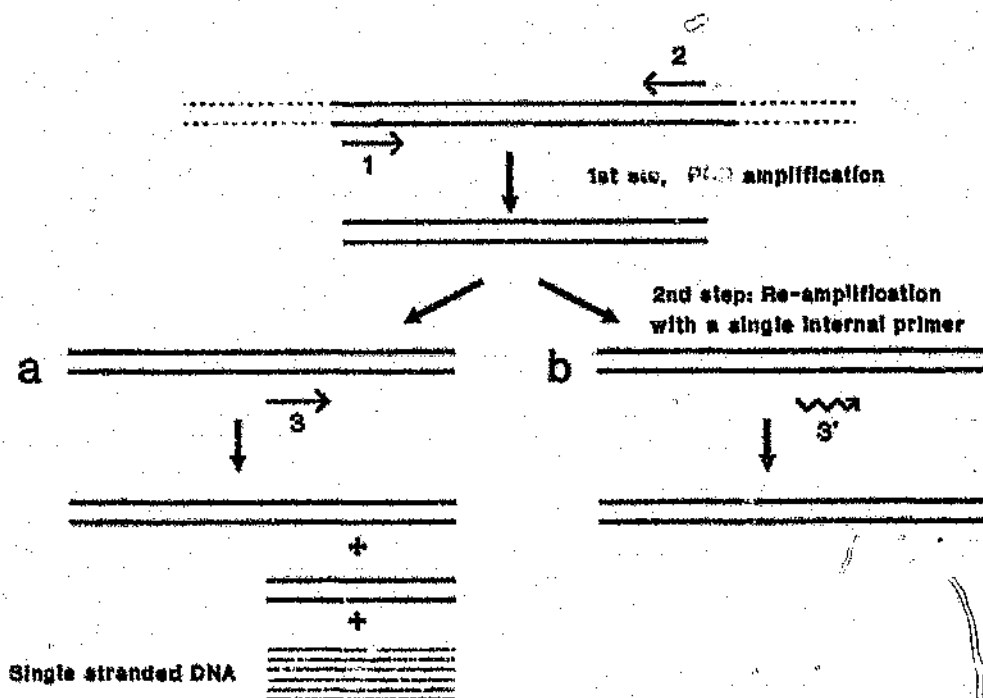


Figure 6.1 Diagrammatic representation of the asymmetric re-amplification protocol. The initial step involves conventional PCR amplification with primers 1 and 2 - each used at a concentration of $0.25\mu\text{M}$. **a** An aliquot of this reaction mixture is then used as template for re-amplification using one internal primer 3 at a concentration of $0.25\mu\text{M}$. Primers 1 and 2 are diluted ($0.005\mu\text{M}$) and do not contribute significantly to the re-amplification. The products of the amplification steps can be electrophoretically resolved and are the double stranded larger and smaller fragments as well as a smaller single stranded fragment. **b** Mismatching of the internal primer (3') results in a lack of hybridisation and no extension. Therefore, only the larger DNA fragment produced during the initial amplification step is discernible after electrophoresis.

The protocol is rapid and simple in that no probe labelling or DNA purification steps are required. Inclusion of a small amount of a radiolabelled deoxynucleotide triphosphate does however make the technique very sensitive.

The methods used were those outlined in Section 2.7 of the Materials and Methods Chapter. The initial template DNA was the recombinant SP64 HBV X-gene plasmid constructed as described in Section 2.4.3. The primers used are indicated in Section 2.7 of the Methods Chapter. Their positions relative to the SP64 HBV X-gene clone and the HBV genome are depicted in Figure 6.2 and Table 6.1 respectively.

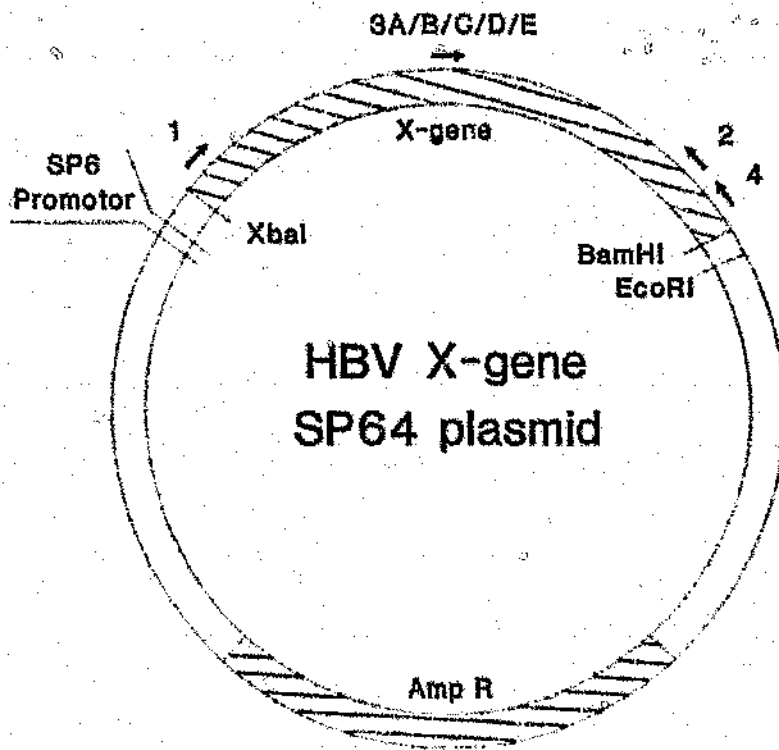


Figure 6.2 Diagrammatic representation of the HBV X-gene SP64 recombinant plasmid and the orientation of the primers (numbered arrows) used in the asymmetric re-amplification protocol. The X-gene fragment is depicted as the shaded area between the *XbaI* and the *BamHI* sites of digestion. The approximate locations of the SP6 RNA polymerase promoter and the ampicillin resistance gene (Amp R) are also indicated. The diagram is not drawn to scale as the length of the X-gene fragment is 396bp and the SP64 plasmid DNA comprises approximately 3000bp.

Primer	Sequence (5' to 3')	HBV Nucleotide positions
1	CCTTTGTTTACGTCCCGTCGGCGC	sense 1420-1443
2	CTACAGCCTCCTAATACAAAGACC	antisense 1788-1765
3	ACGCCCCATCAGATCCTGCCCAAGG	sense 1625-1648
4	GGTGCTGGTGCGCAGACCAATTTC	antisense 1816-1793
3A	AGATCCTGCCCAAGG	sense 1634-1648
3B	AGATCCT <u>Q</u> CCCAAGG	sense 1634-1648
3C	AGATCCT <u>A</u> CCCAAGG	sense 1634-1648
3D	AGATCCT <u>T</u> CCCAAGG	sense 1634-1648
3E	AGATCCTGCCCAAG <u>Q</u>	sense 1634-1648

Table 6.1 Summary of primer nucleotide sequences and orientation on the HBV genome. The bases of primers 3B to 3E indicated in bold print and underlined represent those which were mismatched. The sequence of primer 3A is the normal.

6.2 RESULTS AND DISCUSSION

6.2.1 DEMONSTRATION OF THE METHOD AND IMPROVEMENT OF THE SENSITIVITY BY THE USE OF A RADIOACTIVELY LABELLED NUCLEOTIDE

Figure 6.3 A shows an agarose gel stained with ethidium bromide after electrophoresis of samples prepared according to the amplification procedures outlined in Section 2.7. At least 15 cycles of PCR re-amplification were found to be necessary to detect the smaller DNA fragment by staining. Also, when using a larger amount of template DNA for the re-amplification (5 μ l instead of 1 μ l from the initial 50 μ l PCR reaction mix) the bands were more easily discernible after staining (Figure 6.3, lanes A4 and A5). An indication of the degree of increased sensitivity of the method resulting from inclusion of ^{32} P-dCTP during the PCR reaction is clearly demonstrated by the comparison of lanes A4 and B4 of Figure 6.3.

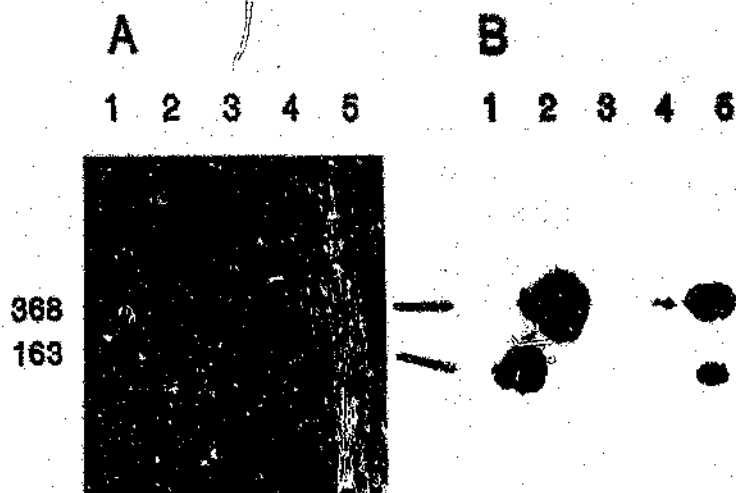


Figure 6.3 A. Electrophoretic separation (ethidium bromide stained 0.8% agarose gel) of hepatitis B virus X-gene DNA. Amplification was with primers 2 and 3 (both $0.25\mu\text{M}$) (lane 1) or primers 1 and 2 (lane 2) and the SP64 X-gene recombinant plasmid as template. The orientation of the primers and their location relative to the nucleotide positions of the HBV genome are described in figure 6.2 and table 6.1. Control re-amplification using original template DNA with primers 3 ($0.25\mu\text{M}$) and 2 ($0.005\mu\text{M}$) is depicted in lane 3. $1\mu\text{l}$ (lane 4) and $5\mu\text{l}$ (lane 5) of DNA amplified as for lane 2 (from a $50\mu\text{l}$ original reaction mixture) was re-amplified using only primer 3 at a final concentration of $0.25\mu\text{M}$. Re-amplification was for 15 cycles. Details of the protocol are described in Section 2.7. The base length of the bands detected is depicted on the left.

B. Autoradiograph of the gel shown in A (15 minute exposure time).

6.2.2 DETERMINATION OF THE NUMBER OF AMPLIFICATION CYCLES REQUIRED FOR EXTENSION OF THE INTERNALLY ANNEALING PRIMER

The 163 base fragment, representing the extended internally annealing primer, was detectable after only 5 cycles when using a radiolabelled precursor (^{32}P -dCTP) during both amplification steps. This is shown in Figure 6.4, lane B2. The fragment was however more easily seen after 10-15 cycles of re-amplification and a markedly shorter autoradiography exposure time (Figure 6.4, lanes A4, A7 and A10).

The use of only one primer in excess during the re-amplification results in asymmetric synthesis. As a result, most of the DNA produced during this step should be single stranded and the amount will increase in a slower almost linear rather than logarithmic fashion. Thus, the template DNA for the re-amplification needs to be present in relative excess for sufficient of the smaller sized DNA fragment to be produced. The production of truncated products by Taq polymerase can be eliminated by increasing the number of cycles of re-amplification. However, because the re-amplification is asymmetric, logarithmic amplification of artefact DNA is minimised.

Electrophoretic mobility of single stranded DNA is not always reliable

according to its molecular weight (Sambrook *et al*, 1989). This forms the basis of the single stranded conformational polymorphism protocols for the detection of point mutations (Orita *et al*, 1989a; Orita *et al*, 1989). The results were similar regardless of electrophoresis under native (figure 6.4) or denaturing (figure 6.5) conditions.

The results of the control experiments depicted in Figures 6.4 and 6.5 (lanes A3, A6, A9, B1, B4 and B7) confirm that there was undetectable amplification when using the original SP64 clone as template and primers 2 and 3 at concentrations as present in the re-amplifications ($0.005\mu\text{M}$ and $0.25\mu\text{M}$ respectively). Being a circular molecule, the 5' and 3' ends of plasmid DNA are not defined as they are in the 368bp template DNA fragment. The *Taq* polymerase enzyme therefore extends the internal primer to a defined point at the 5' end of the initially amplified DNA. Primer extension from the circular plasmid DNA template would be variable.

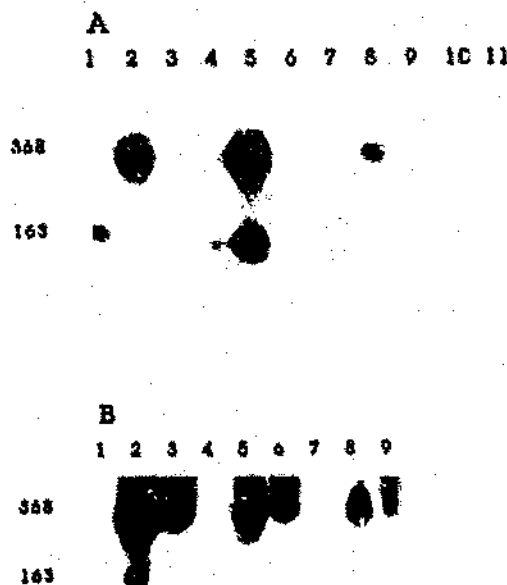


Figure 6.4 Electrophoretic separation (0.8% agarose gel followed by autoradiography) of amplified and re-amplified HBV X-gene DNA prepared using the primers described in figure 6.2 and table 6.1. Lane A1 - amplification using primers 2 and 3 (both 0.25 μ M); Lane A2 - amplification using primers 1 and 2 (both 0.25 μ M); Lanes A3 and A6 - control re-amplification using original SP64 X-gene clone template DNA with primers 3 (0.25 μ M) and 2 (0.005 μ M); Lanes A4 and A7 - 1 μ l (from a 50 μ l original reaction mixture) of DNA amplified as for lane 2, re-amplified using only primer 3 at a final concentration of 0.25 μ M; Lane A5 - DNA prepared as in A4, but using 5 μ l of amplified template DNA; Lane A8 - 1 μ l of DNA prepared as for lane 2 and re-amplified using primer 4 (0.25 μ M). Samples represented in lanes A3 to A8 were subjected to 15 cycles of re-amplification. Those in lanes B7-9, B4-6, B1-3 and A9-11 were similar to A6-8 except that re-amplification was for 1, 2, 5 and 10 cycles respectively. The base lengths of the bands detected is depicted on the left. The autoradiograph shown in A was obtained after exposure of the dried gel for 20 minutes, and that in B after 120 minutes exposure time.

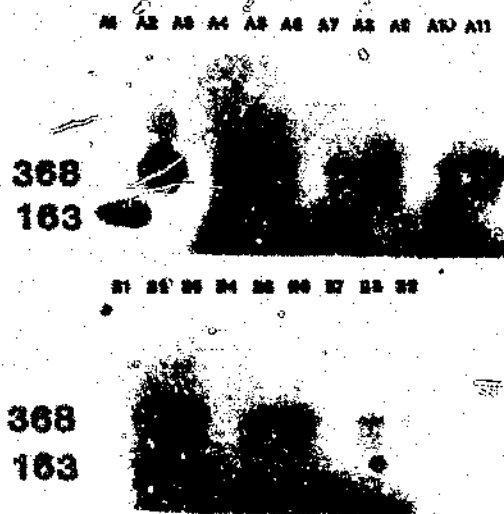


Figure 6.5 The autoradiograph depicted in this figure represents electrophoresis of samples prepared in a similar way to those in figure 6.4. The DNA was however subjected to alkaline denaturing 0.8% gel electrophoresis. The lane numbering is the same as that given in Figure 6.4.

The distinction between single and double stranded DNA is barely discernible using non denaturing polyacrylamide gel electrophoresis. This is depicted in Figure 6.6 where a doublet can be seen in lane 4 and to a lesser extent in lane 5 after 15 and 10 cycles of re-amplification respectively.

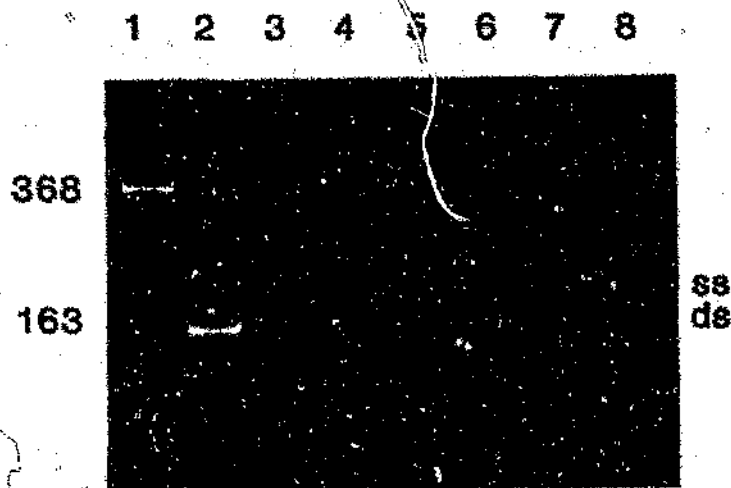


Figure 6.6 Electrophoretic separation (6% polyacrylamide gel) of HBV X-gene DNA amplified using primers 1 and 2 (both $0.25\mu\text{M}$) (lane 1), primers 3 and 2 (both $0.25\mu\text{M}$) (lane 2), control re-amplification using original SP64 plasmid template DNA with primers 3 ($0.25\mu\text{M}$) (lane 3). $1\mu\text{l}$ of DNA amplified as for lane 2 was re-amplified in $50\mu\text{l}$ using only primer 3 at a final concentration of $0.25\mu\text{M}$ for 16, 10, 5, 2 and 1 cycles (lanes 4 - 8 respectively). The sizes of the bands detected is depicted on the left (base pairs), and their single (ss) or double (ds) stranded nature is shown on the right. (The 'stripe' extending from the upper left to the lower right side of the gel is an artefactual effect of the tubes of the UV transilluminator.)

6.2.3 SPECIFICITY OF THE INTERACTION OF THE INTERNAL PRIMER WITH THE INITIALLY AMPLIFIED DNA TEMPLATE

Primer 4 was initially used as internal primer to test the specificity of the re-amplification step. It is located outside the sequence of the DNA initially amplified using primers 1 and 2 (see Figure 6.2 and Table 6.1) but its complementary sequence is present within the SP64 X-gene clone. The absence of detectable re-amplification from this primer is depicted in Figure 6.4 and 6.5 (lanes A8, A11, B3, B6 and B9).

In order to determine the effect of contaminating DNA on re-amplification, a series of experiments was performed using non-specifically amplified DNA as template. Figure 6.7 shows that when used alone, contaminating DNA does not produce extension (lane 4). When the correctly amplified DNA is added to this reaction mixture, there is detectable extension from the internal primer (Figure 6.7, lane 5). This implies that the re-amplification step is dependent on the specific hybridisation of complementary sequences of the internal primer and the amplified template DNA.

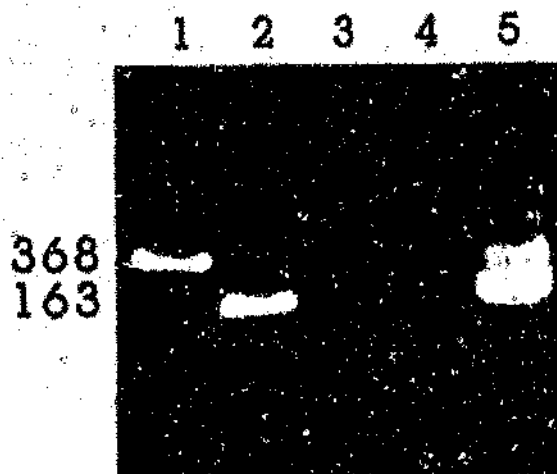


Figure 6.7 Electrophoretic separation (ethidium bromide stained 1.5% agarose gel) of DNA amplified and re-amplified as in Figure 6.4 lanes A2, A1 and A4 represented in lanes 1 to 3. DNA in lanes 4 and 5 was subjected to re-amplification for 15 cycles using non-specifically amplified DNA as template without (lane 4) and with (lane 5) the addition of DNA amplified as for lane 1.

6.2.4 DETERMINATION OF THE EFFECT OF SINGLE MISMATCHES ON EXTENSION OF THE INTERNAL PRIMER

Hybridisation of short oligonucleotides to DNA is a specific method for the detection of small mutations (for example point mutations). This technique is based on the decrease in melting temperature (T_m) caused by mismatches within an oligonucleotide hybrid (Bonner *et al.*, 1973). Manipulating the conditions of hybridisation allows these changes in T_m to be detectable by determining the extent of oligonucleotide annealing (Sambrook *et al.*, 1989).

Conventional symmetric PCR does not allow easy discrimination between the DNA amplified from mismatched and normal primers. Although the efficiency of mismatched primer hybridisation during the early cycles of PCR is low, there is inevitably some annealing followed by logarithmic amplification to produce DNA which includes the mismatched primer/s and complementary sequences. The size and amount of this amplified DNA may thus be indistinguishable from the DNA which is produced by perfectly matched primers.

A different PCR approach, using 3' mismatched primers, has been used for

the detection of small mutations (Okayama *et al*, 1989; Kwok *et al*, 1990; Neubauer *et al*, 1990). This method relies on a conformational change occurring at the site of extension by *Taq* polymerase and this enzyme is inhibited as a result. It has however been found that because of the efficiency of logarithmic DNA amplification during PCR, a single 3' mismatch is not always sufficient to decrease DNA synthesis significantly. More than one mismatch at the 3' end of a primer is often required to detect a mutation (Main *et al*, 1991). The reason for this may be that once a mismatched primer is incorrectly extended, the mistake will be amplified logarithmically, and the presence of at least two mismatches minimises non-specific amplification.

Analysis of the specificity of internal primer annealing during re-amplification was extended to determine the effect of single mismatches on extension.

A rough estimation of the T_m of the perfectly matched primer 3A (Figure 6.2 and Table 6.1) is 48°C (Itakura *et al*, 1984). At annealing temperatures of 50°C and 60°C however there was easily detectable extension from this internal primer as was shown by the production of the 154 base fragment (Figure 6.8, lanes 3 and 4). Mismatched primers 3B, 3C and 3D (see Figure

6.2 and Table 6.1) failed to produce detectable extension when the annealing temperature was 50°C (Figure 6.8, lanes 5-7). Primers 3B, 3C and 3D contained single substitution mismatches at the 8th position of the 15 mer oligonucleotide. Re-amplification with primer 3E, which had a 3' end mismatch (table 6.1), results in failure to produce detectable extension (Figure 6.8, lane 10).

The effect of the linear versus logarithmic amplification on the specificity of the amplification was tested by using two primers for the re-amplification: one internal primer which had the single mismatch at position 8 (primers 3B-D) and the normal primer 2. The results of this experiment are depicted in Figure 6.8. Here it is shown that despite the mismatch at the eighth position, there was easily discernible symmetric amplification at annealing temperatures of 50°C and 60°C (Figure 6.8, lanes 8 and 9). This amplification was on a scale similar to that detectable when using the normal internal primer. The additional largest DNA band seen in lanes 8 and 9 is an artefact which was often produced during symmetric logarithmic PCR using the conditions described in these experiments.

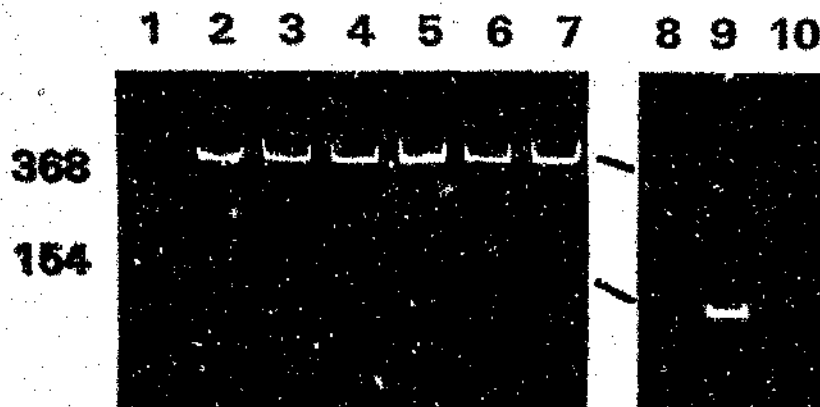


Figure 6.8 Electrophoretic separation (ethidium bromide stained 6% polyacrylamide non-denaturing gel) of amplified and re-amplified hepatitis B virus (HBV) X-gene DNA. DNA depicted in lanes 1 and 2 represents the control size markers with their base lengths indicated on the left of the figure. The band in lane 2 was prepared by standard symmetric amplification of the SP64 X-gene plasmid using primers 1 and 2. Lane 1 represents DNA which was amplified using primer 2 and primer 3 (sense sequence 1634 to 1648, 5' AGATCCTGCCCAAGG 3'). An aliquot (2%) from a PCR amplification reaction of DNA prepared as for lane 2 was re-amplified asymmetrically for 15 cycles using only primer 3 (normal sequence) and annealing temperatures of 50°C (lane 3) and 60°C (lane 4). Similar re-amplification with primer 3 sequences mismatched at the 8th base (C, A or T instead of G) is resolved in lanes 5 to 7. DNA which was produced after symmetric re-amplification using a mismatched primer 3 (similar to that used in lane 5) and normal primer 2 at annealing temperatures of 50°C and 60°C is depicted in lanes 8 and 9 respectively. The DNA in lane 10 depicts separation of DNA re-amplified with the primer 3 sequence mismatched at the 3' end (C instead of G). Two similar gels run independently (lanes 1 to 7 and 8 to 10) are depicted in the figure.

6.3 CONCLUSION

The procedure described here is markedly quicker and simpler than most previously described methods such as Southern blotting, 'hot blot' (Parker and Burmer, 1991) and reverse transcriptase (Kaluz and Reid, 1991) for the confirmation of amplified DNA. Steps such as probe labelling, blot hybridisation and DNA purification are not necessary.

The method also has a distinct advantage over methods which use three primers during the first amplification step (Kai *et al*, 1991). When using such a protocol, one may conceivably get artefactual logarithmic amplification from different points of the template DNA.

Decreased melting temperature . conformational changes at the site of extension by *Taq* polymerase are probably responsible for the observed differences between symmetric and asymmetric re-amplification. The inhibitory effect of single base mismatches on primer extension during asymmetric re-amplification is a useful property which can be exploited to assess oligonucleotide and template DNA sequence complementarity.

With the exclusion of laborious and costly steps, this procedure could be

applied in many PCR protocols where confirmation of the amplified DNA product is required.

Chapter 7

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7. REFERENCES

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