Polyploidy in Murine Hepatocytes

Nicola Claire Martin

Doctor of Philosophy

University of Edinburgh
2002

Declaration

I hereby declare that the work presented in this thesis is my own, except where stated in the text. The work has not been submitted in any previous application for a degree.

Nicola Claire Martin

The author's published papers have been included in this thesis (appendix 6) with the formal permission of the publishers and all joint authors.

With all my love to Gary, Emily and my parents

Table of Contents

Abstract

Polyploidy and binuclearity are important features of the mammalian liver and are associated with ageing. Alterations in the degree of polyploidy and nuclearity occur with the administration of various chemicals and also during regenerative growth. Contradictory hypotheses exist regarding the function and significance of polyploidisation. Early studies suggest that polyploid cells are more resistant to the dominant expression of mutated oncogenes and polyploidisation is therefore a protective mechanism. More recent studies suggest that increasing ploidy is purely associated with terminal differentiation and increased rates of apoptosis. There have also been suggestions that extensive polyploidy could lead to organ failure and development of carcinogenesis. This thesis has looked at various aspects of polyploidisation in the mouse liver to obtain more information about the function of polyploidisation: The effect of the non-genotoxic carcinogen, sodium phenobarbitone (PB) on ploidy and proliferation was determined over 21 days in p53 wild type (+/+), p53 +/- and p53 null (-/-) mice. PB induced a significant increase in the proportion of 8n nuclei and this increase occurred independently of p53. Whether polyploidisation occurs as a protective mechanism or acceleration of ageing in this case is unknown. However, the results confirm that the p53 +/- mouse model, often used in short-term bioassays, may not be suitable for the identification of non-genotoxic carcinogens as the increase in polyploidy occurred equally in all mice and there were no histological abnormalities in any genotype. The function of polyploidy with respect to increasing cell size, receptor expression and susceptibility to apoptosis was also studied. The first accurate measure of volume of isolated hepatocytes differing in ploidy and nuclearity was carried out using confocal image analysis. The increase in volume associated with increasing DNA content was found to be proportional to intercellular adhesion molecule-1 (ICAM-1) surface expression, measured by flow cytometry. Apoptosis induced by interferon-gamma (IFNγ) in culture did not occur preferentially in polyploid hepatocytes. This would suggest that the susceptibility of polyploid cells to apoptosis depends on the circumstances. Evaluation of the effect of fluorescent activated cell sorting (FACS) and Hoechst 33342 on RNA from sorted hepatocytes found that the quality was not affected by

the procedure and the RNA was suitable for subsequent gene expression analysis. Initial experiments using microarray and real-time PCR identified several genes that were induced, including major urinary protein 2 (MUP2) and interferon gamma receptor (IFNR) or repressed, including pterin-4α-carbinolamine dehydratase (PCD), in cells containing greater amounts of DNA. Further biological studies will hopefully determine whether these genes are important to the function or maintenance of polyploidy in hepatocytes.

Abbreviations

Abbreviation	Full Form
2n	diploid
2 x 2n	binuclear diploid cell
4n	tetraploid
2 x 4n	binuclear tetraploid cell
8n	octoploid
2 x 8n	binuclear octoploid cell
a	adenine (base) / (deoxy)adenosine (nucleoside) / (deoxy)adenylate (nucleotide)
2-AAF	2-acetylaminofluorene
Ab	antibody
AIM1	aurora an Ipl1-like midbody-associated protein 1
APC	anaphase promoting complex
b	base
Bir1	baculovirus IAP repeat 1
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin (fractionV)
Bub1	budding uninhibited by benzimidazole 1
C	cytosine (base) / (deoxy)cytidine (nucleoside) / (deoxy)cytidylate (nucleotide) / centigrade
CCl_4	carbon tetrachloride
Ccs52	cell cycle switch 52
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
CEA	chlorendic acid
CNF2	cytotoxic necrotizing factor 2
CPA	cyproterone acetate
cDNA	complementary DNA

cpm	counts per minute
CTO	cell tracker orange
DCB	1,4-dichlorobenzene
DEHP	diethylhexylphthalate
DEN	diethylnitrosamine
DMEM F12	Dulbecco's modified Eagle's medium/Nutrient mixture F12 ham
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dT	deoxythymidine
DTT	dithiothreitol
EDTA	ethylene di-amino tetra-acetic acid
EGF	epidermal growth factor
ERCC-1	excision repair cross complementing-1 gene
ES Cells	embryonic stem cells
ESP1	extra spindle pole 1
ESTs	expressed sequence tags
ETU	ethylene thiourea
FACS	fluorescence activated cell sorter/sorting
Fbl	fumble
FCS	foetal calf serum
FNA	fine needle aspirate
FS	forward scatter (cell size)
Fzr	fizzy-related gene
g	grams
g	acceleration due to gravity ($\sim 10\ ms^{-2}$)
G	guanine (base) / (deoxy)guanosine (nucleoside) / (deoxy)guanylate (nucleotide), gap phase of cell cycle (e.g. G_1 or G_2)
GH	growth hormone
GHR	growth hormone receptor

h	hour/s
HBSS	Hanks' buffered salt solution
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEL	human erythroleukemia
HGF	hepatocyte growth factor
IAP	inhibitor of apoptosis proteins
ICAM-1	intercellular adhesion molecule-1
Ipl1/2	increase in ploidy 1 or 2
IFNγ	interferon gamma
IFNγR	interferon gamma receptor
IGF-1 or 2	insulin-like growth factor 1 or 2
IgG, IgM etc.	immunoglobulin class G, M etc.
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IRF-1	interferon regulatory factor 1
ITS	insulin-transferrin-Sodium selenite
l	litres
LEC	Long-Evans Cinnamon rats
LI	labelling index
m	meter
3'M	3'-methyl-4-dimethylaminobenzene
M	molar
MCP	methylclofenapate
min	minutes
$MgCl_2$	magnesium chloride
µl	microlitres
ml	millilitres
MOB1	mps one binder 1
MPF	mitosis promoting factor
Mps1/2	monopolar spindle 1 or 2

mRNA	messenger RNA
MUP	major urinary protein
N	nucleotide / nucleoside/ base (e.g. in dNTP)
N/A	not applicable
NaOH	sodium hydroxide
NDC1	nuclear division cycle 1
NER	nucleotide excision repair
nm	nanometer
NRS	normal rabbit serum
ORC	origin recognition complex
P	probability value
$p53^{RSC}$	relaxed spindle checkpoint allele
Pop1	polyploidy 1
PB	sodium phenobarbitone or phenobarbital
PBS	phosphate buffered saline
PCD	pterin 4α-carbinolamine dehydratase
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pH	$-\log_{10}[H^+]$
PH	partial hepatectomy
PP	peroxisome proliferator
RNA	ribo(se)nucleic acid
RNase A	ribonuclease A
ROD	rough deal
RPF	replication promoting factor
rpm	revolutions per minute
RTF	real time fluorometer
Rum1	replication uncoupled from mitosis 1
s	second
SCF	Skp1-Cullin-F-box
Scp160p	Saccharomyces cerevisiae protein involved in the control of Ploidy

SD	standard deviation
SDS	sodium dodecyl sulphate
SPA1	spindle pole antigen 1
SS	side scatter (granularity)
T	thymidine (base) / deoxythymidine (nucleoside) / deoxythymidylate (nucleotide)
T_3	tri-iodothyronine
Taq	Thermus aquaticus DNA polymerase
TBE	tris / boric acid / EDTA buffer
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TNFα	tumour necrosis factor alpha
TR	thyroid hormone receptor
TRβ	thyroid hormone receptor beta
Tris	tris (hydroxymethyl) aminomethane
U	uracil (base) / uridine (nucleoside) / uridylate (nucleotide)
UV	ultra violet (radiation)
V	volumes or volts
w/v	weight (mass) per unit volume
ZBP	zipcode binding protein
ZW10	Zeste-White 10

1 Introduction

After the administration of various drugs and chemicals and with increasing age, the rodent and human liver become increasingly polyploid (Brodsky and Uryvaeva, 1977; Hasmall and Roberts, 2000; Kudryavtsev et al., 1993). The significance, mechanism and control of this process remain largely unknown. Contradictory hypotheses exist as to why polyploid cells occur in the liver. Early studies suggest that polyploidisation could be a protective response (Uryvaeva, 1981; Brodsky and Uryvaeva, 1985c), whereas more recent studies suggest that polyploid hepatocytes are associated with tissue differentiation (Biesterfeld et al., 1994; Sigal et al., 1995) and are more likely to undergo apoptosis leading to an increased risk of organ failure or to carcinogenesis (Gupta et al., 2000). Whatever the reason or reasons, polyploidisation is an important feature of the liver worthy of further study. Therefore, this thesis studies various aspects of polyploidisation in the rodent liver. The introduction provides background information about ploidy in the mammalian liver, as well as other polyploid cells and organisms, in order to gain a greater insight into this subject.

1.1 What is Polyploidy?

The majority of mammalian cells are diploid (2n) i.e. they contain two sets of chromosomes. However, many tissues such as the liver, bone marrow, myocardium and pancreas, contain nuclei in which the chromosome number is increased in multiples of n (the normal haploid chromosome number) and are referred to as 'polyploid'. Another phenomenon referred to under the term polyploidisation is multinucleation where a cell contains more than one nucleus. Approximately 80% of rodent adult hepatocytes are binuclear (contain two nuclei). Polyteny, found in a diverse range of tissues and taxa including insects and plants is often referred to as 'polyploidy'. A well-known example is the nuclei of secretory cells in the salivary gland of diptera (Brodsky and Uryvaeva, 1977). These cells undergo a process called endoreduplication, which is different to the process of polyploidisation in

other cells such as hepatocytes and megakaryocytes and will be discussed in more detail in a later section. Aneuploidy, which is often found in malignancies, is sometimes mistakenly referred to as 'polyploidy'. Aneuploidy is the loss or duplication of chromosomes or chromosomal segments but not the duplication of full chromosomal sets (Feldmann, 1992).

The term 'polyploid' is most often used to describe an increase in DNA content within a single nucleus and in some instances has been used to describe binuclear or multinuclear cells. In this thesis 'polyploid' will only be used to describe mononuclear cells and polyploidisation will refer to the mechanism by which a cell obtains a DNA content greater than 2n within the one nucleus. However, in the case of hepatocytes where a binuclear cell contains more than the diploid DNA content, these cells will be called polyploid binuclear cells. The abbreviations used to describe hepatocytes with different ploidy and nuclearity are often confused in the literature. The abbreviations used in this thesis are provided in Table 1.

Table 1. Abbreviations used to describe hepatocytes.

Abbreviation	Type of hepatocyte
2n	Diploid
2 x 2n	Binuclear diploid cell
4n	Tetraploid
2 x 4n	Binuclear tetraploid cell
8n	Octoploid
2 x 8n	Binuclear octoploid cell

1.2 Occurrence of Polyploid Cells

1.2.1 Plants, invertebrates and protozoa

Polyploid cells are found widely in nature, in plants, insects, worms, molluscs and protozoa, as well as in several mammalian tissues (Brodsky and Uryvaeva, 1985a). In insects and higher plants, nearly all normal differentiated cells are polyploid or polytene. In vertebrates polyploidy is much more rare, with about 50 polyploid species within 14 families of fishes, amphibians and reptiles (Otto and Whitton, 2000). High ploidy or polytene cells can be found in the macronucleus of ciliates, in mollusc ganglia, salivary glands, silk gland and many other insect tissues, in rodent trophoblast giant cells and in the haustorial cells of the embryo-suspensor and endosperm of plants (Nagl, 1995; Brodsky and Uryvaeva, 1985b). Many mutant strains of yeast are polyploid and consequently many studies have utilised these organisms to study the growth and control of polyploidisation (Chan and Botstein, 1993). Other organisms widely used in the study of cell growth and development are the nematode *Caernorhabditis elegans, Drosophila* and *Xenopus*. In *Drosophila*, most larval tissues become polytene and adult tissues can either be polyploid or polytene. During larval development in *C.elegans*, endoreduplication cycles occur within the intestinal and hypodermal nuclei (Hedgecock and White, 1985). Endoreduplication is thought to be beneficial to cells as it saves time and energy (Therman et al., 1983). In plants, polyploidy is thought to have evolutionary implications and is thought to have contributed to their success within the environment (Otto and Whitton, 2000; Thompson and Lumaret, 1992; Soltis and Soltis, 1995). It is clear that polyploidisation is important in the growth and development of various organisms and it has been suggested that polyploidisation played an important role in vertebrate evolution (Kobel and Du Pasquier, 1986).

1.2.2 Mammalian cells

Polyploid cells have long been discovered in several human and animal tissues including in the uterus, salivary gland, epidermis, urinary bladder, brain, liver, trachea, bone marrow and myocardium (Brodsky and Uryvaeva, 1985a). More recently, aortic smooth muscle (Barrett et al., 1983; Lee et al., 1992) as well as mouse ovary (Keighren and West, 1993) have also been found to contain polyploid cells. The degree of polyploidy varies greatly in different tissues. In the bone marrow, megakaryocytes synthesise up to 64 times the normal amount of DNA (Biesterfeld et al., 1994). Similarly to the liver, polyploidisation of myocytes in the cardiac ventricle of mice occurs mainly in the first postnatal week, with 70% of cells becoming binuclear and 10% becoming mononuclear with a 4n DNA content (Brodsky et al., 1980). In human artery smooth muscle cells, cerebrum, rectus abdominis and thyroid, approximately 6-7% of cells are 4n with higher polyploid cells being much more rare (Barrett et al., 1983; Bohman et al., 1985). The polyploid nature of megakaryocytes and some other cells is directly related to cell and tissue function and these cells are therefore commonly referred to as obligate polyploids (Zimmet and Ravid, 2000). In other cells including the liver, the function or functions are unknown. However, the possibility remains that polyploidisation is related to cell or tissue function and cell growth in such tissues, as it is usually the tissues that synthesise considerable amounts of tissue proteins and perform vital tissue functions that are polyploid (Brodsky and Uryvaeva, 1977). Polyploidy in various cell types has also been associated with the development of carcinogenesis and disease. However, the significance of polyploidy will be studied in more detail in later sections.

1.2.2.1 Ploidy in the liver

In rats and mice, the formation of polyploid hepatocytes begins shortly after birth with the appearance of binuclear cells (2x2n) and the formation of 4n cells at weaning (Brodsky and Uryvaeva, 1977; Carriere, 1967; Nadal and Zajdela, 1966).

The onset of polyploidy is also associated with independent feeding as prior to weaning there is little diurnal variation in DNA synthesis. However, after weaning, DNA synthesis shows a diurnal pattern, which is affected by a protein deficient diet (Dallman et al., 1974). Interestingly, excess glucose feeding to mice results in polyploidisation of pancreatic β cells (White et al., 1985). In rat liver, the 4n nuclei increase dramatically between 3 to 8 weeks after birth and seem to remain constant thereafter (Bohman et al., 1985). In mice, the level of ploidy continually increases with age and can reach up to 64n in some strains (Epstein, 1967; Severin et al., 1984). The order of polyploid cell formation in the liver is shown in Figure 1. Formation of binuclear cells in the liver is preceded by a burst in mitotic activity (Wheatley, 1972). The mechanism of polyploidisation is described in more detail in a later section but binucleation is thought to be an intermediary stage in the formation of mononuclear polyploid cells (Alfert and Geschwind, 1958; Brodsky and Uryvaeva, 1977). In agreement with this theory is the finding that the formation of binuclear cells in human liver precedes the formation of mononuclear polyploid cells of the next ploidy class (Watanabe and Tanaka, 1982). A study by Carriere, 1967, found that in normal growing rat liver, the majority of cells engaging in DNA synthesis were polyploid. As with mononuclear polyploid cells, binuclear cells increase with age but in some cases have been found to decline at maturity (Gerlyng et al., 1993). The level of binucleation is also species and strain specific. The level is highest in the mouse and can range from 60-80% (Severin et al., 1984), whereas in the rat and in humans the level ranges from 10-30% (Gerlyng et al., 1993; Kudryavtsev et al., 1993; Seglen, 1997; Watanabe and Tanaka, 1982). In human liver, binuclear hepatocytes are present in the embryonic liver and mononuclear polyploid hepatocytes appear between 1 and 5 years of age. Up to 50 years of age further polyploidisation occurs slowly and subsequently intensifies up until 86 to 92 years where the proportion of polyploid cells reaches approximately 27%. As in rats, human 2n hepatocytes can pass several times through the normal mitotic cycles before becoming polyploid (Gerlyng et al., 1993; Kudryavtsev et al., 1993).

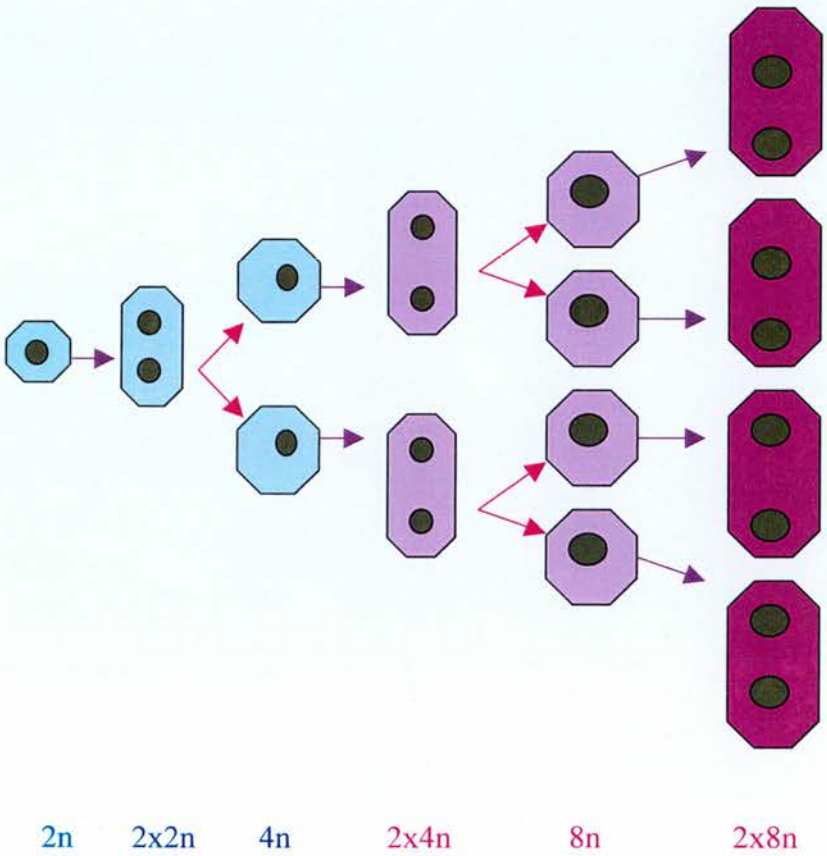


Figure 1. Formation of mononuclear and binuclear hepatocytes. 2n cells become 4n and 4n cells become 8n through the formation of binuclear cells.

The majority of studies on liver ploidy have been carried out in mice, rats and humans. The degree of polyploidy varies from species to species with the degree of polyploidy decreasing in the order of mouse > rat > human > guinea pig > woodchuck (Seglen, 1997). The cellular and nuclear DNA contents of various mammalian liver cells are summarised in Table 2. In the liver of the woodchuck, polyploid cells are absent indicating that polyploidy is not obligatory to liver function (Seglen, 1997). The liver is composed of parenchymal cells (hepatocytes) involved in the detoxification of drugs and toxins, and non-parenchymal cells such as biliary epithelial cells, fenestrated endothelial cells, kupffer cells and Ito cells (Severin et al., 1984) involved in immune responses. Polyploidy in the liver is a feature of the parenchymal population only (Bohman et al., 1985). The parenchymal cell population accounts for 70% of the total cell population in the liver with the remaining 30% made up of non-parenchymal cells. In C3H male mice of 16 weeks of age, the majority of parenchymal cells are 4n (70%) and 80% are binuclear. Diploid and 8n cells make up 20% and 9.5% of the parenchymal population respectively. Some studies have found that polyploidy varies between the sexes, with adult female mice showing higher levels of ploidy than male mice of the same age (Epstein, 1967; Steele et al., 1981a). There is also a variation between different strains of mice (Severin et al., 1984), see Table 2.

Table 2. Ploidy in the mammalian liver: Age, sex and strain variations

Species	Strain	Sex	Age	% Cells*				%	% Nuclei*				Ref.
				2n	4n	8n	16n	BN	2n	4n	8n	16n	(et al.,)
Mouse	C3H	Mi	6 M	15	50	30	5	80	40	50	8	1	Severin 1984
	"	"	24 M	15	25	55	5	50	35	50	10	3	"
	DBA	"	9 M	15	35	42	8	50	38	50	12	0	"
	NZB	"	6 M	22	40	35	8	65	50	38	12	1	"
	Balb/c	F	10W	-	-	-	-	-	58	38	4	-	Steele 1981a
	CBA	F	32 W	-	-	-	-	-	32	47	21	-	"
	"	M	"	-	-	-	-	-	58	34	8	-	"
Rat	Sprague-Dawley	M	200-300g	16	80	4	-	21	-	-	-	-	Gomez-Lechon, 1981
	Wister kyoto	M	150-200g	14	71	15	1	35	-	-	-	-	Mossin 1994
	"	"	50g	47	52	2	-	39	-	-	-	-	Gerlyng 1993
Human	N/A	Mi	PrN, 4.5M	92	8	0	-	5	-	-	-	-	Kudryavt-sev 1993
	"	"	PoN 1D	95	5	0	-	2	-	-	-	-	"
	"	"	51-55y	73	24	3	-	19	-	-	-	-	"
	"	"	86-92y	56	34	10	-	21	-	-	-	-	"

*Values given are averages from several observations and therefore do not always add up to 100%. BN = Binuclear cells, Mi = Mixed, M = Male or Months, F = Female, W = Weeks, y = years, D = day, g = grams, PrN = Prenatal, PoN = Postnatal

The first observations of liver ploidy were described in fixed tissue sections or cells of rodent and human liver several decades ago by several authors using quantitative microspectrophometry (Alfert and Geschwind, 1958; Brodsky and Uryvaeva, 1977; Carriere, 1967; Epstein, 1967; Nadal and Zajdela, 1966). More recent work has utilised newer techniques such as flow cytometry, which allows the accurate and quick determination of ploidy of large numbers of isolated hepatocytes and nuclei (Bohman et al., 1985; Steele et al., 1981a; Steele et al., 1981b; Severin et al., 1984). Other studies have utilised the measurement of cell size and volume as these have been found to be proportional to DNA content (Deschenes et al., 1981; Sigal et al., 1999; Watanabe and Tanaka, 1982; Williams et al., 1997). In mouse tissue section, 2n nuclei ranged from 6.0 to 7.5 μm and 4n nuclei ranged between 7.5 and 9.0 μm (Danielsen et al., 1986). Watanabe and Tanaka, 1982, demonstrated that in human liver, 4n cells were roughly twice the volume of 2n cells and that the volume of binuclear cells was the same as that of mononuclear cells of the same ploidy class e.g. 2x2n and 4n.

1.2.2.1.1 Ploidy of cultured hepatocytes

Many of the studies described above have been carried out on isolated hepatocytes or tissue sections. Studies of hepatocytes in culture can also provide important information about polyploid cells. A study by Gomez-Lechon et al., 1981, showed that the proportion of polyploid nuclei decrease and the proportion of multinucleate cells increase over a period of days in non-proliferating cultures. They suggested that the depletion of 4n and 8n nuclei were due to their instability in culture rather than a transition between ploidy levels. Time-lapse photography demonstrated that multinuclear cells occurred through cell fusion, which is not thought to occur *in vivo* and may therefore be an artefact of culture. The mechanism of polyploidisation is covered in more detail in section 1.4.

1.2.2.1.2 *Ploidy in different zones of the liver*

The proposed lineage model in the liver involving the maturation of stem cells and precursors in the periportal region and ending with maturing hepatocytes near the central vein (Sigal et al., 1992), suggests that different regions of the liver will contain hepatocytes of different ploidy. As mentioned previously, the DNA content of hepatocytes is proportional to cell size and studies have examined either cell size or ploidy when comparing cells from different regions with conflicting findings. Cells from the different regions can be characterised by their function. The cells within the periportal region (zone I) are involved in gluconeogenesis, ureagenesis and amino acid uptake and degradation, whereas the cells in the perivenous area or centrilobular region (zone 3) are involved in glycolysis and glutamine synthesis (Feldmann, 1992; Jungermann and Kietzmann, 1996; Sigal et al., 1992). Cells from each region also differentially express other genes that are summarised in Figure 2, such as alcohol metabolising enzymes and albumin.

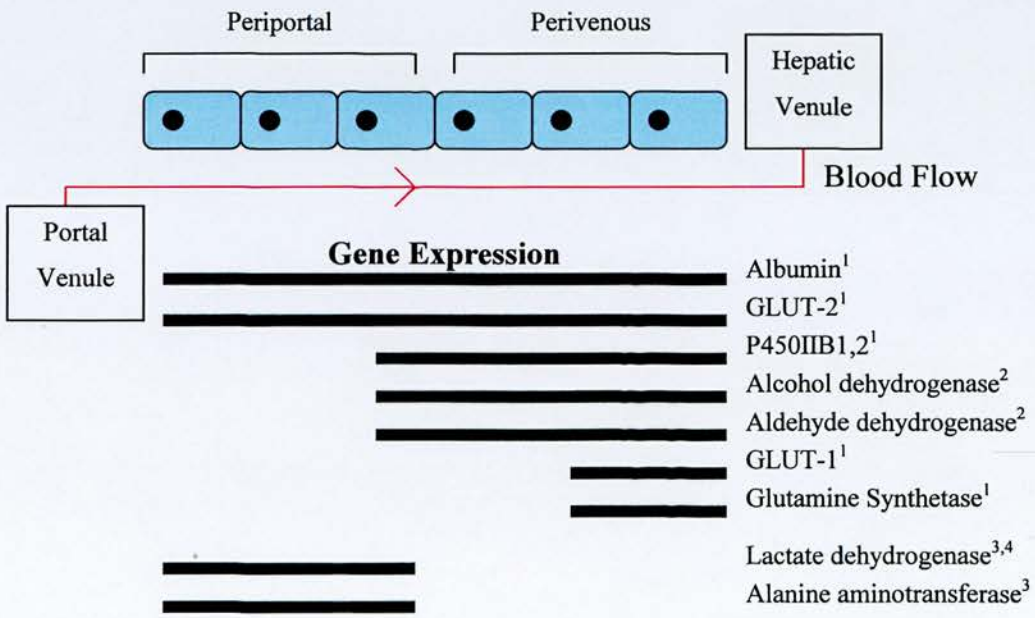


Figure 2. Diagrammatic representation of gene expression in hepatocytes from different zones. Certain genes are expressed in all hepatocytes whereas others are specifically found in the periportal or perivenous region. Adapted from Bilir et al., 1993[1]. Other information taken from Sancho-Tello et al., 1987[2], Rajvanshi et al., 1998[3] and Yamauchi et al., 1988[4].

Vargas et al., 1987, found that cells showing enzymatic properties of the periportal and perivenous areas showed much heterogeneity in regard to ploidy, although the perivenous region was enriched in 2n nuclei whereas the periportal region contained an increased proportion of 4n nuclei. Similarly, Sancho-Tello et al., 1987, demonstrated that the larger neonatal rat hepatocytes showed biochemical and sereological characteristics of the periportal region whereas the smaller, low density cells showed characteristics of the perivenous region. The smaller cells in this study were also found to have higher alcohol-metabolising enzyme activities. Conversely, other studies have demonstrated that cell size was greater in the perivenous region compared to cells from the periportal region (Rajvanshi et al., 1998; Schmucker, 1990). Smaller cells from the periportal region were found to have higher serum albumin or ceruloplasmin biosynthetic rates (Rajvanshi et al., 1998) whereas various studies have shown that the larger cells from the perivenous region have higher cytochrome p450 activity (Ingelman-Sundberg et al., 1988; Rajvanshi et al., 1998; Sweeney et al., 1978b). From these studies, it is obvious that cells in each region express different enzymes and carry out different functions in the liver. However, it is unclear exactly which ploidy populations are found in each region and whether differential gene expression is related to the degree of ploidy in the liver.

1.2.2.1.3 Ploidy during regenerative, non-binucleating growth

The degree of ploidy and nuclearity in the liver has been found to change in many circumstances including after a 2/3 partial hepatectomy (PH). After a PH, the liver has a remarkable capacity to completely regenerate. The response of the liver to such a stimulus is proliferation of all cells types and complete replacement of the removed area (Columbano and Shinozuka, 1996; Michalopoulos and DeFrances, 1997). Early studies of hepatocellular carcinogenesis and more recent studies of regeneration following toxic injury indicated the presence of small, 2n cells, termed oval cells, that could act as progenitor cells. It was suggested that these cells were ultimately responsible for hepatocyte renewal following injury (Sigal et al., 1992; Thorgeirsson, 1993). However, it is now generally accepted that liver regeneration occurs by the division of fully differentiated hepatocytes and that progenitor cells are

only involved in this process when the division of differentiated cells is impaired (Thorgeirsson, 1996; Michalopoulos and DeFrances, 1997). Removal of the liver tissue by PH results in the almost complete disappearance of binuclear cells from the liver (Wheatley, 1972; Melchiorri et al., 1993; Gerlyng et al., 1993). Gerlyng et al., 1993, demonstrated that in rats, the binucleation rate decreased from 27% before PH to 5% 45 h after PH. This study also demonstrated that all (97%) hepatocytes replicated at least once, irrespective of ploidy and nuclearity and therefore all ploidy classes were involved in the regeneration process. The disappearance of binuclear cells does not occur until mitotic activity becomes prominent, indicating that binuclear cells proliferate and form mononuclear progeny (Wheatley, 1972). The 2n hepatocytes exhibit a more sustained proliferative activity during the later phase of liver regeneration (Gerlyng et al., 1993). The growth of the liver after a PH seems to occur in two phases: The first week is where the regenerative growth occurs with an initial loss of binuclear cells and increase in mononuclear cells of all ploidy classes. Subsequently, developmental growth occurs with a high rate of binucleation that eventually restores the fraction of binuclear cells that were initially lost (Saeter et al., 1988). After the first week there is a depletion of 2n cells and an increase in the proportion of polyploid hepatocytes (Brodsky and Uryvaeva, 1977; Melchiorri et al., 1993; Sigal et al., 1999). The significance of alterations in ploidy and nuclearity during regeneration is unclear. The induction of polyploidisation after a PH was suggested to be due to an activation of cell-ageing events such as decreasing the proliferative rate of hepatocytes and advancing the cells through the terminal differentiation process (Sigal et al., 1999). However, it has also been suggested that the switch to non-binucleating growth during regeneration could enable the liver to recover its size without exhausting its growth potential (Gerlyng et al., 1993).

1.2.2.1.4 Drug-induced polyploidisation

Various drugs and chemicals induce DNA synthesis and also alter hepatocyte ploidy and nuclearity. The changes in ploidy after administration of different hepatic mitogens vary. Many studies suggest that the increase in the 2n population is frequently associated with chemical hepatocarcinogenesis. Indeed, administration of thioacetamide to rats increases the 2n population and decreases the polyploid

population as well as increasing S-phase. Long-term administration of thioacetamide produces a high degree of nodule development (Sanz et al., 1995). Other drugs such as carbon tetrachloride (CCl_4) (Steele et al., 1981b) and the liver carcinogens, 2- and 4-acetylaminofluorene (2-AAF, 4-AAF) (Gerlyng et al., 1994; Saeter et al., 1988) and 3'-methyl-4-dimethylaminobenzene (3'M) (Styles et al., 1985) also result in an increase in the proportion of 2n hepatocytes in rodents, although low-doses of 2-AAF were associated with a shift from 2n to 4n (Clawson et al., 1992). Other changes in ploidy occur with the administration of non-genotoxic carcinogens such as phenobarbitone (PB) and the peroxisome proliferators, diethylhexylphthalate (DEHP) and WY-14, 643. Administration of these drugs result in increases in 8n nuclei or cells (Bohm and Noltemeyer, 1981; Hasmall and Roberts, 1997; Hasmall and Roberts, 2000; Miller et al., 1996). A study by Cascales et al., 1994, demonstrated that 7 days administration of cocaine results in an increase in 4n and 8n nuclei and a decrease in 2n nuclei. This effect was even more dramatic when mice were pre-treated with PB. Administration of various drugs also alters the proportion of binuclear cells. After administration of lead nitrate there is an increase in the proportion of binuclear cells, specifically within the 2x4n and 2x8n compartments, with a decrease in 2x2n cells (Melchiorri et al., 1993). However, administration of 2-AAF, 4-AAF, MCP and cyproterone acetate (CPA) induces a pattern of non-binucleating growth similar to the pattern seen during regeneration after a PH (Gerlyng et al., 1994; Saeter et al., 1988). Interestingly, long-term treatment of rats with the antibiotic, rifabutin, induces 'giant' multinuclear cells with some cells containing up to 25 nuclei. The nuclei within these cells appeared to be normal and did not induce any pathological abnormalities (Scampini et al., 1993).

Both the induction of proliferation and alterations in ploidy and nuclearity have been proposed as indicators of the carcinogenic potential of a drug. This hypothesis has been tested by the comparison of the non-genotoxic hepatocarcinogens diethylhexylphthalate (DEHP), chlorendic acid (CEA), MCP and PB with the non-carcinogenic hepatic mitogens 1,4-dichlorobenzene (DCB) and ethylene thiourea (ETU). The studies revealed that all chemicals increased the labelling index (LI) in the mononuclear 8n population. However, it was only the carcinogens that

significantly increased the LI in the binuclear octoploid (2 x 4n) cells. It was therefore suggested that increased DNA synthesis in specific ploidy populations may be significant for subsequent hepatocarcinogenesis (Hasmall and Roberts, 1997; Hasmall and Roberts, 2000).

Various drugs have been found to alter ploidy and injure hepatocytes in particular zones of the liver. Thioacetamide has been shown to induce necrosis in the perivenous region and mainly within the 4n population (Diez-Fernandez et al., 1993). Cocaine and ethanol also preferentially injure cells in the perivenous region as well as altering ploidy (Cascales et al., 1994; Sancho-Tello et al., 1987). Increasing polyploidy associated with high concentrations of the mitotic hepatocarcinogen, dieldrin, occurs only in centrilobular hepatocytes with the periportal region remaining unchanged from controls (Kamendulis et al., 2001). Accumulation of iron in the liver, also associated with an increased risk of cancer, resulted in an increase in 8n nuclei and a decrease in binucleate cells within the centrilobular region of the liver even though the deposition of iron occurred mainly in the periportal region (Madra et al., 1995). Whether injury to certain areas of the liver is directly related to differences in ploidy or whether it is due to different functions of cells within these different regions is unknown.

Clearly studies of regeneration and drug-induced polyploidisation provide vital information about polyploidy in the liver and may help establish the mechanisms and controls involved in the process.

1.2.2.2 Ploidy in cancer and disease

Aneuploid cells are sometimes mistakenly referred to as polyploid. However, aneuploidy is the loss or duplication of chromosomes or chromosomal segments and not the duplicaton of whole chromosomal sets (Feldmann, 1992). The majority of malignant tumours and early stage carcinomas are aneuploid (reviewed by Pihan and Doxsey, 1999). However, hepatocellular carcinomas (HCC), as well as other types of disease, are also associated with changes in the degree of ploidy and nuclearity (Anti et al., 1994; Attallah et al., 1999; Feldmann, 1992), although the findings are

often contradictory. One point to remember is that in the literature, polyploidy is sometimes referred to as aneuploidy and vice versa. However, in this section, care has been taken to ensure that these terms are used separately.

Much work in rodents has used chemically induced carcinogenesis to study ploidy within foci, neoplastic nodules and hepatocellular carcinomas (HCC). The development of HCC requires an inducer such as a PH, an initiating event such as diethylnitrosamine (DEN) and a promoter of growth such as 2-AAF. Several studies in rodents and humans indicate that as well as aneuploidy, a predominance of diploid cells and a decrease in binuclearity are associated with nodular lesions and hepatocarcinogenesis (Anti et al., 1994; Gerlyng et al., 1992; Goolsby and Rao, 1996; Schwarze et al., 1984; Schwarze et al., 1991). Analysis of BrdU incorporation has shown that the 2n cells have a higher proliferative activity than other cells and it has been suggested that this could play a role in the development and progression of the disease (Gerlyng et al., 1992). Schwarze et al., 1984, suggested that the selective outgrowth of 2n cells is an early stage in the development of cancer. The increased diploidy has also been suggested to predispose cells to more severe alterations such as aneuploidy (Anti et al., 1994). Indeed, the hepatocarcinogenic action of the antiestrogen, tamoxifen, is thought to be due to a increase in the proportion of 2n cells in the rat (Dragan et a., 1998).

A predominance of 2n cells in disease is not however the rule. Extensive copper accumulation in the liver in Long Evans Cinnamon (LEC) rats, similar to that seen in patients with Wilson's disease, and an accumulation of iron, results in increasing polyploidy associated with impaired mitotic progression. This can lead to chronic liver injury and to HCC in older rats (Kato et al., 1996; Yamada et al., 1998). Sarafoff et al., 1986, demonstrated that although the majority of foci induced by N-methyl-n-nitrosourea and subsequent feeding with PB consisted of almost exclusively diploid cells, other foci showed an increase in 4n cells and a few large foci contained a mixture of 2n, 4n and 8n hepatocytes. The authors suggest that both 2n and 4n cells are capable of expanding as a homogenous clone. However, the clonal homogeneity is lost when the foci expand. Similarly, Gramantieri et al., 1996,

found that in patients with actual or previous hepatitis B virus (HBV) infection, higher ploidy values and a reduction in binuclearity was associated with the development of HCC. Schwarze et al., 1991, found that long-term treatment of rats with deoxycholic acid resulted in the development of benign neoplastic nodules containing a majority of polyploid cells. However, these nodules failed to progress to the carcinoma stage. Goolsby and Rao, 1996, found that ciprofibrate-induced HCC was associated with the development of aneuploidy and 8n cells. One study of experimental carcinogenesis in mice found no correlation between ploidy and liver carcinogenesis (Danielsen et al., 1988).

The association of ploidy and cancer may be something to do with some alteration in the normal ploidy status in the liver. The nuclei of binuclear cells from normal human liver contain exactly the same amount of DNA (Watanabe and Tanaka, 1982). However, one study of human hepatomas found that binuclear cells obtained from non-cancerous regions and from hepatomas contained nuclei with different DNA contents (Koike et al., 1982). Another study found increases in nuclear deformity as well as increasing DNA content in cases of liver cell dysplasia (Henmi et al., 1985).

Changes in ploidy and nuclearity are also associated with cancer and disease in tissues other than the liver. In atherosclerotic plaques, the degree of tetraploidy was found to be lower than in normal smooth muscle cells (Barrett et al., 1983). However, in spontaneously hypertensive rats, the incidence of polyploidy and multinucleated smooth muscle cells was higher than in normal rats (Lee et al., 1992). In chronic myelocytic leukaemia, there is a marked shift towards lower ploidy in megakaryocytes. However, in patients who have suffered a myocardial infarction, megakaryocyte ploidy is increased (Zimmet and Ravid, 2000). In cervical cancer, both polyploid and aneuploid lesions exist. However, most polyploid lesions regress whereas the aneuploid lesions persist (Reid et al., 1984). High ploidy cells are also found in tumour cell lines and are thought to play a role in the progression of cancer (de la Hoz and Baroja, 1993).

There is a possibility that a change in ploidy is not the sole factor required for disease and that S-phase may also play a role. In a study into chronic viral hepatitis C infection, it was suggested that the suppression of S-phase and the degree of inflammation led to the development of aneuploidy and malignant transformation (Werling et al., 2000). In embryonic tumours, aneuploidy was associated with high levels of S-phase (Rugge et al., 1998).

The significance of ploidy in cancer and disease is unclear and in many cases the hypotheses are contradictory. Development and progression of liver disease may be related to differences in species, strain, age and the agents used in the initiation of cancer (Lin et al., 1989). Other factors such as proliferation and apoptosis are also likely to play a role in the development of disease and alterations in all these processes, not just ploidy, may affect the onset and outcome of disease.

1.3 Significance of Polyploidy

For many cell types such as megakaryocytes and bladder epithelial cells, polyploid cells play a role in tissue function. However, the function and significance of polyploidy in hepatocytes is unclear and as mentioed above, hypotheses are contradictory. Early studies suggest that polyploidisation in hepatocytes is a protective mechanism, whereas more recent work suggests that increased polyploidy is associated with terminal differentiation and could lead to an increased risk of carcinogenesis. This section will examine the function of various polyploid cells and will examine the evidence leading to each of the hypotheses regarding polyploidy in the liver.

1.3.1 A protective mechanism or a 'cheaper' alternative to mitosis?

Brodsky and Uryvaeva, 1977, suggested that when hepatocytes are forced to grow whilst carrying out their tissue function, the cells are incapable of adequate preparative action and as a result replace proliferation with polyploidisation. Studies

of polyteny suggest that by avoiding mitosis, organisms undergoing endoreduplication cycles save a considerable amount of time and energy and suggest that polyploidisation could be an economical alternative to proliferation (Therman et al., 1983). Similarly, non-binucleating growth associated with regeneration after a PH may enable the liver to grow faster whilst retaining its normal function. Therefore there is a possibility that polyploidisation in hepatocytes is an economical alternative to mitosis.

Over the past few decades there have been other suggestions that polyploidisation in the liver could in fact be a protective response. The role of the liver is to absorb and detoxify various toxic substances on a daily basis and therefore, the chance of DNA damage is higher for hepatocytes than for other cells in the body. Polyploidisation avoiding mitosis was therefore proposed as a method that would limit the development of chromosomal abnormalities and reduce the probability of cell death, malignant transformation and other deleterious consequences (Medvedev, 1986; Uryvaeva, 1981). The fact that short-term administration of various chemicals induces polyploidisation in the liver may indicate that polyploidisation is indeed a protective response. Cascales et al., 1994, suggested that the shift to higher ploidy during drug-induced injury occurs as a method of increasing the function of the liver whilst coping with intensive stress injury. Similarly, Sanz et al., 1996, suggested that increasing ploidy associated with ageing results in higher levels of antioxidant enzymes and thereby enhances the defence mechanisms of the cell against oxidative damage. Interestingly, the majority of tumours in the liver are predominantly diploid or aneuploid (Goolsby and Rao, 1996; Saeter et al., 1988; Sargent et al., 1996; Schwarze et al., 1984; Schwarze et al., 1991) (see section 1.2.3.5), which supports the suggestion that cells containing more than the normal number of chromosomes might be protected from the dominant expression of mutated oncogenes (Schwarze et al., 1984). A recent study into the significance of polyploidy in the liver suggested that if polyploidy is a protective response, carnivores, being less exposed to alkaloids and vegatative toxins, may have a lower degree of hepatocyte ploidy than herbivores. Correlation analyses found that there were no significant differences in the ploidy levels between such animals. The results did however suggest that the significant

differences between the levels of mononuclear and binuclear cells could play some role in the detoxification function (Vinogradov et al., 2001).

One obvious question that arises from these hypotheses is 'if beneficial, why has polyploidisation not evolved in the liver of all mammals?' This could be related to longevity of the species, as generally it is the shorter-lived species that have higher levels of polyploid cells. Uryvaeva, 1981, suggested that longer-lived species have better DNA repair mechanisms and because hepatocytes in these species rarely divide, there is sufficient time for chromosomal aberrations to be repaired. Since then, statistical analyses have been carried out on various species to test whether longevity, body weight, rate of development and basal metabolism could be correlated with ploidy. The results indicated that only the basal metabolism and developmental rate correlated significantly. The authors therefore suggested that polyploidisation evolved in relatively short-lived species with rapid development, as a 'cheap' way to cope with toxic insult (Anatskaya et al., 1994). More recently, a comparison of more than 50 mammalian species demonstrated that post-natal growth rate had the strongest effect on ploidy. The authors concluded that somatic polyploidy was therefore a 'cheap' solution to growth problems that appear when an organ is working at the limit of its capabilities (Vinogradov et al., 2001).

1.3.2 Terminal differentiation, senescence and apoptosis

A recent review suggested that advanced polyploidy in mammalian cells is considered to indicate terminal differentiation and cellular senescence and may lead to the loss of replicative activity and eventually to apoptosis (Gupta et al., 2000). Replicative senescence and terminal differentiation are associated with changes in the morphology, biochemistry and genetics of a cell (Smith and Pereira-Smith, 1996). Changes in proliferation, autofluorescence, rate of regeneration and healing have all been studied in ageing animals and have provided evidence that hepatocytes undergo terminal differentiation and senescence. Several studies have shown that cell proliferation is inversely proportional to ploidy and age (Barbason et al., 1974; Brodsky and Uryvaeva, 1977; Mossin et al., 1994). This decrease in proliferative

potential and the finding that polyploid cells show an increase in autofluorescence (from products of lipid peroxidation such as lipofuscin, which represent increased oxidative activity) have indicated that the liver is indeed undergoing a process of terminal differentiation associated with ageing (Sigal et al., 1995). Beyer et al., 1991, demonstrated that regeneration capacity is reduced with age. A reduction in the proliferative response after a PH in older animals was also confirmed by the finding that the induction of $p34^{cdc2}$ and proliferating cell nuclear antigen (PCNA) is reduced (Timchenko et al., 1998). Post et al., 1960, previously showed that there was a decreased rate of healing associated with age after administration of CCl_4. Another study demonstrated that the accumulation of polyploid cells after a PH was associated with an increase in senescence-associated β-galactosidase and p21 expression (Sigal et al., 1999).

Terminal differentiation and senescence have not been properly defined and there is some debate as to whether or not hepatocytes are terminally differentiated. Michalopoulos and DeFrances, 1997, suggested that mature hepatocytes are not terminally differentiated as they are capable of undergoing multiple rounds of DNA synthesis and de-differentiate under the influence of hepatocyte growth factor (HGF) or epidermal growth factor (EGF). Overturf et al., 1997, demonstrated that hepatocytes isolated from adult mouse livers are capable of multiple rounds of division and can undergo greater than 80 rounds following serial transplantation of cells into diseased livers. Separation of cells by centrifugal elutriation also demonstrated that it was in fact the larger, denser cells that had a higher repopulation capacity than smaller cells (Overturf et al., 1999). Similarly, the transplantation of sorted hepatocytes into diseased mouse liver demonstrated that 4n and 8n hepatocytes participate in subsequent repopulation (Weglarz et al., 2000). Therefore, it seems that although polyploid hepatocytes show characteristics of terminal differentiation and senescence with age, these cells are capable of proliferation to compensate for any loss of tissue.

There is some evidence to support the suggestion that polyploid cells are more susceptible to apoptosis. Oren et al., 1999, found that the rate of apoptosis was

increased in polyploid cells after administration of the potent hepatic mitogen, triiodothyronine (T3) to rats pre-treated with the cell cycle inhibitor, retrorsine. Sigal et al., 1999, suggested that apoptosis accounted for the decrease in the proportion of 8n cells 5 days after a partial hepatectomy. Increased rates of apoptosis have also been found in the livers of Long-Evans Cinnamon (LEC) rats where the liver has become populated with megalocytes, containing enormous nuclei (Gupta et al., 2000). Multinucleated giant cells induced in HeLa cells by down regulation of the zinc finger protein, KRC, ultimately died. The authors suggested however that cell death occurred through 'mitotic cell death' due to the unsupportable nature of multiple rounds of DNA synthesis in the absence of complete mitosis (Allen and Wu, 2000). In other studies, apoptosis induced in hepatocytes by the administration of the mitogen, lead nitrate, was not found to occur preferentially in the polyploid population (Melchiorri et al., 1993). Similarly, liver injury induced by aminoguanidine results in a delay in polyploidy, increase in DNA synthesis and is accompanied by the appearance of a hypodiploid population (Diez-Fernandez et al., 1998) suggesting some link between lower levels of ploidy and cell death. Therefore further study is required to determine exactly what circumstances are required to induce apoptosis in polyploid cells.

1.3.3 Other functions of polyploid cells

1.3.3.1 Increasing cell size

In megakaryocytes, polyploidisation is directly related to cell function, resulting in an increase in platelet production (Japa, 1943; Baatout et al., 1998a). Another example of polyploidy relating to cell function is in the development of the butterfly wing. The wing is made up of three different types of scales (cells) that differ in size and position. The actual function of these scales in the construction of the wing depends on their level of ploidy; 8n, 16n or 32n (Brodsky and Uryvaeva, 1985c). Polyploid cells are also important to the function of the urinary bladder. The thick membrane surrounding the bladder is made up entirely of polyploid cells and functions as a barrier between the tissue fluid and the urine (Hicks, 1975). Polyploid

cells are more efficient in this case as they are bigger than 2n cells. The function of polyploidy in cardiomyocytes is unclear. However, the most common consequence of polyploidisation is cell enlargement. Brodsky et al., 1980, suggest that there is a possibility that a small number of polyploid cardiomyocytes has some advantage over a large number of smaller diploid cells.

As mentioned previously, cell size and volume are proportional to DNA content in the rodent and human liver (Danielsen et al., 1986; Deschenes et al., 1981; Watanabe and Tanaka, 1982). The role of increasing size in polyploid hepatocytes has not been investigated. Increasing cell size and volume have been shown to be important in the regulation of many processes including cell proliferation and apoptosis (Lang et al., 1998) and cell size has been implicated in the regulation of the transcription factor, DBP (Schmidt and Schibler, 1995). Therefore, there is a possibility that cell size may play a role in the function of hepatocytes and requires investigation.

1.3.3.2 Increasing RNA and protein synthesis

Increasing RNA transcription and protein synthesis has been associated with increasing DNA content in various polyploid tissues. The 4n cells in the locust testis contain twice the amount of RNA than the 2n cells and the amount of collagen produced by a 4n fibroblast was found to be twice that of a 2n fibroblast in vitro (Brodsky and Uryvaeva, 1985a). Differential expression of various genes in different regions or zones of the liver has already been mentioned (see section 1.2.3.2) and as yet, it is unknown whether gene expression in these zones is related to size or ploidy. Studies which have looked specifically at gene and protein expression within hepatocytes containing different amounts of DNA have found conflicting results. Several studies, using radio-labelling and cytophotometry, have shown that the amount of RNA transcription doubles with increasing ploidy. Therefore, a 4n cell would produce twice as much RNA as a 2n cell and an 8n cell would produce twice that of a 4n cell (Brodsky and Uryvaeva, 1985c). A more recent study found that the levels of catalase and glutathione peroxidase mRNA levels also correlated with the degree of polyploidy (Fogt and Nanji, 1996). However, a study of the level of RNA transcription within a 15 min period in rat hepatocytes found cells

containing double the amount of DNA did not produce significantly more RNA (Collins, 1978). Conflicting findings are also associated with protein synthesis. The amount of albumin produced by 4n rat hepatocytes was found to be approximately twice that produced by 2n hepatocytes (Le Rumeur et al., 1981). The activity of succinate dehydrogenase, NADPH cytochrome *c* and lactate dehydrogenase was found to increase with DNA content (Tulp et al., 1976). However, the activity of glucose-6-phosphate reductase in 2n and 4n rat hepatocytes (Bernaert et al., 1979) was found to be the same. Different techniques and animals were used in these experiments which could, in part, explain some of these contradictory findings. However, it is possible that the expression of only certain genes is increased in proportion to DNA content and as yet it is unknown whether differential gene expression is related to the position and function in the liver and not just ploidy. Further experiments are needed to determine if this is the case.

1.4 Mechanisms of Polyploidisation

Polyploidisation can occur through different mechanisms and most often occurs through a block in mitosis or cell division or through the omission of mitosis. The different mechanisms are often mixed up in the literature. Therefore to avoid confusion, table 3 provides clear definitions of each process. This section will not examine all the mechanisms listed in table 3 but will discuss the mechanisms of polyploidisation which are encountered most frequently including 1) endomitosis, 2) polyploidising mitosis and 3) endoreduplication.

Table 3*. Definitions of the various methods of polyploidisation.

Mechanism of Polyploidisation	Definintion	Example
Acytokinetic mitosis	Cells complete M-phase but do not go through cytokinesis, forming binuclear cells.	Hepatocytes Cardiomyocytes
Endomitosis	Cells enter M-phase but are blocked at prophase, without dissolution of the nuclear membrane.	Megarkaryocyte cell lines, originally thought to occur in primary megakaryocytes
Endomitosis-A	Cells enter M-phase but do not complete anaphase prior to re-entering the cell cycle.	Megakaryocytes
Endoreduplication or endoreplication	Cells do not enter mitosis but proceed from a gap phase to S-phase. Polyteny is a special case where the duplicated chromosomes line up, side by side, forming large polytene chromosomes.	Secretory cells, diptera
Nuclear restitution[•]	Cells enter mitosis and proceed to telophase where there is fusion of chromosomal complexes.	
C-mitosis	Cells enter M-phase but are blocked at metaphase following chromosome condensation and spindle formation. Often results in the formation of several micronuclei[+].	Rarely occurs in non-malignant cells[+].

*Adapted from Zimmet and Ravid, 2000. [•]Definition from Brodsky and Uryvaeva, 1985b. [+]Therman et al., 1983.

1.4.1 Endomitosis

There appear to be at least two types of endomitosis, see Table 3. The first definition of endomitosis suggests that cells enter mitosis and are blocked at prophase. Cells then proceed through to the next gap phase without dissolution of the nuclear membrane. Endomitosis is now more commonly used to describe cells that proceed through anaphase but lack nuclear division and cytokinesis as in endomitosis-A (Edgar and Orr-Weaver, 2001; Therman et al., 1983; Zimmet and Ravid, 2000). Endomitosis occurs in insects, some other invertebrates, plants (Brodsky and Uryvaeva, 1985b) and some mammalian cells (Therman et al., 1983). In mammals, megakaryocytes are perhaps the most widely studied. These cells can reach 128n before fragmenting to form platelets (Baatout et al., 1998a). In primary megakaryocytes it has been shown that the nuclear membrane breaks down during

mitosis and anaphase B, telophase and cytokinesis do not occur (Nagata et al., 1997), see Figure 3. The process of endomitosis in primary megakaryocytes and megakaryocye cell lines (Datta et al., 1996; Kikuchi et al., 1997; Zhang et al., 1996) is different, suggesting that different regulatory mechanisms are involved in each cell type.

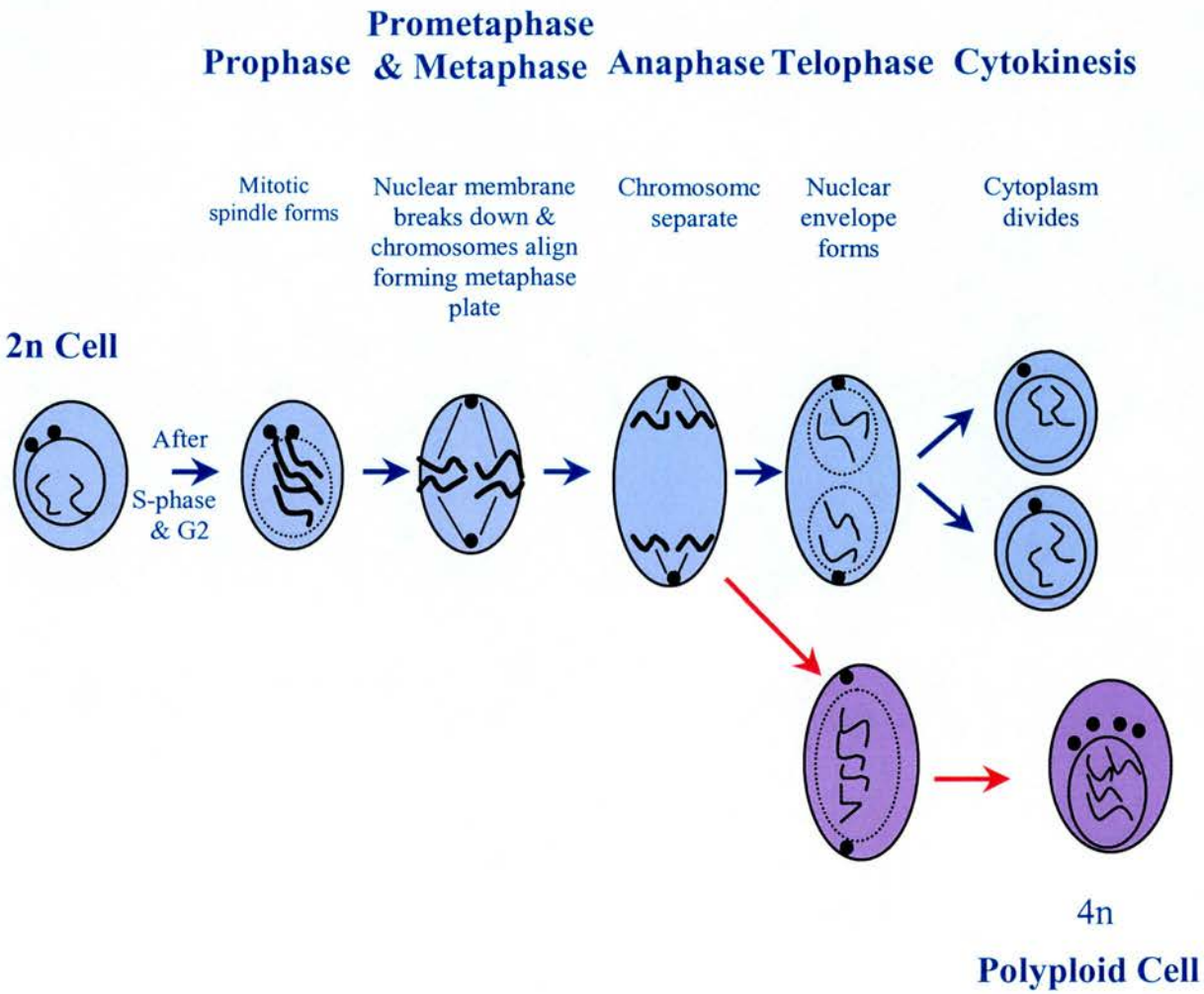


Figure 3. Diagrammatic representation of the process of endomitosis in megakaryocytes. A 2n cell enters mitosis after G2 and continues to anaphase where there is a block. The nuclear membrane reforms at a stage equivalent to telophase and cytokinesis is bypassed completely, resulting in the formation of a polyploid 4n cell.

1.4.2 Polyploidising mitosis

Several decades ago, it was noted that some cells contained more than the normal amount of DNA but had not undergone endoreduplication or endomitosis (Brodsky and Uryvaeva, 1985b). These cells were found to undergo variant mitotic cycles or incomplete mitosis referred to as 'polyploidising mitosis.' Two distinct mechanisms of polyploidising mitoses were subsequently found. The first process was termed 'nuclear restitution' and involved the formation of polyploid cells through the fusion of the chromosomal complexes during telophase. The second process involved the formation of polyploid cells through acytokinetic mitosis and binucleation (Brodsky and Uryvaeva, 1985b). The binuclear cell is thought to be a common intermediate step in the formation of polyploid cells in many mammalian cells including hepatocytes, the myocardium, pigment epithelium of the retina, sympathetic ganglia, fibroblasts of loose connective tissue and in mesothelium. Acytokinesis and binucleation have also been described in the gastric epithelium of starfish, in the intestine of ascarids and in some of the tissues in plants (Brodsky et al., 1980; Brodsky and Uryvaeva, 1985b). In the case of hepatocytes, although it has been accepted that the formation of binuclear cells occurs through acytokinesis, it is unclear exactly how mononuclear polyploid cells arise from these binuclear cells. In vitro studies suggest that S-phase and entry into mitosis occur simultaneously in both nuclei. During mitosis there is fusion of the two spindles and the formation of a single metaphase plate. Anaphase, telophase and cytokinesis follow, resulting in the formation of two mononuclear polyploid cells (Carriere, 1967). Alternatively, time-lapse experiments and electron microscopy studies suggest both nuclei enter S-phase simultaneously then enter mitosis where there is the formation of a common mitotic spindle. Chromosomal segregation and cytokinesis then occur forming mononuclear cells of a higher ploidy class (James, 1977). The proposed mechanisms for the formation of binuclear and polyploid hepatocytes are illustrated in Figure 4. As previously mentioned, the mode of growth in hepatocytes after a liver has undergone a PH or after administration of various drugs, does not involve the formation of intermediary binuclear cells (Wheatley, 1972; Melchiorri et al., 1993). Therefore,

the method of polyploidisation in hepatocytes may differ depending on the circumstances.

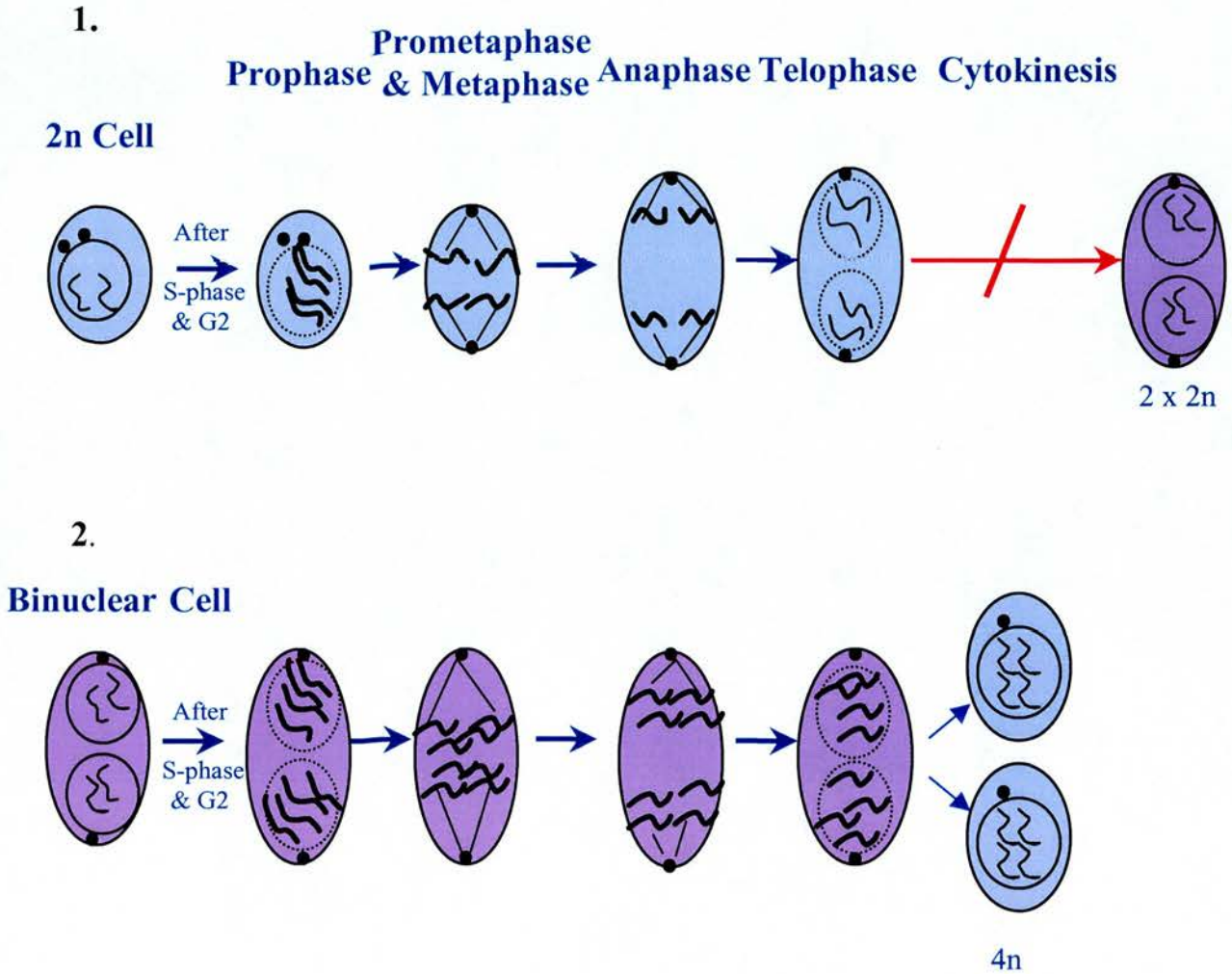


Figure 4. Schematic representation of the proposed mechanisms of polyploidisation in hepatocytes. 1. Formation of binuclear tetraploid cell through acytokinesis, a 2n cell becomes 2x2n; 2. Formation of mononuclear tetraploid cells through either a fusion in the two spindles or formation of a common mitotic spindle at anaphase and the subsequent production of two daughter cells, the 2x2n cell becomes two 4n cells.

1.4.3 Endoreduplication

Endoreduplication or endoreplication is different to the processes occurring in megakaryocytes and hepatocytes. Endoreduplication occurs through repeated DNA synthesis in the absence of any mitotic process. In many organisms the chromatids remain closely associated forming polytene chromosomes (Edgar and Orr-Weaver, 2001; Leitch, 2000). Much of the early work on endoreduplication or polyteny, was published in the 1970's due to the discovery of long banded chromosomes in the larval tissues of *Drosophila*, Chironomidae and some other diptera, reviewed by (Brodsky and Uryvaeva1985b). Other examples are given in section 1.2.1.

1.4.4 Other mechanisms

Fusion of cells is an important mechanism in the formation of multinucleate cells in vivo and in vitro. This process occurs naturally in vivo, during the formation of skeletal muscles and fertilisation of the ovum and is seen in vitro in a variety of cell types (Brodsky and Uryvaeva, 1985b). There seems to be two distinct types of cell fusion: fusion occurring during differentiation when two adjacent cells fuse together and 'refusion' which is more likely to be due to defect in mitosis (Martin, 1972; Wheatley, 1972). Refusion is thought to occur when the two daughter cells reunite after cytokinesis or when a cell undergoes incomplete cytokinesis. As mentioned earlier, adult rat hepatocytes have been shown to undergo spontaneous cell fusion in culture, resulting in the formation of multinuclear cells (Gomez-Lechon et al., 1981). However, it is thought that fusion is an artefact of culture and does not occur in vivo.

Monopolar, multipolar and c-mitoses can result in the formation of polyploid cells or cells containing a reduction in the amount of DNA (Brodsky and Uryvaeva, 1985b). These processes are very rare and will therefore not be discussed in this thesis.

1.5 Control and Regulation of Ploidy

Polyploidisation in the liver is under complex hormonal control. However, the actual molecular mechanisms involved in this process have not been elucidated. Genetic analysis of yeast, *Xenopus*, *Drosophila* and the nematode, *Caenorhabditis elegans* now provide insights into the molecular control of the cell cycle and could provide vital information regarding the molecular mechanisms of polyploidisation. This section will therefore examine the hormonal regulation of this process and try to elucidate some of the factors controlling polyploidisation by examining the regulation of ploidy in the liver and other eukaryotic cells.

1.5.1 Regulation of ploidy and proliferation by hormones, growth factors and cytokines

There is much evidence to suggest that hormonal regulation plays an important role in both polyploidisation and proliferation. In the rat, the ontogeny of 4n cells resembles that of the liver thyroid hormone receptor beta (TR-β) (Rodd et al., 1992). The liver proteins α_{2u}-globulin (Chatterjee et al., 1983), major urinary protein (MUP) (Held et al., 1989) and the liver microsomal protein, LAGS (low affinity glucocorticoid binding site), which are under complex multi-hormone regulation, also appear at this time (Chirino et al., 1991). Several studies have demonstrated that thyroid hormones, growth hormone (GH) and sex steroids modulate polyploidisation and proliferation to varying degrees (Alfert and Geschwind, 1958; Carriere, 1967; Torres et al., 1999). Originally it was thought that the action of thyroid hormone was mediated through the release of growth hormone (Carriere, 1967). However, more recently it was found that GH, unlike thyroid hormone, was not essential for polyploidisation. Torres et al., 1999, demonstrated that the decrease in 4n nuclei induced by a hypophysectomy in rats was reversed by treatment with T_3 but not with GH. Similarly, T_3 significantly increased S-phase and restored the normal adult level of 4n nuclei in the hypothyroid rat but S-phase and level of 4n nuclei were unaffected

by GH. The differences in the degree of ploidy in humans, with males showing a higher proportion of 4n nuclei than females, was found to be due to the effects of gonadal steroids. Oestrogen was said to delay while testosterone promoted the polyploidisation process. The authors therefore suggest that thyroid hormones play an essential regulatory role whereas GH, together with the sex steroids modulate polyploidisation to variable degrees depending on age and gender of the animals.

Other evidence for the importance of hormones in polyploidisation comes from studies on dietary restricted rats. Compared with rats fed ad libitum, dietary restricted rats were found to attain the adult level of ploidy 6 months later (Enesco et al., 1991). Dietary restriction is known to inhibit growth and growth is dependent on levels of thyroid hormone, GH (Carriere, 1967) and the thymus (Pieri et al., 1980; Pieri et al., 1982). Therefore it is possible that modulation of these hormones affects the rate of polyploidisation. However, another possible mechanism of control of this process could be through alterations in thyroid hormone receptor (TR) expression. There are at least three isoforms of the TR, which are present in different tissues at different concentrations (Glass and Holloway, 1990; Mitsuhashi and Nikodem, 1989; Schwartz et al., 1992). During fasting, the levels of the TR $\alpha 2$ isoform mRNA increased in the liver. The TR $\alpha 2$ isoform is unable to bind to T_3 and may act as a competitive inhibitor regulating the effect of T_3 on liver growth (Bakker et al., 1998).

The exact mechanisms by which thyroid hormones exert their effect have not yet been elucidated. In rats, it has been demonstrated that administration of insulin-like growth factor-1 (IGF-1) diminishes the metabolic thyroid hormone action in the liver by at least in part reducing the number to thyroid hormone receptors and their level of expression (Pellizas et al., 1998). T_3 has also been shown to regulate IGF-1 by stimulating hepatic growth hormone receptor gene expression in the chicken (Tsukada et al., 1998). The effect of T_3 was also found to affect the kinetics of thymidine kinase, an enzyme important in DNA synthesis (Maliekal et al., 1997). In replicating hepatocytes, poly (ADP-ribose) polymerase, a nuclear enzyme involved in DNA synthesis, DNA repair, cell replication and transformation is modulated by

thyroid hormones and could be another mechanism of action of thyroid hromones (Cesarone et al., 2000).

Thyroid hormones also influence DNA synthesis during liver regeneration after a PH (Maliekal et al., 1997). Cytokines and growth factors are also involved, including transforming growth factor alpha (TGFα) (Scotte et al., 1997) and interleukin-6 (IL-6) (Cressman et al., 1996). Modulation of DNA synthesis is also achieved in cultured hepatocytes by administration of insulin, EGF (Mossin et al., 1994), dexamethasone, glucagon, norepinephrine (De Juan et al., 1992) and IGF-1 and IGF-2 (Kimura and Ogihara, 1998). Interestingly, administration of IL-6 in mice and IL-11 in rats resulted in increasing megakaryocyte cell size and ploidy (Ishibashi et al., 1989; Yonemura et al., 1993). The importance of cytokines and growth factors in liver growth and in megakaryocytopoiesis may therefore suggest a role for them in the regulation of polyploidisation. Polyploidisation is likely to involve complex interactions with various different molecules and receptors.

1.5.2 Cell cycle

The cell cycle is tightly regulated with many checkpoints ensuring that S-phase does not occur until mitosis is completed and vice versa. In order for polyploidisation to occur, be it endomitosis, endoreduplication of acytokinesis, these checkpoints must somehow be overcome. Some of the main molecules involved in these checkpoints are illustrated in Figure 5. Alterations in several regulatory molecules such as cyclin/cyclin dependent kinase (CDK) complexes and cyclin dependent kinase inhibitors (CDKIs) have been associated with polyploidisation in various cell types. A detailed study of all genes involved in the cell cycle is outwith the scope of this thesis and there are several comprehensive reviews on this subject (Coffman and Studzinski, 1999; Gao and Zelenka, 1997; Nasmyth, 1996b; Pines, 1999). Therefore, the next section examines only genes implicated in the process of polyploidisation.

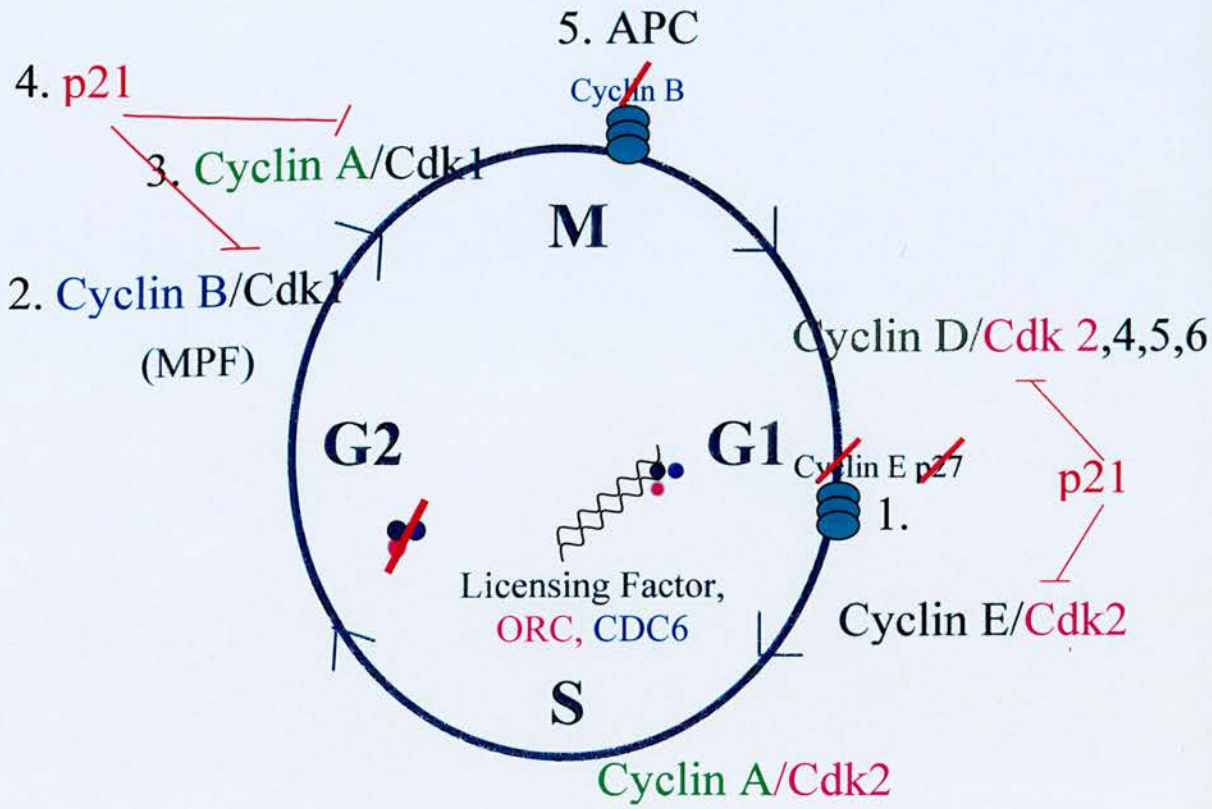


Figure 5. Simplified diagram of the cell cycle representing the main molecules involved in checkpoint controls and polyploidisation. 1. Prevention of SCF activity results in the accumulation of cyclin E and p27 and endoreduplication (Nakayama et al., 2000). 2. Decreasing levels of cyclin B and/or Cdk1 are associated with endoreduplication (Hall et al., 1996; Grafi and Larkins, 1995; Zhang et al., 1998). 3. Overexpression of cyclin A induces multiple rounds of DNA synthesis and the formation of multinuclear cells (Bortner and Rosenberg, 1995; Datta et al., 1998). 4. Overexpression of p21 leads to prevention of mitosis and re-replication of DNA (Kikuchi et al., 1997). 5. Overexpression of APC activators results in reduced levels of cyclin B and endoreduplication (Cebolla et al., 1999).

1.5.2.1 Cyclin/CDK complexes

In mammalian cells, the G1 phase of the cycle is characterised by the presence of G1 cyclins (Cyclin D1, D2, D3) and the CDKs 2, 4, (5) and 6. The cyclin E/CDK 2 complex, sometimes called 'replication promoting factor' (RPF), is required for the G1/S transition and cyclin A/CDK 2 is necessary for the progression through S-phase. Cyclin B and cyclin A bind to CDK 1 ($p34^{cdc2}$) and are involved in the initiation and progression of mitosis. The cyclin B/CDK1 complex is also known as mitosis promoting factor (MPF). Activation of MPF occurs through dephosphorylation of phosphate groups on the tyrosine 15 and threonine 14 residues. Exit from mitosis occurs through ubiquitin-dependent cyclin proteolysis. Changes in the phosphorylation status or expression through increased translation or degradation of any of the above cyclins or CDKs could lead to repeated S-phases without mitosis or mitosis without DNA synthesis resulting in the formation of polyploid or aploid (no DNA) cells, respectively.

Studies in *Xenopus* and yeast have shown that $p34^{cdc2}$ is one of the key regulators of the cell cycle (Dasso and Newport, 1990; Enoch and Nurse, 1990). Mutants of the *cdc2* gene in the fission yeast have been shown to undergo DNA synthesis without an intervening mitosis resulting in the formation of polyploid cells (Broek et al., 1991). Inhibition of $p34^{cdc2}$ by staurosporine in metaphase-arrested mouse cells and the staurosporine analogue, K-252a in rat fibroblasts resulted in the formation of polyploid cells (Hall et al., 1996; Usui et al., 1991). Endoreduplication in maize endosperm has been shown to involve the inhibition of the cyclin B/Cdc2 complex (MPF) (Grafi and Larkins, 1995). Reduction in the levels or activity of MPF is also associated with endoreduplication in budding yeast (Dahmann et al., 1995) and *Drosophila* (Sigrist and Lehner, 1997). Polyploidisation induced by 1,25-dihydroxyvitamin D_3 in HL60 cells was associated with a block in G2/M due to decreased levels of $p34^{cdc2}$ protein and kinase activities. Interestingly, some cells increased other G2 regulators and were able to overcome this block, suggesting that cells have the capability to compensate for decreased $p34^{cdc2}$ activity (Harrison et al., 1999).

Studies using megakaryocyte-derived cell lines have established the importance of cyclin/CDK complexes in endomitosis. A study using the human erythroleukemia (HEL) cell line stimulated with phorbol esters found that elevated and sustained levels of cyclin B1 were found in the nucleus of endomitotic cells and that CDK1 protein levels were reduced. Overexpression of CDK1 did not lead to a normal mitosis and the authors suggested that this was due to a failure in the formation of MPF. This study also demonstrated that cyclin B1, normally localised in the cytoplasm, translocates to the nucleus of polyploid cells (Datta et al., 1996). Conversely, other studies using the MegT (murine megakaryocyte) cell line have found reduced levels of cyclin B1 with apparently normal levels of CDK1. The decrease in cyclin B1 was due to accelerated ubiquitin-dependent degradation (Zhang et al., 1996; Zhang et al., 1998). These studies suggest that endomitotic cells cannot undergo mitosis due to a failure to form MPF either by reduced levels of CDK1 or cyclin B1, resulting in further rounds of DNA synthesis without a complete mitosis. A study into the kinetics of cyclin B1 in primary murine megakaryocytes found that in G1, cyclin B was increased in polyploid cells and in G2/M, levels of cyclin B increased linearly with ploidy. However, there was no definitive change in the polyploid cells and it was therefore suggested that endomitosis in primary megakaryocytes involves alterations of other cell cycle regulators (Carow et al., 2001). Similarly, a study in human megakaryocytes found that endomitosis occurred independently from cyclin B1 regulation and that the ubiquitin-dependent degradation of cyclin B1 occurred normally at anaphase (Roy et al., 2001). The hearts and livers of embryonic or newborn mice, deficient in the winged helix transcription factor, *Trident* (also known as HFH-11, FKL16 and WIN), show extensive polyploidy, with cardiomyocytes gaining up to 50 times the amount of DNA. Disruption of *Trident* results in post-natal death. The authors suggest that *Trident* could play a role in the upregulation of M-phase cyclins or CDKs (Korver et al., 1998). Interestingly, the Trident gene is involved in regulating proliferation induced by a PH (Ye et al., 1999).

Cyclin D3/CDK2 and cyclin E/CDK2 activity have been found to increase during endomitosis in the HEL cell line, as has the specific kinase activity of the cyclin

A/CDK2 complex. This increase in cyclin/CDK complex activity was found to be augmented by reductions in the CDK inhibitory proteins, $p21^{CIP1}$ and $p27^{KIP1}$ (Datta et al., 1998). Antisense experiments in primary megakaryocyte cell cultures have found that although low levels of cyclin B1 are associated with megakaryocyte polyploidy, progression of the polyploidisation process was dependent on the presence of cyclin D3 (Wang et al., 1995). In transgenic mice, overexpression of cyclin D3 increased the level of megakaryocyte ploidy (Zimmet et al., 1997). Therefore, it was suggested that endomitosis involves the upregulation of CDK complexes involved in G1 and S phase progression. *Drosophila* is a particularly interesting model to study as both mitotic and endoreduplication cycles occur during normal development. The endoreduplication cycles are characterised by the absence of cyclins A and B and the periodic expression of cyclin E (Knoblich et al., 1994; Sauer et al., 1995). The switch from mitotic cycles to endoreduplication cycles seems to be regulated by a conserved eukaryotic gene, fizzy-related (*fzr*), which negatively regulates the levels of cyclins A, B and B3 (Sigrist and Lehner, 1997). Loss of the gene encoding a zinc finger transcription factor, *escargot*, is also associated with initiation of an endocycle in *Drosophila*. *Escargot* was found to interact with *Dmcdc2* (the *Drosophila* form of Cdc2 or CDK1) and was therefore proposed to inhibit entry into S-phase by maintaining high level of the cyclin/CDK complex in G2 (Hayashi, 1996).

Cyclin A is critical in the control of DNA replication (Girard et al., 1991; Resnitzky et al., 1995; Rosenberg et al., 1995) and the G2 to M transition (Lehner and O'Farrell, 1990; Ravnik and Wolgemuth, 1996; Pagano et al., 1992) and therefore is another candidate in the control of polyploid cell formation. A comparison of haploid and tetraploid strains of *Saccharomyces cerevisiae* using microarray technology found several genes that were either induced or repressed. One gene that was repressed was *CLN1*, a G1 cyclin with homology to human cyclin A. The authors suggest that lower expression of G1 cyclins allows cells to pass through START at a larger size and remain polyploid (Galitski et al., 1999). Recently it was found that overexpression of the transcription factor, B-myb, a direct physiological target for cyclin A/CDK2, enables various G1 checkpoints to be overcome,

promoting entry into S-phase in certain cell lines (Saville and Watson, 1998). Overexpression of cyclin A in mammary glands of transgenic mice results in the formation of multinucleate cells, suggested to occur through the failure of cytokinesis (Bortner and Rosenberg, 1995). Overexpression of the oncogene, c-myc in colcemid-treated cells is thought to induce DNA re-replication and polyploidy through the activation of CDK2 activity (Kao et al., 1996; Li and Dang, 1999). Therefore, polyploidy seems to occur through alterations in various cyclins, either through overexpression or ubiquitin-dependent degradation and also through altering CDK activity, with different mechanisms occurring in different cells.

The ubiquitin proteasome pathway does in fact play a role in the control of polyploidisation in various cells. Accumulation of cyclin E and $p27^{Kip1}$ in mice lacking the Skp2 protein (an F-box protein and substrate recognition component of a Skp-1-Cullin-F-box protein (SCF) ubiquitin ligase), results in the formation of cells with enlarged nuclei, polyploidy and multiple centrosomes. These enlarged cells show reduced growth rates and increased apoptosis (Nakayama et al., 2000). In *S.cerevisiae*, mutation of the *DOA4* gene, which codes for a ubiquitin hydrolase, results in the overreplication of parts of the genome (Singer et al., 1996). In fission yeast, polyploid *pop1* mutants accumulate high levels of the CDK inhibitor, Rum1 and the S-phase regulator Cdc18. These proteins are not ubiquitinated in the absence of *pop1*, suggesting that *pop1*, may be a recognition factor for Rum1 and Cdc18, targeting these proteins for ubiquitination and degradation through the 26S proteasome pathway (Kominami and Toda, 1997).

In yeast, formation of pre-replication complexes before the initiation of S-phase requires several proteins including an origin recognition complex (ORC), licensing factors such as MCM proteins and Cdc6. Phosphorylation of these proteins by cyclin/CDK complexes has been implicated in the control of S-phase and ensures DNA replication occurs 'once per cycle'. Cyclin B/CDK complexes have been found to inhibit the transition of replication origins to a pre-replicative state in *S.cerevisiae* (Dahmann et al., 1995). The ability of Cyclin A/CDK2 to phosphorylate CDC6 is proposed to be a negative regulatory event, preventing the re-replication of

DNA during S-phase and G2 (Petersen et al., 1999). Experiments on the embryonic cell cycle of frogs indicate that there is a licensing factor that binds DNA during mitosis and is consumed during S-phase, controlling DNA replication (Blow and Laskey, 1988; Leno et al., 1992). In budding yeast, the licensing factor, Swi5, is made in G2 but stays in the cytoplasm due to phosphorylation by Cdc28 (equivalent to Cdc2 in fission yeast). At the end of mitosis, Cdc28 is inactivated, resulting in the dephosphorylation of Swi5 which can then enter the nucleus and bind DNA, allowing replication to occur (Moll et al., 1991). Licensing factors are also thought to be involved in the control of DNA replication in mammalian cells. In megakaryocytes, the licensing factor, MCM3, is found only in polyploidising cells and not in the fully differentiated cells (Nagata et al., 1997). Kinases responsible for the regulation of cyclin/CDK activity through phosphorylation and dephosphorylation determine the timing of G2 and mitosis. One such kinase, Wee1, is responsible for the phosphorylation of CDKs at tyrosine-15 and high levels of this kinase are found during endoreduplication in maize endosperm (Sun et al., 1999). Cyclin/CDK2 acitivity is important for centrosome duplication during the G1/S transition. Failure to coordinate duplication and mitosis results in abnormal numbers of centrosomes and aberrant mitoses (Lacey et al., 1999). Therefore, polyploidy could occur through the modulation of cyclin/CDK complexes at several points in the cell cycle and it is possible that different processes occur in different cells.

1.5.2.2 CDKIs

The gene products, $p21^{CIP1}$ and $p27^{KIP1}$ are structurally related proteins which respond to a diverse set of signals including growth factor depletion, contact inhibition and DNA damage, thus regulating the progression through G1 and S-phase (Polyak et al., 1994; Reynisdottir et al., 1995). Other CDKIs, $p15^{INK4b}$ and $p16^{INK4a}$ respond to exogenous growth factor levels and associate with CDK4 or CDK6 complexes (Serrano et al., 1993; Hannon and Beach, 1994). The *rum1* gene product, p25 (fission yeast) and the *SIC1* gene product, p40 (budding yeast) inhibit B-type cyclins preventing the activation of mitosis during G1 (Benito et al., 1998; Correabordes and Nurse, 1995; Martin-Castellanos et al., 1996; Moreno and Nurse, 1994; Murray, 1994b; Nasmyth, 1996a; Schwob et al., 1994). The actions of these

inhibitors have not been fully established. However, several studies have suggested that they could play a role in polyploidisation by inhibiting CDK complexes involved in G1/S and G2/M progression. Indeed the universal CDKI, p21 has been shown to act on CDK2 and Cdc2 (Pines, 1994; Reynisdottir et al., 1995). Expression of p21 in U2OS-derived cell lines results in an accumulation of 2n and 4n cells, indicating that p21 regulates both G1 and G2 progression in these cells (Medema et al., 1998). In hepatocytes, p21 is of particular interest as increased expression of this CDKI is associated with increasing age and senescence (Sigal et al., 1999). Polyploidisation in the UT-7 megakaryocyte cell line (Kikuchi et al., 1997), HEL cell line (Datta et al., 1998) and in mouse hepatocytes (Wu et al., 1996) is associated with changes in p21 expression. In the UT-7 cell line, overexpression of p21 resulted in an increase in ploidy and it was suggested that p21 acted through the suppression of Cdc2 activity at mitosis. This study also demonstrated that p21 mRNA was induced before polyploidisation in normal human megakaryocytes (Kikuchi et al., 1997). In the liver of p21 transgenic mice, large polyploid nuclei appeared in some of the hepatocytes, indicating the role of p21 in polyploidisation, *in vivo* (Wu et al., 1996). Transfection of p21 in the human breast carcinoma cell line, MCF-7, resulted in the formation of 'giant' polyploid and multinuclear cells (Sheikh et al., 1995). However, overexpression of p21 in other human cell lines was found to induce endoreduplication only in cells lacking pRB (Niculescu III et al., 1998), suggesting that functional pRB is necessary to prevent DNA replication in p21 G_2-arrested cells. Interestingly, in human cell lines treated with microtubule inhibitors, the absence of p21 induces endoreduplication suggesting that p21 is involved in a checkpoint ensuring S-phase does not occur after an aberrant mitosis. In these cell lines the formation of polyploid cells was associated with gross nuclear abnormalities and apoptosis (Mantel et al., 1999; Stewart et al., 1999; Waldman et al., 1996).

Rum1 ('replication uncoupled from mitosis') has recently been identified in fission yeast as a regulator of G1 progression (Labib and Moreno, 1996). This gene has been shown to play a role in the length of G1, the prevention of DNA replication until the completion of mitosis and in the prevention of mitosis until the completion of G1. Overexpression of this gene in yeast was found to induce several rounds of

DNA synthesis without mitosis, resulting in the occurrence of 4n and 8n cells and deletion of *Rum1* resulted in mitosis with no intervening S-phase (Moreno and Nurse, 1994). The *Rum1* gene product, p25 specifically inhibits the B-type cyclins preventing the activation of mitotic complexes in G1 (Correabordes and Nurse, 1995; Martin-Castellanos et al., 1996). $p25^{Rum1}$ accumulates in anaphase and persists until the end of G1. During S-phase, $p25^{Rum1}$ is degraded allowing the activation of mitotic cyclin/Cdc2 complexes and initiation of mitosis. Inhibition of $p25^{Rum1}$ degradation by mutations in phosphorylation sites required for targeted ubiquitin-dependent degradation causes protein stabilisation and polyploidisation (Benito et al., 1998). A similar protein, $p40^{SIC1}$, is found in budding yeast which specifically inhibits Clb (B-Type cyclins)/Cdc28 complexes preventing the initiation of S-phase and mitosis during G1 (Schwob et al., 1994; Nasmyth, 1996a). So far, no CDK inhibitors described have been found to have specificity for the cyclin B/Cdc2 complex in animals (Sherr and Roberts, 1995; Elledge et al., 1996) and it is possible that animal cells use other mechanisms already described to control the proper progression from G1 to S phase.

1.5.2.3 Regulators of mitosis and cytokinesis

Mitosis can be separated into five different stages: Prophase, prometaphase, metaphase, anaphase and telophase. During these stages the chromosomes condense, the nuclear membrane breaks up, the chromosomes are aligned and separated and a nuclear membrane forms around each group of daughter chromosomes. Cytokinesis then ensures that the cytoplasm divides equally, forming two daughter cells (Alberts et al., 1994a). As described previously, polyploidisation can occur if any of these stages are absent or disrupted. Binuclear hepatocytes are thought to result from an absence of cytokinesis (Nadal and Zajdela, 1966) and polyploid megakaryocytes result from a failure to complete anaphase (Nagata et al., 1997). Therefore, the factors controlling mitosis and cytokinesis may be critical in the development or control of polyploidisation in these cells.

Cyclin/CDK complexes such as cyclin B/Cdc2 (MPF) are involved in the progression of mitosis as well as its initiation. MPF kinase activity is directed to

MAPs, among other proteins, and hence plays a role in spindle formation. Dephosphorylation of $p34^{cdc2}$ and cyclin B degradation are required for entry into anaphase and telophase, respectively (Holloway et al., 1993; Jacobs, 1992; Lehner and O'Farrell, 1990). Changes in the normal distribution of these molecules could prevent entry into mitosis and could be involved in polyploidisation where certain phases of mitosis such as anaphase and cytokinesis do not occur. The anaphase promoting complex (APC) is involved in controlling the activity of CDKs by destroying G2 cyclins such as cyclin B during late metaphase. Association of the APC with specific activators seems partially responsible for specific substrate targeting and timing. One APC activator, the human hCDH1 (yeast CDH1) gene shows sequence and functional similarities to the plant homologue, *ccs52.* Overexpression of *ccs52* in yeast triggers mitotic cyclin degradation and induces endoreduplication and cell enlargement, suggesting a role for the APC in the control of endoreduplication (Cebolla et al., 1999). In vascular smooth muscle cells, polyploidisation is associated with overexpression of Cks1, a cdc2 adapter protein that promotes degradation of cyclin B, suggesting that regulation of polyploidisation by the APC may also occur in mammalian cells (Hixon et al., 2000).

Proper chromosome segregation in eukaryotes depends upon the mitotic spindles that assemble at the time of cell division and disassemble upon its completion (Barton and Goldstein, 1996). These spindles are composed largely of microtubules (made up of tubulin subunits) which can attach to the chromosomes through interactions with specialised protein complexes known as kinetochores (Alberts et al., 1994b). Disruption of microtubule assembly or disruption of associated proteins is likely to prevent the completion of mitosis. Inhibition of microtubule assembly by administration of colchicine, which inhibits tubulin polymerisation, results in increasing polyploidisation in megakaryocyte cell lines (Baatout et al., 1998b). Members of the kinesin family play critical roles in cell division. Mitosis kinesin-like protein 1 (MKLP1), a member of this family, causes plus end-directed sliding of microtubules over one another and may mediate anaphase B spindle elongation (Nislow et al., 1992). The mechanism of polyploidisation in thrombopoietin-induced primary megakaryocytes involves a lack of outward movement of the spindle poles

during anaphase B and it has been suggested that this process could result from alterations in the regulation of MKLP1 (Nagata et al., 1997).

Deletions or mutations of various genes involved in duplication of the spindle pole body (mitotic spindle in mammalian cells) in yeast result in a polyploid phenotype including *CDC3* (Schild et al., 1981), *MPS1* and *MPS2* (Winey et al., 1991), *MOB1* (Luca and Winey, 1998), *Kar1* (Rose and Fink, 1987; Vallen et al., 1992). Other mutations preventing the proper separation of chromosomes or cell division are also implicated in polyploidisation. Mutations in *ESP1* result in accumulation of extra spindle pole bodies and asymmetrical segregation of chromosomes (McGrew et al., 1992). Mutational analysis of *SPA1* found that the gene is required for cell growth, spindle segregation and other cellular processes (Snyder and Davies, 1988). Asymmetrical cell division results from mutations in *NDC1* resulting in one daughter cell that doubles in ploidy and a cell that inherits no chromosomes. This gene is required for the attachment of chromosomes to the spindle pole (Thomas and Botstein, 1986). The Scp 160p is also required for the proper segregation of chromosomes (Weber et al., 1997). In fission yeast, *Bub1* is essential in maintenance of the spindle checkpoint, ensuring that chromosomes are properly segregated. However, loss of this gene results in loss of chromosomes often associated with aneuploidy (Bernard et al., 1998). Some of the genes and gene products studied above have been found to be homologous with genes in other eukaryotes. The *ESP1* gene of *S. cerevisiae* is similar to the *cut1* gene of *Schizosaccharomyces pombe* and the *bimB* gene of *Aspergillus nidulans* (McGrew et al., 1992) and Scp160p of *S. cerevisiae* shows some homology with chicken and human vigilin and the product of *C08H9.2* of *Caenorhabditis elegans* (Weber et al., 1997). Therefore, it is possible that homologues of some of these genes may be found to play a role in the polyploidisation of mammalian cells. Other genes involved in the spindle checkpoint in *Drosophila* and in humans are *Rod* and *Zw10* (*hRod* and *hZw10* in humans). The proteins encoded by these genes associate with the kinetochores and ensure anaphase does not occur until all chromosomes are properly orientated on the spindle. Cells lacking these genes are often aneuploid (Basto et al., 2000; Chan et al., 2000). Obviously, genes and gene products involved in the spindle checkpoint are essential

in maintenance of ploidy. However, it should be noted that deletion or mutations in several of the genes mentioned, results in aneuploidy and not polyploidy and may therefore have no role in the process of polyploidisation in mammalian cells.

The Aurora/Ipl1p kinase family is important in the regulation of chromosome segregation and cytokinesis and is therefore important in the maintenance of ploidy and the prevention of aneuploidy (Bischoff and Plowman, 1999). In *S.cerevisiae*, *Ipl1* (increase in ploidy) mutants are defective in chromosome segregation resulting in aneuploidy (Biggins et al., 1999). In *Drosophila*, mutations of *aurora* cause mitotic arrest with chromosomes arranged on a single spindle (Glover et al., 1995). In *C.elegans*, disruption of the *aurora* homologue, *AIR2* results in the production of a single polyploid cell after multiple rounds of DNA replication without cytokinesis (Schumacher et al., 1998). *Xenopus* oocyte and egg extracts contain pEg2, a protein with sequence homology to aurora and Ipl1 enzymes, which is important in the assembly of the mitotic spindle (Roghi et al., 1998). *AIM-1* and *STK15*, also members of the aurora kinase family are continually repressed after the induction of polyploidisation in normal human megakaryocytes and cell lines. The authors suggest that downregulation of *AIM-1* at M-phase may therefore be involved in the abortive mitosis associated with polyploidisation in megakaryocytes (Kawasaki et al., 2001).

Inhibitor of apoptosis proteins (IAPs) suppress apoptotic cell death in several model systems and are highly conserved between insects and mammals. All IAPs contain a baculovirus IAP repeat (BIR) which is essential for the anti-apoptotic activity of IAPs (Fraser er al., 1999). In cultured cells, inhibition of the human BIRP, *survivin*, results in apoptosis, hyperploidy, multinucleation, multipolar mitotic spindles and supernumerary centrosomes. Interestingly, polyploidy induced by *survivin* antagonists is accentuated in p21-deficient cells (Li et al., 1999). In *C.elegans*, the *survivin* homologue, *BIR1*, is important in maintenance of ploidy. Ablation of *BIR1* expression resulted in a defect in cytokinesis and the formation of multinuclear cells (Fraser et al., 1999). *AIR2* was subsequently found to be absent from chromosomes in the absence of *BIR1* and it is proposed that *BIR1* localises *AIR2* to the

chromosomes where *AIR2* phosphorylates proteins and affects chromosome behaviour and spindle organisation (Speliotes et al., 2000). In fission yeast lacking *BIR1*, there is a defect at the metaphase to anaphase transition and a failure to elongate the mitotic spindle (Uren et al., 1999). Downregulation of KRC, a large zinc finger protein that regulates transcription of target genes via the kB gene enhancer element, has recently been found to result in growth without cytokinesis forming giant multinuclear cells in mammalian cell lines. However, it is not known whether KRC acts directly as a growth mediator or indirectly through target genes (Allen and Wu, 2000). Mutations in the *IPL2* (Chan and Botstein, 1993) and *SPA1* (Snyder and Davies, 1988) genes of *S. cerevisiae* also give rise to multinucleate cells, possibly through some failure in bud growth, chromosome segregation or nuclear migration. Recently, there has been a suggestion that cytokinesis is also regulated by centrosomes. Removal of centrosomes from cells results in aborted cytokinesis and the formation of binuclear cells (Hinchcliffe et al., 2001; Piel et al., 2001). However, Hinchcliffe et al., 2001 demonstrated that these cells were blocked in G1. Another protein involved in spindle assembly, chromosome segregation and cytokinesis is the *Drosophila fumble* (*fbl*) protein. Mutations in this gene also result in the formation of multinuclear cells possibly through alterations in membrane synthesis (Afshar et al., 2001). Mutants in the microtubule binding yeast protein, *Bim1* (homologous to human *EB1*) undergo cytokinesis before the spindle is in the correct position and results in a lethal multinucleate phenotype (Muhua et al., 1998).

Separation of daughter cells at cytokinesis is dependent on the formation of a contractile ring, which is composed of actin and myosin-II filaments. Cleavage occurs through the contraction of this ring and results in the formation of two daughter cells with identical copies of the genetic material (Alberts et al., 1994a). The formation of binuclear cells in the liver is thought to occur through a failure in cell division (Nadal and Zajdela, 1966). Therefore, it is possible that factors controlling the formation of the contractile ring during cytokinesis could be disrupted and play a role in this process. Indeed, inhibition of actin polymerisation by cytochalasin B prevents cytoplasmic separation without interfering with DNA replication and induces polyploidisation in megakaryocyte cell lines (Baatout et al.,

1998c), Pacific oyster eggs (Longo et al., 1993) and cells in yeast (Kanbe et al., 1993). Further evidence that deregulation of actin could play a role in polyploidisation comes from an experiment involving cytotoxic necrotizing factor 2 (CNF2). This bacterial exotoxin has been found to induce multinucleation in megakaryocyte cell lines and is thought to act through a modification of rhoA, leading to a dysregulation of actin and disruption of the contractile ring at mitosis (Denko et al., 1997; Hudson et al., 1996).

1.5.2.4 Genes involved in DNA damage and repair

Other proteins involved in the cell cycle checkpoints include those that can sense damage, arrest the cell cycle, initiate repair and cause apoptosis (Murray, 1994a). One of the main proteins involved in this control is p53 (Prost et al., 1998; Bellamy et al., 1997). The p53 onco-suppressor gene is mutated in over half of all human cancers allowing affected cells to avoid death through apoptosis (Hollstein et al., 1991). p53 is involved in several cell cycle checkpoints including at the G1/S and G2/M transitions (Stewart et al., 1995) and has been recently shown to associate with centrosomes and participate in the mitotic spindle checkpoint (Ciciarello et al., 2001). There is conflicting evidence regarding the role of p53 in the control of polyploidisation in normal ageing hepatocytes. Bellamy et al., 1997 found that polyploidisation associated with age in mice was not dependent on p53. Conversely, Yin et al., 1998 found that heterozygous and homozygous p53-knockout mice did not show normal polyploidisation with age and found an increase in the proportion of diploid cells. There is however, general agreement on the role of p53 in polyploidisation after mitotic spindle damage. In experiments involving mouse and human cell lines and mouse fibroblasts, p53-deficient cells become polyploid in response to spindle damaging agents such as nocodazole and colcemid (Casenghi et al., 1999; Minn et al., 1996; Notterman et al., 1998). Administration of mitotic spindle inhibitors to mouse cell lines deficient in p53 and expressing the apoptosis-inhibiting protein, Bcl-x_L also reinitiate DNA replication and become polyploid (Minn et al., 1996). In HeLa cells treated with etoposide, overexpression of Bcl-2 results in multiple rounds of DNA replication and the formation of multinuclear cells, which are inhibited from undergoing apoptosis. The HeLa cell line lacks

functional p53 and pRb checkpoints due to human papillomavirus infection and it therefore suggested that the induction of multinucleation might be due to the loss of the p53 mitotic spindle checkpoint (Elliott et al., 1999). Interestingly, polyploidisation also occurs in human fibroblasts overexpressing a dominant gain of function p53 mutation ($p53^{RSC}$, relaxed spindle checkpoint) when treated with colcemid (Gualberto et al., 1998). Polyploidisation also occurs after DNA damage by irradiation or chemotherapy in p53 mutated lymphoma cells (Illidge et al., 2000).

The excision repair cross complementing gene (ERCC-1) is involved in nucleotide excision repair (NER) after DNA damage. ERCC-1 knockout mice are runted at birth and die before weaning with liver failure. Hepatocytes from these mice demonstrate increasing polyploidy, abnormal nuclei, elevated levels of p53 and p21 and a reduction in DNA synthesis and binucleation suggesting that polyploid cells form due to a block in the cell cycle (McWhir et al., 1993; Nunez et al., 2000; Weeda et al., 1997). The accelerated polyploidisation in ERCC-1 deficient mice was not rescued by p53 deficiency but it was demonstrated that p53 was responsible for the reduction in DNA synthesis and binucleation. However, in mice capable of DNA repair, p53 did not affect binucleation (Nunez et al., 2000).

1.5.2.5 Other molecules associated with polyploidisation

At nonpermissive temperatures, Chinese hamster cells bearing the ts41 mutation accumulate up to 16n. This process of polyploidisation differs from that induced by other mutations discussed above as DNA re-replication occurs without G_2, M-phase and G_1. Therefore, it is suggested that the ts41 gene product participates in the control of entry into mitosis and inhibition of entry into S-phase (Handeli and Weintraub, 1992).

Induction of polyploidisation through inhibition of protein kinases by the staurosporine analogue, K252a, is potentiated by exogenous cyclic AMP. Exogenous cyclic AMP is usually unable to cross the cell membrane. The induction of polyploidisation by K252a, changes the membrane structure, somehow allowing

exogenous cyclic AMP to enter the cell and increase polyploidisation (Zong et al., 2000).

The role of reactive oxygen species (ROS) and antioxidant enzymes in the control of mammalian cell growth and proliferation is well known. One study demonstrated that ROS are capable of inducing proliferation in various cell types, whereas antioxidants reduce this effect (Burdon, 1995). In the liver, ROS and antioxidant enzymes have been shown to modulate cell proliferation (Tsai et al., 1992; Liotti et al., 1987). More recently, it has been suggested that antioxidant enzymes may influence the rate of hepatocyte polyploidisation as PH-induced polyploidy is reduced in transgenic mice overexpressing various antioxidant enzymes (Nakatani et al., 1997).

1.5.3 A gene involved in regulation of cell size

The human tumour suppressor gene *PTEN* (phosphatase and tensin homologue deleted from chromosome 10) encodes a cytoskeleton-associated molecule with both protein phosphatase and phosphatidylinositol 3,4,5-triphosphate (PIP3) 3-phosphatase activities. This gene is particularly interesting due to its role in the regulation of cell size and is thought to inhibit growth through modulating the responses of cells to insulin. In mammalian cell cultures, the lipid phosphatase activity of this protein is involved in regulating cell proliferation, cell survival and cell migration (Huang et al., 1999). During *Drosophila* eye development, inactivation and overexpression of the highly conserved PTEN homologue, *DPTEN*, affects cell size, while overexpression also inhibits cell cycle progression at early mitosis and promotes cell death in a context-dependent manner (Huang et al., 1999). So far, no studies have been carried out to determine whether PTEN plays a role in polyploidisation. Analysis of PTEN expression in hepatocytes of different ploidy would therefore be particularly interesting.

1.6 Summary and Aims

Polyploidisation in the liver occurs naturally with age and after the administration of various drugs and chemicals (Alfert and Geschwind, 1958; Brodsky and Uryvaeva, 1977; Hasmall and Roberts, 1997; Hasmall and Roberts, 2000). The significance of this process is unclear and hypotheses suggest that it is either a protective response or that it is associated with terminal differentiation and senescence, leading to increased rates of apoptosis and possibly to carcinogenesis (Medvedev, 1986; Uryvaeva, 1981; Gupta et al., 2000). By studying polyploidisation induced by PB in hepatocytes differing in p53 expression, the relationship between cell size and receptor expression, the susceptibility of hepatocytes to IFNγ-induced apoptosis and gene expression within different ploidy populations, this thesis hoped to determine possible controls and functions of polyploidisation in mouse hepatocytes.

The mechanisms controlling polyploidisation in hepatocytes are not known. However, there is considerable evidence that p53 is involved in the control of polyploidisation in various cells treated with mitotic spindle inhibitors (Casenghi et al., 1999; Minn et al., 1996; Notterman et al., 1998). There is also evidence to suggest that p53 may be involved in regulation of polyploidisation associated with ageing, although findings are contradictory (Yin et al., 1998; Bellamy et al., 1997). PB is a hepatic mitogen that induces polyploidisation in mice (Bohm and Noltemeyer, 1981). Therefore, the first aim of this thesis was:

- To determine if changes in ploidy and proliferation induced by PB were dependent on p53.

As mentioned above, the function or functions of polyploidisation are unclear and hypotheses are contradictory. Cell size and volume are known to regulate various cell functions including proliferation and apoptosis (Lang et al., 1998). Gene expression in some cells is also influenced by cell size (Schmidt and Schibler, 1995). In hepatocytes, cell size increases in proportion to DNA content (Danielsen et al.,

1986; Deschenes et al., 1981; Epstein, 1967; Sweeney et al., 1978a; Watanabe and Tanaka, 1982). Therefore, the second and third aims were:

- To determine if the increasing size of polyploid hepatocytes played any role in the function of these cells, specifically by determining whether increasing size altered surface receptor expression.
- To determine whether polyploid hepatocytes are more susceptible to apoptosis induced by IFNγ.

The recent development of microarray technology enables thousands of genes to be screened and is particularly useful in cases where there is little information available. This technique has been used to compare haploid and tetraploid strains of yeast and provides new insights into gene expression in different ploidy populations (Galitski et al., 1999). In order to study gene expression within mouse hepatocytes, cells differing only in DNA content must be separated, which has previously been possible using FACS and the DNA dye, Hoechst 33342 (Weglarz et al., 2000). This technique has not been well characterised, but in combination with microarray technology, would provide much information regarding gene expression within different hepatocyte populations. Therefore, the fourth and fifth aims were:

- To sort highly viable and pure populations of hepatocytes differing only in DNA content.
- To ensure that the RNA obtained from these cells was suitable for microarray analysis.

2 Materials and Methods

Unless stated, manufacturers and suppliers are given in Appendix 1. Details of buffers and other solutions are provided in Appendix 2.

2.1 Animals and Animal Procedures

2.1.1 Administration of PB and BrdU

The animal work for the PB study was carried out by AstraZeneca Pharmaceuticals (Alderley Edge). Briefly, 5-6 week old male C57BL/6 p53 +/+, +/- and -/- mice were purchased from Taconic Farms, Germantown, New York and acclimatised for two weeks prior to dosing. Animals were housed five per cage under appropriate conditions of temperature and humidity and with a 12-hour artificial light cycle. Mice were fed powdered irradiated R and M No1 (modified) diet (Special Diet Services) containing PB (1000 ppm) for 21 days; this regimen was chosen based on previous data (not shown). Control groups were fed a similar diet without PB. Food and water were available *ad libitum*. BrdU (0.8mg/ml) was administered continuously in drinking water for 72 h before termination.

2.1.2 Preparation of FNAs for flow cytometry

Mice were euthanased by inhalation of halothane (FLUOTHANE™, AstraZeneca Pharmaceuticals). The mouse body and liver weights were noted. A representative portion of liver tissue was removed from the right lobe, fixed in methacarn overnight and processed to paraffin for histological examination. Fine needle aspirates (FNAs) were then taken using a 20 g needle and making at least 6 passes to include each lobe of the liver; cells were aspirated into citrate buffer. This technique yielded similar numbers of cells from each case. FNAs were stored at -70°C prior to use for the

determination of ploidy and BrdU incorporation; samples were stable for at least 4 months.

2.1.3 Liver perfusion

2.1.3.1 Hepatocyte isolation

Primary hepatocytes from C3H male, 6-12 weeks old (unless otherwise noted), were isolated using a two-step retrograde liver perfusion procedure adapted from the technique described by Bellamy et al., 1997. All perfusion equipment was sterilised with 70% ethanol before use and rinsed with sterile H_2O. Animals were killed by cervical dislocation, mice were dissected and the ribcage was removed. A cannula (OD 1mm, ID 0.5mm) was inserted into the right chamber of the heart and down the thoracic inferior vena cava. All media was heated to and kept at 39.7°C throughout the perfusion. This temperature ensured that the medium entering the liver was 37°C. Perfusion medium was run through at a low flow rate until the liver inflated. The pump was stopped and the portal vein was cut. The flow rate was increased to 9.5 ml/min and the liver was perfused for a further 5 min. The liver was then perfused with digestion medium containing collagenase type 4 (0.5 mg/ml) and DNase type 1 (0.1 mg/ml) for 7 min. HBSS (digestion medium) and collagenase type IV were chosen, as the cell yield was greater than that obtained using other media and collagenase type I. The digested liver held intact by its capsule was removed to a petri dish and the gall bladder was removed. Hepatocytes were dissaggregated by gently scraping cells from the fibrous supporting tissue.

2.1.3.2 Purification of cell suspension

The cell suspension was pipetted a few times with a wide bore pipette to break up clumps and filtered through a 100 μm sieve onto a fresh petri dish. The sieve was washed twice with cold plating medium to collect the rest of the dissaggregated cells. The cell suspension was placed in a falcon tube and made up to 50 ml with ice cold plating medium. The cells were centrifuged at 50 *g* for 5 min at 4°C and the

supernatant (containing debris and non-parenchymal cells) was removed. The cells were then spun over Percoll for 10 min at 50 *g* to purify the hepatocyte population further. The live cell pellet was resuspended in 5 ml cold plating medium and the cell yield and viability was calculated.

2.1.3.3 Assessment of yield and viability

Cell viability was assessed using a trypan blue exclusion assay (Hay, 1992). Cells with damaged membranes take up trypan blue and can be differentiated by light microscopy from live cells that are unstained.

Cell yield and viability were calculated by adding an aliquot of the cell suspension (20 µl) to 50 µl trypan blue and 30 µl phosphate buffered saline (PBS). A drop of this mixture was added to each side of a coverslipped Neubauer haemocytometer (chamber depth 0.1 cm). The number of live and dead (blue) cells in five $1mm^2$ squares on each side of the haemocytometer was counted and the average taken.

Cell Yield ($x10^4$/ml) = cell number in 5 squares x dilution factor (5)
% Viability = number of live cells/total count x 100

Only isolated cells with greater than 90% viability were used in these studies.

2.2 Cell Culture

Hepatocytes isolated by the collagenase perfusion technique were plated onto collagen-coated 8-well glass slides and 6-well culture plates in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM F12) containing 2% FCS at a density of 0.3 – 0.4 x 10^5 cells per cm^2. FCS was added to help cell attachment. Slides were agitated to evenly distribute the cells. After 24 h, cells were washed twice with PBS and the media was replaced with serum-free Chee's modified medium. Cultures were incubated at 37°C in a humid atmosphere containing 5%

CO_2/air. The media was kept less than 2mm deep to maintain optimum oxygen tension and was replaced every 48 h.

2.2.1 Administration of IFNγ

Hepatocytes cultured on 6-well plates or 8-well slides were given IFNγ (100U/ml) in Chee's medium for 1-4 days. This concentration of IFNγ was chosen, as this is the lowest dose that induces maximum apoptosis based on dose response studies carried out in the laboratory (Christian McCullough, personal communication) and studies published in the literature (Kano et al., 1997; Kano et al., 1999; Morita et al., 1995). Controls were set up without IFNγ. Each condition was carried out in 5 wells and the experiment was repeated 3 times. Values are given as mean ± SD.

2.3 Flow Cytometry

2.3.1 Analysis of nuclear DNA content using the Vindelov technique

The Vindelov technique is a robust and reliable method of measuring DNA content of isolated nuclei rather than intact cells; distinguishing mononuclear or binuclear cells is therefore not possible (Vindelov et al., 1983a, b and c). The nuclei from FNA samples (100 μl) taken from PB-treated mice (section 2.1.2) were prepared and the DNA was stained with propidium iodide (PI) by first adding a typsin solution (solution A, 450 μl) for 10 min at room temperature to digest the outer cell membrane. A solution containing trypsin inhibitor and RNase A (solution B, 325 μl) was then added for a further 10 min to prevent excess digestion and to degrade the RNA; samples were mixed by inversion. To stain the DNA, a solution containing PI and spermine tetrahydrachloride (solution C, 250 μl) was added for 10 min at 4°C. Details of all the solutions are in appendix 2. Samples were analysed by flow cytometry (section 2.3.5).

2.3.2 PI staining of whole cells

Another method of analysing DNA content is to stain whole cells rather than nuclei by staining fixed hepatocytes with PI. Fixing permeates the cell membrane allowing PI to enter and bind to DNA. This technique provides a measure of the DNA content of both mononuclear and binuclear cells and therefore, distinguishing these two cell populations is not possible. To analyse the effects of IFNγ on hepatocyte ploidy *in vitro*, hepatocytes were removed from culture plates before treatment and after 4 days treatment by incubating cells at 37°C for 10-20 min in a trypsin (0.12%)/ EDTA (0.02%) solution and removing the rest of the cells with a cell scraper. Freshly isolated hepatocytes were included as controls and cells were fixed in cold 70% ethanol overnight at 4°C and stained with PI (20μg/ml), RNase (0.2mg/ml) and triton X-100 (0.1%) in PBS for 15 min at 37°C. Flow cytometric analysis was carried out as in Section 2.3.5.

2.3.3 Analysis of proliferation in different ploidy classes

To determine if all nuclei undergo proliferation in response to PB, nuclei were double-labelled with PI and FITC-labelled anti-BrdU antibody using the following method: Fine needle aspirates were centrifuged at 300 *g* for 5 min and the supernatant was removed. Absolute ethanol was added for 20 min and samples were centrifuged, as before. The samples were washed twice with PBS. Pepsin (0.2 mg/ml in 2N Hydrochloric acid) was added for 20 min and samples were washed twice with PBS and centrifuged at 1000 *g* for 10 min. Samples were transferred to a round-bottomed 96 well plate, washed in PBS and spun at 1200 *g* for 10 min. Rat monoclonal anti-BrdU antibody (diluted 1:100 in PBS, 0.5% Tween 20 and 0.5% normal rabbit serum [NRS]) was added and the sample incubated overnight at 4°C. Samples were washed twice as above and resuspended in FITC-labelled rabbit anti-rat antibody (diluted 1 in 100 with PBS, Tween 20 and NRS) for 30 min at room temperature. After a further two washes with PBS, PI (100 μg/ml) and RNase

(0.04%) were added to samples on ice for 15 min and analysed by flow cytometry (Section 2.3.5)

2.3.4 Receptor expression

The expression of ICAM-1 and IFNγR on the surface of cells of different ploidy was studied using a double-labelling technique: Isolated hepatocytes were stained with an FITC-conjugated, hamster α-mouse CD54 (ICAM-1) antibody diluted 1 in 10 in PBS with 0.1% sodium azide and 1% BSA for 1 h at 4°C. Ten-fold dilutions of the antibody were used to determine which dilution gave the optimum staining. The addition of sodium azide prevented the internalisation of surface receptors. Isotype controls were included in the analysis (FITC hamster α-IgG, group 1). IFNγR expression was determined using an indirect method. Isolated hepatocytes were stained with purified rat α-mouse CD119 (IFNγR α chain, 0.5 mg/ml) or rabbit α-mouse IFNγR β chain (200 μg/ml) after blocking with appropriate serum in PBS containing 0.1% sodium azide and 1 % BSA for 10 min. Cells were incubated for 1 h at 4°C. Isotype controls were also included (purified rat IgG2a, κ and normal rabbit IgG). Undiluted FITC conjugated secondary antibodies (FITC-α rat IgG2a and FITC-α rabbit IgG) were incubated with cells for 30 min at 4°C. To determine the DNA content, cells were fixed in 70% ethanol and stained with PI, RNase and Triton X-100, as in section 2.3.2. Receptor expression and DNA content was measured by flow cytometry using a BD FACS Calibur (Becton Dickinson, Oxford, UK). PI and FITC were detected as in section 2.3.5. A minimum of 5000 cells was counted and each experiment was carried out in triplicate. The data was normalised by making the intensity of fluorescence of 2n cells equal to 1 and calculating the intensity for 4n and 8n cells accordingly. Values of fluorescence intensity for 2n, 4n and 8n cells were plotted against average cell volume determined by confocal image analysis.

2.3.5 Flow cytometry of DNA content and proliferation

DNA content and proliferation were evaluated using a COULTER®EPICS®XL Flow Cytometer (Beckman-Coulter Electronics, Luton, UK). PI and FITC were both excited at 488nm; the red fluorescence emitted by PI was detected at 620nm and the green fluorescence emitted by FITC was detected at 525nm. Doublet gating was included to ensure only single events were counted. A minimum of 10,000 nuclei was counted to determine nuclear ploidy and BrdU profiles. For whole cells, a minimum of 5000 cells was counted. Mononuclear and binuclear cells with the same DNA content fell within the same gate and were not distinguished in this study. Since PI binds to DNA stochiometrically, the integral red fluorescence recorded by the flow cytometer for each nucleus or cell is proportional to DNA content. Therefore, histograms of FL3 (red fluorescence) are histograms of DNA content. The proportions of 2n, 4n or 8n nuclei or cells were determined by placing gates over the main area of each peak. Gates were set on the first sample analysed and were not changed thereafter.

2.4 Separation of Hepatocytes Based on Size and DNA content

2.4.1 Separation based on cell size using Percoll gradients

Percoll was diluted in PBS containing 11% HBSS to 90, 80, 30 and 20% Percoll solutions. The gradients were made in 15 ml polystyrene centrifuge tubes by first layering 1 ml 90% Percoll followed by 3 ml 80%, 4 ml 30% and 5 ml 20%Percoll from below upwards, respectively. Hepatocytes (~5 x 10^6 cells/ml) suspended in PBS were loaded onto the top of the gradients and the tubes were centrifuged at 300 *g* for 10 min at 4°C. Cell fractions in discrete bands were isolated by pipetting and fixed in 70% ethanol, stained with PI and analysed by flow cytometry as in section

2.3.5. Aliquots of each fraction were taken for cytospins to determine the nuclearity of cells, see section 2.4.2.2.

2.4.2 Sorting of cells based on DNA content

The bis-benzimidazole dye, Hoechst 33342 is a DNA-specific vital cell stain that can be used to separate live cells based on their DNA content. The staining of cells varies dramatically with cell-type, temperature, dye concentration and incubation time. At the time this study was carried out, there was no literature regarding the sorting of live mouse hepatocytes using Hoechst 33342. Therefore, the technique was adapted from methods used to sort other cell types (Arndt-Jovin and Jovin, 1977; Davies et al., 1990; Visser,1980). Cells were obtained by a retrograde perfusion technique (section 2.1.3). Isolated cells were placed in DMEM F12 at a concentration of $<1 \times 10^6$ cells/ml. Hoechst 33342 (10 μg/ml) was added for 30 min at 37°C, in the dark. Cells were centrifuged at 50 *g* for 5 min and 4/5 supernatant was removed to bring the cell concentration to 5×10^6 cells/ml. To reduce clumping, DNase type 1 (0.1 mg/ml) was added and left for 45 min at room temperature. Cells were filtered through a 40 μm filter to remove larger clumps and reduce the size of cells going through the 100 μm nozzle. Cells larger than 40 μm disturb the stream through the nozzle and affect the purity of the sort. Filtering did not remove any of the cell populations of interest. PI (2 μg/ml) was added to help discriminate between live and dead cells.

Hepatocytes were sorted on a BD FACS Vantage (Becton Dickinson) using a dual laser set up. Calibration and alignment of the FACS was carried out by Catherine Simpson (Edinburgh MRC, Centre for Inflammation Research). Hoechst fluorescence was analysed using an Enterprise II Coherent laser (Coherent Inc, CA). The laser power was regulated at 60 mW on the UV line. The cells were sorted using a 100um nozzle at 9PSI at a rate of approximately 2000 event/s. PI fluorescence was collected on the 488 nm line using a 576/26 nm BP filter while Hoechst 33342 emission was collected using a 424/44 nm filter. Cells were gated on

a FSC/SSC bivariate dot plot and dead PI positive cells were removed by subsequent gating. Doublets were removed using pulse processing on the Hoechst signal and the DNA profiles were displayed on a linear histogram using Hoechst area. Sort gates representing 2n, 4n and 8n were applied and sorted using a 2-drop envelope in sort mode Normal R. Collection tubes were lined with FCS and cells were kept on ice after sorting.

2.4.2.1 Determination of sample purity

The purity of each sort was confirmed by fixing an aliquot of cells in 70% ethanol overnight at 4°C and staining with PI (20μg/ml), RNase (0.2 mg/ml) and triton X-100 (0.1%) in PBS for 15 min at 37°C. Cells were analysed by flow cytometry as in section 2.3.5.

2.4.2.2 Proportion of mononuclear and binuclear cells in sorted populations

The proportion of mononuclear and binuclear cells within each sorted population was determined by making cytospins of 1 x 10^3 cells per sample. Slides were fixed in Boum's fixative, stained with Schiff's reagent and light green, as in section 2.7.

2.4.2.3 Cell growth and survival of sorted populations

The number of sorted hepatocytes available for plating was low, as the majority of cells were required for gene expression analysis. However, two initial experiments were carried out to determine if sorting affected hepatocyte growth and survival. After sorting, cells were washed twice in sterile PBS to remove the Hoechst 33342. To assess the growth potential of cells, hepatocytes were first plated on collagen coated 8-well slides at a density of 0.3 – 0.4 x 10^5 per cm^2 in DMEM F12 containing 2% serum (section 2.2). After the cells had adhered, the medium was replaced with Chee's medium and left for 24 or 48 h. Hepatocytes were given BrdU and immunohistochemistry was carried as in section 2.6. On the second occasion, cell growth was examined using time-lapse videomicroscopy.

2.4.2.3.1 *Time-lapse videomicroscopy*

Sorted cells were plated onto small tissue culture flaskettes ($10cm^2$) at the same density as above. BrdU experiments revealed that proliferation occurred in the 2n population between 24 and 48 h. Therefore, to study cells that were actively dividing, cultures were incubated for 24 h prior to the experiment. Cultures were then gassed with 5% CO2/Air prior to their insertion onto the videomicroscope moveable platform (Leica DM IRBE; Leica Microsystems, Cambridge, UK). The cultures were maintained at 37°C throughout the analysis. Using the 10x objective, digital images were taken from 6 fields on each flaskette every 30 min for a further 24 h with the videomicroscope and associated software (QUIPS, Leica). Video analysis of the resultant succession of TIFF files (Microsoft) was carried out using Adobe Premiere and was saved as a QuickTime movie.

2.5 Histological Assessment of PB-Treated Samples

Sections (4μm) of liver from PB-treated samples were stained with haematoxylin and eosin and examined to detect any histopathological abnormalities. The number of cells was calculated, counting the total number of hepatocytes and non-parenchymal cells in 10 high power fields ($0.5mm^2$ per field). The number of hepatocytes was then multiplied by the liver weight to obtain the total number of hepatocytes for each liver (expressed in arbitrary units = hepatocytes per $5mm^2$ x liver weight/mouse weight).

2.6 BrdU Immunohistochemistry

Hepatocytes cultured on 8-well slides were given BrdU (1 μl/ml) for 3 h prior to fixation in 80% ethanol. Slides were incubated in 5M hydrochloric acid (HCl) for 45 min at room temperature. Rat α-BrdU diluted 1 in 100 in blocking solution (PBS, NRS (20%), Tween 20 (0.05%)) was added for 1 h. Negative controls omitted this antibody. Peroxidase-conjugated rabbit α-rat, pre-absorbed with normal mouse

serum (50%) and diluted 1 in 100 in blocking solution, was added for 30 min. Positive cells were visualised using DAB chromagen. Cells were counterstained with haematoxylin and light green. The results are expressed as the percentage of BrdU positive cells. A minimum of 500 cells was counted.

2.7 Analysis of Apoptosis by Feulgen Staining

Apoptotic cells can be distinguished from necrotic cells by their distinct morphology (Bellamy et al., 1997). To determine the amount of apoptosis at each time-point, slides were fixed in Boum's fixative overnight at 4°C and stained according to Feulgen technique (Stevens and Bancroft, 1982) with the following modification: Slides were denatured with 5M HCl for 45 min at room temperature before being stained in Schiff's reagent (diluted 1:3 with water) for 1h and counterstained in light green (0.1%). Schiff's reagent stains DNA a red-purple colour. A minimum of 500 cells was counted and the results were expressed as the percentage of apoptosis.

2.8 Confocal Microscopy

2.8.1 Confocal imaging to measure cell volume

Isolated hepatocytes were plated on collagen-coated glass coverslips (in 6-well plates) in Chees's medium containing 2% FCS for ½ h at 37°C. To distinguish nuclei and cytoplasm, hepatocytes were stained with the nucleic acid stain, SYTO 16 (5 μM) and protein-binding stain CellTracker Orange (CTO; 5 μM) in fresh media for ½ h at 37°C. Glass coverslips were transferred to small petri dishes and covered in fresh media for confocal imaging. Confocal microscopy and calibration of the system for volume determination was carried out as previously described (Bush and Hall, 2001). Confocal images were acquired with the Leica TCS NT confocal system (Leica Microsystems, Heidelberg GmbH, Germany). Linda Sharp (Dept. of Biomedical Sciences, University of Edinburgh) provided the necessary technical

assistance. A x63 water immersion lens, fitted to the upright Leica DMRE microscope was used to collect quantitative fluorescent images of hepatocytes in optical Z-steps of ~1μm. SYTO 16 was excited at 488 nm and CTO at 568nm. A minimum of 300 measurements was taken for nuclear diameter analysis and 70 measurements for cell volume analysis. Measurements were taken from 3 different mice. For the calibration of diameter, images of Fluoresbrite Microspheres (Mean 10 μm ± 0.1) were taken using the same procedure.

2.8.2 Measurement of nuclear diameter and cell volume

Image analysis was performed on a Silicon Graphics O2 workstation (Silicon Graphics Inc., Mountain View, CA) running Bitplane (Bitplane Inc., Zurich, Switzerland), Imaris and VoxelShopPro Software. Volume measurements were carried out as previously described using an intensity threshold technique (Bush and Hall, 2001). The study utilised Calcein AM as the cytoplasmic fluorosphere. Due to the similar spectral response of Syto 16 to Calcein AM, our study utilised the red shifted dye, CTO. To check the validity of CTO, hepatocytes were co-loaded with CTO and Calcein AM and volume analysis was performed. Cell volumes obtained were consistent ($r^2 = 0.78$, $p<0.05$). However, CTO derived volumes were an order of magnitude smaller (x1.7 ± 0.25). Hence, volume measurements were amended to account for this. Nuclear diameter was measured using the same confocal images and software. Measurements were taken at the central plane of each image. Using this technique, the diameter of the 10 μm beads was 9.41 μm ± 0.65. Therefore, there was no need to adjust the nuclear measurements.

2.9 Molecular Techniques

2.9.1 RNA extraction

Sorted cells with the same DNA content were pooled together to obtain larger quantities of RNA that were required for microarray analysis. These sorted samples and cells isolated from whole liver (from young and old mice) were washed twice in sterile PBS and resuspended in RNA extraction buffer (600 µl). Samples were stored at -70°C until required. Total RNA was extracted using a standard phenol/chloroform extraction protocol. Sodium acetate (2M, 60 µl), phenol (300 µl) and chloroform (300 µl) were added to each sample, vortexed and left on ice for 15 min. Protein and other contaminants were removed by micro-centrifugation at 13,000 *g* for 15 min at 4°C. The aqueous phase (top layer) was transferred to fresh eppendorf tubes and an equal volume of 100% ethanol and glycogen (1 µl) was added to precipitate the RNA. The samples were left at -70°C overnight and the RNA was pelleted by centrifugation at 13,000 *g* for 10 min. The pellet was washed twice in ethanol and left to air-dry for 15 min at room temperature. RNA was then resuspended in 30 µl DEPC-treated water and DNase-treated with DNA-free, according to the manufacturers instructions. The RNA was re-precipitated to increase the concentration of RNA by adding 100% ethanol (2 v), 2M Sodium acetate (0.1 v) and glycogen (1 µl) and precipitating as above. RNA was then resuspended in 10 µl TE buffer (pH 7.5). The quantity of RNA was determined by the absorbance reading at 260 nm on a spectrophotometer (Amersham Pharmacia Biotech). The quality of RNA was determined by electrophoresis on a 1% (w/v) agarose gel in TBE buffer. The gel was stained with ethidium bromide and the ribosomal bands were visualised under ultra violet light.

2.9.2 Microarray

2.9.2.1 Spotting of arrays and preparation of cDNA probes labelled with ^{33}P.

cDNA clones from the Incyte mouse GEM™1 set (Incyte Genomics Ltd., Cambridge, UK) were spotted onto nylon membranes at AstraZeneca, Alderley Edge, UK. The GEM™1 set contains known mouse genes from The Institute for Genomic Research Mus.ET database as well as ESTs from GenBank's mouse database. The arrays consist of 8734 genes and each one was spotted in duplicate. Clones contained partial sequences of around 500 bp on average. Sequence verification by AstraZeneca found approximately 96% of sequences were of a good quality. I carried out all microarray and real-time PCR in the Safety Assessment Dept., AstraZeneca, Alderley Edge in collaboration with Dr Jonathan Tugwood.

Probes were prepared from total RNA (15μg where possible) extracted from 2n, 4n and 8n sorted cells and from RNA extracted from whole livers (young and old mice). Details of all sorted and pooled samples are given in Appendix 3. Total RNA was added to 1μl of oligo (dT) primer (0.5μg/μl), the volume was made up to 9.5μl with DEPC-treated water and incubated at 70°C for 10 min then placed on ice. A reaction mixture consisting of first strand buffer, DTT (0.1M), 0.5μl dNTP mix (20mM dATP, dGTP, dTTP and 200μM dCTP) and 3μl alpha ^{33}P dCTP was added and incubated at 42°C for 5 min. Superscript II (200U) was added in a total volume of 20μl and incubated at 42°C for 2 h. Heating at 70°C for 15 min terminated the reaction. RNase H was added and incubated at 37°C for 20 min to degrade template RNA. The solution was made up to a final volume of 50μl with TE buffer and the unincorporated nucleotides were removed using ProbeQuant G-50 Micro columns. The Beckman LS6000SC scintillation counter was used to measure ^{33}P incorporation.

2.9.2.2 Array hybridisation

The arrays were incubated in Church and Gilbert hybridisation buffer for 2 h at 62°C. Mouse COT1 DNA (75μl) (suppresses the hybridisation of rodent repetitive DNA sequences) and the required amount of probe (6 x 10^6 or 1 x 10^7 cpm) were mixed and boiled at 100°C for 5 min, then placed on ice. The COT1 DNA and probe were added to 10 ml buffer and incubated with the array in a Techne bottle at 62°C for 18 h. Following hybridisation, the membranes were rinsed in wash buffer and washed twice for 30 min at 62°C.

2.9.2.3 Visualisation and analysis of arrays

The arrays were placed on Super resolution (type SR) Phosphor screens for 24 to 48 h in the dark and exposed to a phosphoimager using OptiQuant software. Analysis of each spot was carried out using ArrayVision software. The change in gene expression was calculated as $intensity_b/intensity_a$, where b was the sample of higher ploidy and a was the sample of lower ploidy e.g. 4n and 8n samples (b) were compared with 2n samples (a). The actual comparisons and samples that were studied are detailed in Appendix 4. Spots were only included if the intensity was greater than the intensity of the background, calculated from the immediate area surrounding the spots and if the variability between duplicates was less than ± log (0.2) (allowing for the mean variability of duplicates across the whole array). Using custom written macros in Microsoft Excel, the data was sorted and the clones that were induced and repressed more or less than 2-fold were checked on the actual arrays. Three clones; 1) major urinary protein 2 (MUP2), 2) the EST similar to human Pterin 4α-carbinolamine dehydratase (PCD) and 3) IFNγR, were chosen for further analysis on the basis that they met the above criteria and appeared on several different comparisons.

2.9.3 Qualitative and quantitative PCR

2.9.3.1 Primer design and reverse-transcription PCR

Primers were designed from cDNA sequences and included those of the 3 clones that were chosen from the microarray analysis, Pten, a gene involved in regulation of cell size (Huang et al., 1999) and housekeeping genes, G3PDH and β-actin. β-Actin primers (kindly donated by Claire Duggan and Shirley O'Dea, amplicon size ~100 and 400 bp) were used to assess the quality of RNA obtained from cells stained with various fluorescent dyes and cells that had been sorted. All other primer sets were used for quantitative PCR and were first assessed by reverse-transcription PCR to determine if the PCR product was the correct size and to check for primer dimers. Template cDNA was synthesised using 1μg of RNA (from whole mouse liver), RNASEOUT inhibitor (1 μl) Superscript II and Oligo dT primer, according to the manufacturers instructions. Controls for real-time PCR excluded the Superscript II enzyme. RNase H (1 μl) was added at 37°C for 20 min to the samples used for real-time PCR to degrade template RNA. The primers used in this study are shown in Table 4. A typical 50 μl PCR reaction for qualitative PCR contained 1 μM of each primer, 2 μl cDNA, 100 μM dNTPs, 0.1% (w/v) BSA, 0.25 units of *Taq* polymerase and 1.5 mM $MgCl_2$. The following protocol was used for all reactions: 95°C for 5 min; 35 cycles at 95°C for 3 min, 60°C for 1 min, 72°C for 1.5 min.

The products of the β-actin PCR were analysed by electrophoresis using a 1% (w/v) agarose gel that separates products between 500 and 10000 bp. Other products were analysed using a 2% (w/v) agarose gel that enabled the separation of 50 – 2000 bp products. Gels were stained and visualised as in section 2.9.1.

Table 4. Primer sequences used for PCR

Primer	5' sequence (5' to 3')	3' sequence (5' to 3')	Amplicon Size (bp)
MUP	CAAGAAAGACATGGTCCTGACA GA	AAAGTTCCTTCCCGTAGAACT AGCTT	131
PCD	CAGCTGCTTGCAAACCTGAGGG	GAAAGACCGGCACATTCATG GG	216
Pten	TTGTGGTCTGCCAGCTAAAGG	GATATCACCACACACAGGCA ATG	115
IFNγR	TCGGATCCAACCTGTGAGTTT	TACCATAGACTTACGGCTGGC	207
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	451
β-Actin	GTCTAGAGCAACATAGCACAGC	GCACCACACCTTCTACAATGA G	~400

2.9.3.2 Real-time PCR

To confirm the changes in gene expression seen in the microarray analysis, quantitative real-time PCR was carried out using the LightCycler™ on sorted samples and samples from young and old mice. Standards, made from a pool of all cDNAs being tested, were serially diluted as in Table 5. Each 20 µl reaction was set up in glass capillary tubes according to manufacturers instructions and contained 0.5 pmol/µl of each primer, 1 µl cDNA (diluted 1 in 20, 1 in 40 or 1 in 100), 13.4 µl H_2O, 1.6 µl $MgCl_2$ and 2 µl SYBR green (containing Taq DNA polymerase, reaction buffer, 10 mM $MgCl_2$, dNTP mix with dUTP instead of dTTP and SYBR green I dye). The program consisted of four stages: 1) Denaturation at 95°C for 2 min; 2) amplification and quantification; 3) determination of the melt curve where capillaries were raised to 95°C, then cooled to 65°C for 10 s at a transition rate of 20°C/s. The melt curve was then generated by increasing the temperature to 95°C at a temperature transition rate of 0.2°C/s, measuring fluorescence continuously; 4) the chamber was cooled to 40°C for 1 min. The optimal amplification and quantification conditions used for each primer set are given in Table 5.

Table 5. Optimum conditions for real-time PCR

Primers	Annealing Temp.(°C) for 4 s	Time for Elongation[1] (s) at 72°C	Standard Dilutions	Sample Dilutions	Fluorescence Measurement Temp.(°C)
MUP	63	7	1:50-1:1600	1:100	82
PCD	65	11	1:50-1:1600	1:100	83
Pten	60	7	1:10-1:320	1:40	80
G3PDH	62	20	1:50-1:1600	1:100	88

[1]Time for elongation step (s) = (size of the product (bps) / 25) + 2-3s.

The measurements of fluorescence were taken at a temperature just below the melting temperature of each product to ensure specificity. The Real Time Fluorometer (RTF) was used to set the FL1 gains between 10 and 20 on the most concentrated standard. Where possible, triplicate samples were analysed and the experiment was repeated twice.

2.10 Statistics

The majority of the statistical analysis was carried out using GraphPad InStat Software. Linear regression analysis was carried out using Microsoft Excel Software. For all tests a p value less than 0.05 was considered significant.

The following comparisons were done using a 1-way ANOVA:

1) PB-treated samples were compared to relevant controls,
2) The proportion of 2n and 8n cells within each ploidy population after plating, at time 0, after 5 days in culture (Control) and after treatment with IFNγ. Tetraploid cells were compared using a Kruskal-Wallis test as the difference in SDs between each group varied significantly,
3) The amount of RNA extracted from 2n, 4n and 8n sorted samples.

The purity of sorted cells was compared before and after the addition of DNase I and PI using a Kruskal-Wallis test. The induction of apoptosis by IFNγ and the effect of IFNγ on the incorporation of BrdU were compared using a Mann-Whitney U test.

The nuclear diameter of the 2n and 4n populations and the volume of the 2n, 2x2n, 4n and 2x4n cells were compared using an unpaired t-test with a Welch correction for volume. A paired t-test was also used to compare the size of nuclei from the same cell.

3 Phenobarbitone-induced polyploidisation occurs independently of p53

3.1 Objectives

The carcinogenic potential of drugs is of paramount importance in the field of toxicology. In order to assess drugs quickly, it is necessary to develop transgenic animal models which can act as short-term carcinogenicity bioassays and can detect both non-genotoxic and genotoxic drugs (Blain et al., 1998). The p53 tumour suppressor gene that is involved in cell cycle arrest, apoptosis, differentiation and repair (Prives and Hall, 1999) has been found to be mutated in 50% of human tumours (Greenblatt et al., 1994). Transgenic models lacking or containing mutations within this gene are expected to develop tumours more rapidly than wild-type strains (p53 +/+) (Harvey et al., 1993). The p53 homozygous (-/-) mice are not suitable for such studies as they die early in life (Armstrong et al., 1995) and have a high incidence of spontaneous tumours within 3-6 month of age (Donehower et al., 1992). p53 heterozygous (+/-) mice have a much lower incidence of tumours up until 9 months of age (Donehower, 1996). The p53 +/- mouse model has been found to be useful in the identification of genotoxic carcinogens (Blain et al., 1998) but at present, there is no evidence to suggest that it can identify non-genotoxic carcinogens such as Sodium phenobarbitone (PB).

Chronic or long-term administration of PB is associated with the development of hepatocarcinogenesis (Lin et al., 1989; Manjeshwar et al., 1994). Of particular interest to this PhD is the effect of short-term administration of PB on liver ploidy. Administration of PB for 10 days to mice was found to increase the level of ploidy in the liver with no affect on the proportion of mononuclear, binuclear or polynuclear cells (Bohm and Noltemeyer, 1981). Other effects associated with acute administration of this drug include increases in liver weight, RNA and protein synthesis, cytochrome P450 enzymes, increased cell proliferation and a decrease in

the rate of apoptosis (Bohm and Noltemeyer, 1981; Carthew et al., 1998; Lin et al., 1989; Maier and Schawalder, 1993; Wojcik et al., 1988).

One study showed that PB delays and attenuates the G1 checkpoint response in a p53-dependent manner (Gonzales et al., 1998). There is also evidence that p53 could play a role in the control and mechanism of ploidy, although conflicting evidence exists (Bellamy et al., 1997; Yin et al., 1998). Several studies have shown that mutations in p53 can result in the formation of polyploid cells after the disruption of the mitotic spindle (Ciciarello et al., 2001; Gualberto et al., 1998; Minn et al., 1996; Notterman et al., 1998). Induction of polyploidy in fibroblasts from Li-Fraumeni syndrome which contain a congenital mutation in one p53 allele, can lead to aneuploidy through genetic instability and increases the risk of carcinogenesis (Bischoff et al., 1990; Gualberto et al., 1998; Shackney et al., 1989). The possibility that polyploidisation induced by PB could be affected by p53 genotype has not previously been studied. Therefore, the aim of this part of the study was to determine if p53 was involved in the polyploidisation and proliferation of hepatocytes induced by PB. This study utilised C57BL/6 p53 +/+, p53 +/- and p53 -/- male mice and DNA content was measured using flow cytometry. For materials and methods see chapter 2.

3.2 Results

3.2.1 PB caused an increase in liver weight and liver cell number

PB administration for 21 days resulted in a 39-60% increase in liver weight expressed as liver/body weight ratio compared to controls (Table 6: $p<0.05$); no differences were identified relating to p53 genotype. The number of hepatocytes per liver also showed a modest increase after PB administration in p53 +/+ and -/- mice (16% and 21% increase respectively) but not in the p53 +/- group compared to controls ($p<0.05$). The proportion of non-parenchymal cells was the same in PB-treated and control mice for each genotype (Table 6). No histological abnormalities

were identified in any group and in particular no carcinomas were seen; there was no evidence of hepatocyte apoptosis.

Table 6. Effect of phenobarbitone on liver weight and cell number

Group	+/+		+/-		-/-	
	PB	**Con**	**PB**	**Con**	**PB**	**Con**
Liver Weight	0.078* ±0.008	0.050 ±0.002	0.075* ±0.003	0.047 ±0.004	0.077* ±0.004	0.055 ±0.002
Total Cell No.	2244* ±87	1940 ±187	2034 ±300	1848 ±89	2315* ±134	1919 ±166
%NPC	32.78 ±2.38	29.16 ±3.34	33.23 ±2.21	33.42 ±3.08	31.68 ±2.33	28.91 ±2.11

Liver weight is expressed per gram of total mouse weight; total cell number is the total number of hepatocytes per liver (cells per 5mm^2 x liver weight); %NPC is the ratio of non-parenchymal cells to hepatocytes. * Represents a significant increase in PB-treated v. control mice ($p<0.05$).

3.2.2 PB caused liver polyploidisation

Administration of PB for 21 days resulted in a striking increase (250-400%) in the proportion of 8n nuclei compared with control mice (Figure 6), with no differences noted comparing the three p53 genotypes (Figure 6, $p<0.001$). A small but significant reduction in 2n cells was also noted in p53 +/+ and -/- genotypes ($p<0.001$). No changes were identified in the 4n population.

3.2.3 PB increased hepatocyte proliferation

All untreated control samples showed similar baseline levels of BrdU positivity with no differences between p53 genotypes so the controls were combined. The proportion of BrdU positive nuclei was increased in all 3 genotypes after PB treatment (Figure 7; $p<0.001$) with p53 +/+ and -/- mice also showing higher levels of BrdU positivity than p53 +/- mice. Separating BrdU positivity according to ploidy showed increased positivity in 8n cells only for all three p53 genotypes (Figure 7; $p<0.01$).

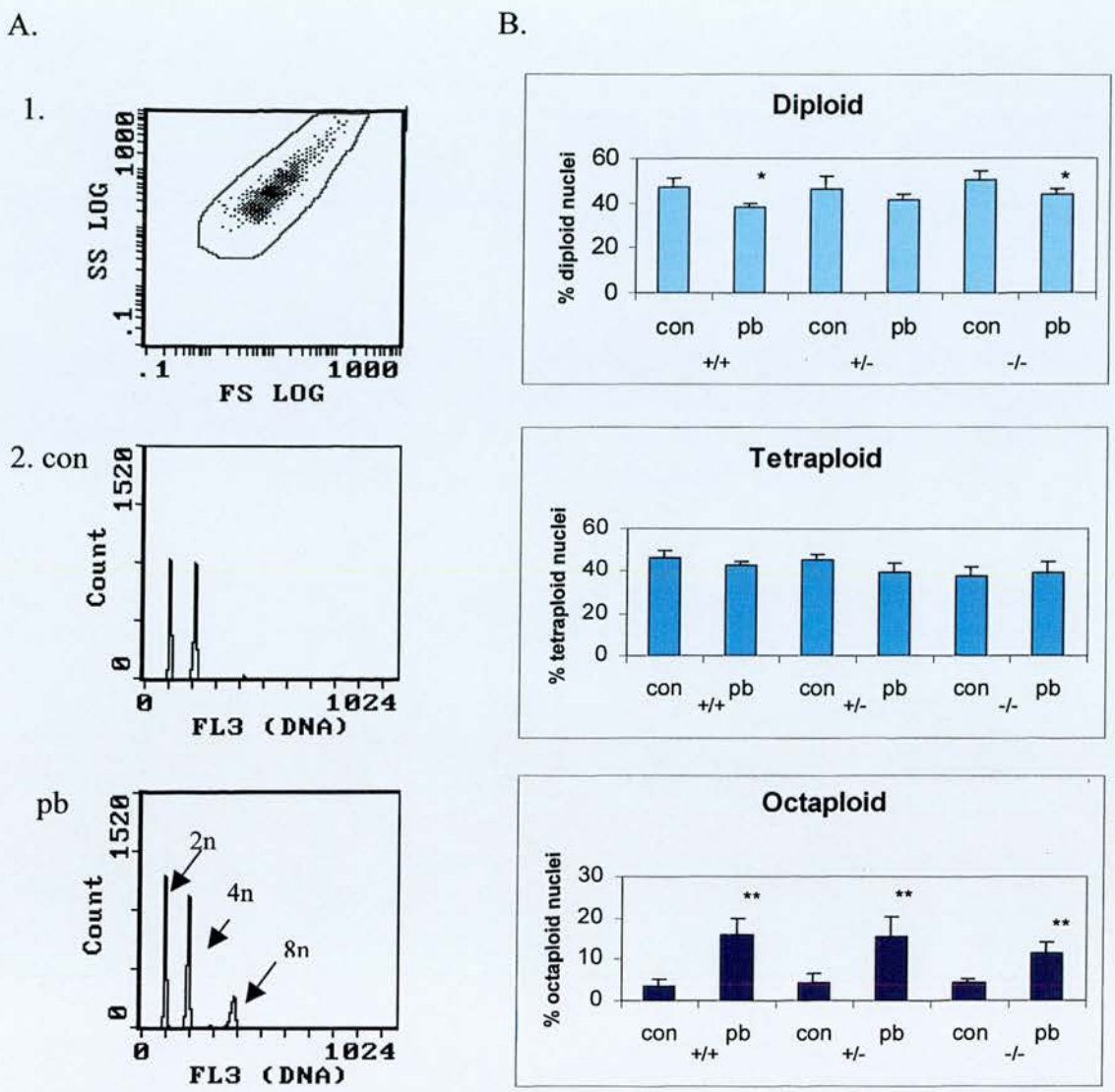


Figure 6. The effect of PB on ploidy. A. Flow cytometry histograms showing (1) side scatter (ss log, granularity) vs. forward scatter (fs log, size) and (2) numbers of events (count, nuclei) vs. ploidy (FL3 (DNA), PI uptake) for control (con) and PB-treated (pb) mice. Arrows indicate 2n, 4n and 8n nuclei. B. Proportion of diploid, tetraploid and octoploid nuclei after 21 days administration of PB. Comparison of proportion of nuclei in each ploidy class in control (con) and PB-treated (pb) groups of p53 +/+, +/- and -/- mice. * Indicates a significant decrease in cell number in PB-treated mice compared with controls ($p<0.001$) ** Indicates a significant increase in cell number in PB-treated mice compared with controls ($p<0.001$).

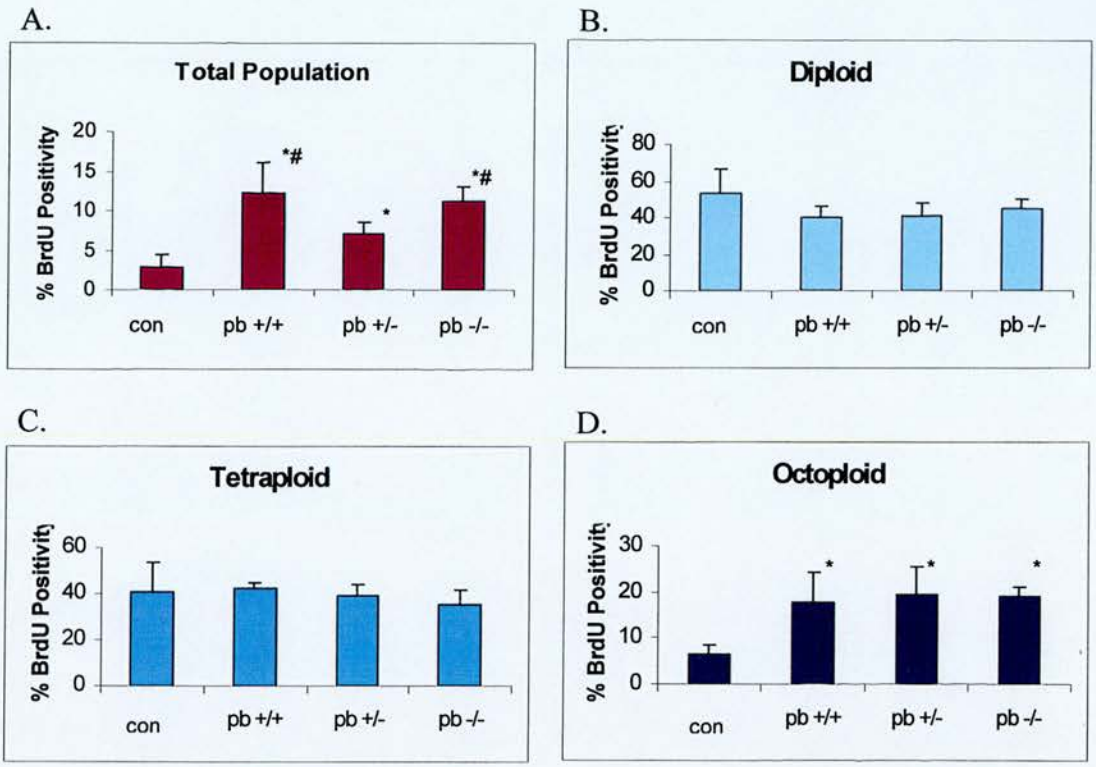


Figure 7. BrdU positivity after 21 days administration of PB. A. Comparison of controls with PB-treated p53 +/+, +/- and -/- mice for the total population of nuclei. Proportion of BrdU positive nuclei in B. 2n, C. 4n and D. 8n ploidy classes. * Indicates an increase compared with controls ($p<0.01$); # indicates an increase compared with p53 +/- mice.

3.3 Discussion

3.3.1 PB induces hepatocyte polyploidisation independently of p53

The results of this study demonstrate that PB induces a large increase in 8n nuclei with no change in 4n and a slight decrease in 2n nuclei by 21 days. This increase in ploidy is not affected by p53. Bohm and Noltemeyer, 1981, found that PB caused an increase in 4n and 8n nuclei with a concomitant reduction in 2n nuclei after 10 days treatment. The reason for the difference between these two studies is not clear but could be related to the different strains of mice studied or the length of time of administration of PB. Age-related polyploidisation varies between strains of mice (Severin et al., 1984) and it could be possible that the degree of polyploidisation in response to PB is strain-dependent.

Some chemicals have been found to increase ploidy predominantly in mononuclear, binuclear cells or both (Melchiorri et al., 1993; Scampini et al., 1993). An increase in 8n mononuclear cells has been described following the administration of the non-carcinogenic hepatic mitogen 1,4-dichlorobenzene (Hasmall and Roberts, 1997) and the peroxisome proliferators WY-14 643 and methylclofenapate (Miller et al., 1996). Although this study has examined 8n nuclei and not cells, immunostaining of BrdU in sections from the same cases as this study showed positivity in both mononuclear and binuclear cells (Orton et al., 1999) indicating that both mononuclear and binuclear cells respond to PB.

3.3.1.1 p53 and polyploidisation

There is conflicting evidence regarding the role of p53 in polyploidisation. Bellamy et al., 1997, demonstrated that p53 deficiency in the normal liver did not affect progression from diploidy to polyploidy with age. Similarly, Gould et al., 2001, found that in p53 +/+, +/- and -/-mice, the changes in the levels of EGFR, M6PB and TGFβ1 in response to PB were the same in all mice and suggested that p53 did not

play a role in early phenobarbitone-induced effects. However, Yin et al., 1998, found that normal polyploidisation associated with age did not occur in p53+/- and -/- mice. The study by Bellamy et al., 1997, did suggest that abnormal ploidy may occur in p53 null livers after DNA damage induced by γ-irradiation, due to the loss of G_1 and G_2 checkpoints. Furthermore, mutations in p53 have been shown to be involved in polyploidisation after administration of spindle depolarising agents by overcoming checkpoint controls and allowing the re-replication of DNA following an incomplete mitosis (Ciciarello et al., 2001; Gualberto et al., 1998; Minn et al., 1996; Notterman et al., 1998). Therefore it remains a possiblity that p53 may be involved in polyploidisation in response to DNA-damaging agents or other drugs that directly affect the cell cycle but may not be involved in age-related polyploidisation nor involved in ploidy induced by non-genotoxic drugs such as PB.

3.3.2 The induction of proliferation by PB may occur through a p53-dependent pathway

PB induced an increase in cell number, liver weight and BrdU positivity compared with controls, confirming PB is mitogenic. The increase in cell number and proliferation in p53 +/+ and -/- mice and not p53 +/- mice, suggests that p53 may play a role in the proliferative response to PB. The increase in cell number was not due to increases in non-parenchymal cells, as PB did not alter the proportion of these cells.

There was an increase in BrdU positivity in PB-treated mice in comparison with untreated controls in the 8n population only. The fact that there was not an increase in BrdU positivity in 2n and 4n nuclei after administration of PB for 21 days indicates that the continuous exposure of cells to PB does not invoke a constant proliferative response in all cells. If this had been the case, the BrdU positivity of 2n and 4n nuclei would also have expected to increase. The increase of BrdU positivity in the 8n population only, together with the increase in proportion of 8n nuclei and slight decrease in 2n nuclei suggests that proliferation is accompanied by polyploidisation with 2n nuclei becoming 8n nuclei by 21 days. Time lapse

experiments in mouse hepatocytes have demonstrated that polyploidisation occurs in 2 stages: Firstly the mononuclear cells become binuclear cells through a process of acytokinesis where the cells replicate their DNA, undergo mitosis but do not divide. The second stage is the formation of mononuclear polyploid cells which occur when the nuclei of a binuclear enter mitosis simultaneously, form a common mitotic spindle and then divide normally, producing two mononuclear cells of a higher ploidy class (Nadal and Zajdela, 1966; Brodsky and Uryvaeva, 1985b). The fact that BrdU positivity was evident in both mononuclear and binuclear cells (Orton et al., 1999) suggests that the transition of 2n nuclei to 8n nuclei could occur through the formation of intermediary binuclear cells.

3.3.3 The p53 +/- mouse is not a good model for the identification of the non-genotoxic drug, PB

The study of drug-induced hepatocarcinogenesis is greatly benefited by the development of transgenic animal models that rapidly develop tumours. The oncosuppressor gene, p53 is mutated in approximately 50% of human tumours (Greenblatt et al., 1994) and p53 transgenic mice have been proposed to be useful models in short-term carcinogenesis studies. Evidence from the literature suggests that p53 is involved in polyploidisation in response to direct DNA damage (Bellamy et al., 1997) and damage to components of the cell cycle such as the mitotic spindle (Ciciarello et al., 2001; Gualberto et al., 1998; Minn et al., 1996; Notterman et al., 1998). However, the results of this study indicate that p53 is not involved in the induction of polyploidy by PB. There were also no apparent histological abnormalities in the PB-treated group compared with controls, suggesting that the p53 +/- mouse model will not be useful in short-term carcinogenesis studies with PB. Further work may help determine if this model may be useful for longer-term studies or to determine if this model may be useful in the identification of other non-genotoxic drugs.

4 Functional Analysis of Mouse Hepatocytes Differing in DNA Content: Volume, Receptor Expression and Effect of IFNγ

4.1 Objectives

The reasons for and functions of polyploidy in the liver remain largely unknown but several hypotheses exist (Chapter 1). The size and volume of polyploid cells is proportional to DNA content (Danielsen et al., 1986; Deschenes et al., 1981; Watanabe and Tanaka, 1982) and the possibility that increasing cell size may play a role in the function of polyploid hepatocytes has not until now been explored. Interestingly, alterations in cell volume in various cell types have been found to participate in a wide variety of functions including cell proliferation and cell death (Lang et al., 1998) and cell size has been implicated in the regulation of the transcription factor, DBP (Schmidt and Schibler, 1995). Therefore, there is a possibility that the size of polyploid cells could modulate their function. One method that increasing size could induce an effect on the cells is by altering the density of surface receptors. To study this hypothesis, the first aim of this chapter was to develop a method of accurately measuring the size of each ploidy population and secondly of determining whether surface receptor expression increased in these cells.

Intercellular adhesion molecule-1 (ICAM-1) and Interferon gamma (IFNγ) have important inflammatory roles in the liver. ICAM-1 is a member of the immunoglobulin supergene family and is expressed on hepatocytes after dissociation of cell-to-cell contact (Ohno et al., 1995) or after induction with pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNFα) and IFNγ (Bumgardner et al., 1998; Oudar et al., 1998). Expression of this adhesion molecule has been found to be important in hepatitis and liver graft rejection (Bumgardner et al., 1998; Schroder et al., 1995). Through the interaction with specific membrane receptors, IFNγ induces G1 cell cycle arrest and apoptosis in hepatocytes (Kano et al., 1997; Morita

et al., 1995; Shinagawa et al., 1991) and is also thought to play a role in hepatitis in humans and mice (Gilles et al., 1992; Peters et al., 1991). Therefore, any differential expression of ICAM-1 or IFNγR on 2n and polyploid hepatocytes could be clinically important.

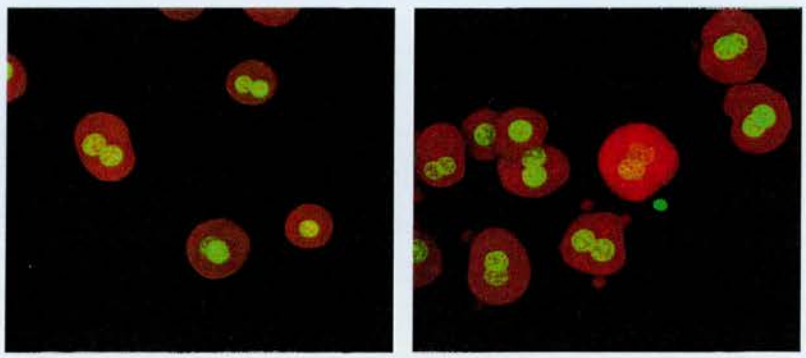
There is some evidence that polyploid hepatocytes are more likely to undergo apoptosis and that extensive polyploidy could lead to organ failure (Gupta et al., 2000). To study this possibility, this chapter has also determined whether polyploid hepatocytes are more likely to undergo apoptosis in response to IFNγ. For materials and methods see Chapter 2.

4.2 Results

4.2.1 Determination of ploidy and volume of hepatocytes using confocal microscopy

Images obtained using confocal microscopy and Imaris imaging software allowed the accurate determination of nuclear ploidy and absolute cell volume. Due to the spherical shape of the nuclei and therefore, the consistent measurements of the nuclei, nuclear diameter was found to be the most accurate determinant of ploidy. The nuclear diameter of all nuclei measured was plotted as a frequency histogram and the diameter of 2n ranged from 7.0 to 9.0 μm and 4n ranged from 9.5 to 11.5 μm (Figure 8). The nuclei falling within each range were found to be significantly different to each other ($p<0.001$). There was no significant difference in the nuclear diameter of nuclei from the same cell. From the 300 nuclei analysed, only one 8n nuclei was measured and therefore the accurate determination of the diameter of 8n nuclei was not possible. The proportion of 2n and 4n nuclei plotted on the frequency histogram was found to be similar to the proportion of these nuclei using flow cytometry (data not shown).

A.



B.

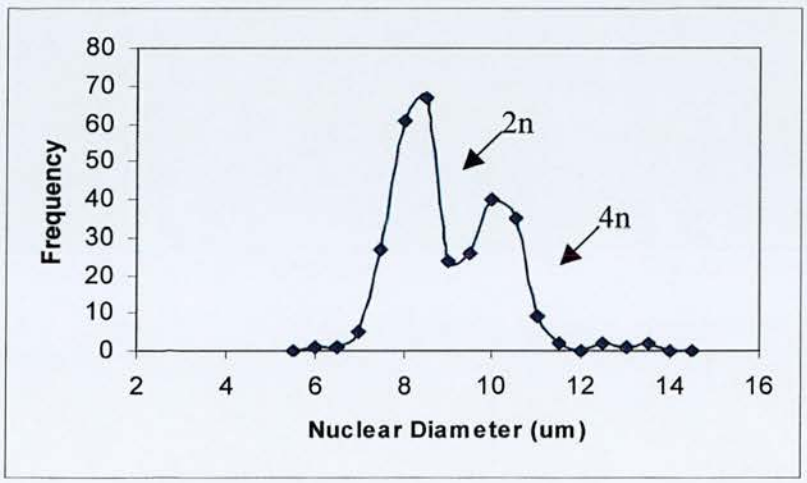


Figure 8. Measurement of nuclear diameter of hepatocytes with different DNA contents. A. Confocal images of hepatocytes stained with dyes SYTO 16 (nucleic acid, green) and CTO (protein, orange). B. Frequency histogram of nuclear diameter measured from confocal images and Imaris imaging software. Arrows indicate the peaks corresponding to 2n and 4n nuclei.

The measurement of nuclear ploidy by measurement of their diameter allowed the subsequent determination of the volume of cells of different ploidy. The volume of 2 x 2n (7035 ± 2391 μm^3) and 4n (7352 ± 1650 μm^3) cells was 1.7-1.8 fold greater than 2n cells (4154 ± 1137 μm^3, $p<0.001$) and 2 x 4n cells (14326 ± 4453 μm^3) was 1.9-2.0 fold greater than 2 x 2n and 4n cells ($p<0.001$). There was no significant difference in the volume of 2 x 2n and 4n cells (Figure 9).

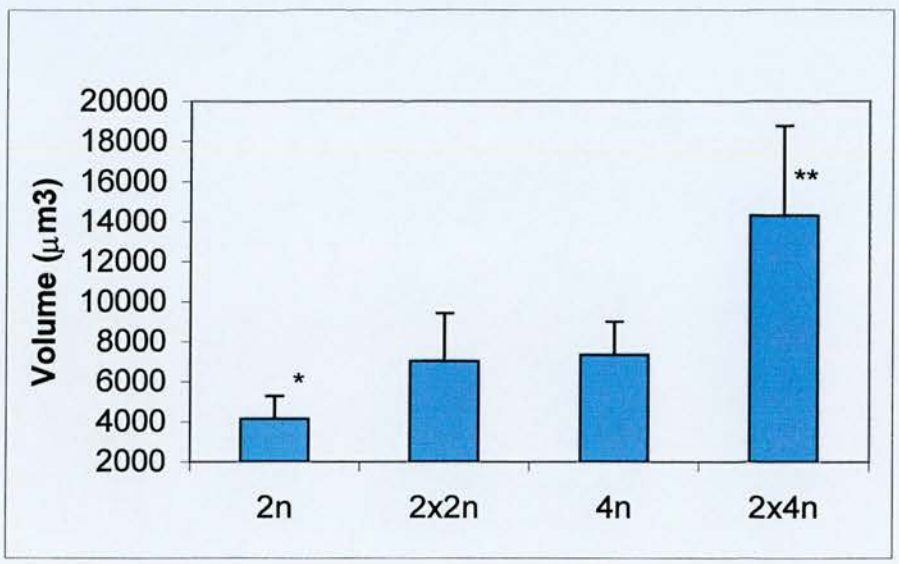


Figure 9. Volume of hepatocytes measured from confocal images and Imaris imaging software. *Significantly lower than 2 x 2n and 4n, $p < 0.05$. **Significantly higher than 2 x 2n and 4n, $p < 0.05$. Values represent mean ± SD.

4.2.2 The fluorescence intensity of ICAM-1 (CD 54) but not IFNγR, increases in relation to DNA content

Alterations in cell size could potentially affect the number of receptors on the cell surface and in turn affect cell signalling and cell function. Two-parameter flow cytometry was used to measure the expression of ICAM-1 or IFNγR and DNA content of hepatocytes. Receptor expression was compared to the volume of

hepatocytes with different DNA contents measured by confocal imaging. Mononuclear and binuclear cells with the same DNA content cannot be distinguished using flow cytometry. Therefore, the average volume of mononuclear and binuclear 4n cells (2x2n and 4n) was used. The linear relationship between fluorescence intensity of ICAM-1 and the average volume of 2n, 4n and 8n cells was highly significant, demonstrating that expression of ICAM-1 increases in proportion to volume and DNA content ($r^2 = 0.907$, $p < 0.0001$) (Figure 10). The fluorescence intensity of IFNγR was the same as the isotype controls indicating that IFNγR was not detectable on these cells. Background fluorescence (isotype controls) was low and increased with increasing DNA content. However, the ratio of fluorescence intensity of ICAM-1 to background was equal for all cells and was therefore excluded from the analysis.

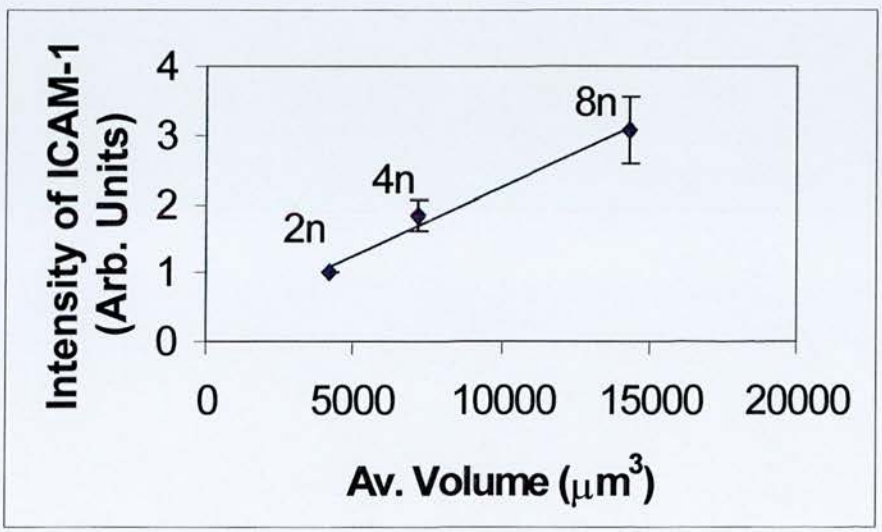


Figure 10. Plot of average volume measured by confocal imaging against fluorescence intensity of ICAM-1 measured by flow cytometry for 2n, 4n and 8n hepatocytes. The values represent mean volume ± SD of 2n, 4n and 8n cells. Graph show the line of best fit, $r^2 = 0.907$, $p < 0.0001$.

4.2.3 IFNγ induces apoptosis in all ploidy populations equally

To determine whether the cells that undergo IFNγ-induced apoptosis are predominantly diploid or polyploid, the cells that remain alive after treatment with IFNγ were studied using flow cytometry. Feulgen staining was used to determine the amount of IFNγ-induced apoptosis. The results from different experiments were not combined due to differences in the baseline levels of apoptosis each time the experiment was performed. Maximal apoptosis occurred 3 – 4 days after administration of IFNγ (Figure 11). Therefore, 4 days was chosen for ploidy analysis. At this time-point, between 10 and 22% of hepatocytes had condensed, fragmented chromatin and were visibly apoptotic (Figure 12).

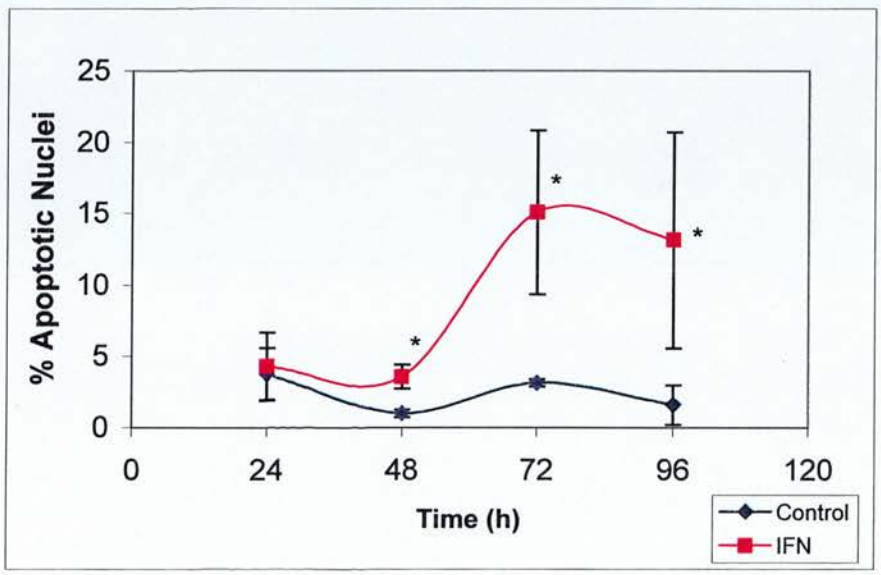


Figure 11. Time course of induction of apoptosis by IFNγ. Graph shows data from one representative experiment, which was carried out 3 times. Values represent mean ± SD, n = 4. *Significantly different to control, p = 0.0286.

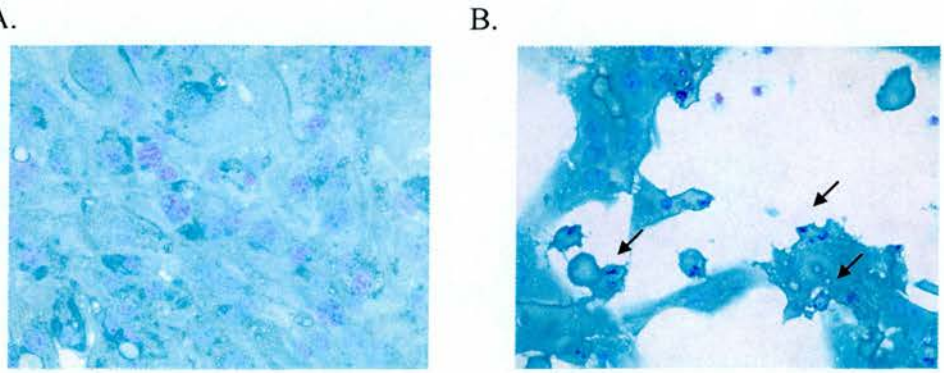


Figure 12. Feulgen staining of hepatocytes after 4 days administration of IFNγ. A. Control and B. IFNγ-treated. Arrows indicate apoptotic nuclei. Images were captured using a x40 objective.

To determine if 2n cells were more resistant to apoptosis after 4 days treatment, flow cytometry was used and showed that the proportion of 2n (8.0 ± 1.6), 4n (65.8 ± 6.0) and 8n (15.1 ± 2.9) cells was the same as controls (2n 8.2 ± 1.4; 4n 67.1 ± 5.2; 8n 16.3 ± 3.0) (Figure 13). This indicates that IFNγ induces apoptosis in all ploidy populations equally. Interestingly, the control hepatocytes showed a significant decrease in 4n cells (67.1 ± 5.2 c.f. 76.7 ± 1.9, $p<0.001$) and increase in 8n cells (16.3 ± 3.0 c.f. 12.7 ± 2.8, $p<0.05$) over 5 days in culture, (Figure 13). The proportion of 2n, 4n and 8n cells did not change after plating.

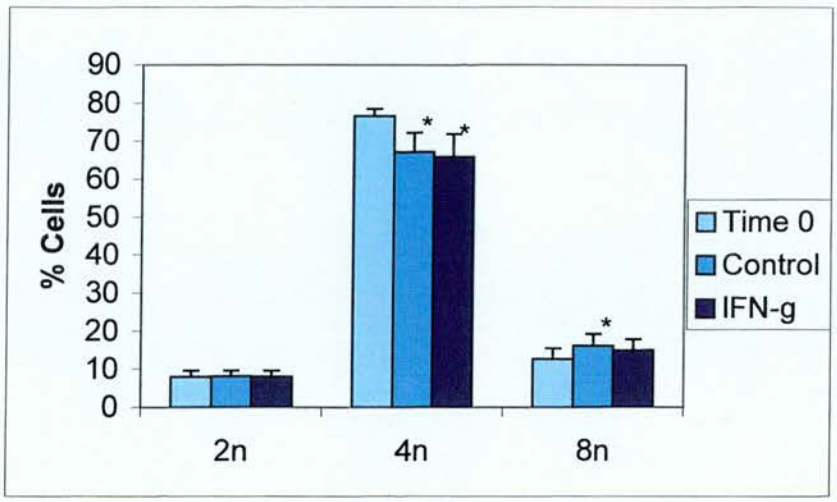
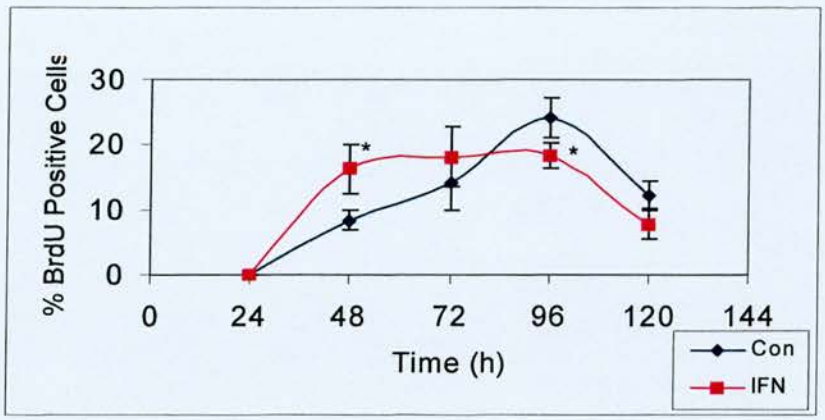


Figure 13. DNA content of cells remaining after IFNγ treatment. Proportion of 2n, 4n and 8n cells in culture before treatment (Time 0) and with (IFN-g) or without (control) IFNγ for 4 days. Administration of IFNγ does not alter the proportion of 2n, 4n and 8n cells. Over time there is decrease in 4n and increase in 8n hepatocytes. *Significantly different to time 0, $p < 0.05$. Values represent mean ± SD, n = 15.

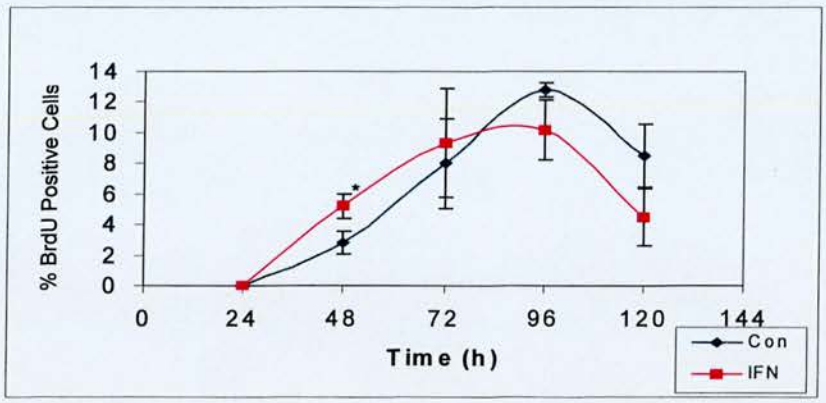
4.2.4 Hepatocytes remaining after IFNγ-treatment did not arrest in G1

The proportion of BrdU positive cells increased over time in control and IFNγ treated cultures. Both mononuclear and binuclear cells incorporated BrdU. After 4 days, IFNγ had no significant effect on the proportion of mononuclear and binuclear BrdU positive cells (Figure 14).

A.



B.



C.

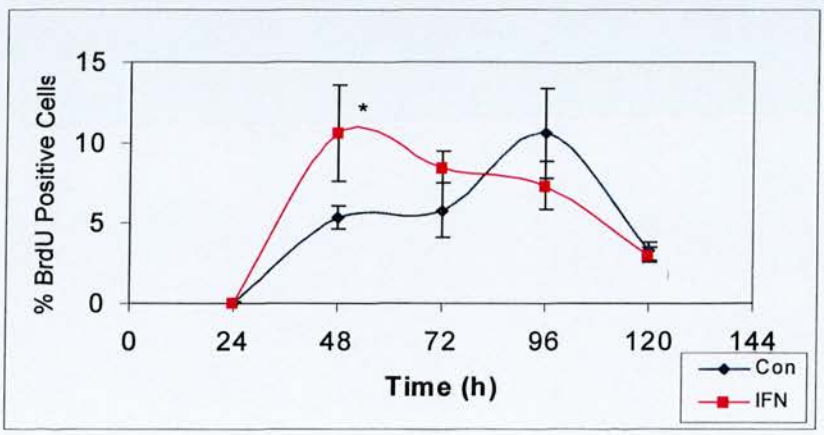


Figure 14. Effect of IFNγ on BrdU incorporation. Hepatocytes were given IFNγ for 4 days in culture and the effect on BrdU incorporation was measured by counting the proportion of BrdU positive cells after a 3 hour pulse. The histograms shown are from one representative experiment that was carried out 3 times. A. Total % BrdU positive cells with IFNγ (IFN) and without (Con). B. Proportion of BrdU positive cells which were mononuclear. C. Proportion of BrdU cells which were binuclear. Values represent mean ± SD, n = 4, *p = 0.0286.

4.3 Discussion

The reasons for increasing polyploidisation as a function of age or after administration of toxic chemicals remain largely unknown. This chapter has studied the differences between 2n, 4n and 8n mouse hepatocytes with respect to cell volume, receptor expression and response to IFNγ to try to determine more about their function.

4.3.1 Confocal microscopy is a useful method for the measurement of cell volume of different ploidy populations

Until now, there was no accurate method of determining the volume of live isolated hepatocytes differing in DNA content. Previous measurements of volume and DNA content have required fixed cell preparations. Confocal microscopy together with appropriate imaging software has been used to determine the volume of live chondrocytes (Bush and Hall, 2001) and can now be applied to hepatocytes. In order to measure the size or volume of hepatocytes of different ploidy using this technique, it was necessary to find a way to distinguish the ploidy populations. Due to the consistently spherical shape of nuclei, nuclear diameter proved to be an accurate measurement of ploidy. The nuclear diameter of 2n nuclei was found to range between 7.0 and 9.0 μm and 4n nuclei between 9.5 and 11.5 μm. Nuclei of the same binuclear cell were also found to be of similar size which has also been demonstrated in human hepatocytes (Watanabe and Tanaka, 1982). These measurements were found to be slightly larger than those found by Danielsen et al., 1986 who measured cells within mouse tissue sections and found the diameter of 2n nuclei ranged between 6.0 and 7.5 μm and 4n nuclei ranged between 7.5 and 9.0 μm. This difference would be expected in fixed tissue sections as fixation shrinks tissues and cells. The advantage of measuring ploidy in this way means that live cells of different ploidy and nuclearity can be visualised along with other parameters such as cell volume. The volume of cells approximately doubled with doubling DNA content and the volume of mononuclear and binuclear cells containing the same

amount of DNA was found to be equal. Other studies in humans, mice and rats, utilising methods such as cytophotometry, microspectrophotometry, stereological image analysis, Coulter counter and sedimentation velocities have shown similar trends (Deschenes et al., 1981; Epstein, 1967; Sweeney et al., 1978a; Watanabe and Tanaka, 1982). However, such studies have relied on mathematical calculations and assumptions that cells are spherical in their determination of cell size and volume. The use of confocal imaging negates these assumptions and calculations and therefore provides the first accurate measurements of mouse hepatocyte cell volume. The only disadvantage is the time required to obtain images using the confocal system, which limits the number of cells that can be analysed.

4.3.2 Expression of ICAM-1 increases with increasing cell size

Cell volume participates in a wide variety of functions including cell proliferation and cell death (Lang et al., 1998) and the possibility that the increase in size of polyploid cells could be involved in the regulation of these cells has not previously been studied. Schmidt and Schibler, 1995 suggested that cell size and volume was a factor controlling the expression of various genes, including the leucine zipper protein, DBP, although the ubiquitous transcription factors, Oct1 and NF-Y, were not regulated by size. This study proposed that one way increasing cell size could modulate the function or response of hepatocytes to signals was by altering the expression of molecules such as ICAM-1 and IFNγR on the cell surface.

ICAM-1 is expressed on hepatocytes after dissociation of cell-cell contact (Ohno et al., 1995) and is important in inflammatory processes, including hepatitis and liver transplant rejection (Bumgardner et al., 1998; Schroder et al., 1995). Therefore cell volume measurements were used in conjunction with flow cytometry to analyse ICAM-1 expression on 2n, 4n and 8n cells. The results indicate that the fluorescence intensity of ICAM-1 increases in proportion to volume and DNA content. As the volume of hepatocytes approximately doubles with doubling DNA content and the fluorescence intensity of ICAM-1 increases accordingly, the polyploid cells actually express a greater number of ICAM-1 molecules. Assuming that fluorescence

intensity approximately equates to the number of ICAM-1 molecules on the surface, the density of ICAM-1 is actually greater on polyploid cells, as surface area (sphere = πr^2) does not increase to the same extent as volume (sphere = $4/3\pi r^3$). Whether or not the number or density of receptors on hepatocytes is functionally important, remains to be seen. There is a possibility that up-regulation of receptors through perfusion injury or in disease, induces the expression of receptors to an extent that is not usually attained in vivo. Therefore, it would also be interesting to study receptor expression in cells of different ploidy in vivo. A comparison of hepatocytes isolated from prenatal, postnatal and adult rats, found that the number of insulin binding sites per cell increased constantly, whereas the affinity of plasma membrane receptors for the hormonal ligand remained unaltered from prenatal to adult hepatocytes (Autuori et al., 1981).

The expression of IFNγR was of particular interest, as this study has also looked at the effects of IFNγ on cells with different DNA content. However, IFNγR α or β chains could not be detected. IFNγR is not expressed at detectable levels on normal human hepatocytes (Volpes et al., 1991) and this could be the case in the mouse. A study by LeClaire et al., 1992, demonstrated by immunocytochemical staining that the majority of immunologically reactive mouse IFNγR protein is intracellular.

4.3.3 All hepatocytes undergo apoptosis in response to IFNγ

Although contradictory evidence exists, several studies have indicated that polyploid cells are more susceptible to apoptosis (Gupta et al., 2000; Oren et al., 1999; Sigal et al., 1999). Therefore, this chapter studied the effects of IFNγ on the different populations of hepatocytes. Although IFNγR was not detectable on isolated hepatocytes, IFNγ did induce apoptosis in culture, in agreement with other studies (Kano et al., 1997; Morita et al., 1995; Shinagawa et al., 1991). The mechanism through which IFNγ exerts its effects is largely unknown, although interferon regulatory factor 1 (IRF-1) has been shown to be essential in IFNγ-induced apoptosis (Kano et al., 1999). Although analysis of gene expression in 2n, 4n and 8n

hepatocytes would have been an interesting way to study the effects of IFNγ on these cells, differences in gene expression would have been undetectable as the majority of cells, at earlier time points, are unaffected by IFNγ. BrdU immunohistochemistry demonstrated that the hepatocytes that remained alive after treatment with IFNγ were able to incorporate BrdU to a similar extent as control cells and were not arrested in G1. In order to determine whether polyploid cells were more susceptible to the induction of apoptosis by IFNγ, flow cytometry was carried out on the cells that remained alive after treatment. The prolonged effects of IFNγ made it necessary to study cells after 4 days treatment.

The proportions of 2n, 4n and 8n hepatocytes were the same as controls indicating that all hepatocytes are equally sensitive to IFNγ-induced apoptosis in vitro. A study by Melchiorri et al., 1993, found that after administration of the mitogen, lead nitrate, the elimination of excess cells by apoptosis did not occur preferentially in the polyploid population. In contrast, Oren et al., 1999, found that the rate of apoptosis was increased in polyploid cells after administration of the potent hepatic mitogen, triiodothyronine (T3) to rats pre-treated with the cell cycle inhibitor, retrorsine. Sigal et al., 1999, suggested that apoptosis accounted for the decrease in the proportion of 8n cells 5 days after a partial hepatectomy. Increased rates of apoptosis have also been found in the livers of LEC rats where the liver has become populated with megalocytes, containing enormous nuclei (Gupta et al., 2000). In the cases where the cell cycle has been disrupted through administration of cell cycle inhibitors like retrorsine or in the case of LEC rats, polyploid cells or megalocytes form through a disruption or block in the cycle. The difference in the susceptibility of polyploid cells to apoptosis may therefore be related to the type of polyploid cell or to the type of injury inflicted on the cell. Susceptibility of hepatocytes to apoptosis may also differ in vivo and in vitro.

There have been reports that polyploid cells are also more susceptible to injury from toxic compounds. Perivenous hepatocytes found to exhibit greater ploidy than periportal cells (Schmucker, 1990) were more susceptible to the cytotoxic effects of carbon tetrachloride and paracetamol (Osypiw et al., 1994). A study by Sancho-

Tello et al., 1987, on young rats, showed that hepatocytes from the perivenous region of the liver were more greatly damaged than periportal cells when exposed to alcohol in vivo. In contrast, the study by Osypiw et al., 1994, found that periportal hepatocytes were more susceptible to methotrexate and 1-naphthylisothiocyanate. Therefore the susceptibility of hepatocytes to injury may be due to their zonal location rather than their degree of ploidy and may be linked to the different function and/or gene expression within cells of these areas.

4.3.4 Hepatocytes become increasingly polyploid in culture

Although there was no change in the proportion of 2n, 4n and 8n hepatocytes after administration of IFNγ, there was a change in the proportion of polyploid cells over time in control cultures. The proportion of 8n hepatocytes increased, 4n hepatocytes decreased and 2n hepatocytes remained the same over 5 days. BrdU immunohistochemistry showed that both mononuclear and binuclear cells proliferate in these cultures. Previous studies have shown that polyploidisation and cell fusion occur in rat hepatocyte cultures (Gomez-Lechon et al., 1981; Mossin et al., 1994). The reasons why the proportions of 4n and 8n populations alter while 2n cells remain the same in this study are unknown. Gomez-Lechon et al., 1981, suggested that polyploid hepatocytes are more unstable in culture. However, this is unlikely to be the case in this study as the 8n population increases. Alterations in the proportions of cells are likely to be due to a combination of cell death, proliferation, polyploidisation and cell fusion and the rate or susceptibility of 2n, 4n and 8n cells to each of these parameters will have an effect on the proportion of each population.

4.4 Summary

This chapter demonstrates that confocal imaging can be used to accurately determine the volume and DNA content of isolated live hepatocytes. The volume of hepatocytes approximately doubles with doubling DNA content and the increase in volume is accompanied by a proportionate increase in ICAM-1 expression. Whether

the number of receptors is biologically relevant and whether all the hepatocyte populations respond equally to the signals from such receptors is unknown. IFNγ-induced apoptosis in all hepatocytes in culture equally although IFNγR was not detectable on isolated cells. Further analysis of receptor expression and susceptibility of polyploid cells to apoptosis are required to help determine the function of polyploid hepatocytes.

5 Comparison of Gene Expression in 2n, 4n and 8n Mouse Hepatocytes

5.1 Objectives

Analysis of gene expression in cells of different ploidy is one way in which more information regarding the control and function of polyploidisation in the liver could be obtained. Given that there is so little information regarding this process in the liver, the ability to screen thousands of genes using microarray technology, within cells differing only in DNA content, would provide a vast amount of information that has not been available before. Such studies have so far only been carried out in yeast. Galitski et al., 1999, compared haploid to tetraploid strains of *Saccharomyces cerevisiae* using microarray technology and found 10 genes that were induced and 7 genes that were repressed. The ability to carry out similar studies in hepatocytes is dependent on a method that separates pure populations of 2n, 4n and 8n cells.

Hepatocytes separated on the basis of size and density using techniques such as centrifugal elutriation (Le Rumeur et al., 1983; Overturf et al., 1999; Watkins III et al., 1992), Percoll gradients (Osypiw et al., 1994; Rajvanshi et al., 1998) and velocity sedimentation (Miller and Phillips, 1969; Sweeney et al., 1978a; Tulp et al., 1976) contain mixed populations of 2n, 4n and 8n cells. Separation of hepatocytes differing only in DNA is only possible by FACS using DNA dyes such as Hoechst 33342. This technique has been used for the separation of various cell types including mouse and rat hepatocytes (Davies et al., 1990; Lydon et al., 1980; VanZandt and Fry, 1983; Weglarz et al., 2000). However, the viability of mouse hepatocytes post-sort was found to be relatively low (Weglarz et al., 2000). The aim of this part of the study was to develop an improved method for separating highly pure populations of live mouse hepatocytes. So far, gene expression analysis of sorted cells differing only in DNA content has not been done in mammalian cells. Therefore, a further aim of this section was to determine if the RNA from sorted

hepatocytes was suitable for gene expression analysis and to carry out initial experiments to compare gene expression using microarray technology in hepatocytes separated on the basis of DNA content by FACS.

5.2 Results

5.2.1 Separation of Hepatocytes

5.2.1.1 Cell sorting

Sorting was carried out using the conditions that achieved the highest percentage purity and viability. Figure 15 shows a typical analysis of mouse hepatocytes stained with Hoechst 33342. Distinct populations of 2n, 4n and 8n cells can be seen. The majority of cells are 4n (73%) and the rest are 2n (7%) and 8n (18%). PI positive (dead cells) made up 30-33% of the total population and were removed from the analysis.

5.2.1.1.1 Purity and viability of sorted hepatocytes

The purity of each sorted population was determined by flow cytometry of PI-stained cells. Purity and viability of sorted cells were affected mainly by doublet gating, addition of DNase type I and the addition of PI. These parameters affected the sorts in different ways. Doublet gating was necessary to gate out clumps, which affected purity greatly. DNase type I was initially added to prevent or reduce cell clumping by reducing the amount of free DNA released from dead cells and thereby increasing the yield of cells post-sort. However together with PI, DNase I also increased the purity of the sorted samples. The purity of all populations increased significantly with the addition of DNase I and PI ($p < 0.05$); the 2n population increased from 59 to 93%, the 4n population increased from 72 to 94% and the 8n population increased from 55 to 82% (Table 7). Figure 16 shows typical flow histograms of pure populations of hepatocytes sorted using PI and DNase I.

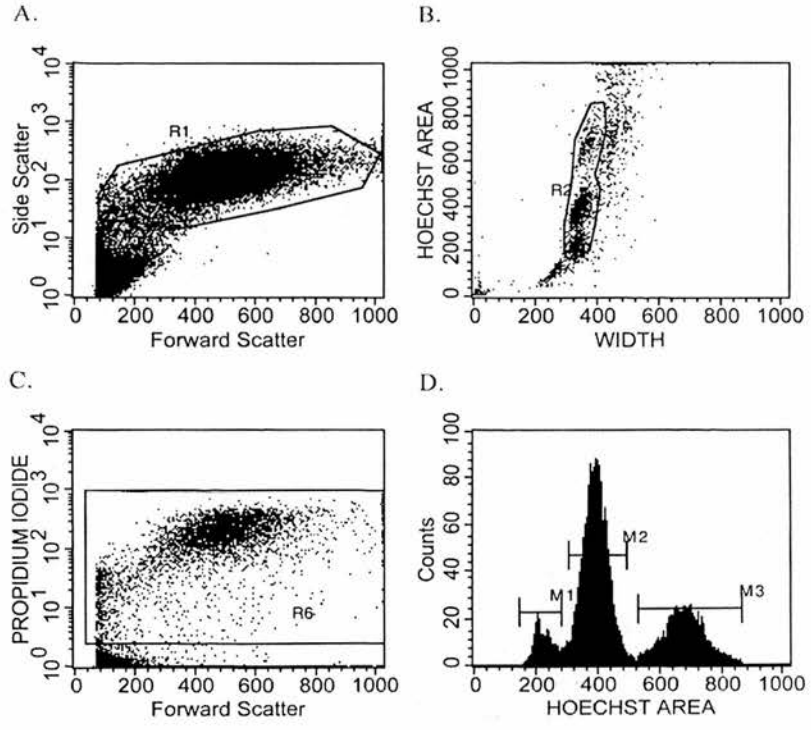


Figure 15. Histograms used for sorting 2n, 4n and 8n hepatocytes. A. Histogram of forward scatter (size) v side scatter (granularity). B. Doublet gating, R3 contains single cells only. C. Histogram of forward scatter v FL2 (PI staining), R6 contains PI positive cells. D. Histogram of Hoechst area (DNA content) v counts. M1, M2 and M3 are the sort gates for the 2n, 4n and 8n populations, respectively.

Table 7. Purity of sorted samples

Doublet Gating	Addition of DNase I	Addition of PI	Av. % Purity of Cells		
			2n	4n	8n
Yes	No	No	59 ± 13.0	72 ± 8.2	55 ± 23.8
Yes	Yes	No	86 ± 0.6	76 ± 1.0	76 ± 5.1
Yes	Yes	Yes	93 ± 2.8*	94 ± 2.6*	82 ± 7.1*

Values represent mean ± SD. *Significantly different to the purity of cells before the addition of DNase I and PI, $p < 0.05$.

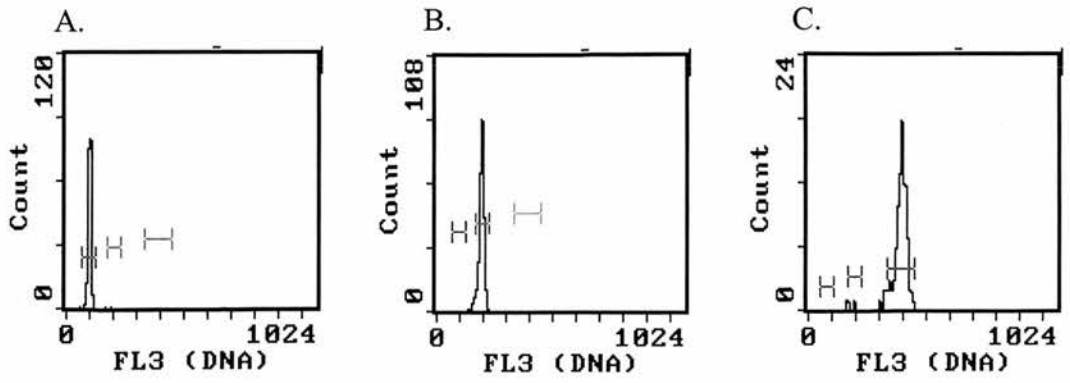


Figure 16. Purity of sorted cells assessed by PI staining and flow cytometry. Histograms of FL3 (PI positivity, DNA content) v counts, A. 2n, B. 4n and C. 8n.

Assessment of cell viability post sort using a trypan blue exclusion assay demonstrated that addition of DNase I as well as doublet gating increased the proportion of viable cells. Viability increased from an average of 35 % to 84 %. The addition of DNase I increased the side scatter of the dead cells (Figure 17).

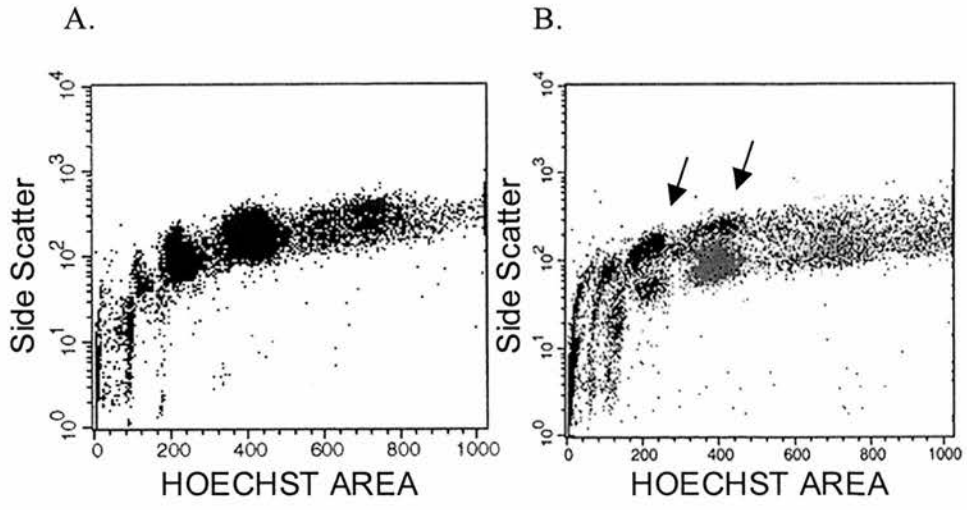


Figure 17. Increase in side scatter of dead cells with the addition of DNase I. A. Before addition of DNase I. B. With the addition of DNase I. Arrows indicate dead cells, which have a higher side scatter.

5.2.1.1.2 *Proportion of mononuclear and binuclear cells in sorted populations*

Cytospins were used to determine the proportions of mononuclear and binuclear cells within the sorted populations. The purity of the 2n population was >92%. The 4n and 8n populations were made up predominantly of binuclear cells (70 – 80%) and 20 – 30% mononuclear cells (Figure 18).

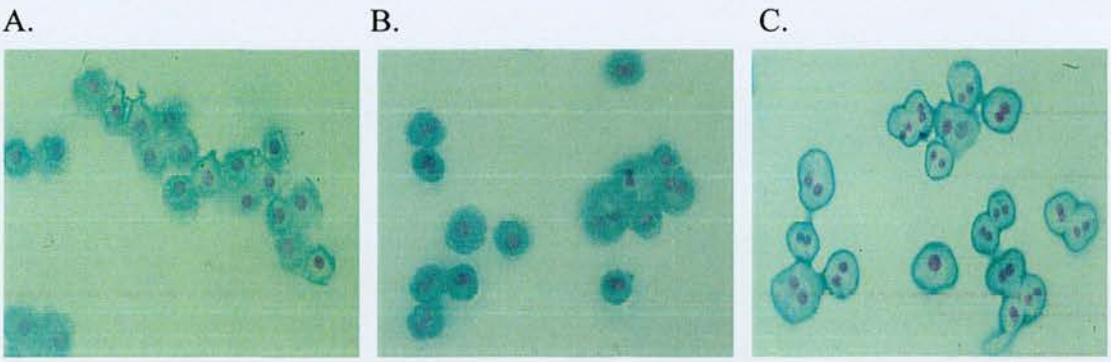


Figure 18. Cytospins of 2n, 4n and 8n cells sorted using PI and DNase I. A. 2n mononuclear cells, B. 4n mononuclear and binuclear cells, C. 8n mononuclear and binuclear cells.

5.2.1.1.3 *Growth capacity and proliferation of sorted cells*

In addition to the trypan blue exclusion assay, viability and growth potential of sorted cells were studied in culture. The ability of cells to attach and spread to the collagen matrix and their ability to progress through S-Phase was examined. After 24 and 48 hr in culture, dead cells rounded up and detached from the surface and the live cells attached and spread. At 48 h, 51% of 2n cells were BrdU positive whereas the 4n population was negative at both time points (Figure 19). Time-lapse experiments showed that sorted cells were capable of cell division (Appendix 5).

A. B.

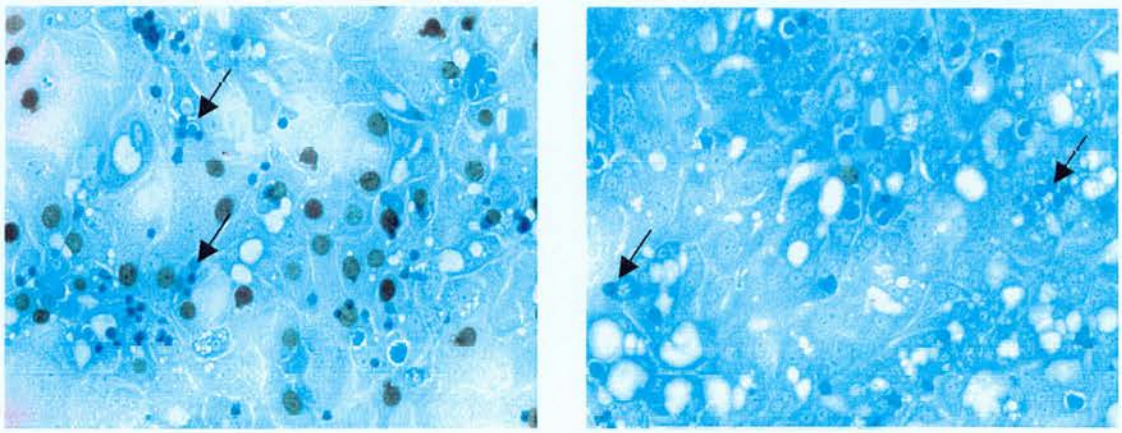


Figure 19. BrdU incorporation of 2n and 4n cells after 48 h in culture. A. 2n and B. 4n. BrdU positive cells stain brown. Arrows indicate dead cells that have rounded up and detached from the culture substratum.

5.2.1.2 Percoll gradients

Percoll gradients were used to determine if mononuclear and binuclear hepatocytes, which could not be separated using FACS, could be separated on the basis of cell density. Percoll gradients resulted in the separation of 3-4 distinct bands of cells. Cytospins of cells from each fraction did not show any enrichment of mononuclear or binuclear cells. However, flow cytometry showed that there was an enrichment of diploid and polyploid cells in different fractions. The lower fractions (H3a and H4) were enriched in polyploid cells (H3a: 4n, 61.8% and 8n, 1.4%; H4: 4n, 53.6% and 8n, 7.3%) compared to the lower fractions (H1a and b) (H1a: 4n, 37.9%, 8n, 0.7%; H1b: 4n, 47.3%, 8n, 1.5%, respectively). These fractions also showed a decrease in the proportion of 2n cells (H3a: 32.1%; H4: 24.2% compared to H1a: 57.3%; H1b: 40.9%, respectively) (Table 8). Similar trends were seen comparing the separated fractions with the cells before separation. Altering the concentration of Percoll did not increase the purity of cells within each fraction with respect to DNA content.

Table 8. DNA content ofcells separated by Percoll gradients.

Mouse / Age	Fraction	% Cells		
		2n	4n	8n
C3H /	Before separation	41.6	45.0	1.0
1.5 months old	H1a	57.3	37.9	0.7
	H2a	38.0	51.6	1.8
	H3a	32.1	61.8	1.4
C3H /	Before separation	19.0	66.6	2.1
2.5 months old	H1b	40.9	47.3	1.5
	H2b	34.3	51.0	3.0
	H3b	37.0	46.2	3.9
	H4	24.2	53.6	7.3

The purity of the separated fractions using Percoll gradients was not suitable for further gene expression analysis and therefore only cells separated by FACS were used.

5.2.2 Analysis of RNA quality

5.2.2.1 Sorting using Hoechst 33342 does not affect RNA quality

The RNA from sorted cells stained with Hoechst 33342 was of good quality and two clear ribosomal bands are visible when the RNA was electrophoresed on 1 % agarose gels (Figure 20).

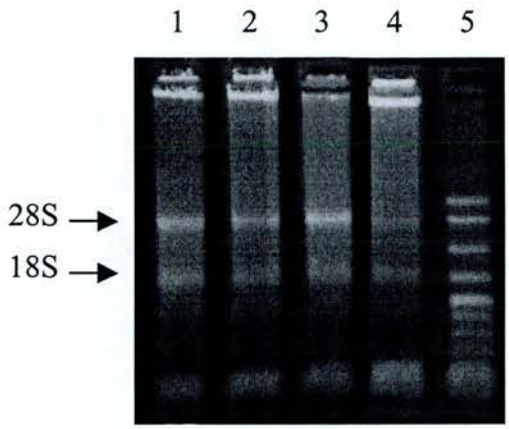


Figure 20. Quality of RNA extracted from sorted hepatocytes. RNA was extracted using phenol/chloroform and was electrophoresed on a 1 % agarose gel. Lane 1. 2n, 2. 4n, 3. 8n, 4. Unsorted cells and 5. Molecular weight marker (Cambio). Arrows indicate the 28S and 18S ribosomal bands.

Reverse transcription PCR using β-actin primers was not affected by staining with Hoechst 33342, sorting or fixation as clear bands are visible. However, PI staining affected the reaction resulting in no product (Figure 21).

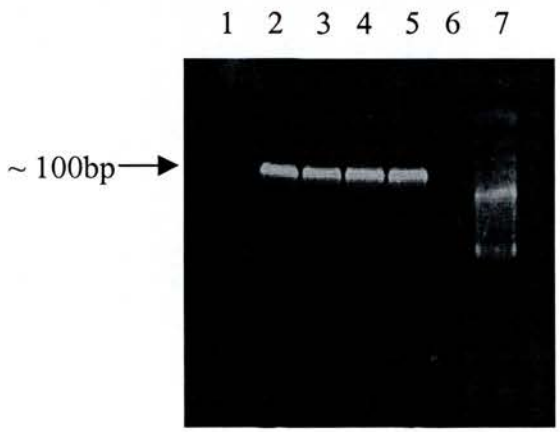


Figure 21. The effect of fixing, sorting or staining with PI or Hoechst 33342 on β-actin reverse transcription PCR. Lane 1. PI staining, 2. Fixed in ethanol, 3. Unstained, unfixed, 4. Hoechst 33342 staining, 5. Sorted cells, 6. Negative control and 7. Molecular weight marker (25 bp ladder; Life Sciences).

5.2.3 Gene Expression Analysis

5.2.3.1 The amount of total RNA from sorted cells increases with ploidy

For analysis of gene expression, it is important to know if the amount of RNA obtained from hepatocytes increases in proportion to DNA content. The results indicate that the total amount of RNA does increase with increasing DNA content and the amount obtained from 8n cells (26.18 μg/1 x 10^6 cells) is approximately 4 times that from 2n cells (6.5 μg/1 x 10^6 cells) (Figure 22, $p < 0.05$).

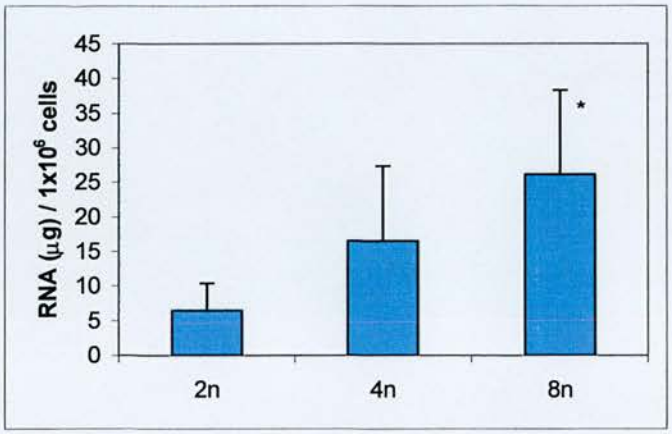


Figure 22. Amount of RNA extracted from sorted hepatocytes. RNA was extracted using a phenol/chloroform extraction protocol and quantified by the absorbance reading at 260 nm on a spectrophotometer. *Significantly greater than 2n, $p < 0.05$.

5.2.3.2 Microarray analysis

Microarray analysis was carried out to screen the expression of over 8500 genes within the sorted 2n, 4n and 8n populations and within hepatocytes from old and

young mice. Figure 23 shows a representative area from a 2n, 4n and 8n array. There are both induced and repressed genes in the 8n sample.

Due to the vast amount of information obtained from microarray analysis, it is necessary to set criteria that will separate the many thousands of genes analysed from the genes of interest. In this case, the genes of interest were those that were induced or repressed more than 2-fold and could therefore not be accounted for by an increase in DNA content. Table 9 shows which genes were induced or repressed more than 2-fold in cells of higher ploidy compared with cells of lower ploidy (details of each comparison are given in Appendix 4). Esterase 31, MUP2 and IFNγR were induced whereas the ESTs similar to PCD and KIAA1265 protein were repressed in the majority of comparisons but induced in 1.

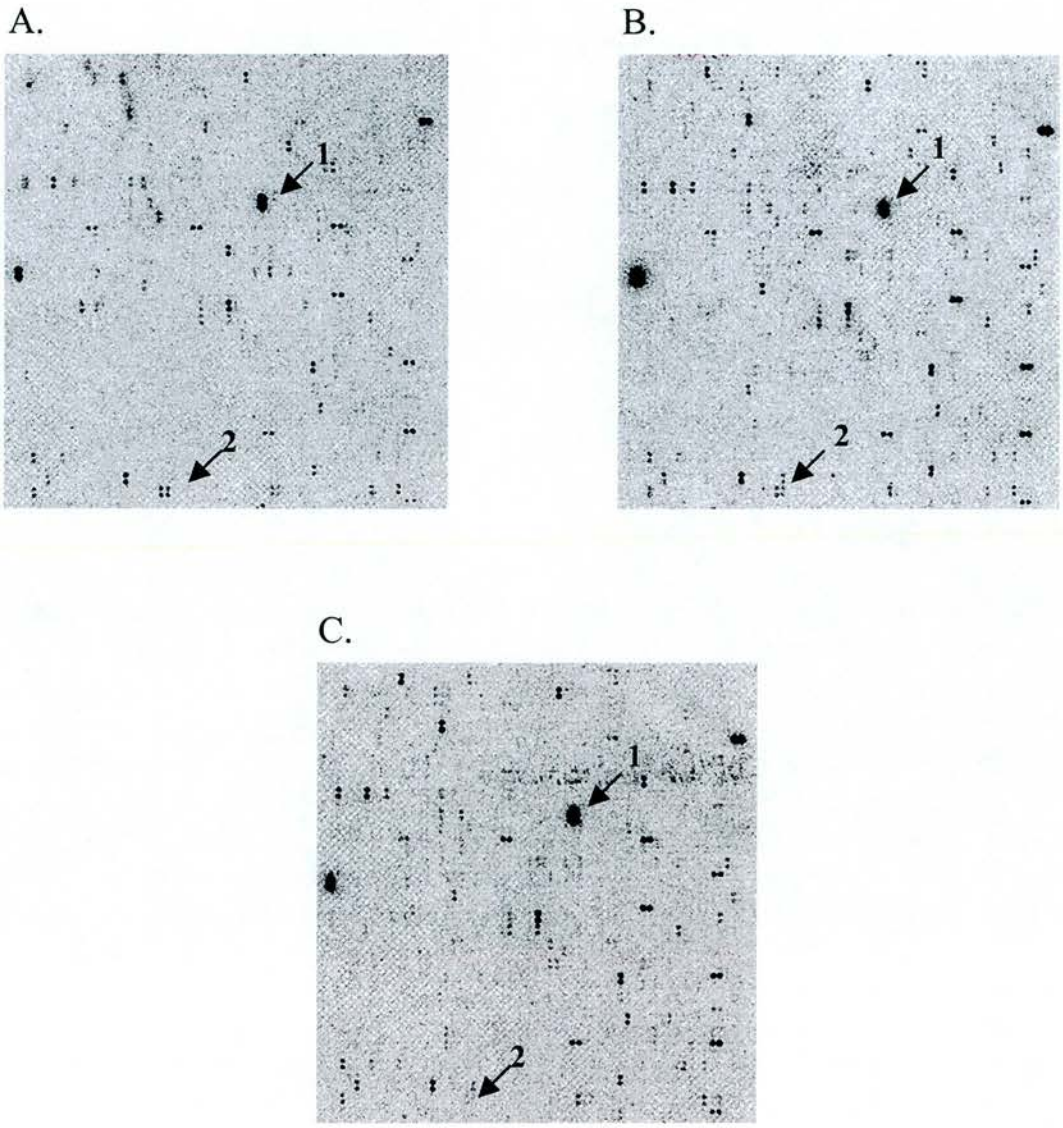


Figure 23. Images of representative areas of nylon microarrays from A. 2n, B. 4n and C. 8n sorted hepatocytes. Highly expressed genes stain darker due to a greater incorporation of P^{33}. Arrow 1 represents the MUP gene, which is more highly expressed in 4n and 8n cells and 2, represents two genes that are repressed in the hepatocytes with greater DNA content. Each gene is spotted in duplicate.

Table 9. The expression of various genes in cells with a higher DNA content.

EMBL Acc. No.	Gene	Comparison (See Appendix 4)/ Fold Induction or Repression							No. of Observ-ations
		1	2	3	4	5	6	7	
AA404079	ESTs		4.1	5.0					2
W83771	ESTs		4.1	4.4					2
AA240258			3.6	4.4					2
AA254921	Esterase 31	5.2	3.3	3.3		2.1			4
AA285921	MUP2	11.6	3.2	5.2		4.1	5.4	5.3	6
AA472426			3.2	5.1					2
AA073514	ZBP1		3.1	3.1					2
AA062198	α1μ-globulin/bikunin	5.5	2.7						2
AA444488	PLA_2	5.6	2.4	4.1					3
AA541842	IFNγR	3.2	2.4	4.0					3
W14332	ESTs highly sim PCD	3.4	3.3	4.0		3.4		3.3	4/1
AA000655	ESTs				2.3			4.4	2
W18585	ESTs, KIAA1265	5.9	5.9	3.8		3.8		5.2	4/1
AA270506	Torsin Family 2, A		3.6	3.3					2
W14540	Histocomp. 2, K		3.3	3.4					2
AA124868	Plasminogen Act.Inh. II		2.5	5.0					2
AA097825			3.0						1
AA273761	DNA Segment, Chr.10		2.9	2.8					2
AA144537	ESTs, Golgi App.		2.7						1

This table illustrates genes that were induced (in blue) and repressed (in red) more that 2-fold in cells of higher DNA content using microarray analysis. Sorted cells with the same DNA content were pooled together (Appendix 3) and cDNA probes prepared using RT-PCR, incorporating p^{33} were hybridised to known sequences on nylon membranes. The expression of each gene in the sorted samples was detected and measured using a phosphoimager and appropriate macros in Microsoft Excel. ESTs = Expressed Sequence Tags, MUP2 = Major Urinary Protein 2, PCD = Pterin 4α-carbinolamine dehydratase, ZBP1 = Zipcode Binding Protein 1, Histocomp. 2,K = Histocompatibility 2, K region, Plasminogen Act. Inh. II = Plasminogen activator inhibitor, type II.

5.2.3.3 Reverse-transcription and real-time PCR

MUP2, IFNγR and the EST similar to PCD, which were either induced or repressed in the microarray analysis were chosen for confirmation by real-time PCR analysis. Pten was also chosen due to its role in the regulation of cell size (Huang et al., 1999; Goberdhan et al., 1999). The primer sets for each gene were first checked by reverse transcription PCR on RNA extracted from whole liver to ensure there were no primer dimers and the PCR products were the correct size. The primers for the IFNγR did not work in any of the conditions that were tried. There were virtually no primer dimers and all bands corresponded to products of the correct size (Figure 24).

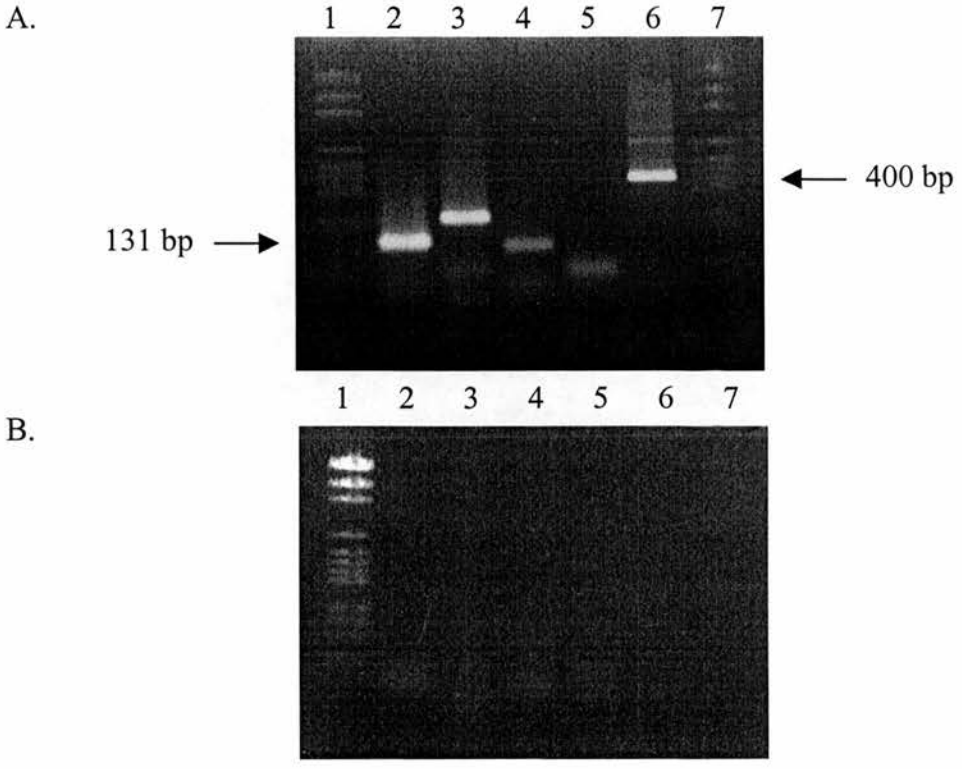


Figure 24. Reverse transcription PCR of primers chosen for real-time PCR analysis. PCR products were electrophoresed on a 2% agarose gel. A. Lane 1. pGEM molecular weight marker, 2. MUP (131bp), 3. PCD (216bp), 4. Pten (115bp), 5. IFNγR (207bp), 6. β-Actin (~400bp) and 7. pGEM molecular weight marker. B. Corresponding controls without the addition of cDNA, run below each sample on the same gel.

Real-time PCR confirmed the expression of MUP2, PCD and Pten in samples extracted from 2n, 4n, 8n sorted cells and in samples from old and young mice; however, the results were so variable, it was not possible to determine if there were any differences between samples.

5.3 Discussion

5.3.1 The separation of hepatocytes differing in DNA content

Sorting using Hoechst 33342 allows the separation of cells differing only in DNA content. This technique has been successfully used in the separation of many cell types including bone marrow (Vanzandt and Fry, 1983), cell lines (Fried et al., 1982), lymphocytes (Loken, 1980), mouse hepatocytes (Weglarz et al., 2000) and rat hepatocytes (Davies et al., 1990). Hoechst 33342 is a DNA-specific dye that binds reversibly to adenine: thymidine rich regions of the DNA (Durand and Olive, 1982). Unlike the nucleic acid dye, PI, Hoechst 33342 did not affect the quality of RNA from sorted cells in these studies. The addition of PI (2 μg/ml) and DNase type 1 (0.1 mg/ml) greatly increased the purity and viability of the sorted populations. The viability was greater than 80% for 4n and 8n populations. This value is higher than the viability of cells presented in the study by Weglarz et al., 2000, who did not use DNase 1. They found that viability was between 50 and 74 %. The addition of DNase 1 increased the side scatter of the dead cells and possibly increased the proportion of dead cells gated out of the analysis.

Although Hoechst 33342 binds quantitatively to DNA, the staining of 2n, 4n and 8n hepatocytes was not linear. On the plot of Hoechst area, the 2n and 4n peaks fall at approximately 200 and 400 respectively, whereas the 8n peaks falls below 800. There are several reasons why this could be the case. Firstly, in the analysis of different types of diploid cells e.g. granulocytes, lymphocytes and macrophages, the position of the 2n peak varies when they are stained with either Hoechst 33342 or PI and this is likely to be due to the different conformation of the DNA within the

nucleus. Therefore, there is a possibility that the conformation of DNA varies in hepatocytes of different ploidy. Secondly, Hoechst 33342 is a live cell stain and is continually being pumped out of the cell. The rate that the dye is pumped out could vary between cell types and affect where the peak will fall.

Other techniques such as centrifugal elutriation (Le Rumeur et al., 1983; Overturf et al., 1999; Watkins III et al., 1992), velocity sedimentation (Miller and Phillips, 1969; Sweeney et al., 1978a; Tulp et al., 1976) and Percoll gradients (Osypiw et al., 1994; Rajvanshi et al., 1995; Rajvanshi et al., 1998) have also been used to separate different populations of hepatocytes. This study has shown that separation of hepatocytes by Percoll gradients enriches the 2n or the polyploid populations; however, separated fractions always contained mixed populations of 2n, 4n and 8n cells and cells of different nuclearity. This was expected, as there is no means of separating cell aggregates. A study by Tulp et al., 1976, used velocity sedimentation to separate mouse and rat hepatocytes on the basis of ploidy. However, they had limited success separating whole cells and the technique was found to be more useful for the separation of isolated nuclei. Techniques used in the separation of cells of different size or density do, however, have other advantages and they have been used to study the function of cells separated from different zones of the liver (Bengtsson et al., 1981; Osypiw et al., 1994; Rajvanshi et al., 1998; Sancho-Tello et al., 1987; Vargas et al., 1987).

5.3.2 Separation of mononuclear and binuclear cells

In adult mice, the majority of hepatocytes are binuclear. Using FACS, mononuclear and binuclear cells containing the same amount of DNA are sorted together. As there may be differences in gene expression between these cells, it was desirable to separate these cells. However, as I have mentioned above, Percoll gradients did not enrich mononuclear or binuclear cell populations. A combination of centrifugal elutriation and flow sorting has been used to enrich populations of mononuclear and binuclear rat hepatocytes (Davies et al., 1990). The purity of the binuclear populations separated in this way was 81%. This was not much greater than the

proportions of binuclear cells obtained from sorting alone in this study. Sorting of mouse hepatocytes resulted in the separation of a predominantly mononuclear 2n population (> 92%) and binuclear 4n and 8n populations consisting of between 70 and 80% binuclear cells. For the separation of mouse cells, it is preferable to use just one technique, as fewer numbers of cells are obtained from a mouse liver compared with a rat liver and one technique will limit the amount of cell loss. Although it has not been possible to separate mononuclear and binuclear hepatocytes of the same ploidy class, information obtained from the comparison of mononuclear 2n cells with predominantly binuclear 4n and 8n cells and the comparison of 2n, 4n and 8n cells themselves will be useful.

5.3.3 Sorted cells were able to grow and proliferate in culture

The growth potential of hepatocytes post-sort was not extensively studied, as the majority of cells were required for gene expression studies. However, initial experiments indicated that 2n and 4n cells were able to attach and spread onto collagen-coated slides. After 48 h, the approximately 50% of 2n cells incorporated BrdU, whereas the 4n cells were negative at 24h and 48 h. BrdU labelling index has been found to be inversely correlated with ploidy (Mossin et al., 1994), and it was therefore expected that the 4n cells would not proliferate at the same time. Although there were too few cells to study later time-points, there is a possibility that 4n hepatocytes would have proliferated after 48 h. As well as incorporating BrdU, the sorted hepatocytes were also able to divide as demonstrated by time-lapse experiments. Hoechst 33342 is a relatively non-toxic and non-mutagenic dye but has been shown to be inhibitory to the cell cycle in some cell types (Arndt-Jovin and Jovin, 1977; Durand and Olive, 1982; Fried et al., 1982; Lydon et al., 1980). Although not studied in this chapter, cell death was noticed in culture by the presence of small, rounded, darker staining cells that detached from the slide. This could be related to effects of the dye or the process of sorting itself. There is also a possibility that there is some deleterious effect on cells from the laser during sorting. More extensive studies on the proliferation of sorted cells over longer periods in

culture as well as assays to quantify cell death would be useful to properly assess the effects of sorting on hepatocytes.

5.3.4 RNA content increases with DNA content and could affect gene expression analysis

The amount of RNA from 8n cells was approximately 4 times that of a 2n cell. Although not significant, the 4n cells contained more RNA than 2n cells and less than 8n cells. The large standard deviation is likely to be due to experimental error. In order to avoid protein contamination after the phenol/chloroform extraction, not all of the RNA can be removed. There are also two RNA precipitation steps in the protocol that increase the chances of losing some of the extracted RNA. Combining the RNA from different sorts also introduces some degree of error, as the amount of RNA extracted will vary each time. In this study, microarray analysis was carried out by comparing an equal amount of RNA from each sample (where possible 15 μg) and an equal amount of cDNA probe. As the amount of RNA from cells was found to increase with increasing DNA content, any changes in gene expression are likely to be much greater when the total amount of RNA in a cell is taken into account. For example, a gene which is induced 3-fold in a 4n cell compared with a 2n cell could express 6 times the amount of that gene if the actual RNA content of the 4n cell was 2-fold greater. Interestingly, a study by Collins, 1978, demonstrated that the rate of RNA synthesis in 2n, 4n and 8n rat hepatocytes was similar and that the rate of RNA synthesis in hepatocytes declines with age. The fact that polyploid cells contain more RNA but do not have increased rates of RNA synthesis could be in some way related to the function of hepatocytes. The increase in RNA in polyploid cells may allow these cells to produce large amounts of protein without expending large amounts of energy. RNA extracted from sorted hepatocytes was not affected by sorting and was suitable for gene expression analysis determined by reverse-transcription PCR.

5.3.5 Gene expression analysis

Due to the lack of information regarding the genes involved in hepatic polyploidisation, the best way of obtaining a lot of information quickly is by screening thousands of genes using microarray technology. Nylon microarrays combined with ^{33}P-labeled radioactive probes are sensitive enough to allow small samples of RNA to be analysed and are therefore suitable for the analysis of flow sorted cells (Bertucci et al., 1999). Comparison of the sorted 2n, 4n and 8n hepatocytes using this technology will provide valuable information about the genes involved in the function and maintenance of polyploidisation in the liver and is the first description of this type of analysis in mammalian cells.

Several studies have demonstrated that various genes and proteins are expressed in proportion to DNA content, including albumin (Le Rumeur et al., 1981), the leucine zipper protein, DBP (Schmidt and Schibler, 1995), succinate dehydrogenase and NADPH cytochrome C (Tulp et al., 1976). These studies have also found genes that are unchanged by increasing ploidy such as the ubiquitous transcription factors, OCT1 and NF-Y (Schmidt and Schibler, 1995) or altered in a way that can not be explained by DNA content such as lactate dehydrogenase, of which the activity per genome was much lower in 2n cells compared with polyploid cells (Tulp et al., 1976). This study was interested in genes that did not solely change relative to DNA content, therefore a cut-off greater than 2-fold was used.

The majority of genes studied either did not vary with increasing ploidy or change relative to DNA content. Several genes were induced or repressed in comparisons of 2n with 4n and 8n cells, unsorted with 8n cells and hepatocytes from young mice compared with those from old mice. The comparison of unsorted with 8n cells and the comparisons of young and old mice were included as controls. If cell sorting had affected gene expression in any way, different gene expression patterns would have been expected in the control samples. However, this did not appear to be the case. The proportion of polyploid cells is greater in older mice. Therefore, the pattern of gene expression in the comparison of young and old mice was also expected to be

similar to comparisons of 2n with polyploid cells. Indeed, several similar genes were detected in these comparisons. Comparing hepatocytes that differ only in DNA content is complicated by the presence of a large proportion of binuclear cells within the 4n and 8n populations. As I have mentioned previously, it is not possible to separate mononuclear and binuclear cells with the same DNA content. However, it was expected that genes important in binucleation as opposed to those involved in the production of mononuclear polyploid cells would also be detected in the comparisons of 2n with 4n and 8n cells, as 2n cells are mononuclear and 4n and 8n cells are predominantly binuclear.

5.3.5.1 Identification of genes involved in the function or maintenance of polyploidy

Three of the genes that appeared on several of the comparisons were of particular interest. The first of these was IFNγR, which was induced in polyploid cells. One of the aims in a previous chapter of this thesis was to determine whether the increase in size of polyploid cells could affect the expression of ICAM-1 or IFNγR. Using flow cytometry it was demonstrated that the fluorescent intensity of ICAM-1 increased in proportion to DNA content. However, IFNγR was not detectable using this technique and it was suggested that IFNγR was mainly intracellular. Microarray analysis suggests polyploid hepatocytes express more IFNγR. However, it is not possible to determine whether polyploid cells translate all IFNγR mRNA into protein or whether polyploid cells just store a larger amount of IFNγR mRNA.

The second gene of interest was the MUP2, also found to be induced in polyploid hepatocytes. The literature regarding the major urinary proteins reveals some striking similarities between MUPs and ploidy. MUP gene expression is first detected in the liver between 2 to 4 weeks of age (Held et al., 1989), at the same time as polyploid cells appear in the liver (Brodsky and Uryvaeva, 1977; Carriere, 1967; Nadal and Zajdela, 1966). MUPs, like ploidy are under complex hormonal control. The synthesis of MUPs is sex-dependent under the influence of androgens and other hormones such as growth hormone, thyroid hormone, the glucocorticoids and insulin

are required for the normal MUP synthesis (Knopf et al., 1983). As described in Chapter 1, the regulation of ploidy is also under the control of hormones with thyroid hormone playing a major role (Torres et al., 1999). The degree of polyploidisation varies between male and female mice with female mice showing higher levels of ploidy than male mice of the same age (Epstein, 1967; Steele et al., 1981a). Similarly, the liver of female mice contains different MUP products and different levels of MUPs to that of the male (Knopf et al., 1983).

MUP gene expression was found to be critical to normal liver development. Suppression of MUP mRNAs in the liver through the expression of a MUP/SV40 T antigen transgene in mice was shown to result in the development of liver hyperplasia and tumours. The livers of these transgenic mice enlarged by 4 weeks of age and the nuclei were found to be irregular in size and shape (Held et al., 1989).

Cavaggioni and Mucignat-Caretta, 2000, have recently provided a comprehensive review regarding the function, control and genetics of the MUPs. The multigene family consists of approximately 35 genes per haploid genome, which are clustered on chromosome 4 and encode small monomeric proteins with M_r of about 18000 (Cavaggioni and Mucignat-Caretta, 2000; McIntosh and Bishop, 1989). The group 1 genes are expressed highly in the liver with MUP mRNA constituting approximately 5% of the total (Shaw et al., 1983). So far, the only known function of MUPs seems to be in chemosensory signalling in rodents. MUPs are secreted in the urine and act as odorant carriers as well as proteins that prime endocrine reactions in females (Cavaggioni and Mucignat-Caretta, 2000). The importance of MUP gene expression in liver development and the striking similarities in hormonal control, expression of MUP and initiation of ploidy make the role of MUP gene expression in polyploidisation worthy of further study.

The third gene of interest was the EST similar to pterin-4α-carbinolamine dehydratase (PCD) that was mainly found to be repressed in polyploid hepatocytes. PCD is also known as phenylalanine hydroxylase-stimulating protein (PHS) and has been found to be identical in structure to a protein of the cell nucleus called

dimerization co-factor of hepatocyte nuclear factor 1 alpha, reported to be involved in transcription (Hauer et al., 1993; Mendel et al., 1991). Another function of PCD is in the cytosol where it is required for efficient tetrahydrobiopterin regeneration after phenylalanine hydroxylase activity (Thony et al., 1998). The fact that this gene seems to be repressed in cells of higher ploidy suggests that it could play some role in the polyploidisation in the liver. However, further studies will be necessary to determine if this is the case.

The level of induction or repression varied between comparisons. This is most likely to be due to inter-animal variation, spotting of the membranes, variability in probe preparation, background variation or the accuracy in the determination of the spot intensity. Therefore, this study did not utilise microarray technology for quantitative analysis but found it particularly useful for screening genes that may otherwise not be associated with ploidy. Quantitative analysis was attempted using real-time PCR. This technique was able to confirm the expression of MUP2, IFNγR and PCD. However, the variation between samples was too great to determine any differences between the 2n, 4n and 8n populations. Real-time PCR uses fluorescence to detect the changes in gene expression and is not as sensitive as radio-labelling used in the microarray analysis.

5.3.5.2 Genes involved in the control of polyploidisation

The only previous attempt to study gene expression in cells of different ploidy using microarray technology was done in yeast. Galitski et al., 1999, compared haploid and tetraploid strains of *S. cerevisiae* in exponential growth and found that 10 genes were induced and 7 genes were repressed greater than 10-fold in the cells of higher ploidy. One of the genes that was repressed, *CLN1*, was found be highly homologous to human cyclin A. Other studies in yeast, megakaryocytes and various cell lines have found that many cell cycle regulators such as cyclin A (Bortner and Rosenberg, 1995; Datta et al., 1998), p21 (Datta et al., 1998; Kikuchi et al., 1997; Sheikh et al., 1995) and genes of the Aurora family (Kawasaki et al., 2001; Schumacher et al., 1998) are involved in the control of polyploidisation. This chapter has focused on the comparison of gene expression within static populations

of hepatocytes and has not looked at the expression of hepatocytes in exponential growth. Therefore genes involved in the cell cycle and others involved in the control of polyploidisation were not expected to vary in the microarray analysis. Studies into the control of polyploidisation in hepatocytes would be of great interest. However, this is much more difficult to do in hepatocytes compared with yeast. There is a possibility that 2n, 4n and 8n hepatocytes could be sorted after the induction of polyploidisation in mice by mitogenic drugs such as PB or peroxisome proliferators. However, the interpretation of the results would be complicated, as there is a possibility that drugs will induce polyploidisation through different pathways or induce the expression of genes not usually associated with normal polyploidisation or proliferation. A study by Chevalier et al., 2000, demonstrated that the protein expression profiles induced by the peroxisome proliferator, nafenopin, were different to those induced by the mitogen, EGF. Another option that would allow the analysis of hepatocytes in exponential growth would be to grow sorted 2n, 4n and 8n cells in culture and extract the RNA when each population is actively proliferating. Further characterisation of the effect of sorting and Hoechst 33342 on hepatocytes would be necessary to determine whether this approach would be suitable.

5.3.5.3 Gene expression in cells of different ploidy and cells in different zonal locations in the liver

This PhD has not studied the ploidy of hepatocytes in situ. However, this area would be interesting to pursue in further studies as the relationship between gene expression within cells of different ploidy and cells in different zones of the liver is unclear. As mentioned earlier, the cell size and degree of ploidy varies in different regions of the liver, with the perivenous cells exhibiting greater polyploidy and the periportal cells less ploidy (Rajvanshi et al., 1998; Schmucker, 1990). There are a number of genes that vary in expression between the different regions of the liver and relate to the different functions of these two zones. The cells within the periportal region (zone I) are involved in gluconeogenesis, ureagenesis and amino acid uptake and degradation, whereas the cells in the perivenous area (zone III) are involved in glycolysis and glutamine synthesis (Feldmann, 1992; Jungermann and Kietzmann, 1996; Sigal et

al., 1992). Perivenous cells express glutamine synthetase, GLUT-1, alcohol dehydrogenase, cytochrome P450, whereas serum albumin, ceruloplasmin and the glucose transporter, GLUT-2 are expressed in hepatocytes throughout the liver lobule (Bilir et al., 1993; Ingelman-Sundberg et al., 1988; Osypiw et al., 1994; Rajvanshi et al., 1998; Yamauchi et al., 1988). As this study has demonstrated, expression of genes such as IFNγR, MUP2 and PCD vary within hepatocytes of different ploidy. Whether the expression of these genes is related to the function of cells within the different zones or whether they are involved more specifically in the function of polyploid cells is unknown. Analysis of 2n, 4n and 8n hepatocytes from different zones of the liver may help determine if this is the case.

This study has demonstrated that microarray technology is a useful method for screening gene expression in sorted 2n, 4n and 8n hepatocytes. The genes induced or repressed more than 2-fold including IFNγR, MUP2 and PCD may play a role in the function or maintenance of polyploidisation. However, further confirmatory studies such as biological assays would be necessary to determine if this is the case. In time, this technique may also be useful in determining which genes are involved in the control of polyploidisation in the liver by studying sorted hepatocytes in exponential growth.

6 General Discussion

As well as having developed novel techniques, this thesis describes some important findings regarding polyploidisation in the rodent liver. One area of particular interest is the significance of polyploidisation in the liver, especially as contradictory hypotheses exist. A review by Gupta et al., 2000, concludes that polyploidisation is an advancement towards terminal differentiation and senescence, with increasing risk of apoptosis, organ failure and hepatocarcinogenesis. In contrast, previous studies suggest that polyploidisation is in fact a protective response (Brodsky and Uryvaeva, 1977; Cascales et al., 1994; Schwarze et al., 1984; Uryvaeva, 1981). Another area of importance is the control of polyploidisation and many studies have been carried out in various cell types to determine what genetic mechanisms are involved. This chapter discusses the results of this thesis with particular regard to the significance and control of polyploidisation.

6.1 The Significance of Polyploidisation

6.1.1 A protective mechanism?

This study found that administration of PB for 21 days increased the proportion and proliferation of 8n nuclei without affecting the proportion of binuclear cells. The increase in ploidy associated with the administration of PB or cocaine has been suggested to enable hepatocytes to carry out their function whilst coping with the intensive stress induced by the drugs (Bohm and Noltemeyer, 1981; Cascales et al., 1994). Other studies suggest that by avoiding mitosis, polyploid cells limit the development of chromosomal abnormalities, which could lead to transformation and cell death (Medvedev, 1986; Uryvaeva, 1981). Whether these suggestions can explain why there is an increase in 8n nuclei after administration of PB is not known. The degree of ploidy has been shown to return to normal after the removal of PB (Bohm and Noltemeyer, 1981). This may indicate that drug-induced

polyploidisation could be a method of coping with drug-induced stress. Administration of PB was not associated with any histological abnormalities and, over this relatively short period, did not induce any signs of disease or apoptosis. Therefore, the induction of polyploidisation did not appear to be deleterious to the cell. Interestingly, the development of carcinogenesis after the administration of many drugs has been associated with long-term rather than short-term administration (Melnick, 1992). Therefore, before any conclusions can be drawn, it would be useful to study the long-term effects of PB on ploidy and disease.

6.1.2 Susceptibility to apoptosis?

There are several conflicting findings regarding the susceptibility of polyploid hepatocytes to apoptosis. Apoptosis occurs in association with increasing polyploidy in rats pre-treated with the cell cycle inhibitor, retrorsine and given T_3 (Oren et al., 1999). The formation of 'giant' polyploid cells in the livers of LEC rats associated with increasing copper accumulation also results in cell death through apoptosis (Yamada et al., 1998). Disruption of cell cycle checkpoints through the deletion, mutation or upregulation of specific genes also results in polyploidy and subsequent apoptosis. Examples include the downregulation of the zinc finger protein, KRC (Allen and Wu, 2000) and transfection of $p21^{Waf1}$ (Sheikh et al., 1995). The livers of Trident (the winged helix transcription factor) and ERCC-1 (involved in NER) knock-out mice show extensive polyploidy with reduced binucleation and the mice die before weaning due to liver failure (Korver et al., 1998; McWhir et al., 1993; Nunez et al., 2000; Weeda et al., 1997). Examination of the polyploid cells in each of the above cases revealed that the nuclei, in the main, were grossly abnormal. These studies certainly provide evidence to link increasing polyploidy with apoptosis. However, can the development of 'giant' or abnormal polyploid cells be related to polyploidy which occurs in normal development or polyploidy induced by the short-term administration of drugs such as PB? Melchiorri et al., 1993, found that polyploid cells induced by administration of Lead nitrate were not preferentially eliminated. Similarly, results form this thesis demonstrated that cultured polyploid cells were equally susceptible to apoptosis induced by IFNγ. As mentioned above,

polyploid cells induced by PB showed no histological abnormalities. These findings may therefore suggest that the susceptibility of polyploid cells to apoptosis may be associated with the degree of polyploidy or induction of 'abnormal' polyploid cells and may depend on whether or not the cell cycle is disrupted. Further studies are necessary to determine whether the abnormal polyploid cells are an advancement of normal polyploidy or whether they occur through different mechanisms.

The increase in polyploidy in the ERCC-1 deficient mice is said to contribute to the progression of severe aneuploidy by 3 weeks (McWhir et al., 1993). Obviously, increasing polyploidy and therefore aneuploidy in this case is likely to lead to an increased risk of hepatocarcinogenesis. Disruption of other cell cycle genes also results in aneuploidy. Mutations or deletions in genes involved in the mitotic spindle checkpoint such as *Bub1* (Bernard et al., 1998) and *Zw10* and *Rod* (Basto et al., 2000; Chan et al., 2000) result in aneuploidy through the misseggregation of chromosomes during anaphase. There is no information at present to indicate that polyploid cells occurring during normal development progress to aneuploidy. However, it would be interesting to determine whether 'normal' polyploid cells contain copies of all chromosomes.

6.1.3 Size, ploidy and receptor expression

Could cell size play a role in the function of polyploid hepatocytes? Large polyploid cells function as a protective barrier in the urinary bladder (Hicks, 1975) and the increasing size of megakaryocytes is important in the production of platelets (Baatout et al., 1998a). Cell size has also been shown to play important roles in various processes including proliferation and apoptosis (Lang et al., 1998). Unlike megakaryocytes and urinary epithelial cells, there is no obvious role of increasing cell size in hepatocellular function. However, differences could be subtle. Increases in volume has the potential to result in a dilution of genes and proteins within the cell and increasing surface area could alter the expression of molecules on the surface. Such changes could lead to alterations in signalling events. On isolated hepatocytes, ICAM-1 expression increased in proportion to cell size and ploidy. Whether or not

all receptors or surface molecules are altered by increasing size or ploidy and whether differences in receptor expression is functionally important remains to be seen.

6.2 The Control of Polyploidisation

Studies in yeast, *Drosophila*, *Xenopus*, *C.elegans* and megakaryocytes have revealed many genes that are important in maintaining ploidy (chapter 1). Overexpression, mutation, deletion or abnormal phosphorylation events of genes involved in the G1/S, G2/M and the mitotic spindle checkpoint can result in the formation of polyploid cells. The regulatory genes involved in polyploidisation in hepatocytes remain unknown. The role of the oncosuppressor gene, p53 in G1/S and G2/M checkpoints (Stewart et al., 1995) and in the mitotic spindle checkpoint (Ciciarello et al., 2001) suggests a possible role for this gene in the control of polyploidisation. The role of p53 in the control of polyploidisation in normal ageing hepatocytes is however contradictory (Bellamy et al., 1997; Yin et al., 1998). In agreement with the study by Bellamy et al., 1997, polyploidisation induced by PB occurred independently of p53. There is however, evidence that p53 plays a role in polyploidisation after mitotic spindle damage (Casenghi et al., 1999; Minn et al., 1996; Notterman et al., 1998), which may suggest that regulation of ploidy may occur through different mechanisms under different circumstances. The mechanisms controlling ploidy in normal ageing hepatocytes may therefore differ from those involved in polyploidisation after cellular or genetic damage.

A valuable technique in the study of genetic regulation in polyploid cells is microarray technology. Galitski et al., 1999, used this technique to determine which genes are involved in the polyploidisation of yeast. A similar study was carried out on sorted hepatocytes in this thesis. However, the cells were not in exponential growth and genes involved in the control of polyploidisation were therefore not expected to change. The genes that were upregulated or repressed in the polyploid cells may however, be important to the function or maintenance of ploidy in

hepatocytes. Biological assays are required to determine if this is the case. Mononuclear and binuclear hepatocytes containing the same DNA content were not possible to separate in this study. Whether binuclear cells are purely an intermediary stage in the formation of polyploid cells is unknown and needs to be examined.

6.3 Future Work

Whether polyploidisation is a protective mechanism or whether it is an advancement of terminal differentiation and senescence resulting in a higher susceptibility to apoptosis requires further study. In either case, understanding the regulation of this process is vital in the control, prevention and treatment of disease. The use of sorting and microarray technology to study hepatocytes in exponential growth will require some characterisation but will provide vital information into the genes involved in polyploidisation. Confocal microscopy and image analysis can be used to visualise genes involved in the process in different hepatocyte populations. The use of biological assays will help to determine the function or role of MUP2, Pterin and IFNγR in polyploid cells.

7 Acknowledgements

I would like to thank my supervisor, David Harrison, for all his support, guidance, encouragement and advice. I now really appreciate the proverbial 'kick up the backside' that made me decide to do a PhD in the first place. The past 3 to 4 years have provided me with many great opportunities that I would otherwise never have had.

Lots of love to Gary and my family, particularly my Mum and Dad for their constant support, good advice, company on 'get-away-from-it-all' holidays and for ensuring I remained as sane as I always have been!! A huge thanks to my Dad for proof-reading. Don't worry you will never have to read it again! Thanks also to Gary's family. The Sunday dinners definitely helped get me through to the end. A big thanks to my Uncle Paul and to Maureen and Jim whose company and kind hospitality in Manchester made my visits a lot of fun.

Thank you to all my friends who provided a trusty ear. Thanks especially to Suzi, Sharon A, Sharon S, Kerry and Kathryn who helped remind me that there was actually life outside a PhD. Thanks also to Ben, Chris M, Chris A, Tim, Jason and Owen (and all the other PhD students who have moved on) who know exactly what it is like! Thanks to June, Jacqui and Lynn for their company at coffee, lunch and occasionally in the pub. Particular thanks to Kim, Gill and all the others who I played korfball with and accompanied me on those fabulous weekends away.

Thanks to all the people I have had the pleasure of working with. A special thanks to Frances Rae, Jane Sadler, Sarah Howie, Cathy Simpson, Helen Caldwell, Angus McGregor, Sharon White, Steve MacKell, Stewart McKenzie, Steven Haig, Dominic Rannie, Peter Bush and Linda Sharp whose patience, technical advice and friendship was greatly appreciated. I could not have done it without you. Thanks also to Shoena and Nick who played a crucial role in the sorting process! Thanks to Elaine for organising the lottery and providing the hope that I may be rich someday.

I would also like to thank AstraZeneca for their contribution to my PhD. A big thanks to all within the Safety Assessment Department, particularly to Jonathan Tugwood, Phil, Mark, Kathy, Jo, Garry and Tom who enabled me to carry out microarray and real-time PCR analysis and who all made me feel very welcome.

I am extremely grateful to the Medical Faculty, University of Edinburgh who provided me with the Whaitt Research Scholarship.

8 APPENDICES

APPENDIX 1

MANUFACTURERS AND SUPPLIERS

Ambion (AMS Biotechnology (Europe) Ltd.,) UK

DNA-free

Glycogen

Amersham Pharmacia Biotech, Little Chalfont, UK

BrdU

Percoll

ProbeQuant G-50 Micro columns

Alpha ^{33}P dCTP

Becton Dickinson, Oxford, UK

40 μm filters

Biogene, Kimbolton, UK

Taq polymerase

Cambio, Cambridge, UK

BioMarker EXT

GraphPad Software Inc., CA, USA

GraphPad InStat Software

Harlan Sera-labs, Loughborough, UK

Rat monoclonal anti-BrdU antibody

Imaging Research, St. Catherines, Ontario, Canada

ArrayVision software

Life Technologies, Paisley, UK

6-well tissue culture plates,
8-well tissue culture glass slides
25 bp DNA ladder
96-well tissue culture plates
Cell scrapers
DMEM F12
DTT
First strand buffer
Flaskettes
Gentamicin
L-Glutamine
HBSS (10x)
IFNγ
Liver perfusion medium
Mouse COT1 DNA
dNTP mix
Oligo (dT) primer
Primers (all others)
RNase H
RNASEOUT
Sterile PBS
Superscript II

Microsoft Corporation, CA, USA

Microsoft Excel Software

Merck, Lutterworth, UK

Light green

Molecular Probes, Cambridge Bioscience, Cambridge, UK

CTO (Cell Tracker Orange)
Hoechst 33342
SYTO 16

NBL Gene Sciences, Cramlington, UK

Agarose

Packard Instruments Company Inc., Il USA

OptiQuant software

Phosphoimager

Super resolution (type SR) Phosphor screens

Pharmingen, San Diego, CA

FITC hamster α-IgG, group 1 antibody

Hamster α-mouse CD54 (ICAM-1) antibody

Purified rat IgG2a, κ antibody

Rat α-mouse CD119 (IFNγR α chain)

Polysciences, Warrington, PA

Fluoresbrite Microspheres

Promega, Southampton, UK

pGEM DNA marker

Roche, Lewes, UK

DNase type I

Glass capillary tubes

LightCycler™

Santa Cruz Biotechnology, Inc., CA, USA

Normal rabbit IgG

Rat α mouse IFNγR β chain antibody

Serotec, Oxford, UK

FITC-sheep α rabbit IgG antibody

FITC-rabbit α rat IgG2a antibody

Sigma, Poole, UK

Boric Acid (For molecular biology) BSA

Carbodiimide

Collagen type 1 (From rat tail)

Collagenase type 4

Chee's modified medium

Chloroform (For molecular biology)

Deionised formamide

Dexamethasone

DMSO (Tissue culture grade)

EDTA (For molecular biology)

EGF

Guanidine isothiocyanate (For molecular biology)

ITS

n-Lauroylsarcosine (For molecular biology)

β-Mercaptoethanol (For molecular biology)

Nonidet P40

PB

Pepsin

Peroxidase-conjugated rabbit α-rat antibody

Phenol, pH 4.3 (For molecular biology)

PI

RNase A

Schiff's reagent

SDS

Sodium acetate

Sodium azide

Sodium hydroxide (1N)

Sodium phosphate

Spermine tetrahydrochloride

Sucrose

Tri-Sodium citrate

TRIZMA BASE (Tris)

Trypan blue (Tissue culture grade)

Trypsin (Tissue culture grade)

Trypsin inhibitor

Tween 20

APPENDIX 2

BUFFERS AND SOLUTIONS

Buffers and solutions are listed in the order they are used in Materials and Methods (Chapter 2). PBS was made in distilled water from tablets obtained from Sigma.

TISSUE CULTURE

Stock Solutions:

Acetic acid (0.1 M)	Add 0.6 ml / 100 ml sterile H_2O.
Carbodiimide	Dissolve 19.5 mg in 100 ml sterile H_2O.
Collagen type 1	Dissolve 10 mg of collagen type 1 (from rat-tail) in 50 ml 0.1M Acetic acid (0.02% w/v). Sterilise solution by adding 5 ml chloroform and leave at 4°C overnight.
Dexamethasone	Dissolve 1 mg in 1ml absolute ethanol and 1 ml PBS (0.04 mg/ml). Store @ -20°C in 500 µl aliquots.
DNase type 1	Dissolve 100 mg in 5 ml sterile H_2O (20 mg/ml). Store @ -20°C in 500 µl aliquots.
EGF	Dissolve 100 µg in 4 ml of Chee's modified medium (25 µg/ml). Store @ -20°C in 500 µl aliquots.
ITS	Dissolve in 5 ml sterile H_2O. Store @ -20°C in 500 µl aliquots.
Linoleic acid	Dissolve 500 mg in 5 ml sterile H_2O. To 5 ml Chee's modified medium, add 250 µl (5 mg/ml). Store @ -20°C in 500 µl aliquots.
Percoll	Add 2.2 ml Hanks' buffered saline to 20 ml Percoll.

Media:

	Amount of Stock (in 500 ml) /Final Concentration

Liver Perfusion Medium

Gentamicin	500 µl / 0.1 mg/ml

Hanks' Buffered Salt Solution (Digestion medium)

Gentamicin	As above

DMEM F12 (Incl. Hepes), pH 7.4 (Plating medium)

Gentamicin	As above
L-Glutamine	10 ml / 4 mM
Dexamethasone	500 µl / 0.04 µg/ml
ITS	500 µl
1M NaOH	1ml

Chee's Modified Medium, pH 7.4 (Culture medium)

Gentamicin	As above
L-Glutamine	"
Dexamethasone	"
ITS	"
EGF	500 µl / 0.025 µg/ml
1M NaOH	As above

Collagen Coating of Slides and Plates

Add 2 volumes carbodiimide solution to 1 volume of collagen solution and coat plates using the following volumes:

Slide/Plate	Surface area (cm^2)	Volume of Carbodiimide/collagen per well (mls)
8-well glass slides	0.69	0.2
6-well plates	9.6	1.0
Small petri dishes	21.5	1.8

FLOW CYTOMETRY

Citrate buffer, pH 7.6

Sucrose	85.5 g	Dissolve in 800 ml
Tri-Sodium citrate	11.76 g	distilled H_2O, adjust pH
DMSO	50 ml	and make up to 1 l.

Stock solution, pH 7.6

Tri-Sodium citrate	2 g	Dissolve in 1.8 l
Tris	121 mg	distilled water, adjust pH
Spermine tetrahydrochloride	1.044 g	and make up to 2 l.
Nonidet P40	2 ml	

Solution A, pH 7.6

Trypsin	15 mg	Dissolve in 500 ml stock solution.

Store at -20°C in 10 ml aliquots, bring to room temperature before use.

Solution B, pH 7.6

Trypsin inhibitor	250 mg	Dissolve in 500 ml stock solution.
RNase A	50 mg	Store as for solution A.

Solution C, pH 7.6

PI	208 mg	Dissolve in 500 ml stock
Spermine tetrahydrochloride	500 mg	soln. Store as for solution A.
		Bring to 0°C before use.

MOLECULAR TECHNIQUES

Buffers:

Church and Gilbert Hybridisation Buffer (100 ml)

1 M Sodium phosphate, pH 7.2	20 ml
0.5 M EDTA	0.2 ml
BSA	1 g
20% SDS	33 ml
deionised formamide	15 ml
H_2O	32 ml

Array Wash Buffer (500 ml)

1 M Sodium phosphate, pH 7.2	20 ml
0.5 M EDTA	1.25 ml
20% SDS	25 ml
H_2O	Up to 500 ml

RNA Extraction Buffer (500 ml), pH 7.0

Guanidine isothiocyanate	23.63 g	Dissolve in 300 ml mQ
0.75 M Sodium citrate	0.37 g	H_2O, adjust pH, make
5% n-lauroylsarcosine	0.25 g	up to 500 ml.
β-mercaptoethanol	360 µl /50 ml Soln.	Add prior to use.

50x TAE Buffer (500 ml), pH 8–8.2

Tris	121 g	Dissolve in 300 ml mQ
Glacial acetic acid	28.6 ml	H_2O, adjust pH, make
0.5 M EDTA (pH 8)	50 ml	up to 500 ml.

TBE Buffer (500 ml)

Tris	54.45 g	Dissolve in 300 ml mQ
Boric acid	24.35 g	H_2O, make up to 500 ml.
EDTA	5.0 g	

OTHER SOLUTIONS

Boum's fixative (500 ml)

Methanol	425 ml
Glacial acetic acid	25 ml
40% Formalin	50 ml

APPENDIX 3

SAMPLES USED FOR GENE EXPRESSION ANALYSIS

Purity and proportion of mononuclear and binuclear cells in samples

Sample (Date)	Sample No.	% Purity (Gated Events)	% Mono-nuclear cells	% Binuclear cells	No. of Sorted Events/No. of cells
2n (12/10/00)	1	90.0	93.1	6.9	262610
2n (17/10/00)	1	92.6	95.1	4.9	355582
2n (8/11/00)	1	97.8	98.0	2.0	381662
2n (10/11/00)	1	95.0	-	-	302081
2n (27/11/00)	2	93.0	97.0	3.0	157309*
2n (30/11/00)	2	91.0	98.0	2.0	43789*
4n (10/10/00)	3	95.0	26.0	74.0	533898
4n (12/10/00)	3	91.3	26.7	73.3	366506
4n (17/10/00)	3	97.1	24.3	75.7	989562
4n (1/11/00)	3	93.5	24.1	75.6	2354231
4n (8/11/00)	4	94.7	32.0	68.2	800334
4n (10/11/00)	4	95.5	20.8	79.0	730062
4n (20/11/00)	4	91.8	18.0	82.0	2417843
4n (22/11/00)	5	90.1	23.0	77.0	162413*
4n (27/11/00)	5	97.0	-	-	300045*
4n (30/11/00)	5	95.0	20.0	80.0	207851*
4n (12/12/00)	5	98.4	21.0	79.0	330028*
8n (10/10/00)	6	78.6	17.9	79.3	162987
8n (17/10/00)	6	69.7	10.8	88.3	571553
8n (1/11/00)	6	82.3	6.8	87.8	733876
8n (8/11/00)	7	89.1	9.3	89.7	458180
8n (10/11/00)	7	84.2	4.4	85.6	627953
8n (20/11/00)	7	87.7	10.0	89.0	341639

Sample (Date)	Sample No.	% Cells*[1]			% Mono-nuclear cells	% Binuclear cells
		2n	4n	8n		
Total Old (4/10/00) 16 weeks	8	14	66	20	28.2	71.8
Total Young (3/2/01) 3 weeks	9	38	35	0.6	41.6	58.2
Total Old (20/11/00) 17 weeks	10	17	72	8	25	75

*[1]Cells in S-phase not included

Details of pooled samples

Sample No./Cell Type	Av. % Purity	Av. % Mono-nuclear cells	Av. % Binuclear cells	Amount of RNA (μg)	Total No. of Events/Cells
1. 2n	93.8 ± 3.3	95.4 ± 2.5	4.6 ± 2.5	5.6	1301935
2. 2n	92.0 ± 1.4	97.5 ± 0.7	2.5 ± 0.7	9.8	201098*
3. 4n	94.2 ± 2.4	25.3 ± 1.3	74.6 ± 1.2	53.1	4244297
4. 4n	94.0 ± 1.9	23.6 ± 7.4	76.4 ± 7.3	49.8	3948239
5. 4n	95.1 ± 3.6	21.3 ± 1.5	78.7 ± 1.5	40.7	1000337*
6. 8n	76.9 ± 6.5	11.8 ± 5.6	85.1 ± 5.1	25.2	1468416
7. 8n	87.0 ± 2.5	7.9 ± 3.1	88.1 ± 2.2	41.7	1427772

Exact counts

APPENDIX 4

COMPARISONS CARRIED OUT USING MICROARRAY ANALYSIS

Comparison	Sample No. (See Appendix 3)	Sample Type	Date of Analysis
1	N/A	Unsorted v 8n	21/8/00
2	1 v 4	2n v 4n	22/01/01
3	1 v 7	2n v 8n	22/01/01
4	2 v 5	2n v 4n	19/02/01
5	3 v 6	4n v 8n	19/02/01
6	10 v 11	TY v TO	19/02/01
7	12 v 11	TY v TO	19/02/01

APPENDIX 5

TIME-LAPSE VIDEOMICROSCOPY

Please see CD attached to back cover.

APPENDIX 6

PUBLISHED PAPERS

The following published papers have arisen out of the work presented in this thesis. They are included with the permission of the joint authors and the publishers of the journals in which they first appeared. Copyright of the following papers remains vested with these publishers as detailed below. Any licence to copy the main text of the thesis does not extend to this appendix which may not be reproduced except with the written permission of the publishers or in accordance with the provisions of the Copyright, Designs and Patents Act 1988, or under the terms of any licence permitting limited copying issued by the Copyright Licensing Agency Limited, 90 Tottenham Court Road, London W1P 9HE.

Reprinted from Toxicology Letters, vol: 119; Martin, NC, McGregor AH, Sansom, N, Gould, S and Harrison, DJ. Phenobarbitone-induced ploidy changes in liver occur independently of p53, pp 109-115, Copyright (2001), with permission from Elsevier Science.

Martin, NC, McCullough, CT, Bush, BG, Sharp, L, Hall, AC and Harrison, DJ. Functional analysis of mouse hepatocytes differing in DNA content: Volume, receptor expression and effect of IFNγ. Journal of Cell Physiology, Copyright (2002, Wiley-Liss, Inc.). Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

9 Bibliography

Afshar K, Gönczy P, DiNardo S, Wasserman SA (2001). *fumble* encodes a pantothenate kinase homolog required for proper mitosis and meiosis in *Drosphila melangaster*. *Genetics* **157**: 1267-1276.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) The cell division cycle. In: Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (eds) Molecular Biology of the Cell. 3 edition. Garland Publishing, Inc., New York, pp 863-910

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) The cytoskeleton. In: Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (eds) Molecular Biology of the Cell. 3 edition. Garland Publishing, Inc., New York, pp 787-862

Alfert M, Geschwind II (1958). The development of polysomaty in rat liver. *Experimental Cell Research* **15**: 230-232.

Allen CE, Wu L-C (2000). Downregulation of KRC induces proliferation, anchorage independence, and mitotic cell death in HeLa cells. *Experimental Cell Research* **260**: 346-356.

Anatskaya OV, Vinogradov AE, Kudryavtsev BN (1994). Hepatocyte polyploidy and metabolism life-history traits - hypotheses testing. *Journal Of Theoretical Biology* **168**: 191-199.

Anti M, Marra G, Rapaccini GL, Rumi C, Bussa S, Fadda G, Vecchio FM, Valenti A, Percesepe A, Pompili M, Armelao F, Gentiloni N (1994). DNA-ploidy pattern in human chronic liver-diseases and hepatic nodular lesions - flow cytometric analysis on echo-guided needle liver-biopsy. *Cancer* **73**: 281-288.

Armstrong J, Kaufman MH, Harrison DJ, Clarke AR (1995). High-frequency developmental abnormalities in p53-deficient mice. *Current Biology* **5**: 931-936.

Arndt-Jovin DJ, Jovin TM (1977). Analysis and sorting of living cells according to deoxyribonucleic acid content. *Journal of Histochemistry and Cytochemistry* **25**: 585-589.

Attallah AM, Tabll AA, Salem SF, El-Sadany M, Ibrahim TA, Osman S, El-Dosoky IM (1999). DNA ploidy of liver biopsies from patients with liver cirrhosis and hepatocellular carcinoma: a flow cytometric analysis. *Cancer Letters* **142**: 65-69.

Autuori F, Baldini P, Ciofi Luzzatto A, Conti Devirgiliis L, Dini L, Incerpi S, Luly P (1981). Insulin binding and internalization in rat hepatocytes during prenatal and postnatal life. *Biochimica et Biophysica Acta* **678**: 1-6.

Baatout S, Chatelain B, Staquet P, Symann M, Chatelain C (1998a). Augmentation of the number of nucleolar organizer regions in human megakaryocyte cell lines after induction of polyploidization by a microtubule inhibitor. *European Journal Of Clinical Investigation* **28**: 138-144.

Baatout S, Chatelain B, Staquet P, Symann M, Chatelain C (1998b). Inhibition of tubulin polymerization in megakaryocyte cell lines leads to polyploidization which affects the metabolism of actin. *Anticancer Research* **18**: 1553-1561.

Baatout S, Chatelain B, Staquet P, Symann M, Chatelain C (1998c). Inhibition of actin polymerization by cytochalasin B induces polyploidization and increases the number of nucleolar organizer regions in human megakaryocyte cell lines. *Anticancer Research* **18**: 459-464.

Bakker O, Razaki H, de Jong J, Ris-Stalpers C, Wiersinga WM (1998). Expression of the α1, α2, and β1 T3-receptor mRNAs in the fasted rat measured using competitive PCR. *Biochemical & Biophysical Research Communications* **242**: 492-496.

Barbason H, Van Cantfort J, Houbrechts N (1974). Correlation between tissular and division functions in the liver of young rats. *Cell and Tissue Kinetics* **7**: 319-326.

Barrett TB, Sampson P, Owens GK, Schwartz SM, Benditt EP (1983). Polyploid nuclei in human artery wall smooth-muscle cells. *Proceedings of the National*

Academy of Sciences of the United States of America-Biological Sciences **80**: 882-885.

Barton NR, Goldstein LSB (1996). Going mobile: Microtubule motors and chromosome segregation. *Proceedings of the National Academy of Science of the United States of America* **93**: 1735-1742.

Basto R, Gomes R, Karess RE (2000). Rough deal and Zw10 are required for the metaphase checkpoint in *Drosophila*. *Nature Cell Biology* **2**: 939-943.

Bellamy COC, Clarke AR, Wyllie AH, Harrison DJ (1997). p53 deficiency in liver reduces local control of survival and proliferation, but does not affect apoptosis after DNA damage. *FASEB Journal* **11**: 591-599.

Bengtsson BG, Kiessling K-H, Smith-Kielland A, Mørland J (1981). Partial separation and biochemical characteristics of periportal and perivenous hepatocytes from rat liver. *European Journal of Biochemistry* **118**: 591-597.

Benito J, Martin Castellanos C, Moreno S (1998). Regulation of the G_1 phase of the cell cycle by periodic stabilization and degradation of the $p25^{rum1}$ CDK inhibitor. *The Embo Journal* **17**: 482-497.

Bernaert D, Wanson JC, Mosselmans R, De Paermentier F, Drochmans P (1979). Separation of adult rat hepatocytes into distinct subpopulations by centrifugal elutriation. Morphological, morphometrical and biochemical characterization of cell fractions. *Biology of the Cell* **34**: 159-174.

Bernard P, Hardwick K, Javerzat J-P (1998). Fission yeast Bub1 is a mitotic centromere protein essential for the spindle checkpoint and the preservation of correct ploidy through mitosis. *Journal of Cell Biology* **143**: 1775-1787.

Bertucci F, Bernard K, Loriod B, Chang Y-C, Granjeaud S, Birnbaum D, Nguyen C, Peck K, Jordan BR (1999). Sensitivity issues in DNA array-based expression measurements and performance of nylon microarrays for small samples. *Human Molecular Genetics* **8**: 1715-1722.

Beyer HS, Sherman R, Zieve L (1991). Aging is associated with reduced liver regeneration and diminished thymidine kinase mRNA content and enzyme activity in the rat. *Journal of Laboratory and Clinical Medicine* **117**: 101-108.

Biesterfeld S, Gerres K, Fischer-Wein G, Böcking A (1994). Polyploidy in non-neoplastic tissues. *Journal of Clinical Pathology* **47**: 38-42.

Biggins S, Severin FF, Bhalla N, Sassoon I, Hyman AA, Murray AW (1999). The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes & Development* **13**: 532-544.

Bilir BM, Gong TWL, Kwasiborski V, Shen CS, Fillmore CS, Berkowitz CM, Gumucio JJ (1993). Novel control of the position-dependent expression of genes in hepatocytes; the GLUT-1 transporter. *Journal of Biological Chemistry* **268**: 19776-19784.

Bischoff FZ, Yim SO, Pathak S, Grant G, Siciliano MJ, Giovanella BC, Strong LC, Tainsky MA (1990). Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome: Aneuploidy and immortalization. *Cancer Research* **50**: 7979-7984.

Bischoff JR, Plowman GD (1999). The aurora/Ip11p kinase family: Regulators of chromosome segregation and cytokinesis. *Trends In Cell Biology* **9**: 454-459.

Blain PG, Battershill JM, Venitt S, Cooper CC, Fielder RJ (1998). Series: Current issues in mutagenesis and carcinogenesis, No. 87: Consideration of short-term carcinogenicity tests using transgenic mouse models. *Mutation Research* **403**: 259-263.

Blow JJ, Laskey RA (1988). A role for the nuclear-envelope in controlling DNA-replication within the cell-cycle. *Nature* **332**: 546-548.

Bohm N, Noltemeyer N (1981). Excessive reversible phenobarbital induced nuclear DNA-polyploidization in the growing mouse liver. *Histochemistry* **72**: 63-74.

Bohman R, Tamura CT, Doolittle MH, Cascarano J (1985). Growth and aging in the rat: changes in total protein, cellularity, and polyploidy in various organs. *Journal Of Experimental Zoology* **233**: 385-396.

Bortner DM, Rosenberg MP (1995). Overexpression of cyclin A in the mammary glands of transgenic mice results in the induction of nuclear abnormalities and increased apoptosis. *Cell Growth & Differentiation* **6**: 1579-1589.

Brodsky WYa, Uryvaeva IV (1977). Cell polyploidy: Its relation to tissue growth and function. *International Review of Cytology* **50**: 275-332.

Brodsky WYa, Arefyeva AM, Uryvaeva IV (1980). Mitotic polyploidization of mouse heart myocytes during the first postnatal week. *Cell and Tissue Research* **210**: 133-144.

Brodsky WYa, Uryvaeva IV (1985a) Genome multiplication in growth and development. The cells of vertebrate animals. In: Barlow PW, Green PB, Wylie CC (eds) Biology of Polyploid and Polytene Cells. Cambridge University Press, Cambridge, pp 20-80

Brodsky WYa, Uryvaeva IV (1985b) Genome multiplication in growth and development. Mechanisms for changing the number of genomes. In: Barlow PW, Green PB, Wylie CC (eds) Biology of Polyploid and Polytene Cells. Cambridge University Press, Cambridge, pp 141-174

Brodsky WYa, Uryvaeva IV (1985c) Genome multiplication in growth and development. The biological significance of polyploidy and polyteny. In: Barlow PW, Green PB, Wylie CC (eds) Biology of Polyploid and Polytene Cells. Cambridge University Press, Cambridge, pp 175-233

Broek D, Bartlett R, Crawford K, Nurse P (1991). Involvement of $p34^{cdc2}$ in establishing the dependency of S-phase on mitosis. *Nature* **349**: 388-393.

Bumgardner GL, Li J, Apte S, Heininger M, Frankel WL (1998). Effect of tumor necrosis factor α and intercellular adhesion molecule-1 expression on immunogenicity of murine liver cells in mice. *Hepatology* **28**: 466-474.

Burdon RH (1995). Superoxide and hydrogen-peroxide in relation to mammalian-cell proliferation. *Free Radical Biology and Medicine* **18**: 775-794.

Bush PG, Hall AC (2001). Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. *Journal of Cellular Physiology* **187**: 304-314.

Carow CE, Fox NE, Kaushansky K (2001). Kinetics of endomitosis in primary murine megakaryocytes. *Journal of Cellular Physiology* **188**: 291-303.

Carriere R (1967). Polyploid cell reproduction in normal adult rat liver. *Experimental Cell Research* **46**: 533-540.

Carthew P, Edwards RE, Nolan BM (1998). The quantitative distinction of hyperplasia from hypertrophy in hepatomegaly induced in the rat liver by phenobarbital. *Toxicological Sciences* **44**: 46-51.

Cascales M, Alvarez A, Gasco P, Fernandez-Simon L, Sanz N, Bosca L (1994). Cocaine-induced liver-injury in mice elicits specific changes in DNA-ploidy and induces programmed death of hepatocytes. *Hepatology* **20**: 992-1001.

Casenghi M, Mangiacasale R, Tuynder M, Caillet-Fauquet P, Elhajouji A, Lavia P, Mousset S, Kirsch-Volders M, Cundari E (1999). p53-independent apoptosis and p53-dependent block of DNA rereplication following mitotic spindle inhibition in human cells. *Experimental Cell Research* **250**: 339-350.

Cavaggioni A, Mucignat-Caretta C (2000). Major urinary proteins, α_{2U}-globulins and aphrodisin. *Biochimica et Biophysica Acta* **1482**: 218-228.

Cebolla A, Vinardell JM, Kiss E, Olah B, Roudier F, Kondorosi A, Kondorosi E (1999). The mitotic inhibitor *ccs52* is required for endoreduplication and ploidy-dependent cell enlargement in plants. *The EMBO Journal* **18**: 4476-4484.

Cesarone CF, Scarabelli L, Demori I, Balocco S, Fugassa E (2000). Poly(ADP-ribose) polymerase is affected early by thyroid state during liver regeneration in rats. *American Journal of Physiology - Gastrointestinal & Liver Physiology* **279**: G1219-G1225.

Chan CSM, Botstein D (1993). Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. *Genetics* **135**: 677-691.

Chan GKT, Jablonski SA, Starr DA, Goldberg ML, Yen TJ (2000). Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nature Cell Biology* **2**: 944-947.

Chatterjee B, Demyan WF, Roy AK (1983). Interacting role of thyroxine and growth hormone in the hepatic synthesis of α_{2u}-globulin and its messenger RNA. *Journal of Biological Chemistry* **258**: 688-692.

Chevalier S, Macdonald N, Tonge R, Rayner S, Rowlinson R, Shaw J, Young J, Davison M, Roberts RA (2000). Proteomic analysis of differential protein expression in primary hepatocytes induced by EGF, tumour necrosis factor α or the peroxisome proliferator nafenopin. *European Journal of Biochemistry* **267**: 4624-4634.

Chirino R, Fernandez L, Lopez A, Navarro D, Rivero JF, Diaz-Chico JC, Diaz-Chico BN (1991). Thyroid hormones and glucocorticoids act synergistically in the regulation of the low affinity glucocorticoid binding sites in the male rat liver. *Endocrinology* **129**: 3118-3124.

Ciciarello M, Mangiacasale R, Casenghi M, Limongi MZ, D'Angelo M, Soddu S, Lavia P, Cundari E (2001). p53 displacement from centromeres and p53-mediated G1 arrest following transient inhibition of the mitotic spindle. *Journal of Biological Chemistry* **276**: 19205-19213.

Clawson GA, Blankenship LJ, Rhame JG, Wilkinson DS (1992). Nuclear enlargement induced by hepatocarcinogens alters ploidy. *Cancer Research* **52**: 1304-1308.

Coffman FD, Studzinski GP (1999). Differentiation-related mechanisms which suppress DNA replication. *Experimental Cell Research* **248**: 58-73.

Collins JM (1978). RNA synthesis in rat liver cells with different DNA contents. *Journal of Biological Chemistry* **253**: 5769-5773.

Columbano A, Shinozuka H (1996). Liver regeneration .8. Liver regeneration versus direct hyperplasia. *FASEB Journal* **10**: 1118-1128.

Correabordes J, Nurse P (1995). $p25^{Rum1}$ orders S-phase and mitosis by acting as an inhibitor of the $p34^{Cdc2}$ mitotic kinase. *Cell* **83**: 1001-1009.

Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, Taub R (1996). Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* **274**: 1379-1383.

Dahmann C, Diffley JFX, Nasmyth K (1995). S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Current Biology* **5**: 1257-1269.

Dallman PR, Spirito RA, Siimes MA (1974). Diurnal patterns of DNA synthesis in the rat: Modification by diet and feeding schedule. *Journal of Nutrition* **104**: 1234-1241.

Danielsen H, Lindmo T, Reith A (1986). A method for determining ploidy distributions in liver tissue by stereological analysis of nuclear size calibrated by flow cytometric DNA analysis. *Cytometry* **7**: 475-480.

Danielsen HE, Steen HB, Lindmo T, Reith A (1988). Ploidy distribution in experimental liver carcinogenesis in mice. *Carcinogenesis* **9**: 59-63.

Dasso M, Newport JW (1990). Completion of DNA-replication is monitored by a feedback-system that controls the initiation of mitosis *in vitro* - studies in xenopus. *Cell* **61**: 811-823.

Datta NS, Williams JL, Caldwell J, Curry AM, Ashcraft EK, Long MW (1996). Novel alterations in CDK1/cyclin B1 kinase complex formation occur during the acquisition of a polyploid DNA content. *Molecular Biology of the Cell* **7**: 209-223.

Datta NS, Williams JL, Long MW (1998). Differential modulation of G_1-S-phase cyclin-dependent kinase 2 cyclin complexes occurs during the acquisition of a polyploid DNA content. *Cell Growth & Differentiation* **9**: 639-650.

Davies R, Cain K, Edwards RE, Snowden RT, Legg RF, Neal GE (1990). The preparation of highly enriched fractions of binucleated rat hepatocytes by centrifugal elutriation and flow cytometry. *Analytical Biochemistry* **190**: 266-270.

De Juan C, Benito M, Alvarez A, Fabregat I (1992). Differential proliferative response of cultured fetal and regenerating hepatocytes to growth factors and hormones. *Experimental Cell Research* **202** : 495-500.

de la Hoz C, Baroja A (1993). Proliferative behaviour of high-ploidy cells in two murine tumour lines. *Journal of Cell Science* **104**: 31-36.

Denko N, Langland R, Barton M, Lieberman MA (1997). Uncoupling of S-Phase and mitosis by recombinant cytotoxic necrotizing factor 2 (CNF2). *Experimental Cell Research* **234**: 132-138.

Deschenes J, Valet JP, Marceau N (1981). The relationship between cell-volume, ploidy, and functional-activity in differentiating hepatocytes. *Cell Biophysics* **3**: 321-334.

Diez-Fernandez C, Bosca L, Fernandezsimon L, Alvarez A, Cascales M (1993). Relationship between genomic DNA-ploidy and parameters of liver-damage during necrosis and regeneration induced by thioacetamide. *Hepatology* **18**: 912-918.

Diez-Fernandez C, Sanz N, Alvarez AM, Wolf A, Cascales M (1998). The effect of non-genotoxic carcinogens, phenobarbital and clofibrate, on the relationship between reactive oxygen species, antioxidant enzyme expression and apoptosis. *Carcinogenesis* **19**: 1715-1722.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery Jr CA, Butel JS, Bradley A (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**: 215-221.

Donehower LA (1996). The *p53*-deficient mouse: a model for basic and applied cancer studies. *Seminars in Cancer Biology* **7**: 269-278.

Dragan YP, Shimel RJ, Bahnub N, Sattler G, Vaughan JR, Jordan VC, Pitot HC (1998). Effect of chronic administration of mestranol, tamoxifen, and toremifene on hepatic ploidy in rats. *Toxicological Sciences* **43**: 129-138.

Durand RE, Olive PL (1982). Cytotoxicity, mutagenicity and DNA damage by Hoechst 33342. *Journal of Histochemistry and Cytochemistry* **30**: 111-116.

Edgar BA, Orr-Weaver TL (2001). Endoreplication cell cycles: More for less. *Cell* **105**: 297-306.

Elledge SJ, Winston J, Harper JW (1996). A question of balance: The role of cyclin-kinase inhibitors in development and tumorigenesis. *Trends In Cell Biology* **6**: 388-392.

Elliott MJ, Murphy KM, Stribinskiene L, Ranganathan V, Sturges E, Farnsworth ML, Lock RB (1999). Bcl-2 inhibits early apoptotic events and reveals post-mitotic multinucleation without affecting cell cycle arrest in human epithelial tumour cells exposed to etoposide. *Cancer and Chemotherapeutic Pharmacology* **44**: 1-11.

Enesco HE, Shimokawa I, Yu BP (1991). Effect of dietary restriction and aging on polyploidy in rat liver. *Mechanisms of Ageing And Development* **59**: 69-78.

Enoch T, Nurse P (1990). Mutation of fission yeast-cell cycle control genes abolishes dependence of mitosis on DNA-replication. *Cell* **60**: 665-673.

Epstein CJ (1967). Cell size, nuclear content, and the development of polyploidy in the mammalian liver. *Proceedings of the National Academy of Science of the United States of America* **57**: 327-334.

Feldmann G (1992). Liver ploidy. *Journal of Hepatology* **16**: 7-10.

Fogt F, Nanji AA (1996). Alterations in nuclear ploidy and cell phase distribution of rat liver cells in experimental alcoholic liver disease: Relationship to antioxidant enzyme gene expression. *Toxicology and Applied Pharmacology* **136**: 87-93.

Fraser AG, James C, Evan GI, Hengartner MO (1999). *Caenorhabditis elegans* inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. *Current Biology* **9**: 292-301.

Fried J, Doblin J, Takamoto S, Perez A, Hansen H, Clarkson B (1982). Effects of Hoechst 33342 on survival and growth of two tumour cell lines and on hematopoietically normal bone marrow cells. *Cytometry* **3**: 42-47.

Galitski T, Saldanha AJ, Styles CA, Lander ES, Fink GR (1999). Ploidy regulation of gene expression. *Science* **285**: 251-254.

Gao CY, Zelenka PS (1997). Cyclins, cyclin-dependent kinases and differentiation. *Bioessays* **19**: 307-315.

Gerlyng P, Grotmol T, Erikstein B, Stokke T, Seglen PO (1992). Reduced proliferative activity of polyploid cells in primary hepatocellular-carcinoma. *Carcinogenesis* **13**: 1795-1801.

Gerlyng P, Abyholm A, Grotmol T, Erikstein B, Huitfeldt HS, Stokke T, Seglen PO (1993). Binucleation and polyploidization patterns in developmental and regenerative rat-liver growth. *Cell Proliferation* **26**: 557-565.

Gerlyng P, Grotmol T, Seglen PO (1994). Effect of 4-acetylaminofluorene and other tumor promoters on hepatocellular growth and binucleation. *Carcinogenesis* **15**: 371-379.

Gilles PN, Guerrette DL, Ulevitch RJ, Schreiber RD, Chisari FV (1992). HBsAg retention sensitizes the hepatocyte to injury by physiological concentrations of interferon-γ. *Hepatology* **16**: 655-663.

Girard F, Strausfeld U, Fernandez A, Lamb NJC (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67**: 1169-1179.

Glass CK, Holloway JM (1990). Regulation of gene expression by the thyroid hormone receptor. *Biochimica et Biophysica Acta* **1032**: 157-176.

Glover DM, Leibowitz MH, McLean DA, Parry H (1995). Mutations in *aurora* prevent centrosome separation leading to the formation of monopolar spindles. *Cell* **81**: 95-105.

Goberdhan DCI, Paricio N, Goodman EC, Mlodzik M, Wilson C (1999). *Drosophila* tumour suppressor *PTEN* controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes & Development* **13**: 3244-3258.

Gomez-Lechon MJ, Barbera E, Gil R, Baguena J (1981). Evolutive changes of ploidy and polynucleation in adult rat hepatocytes in culture. *Cellular and Molecular Biology* **27**: 695-701.

Gonzales AJ, Christensen JG, Preston RJ, Goldsworthy TL, Tlsty TD, Fox TR (1998). Attenuation of G_1 checkpoint function by the non-genotoxic carcinogen phenobarbital. *Carcinogenesis* **19**: 1173-1183.

Goolsby CL, Rao MS (1996). Flow cytometric analysis of neoplastic nodules and hepatocellular carcinomas induced by ciprofibrate in the rat. *British Journal of Cancer* **73**: 197-202.

Gould S, Sidaway J, Sansom N, Betton G, Orton T (2001). Phenobarbitone-induced liver responses in wild type and in p53 deficient mice. *Toxicology Letters* **122**: 131-140.

Grafi G, Larkins BA (1995). Endoreduplication in maize endosperm - Involvement of M-phase-promoting factor inhibition and induction of S-phase-related kinases. *Science* **269**: 1262-1264.

Gramantieri L, Melchiorri C, Chieco P, Gaiani S, Stecca B, Casali A, Bolondi L (1996). Alteration of DNA ploidy and cell nuclearity in human hepatocellular carcinoma associated with HBV infection. *Journal of Hepatology* **25**: 848-853.

Greenblatt MS, Bennett WP, Hollstein M, Harris CC (1994). Mutations in the p53 tumour suppressor gene: Clues to cancer etiology and molecular pathogenesis. *Cancer Research* **54**: 4855-4878.

Gualberto A, Aldape K, Kozakiewicz K, Tlsty TD (1998). An oncogenic form of p53 confers a dominant, gain-of-function phenotype that disrupts spindle checkpoint control. *Proceedings of the National Academy of Science of the United States of America* **95**: 5166-5171.

Gupta S (2000). Hepatic polyploidy and liver growth control. *Cancer Biology* **10**: 161-171.

Hall LL, Th'ng JPH, Guo XW, Teplitz RL, Bradbury EM (1996). A brief staurosporine treatment of mitotic cells triggers premature exit from mitosis and polyploid cell formation. *Cancer Research* **56**: 3551-3559.

Handeli S, Weintraub H (1992). The ts41 mutation in chinese hamster cells leads to successive S phases in the absence of intervening G_2, M, and G_1. *Cell* **71**: 599-611.

Hannon GJ, Beach D (1994). P15(Ink4b) Is a potential effector of TGF-β-induced cell-cycle arrest. *Nature* **371**: 257-261.

Harrison LE, Wang QM, Studzinski GP (1999). 1,25-Dihydroxyvitamin D_3-induced retardation of the G_2 /M traverse is associated with decreased levels of $p34^{cdc2}$ in HL60 cells. *Journal of Cellular Biochemistry* **75**: 226-234.

Harvey M, McArthur MJ, Montgomery Jr CA, Bradley A, Donehower LA (1993). Genetic background alters the spectrum of tumours that develop in p53-deficient mice. *FASEB Journal* **7**: 938-943.

Hasmall SC, Roberts RA (1997). Hepatic ploidy, nuclearity, and distribution of DNA synthesis: A comparison of nongenotoxic hepatocarcinogens with noncarcinogenic liver mitogens. *Toxicology and Applied Pharmacology* **144**: 287-293.

Hasmall SC, Roberts RA (2000). The nongenotoxic hepatocarcinogens diethylhexylphthalate and methylclofenapate induce DNA synthesis preferentially in octoploid rat hepatocytes. *Toxicologic Pathology* **28**: 503-509.

Hauer CR, Rebrin I, Thöny B, Neuheiser F, Curtius H-C, Hunziker P, Blau N, Ghisla S, Heizmann CW (1993). Phenylalanine hydroxylase-stimulating protein/Pterin-4α-

carbinolamine dehydratase from rat and human liver: Purification, characterisation and complete amino acid sequence. *Journal of Biological Chemistry* **268**: 4828-4831.

Hay RJ (1992) Cell line preservation and characterization. In: Freshney RI (ed) Animal Cell Culture: A Practical Approach. 2nd edition. Oxford University Press, Oxford, pp 95-148

Hayashi S (1996). A cdc2 dependent checkpoint maintains diploidy in *Drosophila*. *Development* **122**: 1051-1058.

Hedgecock EM, White JG (1985). Polyploid tissues in the nematode *Caenorhabditis elegans*. *Developmental Biology* **107**: 128-133.

Held WA, Mullins JJ, Kuhn NJ, Gallagher JF, Gu GD, Gross KW (1989). T antigen expression and tumorigenesis in transgenic mice containing a mouse major urinary protein/SV40 T antigen hybrid gene. The *Embo Journal* **8**: 183-191.

Henmi A, Uchida T, Shikata T (1985). Karyometric analysis of liver cell dysplasia and hepatocellular carcinoma: Evidence against precancerous nature of liver cell dysplasia. *Cancer* **55**: 2594-2599.

Hicks RM (1975). The mammalian urinary bladder: An accommodating organ. *Biological Reviews* **50**: 215-246.

Hinchcliffe EH, Miller FJ, Cham M, Khodjakov A, Sluder G (2001). Requirement of a centrosomal activity for cell cycle progression through G_1 into S phase. *Science* **291**: 1547-1550.

Hixon ML, Obejero-Paz C, Muro-Cacho C, Wagner MW, Millie E, Nagy J, Hassold TJ, Gualberto A (2000). Cks1 mediates vascular smooth muscle cell polyploidization. *Journal of Biological Chemistry* **275**: 40434-40442.

Holloway SL, Glotzer M, King RW, Murray AW (1993). Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell* **73**: 1393-1402.

Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991). p53 mutations in human cancers. *Science* **253**: 49-53.

Huang H, Potter CJ, Tao W, Li D-M, Brogiolo W, Hafen E, Sun H, Xu T (1999). PTEN affects cell size, cell proliferation and apoptosis during *Drosophila* eye development. *Development* **126**: 5365-5372.

Hudson KM, Denko NC, Schwab E, Oswald E, Weiss A, Lieberman MA (1996). Megakaryocytic cell line - Specific hyperploidy by cytotoxic necrotizing factor bacterial toxins. *Blood* **88**: 3465-3473.

Illidge TM, Cragg MS, Fringes B, Olive P, Erenpreisa JA (2000). Polyploid giant cells provide a survival mechanism for p53 mutant cells after DNA damage. *Cell Biology International* **24**: 621-633.

Ingelman-Sundberg M, Johansson I, Pentilla KE, Glaumann H, Lindros KO (1988). Centrilobular expression of ethanol-inducible cytochrome P-450 (IIE1) in rat liver. *Biochemical & Biophysical Research Communications* **157**: 55-60.

Ishibashi T, Kimura H, Shikama Y, Uchida T, Kariyone S, Hirano T, Kishimoto T, Takatsuki F, Akiyama Y (1989). Interleukin-6 is a potent thrombopoietic factor in vivo in mice. *Blood* **74**: 1241-1244.

Jacobs T (1992). Control of the cell-cycle. *Developmental Biology* **153**: 1-15.

James J (1977). The genesis of polyploidy in rat liver parenchymal cells. *European Journal of Cell Biology* **15**: 410-419.

Japa J (1943). A study of the morphology and development of the megakaryocytes. *The British Journal of Experimental Pathology* **24**: 73-80.

Jungermann K, Kietzmann T (1996). Zonation of parenchymal and nonparenchymal metabolism in liver. *Annual Review of Nutrition* **16**: 179-203.

Kamendulis LM, Kolaja KL, Stevenson DE, Walborg EFJ, Klaunig JE (2001). Comparative effects of dieldrin on hepatic ploidy, cell proliferation, and apoptosis in rodent liver. *Journal of Toxicology & Environmental Health, Part A* **62**: 127-141.

Kanbe T, Akashi T, Tanaka K (1993). Effect of cytochalasin-a on actin distribution in the fission yeast schizosaccharomyces-pombe studied by fluorescent and electron-microscopy. *Protoplasma* **176**: 24-32.

Kano A, Watanabe Y, Takeda N, Aizawa S-I, Akaike T (1997). Analysis of IFN-γ-induced cell cycle arrest and cell death in hepatocytes. *Journal of Biochemistry* **121**: 677-683.

Kano A, Haruyama T, Akaike T, Watanabe Y (1999). IRF-1 is an essential mediator in IFN-γ-induced cell cycle arrest and apoptosis of primary cultured hepatocytes. *Biochemical and Biophysical Research Communications* **257**: 672-677.

Kao C-Y, Factor VM, Thorgeirsson SS (1996). Reduced growth capacity of hepatocytes from c-myc and c-myc/TGF-α transgenic mice in primary culture. *Biochemical and Biophysical Research Communications* **222**: 64-70.

Kato J, Kobune M, Kohgo Y, Sugawara N, Hisai H, Nakamura T, Sakamaki S, Sawada N, Niitsu Y (1996). Hepatic iron deprivation prevents spontaneous development of fulminant hepatitis and liver cancer in Long-Evans Cinnamon rats. *Journal of Clinical Investigation* **98**: 923-929.

Kawasaki A, Matsumura I, Miyagawa J-I, Ezoe S, Tanaka H, Terada Y, Tatsuka M, Machii T, Miyazaki H, Furukawa Y, Kanakura Y (2001). Downregulation of an AIM-1 kinase couples megakaryocytic polyploidization of human hematopoietic cells. *The Journal of Cell Biology* **152**: 275-287.

Keighren M, West JD (1993). Analysis of cell ploidy in histological sections of mouse tissue by DNA-DNA *in situ* hybridization with digoxigenin-labelled probes. *Histochemical Journal* **25**: 30-44.

Kikuchi J, Furukawa Y, Iwase S, Terui Y, Nakamura M, Kitagawa S, Kitagawa M, Komatsu N, Miura Y (1997). Polyploidization and functional maturation are two distinct processes during megakaryocytic differentiation: Involvement of cyclin-dependent kinase inhibitor p21 in polyploidization. *Blood* **89**: 3980-3990.

Kimura M, Ogihara M (1998). Effects of insulin-like growth factor I and II on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. *European Journal of Pharmacology* **354**: 271-281.

Knoblich JA, Sauer K, Jones L, Richardson H, Saint R, Lehner CF (1994). Cyclin E controls S-phase progression and its down-regulation during *drosophila* embryogenesis is required for the arrest of cell- proliferation. *Cell* **77**: 107-120.

Knopf JL, Gallagher JF, Held WA (1983). Differential, multihormonal regulation of the mouse major urinary protein gene family in the liver. *Molecular and Cellular Biology* **3**: 2232-2240.

Kobel HR, Du Pasquier L (1986). Genetics of polyploid *Xenopus*. *Trends In Genetics* **2**: 314-315.

Koike Y, Suzuki Y, Nagata A, Furuta S, Nagata T (1982). Studies on DNA content of hepatocytes in cirrhosis and hepatoma by means of microspectrophotometry and radioautography. *Histochemistry* **73**: 549-562.

Kominami K-I, Toda T (1997). Fission yeast WD-repeat protein Pop1 regulates genome ploidy through ubiquitin-proteasome-mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18. *Genes & Development* **11**: 1548-1560.

Korver W, Schilham MW, Moerer P, van den Hoff MJ, Dam K, Lamers WH, Medema RH, Clevers H (1998). Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident. *Current Biology* **8**: 1327-1330.

Kudryavtsev BN, Kudryavtseva MV, Sakuta GA, Stein GI (1993). Human hepatocyte polyploidization kinetics in the course of life-cycle. *Virchows Archiv B Cell Pathology Including Molecular Pathology* **64**: 387-393.

Labib K, Moreno S (1996). rum1: A CDK inhibitor regulating G_1 progression in fission yeast. *Trends In Cell Biology* **6**: 62-66.

Lacey KR, Jackson PK, Stearns T (1999). Cyclin-dependent kinase control of centrosome duplication. *Proceedings of the National Academy of Science of the United States of Americal* **96**: 2817-2822.

Lang F, Lepple-Wienhues A, Szabó I, Siemen D, Gulbins E (1998). Cell volume in cell proliferation and apoptotic cell death. *Contrib Nephrol Basel, Karger* **123**: 158-168.

Le Rumeur E, Beaumont C, Guillouzo C, Rissel M, Bourel M, Guillouzo A (1981). All normal rat hepatocytes produce albumin at a rate related to their degree of ploidy. *Biochemical & Biophysical Research Communications* **101**: 1038-1046.

Le Rumeur E, Guguen-Guillouzo C, Beaumont C, Saunier A, Guillouzo A (1983). Albumin secretion and protein synthesis by cultured diploid and tetraploid rat hepatocytes separated by elutriation. *Experimental Cell Research* **147**: 247-254.

LeClaire RD, Basu M, Pinson DM, Redick ML, Hunt JS, Zavodny PJ, Pace JL, Russell SW (1992). Characterization and use of monoclonal and polyclonal antibodies against the mouse interferon-γ receptor. *Journal of Leukocyte Biology* **51**: 507-516.

Ledda-Columbano GM, Pibiri M, Loi R, Perra A, Schinozuka H, Columbano A (2000). Early increase in Cyclin-D1 expression and accelerated entry of mouse hepatocytes into S-Phase after administration of the mitogen 1,4-Bis[2-(3,5-dichloropyridyloxy)] benzene. *American Journal of Pathology* **156**: 91-97.

Lee RMKW, Conyers RB, Kwan CY (1992). Incidence of multinucleated and polyploid aortic smooth muscle cells cultured from different age groups of

spontaneously hypertensive rats. *Canadian Journal of Physiology And Pharmacology* **70**: 1496-1501.

Lehner CF, O'Farrell PH (1990). The roles of *drosophila* cyclin-A and cyclin-B in mitotic control. *Cell* **61**: 535-547.

Leitch AR (2000). Higher levels of organization in the interphase nucleus of cycling and differentiated cells. *Microbiology and Molecular Biology Reviews* **64**: 138-152.

Leno GH, Downes CS, Laskey RA (1992). The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in *Xenopus* egg extract. *Cell* **69**: 151-158.

Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, Villa A, Marchisio PC, Altieri DC (1999). Pleitropic cell-division defects and apoptosis induced by interference with survivin function. *Nature Cell Biology* **1**: 461-466.

Li Q, Dang CV (1999). c-Myc overexpression uncouples DNA replication from mitosis. *Molecular and Cellular Biology* **19**: 5339-5351.

Lin ELC, Klaunig JE, Mattox JK, Weghorst CM, McFarland BH, Pereira MA (1989). Comparison of the effects of acute and subacute treatment of phenobarbital in different strains of mice. *Cancer Letters* **48**: 43-51.

Liotti FS, Menghini AR, Guerrieri P, Mariucci G, Locci P, Bruschelli G (1987). Variations in catalase, glutathione-peroxidase and superoxide- dismutase in regenerating rat-liver. *Cellular and Molecular Biology* **33**: 611-617.

Loken MR (1980). Separation of viable T and B lymphocytes using a cytochemical stain, Hoechst 33342. *Journal of Histochemistry and Cytochemistry* **28**: 36-39.

Longo FJ, Mathews L, Hedgecock D (1993). Morphogenesis of maternal and paternal genomes in fertilized oyster eggs (*Crassostrea gigas*) - Effects of cytochalasin b at different periods during meiotic maturation. *Biological Bulletin* **185**: 197-214.

Luca FC, Winey M (1998). *MOB1*, an essential yeast gene required for completion of mitosis and maintenance of ploidy. *Molecular Biology of the Cell* **9**: 29-46.

Lydon MJ, Keeler KD, Thomas DB (1980). Vital DNA staining and cell sorting by flow microfluorometry. *Journal of Cellular Physiology* **102**: 175-181.

Madra S, Styles J, Smith AG (1995). Perturbation of hepatocyte nuclear populations induced by iron and polychlorinated biphenyls in C57BL/10ScSn mice during carcinogenesis. *Carcinogenesis* **16**: 719-727.

Maier P, Schawalder H (1993). Physiological oxygen-tension modulates the chemically-induced mitogenic response of cultured rat hepatocytes. *Journal of Cellular Physiology* **156**: 119-129.

Maliekal TT, Sudha B, Paulose CS (1997). Kinetic parameters of Thymidine kinase and DNA synthesis during liver regeneration: role of thyroid hormones. *Life Sciences* **60**: 1867-1874.

Manjeshwar S, Laconi E, Sheikh A, Rao PM, Rajalakshmi S, Sarma DSR (1994). *In-vitro* and *in-vivo* response of hepatocytes from hepatic nodules to the mitoinhibitory effects of phenobarbital. *Carcinogenesis* **15**: 1963-1968.

Mantel C, Braun SE, Reid S, Henegariu O, Liu L, Hangoc G, Broxmeyer HE (1999). $p21^{cip\text{-}1/waf\text{-}1}$ deficiency causes deformed nuclear architecture, centriole overduplication, polyploidy, and relaxed microtubule damage checkpoints in human hematopoietic cells. *Blood* **93**: 1390-1398.

Martin BF (1972). Cell replacement and differentiation in transitional epithelium: a histological and autoradiographic study of the guinea-pig bladder and ureter. *Journal of Anatomy* **112**: 433-455.

Martin Castellanos C, Labib K, Moreno S (1996). B-type cyclins regulate G_1 progression in fission yeast in opposition to the $p25^{rum1}$ cdk inhibitor. *The Embo Journal* **15**: 839-849.

McGrew JT, Goetsch L, Byers B, Baum P (1992). Requirement for ESP1 in the nuclear division of *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **3**: 1443-1454.

McIntosh I, Bishop JO (1989). Differential expression in male and female mouse liver of very similar mRNAs specified by two group 1 major urinary protein genes. *Molecular and Cellular Biology* **9**: 2202-2207.

McWhir J, Selfridge J, Harrison DJ, Squires S, Melton DW (1993). Mice with DNA repari gene (*ERCC-1*) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nature Genetics* **5**: 217-224.

Medema RH, Klompmaker R, Smits VAJ, Rijksen G (1998). $p21^{waf1}$ can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. *Oncogene* **16**: 431-441.

Medvedev ZA (1986). Age-related polyploidization of hepatocytes: The cause and possible role. *Experimental Gerontology* **21**: 277-282.

Melchiorri C, Chieco P, Zedda AI, Coni P, Ledda-Columbano GM, Columbano A (1993). Ploidy and nuclearity of rat hepatocytes after compensatory regeneration or mitogen-induced liver growth. *Carcinogenesis* **14**: 1825-1830.

Melnick RL (1992). Does chemically induced hepatocyte proliferation predict liver carcinogenesis? *FASEB Journal* **6**: 2698-2706.

Mendel DB, Khavari PA, Conley PB, Graves MK, Hansen LP, Admon A, Crabtree GR (1991). Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein. *Science* **254**: 1762-1767.

Michalopoulos GK, DeFrances MC (1997). Liver regeneration. *Science* **276**: 60-66.

Miller RG, Phillips RA (1969). Separation of cells by velocity sedimentation. *Journal of Cellular Physiology* **73**: 191-202.

Miller RT, Shah RS, Cattley RC, Popp JA (1996). The peroxisome proliferators WY-14,643 and methylclofenapate induce hepatocyte ploidy alterations and ploidy-specific DNA synthesis in F344 rats. *Toxicology and Applied Pharmacology* **138**: 317-323.

Minn AJ, Boise LH, Thompson CB (1996). Expression of Bcl-x(L) and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. *Genes & Development* **10**: 2621-2631.

Mitsuhashi T, Nikodem VM (1989). Regulation of expression of the alternative mRNAs of the rat α-thyroid hormone receptor gene. *Journal of Biological Chemistry* **264**: 8900-8904.

Moll T, Tebb G, Surana U, Robitsch H, Nasmyth K (1991). The role of phosphorylation and the cdc28 protein-kinase in cell-cycle regulated nuclear import of the *Saccharomyces cerevisiae* transcription factor-swi5. *Cell* **66**: 743-758.

Moreno S, Nurse P (1994). Regulation of progression through the G1 phase of the cell-cycle by the rum1(+) Gene. *Nature* **367**: 236-242.

Morita M, Watanabe Y, Akaike T (1995). Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. *Hepatology* **21**: 1585-1593.

Mossin L, Blankson H, Huitfeldt H, Seglen PO (1994). Ploidy-dependent growth and binucleation in cultured rat hepatocytes. *Experimental Cell Research* **214**: 551-560.

Muhua L, Adames NR, Murphy MD, Shields CR, Cooper JA (1998). A cytokinesis checkpoint requiring the yeast homologue of an APC-binding protein. *Nature* **393**: 487-491.

Murray A (1994a). Cell-cycle checkpoints. *Current Opinion In Cell Biology* **6**: 872-876.

Murray AW (1994b). Rum tale of replication. *Nature* **367**: 219-220.

Nadal C, Zajdela F (1966). Polyploidie somatique dans le foie de rat. I. Le rôle des cellules binucléées dans la genèse des cellules polyploides. *Experimental Cell Research* **42**: 99-446.

Nagata Y, Muro Y, Todokoro K (1997). Thrombopoietin-induced polyploidization of bone marrow megakaryocytes is due to a unique regulatory mechanism in late mitosis. *Journal of Cell Biology* **139**: 449-457.

Nagl W (1995). Cdc2-kinases, cyclins, and the switch from proliferation to polyploidization. *Protoplasma* **188**: 143-150.

Nakatani T, Inouye M, Mirochnitchenko O (1997). Overexpression of antioxidant enzymes in transgenic mice decreases cellular ploidy during liver regeneration. *Experimental Cell Research* **236**: 137-146.

Nakayama K, Nagahama H, Minanishima YA, Matsumoto M, Nakamichi I, Kitagawa K, Shirane M, Tsunematsu R, Tsukiyama T, Ishida N, Kitagawa M, Nakayama K-I, Hatakeyama S (2000). Targeted disruption of *Skp2* results in accumulation of cyclin E and $p27^{Kip1}$, polyploidy and centrosome overduplication. *The EMBO Journal* **19**: 2069-2081.

Nasmyth K (1996a). At the heart of the budding yeast cell cycle. *Trends In Genetics* **12**: 405-412.

Nasmyth K (1996b). Viewpoint: Putting the cell cycle in order. *Science* **274**: 1643-1645.

Niculescu III AB, Chen X, Smeets M, Hengst L, Prives C, Reed SI (1998). Effects of $p21^{CIP1/WAF1}$ at both the G_1/S and the G_2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Molecular and Cellular Biology* **18**: 629-643.

Nislow C, Lombillo VA, Kuriyama R, Mcintosh JR (1992). A plus-end-directed motor enzyme that moves anti-parallel microtubules *in vitro* localizes to the interzone of mitotic spindles. *Nature* **359**: 543-547.

Notterman D, Young S, Wainger B, Levine AJ (1998). Prevention of mammalian DNA reduplication, following the release from the mitotic spindle checkpoint, requires p53 protein, but not p53-mediated transcriptional activity. *Oncogene* **17**: 2743-2751.

Nunez F, Chipchase MD, Clarke AR, Melton DW (2000). Nucleotide excision repair gene (ERCC1) deficiency causes G_2 arrest in hepatocytes and a reduction in liver binucleation: the role of p53 and p21. *FASEB Journal* **14**: 1073-1082.

Ohno A, Mochida S, Arai M, Fujiwara K (1995). ICAM-1 expression in hepatocytes following dissociation of cell-to-cell contact in rats. *Biochemical And Biophysical Research Communications* **214**: 1225-1231.

Oren R, Dabeva MD, Karnezis AN, Petkov PM, Rosencrantz R, Sandhu JP, Moss SF, Wang S, Hurston E, Laconi E, Holt PR, Thung SN, Zhu L, Shafritz DA (1999). Role of thyroid hormone in stimulating liver repopulation in the rat by transplanted hepatocytes. *Hepatology* **30**: 903-913.

Orton TC, Betton GR, Gould S, Jones HB, Sansom NT, Sidaway J (1999). Phenobarbitone-induced liver responses in wild type and p53 deficient mice. *Human & Experimental Toxicology* **18**: 54.

Osypiw JC, Allen RL, Billington D (1994). Subpopulations of rat hepatocytes separated by Percoll density-gradient centrifugation show characteristics consistent with different acinar locations. *Biochemistry Journal* **304**: 617-624.

Otto SP, Whitton J (2000). Polyploid incidence and evolution. *Annual Review of Genetics* **34**: 401-437.

Oudar O, Moreau A, Feldmann G, Scoazec J-Y (1998). Expression and regulation of intercellular adhesion molecule-1 (ICAM-1) in organotypic cultures of rat liver tissue. *Journal of Hepatology* **29**: 901-909.

Overturf K, Al-Dhalimy M, Ou C-N, Finegold M, Grompe M (1997). Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. *American Journal of Pathology* **151**: 1273-1280.

Overturf K, Al-Dhalimy M, Finegold M, Grompe M (1999). The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion. *American Journal of Pathology* **155**: 2135-2143.

Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G (1992). Cyclin A is required at two points in the human cell cycle. *The EMBO Journal* **11**: 961-971.

Pellizas CG, Coleoni AH, Costamagna ME, Di Fulvio M, Masini-Repiso AM (1998). Insulin-like growth factor I reduces thyroid hormone receptors in the rat liver. Evidence for a feed-back loop regulating the peripheral thyroid hormone action. *Journal of Endocrinology* **158**: 87-95.

Peters M, Vierling J, Gershwin ME, Milich D, Chisari FV, Hoffnagle JH (1991). Immunology and the liver. *Hepatology* **13**: 977-994.

Petersen BO, Lukas J, Sorensen CS, Bartek J, Helin K (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *The EMBO Journal* **18**: 396-410.

Piel M, Nordberg J, Euteneuer U, Bornens M (2001). Centrosome-dependent exit of cytokinesis in animal cells. *Science* **291**: 1550-1553.

Pieri C, Giuli C, Del Moro M, Piantanelli L (1980). Electron-microscopic morphometric analysis of mouse liver. II. Effect of ageing and thymus transplantation in old animals. *Mechanisms Of Ageing And Development* **13**: 275-283.

Pieri C, Guili C, Bertoni-Freddari C, Piantanelli L, Nagy IZs (1982). Thymic control of the polyploidization of hepatocytes during aging. *Experientia* **38**: 680-681.

Pihan GA, Doxsey SJ (1999). The mitotic machinery as a source of genetic instability in cancer. *Seminars in Cancer Biology* **9**: 289-302.

Pines J (1994). Cell cycle - p21 inhibits cyclin shock. *Nature* **369**: 520-521.

Pines J (1999). Four-dimensional control of the cell cycle. *Nature Cell Biology* **1**: E73-E79.

Polyak K, Lee MH, Erdjumentbromage H, Koff A, Roberts JM, Tempst P, Massague J (1994). Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**: 59-66.

Post J, Klein A, Hoffman J (1960). Responses of the liver to injury: Effects of age upon the healing pattern after acute carbon tetrachloride poisoning. *Archives of Pathology* **70**: 314-321.

Prives C, Hall PA (1999). The p53 pathway. *Journal of Pathology* **187**: 112-126.

Prost S, Bellamy COC, Cunningham DS, Harrison DJ (1998). Altered DNA repair and dysregulation of p53 in IRF-1 null hepatocytes. *FASEB Journal* **12**: 181-188.

Rajvanshi P, Liu D, Ott M, Gagandeep S, Gupta S (1995). Cell ploidy-based fractionation of hepatocyte subpopulations for highly efficient retroviral gene-transfer. *Hepatology* **22**: 452.

Rajvanshi P, Liu D, Ott M, Gagandeep S, Schilsky ML, Gupta S (1998). Fractionation of rat hepatocyte subpopulations with varying metabolic potential, proliferative capacity and retroviral gene transfer efficiency. *Experimental Cell Research* **244**: 405-419.

Ravnik SE, Wolgemuth DJ (1996). The developmentally restricted pattern of expression in the male germ line of a murine *cyclin A* , *cyclin A2*, suggests roles in both mitotic and meiotic cell cycles. *Developmental Biology* **173**: 69-78.

Reid R, Fu YS, Herschman BR, Crum CP, Braun L, Shah KV, Agronow SJ, Stanhope CR (1984). Genital warts and cervical cancer. VI. The relationship between aneuploid and polyploid cervical lesions. *American Journal of Obstetrics and Gynecology* **150**: 189-199.

Resnitzky D, Hengst L, Reed SI (1995). Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G_1 by $p27^{Kip1}$. *Molecular and Cellular Biology* **15**: 4347-4352.

Reynisdottir I, Polyak K, Iavarone A, Massague J (1995). Kip/Cip and INK4 CDK inhibitors cooperate to induce cell-cycle arrest in response to TGF-β. *Genes & Development* **9**: 1831-1845.

Rodd C, Schwartz HL, Strait KA, Oppenheimer JH (1992). Ontogeny of hepatic nuclear triiodothyronine receptor isoforms in the rat. *Endocrinology* **131**: 2559-2564.

Roghi C, Giet R, Uzbekov R, Morin N, Chartrain I, Le Guellec R, Couturier A, Dorée M, Philippe M, Prigent C (1998). The *Xenopus* protein kinase pEg2 associates with the centrosome in a cell cycle-dependent manner, binds to the spindle microtubules and is involved in bipolar mitotic spindle assembly. *Journal Of Cell Science* **111**: 557-572.

Rose MD, Fink GR (1987). Kar1, a gene required for function of both intranuclear and extranuclear microtubules in yeast. *Cell* **48**: 1047-1060.

Rosenberg AR, Zindy F, Le Deist F, Mouly H, Metezeau P, Brechot C, Lamas E (1995). Overexpression of human cyclin A advances entry into S phase. *Oncogene* **10**: 1501-1509.

Roy L, Coullin P, Vitrat N, Hellio R, Debili N, Weinstein J, Bernheim A, Vainchenker W (2001). Asymmetrical segregation of chromosomes with a normal metaphase/anaphase checkpoint in polyploid megakaryocytes. *Blood* **97**: 2238-2247.

Rugge M, Sonego F, Pollice L, Perilongo G, Guido M, Basso G, Ninfo V, Pennelli N, Gambini C, Guglielmi M, Fabiano A, Leandro G, Keeling JW (1998). Hepatoblastoma: DNA nuclear content, proliferative indices, and pathology. *Liver* **18**: 128-133.

Saeter G, Schwarze PE, Seglen PO (1988). Shift from polyploidizing to nonpolyploidizing growth in carcinogen-treated rat liver. *Journal Of The National Cancer Institute* **80**: 950-958.

Sancho-Tello M, Renau-Piqueras J, Baguena-Cervellera R, Guerri C (1987). A biochemical and stereological study of neonatal rat hepatocyte subpopulations. *Virchows Archiv B Cell Pathology Including Molecular Pathology* **54**: 170-181.

Sanz N, Diezfernandez C, Fernandezsimon L, Alvarez A, Cascales M (1995). Relationship between antioxidant systems, intracellular thiols and DNA ploidy in liver of rats during experimental cirrhogenesis. *Carcinogenesis* **16**: 1585-1593.

Sanz N, Diezfernandez C, Cascales M (1996). Variations of hepatic antioxidant systems and DNA ploidy in rats aged 2 to 8 months. *Biochimica Et Biophysica Acta-Molecular Basis Of Disease* **1315**: 123-130.

Sarafoff M, Rabes HM, Dörmer P (1986). Correlations between ploidy and initiation probability determined by DNA cytophotometry in individual altered hepatic foci. *Carcinogenesis* **7** : 1191-1196.

Sargent LM, Sanderson ND, Thorgeirsson SS (1996). Ploidy and karyotypic alterations associated with early events in the development of hepatocarcinogenesis in transgenic mice harboring c-myc and transforming growth factor alpha transgenes. *Cancer Research* **56**: 2137-2142.

Sauer K, Knoblich JA, Richardson H, Lehner CF (1995). Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic endoreduplication cycles of d*rosophila* embryogenesis. *Genes & Development* **9**: 1327-1339.

Saville MK, Watson RJ (1998). The cell-cycle regulated transcription factor B-Myb is phosphorylated by cyclin A/Cdk2 at sites that enhance its transactivation properties. *Oncogene* **17** : 2679-2689.

Scampini G, Nava A, Newman AJ, Torre PD, Mazué G (1993). Multinucleated hepatocytes induced by rifabutin in rats. *Toxicologic Pathology* **21**: 369-376.

Schild D, Ananthaswamy HN, Mortimer RK (1981). An endomitotic effect of a cell-cycle mutation of *Saccharomyces cerevisiae*. *Genetics* **97**: 551-562.

Schmidt EE, Schibler U (1995). Cell size regulation, a mechanism that controls cellular RNA accumulation: Consequences on regulation of the ubiquitous transcription factors Oct1 and NF-Y, and the liver-enriched transcription factor DBP. *Journal of Cell Biology* **128**: 467-483.

Schmucker DL (1990). Hepatocyte fine structure during maturation and senescence. *Journal of Electron Microscopy Technique* **14**: 106-125.

Schroder AJ, Blaheta RA, Scholz M, Kronenberger B, Encke A, Markus BH (1995). Effects of proinflammatory cytokines on cultivated primary human hepatocytes. *Transplantation* **59**: 1023-1028.

Schumacher JM, Golden A, Donovan PJ (1998). AIR-2: An aurora/Ipl1-related protein kinase associated with chromosomes and midbody microtubules is required for polar body extrusion and cytokinesis in *Caenorhabditis elegans* embryos. *Journal of Cell Biol*ogy **143**: 1635-1646.

Schwartz HL, Strait KA, Ling NC, Oppenheimer JH (1992). Quantitation of rat tissue thyroid hormone binding receptor isoforms by immunoprecipitation of nuclear triiodothyronine binding capacity. *Journal of Biological Chemistry* **267**: 11794-11799.

Schwarze PE, Pettersen EO, Shoaib MC, Seglen PO (1984). Emergence of a population of small, diploid hepatocytes during hepatocarcinogenesis. *Carcinogenesis* **5**: 1267-1275.

Schwarze PE, Saeter G, Armstrong D, Cameron RG, Laconi E, Sarma DSR, Preat V, Seglen PO (1991). Diploid growth-pattern of hepatocellular tumors induced by various carcinogenic treatments. *Carcinogenesis* **12**: 325-327.

Schwob E, Bohm T, Mendenhall MD, Nasmyth K (1994). The B-type cyclin kinase inhibitor $p40^{Sic1}$ Controls the G_1 to S transition in *Saccharomyces cerevisiae* . *Cell* **79**: 233-244.

Scotte M, Laquerriere A, Masson S, Hiron M, Teniere P, Hemet J, Lebreton JP, Daveau M (1997). Transforming growth factor α (TGF-α) expression correlates with DNA replication in regenerating rat liver whatever the hepatectomy extent. *Liver* **17**: 171-176.

Seglen PO (1997). DNA ploidy and autophagic protein degradation as determinants of hepatocellular growth and survival. *Cell Biology and Toxicology* **13**: 301-315.

Serrano M, Hannon GJ, Beach D (1993). A new regulatory motif in cell-cycle control causing specific- inhibition of cyclin D/cdk4. *Nature* **366**: 704-707.

Severin E, Willers R, Bettecken T (1984). Flow cytometric analysis of mouse hepatocyte ploidy. II. The development of polyploidy pattern infour mice stains with different life spans. *Cell and Tissue Research* **238**: 652.

Shackney SE, Smith CA, Miller BW, Burholt DR, Murtha K, Giles HR, Ketterer DM, Pollice AA (1989). Model for the genetic evolution of human solid tumours. *Cancer Research* **49**: 3344-3354.

Shaw PH, Held WA, Hastie ND (1983). The gene family for major urinary proteins: Expression in several secretory tissues of the mouse. *Cell* **32**: 755-761.

Sheikh MS, Rochefort H, Garcia M (1995). Overexpression of $p21^{WAF1/CIP1}$ induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. *Oncogene* **11**: 1899-1905.

Sherr CJ, Roberts JM (1995). Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes & Development* **9**: 1149-1163.

Shinagawa T, Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M (1991). Apoptosis in cultured rat hepatocytes: The effects of tumour necrosis factor α and interferon γ. *Journal of Pathology* **165**: 247-253.

Sigal SH, Brill S, Fiorino AS, Reid LM (1992). The liver as a stem-cell and lineage system. *American Journal of Physiology* **263**: G139-G148.

Sigal SH, Gupta S, Gebhard DF, Holst P, Neufeld D, Reid LM (1995). Evidence for a terminal differentiation process in the rat liver. *Differentiation* **59**: 35-42.

Sigal SH, Rajvanshi P, Gorla GR, Sokhi RO, Saxena R, Gebhard DRJr, Reid LM, Gupta S (1999). Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events. *American Journal of Physiology* **276**: G1260-G1272.

Sigrist SJ, Lehner CF (1997). *Drosophila fizzy*-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* **90**: 671-681.

Singer JD, Manning BM, Formosa T (1996). Coordinating DNA replication to produce one copy of the genome requires genes that act in ubiquitin metabolism. *Molecular and Cellular Biology* **16**: 1356-1366.

Smith JR, Pereira-Smith OM (1996). Replicative senescence: Implications for in vivo aging and tumour suppression. *Science* **273**: 63-67.

Snyder M, Davis RW (1988). SPA1: A gene important for chromosome segregation and other mitotic functions in *S. cerevisiae*. *Cell* **54**: 743-754.

Soltis DE, Soltis PS (1995). The dynamic nature of polyploid genomes. *Proceedings of the National Academy of Science of the United States of America* **92**: 8089-8091.

Speliotes EK, Uren A, Vaux D, Horvitz HR (2000). The survivin-like *C.elegans* BIR-1 protein acts with the aurora-like kinase AIR-2 to affect chromosomes and the spindle midzone. *Molecular Cell* **6**: 211-223.

Steele PRM, Yim APC, Herbertson BM, Watson J (1981a). Some cytofluorimetric studies of the nuclear ploidy of mouse hepatocytes. I. A simple method for isolation of hepatocyte nuclei using *in situ* perfusion of the liver. *British Journal of Experimental Pathology* **62**: 469-473.

Steele PRM, Yim APC, Herbertson BM, Watson J (1981b). Some flow cytofluorimetric studies of the nuclear ploidy of mouse hepatocytes .2. Early changes

in nuclear ploidy of mouse hepatocytes following carbon-tetrachloride administration - evidence for polyploid nuclei arrested in telophase. *British Journal of Experimental Pathology* **62**: 474-479.

Stevens A, Bancroft JD (1982) Proteins and nucleid acids. In: Stevens A, Bancroft JD (eds) Theory and Practice of Histological Techniques. 2nd edition. Churchill Livingstone, Edinburgh, pp 145-154

Stewart N, Hicks GG, Paraskevas F, Mowat M (1995). Evidence for a second cell cycle block at G2/M by p53. *Oncogene* **10**: 109-115.

Stewart ZA, Leach SD, Pietenpol JA (1999). $p21^{Waf1/Cip1}$ inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. *Molecular and Cellular Biology* **19**: 205-215.

Styles J, Elliot BM, Lefevre PA, Robinson M, Pritchard N, Hart D, Ashby J (1985). Irreversible depression in the ratio of tetraploid:diploid liver nuclei in rats treated with 3' -methyl-4-dimethylaminoazobenzene (3' M). *Carcinogenesis* **6**: 21-28.

Sun Y, Dilkes BP, Zhang C, Dante RA, Carneiro NP, Lowe KS, Jung R, Gordon-Kamm WJ, Larkins BA (1999). Characterization of maize (*Zea mays L.*) Wee1 and its activity in developing endosperm. *Proceedings of the National Academy of Science of the United States of America* **96**: 4180-4185.

Sweeney GD, Garfield RE, Jones KG, Latham AN (1978a). Studies using sedimentation velocity on heterogeneity of size and function of hepatocytes from mature male rats. *Journal of Laboratory and Clinical Medicine* **91**: 432-443.

Sweeney GD, Jones KG, Krestynski F (1978b). Effects of phenobarbital and 3-methylcholanthrene pretreatment on size, sedimentation velocity, and mixed function oxygenase activity of rat hepatocytes. *Journal of Laboratory and Clinical Medicine* **91**: 444-454.

Therman E, Sarto GE, Stubblefield PA (1983). Endomitosis - a reappraisal. *Human Genetics* **63**: 13-18.

Thomas JH, Botstein D (1986). A gene required for the separation of chromosomes on the spindle apparatus in yeast. *Cell* **44**: 65-76.

Thompson JD, Lumaret R (1992). The evolutionary dynamics of polyploid plants - origins, establishment and persistence. *Trends In Ecology & Evolution* **7**: 302-307.

Thony B, Neuheiser F, Kierat L, Blaskovics M, Arn PH, Ferreira P, Rebrin I, Ayling J, Blau N (1998). Hyperphenylalaninemia with high levels of 7-biopterin is associated with mutations in the *PCBD* gene encoding the bifunctional protein pterin-4a-carbinolamine dehydratase and transcriptional coactivator (DCoH). *American Journal of Human Genetics* **62**: 1302-1311.

Thorgeirsson S (1993). Commentary. Hepatic stem cells. *American Journal of Pathology* **142**: 1331-1333.

Thorgeirsson SS (1996). Hepatic stem cells in liver regeneration. *FASEB Journal* **10**: 1249-1256.

Timchenko NA, Wilde M, Kosai KI, Heydari A, Bilyeu TA, Finegold MJ, Mohamedali K, Richardson A, Darlington GJ (1998). Regenerating livers of old rats contain high levels of C/EBPα that correlate with altered expression of cell cycle associated proteins. *Nucleic Acids Research* **26**: 3293-3299.

Torres S, Diaz BP, Cabrera JJ, Diaz-Chico JC, Diaz-Chico BN, Lopez-Guerra A (1999). Thyroid hormone regulation of rat hepatocyte proliferation and polyploidization. *Gastrointestinal Liver Physiology* **39**: G155-G163.

Tsai JL, King KL, Chang CC, Wei YH (1992). Changes of mitochondrial respiratory functions and superoxide- dismutase activity during liver-regeneration. *Biochemistry International* **28**: 205-217.

Tsukada A, Ohkubo T, Sakaguchi K, Tanaka M, Nakashima K, Hayashida Y, Wakita M, Hoshino S (1998). Thyroid hormones are involved in insulin-like growth factor-I (IGF-I) production by stimulating hepatic growth hormone receptor (GHR) gene expression in the chicken. *Growth Hormone & IGF Research* **8**: 235-242.

Tulp A, Welagen JJMN, Emmelot P (1976). Separation of intact rat hepatocytes and rat liver nuclei into ploidy classes by velocity sedimentation at unit gravity. *Biochimica et Biophysica Acta* **451**: 567-582.

Uren AG, Beilharz T, O'Connel MJ, Bugg SJ, van Driel R, Vaux DL, Lithgow T (1999). Role for yeast inhibitor of apoptosis (IAP)-like proteins in cell division. *Proceedings of the National Academy of Science of the United States of America* **96**: 10170-10175.

Uryvaeva IV (1981). Biological signigicance of liver cell polyploidy: an hypothesis. *Journal Of Theoretical Biology* **89**: 557-571.

Usui T, Yoshida M, Abe K, Osada H, Isono K, Beppu T (1991). Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, K-252a. *The Journal of Cell Biology* **115**: 1275-1282.

Vallen EA, Scherson TY, Roberts T, Vanzee K, Rose MD (1992). Asymmetric mitotic segregation of the yeast spindle pole body. *Cell* **69**: 505-515.

Van Zandt G, Fry CG (1983). Hoechst 33342 staining of mouse bone marrow: Effects on colony-forming cells. *Cytometry* **4**: 40-46.

Vargas JL, O'Connor E, Roche E, Knecht E, Grisolia S (1987). Analysis by flow cytometry of rat hepatocytes from different acinar zones. *Biochemical And Biophysical Research Communications* **147**: 535-541.

Vindelov LL, Christensen IJ, Keiding N, Spangthomsen M, Nissen NI (1983a). Long-term storage of samples for flow cytometric DNA analysis. *Cytometry* **3**: 317-322.

Vindelov LL, Christensen IJ, Nissen NI (1983b). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **3**: 323-327.

Vindelov LL, Christensen IJ, Jensen G, Nissen NI (1983c). Limits of detection of nuclear-DNA abnormalities by flow cytometric DNA analysis - results obtained by a

set of methods for sample-storage, staining and internal standardization. *Cytometry* **3**: 332-339.

Vinogradov AE, Anatskaya OV, Kudryavtsev BN (2001). Relationship of hepatocyte ploidy levels with body size and growth rate in mammals. *Genome* **44**: 350-360.

Visser JWM (1980). Vital staining of haemopoietic cells with the fluorescent bis-benzimidazole derivatives Hoechst 33342 and 33258. *Acta Pathologica et Microbiologica Scandinavica* **Section A 274 (suppl.)** : 86-90.

Volpes R, van den Oord JJ, De Vos R, Depla E, De Ley M, Desmet VJ (1991). Expression of interferon-γ receptor in normal and pathological human liver tissue. *Journal of Hepatology* **12**: 195-202.

Waldman T, Lengauer C, Kinzler KW, Vogelstein B (1996). Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* **381**: 713-716.

Wang Z, Zhang Y, Kamen D, Lees E, Ravid K (1995). Cyclin D3 is essential for megakaryocytopoiesis. *Blood* **86**: 3783-3788.

Watanabe T, Tanaka Y (1982). Age-related alterations in the size of human hepatocytes. *Virchows Archiv B Cell Pathology Including Molecular Pathology* **39**: 9-20.

Watkins III JB, Thierau D, Schwarz LR (1992). Biotransformation in carcinogen-induced diploid and polyploid hepatocytes separated by centrifugal elutriation. *Cancer Research* **52**: 1149-1154.

Weber V, Wernitznig A, Hager G, Harata M, Frank P, Wintersberger U (1997). Purification and nucleic-acid-binding properties of a *Saccharomyces cerevisiae* protein involved in the control of ploidy. *European Journal of Biochemistry* **249**: 309-317.

Weeda G, Donker I, de Wit J, Morreau H, Janssens R, Vissers CJ, Nigg A, van Steeg H, Bootsma D, Hoeijmakers JHJ (1997). Disruption of mouse *ERCC1* results in a

novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Current Biology* **7**: 427-439.

Weglarz TC, Degen JL, Sandgren EP (2000). Hepatocyte transplantation into diseased mouse liver. Kinetics of parenchymal repopulation and identification of the proliferative capacity of tetraploid and octaploid hepatocytes. *American Journal of Pathology* **157**: 1963-1974.

Werling K, Szepesi A, Szentirmay Z, Schaff Z, Tulassay Z, Szalay F (2000). Effect of hepatitis C virus on hepatocyte proliferation and DNA ploidy in patients with chronic hepatitis C. *Zeitschrift fur Gastroenterologie* **38**: 553-554.

Wheatley DN (1972). Binucleation in mammalian liver. *Exp Cell Res* **74**: 455-465.

White JW, Swartz FJ, Swartz AF (1985). Excess glucose intake induces accelerated β-cell polyploidisation in normal mice: A possible deleterious effect. *Journal of Nutrition* **115**: 271-278.

Williams RA, Baak JP, Meijer GA, Charlton IG (1997). Exploring the possibility of DNA ploidy measurements in tissue sections using liver as a model. *Analytical & Quantitative Cytology & Histology* **19**: 19-29.

Winey M, Goetsch L, Baum P, Byers B (1991). *MPS1* and *MPS2*: A novel yeast genes defining distinct steps of spindle pole body duplication. *Journal of Cell Biology* **114**: 745-754.

Wojcik E, Dvorak C, Chianale J, Traber PG, Keren D, Gumucio JJ (1988). Demonstration by in situ hybridization of the zonal modulation of rat liver cytochrome P-450b and P-450e gene expression after phenobarbital. *Journal of Clinical Investigation* **82**: 658-666.

Wu H, Wade M, Krall L, Grisham J, Xiong Y, VanDyke T (1996). Targeted in vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development, and regeneration. *Genes & Development* **10**: 245-260.

Yamada T, Sogawa K, Kim JK, Izumi K, Suzuki Y, Muramatsu Y, Sumida T, Hamakawa H, Matsumoto K (1998). Increased polyploidy, delayed mitosis and reduced protein phosphatase-1 activity associated with excess copper in the Long Evans Cinnamon rat. *Research Communications in Molecular Pathology & Pharmacology* **99**: 283-304.

Yamauchi M, Potter JJ, Mezey E (1988). Lobular distribution of alcohol dehydrogenase in the rat liver. *Hepatology* **8**: 243-247.

Ye H, Holterman AX, Yoo KW, Franks RR, Costa RH (1999). Premature expression of the winged helix transcription factor HFH-11B in regenerating mouse liver accelerates hepatocyte entry into S phase. *Molecular and Cellular Biology* **19**: 8570-8580.

Yin L, Ghebranious N, Chakraborty S, Sheehan CE, Ilic Z, Sell S (1998). Control of mouse hepatocyte proliferation and ploidy by p53 and p53ser246 mutation *in vivo*. *Hepatology* **27**: 73-80.

Yonemura Y, Kawakita M, Masuda T, Fujimoto K, Takatsuki K (1993). Effect of recombinant human interleukin-11 on rat megakaryopoiesis and thrombopoiesis *in vivo*: comparative study with interleukin-6. *British Journal of Haematology* **84**: 16-23.

Zhang Y, Wang ZG, Ravid K (1996). The cell cycle in polyploid megakaryocytes is associated with reduced activity of cyclin B1-dependent Cdc2 kinase. *Journal of Biological Chemistry* **271**: 4266-4272.

Zhang Y, Wang Z, Liu D, Pagano M, Ravid K (1998). Ubiquitin-dependent degradation of cyclin B is accelerated in polyploid megakaryocytes. *J Biol Chem* **273**: 1387-1392.

Zimmet J, Ravid K (2000). Polyploidy: Occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system. *Experimental Hematology* **28**: 3-16.

Zimmet JM, Ladd D, Jackson CW, Stenberg PE, Ravid K (1997). A role for cyclin D3 in the endomitotic cell cycle. *Mol Cell Biol* **17**: 7248-7259.

Zong Z, Fujikawa-Yamamoto K, Li A, Yamaguchi N, Chang Y-G, Murakami M, Tanino M, Odashima S (2000). Cell membrane changes of structure and function in protein kinase inhibitor-induced polyploid cells. *Cell Prolif* **33**: 29-38.

JOURNAL OF CELLULAR PHYSIOLOGY 191:138–144 (2002)

ORIGINAL ARTICLES

Functional Analysis of Mouse Hepatocytes Differing in DNA Content: Volume, Receptor Expression, and Effect of IFNγ

NICOLA C. MARTIN,[1] CHRISTIAN T. MCCULLOUGH,[1] PETER G. BUSH,[2] LINDA SHARP,[2] ANDREW C. HALL,[2] AND DAVID J. HARRISON[1]*

[1]*Department of Pathology, University of Edinburgh, Medical School, Edinburgh, United Kingdom*

[2]*Department of Biomedical Sciences, University of Edinburgh, Medical School, Edinburgh, United Kingdom*

Polyploidy and binuclearity are characteristics of the mammalian liver. Increasing polyploidisation occurs with age and after administration of various drugs and chemicals. This study was designed to examine the function of ploidy by addressing several questions: (1) Does the increase in size of polyploid hepatocytes have any physiological function by altering surface receptor expression such as intercellular adhesion molecule-1 (ICAM-1, CD54) or IFNγR? and (2) Do polyploid cells respond differently to inflammatory cytokines such as interferon gamma (IFNγ)? We have developed a method to accurately measure the volume of live isolated hepatocytes using confocal microscopy and image analysis. Using flow cytometry, we have shown that the expression of ICAM-1 increases with increasing DNA content and IFNγR is not detectable on isolated mouse hepatocytes. Diploid (2n), tetraploid (4n) and octoploid (8n) hepatocytes were found to be equally susceptible to IFNγ-induced apoptosis in vitro. Although the function of polyploidy remains unanswered, we have described some of the characteristics of polyploidy in isolated hepatocytes and in vitro. J. Cell. Physiol. 191: 138–144, 2002.

The mammalian liver consists of hepatocytes with different DNA content or ploidy (Alfert and Geshwind, 1958; Carriere, 1967; Schulte-Hermann, 1974; Brodsky and Uryvaeva, 1977). Polyploid cells can be mononuclear or binuclear and it is important to distinguish between them. In this study, the term polyploid refers to cells with an increase in DNA content and includes both mononuclear and binuclear cells. However, where we have been able to separate mononuclear from binuclear cells, we make this distinction. In C3H male mice of 16 weeks of age, the majority of parenchymal cells are 4n (70%) and 80% are binuclear. Diploid and 8n cells make up 20% and 9.5% of the parenchymal population, respectively. The degree of polyploidisation varies among mammals. The human liver is predominantly 2n with polyploid hepatocytes occurring in later life (Kudryavtsev et al., 1993). Polyploidisation also increases after partial hepatectomy and after administration of various drugs and chemicals (Steele et al., 1981; Melchiorri et al., 1993; Hasmall and Roberts, 1997; Sigal et al., 1999; Martin et al., 2001).

There is no evidence to suggest why polyploidisation occurs in the liver and conflicting theories exist. One hypothesis suggests that it could be a protective response; cells containing more than the normal number of chromosomes may be protected from dominant expression of mutated oncogenes (Schwarze et al., 1984). Other theories suggest that polyploidisation is purely a terminal differential state associated with ageing (Sigal et al., 1995). A review by Gupta (2000) concluded that polyploid cells might be more susceptible to apoptosis, which could lead to organ failure and oncogenesis. To test these theories, we have examined some of the features of diploid and polyploid mouse hepatocytes, including cell volume, receptor expression and response to IFNγ.

Cell size and volume have previously been examined in mouse, rat and human livers using various methods including cytophotometry and microspectrophotometry (Epstein, 1967; Watanabe and Tanaka, 1982), stereological image analysis (Danielsen et al., 1986) and

Contract grant sponsor: Faculty of Medicine, University of Edinburgh; Contract grant sponsor: Scottish Hospitals Endowment Research Trust; Contract grant numbers: E06004, G30119.

*Correspondence to: David J. Harrison, Department of Pathology, University of Edinburgh, Medical School, Teviot Place, Edinburgh, EH8 9AG, UK. E-mail: david.harrison@ed.ac.uk

Received 14 May 2001; Accepted 7 September 2001

Published online in Wiley InterScience (www.interscience.wiley.com), 12 March 2002.
DOI: 10.1002/jcp.10057

Coulter counter and sedimentation velocities (Sweeney et al., 1979; Deschenes et al., 1981). In all of these studies, there is evidence that increasing cell size is proportional to increasing DNA content. Alterations in cell volume participate in a wide variety of functions including cell proliferation and cell death (Lang et al., 1998). We have used confocal microscopy and image analysis to investigate whether the increase in size of polyploid cells could provide any physiological function by reducing the density of receptors i.e., ICAM-1 or IFNγR on the surface and preventing or reducing injury or cell death.

ICAM-1 is a member of the immunoglobulin supergene family, which is expressed on hepatocytes after dissociation of cell-to-cell contact (Ohno et al., 1995), or after induction with pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNFα) and IFNγ (Bumgardner et al., 1998; Oudar et al., 1998). Expression of this adhesion molecule has been found to be important in certain inflammatory processes in the liver, including hepatitis and liver graft rejection (Schroder et al., 1995; Bumgardner et al., 1998). Therefore, any differential expression of ICAM-1 on 2n and polyploid cells could be clinically important.

IFNγ is thought to play a role in hepatitis in humans and mice (Peters et al., 1991; Gilles et al., 1992) and is known to cause G1 cell cycle arrest and apoptosis in hepatocytes (Shinagawa et al., 1991; Morita et al., 1995; Kano et al., 1997). IFNγ exerts its effects by first interacting with specific membrane receptors (Rubinstein et al., 1987; Langer and Pestka, 1988). Therefore, in addition to ICAM-1 expression, we have looked at the expression of the IFNγR subunits, α and β on 2n, 4n and 8n hepatocytes. We have studied whether the cells that undergo IFNγ-induced apoptosis are predominantly diploid or polyploid by examining the cells that remain alive after treatment with IFNγ using flow cytometry.

The aim of this study was to determine if polyploidy could provide a protective role either by altering the density of surface receptors or by responding differently to IFNγ. We have also developed a method to accurately measure the volume of live isolated hepatocytes of different ploidy and nuclearity.

MATERIALS AND METHODS

Isolation of hepatocytes and cell culture

Primary hepatocytes from C3H male, 6–12 weeks old, were isolated using a retrograde two-step liver perfusion procedure and purified by centrifugation through Percoll (Amersham Pharmacia Biotech, Little Chalfont, UK) (Bellamy et al., 1997). The hepatocytes were plated onto collagen-coated 8-well glass slides and 6-well culture plates (Life Technologies, Paisley, UK) in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM F12; Life Technologies) containing 2% FCS, gentamicin (50 mg/ml, Life Technologies), L-glutamine (4 mM; Life Technologies), dexamethasone (0.04 μg/ml; Sigma, Poole, UK), insulin-transferrin-Sodium selenite media supplement (ITS; Sigma). Cells were plated at a density of $0.3–0.4 \times 10^5/cm^2$. After 24 h, media was replaced with Chee's modified medium (Sigma) containing gentamicin, L-glutamine, dexamethasone, ITS and EGF (0.025 μg/ml; Sigma). IFNγ (100 U/ml) was administered in Chee's medium for 4 days. Controls were set up without IFNγ. Experiments were set up in 5 wells per condition and repeated three times. Each experiment showed the same trend and were combined. Values are given as mean ± standard deviation (S.D.).

Feulgen's staining for apoptosis

Apoptotic cells can be distinguished from necrotic cells by their distinct morphology. To determine the amount of apoptosis at each time-point, slides were fixed in Bouin's fixative (methanol (85%), acetic acid (5%), formalin (10% of 40% solution)) overnight at 4°C. After denaturing with 5N HCl for 45 min at room temperature, slides were stained in Schiff's reagent (Sigma) for 1 h and counterstained in light green (0.1%, Merck, Lutterworth, UK). A minimum of 500 cells was counted and the results were expressed as the percentage of apoptotic cells.

BrdU immunohistochemistry

Hepatocytes cultured on 8-well slides were given BrdU (1 μl/ml, Amersham) for 3 h prior to fixation in 80% ethanol. Slides were incubated in 5N HCl for 45 min at room temperature. Rat α-BrdU (Harlan Sera-labs, Loughborough, UK) diluted 1 in 100 in blocking solution (PBS, NRS (20%), Tween 20 (0.05%)) was added for 1 h. Negative controls omitted this antibody. Peroxidase-conjugated rabbit α-rat (Sigma) pre-absorbed with normal mouse serum (50%) and diluted 1 in 100 in blocking solution, was added for 30 min. Positive cells were visualised using DAB chromagen. Cells were counterstained with haematoxylin and light green. The results are expressed as the percentage of BrdU positive cells. A minimum of 500 cells was counted.

Hepatocyte DNA content

The DNA content of hepatocytes treated with IFNγ and controls was determined by flow cytometry. Isolated hepatocytes were compared with cells plated for 24 h to determine the plating efficiency of the different ploidy populations. Hepatocytes for analysis were fixed after the perfusion or were removed from culture plates using a trypsin (0.12%)/EDTA (0.02%) solution and cell scraper. Cells were fixed in cold 70% ethanol overnight at 4°C and stained with propidium iodide (PI, 20 μg/ml; Sigma), RNase (0.2 mg/ml; Sigma) and Triton X-100 (0.1%; Sigma) in phosphate buffered saline (PBS) for 15 min at 37°C. Flow cytometric analysis was carried out using a COULTER® EPICS® XL Flow Cytometer (Beckman-Coulter Electronics, Luton, UK). PI was excited at 488 nm and the red fluorescence emitted was detected at 620 nm. A minimum of 5,000 cells was counted. Mononuclear and binuclear cells with the same DNA content fell within the same gate and were not distinguished in this analysis.

Confocal imaging to measure cell volume

Isolated hepatocytes were plated on collagen-coated glass coverslips (in 6-well plates) in Chees's medium containing 2% FCS for ½ h at 37°C. The nucleic acid stain, SYTO 16 (5 μM; Molecular Probes, Cambridge Bioscience, Cambridge, UK) and CellTracker Orange (CTO; 5 μM) (Molecular Probes) were added in fresh

media for ½ h at 37°C. Glass coverslips were transferred to small petri dishes and covered in fresh media for confocal imaging. Confocal microscopy and calibration of the system for volume determination was carried out as previously described (Bush and Hall, 2001). Confocal images were acquired with the Leica TCS NT confocal system (Leica Microsystems, Heidelberg GmbH, Heidelberg, Germany). A 63× water immersion lens, fitted to the upright Leica DMRE microscope was used to collect quantitative fluorescent images of hepatocytes in optical Z-steps of ~1 μm. SYTO 16 was excited at 488 nm and CTO at 568 nm. A minimum of 300 measurements was taken for nuclear diameter analysis and 70 measurements for cell volume analysis. For the calibration of diameter, images of Flouresbrite Microspheres (mean 10 μm ± 0.1; Polysciences, Warrington, PA) were taken using the same procedure.

Measurement of nuclear diameter and cell volume

Image analysis was performed on a Silicon Graphics O_2 workstation (Silicon Graphics Inc., Mountain View, CA) running Bitplane (Bitplane Inc., Zurich, Switzerland), Imaris and VoxelShopPro Software. Volume measurements were carried out as previously described using an intensity threshold technique (Bush and Hall, 2001). The study utilised Calcein AM as the cytoplasmic fluorosphere. Due to the similar spectral response of Syto 16 to Calcein AM, our study utilised the red shifted dye, CTO. To check the validity of CTO, hepatocytes were co-loaded with CTO and Calcein AM and volume analysis was performed. Cell volumes obtained were consistent ($r^2 = 0.78$, $P > 0.05$). However, CTO derived volumes were an order of magnitude smaller (1.7× ± 0.25). Hence, volume measurements were amended to account for this. Nuclear diameter was measured using the same confocal images and software. Measurements were taken at the central plane of each image. Using this technique, the diameter of the 10 μm beads was 9.41 μm ± 0.65. Therefore, there was no need to adjust the nuclear measurements.

Receptor expression

ICAM-1 (CD54) expression was studied on isolated hepatocytes by staining cells with a FITC-conjugated, hamster α-mouse CD54 antibody (0.5 mg/ml; Pharmingen, San Diego, CA) diluted 1 in 10 in PBS with 0.1% sodium azide and 1% BSA for 1 h at 4°C. Isotype controls were included in analysis (FITC hamster α-IgG, group 1; Pharmingen). IFNγR expression was determined using an indirect method. Isolated hepatocytes were stained with purified rat α-mouse CD119 (IFNγR α chain, 0.5 mg/ml; Pharmingen) or rabbit α-mouse IFNγR β chain (200 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) after blocking with appropriate serum in PBS with 0.1% sodium azide and 1% BSA for 10 min. Cells were incubated for 1 h at 4°C. Isotype controls were included (purified rat IgG2a, κ; Pharmingen and normal rabbit IgG; Santa Cruz). Undiluted FITC conjugated secondary antibodies (FITC-α rat IgG2a and FITC-α rabbit IgG; Serotec, Oxford, UK) were incubated with cells for 30 min at 4°C. To determine the DNA content, cells were fixed in 70% ethanol and stained with PI, RNase and Triton X-100, as above. Receptor expression and DNA content was measured by flow cytometry using a BD FACS Calibur (Becton Dickinson, Oxford, UK). PI and FITC were both exited at 488 nm; the red fluorescence emitted by PI was detected at 620 nm and the green fluorescence emitted by FITC was detected at 525 nm. A minimum of 5,000 cells was counted and each experiment was carried out in triplicate. The data was normalised by making the intensity of fluorescence of 2n cells equal to 1 and calculating the intensity for 4n and 8n cells accordingly. Values of fluorescence intensity for 2n, 4n and 8n cells were plotted against average cell volume, determined by confocal imaging.

Statistics

GraphPad InStat Software (GraphPad Software Inc., Redfern, CA) was used for the following statistical analyses: The proportion of 2n and 8n cells within each ploidy population at time 0, after 5 days in culture (Control), after treatment with IFNγ (IFN-g) and before and after plating was compared using a one-way ANOVA. The proportion of 4n cells was compared using a Kruskal–Wallis test. The nuclear diameter of the 2n and 4n populations and the volume of the 2n, 2 × 2n, 4n and 2 × 4n cells were compared using an unpaired t-test with a Welch correction for volume. A paired t-test was used to compare the diameter of nuclei from the same cell. Linear regression analysis was carried out using Microsoft Excel Software (Microsoft Corporation, Redfern, CA).

RESULTS

Determination of ploidy and volume of hepatocytes using confocal microscopy

Images obtained using confocal microscopy and Imaris imaging software allowed the accurate determination of nuclear ploidy and absolute cell volume. The nuclear diameter of all nuclei measured was plotted as a frequency histogram and the diameter of 2n ranged from 7.0 to 9.0 μm and 4n ranged from 9.5 to 11.5 μm (Fig. 1). The nuclei falling within each range were found to be significantly different to each other ($P < 0.001$). There was no significant difference in the nuclear diameter of nuclei from the same cell. From the 300 nuclei analysed, only one 8n nuclei was measured and therefore, the accurate determination of the diameter of 8n nuclei was not possible. The proportion of 2n and 4n nuclei plotted on the frequency histogram was found to be similar to the proportion of 2n and 4n nuclei analysed by flow cytometry (data not shown).

The volume of binuclear 2n cells (2 × 2n; 7035 ± 2391 μm^3) and mononuclear 4n cells (4n; 7352 ± 1650 μm^3) was 1.7–1.8-fold greater than mononuclear 2n cells (2n; 4154 ± 1137 μm^3, $P < 0.001$). The volume of binuclear 4n cells (2 × 4n; 14326 ± 4453 μm^3) was 1.9–2.0-fold greater than 2 × 2n and 4n cells ($P < 0.001$). There was no significant difference in the volume of 2 × 2n and 4n cells (Fig. 2).

Fluorescence intensity of ICAM-1 (CD 54), but not of IFNγR, increases in relation to DNA content

Two-parameter flow cytometry was used to measure the expression of ICAM-1 or IFNγR and DNA content of hepatocytes. This was compared with the volume of

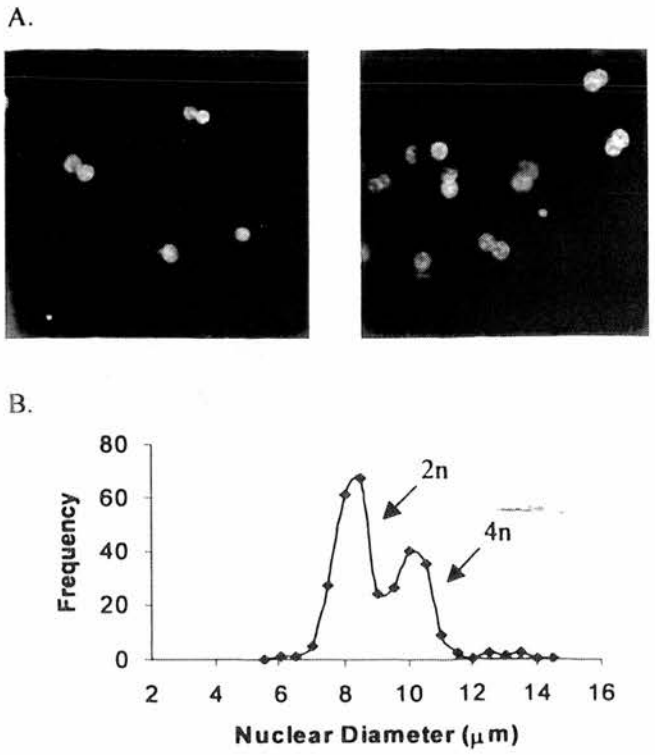


Fig. 1. Measurement of nuclear diameter of hepatocytes with different DNA contents. **A**: Confocal images of hepatocytes stained with dyes CTO (protein) and SYTO 16 (nucleic acid). **B**: Frequency histogram of nuclear diameter measured from confocal images and Imaris imaging software. Arrows indicate the peaks corresponding to 2n and 4n nuclei.

hepatocytes with different DNA contents measured by confocal imaging. Mononuclear and binuclear cells with the same DNA content cannot be distinguished using flow cytometry. Therefore, the volume of binuclear 2n ($2 \times 2n$) and mononuclear 4n cells was combined. The linear relationship between fluorescence intensity of ICAM-1 and average volume of 2n, 4n ($2 \times 2n$ and 4n) and 8n ($2 \times 4n$) cells was highly significant, demonstrating that expression of ICAM-1 increases in proportion to volume and DNA content ($r^2 = 0.907, P < 0.0001$)(Fig. 3). The fluorescence intensity of IFNγR was the same as

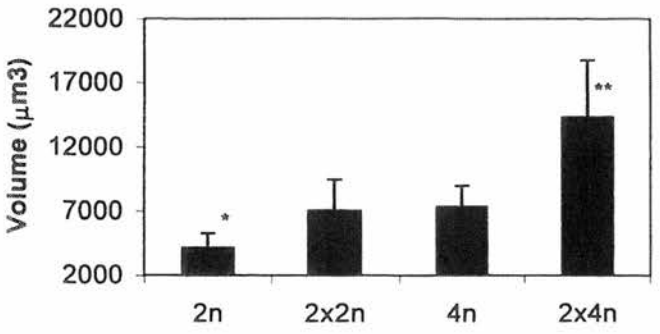


Fig. 2. Volume of hepatocytes measured from confocal images and Imaris imaging software. *Significantly lower than $2 \times 2n$ and 4n, $P < 0.05$. **Significantly higher than $2 \times 2n$ and 4n, $P < 0.05$. Values represent mean ± SD.

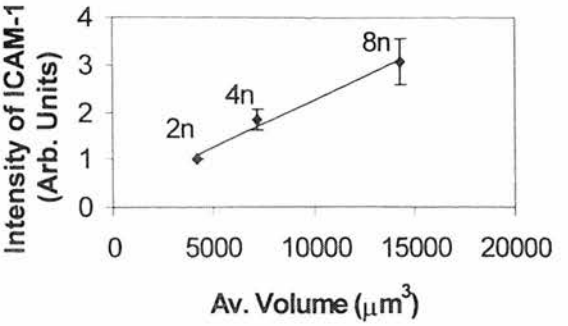


Fig. 3. Plot of average volume measured by confocal imaging against fluorescence intensity of ICAM-1 measured by flow cytometry for 2n, 4n and 8n hepatocytes. The values represent mean volume ± SD of 2n, 4n ($2 \times 2n$ and 4n) and 8n ($2 \times 4n$) cells. Graph shows the line of best fit, $r^2 = 0.907$, $P < 0.0001$.

isotype controls. Background fluorescence (isotype controls) was low and increased with increasing DNA content. However, the ratio of fluorescence intensity of ICAM-1 to background was equal for all cells and was therefore, excluded from the analysis.

IFNγ induces apoptosis in all ploidy populations equally

Feulgen staining was used to determine the amount of IFNγ-induced apoptosis. After 4 days administration of IFNγ, 30%–40% of hepatocytes had condensed fragmented chromatin and were visibly apoptotic (Fig. 4). Analysis of the hepatocytes that were alive after 4 days treatment using flow cytometry showed that the proportion of 2n (8.0 ± 1.6), 4n (65.8 ± 6.0) and 8n (15.1 ± 2.9) cells was the same as controls (2n 8.2 ± 1.4; 4n 67.1 ± 5.2; 8n 16.3 ± 3.0) (Fig. 5). This indicates that IFNγ induces apoptosis in all ploidy populations equally. The control hepatocytes showed a significant decrease in 4n cells (67.1 ± 5.2 c.f. 76.7 ± 1.9, $P < 0.001$) and increase in 8n cells (16.3 ± 3.0 c.f. 12.7 ± 2.8, $P < 0.05$) over 5 days in culture, (Fig. 5). BrdU positivity was apparent in mononuclear (13% ± 0.5) and binuclear cells (11% ± 2.8) after 4 days in culture. The proportion of 2n, 4n and 8n cell did not change after plating.

DISCUSSION

The reasons for increasing polyploidisation as a function of age or after the administration of toxic chemicals remain largely unknown. We have studied the different ploidy populations in the mouse liver to try and determine the differences between 2n, 4n and 8n cells with respect to cell volume, receptor expression and response to IFNγ.

We found that the volume of cells approximately doubled with doubling DNA content, that the volume of mononuclear and binuclear cells containing the same amount of DNA was the same and that the nuclei of binuclear cells were the same size. Other studies in humans, mice and rats, utilising methods such as cytophotometry and microspectrophotometry, stereological image analysis and coulter counter and sedimentation velocities have shown similar trends (Epstein, 1967; Sweeney et al., 1979; Deschenes et al., 1981; Watanabe and Tanaka, 1982). Such studies have

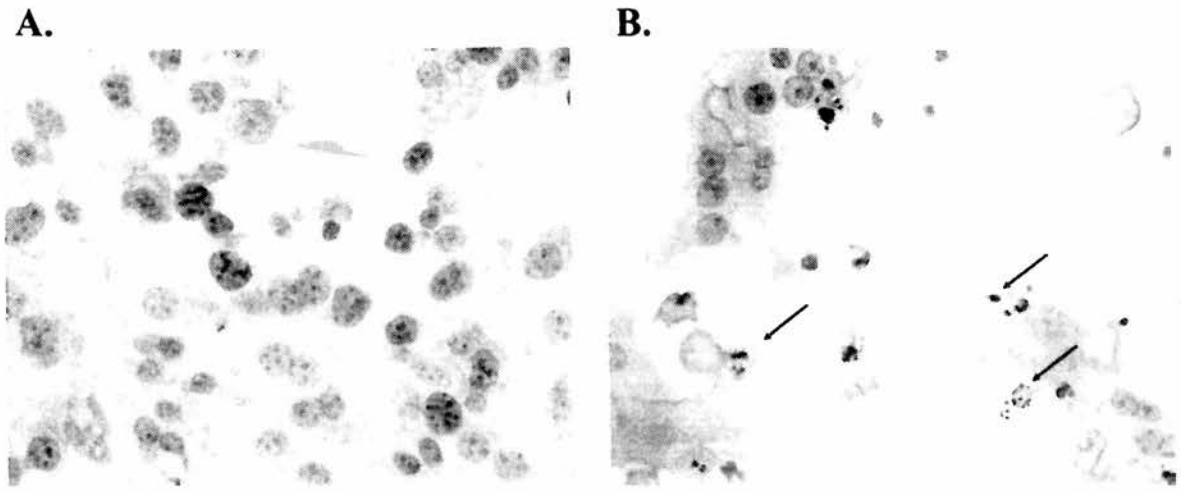


Fig. 4. Induction of apoptosis by IFNγ. Feulgen staining of hepatocytes, arrows indicate apoptotic nuclei. **A**: Control and (**B**) IFNγ-treated. Images were captured using a 40× objective.

relied on mathematical calculations and assumptions that cells are spherical in their calculation of cell size and volume. However, the use of confocal microscopy and image analysis negates these assumptions and calculations and therefore, this study provides the first accurate measurements of mouse hepatocyte cell volume. The actual measurements of nuclear diameter were slightly larger than those described by Danielsen et al. (1986) who measured cells within tissue sections and found the diameter of 2n mouse hepatocytes ranged between 6.0 and 7.5 μm and the diameter of 4n cells was between 7.5 and 9.0 μm. This was expected due to the shrinkage of cells during fixation.

Cell volume participates in a wide variety of functions including cell proliferation and cell death (Lang et al., 1998) and the possibility that the increase in size of polyploid cells could be involved in the regulation of these cells has not been studied. Schmidt and Schibler (1995) suggested that cell size and volume was a factor controlling the expression of various genes, including the leucine zipper protein, DBP although the ubiquitous transcription factors, Oct1 and NF-Y were not regulated by size. A study by Le Rumeur et al. (1981) found 4n hepatocytes produced twice as much albumin as 2n cells. Similarly, the production of succinate dehydrogenase and NADPH cytochrome C were found to be the same. However, the activity of lactate dehydrogenase was much lower in 2n cells (Tulp et al., 1976). This study proposed that one way increasing cell size could modulate the function or response of hepatocytes to signals was by altering the expression of molecules such as ICAM-1 and IFNγR on the cell surface.

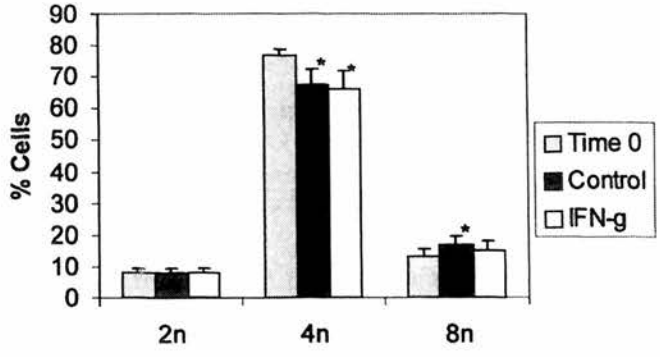


Fig. 5. DNA content of cells remaining after IFNγ treatment. Proportion of 2n, 4n and 8n cells in culture before treatment (Time 0) and with (IFN-g) or without (Control) IFNγ for 4 days. Values represent mean ± SD, n = 15. *Significantly different to Time 0, $P < 0.05$.

ICAM-1 is expressed on hepatocytes after dissociation of cell–cell contact (Ohno et al., 1995) and is important in inflammatory processes, including hepatitis and liver transplant rejection (Schroder et al., 1995; Bumgardner et al., 1998). Therefore, cell volume measurements were used in conjunction with flow cytometry to analyse ICAM-1 expression on 2n, 4n and 8n cells. Our results indicate that the fluorescence intensity of ICAM-1 increases in proportion to DNA content. As the volume of hepatocytes approximately doubles with doubling DNA content and the fluorescence intensity of ICAM-1 increases accordingly, the polyploid cells actually express a greater number of ICAM-1 molecules. Assuming that fluorescence intensity approximately equates to the number of ICAM-1 molecules on the surface, the density of ICAM-1 is actually greater on the polyploid cells, as surface area (sphere $= \pi r^2$) does not increase to the same extent as volume (sphere $= 4/3\pi r^3$). Whether or not the receptor density or actual number of receptors on hepatocytes is functionally important remains to be seen.

The expression of IFNγR was of particular interest as we were also studying the effects of IFNγ on different populations of hepatocytes. However, IFNγR α or β chains could not be detected in this study. IFNγR is not expressed at detectable levels on normal human hepatocytes (Volpes et al., 1991) and this could also be the case in the mouse. A study by LeClaire et al. (1992) demonstrated by immunocytochemical staining that the majority of immunologically reactive mouse IFNγR protein is intracellular.

Although IFNγR was not detectable on isolated hepatocytes, IFNγ did induce apoptosis in culture, in

agreement with other studies (Shinagawa et al., 1991; Morita et al., 1995; Kano et al., 1997). The mechanism through which IFNγ exerts its effects is largely unknown, although interferon regulatory factor-1 (IRF-1) has been shown to be essential in IFNγ-induced apoptosis (Kano et al., 1999). Analysis of gene expression in 2n, 4n and 8n cells would have been an interesting way to study the effect of IFNγ on these cells. However, the time course of effects following IFNγ is quite prolonged and the majority of cells at earlier time points are unaffected by IFNγ. Changes in gene expression before 72 h would be very small or undetectable. Therefore, we have examined the cells that remain alive after treatment with IFNγ for 4 days, to determine if polyploid cells are more susceptible to apoptosis.

The proportion of 2n, 4n or 8n cells was not altered by treatment with IFNγ for 4 days in culture. Therefore, it appears that 2n, 4n and 8n hepatocytes are equally sensitive to IFNγ-induced apoptosis in vitro. There is conflicting evidence regarding the susceptibility of polyploid cells to apoptosis and injury. A study by Melchiorri et al. (1993) found that after administration of the mitogen, lead nitrate, the elimination of excess cells by apoptosis did not occur preferentially in the polyploid population. In contrast, Oren et al. (1999) found that the rate of apoptosis was increased in polyploid cells after administration of the potent hepatic mitogen, triiodothyronine (T3) to rats pre-treated with the cell cycle inhibitor, retrorsine. Sigal et al. (1999) suggested that apoptosis accounted for the decrease in the proportion of 8n cells 5 days after a partial hepatectomy. Increased rates of apoptosis have also been found in the livers of Long-Evans Cinnamon (LEC) rats, where the liver has become populated with megalocytes, containing enormous nuclei (Gupta, 2000). The difference in the susceptibility of polyploid cells to apoptosis may be related to the type of polyploid cell or to the type of injury inflicted on the cell.

The plating efficiency of all hepatocytes was equal. However, after 5 days in culture the proportion of 4n cells decreased, the 8n cells increased and there was no change in the 2n population. BrdU immunohistochemistry showed that both mononuclear and binuclear cells proliferate in these cultures and time-lapse experiments demonstrated that the cells were capable of dividing (data not shown). Rat hepatocytes have been shown to undergo polyploidisation (Mossin et al., 1994) and cell fusion (Gomez-Lechon et al., 1981) in culture. Why the proportions of 4n and 8n cells alter while 2n cells remains the same in this study is unknown. However, it is likely that the proportions are altered through a combination of cell death, proliferation, polyploidisation and cell fusion and the change in each cell population will depend on the susceptibility of cells to each of these processes.

This work illustrates that the volume of hepatocytes approximately doubles with doubling DNA content and this increase in volume is accompanied by a proportionate increase in ICAM-1 expression. Whether the number of receptors is biologically relevant and whether all the hepatocyte populations respond equally to the signals received from such receptors is unknown. IFNγ-induced apoptosis in all hepatocytes in culture equally although IFNγR was not detectable on isolated cells. Further analysis of receptor expression and responses to cytokines such as IFNγ are required to help determine the function of polyploid hepatocytes and to determine whether polyploidisation is a protective response.

ACKNOWLEDGMENTS

The Faculty of Medicine, Whaitt Research Scholarship, University of Edinburgh supported Nicola Martin and Christian McCullough was supported by the Colin and Ethel Gordon Fellowship also from the Faculty of Medicine.

LITERATURE CITED

Alfert M, Geshwind II. 1958. The development of polysomaty in rat liver. Exp Cell Res 15:230–235.

Bellamy COC, Clarke AR, Wyllie AH, Harrison DJ. 1997. p53 deficiency in liver reduces local control of survival and proliferation, but does not affect apoptosis after DNA damage. FASEB J 11:591–599.

Brodsky WYa, Uryvaeva IV. 1977. Cell polyploidy: Its relation to tissue growth and function. Int Rev Cytol 50:275–332.

Bumgardner GL, Li J, Apte S, Heininger M, Frankel WL. 1998. Effect of tumour necrosis factor α and intercellular adhesion molecule-1 expression on immunogenicity of murine liver cells in mice. Hepatology 28:466–474.

Bush PG, Hall AC. 2001. Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. J Cell Physiol 187:304–314.

Carriere R. 1967. Polyploid cell reproduction in normal adult rat liver. Exp Cell Res 46:533–540.

Danielsen H, Lindmo T, Reith A. 1986. A method for determining ploidy distributions in liver tissue by stereological analysis of nuclear size calibrated by flow cytometric DNA analysis. Cytometry 7:475–480.

Deschenes J, Valet JP, Marceau N. 1981. The relationship between cell–volume, ploidy, and functional-activity in differentiating hepatocytes. Cell Biophys 3:321–334.

Epstein CJ. 1967. Cell size, nuclear content and the development of polyploidy in the mammalian liver. Proc Natl Acad Sci USA 57:327–334.

Gilles PN, Guerrette DL, Ulevitch RJ, Schreiber RD, Chisari FV. 1992. HBsAg retention sensitizes the hepatocyte to injury by physiological concentrations of interferon-γ. Hepatology 16:655–663.

Gomez-Lechon MJ, Barbera E, Gil R, Baguena J. 1981. Evolutive changes of ploidy and polynucleation in adult rat hepatocytes in culture. Cell Mol Biol 27:695–701.

Gupta S. 2000. Hepatic polyploidy and liver growth control. Cancer Biol 10:161–171.

Hasmall SC, Roberts RA. 1997. Hepatic ploidy, nuclearity, and distribution of DNA synthesis: A comparison of nongenotoxic hepatocarcinogens with noncarcinogenic liver mitogens. Toxicol Appl Pharmacol 144:287–293.

Kano A, Watanabe A, Takeda N, Aizawa S, Akaike T. 1997. Analysis of IFN-gamma-induced cell cycle arrest and cell death in hepatocytes. J.Biochem 121:677–683.

Kano A, Haruyama T, Akaike T, Watanabe Y. 1999. IRF-1 is an essential mediator in IFN-γ-induced cell cycle arrest and apoptosis of primary cultured hepatocytes. Biochem Biophys Res Commun 25:672–677.

Kudryavtsev BN, Kudryavtseva MV, Sakuta GA, Stein GI. 1993. Human hepatocyte polyploidisation kinetics in the course of life-cycle. Virchows Archiv B Cell Pathol 64:387–393.

Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D. 1998. Functional significance of cell volume regulatory mechanisms. Physiol Rev 78:247–306.

Langer JA, Pestka S. 1988. Interferon receptors. Immunol Today 9: 393–400.

Le Rumeur E, Beaumont C, Guillouzo C, Rissel M, Bourel M, Guillouzo A. 1981. All normal rat hepatocytes produce albumin at a rate related to their degree of ploidy. Biochem Biophys Res Commun 101:1038–1046.

LeClaire RD, Basu M, Pinson DM, Redick ML, Hunt JS, Zavodny PJ, Pace JL, Russell SW. 1992. Characterization and use of monoclonal and polyclonal antibodies against the mouse interferon-gamma receptor. J Leukoc Biol 51:507–516.

Martin NC, McGregor AH, Sansom N, Gould S, Harrison DJ. 2001. Phenobarbitone-induced ploidy changes in liver occur independently of p53. Toxicol Lett 119:109–115.

Melchiorri C, Chieco P, Zedda AI, Coni P, Ledda-Columbano GM, Columbano A. 1993. Ploidy and nuclearity of rat hepatocytes after compensatory regeneration or mitogen-induced liver growth. Carcinogenesis 14:1825–1830.

Morita M, Watanabe Y, Akaike T. 1995. Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. Hepatology 21:1585–1593.

Mossin L, Blankson H, Huitfeldt H, Seglen PO. 1994. Ploidy-dependent growth and binucleation in cultured rat hepatocytes. Exp Cell Res 214:551–560.

Ohno A, Mochida S, Arai M, Fujiwara K. 1995. ICAM-1 expression in hepatocytes following dissociation of cell-to-cell contact in rats. Biochem Biophys Res Commun 214:1225–1231.

Oren R, Dabeva MD, Karnezis AN, Petkov PM, Rosencrantz R, Sandhu JP, Moss SF, Wang S, Hurston E, Laconi E, Holt PR, Thung SN, Zhu L, Shafritz DA. 1999. Role of thyroid hormone in stimulating liver repopulation in the rat by transplanted hepatocytes. Hepatology 30:903–913.

Oudar O, Moreau A, Feldmann G, Scoazec J-Y. 1998. Expression and regulation of intercellular adhesion molecule-1 (ICAM-1) in organotypic cultures of rat liver tissue. J Hepatol 29:901–909.

Peters M, Vierling J, Gershwin ME, Milich D, Chisari FV, Hoofnagle JH. 1991. Immunology and the liver. Hepatology 13:977–994.

Rubinstein M, Novick D, Fischer DG. 1987. The human interferon-γ receptor system. Immunol Rev 97:29–50.

Schmidt EE, Schibler U. 1995. Cell size regulation, a mechanism that controls cellular RNA accumulation: Consequences on regulation of the ubiquitous transcription factors Oct1 and NF-Y and the liver-enriched transcription factor DBP. J Cell Biol 128:467–483.

Schroder AJ, Blaheta RA, Scholz M, Kronenberger B, Encke A, Markus BH. 1995. Effects of proinflammatory cytokines on cultivated primary human hepatocytes. Transplantation 59:1023–1028.

Schulte-Hermann R. 1974. Induction of liver growth by xenobiotic compounds and other stimuli. CRC Crit Rev Toxicol 5:1267–1275.

Schwarze PE, Pettersen EO, Shoaib MC, Seglen PO. 1984. Emergence of a population of small, diploid hepatocytes during hepatocarcinogenesis. Carcinogenesis 5:1267–1275.

Shinagawa T, Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M. 1991. Apoptosis in cultured rat hepatocytes: The effects of tumour necrosis factor α and interferon γ. J Pathol 165: 247–253.

Sigal SH, Gupta S, Gebhard DF Jr, Holst P, Neufeld D, Reid LM. 1995. Evidence for a terminal differentiation process in the rat liver. Differentiation 59:35–42.

Sigal SH, Rajvanshi P, Gorla GR, Sokhi RO, Saxena R, Gebhard DR Jr, Reid LM, Gupta S. 1999. Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events. Am J Physiol 276:G1260–G1272.

Steele PRM, Yim APC, Herbertson BM, Watson J. 1981. Some flow cytofluorimetric studies of the nuclear ploidy of mouse hepatocytes .2. Early changes in nuclear ploidy of mouse hepatocytes following carbon-tetrachloride administration: Evidence for polyploid nuclei arrested in telophase. Br J Exp Pathol 62:474–479.

Sweeney GD, Cole FM, Freeman KB, Patel HV. 1979. Heterogeneity of rat liver parenchymal cells: Cell volume as a function of DNA content. J Lab Clin Med 94:718–725.

Tulp A, Welagen JJMN, Emmelot P. 1976. Separation of intact rat hepatocytes and rat liver nuclei into ploidy classes by velocity sedimentation at unit gravity. Biochim Biophys Acta 451:567–582.

Volpes R, van den Oord JJ, De Vos R, Depla E, DeLey M, Desmet VJ. 1991. Expression of interferon-γ receptor in normal and pathological human liver tissue. J Hepatol 12:195–202.

Watanabe T, Tanaka Y. 1982. Age-related alterations in the size of human hepatocytes: A study of mononuclear and binucleate cells. Vichows Arch B Cell Pathol 39:9–20.

ELSEVIER

Toxicology Letters 119 (2001) 109–115

Toxicology Letters

www.elsevier.com/locate/toxlet

Phenobarbitone-induced ploidy changes in liver occur independently of p53

Nicola C. Martin [a], Angus H. McGregor [a,*], Nigel Sansom [b], Sarah Gould [b], David J. Harrison [a]

[a] *Medical School, Department of Pathology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, UK*
[b] *Safety of Medicines, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK*

Received 28 June 2000; received in revised form 3 November 2000; accepted 6 November 2000

Abstract

Liver polyploidisation, characterised by accumulation of tetraploid and octaploid cells, is found with increasing age and after administration of various drugs. The significance and mechanisms controlling polyploidisation are not understood but p53 is a candidate gene to be involved. We have investigated the effect of p53 on sodium phenobarbitone (PB)-induced liver proliferation and polyploidisation. Using p53 wild type (+/+), heterozygous (+/−) and homozygous (−/−) C57BL/6J mice, we measured ploidy and proliferation (BrdU incorporation) after 21 days oral administration of PB. Administration of PB caused a striking ploidy change compared with untreated controls, with an increase in 8n cells, and no difference noted comparing the p53 genotypes. BrdU positivity also increased significantly compared with controls, with the increase in BrdU positivity occurring in 8n cells. Our results confirm that PB is a hepatic mitogen that causes liver polyploidisation with a striking increase in 8n cells within the liver. p53 status does not appear to have any effect on this PB-induced ploidy change.

Keywords: Ploidy; DNA content; Phenobarbitone; p53

1. Introduction

Increasing ploidy with age characterised by accumulation of tetraploid and octaploid cells is found in both rodent and human liver (Schulte-Hermann, 1974; Brodsky and Uryvaeva, 1977; Gahan and Middleton, 1984). Toxic injury caused by various chemicals and partial hepatectomy also induce an increase in ploidy, usually associated with extensive but transient proliferation (Gerlyng et al., 1993; Melchiorri et al., 1993; Styles, 1993). Furthermore, polyploidisation has been associated with the development of hepatocellular carcinoma although its precise role and significance is uncertain. In some situations polyploidisation may provide protection against the dominant expression of mutated oncogenes (Schwarze et al.,

* Corresponding author. Tel.: +44-131-6502880; fax: +44-131-6506528.
E-mail address: angus.mcgregor@ed.ac.uk (A.H. McGregor).

0378-4274/01/$ - see front matter
PII: S0378-4274(00)00299-X

1984) and therefore, protect against tumour formation. However, the mechanisms regulating polyploidisation are not known and are only now beginning to be investigated in yeasts (Galitski et al., 1999). p53 has well-recognised roles in cell cycle control, mitotic spindle organisation and apoptosis and is a candidate gene to be involved in the control of polyploidisation although conflicting evidence exists regarding the role of p53 in polyploidisation in rodent liver (Bellamy et al., 1997; Yin et al., 1998).

Sodium phenobarbitone (PB) is a hepatic mitogen in rodents that causes liver enlargement by a combination of mitosis, an increase in cell size and an increase in ploidy (Bohm and Noltemeyer, 1981; Lin et al., 1989; Carthew et al., 1998). PB is also a non-genotoxic hepatocarcinogen (Whysner et al., 1996) causing development of hepatocellular carcinomas in male and to a lesser degree in female C57BL/10J mice (Jones, personal communication). However, while PB is a liver mitogen in C57BL/6 mice it is not carcinogenic (Evans et al., 1992) suggesting that the carcinogenic effect is strain dependent and occurs separately from mitogenesis. In vitro exposure of hepatocytes to PB delays and attenuates the G1 checkpoint response in a p53-dependent manner (Gonzales et al., 1998) suggesting that p53 may be one intracellular pathway through which PB acts (Yin et al., 1998).

We have studied PB-induced liver ploidy changes and proliferation in C57BL/6 wild type (+/+), p53 heterozygous (+/−) and p53 homozygous (−/−) male mice.

2. Materials and methods

2.1. Animals and animal procedures

All animal work was carried out by Zeneca Pharmaceuticals. Briefly, 5–6-week-old male C57BL/6 p53 +/+, +/− and −/− mice were purchased from Taconic Farms, Germantown, New York and acclimatised for 2 weeks prior to dosing. Animals were housed five per cage under appropriate conditions of temperature and humidity and with a 12-h artificial light cycle. Mice were fed powdered irradiated R and M No1 (modified) diet (Special Diet Services) containing PB (Sigma, UK; 1000 ppm) for 21 days; this regimen was chosen based on previous data (not shown). Control groups were fed a similar diet without PB. Food and water were available ad libitum. BrdU was administered continuously in drinking water (0.8 mg/ml) for 72 h before termination.

2.2. Preparation of liver cells

Mice were euthanised by inhalation of halothane (FLUOTHANE™, Zeneca Pharmaceuticals). The mouse body and liver weights were noted. A representative portion of liver tissue was removed from the right lobe, fixed in methacarn overnight and processed to paraffin for histological examination. Fine needle aspirates (FNA) were then taken using a 20-g needle and making at least six passes to include each lobe of the liver; cells were aspirated into citrate buffer (8.6% Sucrose, 1.2% trisodium citrate, 5% dimethyl sulfoxide (DMSO) in distilled water, pH 7.6). This technique yielded similar amounts of tissue from each case. FNA were stored at −70°C prior to use for the determination of ploidy and BrdU incorporation; samples were stable for several months.

For ploidy analysis, nuclei were prepared using the method of Vindelov (Vindelov et al., 1983a,b,c). The Vindelov technique is a robust and reliable method of measuring ploidy using isolated nuclei rather than intact cells; distinguishing mononuclear or binuclear cells is, therefore, not possible.

For assessment of proliferation, cells were double-labelled with propidium iodide (PI) and FITC-labelled anti-BrdU antibody. Fine needle aspirates were centrifuged at $300 \times g$ for 5 min and the supernatant was removed. Absolute ethanol was added for 20 min and samples were centrifuged, as before. The samples were washed twice with phosphate buffered saline (PBS). Pepsin (Sigma, UK; 0.2 mg/ml in 2 N hydrochloric acid) was added for 20 min and samples were washed twice with PBS and centrifuged at $1000 \times g$ for 10 min. Samples were transferred to a round-bottomed 96-well plate (NUNC™, Den-

mark), washed in PBS and spun at 1200 × *g* for 10 min. Rat monoclonal anti-BrdU antibody (Harlan Sera-lab, UK; diluted 1:100 in PBS, 0.5% Tween and 0.5% normal rabbit serum (NRS)) was added and the sample incubated overnight at 4°C. Samples were washed twice as above and resuspended in FITC-labelled rabbit anti-rat antibody (Serotec, UK; diluted 1:100 with PBS, Tween and NRS) for 30 min at room temperature. After two washes with PBS, PI (100 μg/ml; Sigma, UK) and RNase (0.04%; Sigma, UK) were added to samples on ice for 15 min.

2.3. Flow cytometry

DNA content and proliferation were evaluated using a COULTER®EPICS®XL Flow Cytometer. PI and FITC were both excited at 488 nm; the red fluorescence emitted by PI was detected at 620 nm and the green fluorescence emitted by FITC was detected at 525 nm. A minimum of 10 000 nuclei was counted to determine ploidy and BrdU profiles.

2.4. Histological assessment

Sections (4 μm) of liver were stained with haematoxylin and eosin and examined to detect any histopathological abnormalities. The number of cells was calculated, counting the total number of hepatocytes and non-parenchymal cells in ten high power fields (0.5 mm^2 per field). The number of hepatocytes was then multiplied by the liver weight to obtain the total number of hepatocytes for each liver (expressed in arbitrary units = hepatocytes per $5mm^2$ × liver weight/mouse weight).

2.5. Statistical analysis

PB-treated samples were compared with relevant controls using one-way ANOVA. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. PB caused an increase in liver weight and liver cell number

Phenobarbitone administration for 21 days resulted in a 39–60% increase in liver weight expressed as liver/body weight ratio compared with controls (Table 1; $P < 0.05$); no differences were identified relating to p53 genotype. The number of hepatocytes per liver also showed a modest increase after PB administration in p53 +/+ and −/− mice (16 and 21% increase, respectively) but not in the p53 +/− group compared with controls ($P < 0.05$). The proportion of non-parenchymal cells was the same in PB-treated and control mice for each genotype (Table 1). No histological abnormalities were identified in any group and in particular no carcinomas were seen; there was no evidence of hepatocyte apoptosis.

3.2. PB caused liver polyploidisation

Administration of PB for 21 days resulted in a striking increase (250–400%) in the proportion of octaploid nuclei compared with control mice (Fig. 1A), with no differences noted comparing the three p53 genotypes (Fig. 1B; $P < 0.05$). A small

Table 1
Effect of PB on liver weight and cell number[a]

Group	+/+		+/−		−/−	
	PB	Control	PB	Control	PB	Control
Liver weight	0.078* ± 0.008	0.050 ± 0.002	0.075* ± 0.003	0.047 ± 0.004	0.077* ± 0.004	0.055 ± 0.002
Total cell number	2244* ± 87	1940 ± 187	2034 ± 300	1848 ± 89	2315* ± 134	1919 ± 166
% NPC	32.78 ± 2.38	29.16 ± 3.34	33.23 ± 2.21	33.42 ± 3.08	31.68 ± 2.33	28.91 ± 2.11

[a] Liver weight is expressed per gram of total mouse weight; total cell number is the total number of hepatocytes per liver (cells per 5 mm^2 × liver weight); % NPC is the ratio of non-parenchymal cells to hepatocytes.* Represents a significant increase in PB-treated vs. control mice ($P < 0.05$).

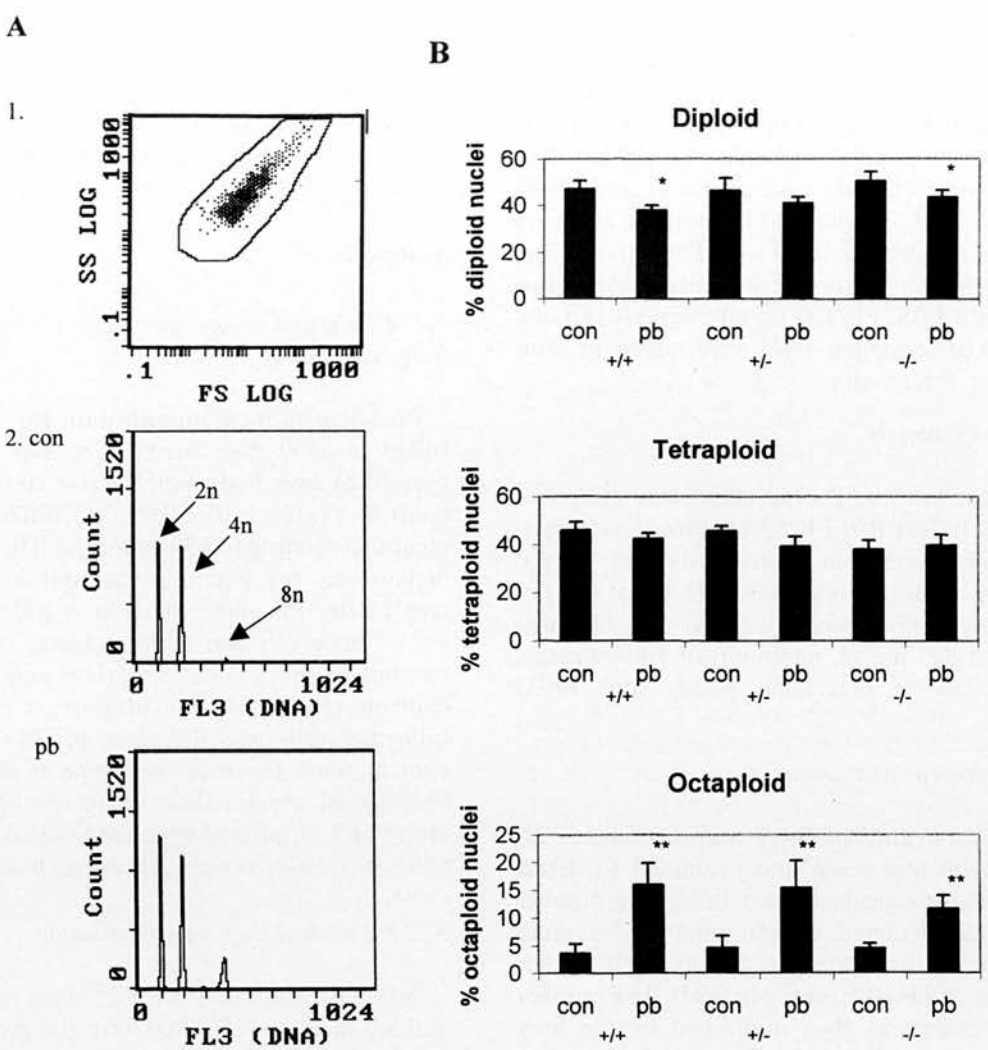


Fig. 1. (A) Flow cytometry histogram showing (1) side scatter (ss log, granularity) vs. forward scatter (fs log, size) and (2) numbers of events (count, nuclei) vs. ploidy (FL3 (DNA), PI uptake) for control (con) and PB-treated (pb) mice. Arrows indicate 2n, 4n,and 8n nuclei. (B) Proportion of diploid, tetraploid and octaploid nuclei after 21 days administration of PB. Comparison of proportion of nuclei in each ploidy class in control (con) and PB-treated (pb) groups of p53 $+/+$, $+/-$ and $-/-$ mice. * Indicates a significant decrease in cell number in PB-treated mice compared with controls ($P < 0.05$) ** Indicates a significant increase in cell number in PB-treated mice compared with controls ($P < 0.05$).

but significant reduction in 2n nuclei was also noted in all three genotypes ($P < 0.05$). No changes were identified in the tetraploid population.

3.3. PB increased hepatocyte proliferation

All untreated control samples showed similar baseline levels of BrdU positivity with no differences between p53 genotypes so the controls were combined. The proportion of BrdU positive nuclei was increased in all three genotypes after PB treatment (Fig. 2; $P < 0.05$) with p53 $+/+$ and $-/-$ mice also showing higher levels of BrdU positivity than p53 $+/-$ mice. Separating BrdU positivity according to ploidy showed increased

positivity in 8n cells only for all three p53 genotypes (Fig. 3; $P < 0.05$).

4. Discussion

We have shown that administration of PB for 21 days results in a striking change in liver ploidy with 250–400% increase in octaploid nuclei with a small reduction in 2n nuclei and no change tetraploid nuclei. No difference was noted whether p53 was present or not. Previous studies in mouse liver showed that PB caused an increase in tetraploid and octaploid cells with a concomitant reduction in diploid cells after 20 days (Bohm and Noltemeyer, 1981). This differs from our findings although the reason for this is not clear, possibly relating to the different strains of mice studied. While our ploidy data for PB relates specifically to octaploid nuclei rather than cells, an increase in mononuclear octaploid cells has been described following administration of a variety of other drugs including the non-carcinogenic hepatic mitogen 1,4-dichlorobenzene (Hasmall and Roberts, 1997), and the peroxisome proliferators WY-14 643 and methylclofenapate (Miller et al., 1996). Nuclearity of cells may also be important as other chemicals cause an increase in ploidy predominantly in binuclear or multinuclear cells (Melchiorri et al., 1993; Scampini et al., 1993) and binucleation has been proposed to be an intermediate stage in the formation of mononuclear polyploid cells (Alfert and Geschwind, 1958). Conflicting evidence exists regarding the effects of PB on hepatocyte nuclearity (Bohm and Noltemeyer, 1981; Maier and Schawalder, 1993).

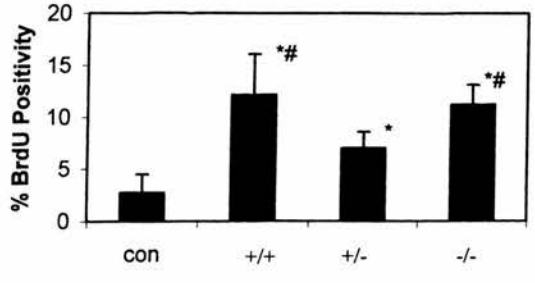


Fig. 2. Proportion of BrdU positive cells after 21 days administration of PB. Comparison of controls with PB-treated p53 +/+, +/− and −/− mice. * Indicates an increase compared with controls ($P < 0.05$); # Indicates an increase compared with p53 +/− mice.

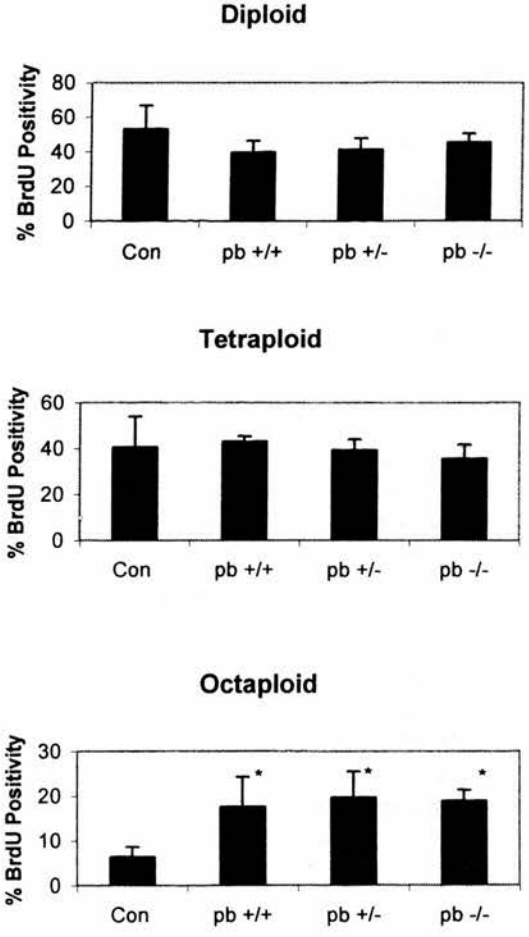


Fig. 3. Proportion of BrdU positive nuclei by ploidy class. Comparison of control and PB-treated mice from different p53 genotypes. * Indicates an increase compared with controls ($P < 0.05$).

Our data confirm that PB is mitogenic, causing an increase in liver weight and total liver cell number and a marked increase in BrdU positivity. Our results suggest that proliferation may be greater in p53 +/+ and −/− than +/− mice, with the increase in BrdU positivity occurring only in 8n nuclei. Similar differences in BrdU

positivity in cells of different ploidy have been described in other models, administration of DCB in rats showing most proliferation in mononuclear octaploid cells (Hasmall and Roberts, 1997). Immunostaining for BrdU in sections from the same cases as our study shows positivity in both mononuclear and binuclear hepatocytes (Orton et al. 1999) suggesting that both mononuclear and binuclear cells are responding to PB.

p53 knockout mice have been shown previously to have reduced age-dependent polyploidisation compared with wild type controls (Yin et al., 1998) while other data has shown that polyploidisation with age is p53-independent (Bellamy et al., 1997). Furthermore, mutations in p53 may control polyploidisation after administration of spindle depolarising agents (Gualberto et al., 1998). However, the role of p53 in polyploidisation induced by non-genotoxic agents is not known. Since the ploidy profile in our study was the same for the three groups of mice, we suggest that p53 is not a critical element in PB-induced liver polyploidisation for this strain of mouse. The mechanisms controlling polyploidisation are complex and it is probable that different pathways to polyploidy exist (for example, age-dependent vs. drug-induced) where different stimuli may activate different intracellular pathways.

References

Alfert, M., Geschwind, I., 1958. The development of polysomaty in rat liver. Exp. Cell Res. 15, 230–270.

Bellamy, C.O.C., Clarke, A.R., Wyllie, A.H., Harrison, D.J., 1997. p53 Deficiency in liver reduces local control of survival and proliferation, but does not affect apoptosis after DNA damage. FASEB J. 11, 591–599.

Bohm, N., Noltemeyer, N., 1981. Excessive reversible phenobarbital induced nuclear DNA-polyploidization in the growing mouse liver. Histochemistry 72, 63–74.

Brodsky, V.Y., Uryvaeva, I.V., 1977. Cell polyploidy: its relation to tissue growth and function. Int. Rev. Cytol. 50, 275–332.

Carthew, P., Edwards, R.E., Nolan, B.M., 1998. The quantitative distinction of hyperplasia from hypertrophy in hepatomegaly induced in the rat liver by phenobarbital. Toxicol. Sci. 44, 46–51.

Evans, J.G., Collins, M.A., Lake, B.G., Butler, W.H., 1992. The histology and development of hepatic nodules and carcinoma in C3H/He and C57BL/6 mice following chronic phenobarbitone administration. Toxicol. Pathol. 20, 585–594.

Gahan, P.B., Middleton, J., 1984. Euploidization of human hepatocytes from donors of different ages and both sexes compared with those from cases of Werners syndrome and progeria. Exp. Gerontol. 19, 355–358.

Galitski, T., Saldanha, A.J., Styles, C.A., Lander, E.S., Fink, G.R., 1999. Ploidy regulation of gene expression. Science 285, 251–254.

Gerlyng, P., Abyholm, A., Grotmol, T., Erikstein, B., Huitfeldt, H.S., Stokke, T., Seglen, P.O., 1993. Binucleation and polyploidization patterns in developmental and regenerative rat-liver growth. Cell Prolif. 26, 557–565.

Gonzales, A.J., Christensen, J.G., Preston, R.J., Goldsworthy, T.L., Tlsty, T.D., Fox, T.R., 1998. Attenuation of G(1) checkpoint function by the non-genotoxic carcinogen phenobarbital. Carcinogenesis 19, 1173–1183.

Gualberto, A., Aldape, K., Kozakiewicz, K., Tlsty, T.D., 1998. An oncogenic form of p53 confers a dominant, gain-of-function phenotype that disrupts spindle checkpoint control. Proc. Natl. Acad. Sci. USA 95, 5166–5171.

Hasmall, S.C., Roberts, R.A., 1997. Hepatic ploidy, nuclearity, and distribution of DNA synthesis: a comparison of nongenotoxic hepatocarcinogens with noncarcinogenic liver mitogens. Toxicol. Appl. Pharm. 144, 287–293.

Lin, E.L.C., Klaunig, J.E., Mattox, J.K., Weghorst, C.M., Mcfarland, B.H., Pereira, M.A., 1989. Comparison of the effects of acute and subacute treatment of phenobarbital in different strains of mice. Cancer Lett. 48, 43–51.

Maier, P., Schawalder, H., 1993. Physiological oxygen tension modulates the chemically induced mitogenic response of cultured rat hepatocytes. J. Cell. Physiol. 156, 119–129.

Melchiorri, C., Chieco, P., Zedda, A.I., Coni, P., Ledda-Columbano, G.M., Columbano, A., 1993. Ploidy and nuclearity of rat hepatocytes after compensatory regeneration or mitogen-induced liver growth. Carcinogenesis 14, 1825–1830.

Miller, R.T., Shah, R.S., Cattley, R.C., Popp, J.A., 1996. The peroxisome proliferators WY-14 643 and methylclofenapate induce hepatocyte ploidy alterations and ploidy-specific DNA synthesis in F344 rats. Toxicol. Appl. Pharm. 138, 317–323.

Orton, T.C., Betton, G.R., Gould, S., Jones, H.B., Sansom, N.T., Sidaway, J., 1999. Phenobarbitone-induced liver responses in wild type and p53 deficient mice. Hum. Exp. Toxicol. 18, 54.

Scampini, G., Nava, A., Newman, A.J., Torre, P.D., Mazue, G., 1993. Multinucleated hepatocytes induced by rifabutin in rats. Toxicol. Pathol. 21, 369–376.

Schulte-Hermann, R., 1974. Induction of liver growth by xenobiotic compounds and other stimuli. CRC Crit. Rev. Toxicol. 5, 1267–1275.

Schwarze, P.E., Pettersen, E.O., Shoaib, M.C., Seglen, P.O., 1984. Emergence of a population of small, diploid hepatocytes during hepatocarcinogenesis. Carcinogenesis 5, 1267–1275.

Styles, J.A., 1993. Measurement of ploidy and cell-proliferation in the rodent liver. Environ. Health Perspect. 101, 67–71.

Vindelov, L.L., Christensen, I.J., Nissen, N.I., 1983a. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3, 323–327.

Vindelov, L.L., Christensen, I.J., Jensen, G., Nissen, N.I., 1983b. Limits of detection of nuclear-DNA abnormalities by flow cytometric DNA analysis — results obtained by a set of methods for sample-storage, staining and internal standardization. Cytometry 3, 332–339.

Vindelov, L.L., Christensen, I.J., Keiding, N., Spangthomsen, M., Nissen, N.I., 1983c. Long-term storage of samples for flow cytometric DNA analysis. Cytometry 3, 317–322.

Whysner, J., Ross, P.M., Williams, G.M., 1996. Phenobarbital mechanistic data and risk assessment; enzyme induction, enhanced cell proliferation and tumour promotion. Carcinogenesis 71, 153–191.

Yin, L., Ghebranious, N., Chakraborty, S., Sheehan, C.E., Ilic, Z., Sell, S., 1998. Control of mouse hepatocyte proliferation and ploidy by p53 and p53ser246 mutation in vivo. Hepatology 27, 73–80.