Co-ordinate Regulation of Cellular Proliferation and Apoptosis in Rodent Liver

by

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Abstract

The balance between cellular proliferation and apoptosis is a co-ordinated process. Rodent hepatocytes proliferate rapidly in response to non-genotoxic liver growth agents such as peroxisome proliferators, a result of both increased DNA synthesis and suppression of apoptosis (Roberts *et al.*, 1995). This study aimed to identify groups of genes that were co-regulated in the response of rodent liver to mitogenic stimuli, and used three compounds known to induce liver growth via different processes: cyproterone acetate (60 mg/kg/day), dexamethasone (100 mg/kg/day) and Wy-14,643 (50 mg/kg/day). Each compound or vehicle was administered to groups of five male rats for up to four days, with animals being sacrificed at time-points between 6 and 96 hours post-initial dose. Total RNA from liver samples was processed and analysed by DNA microarray (Affymetrix). The expression profile for each gene was first compared between the three compounds across all time points, identifying 38 genes that could be clustered into groups showing similar expression profiles.

From these 38 genes, two groups were selected for further investigation and validation by quantitative real-time PCR analysis using a range of samples dosed with the same compounds over the same time-points. One group, composed of malic enzyme 1 (*Me1*) and rat brain acyl CoA hydrolase (*rBACH*), was found to respond only to peroxisome-proliferators, and hence was not investigated further. The second group, comprising seven genes, showed inhibition of gene expression in response to the compounds administered. A similar response was observed during quantitative real-time PCR validation as in the microarray analysis, therefore this group was investigated further using a reporter gene assay.

Promoter sequences immediately upstream of the transcriptional start site (1 kilobase) were successfully cloned into a plasmid upstream of a reporter gene (SEAP) for four of the genes, and were transfected into the FaO rat hepatoma cell line. Exposure of transfected cells to liver growth agents produced a similar profile of SEAP expression to that seen in the original *in vivo* analysis, suggesting regulation of the genes was at the transcriptional level. A deletion construct series was prepared for two of the genes, *HcaRG* and *Nob1p*, and the transcriptional response localised to

within the first ~100 bp of the proximal promoter in each case. Exposure of the ~100 bp reporter gene constructs to β -naphthoflavone (100 μ M), which activates both the aryl hydrocarbon receptor (AhR) and the NF-E2-related factor 2 (Nrf2), elicited a significant ≥ 2 -fold decrease in reporter gene expression, similar to the decrease elicited by the liver growth agents. Dosing with the AhR ligands 3-methylcholanthrene and benzo(a)pyrene failed to mimic the response of the promoter constructs to β -naphthoflavone indicating that AhR was not solely responsible for the inhibition of HCaRG and Nob1p expression observed. However, site-directed mutagenesis of individual potential AhR and Nrf2 response elements within the HCaRG and Nob1p promoter constructs failed to ablate the response to β -naphthoflavone or dexamethasone. This indicated either interplay between the two transcription factors in the response, or that the two transcription factors were capable of compensation.

This study has identified and confirmed a link between two genes exhibiting co-ordinate regulation in their expression in response to liver growth agent administration, with the potential of co-regulation with five other genes. It has identified the mediators of this response as being the AhR, Nrf2 or more likely a combination of the two transcription factors, with the potential of supporting the increasing observation that Nrf2 and the AhR co-operate in the regulation of genes in response to xenobiotic stimuli.

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Abbreviations

ACO	Acyl CoA oxidase
ANOVA	Analysis of variance
AP-1	Activator protein-1
Apaf	Apoptotic protease-activating factor
AhR	Aryl hydrocarbon receptor
AIF	Apoptosis inducing factor
ARE	Antioxidant response element
ARNT	AhR nuclear translocator
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist/killer 1
B(a)P	benzo(a)pyrene
Bax	Bcl-2 associated X protein
Bcl-2	B-cell CLL/lymphoma-2
BH	Bcl-2 homology
BLAST	Basic Local Alignment Search Tool
β -NF	β -naphthoflavone
Bok	Bcl-2-related ovarian killer
bp	base-pairs
BrdU	bromodeoxyuridine
CAT	chloramphenicol acetyltransferase
CAK	Cdk-activating kinase
CBF	CCAAT binding factor
CCD	charge-coupled device
cDNA	complementary DNA
CD	cluster of differentiation
CDC	cell division cycle

Cdk	Cyclin-dependent kinase
CFA	Clofibric acid
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
CHO	Chinese hamster ovary
CHX	cycloheximide
CKI	Cdk inhibitor protein
CLL	Chronic lymphocytic leukaemia
C _n	Copy number
CoA	Coenzyme A
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
CPA	Cyproterone acetate
CPTI	carnitine palmitoyltransferase I
C _t	Threshold cycle
CTD	carboxy-terminal domain
Cx	Connexin
CYP	Cytochrome
dATP	deoxyadenosine triphosphate
DEHP	di(2-ethylhexyl)phthalate
DEN	diethylnitrosamine
DEPC	Diethylpyrocarbonate
Dex	Dexamethasone
DISC	Death inducing signalling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Dnmt	DNA(cytosine-5)-methyltransferase
dNTP	deoxy nucleotide triphosphate
DR	direct repeats
EDTA	Ethylene diamine tetraacetic acid

EGF	Epidermal growth factor
EGFR	EGF receptor
EMSA	Electromobility shift assay
ER	Estrogen receptor
ERK	extracellular signal regulated kinase
EST	Expressed sequence tag
FADD	Fas-associated protein with death domain
FaO	Rat hepatoma cell line FaO
FBS	Foetal bovine serum
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
HAT	Histone acteyltransferase
HCA	Hierarchical cluster analysis
HCaRG	Hypertension-related, calcium-regulated gene
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HEK	Human embryonic kidney
HGF	Hepatocyte growth factor
HNF	Hepatocyte nuclear factor
HO-1	Heme oxygenase-1
IAP	Inhibitor of apoptosis protein
I- κ B	Inhibitor of NF- κ B
IKK	I- κ B kinase
IL	Interleukin
iNOS	inducible nitric oxide synthase
IPTG	isopropyl- β -D-thiogalactopyranoside
IVT	<i>in vitro</i> transcription
kb	kilobases
KEAP1	Kelch-like ECH-associated protein 1
LB	Luria Bertani medium

LCR	Locus control region
LDH	lactate dehydrogenase
LRCH4	Leucine-rich repeats and calponin homology (CH) containing 4
LRF	Liver regeneration factor
LTB4	leukotriene B4
MAPK	mitogen-activated protein kinase
3-MC	3-methylcholanthrene
MDR	Multidrug resistance protein
Me1	Malic enzyme 1
MeCP	methyl-CpG binding protein
MEN	Mitotic exit network
MiRNA	MicroRNA
MKK	MAP kinase kinase
MM	mismatch
mRNA	messenger ribonucleic acid
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear factor kappa B
Nob1p	nin-one binding protein
NPC	non-parenchymal cell
Nrf2	Nuclear factor erythroid 2-related factor 2
NF κ B	Nuclear Factor Kappa B
NQO1	NAD(P)H:quinine oxidoreductase
OGFRL1	Opioid growth factor receptor-like 1
OHdG	hydroxydeoxyguanosine
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCR	Polymerase Chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase

PH	partial hepatectomy
PKA	protein kinase A
PKC	protein kinase C
PM	perfect match
pol	polymerase
PP	Peroxisome proliferator
PPAR	Peroxisome proliferator activated receptor
PPRE	PPAR response element
PT	permeability transition
PUMA	p53-up-regulated mediator of apoptosis
RAR	retinoic acid receptor
Rb	Retinoblastoma
rBACH	rat Brain Acyl CoA Hydrolase
rCOUPg	rat Chicken ovalbumin upstream promoter gamma
RE	restriction enzyme
RIP	Receptor interactive protein
RLU	Relative luminescence unit
ROR α	RAR-related orphan receptor alpha
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	Reverse transcriptase
RXR	Retinoid X receptor
SD	Sprague-Dawley
SDM	Site-directed mutagenesis
SDS	sequence detection system
SEAP	Secreted Alkaline Phosphatase
SEM	Standard error of the mean
siRNA	small inhibitory RNA
SMAC	Second mitochondria-derived activator of caspase
Sp3	Specificity protein 3

STAT	Signal transducer and activator of transcription
TA1	tumour-associated protein 1
TAE	Tris acetate-EDTA (pH 8.3)
TAF	TBP-associated factor
tBHQ	tert-butylhydroquinone
TBP	TATA-binding protein
TCDD	tetrachlorodibenzo- <i>p</i> -dioxin
TE	Tris EDTA
TEF	Transcription elongation factor
TF	Transcription Factor
TGF	Transforming growth factor
T _m	Melting temperature
TNF	Tumour necrosis factor
TNFR	TNF receptor
TR	Thyroid hormone receptor
TRE	T3 response element
TRADD	TNFR-associated death domain
TRAF	TNFR associated factor
TRAIL	TNF-related apoptosis inducing ligand
TSS	Transcription start site
TUNEL	terminal transferase dUTP-biotin nick end labelling
UPGMA	unweighted pair group method with arithmetic averages
UTR	untranslated region
VDAC	voltage-dependent anion channel
wt	Wild-type
WY	Wy-14,643
XRE	xenobiotic response element

Table of Contents

1	Introduction	1
1.1	Gene Expression is regulated and co-ordinated	1
1.1.1	The transcription process	1
1.1.2	Regulation of transcription	3
1.1.2.1	Response elements	3
1.1.2.2	Transcription factors.....	4
1.1.2.3	Enhancers	4
1.1.2.4	Silencers.....	4
1.1.2.5	Locus control regions (LCRs)	5
1.1.3	Examples of co-ordinate gene regulation	5
1.2	Organ size is maintained by a balance between proliferation and apoptosis	7
1.2.1	The cell cycle	7
1.2.2	Control of Apoptosis	9
1.2.2.1	Pathways to apoptosis.....	10
1.2.2.2	The Bcl-2 family.....	13
1.2.3	Regulation of liver growth.....	15
1.2.3.1	Cell cycle priming	17
1.2.3.2	Cell cycle progression.....	20
1.3	Perturbation of liver homeostasis causes organ growth	23
1.3.1	Compounds causing liver growth	23
1.3.1.1	Dexamethasone.....	23
1.3.1.2	Cyproterone Acetate	24
1.3.1.3	Peroxisome Proliferators	25
1.3.1.3.1	Peroxisomes.....	26
1.3.1.3.2	β -oxidation in peroxisomes	26
1.3.1.3.3	Peroxisome Proliferator Activated Receptor- α	27
1.3.1.3.4	Species Differences in response to Peroxisome Proliferators.....	30
1.3.2	Mechanisms of hepatocarcinogenesis - evidence using Peroxisome Proliferators	33
1.3.2.1	Oxidative Stress	33
1.3.2.2	Control of cellular proliferation	34
1.3.2.3	Role in tumour promotion.....	39
1.3.3	Epigenetic mechanisms affecting carcinogenesis	39
1.3.3.1	DNA methylation.....	40
1.3.3.2	MicroRNAs	42

1.3.4	Summary	43
1.4	Aims of the Project.....	44
2	Materials and Methods	45
2.1	Materials.....	45
2.2	Bioinformatic analysis.....	45
2.2.1	Animal treatment and dosing.....	46
2.2.2	Microarray analysis	46
2.2.2.1	Pre-processing of Microarray data.....	47
2.2.2.2	Identification of co-ordinately regulated genes.....	48
2.2.2.3	Investigation of common response elements	50
2.3	Generic Molecular Biology Techniques.....	50
2.3.1	Agarose Gel Electrophoresis	50
2.3.2	Nucleic acid concentration determination.....	51
2.3.3	Growth of Bacterial Cultures.....	51
2.3.4	Preparation of glycerol stocks.....	51
2.3.5	Polymerase Chain Reaction (PCR)	52
2.3.5.1	Template DNA	52
2.3.5.2	Primer Design	52
2.3.5.3	PCR Protocol	53
2.3.6	Cloning	55
2.3.6.1	DNA extraction from agarose gels.....	55
2.3.6.2	Ligation and One Shot Transformation	55
2.3.6.3	Phenol/chloroform check for inserts	56
2.3.6.4	Preparation of plasmid DNA	56
2.3.6.4.1	Preparation of BAC clone DNA.....	56
2.3.6.4.2	Alkaline lysis preparation of plasmid DNA	57
2.3.6.4.3	QIAGEN Endo-free™ preparation of plasmid DNA.....	57
2.3.6.5	Sequence confirmation	58
2.3.6.5.1	Diagnostic restriction enzyme digest.....	58
2.3.6.5.2	DNA Sequencing.....	58
2.3.6.6	Deletion mutant construction	59
2.3.6.7	Site-directed mutagenesis (SDM).....	60
2.4	Cell based techniques.....	61
2.4.1	Recovery from liquid nitrogen.....	61
2.4.2	Maintenance culture	61
2.4.3	Dosing for <i>In vitro</i> experiments.....	62
2.4.4	Transient transfection of the FaO cell line.....	62

2.4.5	Protocol for chemiluminescent detection of SEAP protein.....	63
2.5	Quantitative Real-Time PCR Analysis (TaqMan [®]).....	63
2.5.1	Total RNA Extraction	64
2.5.2	RNA quantitation and integrity determination	64
2.5.3	DNase treatment and First strand cDNA synthesis	65
2.5.3.1	DNase treatment	65
2.5.3.2	cDNA synthesis	66
2.5.4	Primer and Probe Design.....	67
2.5.5	Genomic DNA standards	67
2.5.6	Real-time PCR protocol.....	69
2.6	Lactate Dehydrogenase (LDH) assay.....	70
2.7	Statistical Analysis	71
3	Microarray analysis of gene expression in rat liver following exposure to liver growth agents.....	72
3.1	Introduction	72
3.2	Genes and Expressed Sequence Tags identified exhibiting potential co-ordinate regulation.....	72
3.3	Microarray Analysis - Evidence of positive controls.....	80
3.3.1	Wy-14,643	80
3.3.2	Dexamethasone.....	82
3.3.3	Cyproterone acetate.....	83
3.4	Discussion	84
3.4.1	Genes suppressed in response to liver growth agent administration.....	85
3.4.2	Genes induced in response to liver growth agent administration.....	88
3.4.3	Summary	89
4	Validation and characterization of gene expression by <i>in vivo</i> and <i>in vitro</i> liver growth agent administration.....	91
4.1	Introduction	91
4.2	Validation of the microarray analysis by Quantitative real-time PCR	91
4.2.1	Genes suppressed in response to liver growth agent administration.....	92
4.2.2	Genes induced in response to liver growth agent administration.....	100
4.3	Reporter construct analysis of gene promoter activity.....	104
4.3.1	Cloning of 1 kilobase upstream gene promoters.....	104
4.3.1.1	Identification of gene promoters	104

4.3.1.1.1	HCaRG.....	104
4.3.1.1.2	Nob1p.....	105
4.3.1.1.3	LRCH4.....	106
4.3.1.1.4	Sp3.....	107
4.3.1.1.5	rCOUPg.....	107
4.3.1.1.6	OGFRL1.....	108
4.3.1.1.7	rBACH.....	109
4.3.1.1.8	Me1.....	109
4.3.1.2	Cloning of promoters into vector.....	110
4.3.2	Basal expression of 1 kilobase reporter gene constructs.....	113
4.3.3	Effect of liver growth agents on reporter gene constructs.....	114
4.3.3.1	Assessment of compound toxicity by the lactate dehydrogenase release assay.....	114
4.3.3.2	Dose response analysis.....	116
4.3.3.3	Time response analysis at optimum dose.....	123
4.4	Discussion.....	125
5	Target Examination and Mutagenesis.....	127
5.1	Introduction.....	127
5.2	Cloning and Analysis of deletion mutant constructs.....	128
5.2.1	Basal expression from deletion mutant constructs.....	132
5.2.2	Effect of liver growth agents on deletion mutant constructs.....	134
5.2.3	Discussion.....	137
5.3	Investigation of the key response elements in the control of HCaRG and Nob1p gene expression.....	138
5.3.1	<i>In silico</i> analysis of important regulatory regions.....	138
5.3.2	Dosing with β -naphthoflavone.....	140
5.3.3	Transcription factors exhibiting influence on HCaRG and Nob1p.....	142
5.3.4	Dosing with benzo(a)pyrene and 3-methylcholanthrene.....	146
5.3.5	Assessment of the involvement of Nrf2 and AhR in the control of HCaRG and Nob1p.....	150
5.3.5.1	Dosing with cycloheximide.....	150
5.3.5.2	Site-directed mutagenesis of AhR and Nrf2 regulatory elements.....	154
5.4	Discussion on co-ordinate regulation of HCaRG and Nob1p.....	159
6	General discussion.....	162
6.1	Discussion.....	162

6.2	Future directions	174
7	References.....	176
8	Appendix.....	191
8.1	Vector Maps.....	191
8.2	Oral presentations of this research.....	193
8.3	Poster presentations of this research	193

List of Figures

Figure 1.1: Pathways to caspase activation in apoptosis.....	12
Figure 1.2: Interactions between members of the Bcl-2 family of proteins.....	15
Figure 1.3: Model for the stages in liver regeneration	17
Figure 1.4: Proposed sequence of events in liver regeneration.....	19
Figure 1.5: β -oxidation pathway in peroxisomes.....	27
Figure 1.6: Scheme depicting the possible relationship between PPAR α and p38 MAP kinase in the control of hepatocyte growth	38
Figure 2.1: T7 primer for First strand cDNA synthesis from mRNA.....	46
Figure 2.2: Scheme outlining data mining process following Microarray analysis of SD rat liver transcript levels in response to liver growth agents.....	49
Figure 3.1: Dendrogram showing clustering of genes of interest using Hierarchical Cluster Analysis (HCA).....	77
Figure 3.2: Dendrogram showing clustering of the two groups of genes selected for further investigation.....	78
Figure 3.3: Summary of known literature on potentially co-regulated genes.....	88
Figure 4.1: Expression of HCaRG in response to administration of liver growth agents.....	94
Figure 4.2: Expression of Nob1p in response to administration of liver growth agents.....	95
Figure 4.3: Expression of rCOUPg in response to administration of liver growth agents.....	96
Figure 4.4: Expression of Sp3 in response to administration of liver growth agents.....	98
Figure 4.5: Expression of LRCH4 in response to administration of liver growth agents	99
Figure 4.6: Expression of OGFRL1 and AI044183 in response to administration of liver growth agents.....	99
Figure 4.7: Expression of Me1 in response to administration of liver growth agents.....	102
Figure 4.8: Expression of rBACH in response to administration of liver growth agents.....	103
Figure 4.9: Chromosomal contig references for the cloned region of the HCaRG promoter.....	105
Figure 4.10: Chromosomal contig references for the cloned region of the Nob1p promoter.....	106
Figure 4.11: Chromosomal contig references for the cloned region of the LRCH4 promoter.....	106
Figure 4.12: Chromosomal contig references for the cloned region of the Sp3 promoter	107
Figure 4.13: Chromosomal contig references for the cloned region of the rCOUPg promoter.....	108
Figure 4.14: Chromosomal contig references for the cloned region of the OGFRL1 promoter.....	108
Figure 4.15: Chromosomal contig references for the cloned region of the rBACH promoter.....	109
Figure 4.16: Chromosomal contig references for the cloned region of the Me1 promoter	110
Figure 4.17: Diagnostic digests of pHCaRG-SEAP using <i>Bgl</i> II and <i>Not</i> I.....	111
Figure 4.18: Diagnostic digests of pNob1p-SEAP using <i>Bgl</i> II and <i>Not</i> I.....	111

Figure 4.19: Diagnostic digests of pSp3-SEAP using <i>Spe I</i> and <i>Not I</i>	112
Figure 4.20: Diagnostic digests of pLRCH4-SEAP using <i>Spe I</i> and <i>Not I</i>	112
Figure 4.21: Diagnostic digests of pRBACH-SEAP using <i>Pst I</i>	113
Figure 4.22: Basal expression of SEAP under control of cloned gene promoters of interest.....	114
Figure 4.23: Effect of exposure of test compounds on cultured FaO cell viability by LDH assay.....	116
Figure 4.24: Dose response of HCaRG and Nob1p promoter constructs to dexamethasone	118
Figure 4.25: Dose response of HCaRG and Nob1p promoter constructs to CPA.....	120
Figure 4.26: Dose response of HCaRG and Nob1p promoter constructs to CFA.....	121
Figure 4.27: Dose response of HCaRG and Nob1p promoter constructs to Wy-14,643.....	122
Figure 4.28: Effect of liver growth agents on HCaRG and Nob1p promoter constructs with time.....	124
Figure 5.1: Strategy for generation of deletion constructs.....	128
Figure 5.2: Generation of deletion constructs and diagnostic analysis using <i>Not I</i>	130
Figure 5.3: Basal expression of SEAP from HCaRG and Nob1p deletion constructs.....	133
Figure 5.4: HCaRG and Nob1p deletion construct analysis following dosing with liver growth agents.....	136
Table 5.2: Potential transcription factor binding sites common to HCaRG and Nob1p proximal promoter regions.....	139
Figure 5.5: Effect of β -naphthoflavone on SEAP expression controlled by HCaRG and Nob1p proximal promoter regions.....	141
Figure 5.6: Location of potential Nrf2 and AhR response elements for HCaRG and Nob1p.....	143
Figure 5.7: Structure of mRNA for HCaRG and Nob1p	145
Figure 5.8: Dose response curve for 3-methylcholanthrene administration to FaO cells transfected with HCaRG and Nob1p promoter constructs.....	147
Figure 5.9: Dose response curve for benzo(a)pyrene administration to FaO cells transfected with HCaRG and Nob1p promoter constructs.....	148
Figure 5.10: Effect of CHX dosing on SEAP expression from HCaRG-93 and Nob1p-71 constructs	151
Figure 5.11: Effect of CHX dosing on HCaRG and Nob1p expression in FaO cells by quantitative RT-PCR	153
Figure 5.12: Strategy for generation of mutant constructs by site-directed mutagenesis.....	154
Figure 5.13: Basal expression of SEAP from HCaRG and Nob1p wild-type and mutant constructs	156
Figure 5.14: Effect of mutation of the potential Nrf2 response element in the HCaRG-93 bp construct.....	157
Figure 5.15: Effect of mutation of the potential AhR response element in the Nob1p-71 bp construct.....	157
Figure 5.16: Effect of mutation of the potential Nrf2 response element in the Nob1p-312 bp construct.....	158
Figure 6.1: Summary of findings for co-ordinate regulation in response to liver growth agents.....	164

Figure 6.2: Proposed model for the pathway from liver growth agent administration to
liver growth 171

Figure 8.1: pSEAP plasmid 191

Figure 8.2: TOPO® pCR®2.1 plasmid (Invitrogen) 192

Figure 8.3: pCR®-Blunt plasmid (Invitrogen) 192

List of Tables

Table 1.1: PPRE sequences identified for a number of genes induced by PPs.....	29
Table 2.1: List of suppliers of reagents and materials used in the project.....	45
Table 2.2: Bacterial Artificial Chromosome clones (CHORI) used as PCR templates	52
Table 2.3: Primer sequences for PCR of genes of interest	53
Table 2.4: Volumes used in PCR reactions (Taq polymerase).....	54
Table 2.5: Volumes used in PCR reactions (<i>Pfx</i> polymerase).....	54
Table 2.6: Example temperature program for PCR amplifications.....	54
Table 2.7: Optimised annealing temperatures and enzymes for genes cloned.....	54
Table 2.8: pCR [®] 2.1-TOPO ligation reaction	55
Table 2.9: Reagent volumes for confirmatory Restriction Enzyme digests	58
Table 2.10: Primers and restriction endonucleases used to generate deletion constructs.....	59
Table 2.11: Primer annealing temperatures (T_m) for HCaRG and Nob1p deletion mutants.....	59
Table 2.12: Primer sequences for HCaRG and Nob1p SDM mutants.....	60
Table 2.13: Primer annealing temperatures (T_m) and enzymes for SDM mutants.....	61
Table 2.14: Temperature program for cDNA synthesis.....	67
Table 2.15: ABI Prism Primer Express design criteria	68
Table 2.16: Primer and probe sequences for TaqMan Real-Time PCR.....	68
Table 2.17: Reagent volumes for preparation of TaqMan bulk mix	69
Table 2.18: Thermal cycling conditions for TaqMan real time PCR.....	69
Table 3.1: Fragments identified as showing potential importance in liver growth regulation (across 3 compounds).....	74
Table 3.2: Expression changes for genes selected for further investigation	79
Table 3.3: Positive controls from Microarray analysis of rat liver following WY administration	81
Table 3.4: Positive controls from Microarray analysis of rat liver following DEX administration	83
Table 3.5: Positive controls from Microarray analysis of rat liver following CPA administration	84
Table 5.1: PCR primers and restriction enzymes used to generate deletion constructs.....	129
Table 5.3: EC ₅₀ values for β -NF, 3-methylcholanthrene and benzo(a)pyrene	149

1 INTRODUCTION

1.1 GENE EXPRESSION IS REGULATED AND CO-ORDINATED

In prokaryotes, genes encoding proteins required for a co-ordinated response to a signal are found close together in the genome and form an operon. Transcription of the operon forms a single, multi-gene mRNA with internal initiation codons defining the start of individual genes. An example is the *lac* operon that is transcribed in response to lactose. The *lac* operon contains genes encoding the enzyme β -galactosidase that is required for cleavage of lactose, a permease enzyme to allow lactose uptake by the cell, and a trans-acetylase to modify the molecule. All three genes are under the control of the *lac* operator whose repressor protein is inactivated by the presence of lactose (reviewed in Lewis, 2005). This form of co-ordinated response is very simple and is suitable for single-celled organisms. However, in higher eukaryotic organisms that are composed of many cells requiring intercellular communication to exist, this simple co-ordination does not occur. In these organisms individual genes are transcribed producing separate mRNAs from each gene. This allows greater flexibility in the regulation of gene expression, unlike prokaryotes where the need of one gene will result in the expression of several others. In addition, co-ordinately expressed genes in eukaryotes are not frequently found close together in the genome.

A mechanism for eukaryotic co-ordinate gene expression was proposed by Britten and Davidson (1969). They suggested that genes co-ordinately regulated in response to a signal would contain a common regulatory element that would cause activation of the gene. Genes could contain many such regulatory elements and could therefore be activated by a variety of stimuli. It is now known that signals result in the expression or activation of transcription factors, which can then interact with DNA sequence motifs termed response elements, located within or adjacent to gene promoters. Binding of a transcription factor leads to activation of the gene(s) containing the response element, resulting in production of functional protein that can produce an effect.

1.1.1 THE TRANSCRIPTION PROCESS

Transcription of genes involves the enzyme RNA polymerase. In eukaryotes there are three classes of RNA polymerase that are involved in the formation of different types of RNA. RNA polymerase

II (RNA pol II) is responsible for the transcription of genes capable of encoding a protein, and produces an mRNA transcript. RNA pol I and III are involved in the transcription of genes encoding the ribosomal and transfer RNAs.

Eukaryotic RNA polymerases do not directly bind to DNA. Instead they require transcription factors that recognise specific DNA sequences and target the polymerase to the site of transcriptional initiation. This is achieved through the formation of an initiation complex. In many eukaryotic genes transcribed by RNA pol II a short DNA sequence containing A and T nucleotides can be found, existing around 30 bp upstream of the transcriptional start site. This is known as a TATA box and is important in determining the point of transcriptional initiation. The TATA box acts as a target sequence on which the transcription complex for RNA pol II can assemble.

The first factor to bind the TATA box is the transcription factor TFIID and this is facilitated by another factor TFIIA. TFIID is actually composed of the TATA-binding protein (TBP) that is responsible for the binding to the TATA box, and TBP-associated factors (TAFs) that are thought to respond to transcriptional activators. Following formation of the TFIID/TFIIA complex (DA complex), this is recognised by TFIIB (Maldonado *et al.*, 1990) that binds to RNA pol II via TFIIF, recruiting it to the pre-initiation complex (PIC). A further two factors, TFIIIE and TFIIH then bind to the complex. TFIIH has an intrinsic kinase activity that phosphorylates RNA pol II leading to initiation of transcription (Woychik and Hampsey, 2002). The polymerase and its associated proteins then move along the DNA strand towards the termination site. However, TFIID and TFIIA remain at the promoter allowing further recruitment of TFIIB and RNA pol II, to allow repeated rounds of transcription of the same gene.

Recently a complex termed Mediator was identified that binds RNA pol II following formation of the DA complex in response to an activator i.e. a DNA-binding protein that regulates genes by affecting the rate of transcription (Myers and Kornberg, 2000). Mediator does not interact directly with DNA, but binds the RNA pol II and also influences the kinase activity of TFIIH. Mediator is a modular complex consisting of multiple proteins that serve as an interface between the RNA pol II machinery and gene-specific regulatory proteins for both positive and negative regulation of the core transcriptional machinery (Myers and Kornberg, 2000).

As well as initiation of transcription via the TATA box, additional response elements have been identified including the CCAAT box and the GC box, which are located upstream of the TATA box. These motifs bind specific factors that can enhance the rate of transcription. This may be through increasing the stability or the activity of the basal transcription complex. The transcription factors Sp1 and Sp3 bind GC boxes and are widely expressed with Sp1 being able to directly interact with TBP and influence the basal transcription machinery (Bouwman and Philipson, 2002). The CCAAT binding factor (CBF) also termed nuclear factor Y (Coustry *et al.*, 1995) is one transcription factor that can bind the CCAAT box and is believed to be important for the expression of genes that are specific to differentiated cells (Marziali *et al.*, 1999).

1.1.2 REGULATION OF TRANSCRIPTION

Promotion of gene transcription can involve a number of different sequences. Promoters are composed of an array of sequence motifs, many of which are binding sites for transcription factors (reviewed in Muller *et al.*, 1988). The core promoter is the site where the initiation complex is assembled and is composed of the transcription start site and in the majority of cases an upstream TATA box. Further control can be exerted from a greater distance by enhancer and silencer elements, which can bind many factors and may be important in the co-ordination of gene expression. Locus control regions (LCRs) also influence the rate of transcription from a great distance.

1.1.2.1 Response elements

Response elements are usually short sequences of around 10 to 30 bp in length. As mentioned these sequences can either be within the promoter itself or located near to the promoter. Response elements within the promoter can be either palindromic or direct repeat sequences. For example, the estrogen response element is a 20 bp palindrome whereby the sequence is the same in either the 5' to 3' or the 3' to 5' direction. The presence of three random bases located in the centre of the palindrome confers specificity for oestrogen, since the thyroid hormone response element is identical except for these three bases (Hirst *et al.*, 1992). Direct repeat elements consist of two repeats of a short sequence and as with the estrogen response element, the spacing between the repeats regulates which hormone can bind.

1.1.2.2 Transcription factors

Transcription factors bind to specific response elements leading to activation of transcription of specific genes. They contain structural binding motifs for binding to DNA such as zinc fingers, and for binding to associated proteins via leucine zippers. Nuclear receptors are a class of ligand-activated transcription factors that can be cytosolic or nuclear in localisation, and are classified according to their sequence homology. Nuclear receptors contain several structural domains including DNA and ligand binding domains, and a flexible hinged region that contains a nuclear localisation signal (NLS) (Robinson-Rechavi *et al.*, 2003). The classical mechanism involves a ligand entering the cell and binding its respective nuclear receptor, inducing the dissociation of heat shock proteins, homodimerisation and translocation to the nucleus where the receptor can bind specific response elements within the DNA. This allows recruitment of the transcription machinery and associated proteins. It is now accepted that many nuclear receptors remain in the nucleus bound to DNA where they serve as repressors prior to ligand binding (Giguère, 1999).

1.1.2.3 Enhancers

Enhancer elements may facilitate gene activation by the same mechanisms as response elements. Enhancers are regulatory DNA sequences often similar to response elements but which are located at a distance from the transcriptional start site. They are often necessary for high-level transcription of a gene, and are frequently specific to tissues in which their particular gene is normally active. An enhancer can activate a promoter when several thousand bp distant and from either orientation relative to the promoter. Furthermore, enhancers have been shown to activate a promoter whether positioned upstream, downstream or within a region of the transcription unit that is later removed by splicing. Several models exist to explain the mechanism of action of enhancers on transcription. The most probable is the looping model whereby the enhancer and promoter for a particular gene interact via DNA binding proteins forming a loop of intervening DNA (Studitsky, 1991). This allows interactions between regulatory proteins that are usually distant from each other.

1.1.2.4 Silencers

As well as enhancers, certain negative regulators of gene activation termed silencer elements have been identified. These elements are similar in characteristics to enhancers, except that their effect is

inhibitory to gene expression. Silencers may act by causing further condensation of the chromatin to prevent the transcription complex binding the DNA, or by binding a factor that inhibits promoter activity.

1.1.2.5 Locus control regions (LCRs)

A further element important in regulation of gene expression is the LCR, which is thought to affect chromatin structure allowing transcription of surrounding genes (Felsenfeld, 1992). LCRs confer high-level gene expression that is position independent, and can function from many kilobases away. It is possible that LCRs are responsible for tissue-specific expression of genes, altering the chromatin structure over a large region of DNA, and allowing binding of specific transcription factors to the promoter and enhancer regions of individual genes by preventing the inhibitory effect of histones on transcription (Felsenfeld, 1992).

1.1.3 EXAMPLES OF CO-ORDINATE GENE REGULATION

Co-ordination of the response to a stimulus involves interplay between different pathways involved in homeostatic control, with the purpose of increasing the efficiency of that response. In order to understand the molecular mechanisms behind co-ordinate responses the individual interactions must first be identified. This information can then be compiled to determine the network of interactions. Several examples exist where co-ordinate regulation has been investigated, including control of gene expression and cellular differentiation, and will be discussed below.

Gene expression in eukaryotes involves a coupled network that co-ordinates the many pathways involved. Formation of mRNA is a stepwise process involving 5' capping, polyadenylation of the 3' end, and intron removal by splicing. The mRNA is then exported to the cytoplasm where it is translated into a protein. Alongside this process an RNA surveillance system ensures removal of any mutant pre-mRNA and mRNA prior to translation. However, this stepwise system is not as simple as first thought, with the different steps in the expression pathway interconnecting to form a complex network. This can be either through the tethering of components in close proximity to each other, or through functional interactions, both facilitating the production of mRNA. A model has been proposed consisting of gene expression factories whereby the proteins involved in transcription, capping, polyadenylation and splicing are anchored together in the nuclear

substructure (Maniatis and Reed, 2002). This solves several logistical problems associated with processing of pre-mRNA. For example, tight coupling of the 5' capping and transcription machinery allows rapid capping of the pre-mRNA as it is extended by RNA pol II, therefore protecting the pre-mRNA from degradation. Similarly, coupling of the splicing factors close to the pre-mRNA as it is formed allows for efficient recognition of intron-exon junctions. It is thought that these processes are coupled via distinct domains of the carboxy-terminal domain (CTD) of the RNA pol II large subunit. Deletions of this region do not affect transcription, but have significant implications on the efficiency of capping, polyadenylation and splicing.

In addition to coupling via the CTD of RNA pol II, coupling has also been observed between the different processing steps (Maniatis and Reed, 2002). Proteins that bind to the 5' cap of pre-mRNA have been shown to interact with splicing factors, facilitating recognition of the first 5' splice site. Similarly, the splicing machinery at the terminal 3' intron is in turn coupled to polyadenylation factors and promotes 3' end cleavage and polyadenylation. These interactions increase the efficiency and accuracy of the individual processes. In addition, co-ordination has been shown between the RNA surveillance system, the splicing machinery and export of the mRNA from the nucleus, ensuring that mRNA containing mutations is detected and degraded prior to translation.

In a study by Lam *et al.* (2001) a link was made between gene function and mRNA half-life. Using the anti-cancer drug flavopiridol to broadly inhibit transcription, genomic-scale mRNA turnover was assessed. Flavopiridol is a cyclin-dependent kinases (Cdk) inhibitor that arrests cells *in vitro* at the Gap 1 (G₁) to Synthetic (S), and Gap 2 (G₂) to Mitosis (M) transitions, as well as slowing the progression through S phase. Flavopiridol also inhibits the activity of the transcription elongation factor P-TEFb that phosphorylates the CTD of the RNA pol II complex, facilitating transcriptional elongation of cellular genes. Lam *et al.* (2001) showed that the action of flavopiridol was mediated largely through inhibition of P-TEFb, and that P-TEFb regulates the transcriptional elongation of the majority of cellular genes. DNA microarray analysis was used to assess cellular mRNA turnover rates in response to a range of stimuli, as compared with results from cells pre-treated with flavopiridol. Interestingly, genes with labile mRNAs included transcription factors from the NF- κ B family, members of the inhibitor of apoptosis (IAP) family and cell cycle regulators such as c-Myc.

Transcriptionally inducible genes appear to preferentially encode mRNAs that are rapidly degraded. Adenylate-uridylylate-rich elements (AREs) that are one type of element involved in mediating rapid turnover of mRNA were found in the majority of unstable mRNAs, with decreasing frequency in the more stable mRNAs. Lam and colleagues categorised genes by the function of their proteins and found certain classes were enriched for unstable mRNAs. These included cytokine-encoding genes, immediate early transcription factor genes, regulators of apoptosis and regulators of M phase of the cell cycle. This indicates that levels of mRNA can be rapidly decreased following termination of transcriptional activation, representing a co-ordinated strategy to rapidly modulate the mRNA levels of transcriptionally inducible genes. This would be particularly important in such functions as cell cycle control and apoptosis.

1.2 ORGAN SIZE IS MAINTAINED BY A BALANCE BETWEEN PROLIFERATION AND APOPTOSIS

Organ size is controlled by cellular proliferation and cell death and represents a closely regulated process. A high level of control is required to ensure that cells are accurately replicated and that any cells containing potentially deleterious mutations in their DNA are not allowed to progress through the cell cycle. Cells containing incorrectly replicated DNA can be detected at one of numerous checkpoints throughout the cell cycle, leading to removal of the cell by the process of programmed cell death (apoptosis).

1.2.1 THE CELL CYCLE

The cell cycle is composed of the G₁, S, G₂ and M phases, with the majority of cells being quiescent in the G₀ phase where they are not dividing. Together the G₁, S and G₂ phases are termed interphase and represent around 95-percent of the total cell cycle time. Mitosis is the most dramatic phase involving separation of the cell into two daughter cells, however this process only takes a short time in comparison to the other phases. The majority of cell cycle research has been conducted in the unicellular eukaryote *Schizosaccharomyces pombe* with the selection of mutants with altered cell division that define cell division cycle (CDC) genes. It was this type of analysis that led to the discovery of Cdks, from identification of the yeast *cdc2* gene that was crucial in promoting cell cycle progression (Nurse and Thariaux, 1980). A second gene termed *wee* was also identified as a

negative regulator of mitosis and later identified in numerous species including vertebrates where it was termed Wee1. Cdks are composed of a 34 kDa catalytic subunit, complexed with a cyclin component and are regulated at three levels (Nurse, 1997). These are (i) complex between cyclin and Cdk that can be reversed by degradation of the cyclin, (ii) protein phosphorylation of a tyrosine residue within the kinase ATP-binding site by enzymes such as Wee1 that inactivate the Cdk, and (iii) inhibition by specific Cdk inhibitors (CKIs) that are particularly active in G₁ phase. In mammals these include p21^{CIP1/WAF1} and p27 that inhibit the G₁ to S transition, and members of the INK4 family that inhibit Cdk4 and Cdk6.

After a cell has been signalled to divide, either by a physiological stimulus (such as following partial hepatectomy) or by a mitogenic substance, it leaves the resting G₀ state and enters G₁. This phase occupies approximately half of the total cell cycle time and is the longest of the phases. During G₁ the main drivers of cell cycle progression, the Cdks, are expressed in a highly regulated, time-dependent manner. Combinations of Cdks and their regulators the cyclins operate in specific parts of the cell cycle and can be regulated by ubiquitination and subsequent proteolysis. Cdks not only require binding of cyclins, but also require subsequent phosphorylation by Cdk-activating kinases (CAKs). The first combination is cyclin D (cyclin D1, 2 and 3) and Cdk4 and 6 that increase in late G₁ phase, and are joined by cyclin E and Cdk2, all of which drive the cell into the phase of DNA replication, S phase. The G₁ DNA damage checkpoint is encountered towards the end of G₁ phase and is where DNA damage can be detected. The tumour suppressor protein p53 is expressed at this point and governs expression of downstream proteins to influence the fate of the cell. Following this the cell passes through the so-called restriction point (R) after which the cell is committed to enter S phase. Cdk activity can be negatively regulated by inhibitory phosphorylation by Wee1 and subsequent dephosphorylation to activate the Cdk by Cdc25 phosphatases (Donzelli and Draetta, 2003).

S phase has three checkpoints all concerned with the process of DNA replication. The cell cycle can arrest here if the DNA is incorrectly replicated and not suitable to proceed to G₂. At the end of S phase there are two duplicate copies of the genome. The cell is then driven into G₂ phase by cyclin A and Cdk2. G₂ phase is shorter than G₁ and is concerned largely with preparation for cell

division, including replication of cellular organelles. Cyclins A and B in combination with Cdk1 drive the cell through G₂ and M phase. The G₂ checkpoint is the last point before the cell commits to division. It is responsible for monitoring that there is no unreplicated DNA and that the two sets of the genome are intact. Any badly damaged DNA will trigger the pathway to programmed cell death (apoptosis). Cdc25 phosphatases are required for activation of cyclin B/Cdk1 to allow progress to M phase, and the Cdc25 phosphatases can be deactivated by phosphorylation by the checkpoint kinase Chk1 protein that allows subsequent binding of 14-3-3 proteins (Peng *et al.*, 1997). It is thought that binding of 14-3-3 proteins creates a nuclear export signal that removes Cdc25 phosphatases from the nucleus allowing time for repair of damaged DNA prior to mitosis (Lopez-Girona *et al.*, 1999).

M phase is composed of four phases: prophase, metaphase, anaphase and telophase, and has a checkpoint during metaphase that identifies misaligned chromosomes and that the microtubules are attached to the kinetochores prior to the start of anaphase. Following completion of telophase the process of cytokinesis occurs whereby the cytoplasm is divided, completing the cycle with the formation of two daughter cells. These cells can then enter G₀ or re-enter G₁ if repeated rounds of replication are required. A signalling cascade known as the mitotic exit network (MEN) is responsible for driving cells from mitosis into the next cell cycle, and converges on the activation of Cdc14 phosphatase (Jensen *et al.*, 2002). Cdc14 promotes proteolytic degradation of mitotic cyclins, and dephosphorylates and activates the Cdk inhibitor Sic1 that in turn lead to inactivation of Cdk activity, which is a requirement for progression of the cell through telophase and cytokinesis.

1.2.2 CONTROL OF APOPTOSIS

Apoptosis is the controlled deletion of cells to counterbalance mitotic cell division. It is an active process that is controlled by cellular components in a pre-programmed manner. Without this negative control there would be an overabundance of cells, which would result in sub-optimal function of the tissue or organ. The process of apoptosis is highly regulated and morphologically characteristic. The nucleus and the cytoplasm first condense and the cell forms discrete fragments that are each membrane-bound and termed apoptotic bodies. These are then ingested by phagocytic cells and degraded by their lysosomal proteolytic enzymes. This prevents spillage of

cellular contents that would otherwise trigger an immune response, and also allows recycling of the cellular constituents.

1.2.2.1 Pathways to apoptosis

An apoptotic signal is mediated and executed via the activation of a family of cysteinyl aspartate-specific proteases termed caspases, which cleave and activate each other in a cascade, leading to the activation of so-called execution caspases (Thornberry and Lazebnik, 1998). Caspases are synthesised as inactive pro-enzymes, requiring cleavage at internal aspartic acid residues, producing the active form of the enzyme. This cleavage can be accomplished by other caspases upstream in the signalling pathway, but in some cases autoproteolytic activation can occur. Control of caspase activation is achieved through their presence as precursor enzymes that are largely inactive. Activation requires either close proximity with other caspase molecules enabling auto-activation, or cleavage by a caspase further upstream in the cascade. Caspase activation also involves Inhibitor of apoptosis proteins (IAPs) that are capable of inhibiting caspases via occlusion of the active site (Crook *et al.*, 1993).

Apoptosis can be initiated by internal or external signals. These include DNA damage, oxidative stress, and growth factor removal. The pathways to activation of caspases can be mediated via receptors, induced by toxic chemicals, or triggered by the involvement of cytotoxic T lymphocytes and NK cells. These pathways vary between cell types in their relative use, however amplification of the response can be achieved by overlap between the pathways (Figure 1.1).

Cytokines are able to induce apoptosis via binding to cell surface receptors, resulting in downstream caspase activation. Two death receptors (DR) in particular have been researched intensively, namely TNFR-1 and Fas (also termed CD95 or Apo-1). Activation of either receptor via binding to their respective ligands, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL), results in apoptotic cell death within a matter of hours. Binding of TNF α to TNFR-1 causes receptor trimerisation allowing recruitment of the adaptor protein TNFR-associated death domain (TRADD), followed by TNFR associated factor-2 (TRAF-2), receptor interactive protein (RIP) and the Fas-associated protein with death domain (FADD). FADD can then mediate activation of caspase-8. The Fas receptor responds to FasL binding in a similar way to TNFR-1. Aggregation of

pro-caspase-8 molecules is thought to form a death inducing signalling complex (DISC), which causes auto-activation of caspase-8 (Ashkenazi and Dixit, 1998). Caspase-8 can then cleave and activate caspase-3, initiating a downstream caspase cascade.

Apoptosis can be induced without the requirement for receptor activation at the plasma membrane. This pathway involves proteins contained within the mitochondria, and can lead to caspase activation, and caspase-independent cell death via proteins such as apoptosis inducing factor (AIF). It is now known that the release of cytochrome c into the cytosol is a central step in the progression of the apoptotic pathway. Cytochrome c is contained within the inter-membrane space of mitochondria and serves to transfer electrons between complex III and complex IV of the electron transport chain (Bernardi and Azzone, 1981). Liu *et al.* (1996) first demonstrated that cytochrome c forms a component of the apoptosome complex that leads to the activation of caspase-3. Using cytosolic extracts from apoptotic cells *in vitro*, they were able to demonstrate the presence of cytochrome c, and that removal of the protein diminished the apoptotic activity. Li *et al.* (1997) then purified a protein factor required for apoptosis, termed apoptotic protease-activating factor 3 (Apaf-3). Analysis of the structure of Apaf-3 showed it to be pro-caspase-9. It was found that in the presence of cytochrome c and deoxyadenosine triphosphate (dATP), pro-caspase-9 and the cytosolic protein Apaf-1 were bound to each other, forming a complex termed the mitochondrial apoptosome, leading to caspase-9 activation. Mitochondrial release of cytochrome c is accompanied by the release of other pro-apoptotic proteins, for example the second mitochondria-derived activator of caspase (SMAC, also termed DIABLO). SMAC was identified by Verhagen *et al.* (2000) as a mediator in the formation of the apoptosome. SMAC binds to and prevents IAP inhibition of caspase activation.

It is thought that agents that damage DNA such as etoposide, lead to upregulation of the cellular levels of the pro-apoptotic protein p53. p53 induces cell cycle arrest by regulating Cdk activity. p53 also translocates to mitochondria where it is thought to increase the transcription of Bax, a pro-apoptotic member of the Bcl-2 family of proteins (Miyashita *et al.*, 1994). Bax is thought to localise to the mitochondrial membrane and facilitate the release of cytochrome c, leading to the formation of the apoptosome with subsequent activation of caspase-9.

In some cell types it has been demonstrated that caspase-8 can cause the release of cytochrome c from the mitochondrial inter-membrane space (Desagher *et al.*, 1999), allowing amplification of the death receptor pathway. This is achieved through cleavage of the pro-apoptotic protein Bid forming a 15 kDa C-terminal fragment termed truncated Bid (tBid). Bid and tBid translocate to mitochondria and activate Bax, which inserts itself into the outer mitochondrial membrane. In a similar way it has been suggested that caspase-3 can activate caspase-8 (Slee *et al.*, 1999, Tang *et al.*, 2000) leading to cleavage of Bid and amplification of the mitochondrial response.

Figure 1.1: Pathways to caspase activation in apoptosis

(A) Binding of plasma membrane death receptors such as the Fas receptor to their ligand results in activation of pro-caspase 8 via adaptor molecules such as FADD. Aggregation of pro-caspase-8 molecules on the cytoplasmic region of the Fas receptor leads to autoactivation of caspase-8. This can then activate caspase-3 directly (black lines) or cleave Bid forming tBid, which translocates to mitochondria and causes release of cytochrome c and SMAC, with subsequent activation of caspase-9 (red lines). This provides an amplification system from the initial stimulus. (B) Cellular stress induced by chemicals or UV-irradiation can lead to expression of the pro-apoptotic protein p53. This increases expression of Bax that could mediate the release of cytochrome c and SMAC from the mitochondrial inter-membrane space. This results in the formation of the apoptosome leading to caspase-9 activation (red lines). Caspase-9 can then activate caspase-3, which can either cleave downstream caspases, or possibly activate pro-caspase-8 allowing amplification of the response via cleavage of Bid. (Adapted from Adrain and Martin, 2001.)

1.2.2.2 The Bcl-2 family

The Bcl-2 family of proteins have a major role in the control of apoptosis with twenty-five members having been identified to date. There are three subfamilies that contain at least one of four α -helical domains (BH1 to BH4). Anti-apoptotic members such as Bcl-2 and Bcl-xL show a high degree of sequence homology. Pro-apoptotic members show either sequence homology at BH1, BH2 and BH3 domains (for example Bax and Bak), or sequence homology at BH3 domains only (for example Bid). Bcl-2 family members are found in the cytosol, attached to cytoskeleton components and membranes. They can dimerise forming hetero- or homodimers (Figure 1.2), the resulting ratio determining pro- or anti-apoptotic effects (Hunter and Hawarth, 1979). Bcl-2 family members respond to a diverse range of apoptotic stimuli, including growth factor deprivation and exposure to cytotoxic drugs.

A proposed major site of action of Bcl-2 family members is at the mitochondrial permeability transition (PT) pore to modulate cytochrome c release. This is formed from a complex of the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), and cyclophilin D. ANT and VDAC form the relatively non-selective PT pore that allows passage of molecules up to 1.5 kDa (Hunter and Hawarth, 1979). There is evidence for the release of cytochrome c from this pore in response to apoptotic stimuli. Although the exact mechanism is not clear Bcl-2 family members are known to interact with this process.

Over-expression of the anti-apoptotic proteins Bcl-2 or Bcl-xL inhibits cytochrome c release, possibly via PT pore modulation (Yang *et al.*, 1997), whereas over-expression of the pro-apoptotic Bax leads to enhanced cytochrome c release (Susin *et al.*, 1996). These effects could be due to the dimerisation states of the two proteins (Figure 1.2), with the relative levels of pro- and anti-apoptotic proteins determining the outcome for the cell. Bcl-2 is thought to translocate to the outer mitochondrial membrane (Shimizu *et al.*, 1999) but the exact mechanism for its anti-apoptotic effects is unknown. Overexpression of Bcl-2 was found to prevent staurosporine or etoposide-induced apoptosis via reduced translocation of pro-apoptotic Bax to mitochondria and subsequent caspase activation (Murphy *et al.*, 2000). Bcl-2 may also interact with the inner mitochondrial membrane phospholipid cardiolipin (Lutter *et al.*, 2000). This is found at contact sites between the

inner and outer mitochondrial membrane, where the PT pore is believed to be located. Another possible mechanism for the anti-apoptotic effects of Bcl-2 and Bcl-xL is via their BH4 domain that is required for activity. Mutations in the BH4 region prevent function of all anti-apoptotic Bcl-2 proteins. It is thought that via its BH4 domain, Bcl-xL can induce VDAC closure (Shimizu *et al.*, 1999) and prevent cytochrome c release.

Pro-apoptotic Bax directly interacts with the VDAC to induce release of cytochrome c and membrane potential loss in isolated mitochondria. This was confirmed in mitochondria from VDAC-deficient yeast, whereby Bax-induced cytochrome c release and loss of membrane potential did not occur (Shimizu *et al.*, 1999). Overexpression of Bax in Jurkat T cells caused apoptosis via induction of the PT pore (Pastorino *et al.*, 1998). Inhibition of the PT pore by cyclosporin A prevented all biochemical features of apoptosis, whereas caspase inhibition prevented all except cytochrome c release, demonstrating that caspases are not required in this process (Pastorino *et al.*, 1998).

Pro-apoptotic Bid is cleaved by activated caspase-8 forming tBid that is thought to induce cytochrome c release from mitochondria (Li *et al.*, 1998), and to cause perinuclear clustering of mitochondria in apoptosis (Li *et al.*, 1998). Bid has been shown to induce cytochrome c release but not via loss of mitochondrial membrane potential, or VDAC activity (Shimizu and Tsujimoto, 2000). tBid is thought to induce lipid destabilisation at contact sites between the inner and outer mitochondrial membrane (Lutter *et al.*, 2001), but cannot induce cytochrome c release alone, requiring dimerisation with Bax. It is therefore thought that tBid in conjunction with Bax interact with the PT pore (Kroemer and Reed, 2000), and mediate death receptor-induced cytochrome c release from mitochondria (Li *et al.*, 1998).

In non-apoptotic cells, the pro-apoptotic Bcl-2 family member Bad is inactive due to phosphorylation by protein kinases such as protein kinase A (PKA) and mitogen-activated protein kinase (MAPK). This phosphorylation occurs at two serine residues within a 14-3-3 consensus site, and results in the binding of Bad to the regulatory protein 14-3-3 within the cytoplasm, preventing its pro-apoptotic effects (Downward, 1999). When apoptosis is stimulated, Bad is dephosphorylated by phosphatases such as calcineurine and can then translocate to the

mitochondria where it binds Bcl-xL, allowing release of Bax from Bcl-xL-Bax heterodimers (Downward, 1999). Bax can then induce release of cytochrome c causing activation of the caspase cascade.

Figure 1.2: Interactions between members of the Bcl-2 family of proteins

The dimerisation state of anti-apoptotic proteins such as Bcl-2 and Bcl-xL and anti-apoptotic Bax determines the fate of the cell and is dependent on the relative concentrations of the proteins. The pro-apoptotic protein Bad can influence this equilibrium by forming heterodimers with Bcl-2, relieving its inhibitory effect on Bax. This allows Bax to interact with the mitochondrial membrane in conjunction with Bid leading to cytochrome c release and apoptosis.

1.2.3 REGULATION OF LIVER GROWTH

Control of liver growth involves a balance between cellular proliferation and apoptosis, however the mechanism for maintenance of this balance is not fully understood at present. There is no better model for examining the mechanisms behind growth control than in the liver, due to its remarkable regenerative capacity and the ability of the organ to rapidly terminate growth and finely control its size. This growth is interesting as it occurs via replication of adult hepatocytes and not precursor cells (Fausto, 2000). The liver appears to maintain its optimal mass by the balance of proliferation of healthy hepatocytes, and removal of excess or damaged cells by apoptosis. Under physiological conditions the turnover of hepatocytes is a slow process, with the half-life estimated at 6 months (Michalopoulos and DeFrances, 1997). Liver is remarkable in its ability to regenerate following partial hepatectomy (PH), a process that has been widely used for studying the control of

liver growth. This compensatory regeneration is known to occur following removal of up to 70-percent of the liver, and within minutes the remaining hepatocytes leave the quiescent G_0 state and enter the G_1 phase of the cell cycle in preparation for cell replication, restoring the liver to its original mass in 10 to 14 days (Haber *et al.*, 1993). This is accomplished by hyperplasia of the remaining hepatocytes, with most hepatocytes undergoing at least one round of replication, with some replicating twice producing a later increase in proliferation that is smaller than the first (Haber *et al.*, 1993). Hepatocytes rapidly enter the cell cycle, with onset of S-phase (DNA synthesis) within 14 and 24 hours of PH, with the peak of DNA replication occurring at 24 hours post-PH in rats (Fausto, 2000). Mitosis occurs around 8 hours after DNA synthesis, although this period is increased in older animals. Non-parenchymal cells of the liver also replicate although this can be delayed for approximately 24 hours and is due to a longer G_1 phase (Haber *et al.*, 1993).

Once the original mass of the liver has been restored proliferation ceases abruptly. In addition, any excess cells are removed by apoptosis. This can be observed following transplantation of an oversized liver, where the organ mass decreases by apoptotic cell death until it is a more suitable size for the recipient (Fausto, 2000). This level of control is unlikely to be co-ordinated by a single factor, and is known to involve several pathways leading to different genes being up- and down-regulated. Since the liver can alter its growth state so rapidly, it provides a useful model in which to study both normal and abnormal cell growth. Haber *et al.* (1993) identified over 70 genes that were induced rapidly following PH. In analysing the expression of these and other genes they defined three basic patterns of gene induction. These were immediate-early, delayed-early and liver-specific genes. It is thought that for hepatocytes to proliferate after PH they must first be primed to increase their responsiveness to growth factors (Fausto, 2000). Priming is thought to involve expression of immediate-early genes and represents the G_0 to G_1 transition. The proteins encoded by immediate-early genes then increase the expression of the delayed-early genes. Priming allows hepatocytes to respond fully to growth factors such as Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF), and Transforming Growth Factor α (TGF α). This allows progression of the cell through the cell cycle by allowing the G_1 to S transition to occur (Fausto, 2000) (Figure 1.3).

Figure 1.3: Model for the stages in liver regeneration

In response to a stimulus cells can leave the resting state of G₀ and enter the G₁ gap phase that prepares the cell for DNA replication (S phase). Following S phase the cell enters the G₂ phase where it prepares for mitosis (M phase). The G₁ and G₂ phases allow time for cell enlargement prior to cell division. The G₁ checkpoint is one of several control points that allow the cell cycle to be arrested if cell division is considered unfavourable. Priming is thought to be initiated by cytokines that sensitise the cell to growth factors, which are required for progression past the G₁ checkpoint in the cell cycle. (Adapted from Fausto, 2000.)

1.2.3.1 Cell cycle priming

Priming involves the expression of immediate-early genes, a process that occurs independently of *de novo* protein synthesis, relying on pre-existing transcription factors within the quiescent cell (Mohn *et al.*, 1990). The immediate-early genes encode transcription factors, growth factors and regulators of signal transduction, which regulate the induction of the delayed-early response. Several of these genes are known proto-oncogenes, and although their exact role is not known, they are important in the regulation of liver growth. The levels of c-fos and c-jun mRNA increase immediately after PH with the peak of expression occurring after 30 minutes, and returning to normal within 2 hours post-PH (Morello *et al.*, 1990). Levels of c-myc mRNA increase more slowly with peak expression around 2 to 4 hours post-PH (Morello *et al.*, 1990). Activator Protein-1 (AP-1) is a transcription factor composed of c-Fos and c-Jun proteins bound via a leucine zipper protein termed Liver Regeneration Factor-1 (LRF-1). AP-1 is thought to control delayed-early gene expression during the G₁ phase of the cell cycle (Hsu *et al.*, 1992) such as those associated with cell cycle progression, and also to increase expression of the c-myc gene. The protein product of the c-myc gene heterodimerises with a protein Max to form a transcription factor that can activate genes required for DNA synthesis and cell cycle control (Hsu *et al.*, 1992).

The role of cytokines in priming cells for entry into the cell cycle has been investigated. Tumour Necrosis Factor α (TNF α), a multi-functional cytokine, is thought to cause production of reactive oxygen species (ROS) through interaction with one of its receptors TNFR-1 (Yamada *et al.*, 1997b), leading to activation of the transcription factor Nuclear Factor- κ B (NF- κ B). NF- κ B can then migrate to the nucleus and bind to genes containing NF- κ B recognition sites, such as those involved in inflammation, stress response and cell replication. NF- κ B is activated very rapidly and is detectable within 30 minutes following PH. The levels are transient however, and are no longer detectable at 4 to 5 hours post-PH (Fausto, 2000). Interleukin-6 (IL-6) is also thought to be involved in the immediate-early response, activating the Signal Transducer and Activator of Transcription, STAT-3. This protein also translocates to the nucleus where it regulates the expression of genes involved in inflammation and cell proliferation. Studies in IL-6 knockout mice showed impaired liver regeneration following PH, and this was reversed by injection of IL-6 (Cressman *et al.*, 1996). Activation of STAT-3 occurs at a slower rate than that of NF- κ B, with STAT-3 being detectable between 1 to 2 hours post-PH (Cressman *et al.*, 1995).

Interestingly, TNF α can function as an anti-apoptotic or a pro-apoptotic agent, the latter effect being opposed to liver regeneration, occurring in the absence of NF- κ B (Fausto, 2000). Blockage of NF- κ B expression has been shown to result in apoptosis following DNA replication (Iimuro *et al.*, 1998), suggesting the presence of NF- κ B in the regenerating liver alters the activity of TNF α . Furthermore, in knockout mice for the TNF α /IL-6 inducible nitric oxide synthase (iNOS) gene, hepatic regeneration was severely impaired with increased apoptosis, despite the normal induction of TNF α and IL-6, and activation of NF- κ B and STAT-3 (Rai *et al.*, 1998). This suggests a requirement for nitric oxide in the proliferation observed following PH, although further investigation is required.

TNF α and IL-6 signalling through NF- κ B and STAT-3 may represent the initial signal for proliferation following PH, activating expression of immediate-early genes. Liver-specific gene expression is also necessary around this time to maintain metabolic homeostasis during regeneration, and an increase in glucagon with decreased insulin has been observed following PH.

This induces glycogenolysis and gluconeogenesis in the remaining hepatocytes, counteracting the hypoglycaemia observable following PH, with the levels of both hormones normalising after a few hours (Bucher and Swaffield, 1975). Also changes in the levels of expression of genes involved in glucose homeostasis have been observed, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (Rosa *et al.*, 1992). These results have led to the suggestion that the increased metabolic demand following PH causes the increased TNF α and IL-6 levels, which in turn are thought to be responsible for cell priming (Fausto, 2000). Figure 1.4 illustrates a possible scheme for liver regeneration with respect to metabolic demands on the organ.

Figure 1.4: Proposed sequence of events in liver regeneration

Initiation and termination of liver regeneration are possibly linked to metabolic demands on the liver. Loss of tissue leads to increased metabolic load on the remaining hepatocytes that could be the trigger for activation of NF- κ B and cell cycle priming. This process is thought to sensitise cells to growth factors that allows cell cycle progression and proliferation to restore the original functional capacity of the tissue. (Adapted from Fausto, 2000.)

1.2.3.2 Cell cycle progression

Delayed-early genes are involved in progression, and require protein synthesis for their expression to occur, indicating that this is secondary to that of immediate-early gene expression. Delayed-early genes include the anti-apoptotic gene Bcl-xL that could be involved in the control of ROS generation as well as inhibiting apoptosis (Fausto, 2000). A study by Loyer *et al.* (1996) using cultured hepatocytes, characterised the changes in cyclin levels throughout the cell cycle, and investigated the role of growth factors in progression. They found that progression through G₁ occurred regardless of growth factor stimulation until the R point in G₁, after which EGF was required for continuation through the cell cycle. Cells grown in media containing serum/growth factors showed elevated Cyclin D1/cdk4 levels, whereas those grown in serum-free media did not, indicating a requirement for growth factors to allow progression from G₁ to S phase (Loyer *et al.*, 1996).

Although TNF α is involved in the priming of hepatocytes, it is not a complete mitogen, as shown by the inability of cultured hepatocytes to proliferate in serum-free medium regardless of the presence of TNF α (Fausto, 2000). Therefore it is clear that growth factors are required for progression through the cell cycle. Whether growth factors are involved in the initial priming of hepatocytes in response to PH is not clear, however evidence indicates that growth factors such as HGF, EGF and TGF α are not involved in this early stage. Direct infusion of these growth factors along with the co-mitogen insulin into the mesenteric vein of rats failed to induce significant DNA synthesis at 24 hours as determined by pulse-labelling with bromodeoxyuridine (BrdU) (Webber *et al.*, 1994a). Hepatectomy was performed on the animals whereby 30-percent of the liver was removed, however this alone did not induce DNA synthesis. Infusion of growth factors into these animals caused a significant increase in DNA synthesis (Webber *et al.*, 1994a). This indicates that hepatectomy causes the transition from the G₀ to G₁ phase, but that growth factors are required for progression into S-phase.

The role of growth factors in progression can be both stimulatory and inhibitory, thus providing a restraint on proliferation. Stimulatory growth factors include HGF, EGF and TGF α , with negative control involving TGF- β 1 and activin. HGF is a heterodimeric glycoprotein and a known

stimulator of DNA synthesis (Michalopoulos and DeFrances, 1997). HGF levels increase rapidly following PH, with circulating levels increasing during the initial 4 hours post-PH (Fausto, 2000). HGF is the most powerful mitogen known for hepatocytes, and its expression is correlated with the extent of liver damage (Wu *et al.*, 2006). HGF is produced by both extra-hepatic and hepatic mesenchymal cells (such as Kupffer cells) and acts in an endocrine and/or paracrine fashion, stimulating hepatocyte replication. HGF signals through its receptor, a tyrosine kinase membrane receptor encoded by the proto-oncogene c-met. Inactivation of either the HGF gene or c-met in mice leads to embryonic lethality by day 16 (Huh *et al.*, 2004). However, prior to this a reduction in liver size by up to 55-percent has been observed (Pistoi and Morello, 1996). Levels of HGF mRNA in non-parenchymal cells reach a maximum between 18 and 24 hours post-PH, around the time that onset of DNA synthesis occurs. This indicates the role of HGF in progression of a primed cell from G₁ into S phase of the cell cycle (Fausto *et al.*, 1995).

EGF functions as an endocrine factor in the mouse, and is produced largely in the salivary glands. Removal of salivary glands delays the peak of DNA synthesis after PH by 24 hours (Fausto *et al.*, 1995). Analysis of rat hepatocytes post-PH has shown rapid synthesis of EGF mRNA and protein, suggesting an autocrine in addition to endocrine action of EGF (Mullhaupt *et al.*, 1994). Binding of EGF to its receptor EGFR causing activation of an intrinsic tyrosine kinase that activates downstream signalling leading to induction of mitosis and differentiation of certain cell types.

TGF α is produced in hepatocytes and is elevated after PH, reaching a maximum just prior to the peak of DNA synthesis (Fausto, 2000). TGF α appears to be a more effective stimulator of DNA synthesis than EGF, even though it shares 35-percent sequence homology with EGF and acts through the EGFR. TGF α differs from EGF and HGF in that it acts in an autocrine manner. Binding of either EGF or TGF- α to the EGFR allows autocrine amplification of TGF- α synthesis both *in vitro* and *in vivo* (Fausto *et al.*, 1995). Constitutive expression of TGF- α in hepatocytes was studied using transgenic mice that overexpress human TGF- α (Webber *et al.*, 1994b). This resulted in increased hepatic proliferation with 25- to 40-percent increases in liver size during the first month of life, even though hepatocyte differentiation was maintained as normal. After one month of age liver size stabilised through increased cell turnover, and the appearance of tumours was not

seen until after 12 months of age. Cultures of hepatocytes from these transgenic animals can be maintained in serum-free medium for a number of rounds of replication, without the requirement for other growth factors. Addition of the medium from these cells to cultures of normal hepatocytes stimulates DNA synthesis, which can be inhibited using anti- TGF- α antibodies (Fausto *et al.*, 1995). This indicates the mitogenic potency of TGF- α in hepatocytes.

As well as the stimulatory effects of HGF, EGF and TGF- α , negative control of progression is exerted by expression of factors such as TGF- β and activin (Ichikawa *et al.*, 2001). Hepatocytes and non-parenchymal cells produce TGF- β as one of three possible isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. TGF- β expression is low in normal liver, but increases significantly a few hours following PH (Fausto *et al.*, 1995). The elevated levels of TGF- β 2 and TGF- β 3 mRNA are relatively short lived, with TGF- β 1 mRNA showing prolonged elevation with a maximum between 36 and 72 hours post-PH. TGF- β is known to inhibit DNA synthesis *in vitro* and *in vivo*, and can prevent liver regeneration in response to mitogenic stimuli (Russell *et al.*, 1988). Therefore it has been proposed that expression of TGF- β is low initially to allow the first round of DNA synthesis to occur, but then increases in order to provide finer control on proliferation as the liver approaches its original mass (Fausto *et al.*, 1995). In addition, TGF- β is thought to be pro-apoptotic, preventing excess proliferation (Caja *et al.*, 2007). Apoptosis is infrequent in quiescent liver, however it is observable following removal of growth stimuli and during liver atrophy associated with starvation. A study by Oberhammer *et al.* (1992) showed expression of TGF- β 1 in apoptotic hepatocytes by immunostaining for the epitope of the protein, and demonstrated increased incidence of apoptosis in hepatocytes following treatment with TGF- β 1 both *in vitro* and then *in vivo* after pre-treatment with the mitogenic compound cyproterone acetate.

Further control may involve activin, a potent inhibitor of proliferation that is undetectable in quiescent liver. Activin levels have been reported to increase dramatically in the initial hours following PH (Yasuda *et al.*, 1993). Similarly, proteins involved in the control of apoptosis such as members of the Bcl-2 family of proteins, may play a role in refining the extent of proliferation.

1.3 PERTURBATION OF LIVER HOMEOSTASIS CAUSES ORGAN GROWTH

Maintenance of organ size is regulated by the balance between proliferation and apoptosis involving many of the factors identified through investigation of compensatory regeneration using the PH model. Compensatory regeneration involves loss of cells as a stimulus for proliferation. However, liver cell proliferation can be induced by mitogenic compounds without requiring cell loss, this process being termed direct hyperplasia. Investigation of this chemically-induced form of growth has demonstrated the involvement of different signal transduction pathways and growth factors as compared to compensatory regeneration. Several examples of xenobiotics known to cause liver enlargement and hepatocarcinogenesis will be discussed further, with particular emphasis on the peroxisome proliferator (PP) class of compounds.

1.3.1 COMPOUNDS CAUSING LIVER GROWTH

The rodent liver is sensitive to the effects of a variety of xenobiotics that perturb normal liver homeostasis, affecting the balance between hepatocyte proliferation and apoptosis that can ultimately result in tumourigenesis. Compounds that cause liver enlargement can do so through either one or a combination of increasing cell number (hyperplasia), or increasing cell size (hypertrophy). Examples of compounds causing liver growth predominantly by hyperplasia include the anti-androgen cyproterone acetate and the peroxisome proliferators, so named because of their effects on the number and size of peroxisomes. In contrast, the glucocorticoid dexamethasone causes liver growth predominantly via hypertrophy. These compounds will be discussed further with particular emphasis on the peroxisome proliferators as they provide a good model for the characterisation of abnormal growth mechanisms, and have been intensively researched due to the differential response of rodents and humans to these compounds. Similarly, the current understanding regarding the species differences in the response to PPs and the mechanisms postulated for the rodent response to these compounds will also be discussed.

1.3.1.1 Dexamethasone

Dexamethasone is a synthetic glucocorticoid used as an immunosuppressive agent in the field of clinical transplantation. Glucocorticoids bind the intracellular glucocorticoid receptor (GR) and suppress the expression of proinflammatory genes that can be causative in the rejection of a

transplant. Dexamethasone is known to induce hypertrophy of hepatocytes. Nagy and colleagues (2001) showed that following PH, treatment with dexamethasone led to the restoration of rodent liver mass despite the finding that DNA synthesis, hepatocyte hyperplasia and stem cell activation were inhibited. The increase in liver mass was accomplished by hypertrophy of the remaining hepatocytes, and following withdrawal of dexamethasone the cells returned to a replicative state, restoring the normal DNA content and structure of the liver. This inhibition was shown to be via suppression of the expression of TNF α and IL-6 (Nagy *et al.*, 1998), which resulted in reduced activation of the downstream effectors NF- κ B and STAT3 (Debonera *et al.*, 2003).

In partial explanation of the effect of glucocorticoid treatment on the inflammatory response was the finding that dexamethasone reduced the activity of Jun N-terminal kinase (Jnk) in fetal hepatocytes as induced by TNF α (Ventura *et al.*, 1999). This would in turn prevent the downstream effects of Jnk including formation of AP-1, a transcription factor required for liver development. Glucocorticoids are also known to induce deposition of glycogen via both direct induction of glycogen synthase, and induction of the phosphatase that activates this enzyme (Vanstapel *et al.*, 1980). It is thought that the resultant increase in deposition of glycogen in hepatocytes is causative of the hypertrophy in response to dexamethasone treatment (Crunkhorn *et al.*, 2004).

Dexamethasone can also inhibit apoptosis in rat hepatoma cells as induced by TGF- β 1 that was found to be via induction of the anti-apoptotic protein Bcl-xL (Yamamoto *et al.*, 1998), which occurred by increasing both the mRNA and protein levels of Bcl-xL, and was found to be via the mitochondrial pathway to activation of the apoptotic cascade and not via CD95/Fas ligand (Scoltock *et al.*, 2006).

1.3.1.2 Cyproterone Acetate

Cyproterone acetate (CPA) is an anti-androgenic compound developed for treatment of prostate cancer, which acts by preventing the binding of dihydrotestosterone to specific receptors in prostatic carcinoma cell. CPA induces hepatomegaly in rodents through a combination of hyperplasia and hypertrophy, the hyperplasia being reversible following cessation of treatment with CPA with removal of excess cells by apoptosis (Bursch *et al.*, 1986). CPA was found to be a carcinogen in rodents, causing adduct formation with hepatic DNA *in vitro* and *in vivo*, leading to the

formation of hepatic tumours following long-term treatment with the compound (Topinka *et al.*, 1993). The DNA adducts formed were found to be highly persistent in rats leading to accumulation of adducts within the liver (Werner *et al.*, 1995) with binding of CPA to DNA being higher in the female than the male. CPA was therefore classified as a genotoxic carcinogen in rodents since it could bind to DNA potentially causing gene mutations that could result in cell transformation to a neoplastic state. The finding that CPA was genotoxic in rat liver was also confirmed in primary cultures of human hepatocytes (Werner *et al.*, 1997) although the induction of DNA repair was similar between male and female in humans. Reduction and sulfonation reactions are required for production of the primary metabolite of CPA that is responsible for the formation of DNA adducts, however this metabolite has a short half-life and can only react with DNA within the cell in which it is formed (Brambilla and Martelli, 2002). The International Agency for Research on Cancer (IARC) has categorised the family of progestins (of which CPA is a member) as being possibly carcinogenic to humans, and as carcinogenic specifically concerning the use of CPA in combination with estrogen as an oral contraceptive, due to an increased risk of hepatocellular carcinoma (Brambilla and Martelli, 2002).

1.3.1.3 Peroxisome Proliferators

Peroxisome proliferators are non-genotoxic hepatocarcinogens that cause tumour induction without directly binding to or damaging DNA. Peroxisome proliferation was first described in the 1960s by Hess *et al.* (1965) following administration of the hypolipidaemic drug clofibrate to rats and mice. Common features are an increase in both the number and size of hepatocellular peroxisomes, hepatomegaly, and ultimately liver cancer (Reddy *et al.*, 1980). Many other compounds were found to produce similar effects in rodents including plasticisers (e.g. 2-diethylhexylphthalate), herbicides (e.g. lactofen), solvents (e.g. trichloroethylene) and other hypolipidaemic drugs (e.g. nafenopin). These compounds together form a structurally diverse group with the common feature of causing carcinogenesis in rodents with the liver being the major target. The following describes the phenomenon of peroxisome proliferation and the potential mechanisms leading to hepatocarcinogenesis in rodents following exposure to these compounds.

1.3.1.3.1 Peroxisomes

Peroxisomes are specialised intracellular organelles found in all eukaryotic organisms, which contain oxidative enzymes necessary for the breakdown of fatty acid molecules (reviewed in Wanders *et al.*, 2001). They are single-membrane bound organelles and do not contain DNA or ribosomes, and hence require import of proteins produced in the cytosol (Kunau, 1998). These include enzymes such as catalase and urate oxidase, which are often present at high concentrations. Peroxisomes are most abundant in liver and kidney. They utilise molecular oxygen to remove hydrogen atoms from organic substrates resulting in the formation of a shortened acyl CoA, acetyl CoA, NADH and hydrogen peroxide (Figure 1.5). The hydrogen peroxide is then utilised by catalase to oxidise substrates including alcohol and various toxic molecules, with any excess being converted to oxygen and water. The major function of the peroxisomal oxidative reactions is the breakdown of fatty acid molecules to acetyl CoA, which is exported from the peroxisome for reuse in biosynthetic reactions. Peroxisomal enzymes are also important for alcohol oxidation, cholesterol and bile acid synthesis, and amino acid metabolism. The importance of peroxisomal reactions is demonstrated by inherited disorders such as Zellweger syndrome, which results from defective protein import into peroxisomes (Steinberg *et al.*, 2006). This results in severe peroxisomal deficiency and is fatal at birth.

1.3.1.3.2 β -oxidation in peroxisomes

β -oxidation was found to occur in peroxisomes in 1976 (Lazarow and De Duve, 1976), and involves enzymes distinct from those found in mitochondrial β -oxidation. In addition, peroxisomal β -oxidation is not coupled to ATP production. Free fatty acids must first be converted to a CoA derivative before oxidation can occur. This is carried out by acyl CoA synthetases located on the cytoplasmic side of the peroxisomal membrane. The acyl CoA derivative is then transported passively into the peroxisome and oxidised by the FAD-containing enzyme ACO generating hydrogen peroxide. The bifunctional enzyme then carries out hydration and then dehydrogenation reactions, followed by thiolytic cleavage to yield acetyl CoA and the shortened acyl CoA. This can then be repeatedly shortened or can exit the peroxisome for use elsewhere. Although mitochondria and peroxisomes are both capable of β -oxidation, the mitochondrial carnitine shuttle cannot import

very-long-chain fatty acids (>C22). Hence the function of peroxisomal β -oxidation may be to shorten very-long-chain fatty acids for subsequent oxidation in mitochondria.

Figure 1.5: β -oxidation pathway in peroxisomes

Free fatty acids are converted to a CoA derivative by acyl CoA synthetases located on the cytoplasmic side of the peroxisomal membrane prior to oxidation by Acyl CoA oxidase (ACO), a process that generates hydrogen peroxide. Hydration and dehydrogenase reactions are performed by the bifunctional enzyme, followed by thiolytic cleavage to yield acetyl CoA and the shortened acyl CoA. This process can then be repeated generating shorter fatty acid chains, or the acyl CoA can exit the peroxisome for use elsewhere, for example β -oxidation in mitochondria.

1.3.1.3.3 Peroxisome Proliferator Activated Receptor- α

PPs activate the nuclear receptor PPAR α , a member of the steroid hormone receptor superfamily. PPAR α was first cloned in mouse liver by Isseman and Green (1990). A mouse liver complementary DNA (cDNA) library was screened with a series of oligonucleotide probes based on a highly conserved region within the DNA binding domain of several nuclear receptors, and identified a novel nuclear receptor that responded to PPs. The PPAR α cDNA encoded a 468 amino acid polypeptide with a predicted molecular weight of 52 kDa. PPAR α was identified as a putative transcription factor activated specifically by a range of PPs tested, as determined using a

chimaeric receptor construct of the putative ligand-binding domain of PPAR α , and the N-terminal sequence and DNA-binding domain of the human estrogen receptor (hER) (Issemann and Green, 1990). In the presence of a PP, the hER-PPAR α construct transcriptionally activated a reporter gene under the control of hER. The level of PPAR α activation was shown to correlate with the potency of various PPs (Issemann and Green, 1990). Expression of murine PPAR α was located principally to the liver, the kidney and the heart (Issemann and Green, 1990). PPAR α was also identified in the rat with expression primarily in hepatocytes, cardiomyocytes and in the proximal tubule cells of the kidney (Braissant *et al.*, 1996).

Important evidence for the role of PPAR α in peroxisome proliferation was provided by the generation of a PPAR α -null mouse (Lee *et al.*, 1995). Homologous recombination was used to disrupt the ligand-binding domain of the receptor, and resulted in abolishment of the pleiotropic effects of PPs in the mouse. PPAR α null mice on chronic exposure to potent PPs such as Wy-14,643 and clofibrate, do not show a transcriptional response, neither is there an increase in DNA synthesis as normally seen (Lee *et al.*, 1995). This proved that the response to PPs is mediated through PPAR α .

The mechanism by which PPAR α interacts with DNA to modulate gene expression has been identified. As with other members of the steroid hormone receptor family, heterodimerisation with the retinoid X receptor (RXR) increases the strength of interaction of the receptor with DNA (Green, 1993). Three RXRs have been identified (RXR α , β and γ) with 9-cis-retinoic acid being the common ligand. RXR β was found to heterodimerize with PPAR α , and to cooperatively stimulate the ACO gene promoter (Keller *et al.*, 1993). Studies have shown activation of the ACO gene in response to either the PP clofibrate or to 9-cis-retinoic acid, with increased activation upon simultaneous exposure to both ligands (Kliwer *et al.*, 1992). The PPAR α -RXR heterodimers interact with DNA at specified sites termed peroxisome proliferator response elements (PPRE) (Issemann and Green, 1990), which are located upstream of the target gene and result in specific gene transcription. Tugwood *et al.* (1992) proposed that PPREs should be found in the 5' flanking region of genes regulated by PPs. They examined the region upstream of the rat ACO gene that is

up regulated in response to PP administration, and identified an element 570 base-pairs (bp) from the ACO gene. The response to the potent PP Wy-14,643 was abolished upon removal of this region, and the specific binding of PPAR α to the PPRE was also demonstrated. The element contains a direct repeat of two hexameric sequence motifs TGACCT and TGTCCT, spaced by a single nucleotide termed direct repeat 1 (DR1). The importance of the DR1 element in binding PPAR α -RXR heterodimers was demonstrated by Issemann *et al.* (1993), who showed reduced or absent binding if the spacing was altered by even 1 bp. Following on from this, PPRES were identified near to many other PP-induced genes that are largely involved in lipid metabolism and transport (Table 1.1).

Table 1.1: PPRE sequences identified for a number of genes induced by PPs

Gene	PPRE sequence	Reference
Rat acyl CoA oxidase	TGACCT T TGTCCT	Tugwood <i>et al.</i> , 1992
Rat CYP4A1	TCCCCT C TGACCT	Aldridge <i>et al.</i> , 1995
Rat Bifunctional enzyme	TGAACT A TTACCT	Zhang <i>et al.</i> , 1992
Human fatty acyl CoA oxidase	AGGTCA C TGGTCA	Varanasi <i>et al.</i> , 1996
Human apolipoprotein A-I	TGACCC C TGCCCT	Vu-Dac <i>et al.</i> , 1994
Human apolipoprotein C-III	TGACCT T TGCCCA	Hertz <i>et al.</i> , 1995
Human lipoprotein lipase	TGCCCT T TCCCCC	Schoonjans <i>et al.</i> , 1996

PPRE sequences are shown with corresponding references. Sequences represent the minimum required for gene activation to occur.

The physiologic role of PPAR α has been determined largely from the identification of its target genes. PPAR α is expressed primarily in tissues that perform high levels of fatty acid catabolism, such as in the liver. Genes involved with the uptake and binding of fatty acids within cells, fatty acid oxidation and lipoprotein assembly have been identified as containing PPRES. Fasting has been shown to induce hepatic expression of PPAR α target genes involved in fatty acid oxidation. These include the mitochondrial enzymes medium-chain acyl-CoA dehydrogenase and carnitine palmitoyltransferase I (CPTI), and the extra-mitochondrial enzymes ACO and cytochrome P450 4A3 (Leone *et al.*, 1999). PPAR α -null mice show a significant increase in basal levels of serum

cholesterol, particularly high-density lipoprotein (HDL) cholesterol, and in hepatic apolipoprotein A-I mRNA and serum apolipoprotein A-I levels (Peters *et al.*, 1997). Administration of Wy-14,643 to wild-type mice typically results in reduced serum triglycerides, however this did not occur in the PPAR α -null mice (Peters *et al.*, 1997). This suggests that PPAR α is important in the uptake and metabolism of lipids and in lipoprotein metabolism. In response to fasting, PPAR α mRNA levels increase in wild-type mice to accommodate the increased need for hepatic fatty acid oxidation (Kersten *et al.*, 1999). Up-regulation of genes involved in fatty acid transport in response to PPs has also been attributed to PPAR α (Motojima *et al.*, 1998). Endogenous ligands for PPAR α have been identified, including unsaturated long-chain fatty acids (Keller *et al.*, 1993) and the inflammatory mediator and chemotactic agent leukotriene B₄ (LTB₄), which on binding to PPAR α causes activation of genes encoding enzymes involved in fatty acid catabolism (Devchand *et al.*, 1996). This leads to breakdown and deactivation of LTB₄ thus controlling inflammation.

Several receptors demonstrating a high degree of sequence homology to PPAR α have also been identified. PPAR γ is highly expressed in macrophages and white and brown adipose tissue (Braissant *et al.*, 1996). It is involved in the differentiation and regulation of adipocytes, and the thiazolidinediones (glitazones) used to treat Type 2 Diabetes Mellitus target this receptor (Lehmann *et al.*, 1995). PPAR δ was first discovered by Schmidt and colleagues (1992) and was found to be expressed ubiquitously at a higher level than PPAR α or PPAR γ with the exception of adipose tissue. Less is known regarding the function of PPAR δ , but it is currently under investigation as a therapeutic target in the treatment of Type 2 Diabetes Mellitus (Takahashi *et al.*, 2006).

1.3.1.3.4 Species Differences in response to Peroxisome Proliferators

The relevance of peroxisome proliferator-induced carcinogenesis to humans has been investigated since the 1980s. Marked species differences in the response to non-genotoxic carcinogens have been reported, with several studies indicating that human hepatocytes are refractory to the effects of PPs as seen in rodents. Perrone *et al.* (1998) compared the response of cultured human and rat hepatocytes to clofibrate and ciprofibrate, which are known rodent peroxisome proliferators. Rat

hepatocytes were found to express peroxisomal ACO at a 10-fold greater level than human hepatocytes. Suppression of TGF α -induced apoptosis was achieved in rat hepatocytes using clofibrate, as determined using TUNEL. However, the same dose failed to suppress apoptosis in human hepatocytes. Similarly, ciprofibrate caused a two-fold increase in DNA synthesis in rat hepatocytes, but inhibited DNA synthesis in human hepatocytes (Perrone *et al.*, 1998). Human studies have been performed on patients treated with hypolipidaemic drugs such as clofibrate and ciprofibrate. These studies have shown an absence of significant adverse effects (Ashby *et al.*, 1994). Several large epidemiological studies have confirmed the lack of carcinogenic response to PPs. Long-term administration of clofibrate and gemfibrozil to hypolipidaemic patients resulted in no significant increase in the incidence of cancer in studies with follow-up periods of up to nine years (reviewed in Stott and Hawkins, 1993). Taken together, this evidence indicates that humans are resistant to the effects of peroxisome proliferators. Research has now centred on discovering the cause of this resistance.

The possibility of non-responsive species lacking functional PPAR α was refuted by the cloning of fully functional receptors in both human (Sher *et al.*, 1993) and guinea-pig (Bell *et al.*, 1998), a species that shows similar resistance to PPs. Sher *et al.* (1993) used a reporter gene assay to compare the response of human PPAR α with mouse PPAR α in response to the PPs Wy-14,643, clofibrate and nafenopin. Induction of chloramphenicol acetyltransferase (CAT) activity via the PPRE of the ACO gene was used to compare the functionality of human and mouse PPAR α cDNAs. Human PPAR α (hPPAR α) exhibited the ability to activate the ACO gene promoter and was comparable to the mouse PPAR α (mPPAR α) in this respect, indicating that PPAR α activity was not responsible for the species differences observed.

The quantity of PPAR α mRNA was shown to be 10-fold less in human (Palmer *et al.*, 1998) and guinea-pig (Tugwood *et al.*, 1998) compared to rodents. Following on from this, the possibility of mutant forms of PPAR α was investigated. Two such forms have been cloned from human liver. The first mutant receptor to be discovered was hPPAR α 8/14, a truncated form of hPPAR α produced by incorrect splicing of the PPAR α mRNA at exon 6 (Roberts *et al.*, 2000). The splice

defect producing hPPAR α 8/14 results in the generation of a premature stop codon, producing a 174 amino acid truncated receptor instead of the full-length 409 amino acid receptor. The truncated mRNA encoding this mutant receptor appears to be universally expressed in human liver, with incidence between 10- and 40-percent of total PPAR α mRNA (Roberts *et al.*, 2000). The second mutant receptor reported was hPPAR α 6/29, a full-length receptor with normal DNA-binding capacity, but which is unresponsive to PPs. hPPAR α 6/29 has four amino acid substitutions with two of these affecting the ability of hPPAR α 6/29 to respond to PPs (Myers *et al.*, 1997). Roberts and colleagues (1998) used electrophoretic mobility shift assays (EMSA) to demonstrate the ability of hPPAR α 6/29 to bind the rat ACO PPRE in the presence of RXR α . hPPAR α 6/29 was found to be unresponsive to a number PPs, and acted as a dominant negative repressor of mPPAR α activity (Roberts *et al.*, 1998). This was demonstrated by adding increasing amounts of hPPAR α 6/29 to a reporter gene assay containing Wy-14,643 and a constant amount of mPPAR α . hPPAR α 6/29 caused inhibition of mPPAR α activation of the PPRE-containing reporter gene in a concentration-dependent manner. This was further proved when hPPAR α 6/29 was introduced into rat hepatocytes *in vitro*, where it prevented the suppression of apoptosis in response to nafenopin (Roberts *et al.*, 1998). However, humans do respond to the hypolipidaemic properties of certain PPs, altering expression of enzymes involved in lipid homeostasis. One hypothesis to explain this selectivity in response is that the expression of hPPAR α is sufficient to enable the beneficial effects of therapeutic PPs, but is insufficient to affect the number of genes required to produce the pleiotropic effects observed in rodents (Roberts *et al.*, 2000).

Differences in the promoter sequences of PP-responsive genes may cause species differences. Activity of ACO in response to PPs is reported to be 10-fold lower in human hepatocytes than in rat hepatocytes (Perrone *et al.*, 1998). Varanasi *et al.* (1996) identified a PPRE upstream of the human ACO gene and showed it to be active in reporter gene assays. However, in an attempt to verify this observation, Woodyatt *et al.* (1999) looked for evidence of the ACO PPRE in 22 unrelated individuals. They found the PPRE to be inactive in every case. Therefore it is likely that a combination of reduced quantity of PPAR α and reduced efficacy contribute to the lack of response to PPs seen in humans.

1.3.2 MECHANISMS OF HEPATOCARCINOGENESIS - EVIDENCE USING PEROXISOME PROLIFERATORS

Since many of the compounds displaying peroxisomal proliferative effects in rodents are frequently exposed to humans, research has focussed on defining the underlying mechanisms to their hepatocarcinogenicity, and the investigation of PP effects in humans. Despite the differential response of humans and rodents to peroxisome proliferators, this class of compounds has been useful in advancing the understanding of mitogen-induced hepatocarcinogenesis. The exact mechanism leading to carcinogenesis is unknown, although a number have been proposed and will be discussed here. These include the generation of reactive oxygen species (ROS), tumour promotion, and induction of cellular proliferation through increased DNA synthesis and suppression of apoptosis.

1.3.2.1 Oxidative Stress

This theory describes the indirect formation of PP-induced mutations through intracellular hydrogen peroxide generation (Lake, 1995). Hydrogen peroxide can cause DNA damage possibly leading to tumour initiation. Some evidence exists in support of this theory. PPs are known to increase peroxisomal β -oxidation particularly the activity of ACO, which generates hydrogen peroxide as a by-product. This increase in ACO activity is not mirrored by an increase in catalase, which is a peroxidase that allows breakdown of potentially harmful excess hydrogen peroxide (Yeldandi *et al.*, 2000). Therefore through repeated cycles of β -oxidation large amounts of hydrogen peroxide could be generated. Peroxide can diffuse freely out of peroxisomes where it could cause oxidative damage to DNA and cellular membranes, potentially leading to DNA mutations. Levels of the cytosolic enzyme glutathione peroxidase have been shown to decrease on long-term exposure to PPs, and hence another mechanism for removal of hydrogen peroxide is reduced (Conway *et al.*, 1989). In common with this, peroxide-modified lipids have been described in hepatocytes of rats treated with PPs (Conway *et al.*, 1989), and increased levels of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative DNA damage, have been observed in hepatic DNA following chronic exposure to PPs (Kasai *et al.*, 1989).

However, a weight of evidence exists against the theory of PP-induced hepatic oxidative damage. The measurement of 8-OHdG in response to PP exposure by Kasai and colleagues (1989) was performed using total cellular DNA. This experiment was repeated using hepatic nuclei only, and found no significant increase in 8-OHdG levels (Cattley and Glover, 1993). This difference can be explained since total cellular DNA includes both nuclear and mitochondrial DNA, however mitochondrial DNA is known to be more susceptible to oxidative damage (Richter *et al.*, 1988). Soliman *et al.* (1997) used measurement of hepatic F2-Isoprostanes, a sensitive indicator of oxidative stress *in vivo*, to determine the response to PPs. They found Wy-14,643 caused a 16-fold increase in peroxisomal β -oxidation, with minimal effects on catalase activity. However, the hepatic levels of esterified F2-Isoprostanes were unaltered, indicating that oxidative stress does not occur in response to PP administration. This was further substantiated by Conway and Popp (1995) who found no increase in ethane exhalation or hepatic conjugated diene levels (both indicators of oxidative stress) using a higher dose of Wy-14,643 in a longer study. Perhaps the most crucial evidence disproving the oxidative stress theory was found using ACO-null mice, whereby peroxisome proliferation and hepatocarcinogenesis in response to PP administration were unaltered (Fan *et al.*, 1998). Also, in studies comparing the response to Wy-14,643 and less potent PPs, little difference in the effect on peroxide formation was found, even though Wy-14,643 is known to be more carcinogenic (Lake, 1995). Therefore it seems unlikely that oxidative stress is the sole cause for PP-induced hepatocarcinogenesis.

1.3.2.2 Control of cellular proliferation

Balancing cell proliferation and cell death by apoptosis finely controls cell number. It has been observed by Berman and colleagues (1983) that PPs cause cellular proliferation in the liver in common with the liver growth agent phenobarbital. Rodent hepatocytes proliferate rapidly in response to PPs, an effect of both increased DNA synthesis and suppression of apoptosis (Roberts *et al.*, 1995). In response to most PPs there is induction of DNA synthesis, but this frequently returns to basal levels (Ashby *et al.*, 1994), however some more potent carcinogenic PPs such as Wy-14,643 can cause sustained elevation of hepatocyte proliferation (Marsman *et al.*, 1988). Therefore it appears that the degree of hepatocarcinogenicity of a compound is more related to its

ability to induce a persistent increase in DNA synthesis rather than inducing an acute phase of growth that quickly returns to basal levels.

As well as stimulating proliferation, there is evidence that PPs suppress apoptosis. Apoptosis can be triggered by TGF β 1, genotoxic agents such as the anti-cancer drug etoposide, and by activation of the CD95 death receptor. Gill *et al.* (1998) showed that the effects of the afore-mentioned apoptotic stimuli could be suppressed *in vitro* by the PP nafenopin using cultured rodent hepatocytes. This suppression has been shown to be reversible, with withdrawal of non-genotoxic agents causing increased apoptosis and regression of hepatic hyperplasia *in vivo* (Bursch *et al.*, 1984).

The mechanism for PP-induced DNA synthesis and suppression of apoptosis is not fully understood, though it is likely to involve the combination of several pathways. Recent interest has centred on the role of hepatic non-parenchymal cells, particularly Kupffer cells. Nafenopin and Wy-14,643 caused increased phagocytic activity of Kupffer cells *in vivo* (Bojes and Thurman, 1996), a marker of Kupffer cell activation. Kupffer cells are hepatic macrophages that represent approximately 10-percent of the liver cell population. They secrete a range of cytokines including TNF α . A role for TNF α has been proposed following the observation of reduced hepatic regeneration in TNFR1-null mice following partial hepatectomy (Yamada *et al.*, 1997b). Rolfe and colleagues (1997) investigated whether treatment of cultured rodent hepatocytes with TNF α gave analogous results to treatment with PPs. They stimulated apoptosis using TGF α and found significant suppression of apoptosis and stimulation of DNA synthesis following TNF α treatment. The suppression of apoptosis was significantly reduced following addition of anti-TNF α antibodies to the medium (Rolfe *et al.*, 1997). Decreased DNA synthesis following administration of anti-TNF α antibodies has been observed *in vivo* (Bojes and Thurman, 1996), and inactivation of Kupffer cell TNF α production using glycine prevented Wy-14,643-induced hepatocyte proliferation (Rose *et al.*, 1997).

Further experiments by Hasmall *et al.* (2000) showed the dependency of PP-induced hepatocyte changes on non-parenchymal cells. Addition of non-parenchymal cells or medium from non-parenchymal cell cultures increased hepatocyte DNA synthesis and suppressed TGF α 1-induced

apoptosis. Without these cells the response of hepatocytes to nafenopin was prevented, and was restored by re-addition of non-parenchymal cells to the hepatocyte culture. This evidence indicates a role for non-parenchymal cells and TNF α in mediating PP effects on the liver. However, TNF α alone cannot be responsible for the effects of PPs, since levels of TNF α produced by cultured non-parenchymal cells (NPCs) in response to nafenopin treatment were not high enough to elicit an effect on cultured hepatocytes (Hasmall *et al.*, 2000). Peters *et al.* (2000) demonstrated that PPAR α is not expressed in Kupffer cells, suggesting that Kupffer cells either respond generally to toxic stimuli by secreting TNF α , or that this is somehow dependent on PPAR α in hepatocytes.

The proliferative effects of TNF α are possibly mediated by activation of NF- κ B. This is usually bound to and inactivated by the inhibitory proteins I- κ B α or I- κ B β . The most efficient activators of NF- κ B are TNF α and IL-1, which rapidly stimulate I- κ B kinase (IKK) that phosphorylates I- κ B inhibitory proteins (DiDonato *et al.*, 1997). Phosphorylation of these proteins leads to their rapid degradation, allowing nuclear translocation of NF- κ B, where it is involved in the production of cytokines for control of cell growth and differentiation (Baldwin, 1996). This includes IL-6 that is essential for liver regeneration following partial hepatectomy (Cressman *et al.*, 1996). Rusyn and colleagues (1998) showed that the PP Wy-14,643 activated NF- κ B in rat liver and that early activation occurred predominantly in Kupffer cells by as much as 25-times greater than in hepatocytes. This suggests that PP-induced hepatocyte activation of NF- κ B occurs following early activation of NF- κ B in Kupffer cells. Rusyn *et al.* (1998) hypothesised that activation of NF- κ B in Kupffer cells leads to induction of TNF α and other cytokines, which then cause activation of NF- κ B in hepatocytes leading to hepatocyte proliferation (Rose *et al.*, 1999b). The importance of NF- κ B activation in suppressing apoptosis has also been demonstrated by the inhibition of NF- κ B using a dominant mutant of I- κ B, which resulted in hepatocyte cell death by apoptosis (Hellerbrand *et al.*, 1998). This effect could contribute to hepatocyte proliferation in response to PP treatment.

The mechanism for activation of NF- κ B in Kupffer cells remains unclear. There is evidence for a mechanism involving reactive oxygen species. Xanthine oxidase is present at a significant level in Kupffer cells, and is activated rapidly in response to cellular stress. It is possible that PPs directly

increase the activity of this enzyme leading to the generation of ROS (Rose *et al.*, 1999a). Pre-treatment of rats with the free radical scavenger and xanthine oxidase inhibitor allopurinol, prevented the rapid activation of NF- κ B in non-parenchymal cells (Rusyn *et al.*, 1998).

Protein kinase C (PKC) may be involved in NF- κ B activation, either through direct phosphorylation and removal of I- κ B (Diaz-Meco *et al.*, 1994), or through increasing oxidant levels in the Kupffer cell that indirectly modulate NF- κ B activity (Flohe *et al.*, 1997). PKC is activated by free fatty acids (Nishizuka, 1988) and these could accumulate in the cell due to inhibition of acyl CoA synthetase, which has been demonstrated *in vitro* using the PP Wy-14,643 (Bojes and Thurman, 1994). Rose and colleagues (1999a) assessed *in vitro* superoxide production from direct exposure of Kupffer cells to Wy-14,643. They found a dose-dependent 7-fold increase in superoxide production compared with controls. This could be inhibited using glycine, which has been shown to inhibit intracellular calcium signalling (Ikejima *et al.*, 1997), on which PKC activity is dependent. Similarly, the PKC inhibitor staurosporine inhibited superoxide production in a dose-dependent manner, indicating the production of oxidants involves PKC. In support of PKC involvement, Bojes and Thurman (1994) demonstrated that PKC activity increased 3-fold in response to long-term administration of Wy-14,643, and that the levels of PKC elevated in proportion to the hepatocarcinogenic potency of a number of PPs tested.

It has been shown that PKC activity up-regulates expression of PPAR α in response to administration of certain PPs *in vitro* (Yaacob *et al.*, 2001). However, PKC was not found to be involved in the transcriptional activity of PPAR α . This suggests that PPs activate responsive genes in hepatocytes via PPAR α , but also lead to increased PPAR α expression and possibly other effects via PKC.

Cosulich and colleagues (2000) demonstrated a role for mitogen-activated kinases (MAP kinases) in the induction of cell proliferation by PPs. Using inhibitors to p38 MAP kinase and MAP kinase kinase (MKK) they found the nafenopin-induced induction of DNA synthesis and suppression of TGF β -induced apoptosis were prevented. However peroxisome proliferation was unaffected as shown by induction of palmitoyl CoA oxidation, a measure of peroxisome proliferation. The p38

and extracellular signal regulated kinase (ERK) families of MAP kinases are known to be involved in NF- κ B activation (Vanden-Berghe *et al.*, 1998) and IL-6 release (Beyaert *et al.*, 1996). Based on this evidence, Roberts and colleagues (2002) proposed that cross talk between MAP kinases and PPAR α could lead to regulation of hepatocyte growth (Figure 1.6).

Figure 1.6: Scheme depicting the possible relationship between PPAR α and p38 MAP kinase in the control of hepatocyte growth

In response to peroxisome proliferators there may be cross-talk between MAP kinases as induced by the response of non-parenchymal cells via TNF α and IL-6, and PPAR α in hepatocytes. The interplay between MAP kinases and PPAR α could lead to deregulation of hepatocyte proliferation, peroxisome proliferation and potentially carcinogenesis. (Adapted from Roberts *et al.*, 2002.)

Therefore evidence suggests that PPs act through PPAR α that is expressed in hepatocytes but not in non-parenchymal cells. Cytokines such as TNF α produced by non-parenchymal cells are involved in the proliferative response to PPs, most probably through activation of NF- κ B either through generation of ROS by xanthine oxidase and PKC, or through PKC-mediated removal of I- κ B proteins. Proliferation may also involve signalling through MAP kinases, although the exact mechanisms are not fully understood.

1.3.2.3 Role in tumour promotion

Promotion describes the acceleration of pre-initiated cells to form tumours. PPs have a mitogenic effect particularly early in administration (Eldridge *et al.*, 1990), and could cause increased replication of pre-initiated cells. Older animals have a higher frequency of hepatic pre-neoplastic lesions, which probably arise through accumulation during the life of the animal. Studies using Wy-14,643 (Cattley *et al.*, 1991) and nafenopin (Kraupp-Grasl *et al.*, 1991) showed higher incidence of hepatic carcinomas and adenomas in older animals, suggesting a requirement for initiated cells to be present for PP-induced hepatocarcinogenesis. It has been demonstrated that Wy-14,643 administration following prior initiation with a genotoxic carcinogen such as diethylnitrosamine (DEN) results in significantly increased numbers of hepatic tumours compared to controls (without PP administration), suggesting a role for PPs in tumour promotion (Cattley and Popp, 1989).

Intercellular communication is essential for the normal functioning of cells, and may be affected by the action of PPs at gap junctions. It is known that neoplastic cells show reduced or absent intercellular signalling, losing contact inhibition and proliferating despite this restraint. Loss of connexin (Cx) molecules that form gap junctions has been associated with the cancer process. Mally and colleagues (2002) showed significant reduction in expression of Cx32 in liver following Wy-14,643 treatment. This correlated with induction of hepatocyte proliferation and suppression of apoptosis. It has been suggested that this loss of intercellular signalling involves PKC (Elcock *et al.*, 2000). Therefore it is possible that reduced intercellular communication plays an important part in PP-induced hepatocarcinogenesis.

1.3.3 EPIGENETIC MECHANISMS AFFECTING CARCINOGENESIS

As well as the effects of carcinogens on the process of transcription already described, recent advances have highlighted the role of epigenetic mechanisms in carcinogenesis. Epigenetics refers to the study of changes in gene function occurring without a change in the actual sequence of the DNA. Numerous mechanisms have been implicated as causative in carcinogenesis and the following describes the known influence of two such mechanisms.

1.3.3.1 DNA methylation

Examples of epigenetic phenomena are chromatin remodelling and DNA methylation that both contribute to the regulation of gene expression. Chromatin remodelling involves the posttranslational acetylation of histones that make up the nucleosomes of chromatin by histone acetyltransferases (HATs), and the subsequent deacetylation by histone deacetylases (HDACs). Chromatin is usually restrictive to transcription and as such does not allow binding of the transcriptional machinery (TFIID and RNA pol II). It requires a conformational change via acetylation of key lysine residues in the histone N-terminal tail domains that weakens the histone-DNA interactions and allows transcription to occur (Hassig and Schreiber, 1997). HDACs reverse this acetylation and hence restrict access to the DNA, preventing transactivation of gene promoters.

DNA methylation facilitates the process of histone deacetylation, resulting in transcriptional silencing of genes via methylation of CpG islands in gene promoters. CpG islands are regions of DNA composed of dinucleotides of cytosine and guanine, and are so-called as a single site is composed of a cytosine linked to a guanine by a phosphodiester bond. Methylation of CpG islands by DNA methyltransferases occurs on the cytosine producing 5-methylcytosine, and is a critical event in determining the expression of a gene (Pradhan and Esteve, 2003). Following methylation of the CpG island by a methyltransferase enzyme such as the DNA(cytosine-5)-methyltransferase 1 (Dnmt1), the most abundant methyltransferase in mammals, methyl-CpG binding proteins (MeCPs) are recruited that can bind either naked DNA or within chromatin (Nan *et al.*, 1997). Transcriptional repression is then allowed by recruitment of a HDAC by MeCPs such as MeCP2 (Nan *et al.*, 1998). The HDAC then removes acetyl groups from the N-terminal tails of histones, resulting in gene silencing. It has been postulated that methylation of CpG islands by the methyltransferase Dnmt1 causes repression of gene expression not only by binding of MeCP2, which in turn binds to a histone deacetylase such as HDAC1, but also by direct binding of Dnmt1 to HDAC1 via its transcriptional repression domain (Fuks *et al.*, 2000). MeCP2 has also been linked to histone methylation, another epigenetic modification that regulates chromatin organisation and subsequently gene expression (Fuks *et al.*, 2003).

The relevance of DNA methylation and chromatin remodelling to carcinogenesis has been implicated in numerous studies and is the focus of much research as a target for chemotherapeutic intervention (Worm and Guldberg, 2002). Abnormal DNA methylation has been observed for over 50 genes in various cancers (Bombail *et al.*, 2004), including the *k-ras* oncogene for which hypermethylation has been implicated in the development of colon tumours (Esteller, 2002). Cancer cells commonly present with global hypomethylation with regions of hypermethylation when compared to normal cells, and this has been most intensively researched with respect to hypermethylation of tumour suppressor and tumour-related genes. In the normal cell the Rb tumour suppressor protein (pRb) is known to physically interact with HDAC1 and is recruited by the transcription factor E2F1 to target promoters where it masks the E2F1 transactivation domain, suppressing expression of genes that could promote progression through the cell cycle (Magnaghi-Jaulin *et al.*, 1998). Robertson and colleagues (2000) showed that Dnmt1 also interacts with pRb, E2F1 and HDAC1 to repress transcription from gene promoters containing E2F1 binding sites, indicating that in the normal cell the process of DNA methylation was co-operating with pRb repression of downstream transactivation to regulate progression through the cell cycle. McCabe and colleagues (2005) found that the mouse and human *Dnmt1* gene promoters contained E2F binding sites, and that in the absence of pRb the cellular levels of Dnmt1 increase, which correlates with inactivation of a tumour suppressor gene by DNA hypermethylation. This suggests that disruption of the pRB/E2F pathway that is a common finding in human cancer can lead to hypermethylation and gene silencing of important cell cycle regulators.

The effect of DNA methylation on gene expression has also provided a potential mechanism for the action of non-genotoxic carcinogens (Goodman and Counts, 1993) since these compounds do not directly damage the DNA. Pereira and colleagues (2004) evaluated the ability of certain bile acids known to be non-genotoxic carcinogens in rats to induce hypomethylation in colon DNA, measured as the 5-methylcytosine content. They observed a correlation between the carcinogenicity of the bile acid and the degree of hypomethylation observed, with no evidence of hypomethylation found in response to administration of the anti-carcinogenic bile acid ursodeoxycholic acid. Watson and Goodman (2002) compared the response of liver tumour-sensitive and a relatively resistant strain of mice to treatment with the non-genotoxic rodent carcinogen phenobarbital. They observed

more methylation changes, particularly hypermethylation, in the tumour-sensitive animals compared to the resistant animals, indicating that the ability to maintain normal methylation patterns is inversely related to tumour susceptibility. Similarly the effect of the PP Wy-14,643 on DNA methylation has been determined in mouse liver where it was proposed that hypomethylation of key regulators of proliferation such as *c-myc* were involved in the increased proliferation in response to the compound (Ge *et al.*, 2001).

1.3.3.2 MicroRNAs

MicroRNAs (miRNAs) are an abundant class of small, non-coding RNAs (~22 nucleotides) that are thought to regulate expression of protein-coding genes in animals and plants. The first of these regulatory RNAs to be identified was the *lin-4* RNA of *Caenorhabditis elegans* that controls the timing during larval development. It does so by pairing with sites in the 3' untranslated region (UTR) of target mRNAs and negatively regulating their translation via this RNA-RNA interaction (Lee *et al.*, 1993). Following on from this discovery, over 300 miRNA genes have been identified in the human genome, and the predictions of interactions with mRNA estimates that between dozens and hundreds of mRNAs may be regulated by each miRNA, due to the short nature of the sequences, and the fact that silencing is possible without requiring an exactly complementary sequence (Tang *et al.*, 2003).

miRNAs have been implicated as possessing both tumour suppressor and oncogenic properties. miRNAs have been linked to numerous cancers including chronic lymphocytic leukaemia (CLL) that is characterised by malignant B cells overexpressing the anti-apoptotic protein Bcl-2. The expression of two miRNAs, *miR-15a* and *miR-16-1* were found to be down-regulated or deleted in the majority of CLLs, and consequently it was shown that both miRNAs negatively regulate Bcl-2 levels at a post-transcriptional level (Cimmino *et al.*, 2005). This suggests that the normal apoptotic response may be potentiated by *miR-15a* and *miR-16-1* via reduction of translation of Bcl-2. Another miRNA shown to be suppressed in cancer is *let-7* (*let-7*), as demonstrated in lung tumours when compared to normal lung tissue. The *let-7* miRNA is expressed in *C. elegans* and has been postulated as a suppressor of cellular proliferation, since this miRNA targets and negatively regulates *let-60*, the *C. elegans* ortholog of the oncogene Ras (Johnson *et al.*, 2005). Target sites for *let-*

7 were also identified in the mammalian Ras genes allowing *let-7* to negatively regulate Ras expression.

The majority of miRNA genes are down-regulated in cancer, however several exceptions have been investigated, including those of the *miR-17-92* cluster. The expression of these miRNAs was found to be increased in primary lymphoma and a wide range of tumour-derived cell lines (Hammond, 2005), and a direct role in tumourigenesis has been demonstrated for these miRNAs in a mouse model for Burkitt's lymphoma where forced expression of the *miR-17-92* cluster acted with c-myc expression to accelerate the development of tumours (He *et al.*, 2005).

1.3.4 SUMMARY

The mechanisms controlling the response to liver growth agents are complex, involving signalling through many classical mediators including cytokines, nuclear receptors and members of the MAP kinase family. The response to PPs has been extensively researched although the exact mechanism underlying this response is still unknown. PPAR α is clearly a requirement in the response although the interplay between this and other regulators such as p38 MAP kinase requires further investigation. It is clear that non-parenchymal cells are involved in the response, however the mechanisms involved are not clear. Similarly the extent to which ROS generation is involved is unknown, with further investigation of the involvement of TNF α and IL-6 in the initial response to mitogenic stimuli being required. The current challenge is therefore to elucidate how these factors interact both within hepatocytes and between non-parenchymal cells and hepatocytes, allowing co-ordination of the suppression of apoptosis and cell cycle progression as observed in response to liver growth agents. Similarly the involvement of epigenetic mechanisms such as alterations in DNA methylation patterns, or the expression of microRNAs may play a role in the cellular response to carcinogens.

This project seeks to further investigate the regulation of cellular proliferation and apoptosis using the rodent response to liver growth agents as a model, with the view to identifying central regulators in the co-regulation of these two homeostatic processes.

1.4 AIMS OF THE PROJECT

DNA synthesis and apoptosis are related in their regulation, as evidenced by the response of rodent liver to peroxisome proliferators (section 1.3.2.2), however the molecular mechanisms underlying this regulation are not fully understood. The main hypothesis of this project is that genes exhibiting similar expression profiles in response to mitogenic compounds will share common pathways to their activation. Therefore the aims of this project were to identify genes involved in cellular proliferation and apoptosis that show evidence of co-ordinate regulation, and to characterise the central regulators in the expression of these genes. This in turn will improve the understanding of the mechanisms involved in liver growth, and potentially provide molecular markers for future use in analysis of the effects of xenobiotics on liver growth.

The research objectives necessary to achieve these aims were as follows:

1. To identify co-ordinately regulated gene pairs through inspection of gene expression profiles produced by DNA microarray analysis of a number of genes following exposure to xenobiotics known to cause liver growth such as the peroxisome proliferator Wy-14,643.
2. To verify initial data using (i) Real-Time Polymerase Chain Reaction (RT-PCR) and (ii) reporter gene assay of upstream promoter sequences.
3. To identify common regulatory pathways in response to different xenobiotics by analysing the promoters of gene pairs to identify common transcription factor binding sites.
4. To prove experimentally the co-ordinate regulation using site-directed mutagenesis (SDM) to disrupt these binding sites and determine their effect on the co-ordinate regulation.
5. To build an interaction network to allow identification of genes central to the regulation of liver homeostasis and the response to xenobiotic exposure. The central regulators may then be used as molecular markers of liver growth and would be useful in the assessment of the xenobiotic effect of compounds under development.

2 MATERIALS AND METHODS

2.1 MATERIALS

Table 2.1 shows the list of reagents and materials used in the course of the project, and the suppliers used in each case. All other chemicals were purchased from Sigma-Aldrich (Gillingham, UK) and were of molecular biology standard.

Table 2.1: List of suppliers of reagents and materials used in the project

Applied Biosystems (Foster City, USA)	TaqMan [®] Universal PCR Master Mix (4364340) Ambion [®] siRNAs
Canberra Packard (Schwadorf, Austria)	96-well Optiplates
Invitrogen Ltd (Paisley, UK)	<i>Pfx</i> polymerase (11708-013) dNTPs (10297-018) TA cloning kit (K4500-01) cDNA synthesis reagents Dulbecco's Modified Eagle Medium: with L-glutamine (41965-039) Gentamicin (10 mg/mL) (15710-049) Foetal Bovine Serum (10108-165) MEM Non-essential amino acids (11140-035) Trypsin-EDTA (15400-054)
MP Biomedicals (Illkirch, France)	Aurora Chemiluminescent Reporter gene assay kit
MWG (Milton Keynes, UK)	PCR primers TaqMan probes
Novagen (Nottingham, UK)	Rat genomic DNA (69238)
Oxoid (Basingstoke, UK)	Tryptone Yeast Extract Bacterial Agar Phosphate buffered saline (PBS) tablets
Promega UK (Southampton, UK)	DNA modifying enzymes Taq polymerase (M1861) 1 kb DNA step ladder (G6941) Transfast transfection reagent (E2431)
Qiagen Ltd (Crawley, UK)	MinElute Gel Extraction Kit (28604) QIAprep Spin Miniprep Kit (27106) EndoFree Plasmid Maxi Kit (12362)
Roche (Welwyn Garden City, UK)	Agarose Cytotoxicity Detection Kit (4744926001)

2.2 BIOINFORMATIC ANALYSIS

For the microarray analysis, all steps up to, and including the raw data production from DNA Microarrays was performed prior to the commencement of the project at GlaxoSmithKline

(Welwyn, UK). Methods for these steps are included for reference only. All stages of Bioinformatic analysis following from this were carried out at the University of Surrey by Elizabeth Anderson.

2.2.1 ANIMAL TREATMENT AND DOSING

Three compounds known to induce liver enlargement in rodents were administered to groups of male Sprague-Dawley (SD) rats (n=5). The compounds (and doses) were cyproterone acetate (60 mg/kg/day), dexamethasone (100 mg/kg/day) and Wy-14,643 (50 mg/kg/day). All compounds were administered in corn oil. Three doses of each compound (or corn oil as vehicle control) were administered at 9am in the morning at 0, 24 and 48 hrs from the start of the study. Groups of animals were sacrificed at 6, 12, 24, 48, 72 and 96 hrs post-initial dose, the livers were excised, minced and stored at -80°C until analysis.

2.2.2 MICROARRAY ANALYSIS

Collected liver samples were analysed using Rat Genome U34 GeneChip[®] microarray chips (Affymetrix), containing probe sets for over 24,000 mRNA transcripts and ESTs across three arrays. Total RNA was extracted from the tissue samples using the QIAGEN RNeasy kit, and RNA integrity checked by gel electrophoresis. RNA was quantified to determine RNA concentration and protein contamination using absorbance spectroscopy (A_{260}/A_{280} ratio between 1.9 and 2.1 required). First Strand Synthesis was performed using the T7 primer (Figure 2.1) to hybridise to mRNA strands and prime the first strand synthesis by Superscript RT II (Invitrogen). The T7 primer incorporates an oligo(dT) region that allows binding to the poly(A) tail of mRNA.

Figure 2.1: T7 primer for First strand cDNA synthesis from mRNA

5' - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄ - 3'

Extra bases

T7 promoter sequence

Transcription Initiation Sequence

Oligo(dT) for mRNA poly-A tail (or gene specific sequence if required)

The transcription initiation sequence is necessary for the *in vitro* transcription stage to synthesise biotin-labelled cRNA prior to fragmentation and hybridisation to the array.

A second cDNA strand was then synthesised by RNA replacement (Nick translation) using the cDNA produced in the first reaction. The double-stranded cDNA was cleaned prior to *in vitro* transcription (IVT). The IVT reaction allows high yield biotin-labelling of transcripts. T7 RNA polymerase catalyses IVT in the presence of biotin-labelled UTP, using the transcription initiation sequence incorporated in the T7 primer. RNA polymerase binds its double-stranded DNA promoter and separates the two strands. It then uses the 3' to 5' (antisense) strand as a template for synthesis of a complementary 5' to 3' RNA strand (cRNA). The biotin-labelled cRNA was cleaned in order to remove RNA from the sample, and quantitated so that the same amount could be applied to each array. The cRNA yield from the IVT reaction was then adjusted in order to reflect carryover of unlabelled RNA (minimum concentration for cRNA fragmentation was 0.6 µg/mL).

The cRNA was then fragmented prior to hybridisation onto the array, using metal-induced hydrolysis/"Hammerhead cleavage" to produce 35 - 200 base-pair fragments. A hybridisation cocktail containing fragmented cRNA, synthetic control oligonucleotide and hybridisation controls was then applied to a primed array, and incubated overnight at 45°C in a rotating oven. Arrays were washed and stained using a three-stage system using a Genechip® fluidics station 400 operated by Affymetrix Microarray Suite version 5.0:

- streptavidin phycoerythrin (SAPE) conjugate was applied to the array - biotin-labelled fragments bind the SAPE conjugate
- an anti-streptavidin biotinylated antibody then binds the streptavidin
- further SAPE was added that binds the biotinylated antibody

Each array was then read twice at 3µm resolution with an emission of 578 nm by an Agilent GeneArray® confocal scanner (excitation at 488 nm). The signal was converted to an electrical current and hence to a numerical value using Affymetrix Microarray Suite version 5.0.

2.2.2.1 Pre-processing of Microarray data

The data generated from the microarray analysis was normalised using an Affymetrix algorithm that allows reliable comparison of multiple arrays, minimising differences of both biological and technical origin. Biological variation can be due to differences for example in age, weight, genetic

background of test subjects. Technical variations could be due to for example, handling error, or differences in the quality or quantity of the target hybridised or the reagents used. The normalisation step is necessary to correct for such variations between any two arrays, allowing comparison of the results between multiple arrays. The normalisation factor is shown in equation 1. Each 25-mer oligonucleotide probe on the array has a corresponding mismatch probe (MM) that contains a single mismatch at position 13, providing a specificity control when compared to the corresponding perfect match (PM) probe. Hence if there are no mismatches on either array, the normalisation factor is 1. Any difference between the two chips provides a scaling factor that can be applied to normalise chip₂ to chip₁.

$$\text{Normalisation factor} = \frac{\sum \text{chip2}(PM - MM)}{\sum \text{chip1}(PM - MM)} \quad \text{Equation 1}$$

2.2.2.2 Identification of co-ordinately regulated genes

Following normalisation, it was necessary to mine the data, identifying those genes that showed co-ordinate regulation in response to drug exposure: The general schema for this analysis is shown in Figure 2.2, and described in detail below. Initially, only gene fragments showing a minimum three-fold change in expression (in either direction, at least one time point for any compound) were further considered. This cut-off was chosen to increase the likelihood that the genes included would reflect expression changes of biological importance. Once gene fragments had been filtered by their maximal change (≥ 3 -fold), data was sorted to identify those fragments that showed similar patterns of expression across the six time-points measured.

Firstly, the data was sorted by accession number using Microsoft® Excel 2000 thus grouping all gene profiles together. The direction of change of gene expression for each gene fragment was labelled for each time point (up, down or unchanged). The CONCATENATE function was then used to link fragment accession number and direction of change together producing a single description for each profile i.e. **D88890_at(11)upupupupupup** where **D88890_at(11)** is the fragment accession number and the repeated **up** represents the direction of change for all six time points. The IF function was then used to identify whether one description was the same as for the following row i.e. same accession number and same direction of change for all time points: If

descriptions were identical the statement **KEEP** was entered in the subsequent column, and if non identical then **DISCARD** was entered. Wherever three **KEEP** statements were found adjacent (i.e. all three compounds producing the same description) the fragment accession number was noted.

Secondly, fragments with similar profiles across multiple compounds were clustered manually into groups showing potential co-ordinate regulation. Clustering was verified using the Multi-Variate Statistical Package (MVSP 3.13b© Kovach Computing Services). Clustering was by Pearson Correlation Coefficient (unweighted pair group average linkage technique with randomised input order).

Figure 2.2: Scheme outlining data mining process following Microarray analysis of SD rat liver transcript levels in response to liver growth agents

Data Mining Process	Number of genes
Total number of genes on Microarray	~ 20,000 genes
Screened for genes showing ≥ 3 fold change in transcript level in response to compounds (in either direction and at any time point permitted)	CPA → 2420 genes DEX → 3072 genes WY → 1992 genes
Screened for genes showing similar profile across all three compounds used	38 genes
Genes with similar profiles clustered together to form potentially co-regulated groups	10 groups

The number of gene fragments showing response to all three liver growth agents was determined by expression profile comparison, the profiles for these 38 genes were then clustered.

2.2.2.3 Investigation of common response elements

Promoter regions immediately adjacent to the putative transcriptional start site (TSS) for each gene showing co-ordinate regulation were obtained from the NCBI website (www.ncbi.nlm.nih.gov) as follows. The gene related to each gene fragment under investigation was obtained using both the NCBI BLAST tool to probe the blastn database (www.ncbi.nlm.nih.gov/BLAST, Altschul *et al.*, 1990), and the NetAffix analysis database (www.affymetrix.com). The mRNA sequence for each gene was then used to probe the rat genome using the NCBI BLAST tool. The one kilobase region adjacent to the first exon was then entered into the Match tool (<http://transfac.gbf.de/TRANSFAC>) and MatInspector (available at <http://www.genomatix.de>) that interrogate the TRANSFAC database of transcription factor binding sites (Quandt *et al.*, 1995). The output from these searches is in the form of matrix numbers that define a single transcription factor binding site, or that of a group of related transcription factors. In both cases the core similarity and matrix similarity were both set at 0.7 (a low stringency search to minimise false negatives) and the analysis performed. The matrix similarity describes the similarity between a matrix and a part of the input sequence, with the core similarity representing the similarity between only the five most conserved nucleotides in the matrix and the input sequence. From the results for each individual gene, a list of the matrix numbers common between the genes was compiled and the corresponding response elements identified using the Genecards website (<http://bioinfo.weizmann.ac.il/cards>) to provide information on the known function of the transcription factors.

2.3 GENERIC MOLECULAR BIOLOGY TECHNIQUES

2.3.1 AGAROSE GEL ELECTROPHORESIS

All electrophoresis was performed on 1% agarose gels prepared by dissolving 1 g agarose (Promega) in 100 mL 1xTAE (0.04 M Tris Acetate, 0.01 M EDTA, pH 8.3) plus ethidium bromide (0.5 µg/mL). Once set, gels were placed in RunOne™ electrophoresis tanks filled with 1xTAE buffer. Samples were mixed with loading buffer (0.25% Orange G, 50% glycerol) and were loaded (maximum 20 µL per well) along with 5 µL 1 kb DNA ladder (100 µg/mL, Promega) as size marker. Electrophoresis was performed at 5 V/cm until the loading dye had moved three-quarters

of the gel length. Bands were visualised using a 302 nm medium wavelength ultra-violet transilluminator (Syngene Gene Genius BioImaging System) at 0.04 second exposure, and GeneSnap 4.01 (Synoptics) software.

2.3.2 NUCLEIC ACID CONCENTRATION DETERMINATION

DNA concentration was determined by spectrophotometry using a GeneQuant II RNA/DNA calculator (Pharmacia Biotech). One absorbance unit at 260 nm correlates to 50 µg/mL of double stranded DNA. The A_{260}/A_{280} ratio was determined to estimate DNA purity with respect to protein contamination. Pure DNA has a ratio of between 1.7 and 1.9 in 10 mM Tris (pH 7.5).

2.3.3 GROWTH OF BACTERIAL CULTURES

Plasmid-containing bacteria from recent transformations (less than 1 week old) or a glycerol stock were spread across the surface of an antibiotic-containing agar plate (100 µg/mL ampicillin or 50 µg/mL kanamycin as required) and incubated overnight at 37°C. A single colony was picked using a sterile toothpick and inoculated into 5 mL LB medium containing the appropriate antibiotic. This culture was incubated overnight at 37°C on a rotary incubator at 225 rpm. For subsequent maxi-preparation 100 µL of mini-culture was added to 250 mL antibiotic-containing LB medium and grown overnight at 37°C with shaking.

2.3.4 PREPARATION OF GLYCEROL STOCKS

From mini-cultures (section 2.3.3) glycerol stocks were prepared for long-term storage, whereby 150 µL of sterile glycerol was added to 850 µL of bacterial culture in a sterile 1.5 mL tube. The tube was vortexed briefly and stored at -20°C.

2.3.5 POLYMERASE CHAIN REACTION (PCR)

2.3.5.1 Template DNA

For cloning of target genes/promoters polymerase chain reaction (PCR) was performed using either rat genomic DNA (Novagen) as template. If no suitable PCR product was amplified, bacterial artificial chromosome (BAC) clone DNA was used as an alternative (Children's Hospital Oakland Research Institute, CHORI) (Table 2.2). These are rat genomic DNA clones containing approximately 200 kilobase-pairs (kbp) each. The exact clones required were selected using the clone-finder available at the Human Genome Sequencing Center (HGSC) from Baylor College of Medicine (www.hgsc.bcm.tmc.edu/projects/rat). BAC clone DNA was prepared as described in section 2.3.6.4.1.

Table 2.2: Bacterial Artificial Chromosome clones (CHORI) used as PCR templates

Gene	Project	Clone	Accession No.
HCaRG	kaxx	CH230-335N13	AC124883
Nob1p	gjld	CH230-110O21	AC106171
Me1	gbwr	CH230-6H6	AC124868
RBACH	gtso	CH230-229D4	AC118359

BAC clones ordered for genes where PCR was unsuccessful using rat genomic DNA as template. The reduced size of template improved the specificity of the PCR.

2.3.5.2 Primer Design

The published sequence for the 5' flanking regions of HCaRG, LRCH4, Ces2 and rBACH, and the 3' flanking regions of Nob1p, Sp3, Me1 and rCOUPg were obtained as in section 2.2.2.3 and imported into Vector NTI 4.0 (InforMax Inc.). PCR primers were then designed using this program with the following parameters: Product length 900 to 1100bp, % GC to between 40 and 60% and primer length 17 to 23 bp. Derived primer sequences with the expected amplicon sizes are shown in Table 2.3.

Table 2.3: Primer sequences for PCR of genes of interest

Gene	Size (bp)	Sense/ Antisense	Primer Name	Sequence (5' to 3')
HCaRG	1006	Sense	HCaRG-949s	5'- TAAGCAGCTGGGCAGCTCTTC-3'
HCaRG		Antisense	HCaRG+57a	5'- CAAGCCTTTACCGCCCTCTG-3'
Nob1p	1087	Sense	Nob1p-26s	5'- AGCCACAACGTGCTCCACAG-3'
Nob1p		Antisense	Nob1p+1061a	5'- TTTTGGGTATATGCCCAGGAGAG-3'
LRCH4	1138	Sense	LRCH4-986s	5'- GTCCTAGCTGGGCGGTGATG-3'
LRCH4		Antisense	LRCH4+152a	5'- ACGGAGACTGAAGCTGCAGC-3'
Sp3	1049	Sense	Sp3-61s	5'- TGTGTTGGGCAGCGGTATTCTG-3'
Sp3		Antisense	Sp3+988a	5'- TGGGCAGTATGTCCTTCCCC-3'
rBACH	970	Sense	rBACH-800s	5'- TTCTGTGGCCTGAAACTTGG-3'
rBACH		Antisense	rBACH+170a	5'- CATCCTTGTGAGCCTATAGCATC-3'

Upon receipt, lyophilised primers (MWG) were resuspended in 1x TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) to a stock concentration of 100 mM. The stock primers were diluted with nuclease-free water to 10 mM. Stock and working concentrations were both stored at -20°C.

2.3.5.3 PCR Protocol

PCR reactions were performed using 200 ng of BAC plasmid DNA as template (section 2.3.5.1) or 250 ng of rat genomic DNA (Novagen). Reactions were performed in duplicate using thin-walled GeneAmp reaction tubes (Perkin Elmer). Tables 2.4 (Taq polymerase) and 2.5 (*Pfx* polymerase) show the volumes used for PCR reactions with low fidelity and high fidelity polymerase respectively. Table 2.6 shows the temperature cycles used for each primer pair, and Table 2.7 shows the optimised annealing temperatures.

PCR products were visualised by separation in a 1% agarose gel containing ethidium bromide (section 2.3.1) and size estimated by comparison to a 10 kb step ladder (Promega).

Table 2.4: Volumes used in PCR reactions (Taq polymerase)

Reagent	Volume (μL)	Working concentration
10x PCR Buffer	5	1x
MgCl ₂ (25 mM)	3	1.5 mM
Primers (20 mM)	1.5 (1:1 combined mixture)	30 pmol/μL
dNTPs (10 mM)	1.5	300 μM
Betaine (5x)	10	1x
Taq Polymerase (5 U/μL)	0.5	2.5 U
DNA	200 ng	4 ng/μL
Nuclease-free water	Volume up to 50 μL	

Table 2.5: Volumes used in PCR reactions (Pfx polymerase)

Reagent	Volume (μL)	Working concentration
10x PCR Buffer	5	1x
MgSO ₄ (50 mM)	1	1 mM
Primers (20 mM)	1.5 (1: 1 combined mixture)	30 pmol/μL
dNTPs (10 mM)	1.5	300 μM
Enhancer (10x)	5	1x
Pfx Polymerase (2.5 U/μL)	0.5	1.25 U
DNA	200 ng	4 ng/μL
Nuclease-free water	Volume up to 50 μL	

Table 2.6: Example temperature program for PCR amplifications

Stage	Temperature (°C)	Time (minutes)	Number of cycles
1	94	5	1
2	94	0.5	30
	55	1	
	72	2	
3	72	5	1

Table 2.7: Optimised annealing temperatures and enzymes for genes cloned

Gene	Enzyme for PCR	Annealing Temperature (°C)
HCaRG	Taq polymerase	50
Nob1p	Taq polymerase	50
LRCH4	Taq polymerase	57
Sp3	Taq polymerase	57
rBACH	Pfx polymerase	47

2.3.6 CLONING

2.3.6.1 DNA extraction from agarose gels

PCR reactions (50 μL) were separated using a 1% agarose gel containing 100 $\mu\text{g}/\text{mL}$ crystal violet DNA stain. Gels were run as section 2.3.1 in 1 x TAE buffer. Bands of expected size (approximately 1000 bp) were excised from the gel using a scalpel and the DNA extracted using the MinElute Gel Extraction Kit (Qiagen). The columns contain a silica membrane for binding of DNA in high-salt buffer, and elution with low salt buffer or water. This allows removal of agarose, primers, enzymes and other impurities from DNA. The manufacturer's protocol was followed, with a final elution volume of 10 μL .

2.3.6.2 Ligation and One Shot Transformation

Taq amplified flanking sequences were ligated into the pCR[®]2.1-TOPO vector (Invitrogen). Reactions were set up as shown in Table 2.8 and allowed to proceed overnight at 14°C before transformation into bacteria. Alternatively, PCR products from *Pfx* polymerase were cloned into the pCR Blunt cloning vector (Invitrogen) using the same protocol, but with ligation at 16°C for 1 hour.

Table 2.8: pCR[®]2.1-TOPO ligation reaction

Reagent	Volume (μL)
Linear vector (25ng/ μL)	1
PCR product	5
10x ligation buffer	1
T4 DNA ligase (4U/ μL)	1
Nuclease-free water	2

Resultant ligation mixtures were transformed into competent TOP10 *E.coli* cells using the One Shot Transformation kit (Invitrogen). Bacterial cells were stored at -80°C until required, then thawed on ice for 10 minutes. 5 μL of each ligation reaction was added to a vial of cells and incubated on ice for 30 minutes. Cells were subjected to a brief heat shock (42°C for 45 seconds) and recovered on ice for 2 minutes. 200 μL LB broth was added to each vial, followed by incubation at 37°C for 1 hour with shaking (200 rpm). The pCR 2.1-transformed cells were plated on agar containing 50

µg/mL ampicillin, 0.012 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to allow blue/white selection. The pCR Blunt-transformed cells were plated on agar containing 50 µg/mL kanamycin. These cells did not require blue/white screening due to an integral *cadB* lethality gene. All plates were incubated overnight at 37°C. For each vector ten white colonies were picked and inoculated in 5 mL LB containing the appropriate antibiotic. For pCR 2.1-transformed cells, one blue colony was picked to provide a vector only control. Cultures were grown overnight at 37°C with shaking (200 rpm) and then stored at 4°C.

2.3.6.3 Phenol/chloroform check for inserts

To rapidly assess for the presence of inserts, 60 µL of overnight LB culture was mixed with 60 µL phenol/chloroform (1:1, pH 7.9) and 10 µL 5 x Orange G loading dye (0.25 % Orange G, 50 % Glycerol). Samples were vortexed and centrifuged for 1 minute at 10,000 rpm. 20 µL of the top phase was analysed by agarose gel electrophoresis (section 2.3.1) along with 10 µL of starting plasmid (5 ng/µL). The presence of an insert was confirmed by comparison with the original plasmid.

2.3.6.4 Preparation of plasmid DNA

2.3.6.4.1 Preparation of BAC clone DNA

BAC clones were cultured on agar containing 25 µg/mL chloramphenicol. Individual colonies were picked and incubated overnight at 37°C in 5 mL LB broth containing 25 µg/mL chloramphenicol. The BAC vector was isolated using a modified alkaline lysis method and QIAGEN mini-prep solutions (1 to 3). Briefly, 1 mL of culture was centrifuged at 3,000 rpm for 10 minutes. Supernatants were discarded and pellets resuspended in 300 µL solution 1 and vortexed briefly. 300 µL solution 2 was added to each sample and mixed gently. Samples were incubated at room temperature for up to 5 minutes. 300 µL solution 3 was added and mixed gently. Samples were placed on ice for 5 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatants were transferred to fresh microcentrifuge tubes containing 800 µL ice-cold isopropanol and incubated

on ice for 5 minutes. Samples were centrifuged at 10,000 rpm for 15 minutes at 4°C. Pellets were washed with 500 µL 70% ethanol and centrifuged at 10,000 rpm for 5 minutes. Pellets were then air-dried at room temperature and resuspended in 40 µL 1x TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8) by gentle tapping. Clones were verified to confirm the target sequence using real-time quantitative PCR (TaqMan) with probe/primer sets appropriate for each target gene. Clone preparations were diluted 1000-fold for PCR reactions and 5 µL was used for quantitative real-time PCR analysis (section 2.5.6) to confirm the correct BAC clone.

2.3.6.4.2 Alkaline lysis preparation of plasmid DNA

Plasmid DNA was extracted from cultured cells using the alkaline lysis method (personal communication, Dr K. Plant). 1 mL of each culture was centrifuged for 1 minute at 10,000 rpm. Pellets were re-suspended in 100 µL solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8) and vortexed. 200 µL solution 2 (0.2M NaOH, 1% SDS) was added and mixed gently by inverting. Samples were incubated on ice for 2 minutes, followed by addition of 150 µL solution 3 (3M potassium acetate, 10% glacial acetic acid). Samples were mixed gently and left on ice for 5 minutes. Samples were centrifuged for 5 minutes at 10,000 rpm. The supernatant was transferred to a clean microcentrifuge tube together with an equal volume of phenol/chloroform (1:1, pH 7.9). Samples were vortexed and centrifuged for 5 minutes at 10,000 rpm. The aqueous phase was added to 800 µL ethanol and immediately centrifuged for 5 minutes at 13,000 rpm. Pellets were washed with 800 µL 70% ethanol solution, and centrifuged for 2 minutes at 13,000 rpm. Pellets were air-dried for 5 minutes and re-dissolved in 50 µL 1x TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Preparations were stored at -20°C.

2.3.6.4.3 QIAGEN Endo-free™ preparation of plasmid DNA

For sequencing and sub-cloning of pCR Blunt and pCR 2.1 constructs, DNA was isolated from *E.coli* DH5α cells using the QIAGEN Mini-prep kit. Generation of µg quantities of DNA was achieved for pSEAP constructs using the QIAGEN Endofree™ Maxiprep kit to isolate plasmid DNA from *E.coli* DH5α cells. The manufacturer's protocols were followed in each case with the

DNA being re-dissolved in between 250 µL and 500 µL of endotoxin-free buffer TE. DNA concentrations were determined by spectrophotometry (section 2.3.2) and the integrity of the plasmid DNA checked by electrophoresis (section 2.3.1).

2.3.6.5 Sequence confirmation

2.3.6.5.1 Diagnostic restriction enzyme digest

Restriction enzymes suitable to produce two fragments of differing size were selected using restriction maps (Vector NTI). Reagent volumes are shown in Table 2.9 and represent reactions requiring two enzymes. Where a single enzyme was used an extra 0.5 µL water was added per sample. A master-mix was prepared for multiple reactions.

Table 2.9: Reagent volumes for confirmatory Restriction Enzyme digests

Reagent	Volume (µL)
Enzyme 1 (10 U/µL)	0.5
Enzyme 2 (10 U/µL)	0.5
Buffer	1.5
Nuclease-free water	7.5
Plasmid mini-prep	5

The digests were incubated at 37°C for 1 to 2 hours. The results were visualised by electrophoresis on a 1% agarose gel containing ethidium bromide (section 2.3.1).

2.3.6.5.2 DNA Sequencing

Sequencing was carried out using a Beckman Coulter 2000XL instrument by Mrs Victoria Leggett using the “termination reaction” method. Four different fluorescent dye labels were incorporated into extended DNA products using 3’ dye-labelled dideoxynucleotide triphosphates, products separated by capillary electrophoresis and detected by fluorescence emission. Plasmids were sequenced from 250 ng DNA per reaction, using M13 forward and reverse primers for pCR Blunt and pCR 2.1 vectors, and pSEAP 5’ (CTAGCAAAATAGGCTGTCCC) and 3’ (CCTCGGCTGCCTCGCGGTTCC) primers. Sequence identity was then confirmed using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast>).

2.3.6.6 Deletion mutant construction

For the HCaRG and Nob1p promoters, deletion constructs were produced to allow closer examination of the 1 kb promoter sequences that were initially cloned. Deletion constructs were produced using PCR primers designed to include restriction enzyme sites, allowing the production of smaller daughter fragments using the initial 1 kb as target sequence. Primer names represent the gene name and the entire region cloned (including some vector sequence). PCR from deletion mutant primers was carried out using Taq polymerase (Invitrogen). Table 2.10 shows the primer sequences used to generate the deletion constructs. Table 2.11 shows the annealing temperatures for each primer pair with the corresponding restriction endonuclease sites generated. Following amplification by PCR, fragments were purified by gel extraction, digested with the appropriate restriction endonucleases and ligated into pSEAP (pre-digested with the same enzymes).

Table 2.10: Primers and restriction endonucleases used to generate deletion constructs

Primer Name	Sense primer	Antisense primer
HCaRG-158 bp	gcgctcgg <u>ttccggga</u> accac	atctcgagcggccgcca <u>gtg</u>
HCaRG-537 bp	taaggcaggtaccacacaaattacgaccc	atctcgagcggccgcca <u>gtg</u>
HCaRG-765 bp	gaactcacagagatctgtctgcct	gcttacttagatcgagatctcgag
Nob1p-144 bp	gttcctctcgagtcgggaggagtg	atctcgagcggccgcca <u>gtg</u>
Nob1p-382 bp	atcgaaggta <u>cttactg</u> atttatatgtag	gcttacttagatcgagatctcgag
Nob1p-797 bp	tagctcggtacccatttttaatagg	gcttacttagatcgagatctcgag

Restriction endonuclease recognition sequences are underlined, with mutated nucleotides shown in bold. Constructs were verified using restriction digests and by direct sequencing.

Table 2.11: Primer annealing temperatures (T_m) for HCaRG and Nob1p deletion mutants

Primer Pair	T _m (°C)	Restriction Endonucleases
HCaRG-158 bp	61	Acc65 I/Xho I
HCaRG-537 bp	50	Acc65 I/Xho I
HCaRG-765 bp	50	Bgl II
Nob1p-144 bp	61	Xho I
Nob1p-382 bp	50	Bgl II
Nob1p-797 bp	50	Acc65 I/Bgl II

2.3.6.7 Site-directed mutagenesis (SDM)

SDM was used to introduce point mutations into the HCaRG-158 bp and Nob1p-144 bp constructs to knock-out the effects of potential transcription factor binding sites. In each case two sets of PCR primers were produced. One set amplified a large region upstream of the putative binding site, while the second set amplified the downstream region. In each case a minimum of four bases were mutated to convert the sequence to the required restriction endonuclease recognition sequence (Table 2.12). PCR from SDM primers was carried out using Taq polymerase (Invitrogen). Primer annealing temperatures and the restriction endonucleases appropriate to each primer pair are shown in Table 2.13.

Table 2.12: Primer sequences for HCaRG and Nob1p SDM mutants

Gene/ Distance from TSS	Fragment ID	Sense primer	Antisense primer
HCaRG/ Nrf2-28 bp	Upstream	cgccattcaggctgcgcaac	cgggtcc gg agaagtggacca
	Downstream	cacttctcc gg accgcccaac	tggctgtggctccactgtctgg
Nob1p/ Nrf2+27 bp	Upstream	gcagcgtgaccgctacacttgcca	agctccgg tgat caccacaacgtgct
	Downstream	ttgtgt gat caccggagctttct	acaggtaggccgtggctgtggctcca
Nob1p/ Nrf2-281 bp	Upstream	tggagtccacgttcttaatagtggact	tgaaag ggccc agctagaaagag
	Downstream	ctagct gggccc tttcaacca	acaggtaggccgtggctgtg
Nob1p/ AhR+17 bp	Upstream	gcagcgtgaccgctacacttgcca	agccacact ag tctccacaggcgccatgt
	Downstream	tgtggag actag tgtggctgac	tggctgtggctccactgtctggc
Nob1p/ AhR-66 bp	Upstream	aggctgcgcaactgttgggaagggcgatc	ccaca gggccc ctccacaggcgccat
	Downstream	gtggag ggccc ctgtggctgac	tggctgtggctccactgtctgg

Potential transcription factor binding site and the distance of the site from the TSS (upstream and downstream) are shown. Mutated bases are shown in bold corresponding to restriction endonuclease recognition sequences.

PCR amplification of the primers sets produced two overlapping products per binding site. PCR products were gel extracted (section 2.3.6.1) and digested with the appropriate enzyme (Table 2.13). The two corresponding products were then ligated to form a single section with the binding site mutated to the restriction endonuclease recognition sequence. The ligated product was then diluted 1 in 100 prior to PCR amplification using either HCaRG-158 or Nob1p-144 primers (Table 2.10) in order to generate sufficient quantities of the mutated region to allow ligation into pSEAP and subsequent transformation of *E.Coli* DH5 α cells as described in section 2.3.6.6.

Table 2.13: Primer annealing temperatures (T_m) and enzymes for SDM mutants

Gene/Distance from TSS	Primer Pair	T_m (°C)	Restriction Endonuclease
HcARg/Nrf2-28 bp	Upstream fragment	64	Acc III
	Downstream fragment	66	Acc III
Nob1p/Nrf2+27 bp	Upstream fragment	71	Bcl I
	Downstream fragment	65	Bcl I
Nob1p/Nrf2-281 bp	Upstream fragment	63	Apa I
	Downstream fragment	63	Apa I
Nob1p/AhR+17 bp	Upstream fragment	70	Spe I
	Downstream fragment	57	Spe I
Nob1p/AhR-66 bp	Upstream fragment	68	Apa I
	Downstream fragment	68	Apa I

2.4 CELL BASED TECHNIQUES

2.4.1 RECOVERY FROM LIQUID NITROGEN

FaO hepatoma cells (Moore and Weiss, 1982; ECACC number 89042701) were stored frozen (in liquid nitrogen) in 90% foetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO). A vial containing approximately 1×10^6 cells was thawed at 37°C for 10 minutes, and mixed gently with 10 mL of Dulbecco's modified eagle medium [with phenol red and L-glutamine, supplemented with 10% FBS, 1x non-essential amino acids (NEAA) and 10 µg/mL gentamycin (Gibco BRL)]. Cells were centrifuged at 150 x g for 5 minutes, and resuspended in 10 mL fresh medium. The cell suspension was transferred to a 25 cm² vented culture flask (NuncTM, Nunc Brand Products), and incubated at 37°C in 4% CO₂.

2.4.2 MAINTENANCE CULTURE

Cells were passaged every five days in the ratio of 1:4. When confluent cells were washed with 10 mL 1x phosphate-buffered saline (PBS), and incubated with 2 mL Trypsin-EDTA solution (1x) for approximately 5 minutes (until the monolayer had detached). The TE was neutralised with 2 mL of medium, and the cells resuspended with a pipette. Then 1 mL of cell suspension was transferred to each of four fresh 75 cm² vented culture flask (NuncTM, Nunc Brand Products) containing 14 mL of complete medium (section 2.4.1), and incubated at 37°C in 4% CO₂.

2.4.3 DOSING FOR *IN VITRO* EXPERIMENTS

FaO cells were seeded onto plates (NuncTM, Nunc Brand Products) at a density of 5×10^5 cells per mL. Cells were passaged to a maximum of 12 times following which fresh cells were recovered (section 2.4.1) and cultured. For 6-well plates 2.5 mL of cell suspension was transferred to each well, and for 96-well plates this volume was 100 μ L. Plates were incubated for 48 hours to allow the cells to attach and reach approximately 70% confluence. Stock solutions of xenobiotics were prepared in DMSO (0.5 mL) at 1000x concentration with all drug stocks being prepared daily to prevent degradation during storage. Working solutions were prepared by 1000-fold dilution in complete medium, resulting in a working DMSO concentration of 0.1 %. The xenobiotics used were cyproterone acetate (Sigma C3412), clofibrate acid (ICN Biochemicals no.150701), dexamethasone (Sigma D-1756), Wy-14,643 (Sigma C7081), β -naphthoflavone (Sigma N3633), 3-methylcholanthrene (Sigma 213942) and benzo(a)pyrene (Sigma 48564). Medium (with or without compound/DMSO) was replaced every 48 hours.

2.4.4 TRANSIENT TRANSFECTION OF THE FAO CELL LINE

Transfast (Promega) was chosen as the transfection reagent for FaO cells as it was used previously in this laboratory (Swales *et al.*, 2003). The method was optimised for the amount of DNA transfected, using 0.05 μ g, 0.1 μ g and 0.25 μ g with a reagent:DNA ratio of 3:1. Cells were seeded into clear 96-well plates (NuncTM, Nunc Brand Products) at a density of 5×10^5 cells/mL (100 μ L per well). Cells were incubated overnight and were hence 50 to 70 % confluent at the time of transfection. DNA (0.25 μ g per well) was added to serum-free medium (for a total volume of 40 μ L per well) and vortexed briefly. Transfast (9 μ L per μ g DNA) was then added to the DNA/medium mixture and vortexed immediately. The transfection mixture was incubated at room temperature for 15 minutes. The medium was then removed from the cells, and 40 μ L of transfection mixture was added to each well. Cells were incubated for 1 hour at 37°C. The transfection mixture was then directly overlaid with 80 μ L of complete medium, and the cells were mixed gently by swirling the plate. Cells were incubated overnight at 37°C (section 2.4.2) prior to assay for basal activity of the promoter, or dosing with liver growth agents in growth medium for the required incubation time.

2.4.5 PROTOCOL FOR CHEMILUMINESCENT DETECTION OF SEAP PROTEIN

Luminescence of secreted alkaline phosphatase was measured using the Aurora™ AP chemiluminescent assay kit (MP Biochemicals). For each well, 100 µL of medium was removed and transferred to a fresh 96-well plate and stored at -20°C until analysis. The assay was performed using the following adaptation of the manufacturer's protocol. Medium to be analysed (10 µL) was transferred to a 96-well Optiplate containing 18 µL of 1x dilution buffer and incubated at 65°C for 1 hour to destroy endogenous alkaline phosphatase activity (pSEAP produces placental alkaline phosphatase that is stable at this temperature). The plate was cooled on ice for 2 minutes and left to equilibrate to room temperature for 15 minutes. 24 µL of assay buffer was added and left for 5 minutes at room temperature. Then 24 µL of reaction buffer (containing the substrate disodium 3-(4-methoxyspiro [1,2-dioxetane-3,2'-(5'-chloro)tricyclo(3.3.1.1)decan-4-yl)phenyl phosphate]) (CSPD) was added and left for 10 minutes at room temperature. Dephosphorylation of the substrate by alkaline phosphatase yields luminescence, which was read using a Packard LumiCount™ (Packard Bioscience) luminometer with PlateReader version 2.10 (Packard Instrument Co. Inc.) software. Results were expressed as relative luminescence units (RLU) for direct comparison between reporter constructs.

2.5 QUANTITATIVE REAL-TIME PCR ANALYSIS (TAQMAN®)

Quantitative real-time PCR is a technique that uses the kinetics of the early amplification phase of a PCR reaction to quantify the level of mRNA transcript in a sample. Samples are compared to genomic DNA standards of known copy number to provide a final quantitation. Therefore, in order for successful quantitation the primers must be designed within exons, and DNA contamination must be avoided.

The method used in this experiment used fluorescent-labelled target specific probes termed TaqMan probes (Heid *et al.*, 1996). The probe is dual-labelled with a reporter dye (FAM) covalently linked to the 5' end of the probe, and a quencher molecule (TAMRA) linked to the 3' end. The probe and its specific primer pair hybridise to the target sequence, allowing priming of Taq DNA polymerase. The 5' to 3' exonuclease activity of Taq DNA polymerase allows the enzyme to cleave

the terminal nucleotides of double-stranded DNA. Therefore during the extension cycle of the PCR, the reporter dye is cleaved from the probe. The quencher molecule and the fluorescent dye are then distanced and hence the peak fluorescence emission increases. The fluorescence is measured by an ABI Prism 7900 sequence detection system (SDS) that uses a charge-coupled device (CCD). The fluorescence is then normalised to a reference dye by the SDS software to obtain a ratio for each sample at the end of each thermal cycle. A baseline value is produced by measuring the fluorescence between cycles 3 and 15, unless there is an increase in the amplification curve prior to cycle 15. A threshold (Th) is set above the baseline so that it falls within the exponential phase of amplification. The threshold cycle (C_t) for any sample is the fractional cycle number at which the normalised ratio crosses the Th. The copy number (C_n) for a given target gene is calculated by comparison of sample C_t values with those of standards with known copy number. A lower C_t value corresponds to a higher C_n value, since the higher the level of starting material in the sample, the sooner the amplification would reach exponential phase.

2.5.1 TOTAL RNA EXTRACTION

Following incubation cells were washed with 2.5 mL 1x PBS, and lysed on the plate using 350 μ L Buffer RLT (QIAGEN RNeasy[®] extraction kit) containing 1% β -mercaptoethanol (Sigma). Cell lysates were homogenised by passing through a 21-gauge needle ten times. Samples were then stored at -80°C until required. Samples were then thawed at in a water-bath at 37°C for 15 minutes, and added to 350 μ L isopropanol. The mixture was transferred directly to an RNeasy[®] column and centrifuged according to the manufacturer's protocol. Lysates were passed through the column twice, and the RNA was eluted from the column using 50 μ L RNase-free water that was re-eluted a second time to increase the RNA yield. All centrifugation steps were performed at 13,000 rpm, and the extracted RNA was stored at -20°C until required.

2.5.2 RNA QUANTITATION AND INTEGRITY DETERMINATION

RNA quality was assessed by agarose gel electrophoresis (section 2.3.1). 5 μ L extract was separated on a 1% agarose gel and visualised by UV. The RNA was considered of sufficient integrity if the

ribosomal RNA (rRNA) bands were sharp, and the 28S rRNA band was roughly twice as intense as the 18S rRNA band.

RNA quantitation was performed using the Ribogreen™ RNA Quantitation Assay kit (Molecular Probes, R-11490). The Ribogreen™ reagent is a fluorescent nucleic acid stain for quantitation of RNA in solution with a 1000-fold greater sensitivity than UV spectrophotometry (Molecular Probes datasheet MP11490). The low range assay was used in each case. Briefly, a 1xTE working solution was prepared from the provided 20xTE stock solution. This was used to dilute the provided 100 µg/mL rRNA standard producing a 1000 ng/mL RNA solution. A standard curve was prepared using this 1000 ng/mL RNA solution. 100 µL was added to two neighbouring wells on the 96-well plate. 100 µL 1xTE was added to well 2 and to six other wells. Double dilutions were made by transferring 100 µL of well 2 into well 3, mixing by pipetting, and so on until the seventh well. The final well was a blank containing only 1xTE. The excess 100 µL in well 7 was discarded. This provided a standard curve from 1000 ng/mL to 15.62 ng/mL. Samples were diluted 20-fold using 1xTE and 5 µL of each diluted sample was transferred to a well of black 96-well plate containing 95 µL 1xTE. This provided concentrations within the range of the standard curve (approximately 125 ng/mL from spectrophotometric assessment). The Ribogreen™ RNA quantitation reagent was warmed to room temperature and protected from light to avoid photo-degradation. It was diluted 200-fold with 1xTE, and 100 µL was added to each well. The plate was incubated at room temperature for 5 minutes, and the fluorescence was measured using a fluorescent plate reader (Molecular Devices SpectraMax Gemini XS) and Softmax® Pro 4.0 software at 485 nm excitation and 530 nm emission. Sample concentrations were calculated using the standard curve after subtraction of the background fluorescence.

2.5.3 DNASE TREATMENT AND FIRST STRAND CDNA SYNTHESIS

2.5.3.1 DNase treatment

The total RNA samples were treated with DNase I (Promega) to remove all genomic DNA contamination. For each sample, 5 µg RNA was transferred to a 0.5 mL microcentrifuge tube along with 2 µL 10x DNase reaction buffer, 2 µL DNase I (1 U/µL). The volume in each tube was made

up to 20 μL with RNase-free water. Samples were incubated in a PCR machine at 37°C for 30 minutes. The reaction was stopped by addition of 2 μL DNase stop solution (20 mM EGTA, pH 8, Promega) and incubation at 65°C for 10 minutes.

2.5.3.2 cDNA synthesis

To ensure DNase treatment was successful, cDNA synthesis was performed on two aliquots of each sample. One aliquot was treated with the Reverse Transcriptase (RT) enzyme and the other was not. Therefore any DNA remaining after DNase treatment was detectable in the RT negative sample during subsequent quantitative real-time PCR analysis. Any samples showing evidence of DNA contamination were not included in analysis. For cDNA synthesis, 1 μg of DNase treated RNA (equivalent to 4 μL) was transferred to each of two 0.5 mL microcentrifuge tubes. To each tube were added 1.5 μL RNase-free 10 mM dNTP mixture (Invitrogen) and 1.5 μL random hexamer primer mixture (3 $\mu\text{g}/\mu\text{L}$ diluted 20-fold in RNase-free water, Invitrogen) and 4 μL RNase-free water. Samples were incubated at 65°C for 5 minutes, and then cooled on ice for at least 2 minutes.

The cDNA synthesis used the Invitrogen Superscript II First-strand synthesis system for RT-PCR (catalogue no. 11904-018) that allows cDNA synthesis from between 1 ng and 5 μg total RNA. An RT-positive reaction mixture was prepared for $n+2$ samples:

- 4 μL 5x First strand buffer
- 2 μL DTT (0.1 M)
- 1 μL RNaseOUT RNase inhibitor
- 0.25 μL Superscript II RNase H-RT (200 U/ μL)
- 0.75 μL Diethylpyrocarbonate (DEPC)-treated water

An RT-negative reaction mixture was also prepared containing:

- 4 μL 5x First strand buffer
- 2 μL DTT (0.1 M)
- 2 μL DEPC-treated water

To each sample 8 μL of the appropriate mixture was added, and samples were mixed gently and collected by brief centrifugation. The samples were then incubated as Table 2.14.

Table 2.14: Temperature program for cDNA synthesis

Temperature ($^{\circ}\text{C}$)	Time (minutes)
25	10
42	50
70	15

Samples were made up to 100 μL total volume with RNase-free water giving an approximate concentration of 10 $\mu\text{g}/\text{mL}$. Samples were stored at -20°C until required.

2.5.4 PRIMER AND PROBE DESIGN

Primers and probes were designed within a single exon using the ABI Primer Express software and following the selection criteria provided by the program (Table 2.15). The chosen primer and probe sequences (Table 2.16) were ordered from MWG. Primers were supplied lyophilised and were diluted to a stock concentration of 100 mM with 1x TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) for prolonged storage. The stock primers were diluted with nuclease-free water to a working concentration of 10 mM. Probes were similarly diluted to a stock concentration of 100 mM with 1x TE, and a working concentration of 5 mM using nuclease-free water. Stock and working concentrations were stored at -20°C .

2.5.5 GENOMIC DNA STANDARDS

Rat genomic DNA (Novagen) was supplied at a concentration of 285 $\text{ng}/\mu\text{L}$ (equivalent to 1.4×10^6 single stranded copies per μL). This was diluted to 143 $\text{ng}/\mu\text{L}$ (equivalent to 2×10^5 single stranded copies per μL). In each analysis 5 μL of this standard was analysed giving a top standard of 1×10^6 copies. This standard was diluted to give 1×10^5 , 1×10^4 , 1×10^3 and 1×10^2 copies by a 10-fold serial dilution. All standards were prepared using DEPC-treated water.

Table 2.15: ABI Prism Primer Express design criteria

Primer
Tm 58 to 60 °C
20 to 80 % GC
9 to 40 bases in length
Less than 2 °C difference in Tm
Maximum 2 G/C bases at 3' end
Probe
Tm 10 °C higher than primer Tm
20 to 80 % GC
9 to 40 bases in length
No G on 5' end
Less than four contiguous G bases
Less G than C bases
Amplicon
50 to 150 bases in length
3' end of primer as close to 5' end of probe as possible

Table 2.16: Primer and probe sequences for TaqMan Real-Time PCR

Gene	Forward Primer	Reverse Primer	Probe
HcaRG	5'-TGGCATTCCTCAGGATCTAATTG-3'	5'-AGTCGAGAAAGAGGGCGTTGA-3'	5'-TCCCAAATGCCAAACTGGCCAAATCT-3'
Nob1p	5'-CTAGCTGCTGAGGAGGCTGTAGT-3'	5'-GGCCCACTGCTTCTGTCTAGA-3'	5'-CCCCAGTGTCTGTCTGCAAAACCA-3'
rCOUPg	5'-TGCCTGCATCCCTCATCTC-3'	5'-GAGCGTCTCGATGGGTGTCT-3'	5'-CCACCAGGGCGCATGAAGAAAAAGCT-3'
LRCH4	5'-GTGTGTAGTAATTGGTCTTGGGTATTCC-3'	5'-CACAAACACTCTTCACCCAGATAGG-3'	5'-CTGCCCCITAGGCTGCTTCGAGTGCA-3'
Sp3	5'-CTGTGGAAAGCTGGGAGAGATG-3'	5'-CCCTGTTGAATATTTGCAAGGATA-3'	5'-TGTTGTTCCTCTCTGCAGTAATCAAGGCA-3'
Me1	5'-TCAAGGATATGGCTGCCITTCA-3'	5'-AGCITTGCTGTCGGATTACTC-3'	5'-CGAGCGGCCCCATCATCTTTGCTT-3'
RBACH	5'-AAGAAGGCAAAGGCCGCTAT-3'	5'-CTAGGGCTGAGGCTCTGTATGG-3'	5'-CCTGTGCTTCGCCCTTCATCTGCA-3'

2.5.6 REAL-TIME PCR PROTOCOL

To minimise pipetting errors a bulk mix was prepared containing the probe and primers, water and TaqMan universal PCR Master Mix (Applied Biosystems). Table 2.17 shows the volumes per reaction. 20 µL of the bulk mix was aliquoted into each required well of a 96 well optical reaction plate (ABgene® Thermo-Fast®). 5 µL of each cDNA sample or standard solution was then added to the appropriate well on the plate. Two wells per plate contained 5 µL of DEPC-treated water as sample blanks. Repeat analysis of samples was not undertaken due to the high reproducibility of the quantitative real-time PCR analysis, typically under 2 % (K. Swales, personal communication); in addition, as a minimum of three biological repeats were present for each data point this provided a more robust measure of the variation within the biological system.

Table 2.17: Reagent volumes for preparation of TaqMan bulk mix

Component	Volume (µL)
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Probe (5 µM)	0.5
DEPC-treated water	5
TaqMan universal PCR Master mix	12.5

Following sample application the plate was sealed with an optical adhesive cover (ABgene®). The plate was centrifuged at 1000 rpm for 1 minute. The plate was placed in the ABI Prism® 7000 sequence detection system (Applied Biosystems). The plate layout was assigned within the SDS software and the appropriate detector (FAM/TAMRA) selected. The reaction volume per well was 25 µL. The thermal cycling conditions were as Table 2.18.

Table 2.18: Thermal cycling conditions for TaqMan real time PCR

Stage	Temperature (°C)	Time (minutes)	Repetitions	Function
1	50	2	1	
2	95	10	1	Denaturation
3a	95	0.25	40	Annealing/Extension
3b	60	1		

Once completed the run was analysed by adjusting the baseline (default setting 0.2) to the mid point of the exponential phase of the amplification plots for all the standards. All samples were then checked to fall within the high and low samples of the standard curve to ensure the baseline setting was adequate for all samples, and that measurements would fall within the linear range of the analysis. The program (ABI Prism 7000 Sequence Detection System version 1.0[®] Applied Biosystems) automatically converted the C_t values for the samples into target copies using the predefined standard curve. The results were exported for analysis that involved calculation of mRNA copies per ng total RNA and the mean and standard error calculated for each sample group. This was used to calculate fold-change values compared to vehicle-control (DMSO) and basal expression values.

2.6 LACTATE DEHYDROGENASE (LDH) ASSAY

The cytotoxic potential of the compounds used in the study was determined using the Cytotoxicity Detection Kit (Roche), a colorimetric assay for quantitation of LDH release from damaged/dying cells. Cells were seeded in 96-well plates and dosed as for transfection experiments (section 2.4.3) over a 96 hour period. Growth medium was removed every 24 hours and replaced immediately with fresh medium containing compound of interest (prepared as section 2.4.3). As positive control for each time-point, untreated cells were subjected to complete lysis as induced by addition of Triton X-100 (2%) for 15 minutes. The negative control at each time-point was FaO cells incubated alone. All samples were stored at 4°C in microplates until time of assay.

Prior to assay, microplates were centrifuged at 250 g for 10 minutes to pellet cellular debris. Supernatant was transferred to a fresh 96-well plate and diluted 1 in 4 with fresh growth medium. Reaction buffer (100 µL) was added to each well containing sample (100 µL) and incubated at room temperature in the absence of light for 20 minutes, prior to analysis using a multi-well ELISA reader. Cytotoxicity was calculated as follows: cytotoxicity (%) = (test value - negative control value)/(positive control value - negative control value) x 100.

2.7 STATISTICAL ANALYSIS

Results were analysed for statistical significance using one-way analysis of variance (ANOVA) with Bonferroni all means Post Hoc test (GraphPad Prism Software version 4.0, San Diego, CA).

3 MICROARRAY ANALYSIS OF GENE EXPRESSION IN RAT LIVER FOLLOWING EXPOSURE TO LIVER GROWTH AGENTS

3.1 INTRODUCTION

DNA Microarray analysis can provide information on gene expression on a genome-wide scale. Although not fully quantitative, arrays allow rapid identification of key gene expression changes that can then be validated and characterised. This experiment sought to discover genes involved in the response to administration of liver growth agents, potentially identifying biomarkers of liver growth that could be useful in candidate-drug toxicity screening. The sample generation, collection and raw data production were performed at GlaxoSmithKline (Welwyn, UK) prior to the commencement of this project. All subsequent bioinformatic analysis was carried out at University of Surrey by Elizabeth Anderson.

3.2 GENES AND EXPRESSED SEQUENCE TAGS IDENTIFIED EXHIBITING POTENTIAL CO-ORDINATE REGULATION

Affymetrix gene arrays are composed of 25-mer oligonucleotide probes complementary to a section of an mRNA or expressed sequence tag (EST). The sequences of the probes are represented by accession numbers and are termed *gene fragments*. For each potential gene, several probes are used spanning the length of the expressed sequence, thus increasing the confidence in any change measured in the expression of a gene. In this experiment, from the initial $\approx 20,000$ genes represented on the array, the expression of approximately 6000 gene fragments were selected as being altered in response to the liver growth agents administered (Figure 2.2), based on an arbitrary cut-off of a minimum 3-fold change versus control. Next, it was necessary to identify genes showing consistent expression profiles in response to all three liver growth agents. Wy-14,643, dexamethasone and CPA display different effects on the liver such as hypertrophy, hyperplasia or a combination of both. It was hypothesised that any gene demonstrating similarity in response to all three compounds was likely to be a key regulator in the control of liver growth.

From the gene fragments identified from the multi-compound comparison, several were already associated with known genes. For the remaining gene fragments, association with genes was achieved by probing the rat genome sequence with the fragment sequence (obtained using the

NCBI accession number) using the BLAST alignment tool (Altschul *et al.*, 1990) available at NCBI (www.ncbi.nlm.nih.org/BLAST). If this failed to confirm the gene identity, the fragment sequence was used to probe the *est_others* database (www.ncbi.nlm.nih.org/BLAST/blastn) in order to obtain a longer sequence. This extended sequence could then be searched against the rat genome sequence with increased probability of identifying the associated gene. In many cases it was not possible to identify the gene at the time. The rat genome sequence is currently under constant update, and as the sequence nears completion it will become possible to identify the remaining gene fragments.

From this multi-compound comparison, 38 gene fragments were identified as showing similar expression profiles with time across all three compounds used in the study (Table 3.1).

The 38 genes/ESTs showing potential importance in a co-ordinated response to liver growth agents were next clustered manually into groups according to the shape of their expression profile, removing the importance of scale of change from the data. This method was used to reveal genes that were potentially related to each other in response to the same stimuli. The clustering identified many groups of which two were selected for further investigation. The criteria for selection included similarity of expression profile between gene fragments, and any evidence of a functional connection that could be found. This was only possible where a gene was identifiable from the fragment sequence.

The manual clustering was verified using the Multi-Variate Statistical Package (MVSP 3.1 © Kovach Computing Services) analysing similarity between gene expression profiles by hierarchical cluster analysis (HCA) with random input order using an unweighted pair group method with arithmetic mean (UPGMA) linkage technique with Pearson Coefficient to score similarities. This gives equal weight to all points in each cluster and is therefore a more balanced clustering technique, with a Pearson Coefficient of 1 indicating complete similarity, a value of zero showing no similarity, and -1 indicating an inverse relationship.

Table 3.1: Fragments identified as showing potential importance in liver growth regulation (across 3 compounds)

Accession No	Direction of expression change at time-point (hr post-dose)							Gene Details
AA849767	↑	↑	↑	↑	↑	↑	↑	brain-enriched SH3-domain protein (Besh3)
AI229638	↑	↑	↑	↑	↑	↑	↑	Thymidine kinase 1 (TK1)
AB010635	↑	↑	↑	↑	↑	↑	↑	Carboxylesterase 2 (intestine and liver)
AI171506	↓	↑	↑	↑	↑	↑	↑	Malic enzyme 1 (Me1)
D88890	↑	↑	↑	↑	↑	↑	↑	rat Brain acyl-CoA hydrolase (rBACH)
AI235320	↑	↑	↑	↑	↑	↑	-	Aconitase 2 (Aco2)
J03190	↑	↑	↑	↑	↑	↑	↓	Aminolevulinatase delta synthase 1 (Alas1)
L00320	↑	↑	↑	↑	↑	↑	↓	cytochrome P450b gene, exon 9 (phenobarbital-inducible)
AA944386	↓	↓	↓	↓	↓	↓	↓	
AI044411	↓	↓	↓	↓	↓	↓	↓	
AI228798	↓	↓	↓	↓	↓	↓	↓	
AI013876	↓	↓	↓	↓	↓	↓	↓	
AI029007	↓	↓	↓	↓	↓	↓	↓	
AA850675	↑	↑	↑	↑	↑	↑	↑	Cd63 antigen
AA997844	↑	↑	↑	↑	↑	↑	↑	
AI009800	↓	↓	↓	↓	↓	↓	↓	
AI011082	↓	↓	↓	↓	↓	↓	↓	Highly similar to mouse importin alpha-3
D49653	↑	↑	↑	↑	↑	↑	↓	Leptin homologue
AA900612	-	-	-	-	-	-	-	
AA955109	-	-	-	-	-	-	-	fatty acid Coenzyme A ligase, long chain 6 (FACL6)
AI030404	-	-	-	-	-	-	-	

Accession No	Direction of expression change at time-point (hr post-dose)						Gene Details
	6	12	24	48	72	96	
AI044121	-	-	-	-	→	-	
AI060162	-	-	-	-	→	-	
AI069943	-	-	-	-	→	-	
AI137528	-	-	-	-	-	→	
AA899199	↑	↑	→	→	→	→	hypertension-related, calcium regulated gene (HCaRG)
AA956531	↑	↑	→	→	→	→	Nob1p/human ART-4
AI008907	↑	↑	→	→	→	→	Sp3
AI009019	↑	↑	→	→	→	→	rCOUPg (rat orphan nuclear receptor)
AI044183	-	↑	→	→	→	-	
AI045104	↑	↑	→	→	→	→	opioid growth factor receptor-like 1 (OGFRL1)
AI045165	↑	↑	→	→	→	→	leucine-rich repeats and calponin homology domain-containing 4 (LRCH4)
AA818887	↑	→	→	→	→	→	
AI180412	↑	→	→	→	→	→	
M18529	↑	→	→	→	→	→	
AA851401	→	→	→	→	→	→	
AI043805	→	→	→	→	→	→	Kidney superoxide-producing NADPH oxidase (Kox1)
E01184	→	→	→	→	→	→	CYP1A2

Fragments are listed in order according to profile characteristics starting from induction (yellow) through to inhibition (green) across all time-points. (Dashed fields reflect <3-fold change in response to liver growth agent administration.) Details of the associated genes are included where known.

Figure 3.1 shows the results of the HCA for all 38 genes or ESTs. It shows two main regions where the expression profiles for gene fragments were (i) consistent over all three compounds administered and (ii) consistent with the expression profiles of another gene fragment. The Pearson Coefficient for expression profiles for the gene fragments corresponding to Malic enzyme 1 (Me1) and rat Brain Acyl CoA Hydrolase (rBACH) is 0.74. This indicates a close similarity in the expression profiles for these genes in response to each of the three liver growth agents administered. Me1 and rBACH share a common involvement in lipid homeostasis and both enzymes are cytoplasmic (section 3.3.2).

The second interesting cluster in Figure 3.1 includes seven gene fragments of which six could be linked to genes (section 3.3.1). This cluster has a Pearson Coefficient of 0.75 indicating a similarity in the expression profiles for these genes. As with Me1 and rBACH, all seven gene fragments cluster together across all three compounds.

Figure 3.2 shows the clustering of the two groups of genes selected for further investigation. The profiles for one of the groups comprising HCaRG, Nob1p, Sp3, LRCH4, rCOUPg, OGFRL1 and an unidentified gene (labelled EST) were clustered against the profiles for Me1 and rBACH. This highlights the similarities between the expression profiles within each cluster, and also the difference between the two clusters (Pearson Coefficient of -0.3 between groups). Each of these two clusters of genes show potential for co-ordinate regulation and were investigated further.

Table 3.2 shows the changes in expression for the genes selected for further investigation in response to each of the three compounds. This highlights both the similarity in expression profile between compounds for each gene, and also the similarity between potentially co-regulated genes. For HCaRG, Nob1p, Sp3, LRCH4, rCOUPg, OGFRL1 and the EST there is a consistent initial increase in expression at 6 and 12 hours post-initial dose followed by a prolonged decrease in expression. It is possible that the initial 12 hours post-dose represents a stress response to the addition of xenobiotic and is not the true response. The crucial phase of the response is likely after this initial period, as indicated by the period of inhibition being sustained over a much longer period (≥ 72 hours).

Figure 3.1: Dendrogram showing clustering of genes of interest using Hierarchical Cluster Analysis (HCA)

HCA of expression profiles for all 38 genes/ESTs exhibiting ≥ 3 fold change in response to liver growth agent administration. Gene fragment accession numbers are shown with appropriate compound administered. The two regions labelled indicate genes showing high similarity in gene expression profile.

Figure 3.2: Dendrogram showing clustering of the two groups of genes selected for further investigation

HCA of the gene fragments from the two groups of interest displaying similar profiles and hence potential co-ordinate regulation. Details of the associated genes are included where known. Similarity scoring is by Pearson Coefficient with a value of 1 indicating identical expression profiles.

Table 3.2: Expression changes for genes selected for further investigation

Gene	Drug	Direction of expression change at time-point (hr post-dose)					
		6	12	24	48	72	96
HcARG	WY	↑	↑	↓	↓	↓	↓
	Dex	↑	↑	↓	↓	↓	↓
	CPA	↑	↑	↓	↓	↓	↓
Nob1p	WY	↑	↑	↓	↓	↓	↓
	Dex	↑	↑	↓	↓	↓	↓
	CPA	↑	↑	↓	↓	↓	↓
Sp3	WY	↑	↑	↓	↓	↓	↓
	Dex	↑	↑	↓	↓	↓	↓
	CPA	↑	↑	↓	↓	↓	↓
LRCH4	WY	↑	↑	↓	↓	↓	↓
	Dex	↑	↑	↓	↓	↓	↓
	CPA	↑	↑	↓	↓	↓	↓
rCOUPg	WY	↑	↑	↓	↓	↓	↓
	Dex	↑	↑	↓	↓	↓	↓
	CPA	↑	↑	↓	↓	↓	↓
OGFRL1	WY	↑	↑	↓	↓	↓	↓
	Dex	↑	↑	↓	↓	↓	↓
	CPA	↑	↑	↓	↓	↓	↓
EST	WY	-	↑	↓	↓	↓	-
	Dex	-	↑	↓	↓	↓	-
	CPA	-	↑	↓	↓	↓	-
rBACH	WY	↑	↑	↑	↑	↑	↑
	Dex	↑	↑	↑	↑	↑	↑
	CPA	↑	↑	↑	↑	↑	↑
Me1	WY	↑	↑	↑	↑	↑	↑
	Dex	-	↑	↑	↑	↑	↑
	CPA	-	↑	↑	↑	↑	↑

Fragments are listed in order according to profile characteristics starting from inhibition (green) through to induction (yellow) across all time-points. (Dashed fields represent <3-fold change in response to liver growth agent administration.) EST represents gene fragment AI044183 that could not be associated with a gene at the time of investigation.

3.3 MICROARRAY ANALYSIS - EVIDENCE OF POSITIVE CONTROLS

The identification of transcripts previously shown to be modulated by liver growth agents in rodent liver adds to the confidence in the robustness of the microarray analysis performed at GSK. However, the genes selected for further investigation do not include any classical mediators of proliferation or apoptosis, the most likely reason for this being the minimum 3-fold change (versus control) threshold. This would remove many genes that alter by only a small amount from the analysis. Alternatively, selection of fragments of interest using profile similarity may exclude some gene fragments that are associated with cell proliferation/apoptosis, but which do not form part of a co-ordinated response.

The arrays included controls to ensure the technique was carried out correctly, however it was necessary to identify some compound-specific expression changes as controls to verify that the doses of the compounds used were appropriate. Such controls could include drug metabolising enzymes such as a specific member of the Cytochrome P450 family that would be induced in response to levels of a certain xenobiotic in the body. Similarly, the expression of genes linked to cell cycle control such as the cyclins and Cdks, or genes involved in apoptosis such as member of the Bcl-2 family of proteins would be expected to alter in response to administration of liver growth agents. The following describes some examples of positive controls for each of the compounds administered.

3.3.1 WY-14,643

Wy-14,643 (WY) is known to induce the expression of CYP4A1 (Aldridge *et al.*, 1995) with 20-fold induction observed in precision cut rat liver slices dosed for 24 hours with 50 μ M WY (Pan *et al.*, 2002). The Affymetrix array contained several probe sets for CYP4A1 (probe set accession numbers M57718, AA924267, X07259) each containing sixteen different 25-mer oligonucleotide probes. Table 3.2 shows the results for the expression of CYP4A1 in response to WY (50 mg/kg/day). The results for the WY-treated samples indicate a similar response to that observed previously (29-fold induction at 24 hours post-dose), with similar induction profiles across the three probes. Hence the dose of Wy-14,643 used in this study was sufficient to induce an appropriate response by the major liver detoxifying enzyme for this compound.

Genes associated with cellular proliferation and apoptosis were also altered in response to WY administration. The pro-apoptotic protein Bcl-2-related ovarian killer (Bok) was first identified by Hsu and colleagues (1997) who found overexpression of the protein in Chinese hamster ovary (CHO) cells resulted in apoptosis. In response to WY administration the expression of Bok (AF027954) was reduced to 30 percent of control at 24 hours post-dose (Table 3.3). The decreased expression of Bok and hence the potential reduction in apoptosis provides possible evidence that liver growth had been induced by WY.

The gene CcnA2 encodes the cell cycle-related protein cyclin A2 that binds to Cdk2 allowing G₁/S and G₂/M cell cycle transitions. Expression of CcnA2 is known to be up-regulated in mouse liver tumours (Haddad *et al.*, 2000), with cyclin A2 expression increasing proportionally to the proliferative response of rat liver following induction of transient liver proliferation (Payraudeau *et al.*, 1998). Following administration of WY the expression of CcnA2 (AA998516) increased between 48 and 96 hours post-dose (Table 3.3) as would be expected during cellular proliferation. Taken together the results for CYP4A1, Bok and CcnA2 indicate that the dosing of WY used in the experiment was appropriate to induce cellular proliferation and a metabolic response to the compound.

Table 3.3: Positive controls from Microarray analysis of rat liver following WY administration

Fragment Accession Number	Gene	Gene expression (drug/control) hours post initial dose					
		6	12	24	48	72	96
M57718	CYP4A1	2.5	6.5	4.2	5.6	3.6	5.7
AA924267	CYP4A1	4.7	21.6	29.2	38.5	19.8	61.3
XO7259	CYP4A1	3.3	14.9	14.8	17.5	14.5	9.5
AF027954	Bok	0.9	1.6	0.3	1.1	0.6	0.5
AA998516	CcnA2	1.0	0.2	1.6	3.6	4.0	18.7

Fragment accession number and corresponding gene are shown with expression profile across the time-points analysed. Values represent treated/control with values in bold representing ≥ 3 -fold change versus control, indicating potential biological significance. WY dosing scheme described in section 2.2.1.

3.3.2 DEXAMETHASONE

Dexamethasone is a steroidal compound known to induce a range of CYP genes most notably the gene CYP3A23 (alias CYP3A1, CYP3A3; 98-percent nucleotide sequence identity). Meredith and colleagues (2003) showed a 97-fold increase in CYP3A1 expression by real-time quantitative PCR analysis following 50 mg/kg/day dexamethasone over a four day period. In this experiment CYP3A1 (L24207) gene expression was induced to a maximum of 12.8-fold 12 hours post initial dose (Table 3.3). This indicates that the dose of dexamethasone used was sufficient to induce a metabolic response.

Expression of the pro-apoptotic protein Bad (Bcl2-associated death promoter) was down-regulated to 30-percent of control following dosing with dexamethasone (Table 3.4). This could indicate a proliferative response to administration of the liver growth agent. Cyclin G2 (AI171262) expression decreased to 20-percent of control at 24 hours post initial dose (Table 3.3). Cyclin G2 was proposed as a negative regulator of cell cycle progression in the gastric adenocarcinoma cell line SGC-7901 (Liu *et al.*, 2004), and was found to induce cell cycle arrest via complex formation with protein phosphatase 2A (Bennin *et al.*, 2002). The expression of cyclin G1 (AI178559) was increased at maximum 3.5-fold in response to dexamethasone administration (Table 3.4). Jensen and colleagues (2003) reported reduced tumour incidence in cyclin G1-deficient mice that was linked to negative feedback on cellular levels of the tumour suppressor protein p53.

Dexamethasone also induced expression of the tumour-associated protein 1 (TA1) to a maximum 6.4-fold at 12 hours post initial dose in this study. TA1 is not highly expressed in adult rat liver, but is abundant in rat hepatoma (Sang *et al.*, 1995), indicating TA1 may have a role in tumour progression. Hence the induction of TA1 by dexamethasone observed herein may be indicative of a biological response leading to tumour initiation.

Table 3.4: Positive controls from Microarray analysis of rat liver following DEX administration

Fragment Accession Number	Gene	Gene expression (drug/control) hours post initial dose					
		6	12	24	48	72	96
L24207_r_at*	CYP3A1	1.4	10.6	5.6	5.3	11.2	4.1
L24207_j_at*	CYP3A1	0.4	12.8	3.4	3.5	5.0	2.0
AA818072	BAD	0.3	0.5	1.3	0.9	0.6	1.7
AI171262	CcnG2	0.5	0.5	0.2	0.6	1.5	1.1
AI178559	CcnG1	3.5	1.3	1.7	1.2	1.2	1.2
AB015432	TA1	1.3	6.4	0.3	0.4	1.1	0.7

Fragment accession number and corresponding gene are shown with expression profile across the time-points analysed. Values represent treated/control with values in bold representing ≥ 3 -fold change versus control, indicating potential biological significance. Dexamethasone dosing scheme described in section 2.2.1. (*L24207_r_at and L24207_j_at represent two separate probe sets for CYP3A1.)

3.3.3 CYPROTERONE ACETATE

CPA is known to induce expression of CYP3A23 (CYP3A1). Schuetz and colleagues (1998) found the induction of CYP3A23 by CPA was via a DR3 element in the gene promoter, and was similar to the induction by dexamethasone. The maximal alteration in expression of CYP3A1 (L24207) was an induction of 10.3-fold following CPA administration (Table 3.4) indicating an appropriate response by the liver.

Similar to the results following administration of WY (section 3.4.1), the expression of Bok (AF027954) and CcnA2 (AA998516) were altered in response to CPA administration. Bok expression was reduced to 30 percent of control at 48 hours post initial dose and to 10-percent of control after 96 hours (Table 3.5). CcnA2 expression increased 18-fold over control at 48 hours post initial dose of CPA (Table 3.5). These results are similar to the expression changes induced by WY administration, indicating that the dosing scheme for CPA was appropriate.

Table 3.5: Positive controls from Microarray analysis of rat liver following CPA administration

Fragment Accession Number	Gene	Gene expression (drug/control) hours post initial dose					
		6	12	24	48	72	96
L24207_r_at*	CYP3A1	1.5	5.7	10.3	4.5	10.3	3.4
L24207_j_at*	CYP3A1	0.6	6.3	2.7	3.1	4.2	2.2
AF027954	Bok	0.9	0.5	0.5	0.3	0.5	0.1
AA998516	CcnA2	0.6	0.6	2.3	18.0	4.0	6.2

Fragment accession number and corresponding gene are shown with expression profile across the time-points analysed. Values represent treated/control with values in bold representing ≥ 3 -fold change versus control, indicating potential biological significance. CPA dosing scheme described in section 2.2.1. (*L24207_r_at and L24207_j_at represent two separate probe sets for CYP3A1.)

3.4 DISCUSSION

In this section, microarray analysis was used to identify gene fragments significantly altered in rat liver by exposure to one of three liver growth agents, and then groups of co-ordinated gene fragments selected for further analysis. The positive controls highlighted in section 3.3 represent a selection of gene expression changes from the microarray data. They increase confidence that the change in expression of the genes of interest were in response to a dose of each compound that could be expected to induce liver growth. Many of the gene fragment accession numbers were not linked to known genes at the time of analysis, due in part to the incompleteness of the rat genome sequence. Although this linkage would be possible for any single gene from an individual fragment or its related EST, it would be unfeasible to do so with the large number of ‘unknown’ fragments present on the microarray. Hence many interesting positive controls would have been missed through lack of gene annotation for the array. Similarly the use of a minimum 3-fold change to refine the data, may have missed some mediators of growth that did not alter in their expression to this extent. However the positive controls described are sufficient to consider the data suitable for the identification of genes involved in liver growth.

The cluster analysis identified several groups demonstrating potential co-ordinate regulation, of which two were selected for further investigation. One group of genes were found to be down-

regulated in response to liver growth agent administration, the second group being up-regulated. Current information on these genes is summarised below wherever published work existed.

3.4.1 GENES SUPPRESSED IN RESPONSE TO LIVER GROWTH AGENT ADMINISTRATION

A group of seven gene fragments were identified as showing potential co-ordinate regulation, exhibiting inhibition of gene expression in response to liver growth agent administration. Six of these could be linked to genes, with one gene fragment being unidentifiable at present (Figure 3.3). From the six genes identified only four have been investigated previously and these are detailed below.

Hypertension-related, calcium-regulated gene (HCaRG) is a calcium-regulated nuclear protein first identified by Solban *et al.* (2000). The group were attempting to identify gene(s) responsible for the negative calcium balance observed in the spontaneously hypertensive rat. Solban and colleagues went on to clone both the rat and human orthologues of HCaRG. The gene is expressed in many tissues including the liver and its expression is greatly increased in the adult. The expression of HCaRG was greatly reduced in tumour cells and cancerous cell lines (Solban *et al.*, 2000).

HCaRG was found to be a potential regulator of cellular proliferation. Overexpression of rat HCaRG in the HEK293 cell line (human embryonic kidney) resulted in decreased proliferation as measured by cell counting and [³H]thymidine incorporation as compared to vector only (Solban *et al.*, 2000). Cell cycle analysis showed the reduced proliferation was via G₂M cell cycle arrest (Devlin *et al.*, 2003). HCaRG has since been implicated in cellular repair after injury, with overexpression of HCaRG in two renal cell lines leading to increased cellular migration, extracellular matrix adherence and expression of genes involved in cellular migration such as TNF α (Hader *et al.*, 2005). This evidence suggests that HCaRG may be part of a protective mechanism against cellular injury and unwanted cellular proliferation.

The nin one binding protein (Nob1p) is a nuclear protein first identified in *Saccharomyces cerevisiae* (*S.cerevisiae*) by Tone and colleagues (2000). It has since been identified in many species including most recently human where it was found to be expressed primarily in the liver, lung and spleen (Zhang *et al.*, 2005). The *NOB1* gene in *S.cerevisiae* that encodes Nob1p, a 459 amino acid protein,

was detectable in growing cells but not in cells in the stationary phase. Nob1p was found in the proteasomal band following glycerol gradient centrifugation and was therefore postulated to be involved in proteasome function in growing cells (Tone *et al.*, 2000). Nob1p was found to function in proteasome assembly, forming a complex with the 19S regulatory particle, facilitating its binding to the 20S proteasome in the nucleus (Tone *et al.*, 2002). Nob1p is then internalised and degraded by the newly formed 26S proteasome. Overexpression of NOB1 overcame the defects in maturation of the 20S proteasome caused by the Ump1Delta mutation (Tone *et al.*, 2002), which usually results in the formation of functionally impaired proteasomes (Ramos *et al.*, 1998), indicating that Nob1p is essential to functional proteasome assembly.

Nob1p was recently found to have a second important role in the cell. Nob1p is essential for the processing of 20S pre-rRNA to the mature 18S rRNA in ribosome synthesis (Fatica *et al.*, 2003). Eukaryotic ribosome synthesis occurs to a large extent in the nucleolus, with transcription of a single transcript that is cleaved forming the mature 18S rRNA (small ribosomal unit), 5.8S and 28S rRNAs (large ribosomal unit). The transcription, pre-rRNA cleavage and ribosome assembly events occur in the nucleolus, with pre-40S and pre-60S ribosomal subunits being subsequently transported to the cytoplasm. In *S.cerevisiae* the maturation of these ribosomal subunits includes processing of 20S pre-rRNA to form the mature 18S rRNA via cleavage by Nob1p. Fatica and colleagues (2003) showed that depletion of Nob1p led to accumulation of 20S pre-rRNA. They found Nob1p to be associated with the pre-40S particle, and present in both the nucleus and cytoplasm, suggesting that Nob1p accompanies the pre-40S particle during export from the nucleus to the cytoplasm.

The Sp (specificity protein) family of transcription factors are known to bind to GC-rich regions of DNA, and are ubiquitously expressed (Kolell and Crawford, 2002). Sp1 and Sp3 are closely related, are thought to have been formed from a gene duplication event from Sp4 (Kolell and Crawford, 2002), and compete for the same DNA binding sites (Hagen *et al.*, 1994). Expression of Sp3 is self-regulated with the human Sp3 promoter containing two binding sites for Sp1/Sp3, with binding to both sites necessary for full promoter activity (Lou *et al.*, 2005). Self-regulation is achieved through

the C-terminal D-domain of Sp3, which decreases transactivation of the Sp3 promoter (Hammill *et al.*, 2005).

Sp3 is increased in cells undergoing differentiation and is a positive regulator of p21^{CIP1/WAF1}, a CDK inhibitor and key negative regulator of the cell cycle (Koutsodontis *et al.*, 2002). The proliferative action of c-Myc in alleviating cell cycle arrest has been linked to sequestration of Sp1/Sp3, preventing Sp1/Sp3 binding and transactivation of the p21^{CIP1/WAF1} promoter (Gartel *et al.*, 2001). Functional cooperation between the tumour suppressor protein p53 and Sp1/Sp3 was shown to up-regulate transcription of p21^{CIP1/WAF1} and the p53-up-regulated mediator of apoptosis (PUMA), and hence Sp1/Sp3 may be essential to the cellular response to p53 activation (Koutsodontis *et al.*, 2005). Hence a reduced level of cellular Sp3 (as observed in the microarray data herein) could remove the negative regulation of growth by p21^{CIP1/WAF1}, and therefore contribute to the proliferative effect of a liver growth agent.

rCOUPy also known as Ear2 or Nr2F6 (subfamily 2, group F, member 6) is an orphan nuclear receptor and a member of the chicken ovalbumin upstream promoter family of transcription factors (COUP-TFs), which are involved in the transcriptional regulation of a variety of genes. Ear2, the human orthologue of rCOUPy, is highly expressed in human foetal liver and is predicted to be similar in primary structure to steroid and thyroid hormone (T3) receptors (Miyajima *et al.*, 1988). Zhu and colleagues (2000) showed that Ear2 interacts with the T3 receptor TRβ1, a mediator of the effects of T3 in cells. The interaction with Ear2 caused repression of the binding of TRβ1 to the T3 response element (TRE) in effector gene promoters. This negative regulation of transcriptional activity by Ear2 also extended to the glucocorticoid receptor (GR) and the estrogen receptor (ER). Similarly, COUP-TFs have been shown to interact with and negatively regulate binding of other receptors to target promoters, including the Vitamin D receptor, the retinoic acid receptor, PPARs, RXR and HNF4 (Qiu *et al.*, 1996). Therefore down-regulation of rCOUPy expression in response to a liver growth agent (as observed in the microarray data herein) may contribute to liver growth through removal of its negative influence on other receptors.

Figure 3.3: Summary of known literature on potentially co-regulated genes

Summary of the literature as described in text indicating potential co-regulation of HCaRG, Nob1p, Sp3, rCOUPg, LRCH4, OGFRL1 and the gene fragment AI044183 (EST in diagram). The inhibition of gene expression observed for each of the genes/EST in response to exposure to liver growth agents may be causative of liver growth through the removal of a negative influence on growth.

3.4.2 GENES INDUCED IN RESPONSE TO LIVER GROWTH AGENT ADMINISTRATION

Rat Brain acyl CoA hydrolase (rBACH) is a cytosolic enzyme that catalyses the hydrolysis of palmitoyl CoA to palmitate and coenzyme A (CoASH), and could therefore regulate intracellular levels of acyl CoAs, free fatty acids and CoASH (Hunt and Alexson, 2002). Hence rBACH may have an important regulatory role by modulating the levels of fatty acyl CoA, which can either be ligands for certain transcription factors or substrates for fatty acid metabolising enzymes. rBACH is a Type II thioesterase (high molecular mass group) and has a broad specificity with activity towards C₆ to C₂₀ fatty acyl CoAs (Hunt and Alexson, 2002). rBACH expression is constitutively active in brain and testis, whereas its expression could be induced in liver using the peroxisome proliferator

di(2-ethylhexyl)phthalate (DEHP) (Yamada *et al.*, 1997a). The induction of rBACH expression by liver growth agent administration observed in the microarray data herein is therefore in agreement with published results using peroxisome proliferators, providing increased confidence in the analytical approach used herein.

Malic enzyme 1 (Me1) is a cytosolic, NADP-dependent enzyme that generates NADPH for fatty acid biosynthesis through oxidative decarboxylation of malate to pyruvate. A regulatory PPAR/RXR response element (PPRE) was identified in the malic enzyme promoter, conferring responsiveness to peroxisome proliferators (Castelein *et al.*, 1994). It was found that the orphan nuclear receptor COUP-TF competes with PPAR/RXR for this PPRE, therefore preventing transactivation of the Me1 promoter in the absence of exogenously added ligands (Baes *et al.*, 1995). Human malic enzyme expression is induced in response to T3 via TR β binding to a TRE in the ME promoter (Gonzalez-Manchon *et al.*, 1997). The identification of a PPRE in the promoter of Me1 is confirmatory to the results seen herein where Wy-14,643 administration induced Me1 expression.

3.4.3 SUMMARY

In this chapter a bioinformatic approach has been used to identify a subset of gene fragments that show co-ordinated expression profiles in response to multiple, chemically distinct, liver growth agents. The expression of HCaRG, Nob1p, Sp3, rCOUP γ , LRCH4 and OGFRL1 despite showing an initial increase in expression that was likely a stress response, showed a prolonged decrease in response to liver growth agent administration that was observable between 12 and 24 hours post-initial dose. This time-point most likely represents a regulatory phase in the response to the xenobiotics, since treatment of rats with a single dose of Wy-14,643 (100 mg/kg) results in increased expression of Cdk genes between 24 and 48 hours post-dose with entry into DNA synthesis also observable at this time-point (Rininger *et al.*, 1997). Also, rats treated with Wy-14,643 only show altered hepatocyte foci following 22 weeks of treatment, with neoplastic liver nodules observable between 26 and 50 weeks, and hepatocellular carcinoma progressing thereafter (Rao and Reddy, 1987). Given that liver tumours are only observable in rats following long-term treatment, it is likely that expression changes at 24 hours post-initial dose are not purely a consequence of a pathological state.

Although little information exists at present on the function of LRCH4 and OGFRL1, the other genes identified in this cluster indicate a protective role against deleterious proliferation. HCaRG has been identified as a suppressor of growth through G₂M cell cycle arrest (Devlin *et al.*, 2003). Nob1p is essential for proteasome assembly (Tone *et al.*, 2002), and its suppression could be influential on the levels of proteins that would otherwise be degraded by the 26S proteasome. Sp3 transcriptionally regulates a wide variety of genes including the up-regulation of anti-proliferative genes such as p21^{CIP1/WAF1} (Koutsodontis *et al.*, 2002). rCOUP γ may negatively affect other receptors that influence proliferation such as PPARs (Qiu *et al.*, 1996), hence its suppression would remove this negative control.

The response of rBACH and Me1 to liver growth agent administration is in agreement with published data where administration of peroxisome proliferators induced their expression (Yamada *et al.*, 1997a; Castelein *et al.*, 1994). rBACH and Me1 are both involved in lipid homeostasis, and this response to liver growth agents may represent a direct response to the effect of the compounds on nuclear receptors such as PPAR α , or due to the increased metabolic demand from proliferative cells.

Further investigation of the effect of liver growth agent administration on the transcriptional activation of all these genes is necessary to prove co-ordination in their regulation, and whether the expression changes observed are a cause or an effect of chemically-induced growth. In the following chapters these changes will be confirmed (Chapter 4) and the molecular mechanisms underlying such a co-ordinated response examined (Chapter 5).

4 VALIDATION AND CHARACTERIZATION OF GENE EXPRESSION BY *IN VIVO* AND *IN VITRO* LIVER GROWTH AGENT ADMINISTRATION

4.1 INTRODUCTION

Validation of the microarray analysis in the preliminary study was required in order to confirm that the genes selected in Chapter 3 were suitable for further investigation, as microarray analysis is not considered fully quantitative. Two approaches were followed to confirm the changes observed in gene expression: First, real-time PCR was used to quantify the changes in gene transcript level in response to administration of liver growth agents with a dosing scheme similar to that used in the preliminary experiment. Samples were analysed from rat liver, primary rat hepatocytes and from the immortalised rat hepatoma FaO cell line. Second, assessment of promoter activity in response to *in vitro* dosing with liver growth agents was undertaken. The upstream promoter sequences from each gene of interest were cloned wherever possible into a reporter plasmid upstream of the secretable alkaline phosphatase gene (SEAP). The reporter gene assay allows confirmation of the preliminary study data, and is also used in the dissection of gene promoters, as will be demonstrated in Chapter 5.

4.2 VALIDATION OF THE MICROARRAY ANALYSIS BY QUANTITATIVE REAL-TIME PCR

Following analysis of the *in vivo* microarray data, it was necessary to validate the results from the preliminary study using a fully quantitative technique. The expression of the selected genes was assessed using quantitative real-time PCR following the method described in section 2.5.

A range of samples were tested including *in vivo* samples from rat liver (at 24, 48 and 72 hours post-dose), samples from primary rat hepatocytes in culture (12 to 96 hours post-dose), and from cultured FaO cells (12 to 96 hours post-dose). For the samples produced from rat liver, all samples and subsequent cDNA synthesis were produced for a previous experiment by Dr K. Swales at the University of Surrey. For the samples produced from primary rat hepatocytes, all steps from dosing through to cDNA synthesis were performed by Dr K. Plant at the University of Surrey. For the samples produced from FaO cells, all steps were performed by E. Anderson. FaO cells were dosed

using the peroxisome proliferator clofibric acid (CFA) instead of Wy-14,643 due to a lack of commercial availability at the time. The dose was chosen based on published *in vitro* work using peroxisome proliferators (Meredith *et al.*, 2003). All analysis using real-time PCR was performed by E. Anderson.

In each case, samples were produced with and without Reverse Transcriptase (RT) enzyme in order to determine genomic DNA contamination. All RT negative controls were free of this contamination (data not shown), hence all results represented amplification from RNA templates only. The results for each gene are shown in the following sections, with any significant changes in expression highlighted. All results are normalised to the levels of 18S rRNA in the sample, and expressed as fold-change against control (DMSO) values. 18S rRNA was used for normalisation as substantial variation had been noted following normalisation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels (data not shown) as has been observed by others (Goidin *et al.*, 2001, Bas *et al.*, 2004). Due to the abundance of 18S rRNA all samples required prior dilution with nuclease-free water (1 in 1000). For all points the standard error of the mean (SEM) was calculated across repeat samples and was less than 10% in each case.

4.2.1 GENES SUPPRESSED IN RESPONSE TO LIVER GROWTH AGENT ADMINISTRATION

Analysis of the results of the microarray experiment identified a group of seven fragments showing a reduction in expression in response to administration of liver growth agents. Of these seven fragments, six could be positively identified, and hence their expression validated using quantitative real-time PCR. It should be noted that analysis of the FaO cell line and the primary hepatocytes was not undertaken for the expression of all six genes due the identification of some of the microarray fragments after these analyses had been carried out.

Figure 4.1 shows the expression profiles for HCaRG transcript levels analysed by microarray and quantitative real-time PCR. The microarray analysis showed an initial increase in gene expression followed by a rapid decrease in expression between 12 and 48 hours post initial dose, reaching a maximal change at 48 hours. The response to all three compounds was very similar in profile, with dexamethasone resulting in the largest decrease to 10-percent of control. The results of the real-

time PCR analysis of rat liver (Figure 4.1) show a greatly decreased response to all three compounds compared to the microarray analysis, however the expression profile was maintained, with maximal decrease in expression occurring at 48 hours post initial dose. This was true of the response of cultured primary hepatocytes (Figure 4.1) and of cultured FaO cells (Figure 4.1) as measured by quantitative real-time PCR. For each sample type the response was greatly reduced compared to the microarray analysis, which could be due to the increased specificity and dynamic range afforded by real-time quantitative PCR, or due to differences in the probe designs between the analyses. Despite this, validation of the decreased expression of HCaRG was achieved for each compound tested in at least one sample type, verifying the inhibition of expression shown in the microarray analysis.

The expression profiles generated for Nob1p by microarray analysis (Figure 4.2) show an initial increase in gene expression between 6 and 12 hours post dose, similar to that observed with HCaRG (Figure 4.1). Nob1p expression then decreased gradually reaching the peak of inhibition of expression at 72 hours post dose (13-percent of control following CPA administration). This inhibition of expression was confirmed by quantitative real-time PCR analysis of liver from xenobiotic-exposed rats, with dosing regimes as used previously in the microarray experiment (Figure 4.2); however, a decreased fold change (2-fold decrease versus control) at 48 hours post dose was observed with this analysis. The real-time PCR analysis of primary hepatocytes also confirmed the inhibition of gene expression observed in the microarray analysis, however the profiles differed in the time point of maximal inhibition. Dexamethasone (100 μ M) caused Nob1p expression to decrease to 80-percent of control at 24 hours post dose, Wy-14,643 (100 μ M) to 85-percent of control at 48 hours post dose, and CPA (100 μ M) to 70-percent of control by 96 hours post dose. However the results from the cultured primary hepatocytes reflected different conditions and different dosing to the *in vivo* experiments, with removal of the influence of non-parenchymal cells potentially influencing the degree of change of gene expression. Also, since Nob1p and the other genes suppressed by liver growth agent administration are nuclear proteins, it may be that small changes in expression level could have drastic effects on the cellular response.

Figure 4.1: Expression of HCaRG in response to administration of liver growth agents

In vivo gene expression of HCaRG as generated by microarray analysis and by quantitative real-time PCR analysis of rat liver (n=5) when dosed with liver growth agents (section 2.2.1), and *in vitro* expression of HCaRG in primary hepatocytes (n=5) and FaO cells (n=3) dosed with liver growth agents and generated by quantitative real-time PCR analysis. Variation in repeat measurements was less than 10-percent in each case. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ relative to vehicle control using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

The expression profiles for rCOUPg in response to xenobiotics are shown in Figure 4.3. Microarray analysis demonstrates a similar response by rCOUPg to all three compounds, with the response in the initial 12 hours post dose also being similar as for HCaRG and Nob1p (Figure 4.3). The expression then decreases rapidly to around 50-percent of control by 24 hours, and remains inhibited for the following 72 hours. The quantitative real-time PCR analysis of rat liver confirms this inhibition, with rCOUPg expression decreasing to 60-percent of control at 48 hours post initial

dose. The results showed high similarity between the expression profiles for the three compounds tested. The quantitative real-time PCR analysis of cultured FaO cells also confirms the inhibition of rCOUPg expression (Figure 4.3), showing high similarity to the microarray results, with a prolonged decrease in gene expression from 24 to 96 hours post initial dose. The extent of this decrease varied from 40-percent of control in response to dexamethasone (100 μ M) to 65-percent of control in response to CFA (50 μ M) at 24 hours post initial dose.

Figure 4.2: Expression of Nob1p in response to administration of liver growth agents

In vivo gene expression of Nob1p as generated by microarray analysis and by quantitative real-time PCR analysis of rat liver (n=5) when dosed with liver growth agents (section 2.2.1), and *in vitro* expression of Nob1p in primary hepatocytes (n=5) and FaO cells (n=3) dosed with liver growth agents and generated by quantitative real-time PCR analysis. Variation in repeat measurements was less than 10-percent in each case. *=p<0.05, **=p<0.01, ***=p<0.001 relative to vehicle control using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

Figure 4.3: Expression of rCOUPg in response to administration of liver growth agents

In vivo gene expression of rCOUPg as generated by microarray analysis and by quantitative real-time PCR analysis of rat liver (n=5) when dosed with liver growth agents (section 2.2.1), and *in vitro* expression of rCOUPg in FaO cells (n=3) dosed with liver growth agents and generated by quantitative real-time PCR analysis. Variation in repeat measurements was less than 10-percent in each case. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ relative to vehicle control using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

The microarray analysis of Sp3 expression in response to liver growth agent administration (Figure 4.4) showed high similarity to the results for Nob1p (Figure 4.2). Sp3 expression increased initially between 6 and 12 hours post initial dose, and then decreased gradually to 48 hours. This expression then decreased rapidly in the next 24 hours, with a maximal inhibition of gene expression (10-percent of control) in response to CPA (60 mg/kg/day) occurring at 72 hours post initial dose. The quantitative real-time PCR analysis of primary hepatocytes in culture (Figure 4.4) confirmed the

inhibition of Sp3 expression observed in the microarray experiment. The response to CPA (100 μ M) and dexamethasone (100 μ M) showed a decrease in expression of Sp3 to 85-percent of control at 24 hours post dose, decreasing to 70-percent of control in response to CPA (100 μ M) at 48 hours post dose. The response to CPA by the primary hepatocytes reflects the microarray generated expression profile for this compound, with CPA causing the greatest extent of inhibition of Sp3 expression in both experiments.

Figure 4.5 shows the expression profiles for LRCH4. The microarray analysis of LRCH4 expression shows high similarity to that of rCOUPg (Figure 4.3), with an initial increase in gene expression at 12 hours, followed by a rapid decrease to 24 hours post initial dose in response to all three compounds, with maximum inhibition to 30-percent of control at this time-point in response to CPA (60 mg/kg/day). The inhibition LRCH4 expression was then maintained to 96 hours post initial dose in response to each of the compounds, with dexamethasone (100 mg/kg/day) causing a maximal decrease to 16-percent at 72 hours post initial dose. The quantitative real-time PCR analysis of LRCH4 expression (Figure 4.5) confirmed the microarray profiles, showing a consistent inhibition of LRCH4 expression across the time-points analysed, reaching a maximal decrease to approximately 55-percent of control for all three compounds at 96 hours post initial dose.

The microarray analysis of the expression of OGFRL1 (Figure 4.6) shows similar results to the other genes, particularly for rCOUPg (Figure 4.3) and LRCH4 (Figure 4.5) with OGFRL1 expression increasing initially and then rapidly decreasing between 12 and 24 hours post initial dose. The profiles were similar across the three compounds tested, with a consistent decrease in OGFRL1 expression between 24 and 96 hours, with maximal decrease to 21-percent of control at 72 hours post initial dose in response to CPA (60 mg/kg/day). The expression of OGFRL1 was not validated by quantitative real-time PCR as the gene was identified late on in the project and attempts to clone the promoter were unsuccessful due to the high GC content of the sequence and hence an inability to produce a clean PCR amplification of the promoter.

Figure 4.7 shows the microarray results for the gene fragment AI044183. It was not possible to identify the gene associated with fragment AI044183 (which was derived from an EST) during the period of the project and hence validation could not be undertaken. The results show an initial increase in expression between 6 and 12 hours, with a gradual decrease in expression in response to dexamethasone (100 mg/kg/day) and CPA (60 mg/kg/day) to 72 hours post initial dose (causing maximal inhibition of 5-percent of control). The expression profile in response to WY-14,643 (50 mg/kg/day) only differed from the other two compounds at 24 hours post initial dose where it caused inhibition of expression of the gene associated with AI044183 to 8-percent of control.

Figure 4.4: Expression of Sp3 in response to administration of liver growth agents

In vivo gene expression of Sp3 as generated by microarray analysis and *in vitro* expression of Sp3 in FaO cells (n=3) dosed with liver growth agents and generated by quantitative real-time PCR analysis. Variation in repeat measurements was less than 10-percent in each case. *=p<0.05, **=p<0.01, ***=p<0.001 relative to vehicle control using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

Figure 4.5: Expression of LRCH4 in response to administration of liver growth agents

In vivo gene expression of LRCH4 as generated by microarray analysis and *in vitro* expression of LRCH4 in FaO cells (n=3) dosed with liver growth agents and generated by quantitative real-time PCR analysis. Variation in repeat measurements was less than 10-percent in each case. *=p<0.05, **=p<0.01, ***=p<0.001 relative to vehicle control using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

Figure 4.6: Expression of OGFRL1 and AI044183 in response to administration of liver growth agents

In vivo gene expression of OGFRL1 (A) and AI044183 (B) as generated by microarray analysis when dosed with liver growth agents (section 2.2.1). Variation in repeat measurements was less than 10-percent in each case.

4.2.2 GENES INDUCED IN RESPONSE TO LIVER GROWTH AGENT ADMINISTRATION

The preliminary study identified Me1 and rBACH as showing potential co-ordinate regulation, with the expression of these genes increasing in response to liver growth agent administration. Figure 4.7 shows the expression profiles for Me1 from the microarray analysis and from the quantitative real-time PCR analysis of both *in vivo* liver samples and samples from FaO cells. The results of the microarray analysis (Figure 4.7) show a pronounced effect of Wy-14,643 administration at 12 hours post initial dose that decreases rapidly, before steadily increasing between 48 and 72 hours with maximal expression (15-fold over control) at 72 hours. Administration of CPA resulted in a similar pattern of expression with a maximum 9-fold increase over control at 72 hours post initial dose. Dexamethasone administration gave similar results but with a greatly reduced fold change over control (4-fold maximum at 72 hours post initial dose).

Figure 4.7 also shows the results of the quantitative real-time PCR analysis of rat liver dosed as in the initial microarray experiment. The expression of Me1 shows similar pattern of change but at a reduced level compared to the microarray analysis. Administration of Wy-14,643 produced a gradual increase in expression over control between 24 and 72 hours reaching 5-fold at 72 hours post initial dose. However, this may not represent the maximum response since only three time-points were taken for analysis during this experiment: It is possible that a longer exposure time (96 hours) would have seen a greater response. CPA and dexamethasone produced similar results at 24 and 48 hours post-dose, however the expression never increased above two-fold for the time-points measured. Once again, it is possible that a larger response occurring at a later time point that was not measured during the *in vivo* real-time quantitative PCR experiment.

Examination of the response to xenobiotics in cultured FaO cells showed greatly reduced fold-changes in the expression of Me1 over control treated cells (Figure 4.7). The administration of the peroxisome proliferator CFA resulted in a 1.5-fold change in expression at 72 hours. However the response of the cultured cells was consistently reduced across all the genes analysed, indicating that the cells were not sufficiently similar to *in vivo* hepatocytes to allow measurement of the effects of such compounds. Although the FaO cell line is generally regarded to be the most accurate rat hepatocyte model, loss/reduction of genomic activation in response to xenobiotics is a common

phenomenon for many hepatocyte cell lines, usually as a result of the reduced expression of important transcription factors. Indeed, the response of FaO cells to peroxisome proliferators such as nafenopin has previously been shown to be reduced when compared to the effect *in vivo* (Bayly *et al.*, 1993).

Figure 4.8 shows the expression profiles for rBACH in response to liver growth agents. The microarray analysis revealed similar results to the profiles for Me1, with a maximal 17-fold increase in expression over control in response to Wy-14,643. The profiles for both CPA and dexamethasone are again reduced compared to the effect of Wy-14,643, increasing to 6-fold and 8-fold respectively at 96 hours post initial dose. The quantitative real-time PCR analysis of rat liver (Figure 4.8) is similar in profile to the microarray data for Wy-14,643 administration, but with a diminished response (maximal 6-fold at 72 hours post-dose). This response is similar to that observed for Me1, indicating that it is either a difference between analysis by microarray and by real-time PCR, or that there was some RNA degradation during storage of the samples used in the real-time PCR experiment, which could cause a narrowing in the difference between control and treated tissue. The results for the FaO cell line (Figure 4.8) show a 2.2-fold change in rBACH expression over control following administration of CFA at 72 hours post initial dose. Dexamethasone and CPA produced no significant increases in expression of rBACH in the period 48 to 96 hour post-dose.

Taken together, the results confirm a similarity between these two genes, although the similarity across the three compounds has not been confirmed. The relationship between rBACH and Me1 could therefore be specific to Wy-14,643 and CFA, and hence to peroxisome proliferators in general.

Figure 4.7: Expression of Me1 in response to administration of liver growth agents

In vivo gene expression of Me1 as generated by microarray analysis and by quantitative real-time PCR analysis of rat liver (n=5) when dosed with liver growth agents (section 2.2.1), and *in vitro* expression of HCaRG in FaO cells (n=3) dosed with liver growth agents and generated by quantitative real-time PCR analysis. Variation in repeat measurements was less than 10-percent in each case. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ relative to vehicle control using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

Figure 4.8: Expression of rBACH in response to administration of liver growth agents

In vivo gene expression of rBACH as generated by microarray analysis and by quantitative real-time PCR analysis of rat liver (n=5) when dosed with liver growth agents (section 2.2.1), and *in vitro* expression of HCaRG in FaO cells (n=3) dosed with liver growth agents and generated by quantitative real-time PCR analysis. Variation in repeat measurements was less than 10-percent in each case. *=p<0.05, **=p<0.01, ***=p<0.001 relative to vehicle control using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

4.3 REPORTER CONSTRUCT ANALYSIS OF GENE PROMOTER ACTIVITY

An alternate strategy to validate the microarray results was the cloning of the upstream promoter sequences for the genes of interest. Examination of the response of these regulatory sequences to the test chemicals allows a direct measure of the effect of each xenobiotic on the transcriptional activity of the gene, further confirming the changes seen in the initial microarray analysis. For each gene, the region immediately adjacent to the transcriptional start site was identified and the approximately 1 kilobase of sequence amplified by PCR. The resulting approximate 1 kilobase of regulatory region was cloned into a reporter vector upstream of the SEAP gene. Transfection into the FaO cell line allowed quantitation of the control exerted by the cloned regulatory region, through the production of a secretable alkaline phosphatase into the growth medium for easy assay using a chemiluminescent method.

4.3.1 CLONING OF 1 KILOBASE UPSTREAM GENE PROMOTERS

The following section describes the details of the cloning of each promoter and the results of the subsequent transfections. Although attempts were made to clone all of the genes of interest, I was not able to clone the proximal regulatory regions for rCOUPg, OGFRL1 and Me1 within the time constraints of the project. This is most likely due to the high GC content of these regulatory regions, a consequence of the CpG islands often associated with gene regulatory regions. Successful cloning of promoter sequences into pCR[®]2.1 or pCR[®]-Blunt (Invitrogen) and subsequently into pSEAP was achieved for rBACH, HCaRG, Nob1p, Sp3 and LRCH4.

Since rBACH and Me1 showed a response specific to both Wy-14,643 and CFA (Figures 4.7 and 4.8), only the group of genes suppressed in response to liver growth agents were investigated beyond their basal promoter activity.

4.3.1.1 Identification of gene promoters

4.3.1.1.1 HCaRG

The fragment accession number for HCaRG (AA899199) used on the microarray refers to a 543 bp sequence. This sequence was used to interrogate the blastn database at NCBI using the BLAST alignment tool, and aligned to the 1100 bp RefSeq mRNA (NM_139108). This mRNA sequence

was then used to interrogate the rat genome, producing a single hit with the chromosome 8 contig (accession number NW_047780). The gene was found to run parallel with the contig, with the initial ~1 kilobase of proximal promoter sequence located at positions 9551251 to 9552200 on the contig. PCR primers were designed encompassing this region and part of the first exon to ensure that all potential regulatory elements around the transcription start site were included (Figure 4.9).

Figure 4.9: Chromosomal contig references for the cloned region of the HCaRG promoter

Chromosomal mapping of the rat HCaRG mRNA (NM_139108) to the rat genomic contig NW_047780 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.1.2 Nob1p

The fragment accession number for Nob1p (AA956531) from the microarray was used to interrogate the BLAST tool against the blastn database at NCBI, and aligned to the 1547 bp RefSeq mRNA (NM_199086). The mRNA sequence was searched against the rat genome and produced a single hit with the chromosome 19 contig (accession number NW_047535). The gene was found to run opposite with the contig, and hence the 1 kilobase upstream promoter was located at positions 8856871 to 8857932 on the contig. PCR primers were designed encompassing this region and part of the first exon (Figure 4.10).

Figure 4.10: Chromosomal contig references for the cloned region of the Nob1p promoter

Chromosomal mapping of the rat Nob1p mRNA (NM_199086) to the rat genomic contig NW_047535 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.1.3 LRCH4

The fragment accession number for LRCH4 (AI045165) was searched using the BLAST tool against the blastn database at NCBI, and aligned to the 2581 bp mRNA (XM_001068283). The mRNA sequence was searched against the rat genome and produced a single hit with the chromosome 12 contig (accession number NW_047369). The gene was found to run parallel with the contig, and hence the 1 kilobase upstream promoter was located at positions 7675201 to 7676187 on the contig. PCR primers were designed encompassing this region and part of the first exon (Figure 4.11).

Figure 4.11: Chromosomal contig references for the cloned region of the LRCH4 promoter

Chromosomal mapping of the rat LRCH4 mRNA (XM_001068283) to the rat genomic contig NW_047369 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.1.4 Sp3

The fragment accession number for Sp3 (AI008907) was searched using the BLAST tool against the blastn database at NCBI, and aligned to the 2661 bp mRNA (NM_001029905). The mRNA sequence was searched against the rat genome and produced multiple hits, the most identical falling within a chromosome 3 contig (accession number NW_047656). The gene was found to run opposite to the contig, and hence the 1 kilobase upstream promoter was located at positions 1871491 to 1872479 on the contig. PCR primers were designed encompassing this region and part of the first exon (Figure 4.12).

Figure 4.12: Chromosomal contig references for the cloned region of the Sp3 promoter

Chromosomal mapping of the rat Sp3 mRNA (NM_001029905) to the rat genomic contig NW_047656 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.1.5 rCOUPg

The fragment accession number for rCOUPg (AA009019) was searched using the BLAST tool against the blastn database at NCBI, and aligned to the 1775 bp mRNA (NM_139113). The mRNA sequence was searched against the rat genome and produced a single hit with the chromosome 16 contig (accession number NW_047470). The gene was found to run opposite to the contig, and hence the 1 kilobase upstream promoter was located at positions 881259 to 882253 on the contig. PCR primers were designed encompassing this region and part of the first exon (Figure 4.13).

Figure 4.13: Chromosomal contig references for the cloned region of the rCOUPg promoter

Chromosomal mapping of the rat rCOUPg mRNA (NM_139113) to the rat genomic contig NW_047470 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.1.6 OGFRL1

The fragment accession number for OGFRL1 (AI045104) was searched using the BLAST tool against the blastn database at NCBI, and aligned to the 1728 bp mRNA (NM_001025708). The mRNA sequence was searched against the rat genome and produced a single hit with the chromosome 9 contig (accession number NW_047813). The gene was found to run opposite to the contig, and hence the 1 kilobase upstream promoter was located at positions 22247453 to 22248425 on the contig. PCR primers were designed encompassing this region and part of the first exon (Figure 4.14).

Figure 4.14: Chromosomal contig references for the cloned region of the OGFRL1 promoter

Chromosomal mapping of the rat OGFRL1 mRNA (NM_001025708) to the rat genomic contig NW_047813 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.1.7 rBACH

The fragment accession number for rBACH (D88890) was searched using the BLAST tool against the blastn database at NCBI, and aligned completely to the 1523 bp mRNA (NM_013214). The mRNA sequence was searched against the rat genome and produced three hits. From these three hits, one was identified as an active gene, aligning to the chromosome 5 contig (accession number NW_047727). The other two hits were confirmed as inactive pseudo-genes by searching against the EST_others database at NCBI, whereby no alignments were found. The gene was found to run parallel with the contig, and hence the 1 kilobase upstream promoter was located at positions 9228940 to 9229834 on the contig. PCR primers were designed encompassing this region and part of the first exon (Figure 4.15).

Figure 4.15: Chromosomal contig references for the cloned region of the rBACH promoter

Chromosomal mapping of the rat rBACH mRNA (NM_013214) to the rat genomic contig NW_047727 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.1.8 Me1

The fragment accession number for Me1 (AI171506) was searched using the BLAST tool against the blastn database at NCBI, and aligned to exon 14 of the 1761 bp mRNA (NM_012600). The mRNA sequence was searched against the rat genome and produced a single hit with the chromosome 8 contig (accession number NW_044800). The gene was found to run opposite with relation to the contig, and hence the 1 kilobase upstream promoter was located at positions 8233072 to 8234080 on the contig. PCR primers were designed encompassing this region and part of the first exon (Figure 4.16).

Figure 4.16: Chromosomal contig references for the cloned region of the Me1 promoter

Chromosomal mapping of the rat Nob1p mRNA (NM_012600) to the rat genomic contig NW_044800 showing the position of exon 1, the promoter region cloned and the genomic references for the region (blue).

4.3.1.2 Cloning of promoters into vector

Following PCR amplification of the proximal promoters of the genes of interest, the products were cleaned by gel extraction (section 2.3.6.1), and cloned into an initial vector pCR[®]2.1 (Invitrogen) that allowed blue/white screening of colonies. Following confirmation of the presence of the correct insert by diagnostic digest and direct sequencing (section 2.3.6.5) the inserts were cloned into the expression vector pSEAP. Figures 4.17 to 4.21 show the diagnostic digests for each of the promoter constructs that was cloned. Digests were designed to cut at one point within the cloned region using a specific restriction enzyme cutting site, with the other site falling outside the cloned region, allowing confirmation of both the identity of the cloned region and its orientation. In each case the appropriate sized fragments have been produced as compared to either a 10 kb or a 100 bp step ladder. Therefore from the nine genes that had been selected for further investigation, four of the genes whose expression was suppressed and one of the genes whose expression was induced in response to liver growth agent administration were successfully cloned into pSEAP. These were rBACH (induced) and HCaRG, Nob1p, LRCH4 and Sp3 (suppressed).

Figure 4.17: Diagnostic digests of pHCaRG-SEAP using *Bgl* II and *Not* I

Diagnostic digest of pHCaRG-SEAP using two separate enzymes for confirmation of cloned region. Lane 1 contains the construct digested with *Bgl* II producing two bands of size 805 bp and 4940 bp. Lane 2 contains the construct digested with *Not* I producing two bands of size 1261 bp and 4484 bp.

Figure 4.18: Diagnostic digests of pNob1p-SEAP using *Bgl* II and *Not* I

Diagnostic digest of pNob1p-SEAP using two separate enzymes for confirmation of cloned region. Lane 1 contains the construct digested with *Bgl* II producing two bands of size 739 bp and 5087 bp. Lane 2 contains the construct digested with *Not* I producing two bands of size 1342 bp and 4484 bp.

Figure 4.19: Diagnostic digests of pSp3-SEAP using *Spe I* and *Not I*

Diagnostic digest of pSp3-SEAP using two separate enzymes for confirmation of cloned region. Lane 1 contains the construct digested with *Spe I* producing two bands of size 703 bp and 5085 bp. Lane 2 contains the construct digested with *Not I* producing two bands of size 1304 bp and 4484 bp.

Figure 4.20: Diagnostic digests of pLRCH4-SEAP using *Spe I* and *Not I*

Diagnostic digest of pSp3-SEAP using two separate enzymes for confirmation of cloned region. Lane 1 contains the construct digested with *Spe I* producing two bands of size 933 bp and 4842 bp. Lane 2 contains the construct digested with *Not I* producing two bands of size 1291 bp and 4484 bp.

Figure 4.21: Diagnostic digests of pRBACH-SEAP using *Pst* I

Diagnostic digest of pRBACH-SEAP using the enzyme *Pst* I for confirmation of cloned region (lane 1) producing two bands of size 870 bp and 4665 bp.

4.3.2 BASAL EXPRESSION OF 1 KILOBASE REPORTER GENE CONSTRUCTS

Figure 4.22 shows the levels of basal expression of SEAP following transfection of each of the promoter constructs generated. Of the four promoter constructs generated from the suppressed group of genes, only the HCaRG and Nob1p promoters caused significant SEAP expression over control (pSEAP). Sp3 and LRCH4 promoter constructs failed to show significant basal activity, hence it would be impossible to investigate inhibition of SEAP expression using these constructs. It is possible that the key regulatory element(s) for these genes are not contained within the proximal 1 kb of promoter sequence, but further upstream, or that an enhancer sequence not present in the reporter constructs, is required for maximal expression of Sp3 and LRCH4. Hence only HCaRG and Nob1p were investigated further, with the hypothesis that they would form a model for the rest of the group of seven co-regulated genes. rBACH proved to be a strong promoter inducing SEAP expression at a higher level than the SV40 promoter, however the suppression of gene expression observed for the group of seven genes represented a more interesting group to investigate further due to the similarity across all three compounds tested. Therefore the potential co-ordinate regulation of rBACH and Me1 was not investigated further.

Figure 4.22: Basal expression of SEAP under control of cloned gene promoters of interest

Basal levels of SEAP activity in growth medium were measured 48 hours post-transfection into FaO cells for each promoter construct. ***= $p < 0.001$, ns = not significant relative to control (empty pSEAP vector) using one-way ANOVA with Bonferroni's multiple comparison post hoc test. pSEAP under the control of the SV40 promoter was included as a positive control. Basal level for rBACH promoter construct is included but was not investigated further (see section 4.2.2).

4.3.3 EFFECT OF LIVER GROWTH AGENTS ON REPORTER GENE CONSTRUCTS

Following assessment of the basal activity of the gene promoters it was necessary to investigate the effect of liver growth agent administration on FaO cells transfected with the 1 kb promoter constructs. Due to the low basal activity of the Sp3 and LRCH4 promoter constructs it was impossible to investigate the inhibitory effects of liver growth agents on these genes. Hence only the constructs containing the promoters of HCaRG and Nob1p were investigated further.

4.3.3.1 Assessment of compound toxicity by the lactate dehydrogenase release assay

To confirm that any inhibition of gene expression observed was not due to cellular toxicity, FaO cells were first dosed with liver growth agents according to the dosing scheme for the reporter gene assay and assayed for LDH release, to examine for toxicity.

Figure 4.23 shows the results of the LDH assay. In untreated cells a small degree of cell death is seen over the culture time, with the percentage of LDH release increasing from 9-percent at 24 hours to 13-percent at 96 hours post initial dose. The DMSO (vehicle) control shows no significant effect on LDH release compared to untreated cells across all the time-points measured, demonstrating that it has no effect on cell viability.

All the drugs caused only minor, if any cell death, with the maximal effect being a 4-percent increase in the level of LDH release in response to CPA (100 μ M) at 48 hours post initial dose. The peroxisome proliferators CFA and Wy-14,643 showed a protective effect by causing a small but significant decrease in LDH release at 48 and 72 hours post initial dose (CFA) and 96 hours post initial dose (Wy-14,643). No increase in LDH release was observed for any of the compounds at 24 hours post initial dose. At this time-point a measurable decrease in the expression of the genes of interest had occurred as shown by microarray and quantitative real-time PCR, indicating that the decreases observed for these genes were not due to xenobiotic toxicity.

The physiological relevance of these doses is difficult to determine due to the differences between *in vivo* and *in vitro* test systems. A qualitative similarity has been noted between primary cultures of rat hepatocytes and *in vivo* liver (de Longueville *et al.*, 2003, Jessen *et al.*, 2003). In a microarray experiment to assess gene expression between the two systems, the response of primary hepatocytes dosed with Wy-14,643 (500 μ M) was 80-percent of *in vivo* liver dosed at 100 mg/kg (Jessen *et al.*, 2003). At this dose, Wy-14,643 is known to cause hepatocarcinogenesis in rodents in long-term studies (Motoki *et al.*, 1997).

Figure 4.23: Effect of exposure of test compounds on cultured FaO cell viability by LDH assay

Toxicity of all the compounds used in transfection assays was assessed by assay of LDH release. Cells were incubated with compound in growth medium at the maximum dose used in transfection assays for the appropriate time. Medium was removed for assay and fresh medium containing control or compound replaced. Values expressed are percentage of total LDH release as induced by incubation of cells with 2% Triton X-100 in growth medium for 10 minutes. $\ast = p < 0.05$, $\ast\ast\ast = p < 0.001$ relative to vehicle control (DMSO) at each time-point using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

4.3.3.2 Dose response analysis

Following transfection with the promoter constructs to confirm basal activity for the gene promoter sequences, the constructs were transfected into FaO cells and exposed to drug or control for 48 hours prior to assessment of SEAP expression. In this way it was possible to determine the effect of the compounds on the 1 kb proximal promoter sequences and their effect on gene expression, as inferred from their ability to drive expression of SEAP. The 48 hour time-point was chosen since all the genes suppressed in response to liver growth agent exposure showed evidence of suppression by this time-point, particularly in the microarray results. Since the pHCaRG-SEAP and pNob1p-SEAP were the only constructs to show significant basal activity for assessment of

inhibition, it was most important that this time-point would be relevant for them, as is the case for both genes following quantitative real-time PCR assessment of rat liver (Figures 4.1 and 4.2).

The compounds tested were the peroxisome proliferators CFA and Wy-14,643, CPA and dexamethasone as used in the previous experiments. A range of doses from 1 μ M to 100 μ M were selected to analyse the dose response of each construct, allowing assessment of the optimal dose for future experiments. In each case the vehicle control was DMSO, with results representing repeat samples (n=5) expressed as mean $\pm$ SEM. Dose response analysis allowed validation of the response observed in the microarray data, with the ability to define the most appropriate dose and hence the maximal response by each promoter, which was not the case for the *in vitro* quantitative real-time PCR analysis.

Figure 4.24 shows the dose response results for the two constructs in response to dexamethasone. In both cases a highly significant response was observed, reaching a maximal 2.5-fold decrease in SEAP expression for HCaRG at 100 μ M, and maximal 3.5-fold decrease for Nob1p at 100 μ M. The HCaRG promoter (Figure 4.24a) caused consistent suppression of SEAP expression across the range of doses, indicating that the dose required for maximal suppression was potentially in the nM range. In comparison, the Nob1p promoter (Figure 4.24b) exhibited increasing inhibition of SEAP expression with increasing dose, and the dose causing maximal suppression may have been in excess of 100 μ M. Hence the HCaRG promoter appears to have reached the maximal suppression using a concentration of 1 μ M, whereas the maximal suppression of Nob1p may be at a concentration higher than 100 μ M. Therefore the HCaRG promoter may be more sensitive to the presence of dexamethasone than the Nob1p promoter, but the Nob1p promoter is capable of suppressing SEAP expression to a greater degree than the HCaRG promoter (3.5-fold decrease for Nob1p versus 2.5-fold for HCaRG). From the range of doses tested the dose of 100 μ M was chosen for future experiments since this dose did not produce toxic effects in the FaO cells at 24 and 48 hours post dose (Figure 4.23) and produced a significant degree of suppression by both promoter constructs.

Figure 4.24: Dose response of HCaRG and Nob1p promoter constructs to dexamethasone

Promoter constructs for HCaRG (A) and Nob1p (B) were transfected into FaO cells (n=5) and following a 24 hour incubation were dosed with compound at the appropriate dose or DMSO (control). Growth medium was removed following 48 hour incubation and assayed for SEAP using the chemiluminescent method described in section 2.4.5. ***= $p < 0.001$ relative to vehicle control (DMSO) using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

Figure 4.25 shows the dose response curves for administration of CPA. The HCaRG promoter significantly suppressed SEAP expression at 1 μM but failed to in response to 10 or 25 μM CPA (Figure 4.25a). However, at 50 μM CPA the expression of SEAP was suppressed to a similar extent as the 1 μM dose, and at 100 μM it was reduced to 25-percent of control. Therefore, for HCaRG

there is a biphasic response to CPA that could be due to the involvement of different nuclear receptors at low and high doses. An example is in the expression of glutathione *S*-transferase A2 that is suppressed in response to low doses of dexamethasone in a GR-dependent manner, and induced by high doses via PXR activation producing a biphasic expression profile (Falkner *et al.*, 2001). For the Nob1p promoter all the doses resulted in significant suppression of SEAP expression, however there was a notable increase in suppression at 10 μ M that decreased at 25 μ M, prior to increasing at 50 and 100 μ M (Figure 4.25b). For both constructs 100 μ M CPA produced the greatest degree of suppression of SEAP expression, with administration of this dose resulting in a ~5-fold decrease in the expression of SEAP for both promoter constructs. For pHCaRG-SEAP the difference in expression of SEAP between 50 and 100 μ M CPA was ~2-fold versus 5.5-fold, and for pNob1p-SEAP this difference was ~3-fold versus 5-fold. This difference cannot be accounted for by the 17-percent increase in LDH release at 48 hours post dose with 100 μ M CPA versus control treated cells (Figure 4.23), and hence it was decided to dose with 100 μ M CPA in future experiments, despite the increase in LDH release observed at this dose.

Figure 4.26 shows the dose response results for administration of CFA. For both the HCaRG construct (Figure 4.26a) and the Nob1p construct (Figure 4.26b) there was a correlation between dose and suppression of SEAP expression. In both cases 100 μ M CFA produced the greatest degree of suppression, reaching a 2-fold decrease for each construct. It is likely that this dose produced near to the maximal suppression since the results for 25 and 50 μ M were very similar to that for 100 μ M CFA. However, since there was no significant increase in LDH release in response to 100 μ M CFA, and in fact a reduction in LDH release was observed at 48 and 72 hours post initial dose, this dose was used for future experiments.

Figure 4.27 shows the dose response results for administration of Wy-14,643. For the pHCaRG-SEAP construct a maximal suppression of SEAP expression of 2.6-fold versus DMSO control was observed using 50 μ M Wy-14,643 (Figure 4.27a). For the Nob1p-SEAP construct this compound produced the least suppression of SEAP expression compared to the other liver growth agents administered, reaching a maximal suppression of 1.7-fold at 100 μ M Wy-14,643 (Figure 4.27b).

Since Wy-14,643 (100 μ M) caused the maximal effect on the Nob1p promoter, and a statistically significant effect on the HCaRG promoter (~2-fold decrease versus DMSO control), but did not cause any detrimental effects on LDH release at any of the time-points assessed (Figure 4.23), this dose was used in future experiments.

Figure 4.25: Dose response of HCaRG and Nob1p promoter constructs to CPA

Promoter constructs for HCaRG (**A**) and Nob1p (**B**) were transfected into FaO cells (n=5) and following a 24 hour incubation were dosed with compound at the appropriate dose or DMSO (control). Growth medium was removed following 48 hour incubation and assayed for SEAP using the chemiluminescent method described in section 2.4.5. ***=p<0.001 relative to vehicle control (DMSO) using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

Figure 4.26: Dose response of HCaRG and Nob1p promoter constructs to CFA

Promoter constructs for HCaRG (**A**) and Nob1p (**B**) were transfected into FaO cells (n=5) and following a 24 hour incubation were dosed with compound at the appropriate dose or DMSO (control). Growth medium was removed following 48 hour incubation and assayed for SEAP using the chemiluminescent method described in section 2.4.5. $\ast=p<0.05$, $\ast\ast=p<0.01$, $\ast\ast\ast=p<0.001$ relative to vehicle control (DMSO) using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

Figure 4.27: Dose response of HCaRG and Nob1p promoter constructs to Wy-14,643

Promoter constructs for HCaRG (**A**) and Nob1p (**B**) were transfected into FaO cells (n=5) and following a 24 hour incubation were dosed with compound at the appropriate dose or DMSO (control). Growth medium was removed following 48 hour incubation and assayed for SEAP using the chemiluminescent method described in section 2.4.5. **= $p < 0.01$, ***= $p < 0.001$ relative to vehicle control (DMSO) using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

4.3.3.3 Time response analysis at optimum dose

After the dose response experiments it was necessary to analyse the time response to administration of the compounds. The constructs were transfected into FaO cells and following 24 hours incubation were dosed with each compound at the appropriate dose. Medium was removed for assay every 24 hours for a 72 hour period, and fresh medium containing the compound was subsequently replaced and incubated for the next 24 hours.

Figure 4.28 shows the results of the time response experiment. The results for the HCaRG promoter construct (Figure 4.28a) show a significant suppression of SEAP expression (~2-fold versus DMSO control) across the entire 72 hour period measured, with similar results between the four liver growth agents tested. This was not attributable to drug toxicity since measurement of LDH release of cells under the same conditions showed no toxicity at 24 hours, and only a marginal effect at 48 hours post-dose (section 4.3.3.1).

Figure 4.28b shows the results for the Nob1p promoter construct. The effect of the compounds was less consistent than for HCaRG but produced the same magnitude of suppression, indicating that the two promoters may be under the same control in response to liver growth stimuli. The main differences between the two constructs were that at 24 hours post-dose Wy-14,643 (100 μ M) failed to significantly suppress SEAP expression and that at 72 hours dexamethasone (100 μ M) produced a 20-percent decrease in SEAP expression compared to 50-percent for HCaRG at the same time point. However, the suppression of SEAP expression exhibited at 48 hours for both promoter constructs across all four compounds is similar to that from the real-time PCR analysis in rat liver (Figure 4.1b and 4.2b), and indicates that the promoters are potentially co-regulated in response to liver growth stimuli.

Figure 4.28: Effect of liver growth agents on HCaRG and Nob1p promoter constructs with time

Promoter constructs for HCaRG (**A**) and Nob1p (**B**) were transfected into FaO cells and following 24 hour incubation were dosed with either compound or DMSO (control). Growth medium was removed every subsequent 24 hours for a 72 hour period and assayed for SEAP using the chemiluminescent method described in section 2.4.5. $\ast = p < 0.05$, $\ast\ast = p < 0.01$, $\ast\ast\ast = p < 0.001$ relative to vehicle control (DMSO) at each time-point using one-way ANOVA with Bonferroni's multiple comparison post hoc test.

4.4 DISCUSSION

The genes identified from the bioinformatic analysis as showing co-ordinate regulation by liver growth agents in Chapter 3 have been validated in this chapter. In addition, the response has been characterised using a range of liver growth agents, exposure periods and doses, using both quantitative real-time PCR and reporter gene assay. For rBACH and Me1 the response was found to be specific to the peroxisome proliferators Wy-14,643 and CFA. Although the two genes may represent an important part of the response to peroxisome proliferator exposure, the changes in their expression do not represent a central event in the cellular response to varied liver growth stimuli, and hence were not investigated further herein.

For the genes originally identified as being suppressed in response to liver growth agent administration there was a high similarity in expression profile across all seven genes. Despite the range of sample types and dosing schemes examined within this chapter the suppression observed *in vivo* by microarray analysis was confirmed throughout the quantitative real-time PCR validation experiments, although to a varying extent as might be expected from comparison between *in vivo*, primary and immortalised cells. The FaO cell line is a well differentiated hepatoma cell line (Moore and Weiss, 1982) that has previously been used to assess the effect of both Wy-14,643 and dexamethasone on PPAR α expression (Sterchele *et al.*, 1996). The cell line also displays inhibition of apoptosis as induced by the peroxisome proliferator nafenopin (50 μ M) as is observable *in vivo* (Bayly *et al.*, 1994). However, the transcription profile of the cell line is known to decrease with increasing passage number (M. Weiss, personal communication) and this may have contributed to the reduced response compared to samples from liver and primary hepatocytes.

The microarray data suggested the presence of two sub-groups within the group of seven genes whose expression was suppressed by liver growth agents: HCaRG, rCOUPg, LRCH4, OGFRL1 and AI044183 showing maximal inhibition of gene expression at 48 hours, and Sp3 and Nob1p at 72 hours post initial dose. However the quantitative real-time PCR data from rat liver administered the same dosing regime showed a highly similar response between HCaRG, rCOUPg and Nob1p, indicating that the response of the seven genes may be more similar than the microarray data initially suggested.

Throughout the dosing experiments for HCaRG and Nob1p a consistent suppressive effect has been observed. This was true whether by microarray, quantitative real-time PCR or reporter gene assay analysis of the suppression of gene expression. The magnitude of suppression was ≥ 2 -fold versus control in the majority of the results, with similar profiles of suppression in response to the variety of liver growth agents used. Since a wide variety of sample types and techniques were used in the validation of this suppression, there is a high certainty that it represents a genuine response to liver growth stimuli, since it is not due to toxic insult from such compounds as proved by the assessment of cellular LDH release.

The successful cloning of the HCaRG and Nob1p promoters and the validation of their inhibitory response to liver growth stimuli by reporter gene assay confirms the potential co-ordinate regulation of these two genes in the response to liver growth stimuli. Despite the difficulties in cloning the rCOUPg and OGFRL1 promoters, and the lack of basal activity of the Sp3 and LRCH4 1 kb proximal promoter constructs, validation of the co-regulation of all seven genes would be possible following identification of the key regulatory transcription factor(s) governing the response of HCaRG and Nob1p to liver growth agents (Chapter 5).

5 TARGET EXAMINATION AND MUTAGENESIS

5.1 INTRODUCTION

The response of HCaRG and Nob1p to liver growth agent administration *in vitro* (Chapter 4), confirmed the similarity in the expression of these two genes in response to these agents first observed with the *in vivo* microarray experiment (Chapter 3). Therefore, further analysis is required to explore the regulation of HCaRG and Nob1p with the view to understanding the molecular mechanism controlling the response to agents causing liver growth. Little is known about the regulation of either HCaRG or Nob1p since both genes were identified recently (Solban *et al.*, 2000; Tone *et al.*, 2000) and the majority of the research on these genes has centred on their downstream effects. Similarly, no previous link has been suggested between these two genes and the other five genes identified as exhibiting potential co-ordinate regulation from the *in vivo* microarray study (Chapter 3).

HCaRG is a potential regulator of cellular growth and differentiation as shown by its overexpression in the human embryonic kidney cell line HEK-93, resulting in reduced proliferation of the cells (Solban *et al.*, 2000), which was later shown to be via cell cycle arrest (Devlin *et al.*, 2003). In contrast, no direct link between Nob1p and cellular growth has previously been suggested, with the major role of Nob1p being as an essential component for the formation of the 26S proteasome (Tone *et al.*, 2000) and for pre-rRNA synthesis (Fatica *et al.*, 2003). The effect of decreasing expression of these genes could be detrimental to the cell, since the 26S proteasome is important in the breakdown of ubiquitinated proteins and decreased removal of ubiquitinated proteins could result in the maintenance of intracellular signals that should have been removed, potentially impacting upon cell growth. Similarly, removal of a protein such as HCaRG that acts to prevent cellular division through the promotion of differentiation and the ability to induce cell cycle arrest would be detrimental to the cell with regard to transformation.

The following chapter presents the results of dissection of the promoters of HCaRG and Nob1p including identification of the important regions controlling the response to liver growth agents. It describes the *in silico* analysis performed on the relevant sections of the gene promoters as identified by deletion construct analysis, and outlines the approach taken to reduce the number of potential

response elements implicated in the response of HCaRG and Nob1p. This in turn allows presentation of a model on the role of upstream transcription factors in the regulation of the two genes and their response to liver growth agents (Chapter 6).

5.2 CLONING AND ANALYSIS OF DELETION MUTANT CONSTRUCTS

Following analysis of the 1 kilobase upstream promoter regions of HCaRG and Nob1p, the promoter constructs were each used as templates for generation of three smaller daughter constructs. The generation of these deletion constructs was accomplished using PCR amplification from primers containing existing restriction enzyme cutting sites, or where not possible point mutations that were created to produce cutting sites (Figure 5.1). The PCR products were then digested with the appropriate enzyme(s) and sub-cloned in to pSEAP. Table 5.1 shows the PCR primers and the enzymes for each deletion construct generated.

Figure 5.1: Strategy for generation of deletion constructs

The stages in the generation of deletion constructs for HCaRG and Nob1p. Firstly PCR amplification is performed using nested primers containing a restriction enzyme (RE) cutting site unique to the region of interest, producing daughter fragments. Secondly the PCR products are purified by gel extraction to remove any interfering non-specific bands, and the product is digested with the appropriate enzyme(s). Thirdly the digested product is ligated into pSEAP that has been digested with the same enzyme(s) and purified by gel extraction. The ligation mixture can then be transformed into *E.Coli* DH5 α for generation of large quantities of the construct.

Table 5.1: PCR primers and restriction enzymes used to generate deletion constructs

Gene name/ Fragment length (bp)	Forward primer	Reverse primer
HCaRG - 701	gaactcacag agatct gtctgcct ↑ <i>Bgl</i> II	gcttacttagatcg cagatct cgag ↑ <i>Bgl</i> II
HCaRG - 474	taaggcag gtacc acacaaatttacgaccc ↑ tgtacc → <i>Acc65</i> I	atctc gagc ggccgcccagtg ↑ <i>Xho</i> I
HCaRG - 93	gcgctc gtacc gggaaccac ↑ ggttcc → <i>Acc65</i> I	atctc gagc ggccgcccagtg ↑ <i>Xho</i> I
Nob1p - 725	tagctc gtacc ccatttttaatagg ↑ tgtacc → <i>Acc65</i> I	gcttacttagatcg cagatct cgag ↑ <i>Bgl</i> II
Nob1p - 312	atcga gtacc ttactgatttatatgatag ↑ cgccacc → <i>Acc65</i> I	gcttacttagatcg cagatct cgag ↑ <i>Bgl</i> II
Nob1p - 71	gttcctc tcgag tccgggaggagtg ↑ ctccag → <i>Xho</i> I	atctc gagc ggccgcccagtg ↑ <i>Xho</i> I

PCR primers for generation of deletion constructs from 1 kb promoter sequences. Restriction enzyme cutting sites are shown in bold with relevant enzyme, mutated bases are underlined where relevant. Forward primers were designed within the promoter region to produce smaller promoter constructs, in combination with a reverse primer located 3' to the promoter i.e. within the pSEAP vector sequence.

Figure 5.2 illustrates the design of the deletion constructs for the HCaRG (Figure 5.2a) and Nob1p (Figure 5.2c) promoters. Each daughter fragment is contained within the larger fragment, allowing removal of a portion of the sequence in each case, and assessment of the intervening sequence for its input into regulation of gene expression. It should be noted that the format of the labelling of constructs, for example Nob1p-71 bp, indicates the promoter in question and the size of the promoter region cloned into pSEAP, and does not reflect the total number of base-pairs of promoter cloned. The choice of such a nomenclature is because along with the regulatory regions, a portion of exon 1 was amplified in the initial PCR. For the HCaRG constructs this represented 57 bp of the 73 bp in exon 1, and for the Nob1p constructs this was the entire 72 bp in exon 1. The position of the *Not* I site used in the diagnostic digest is shown in each case, with the second *Not* I cutting site being located elsewhere within the pSEAP construct sequence. The diagnostic digests

for the HCaRG (Figure 5.2b) and Nob1p (Figure 5.2d) deletion constructs indicate the presence of the correct insert in each case.

Figure 5.2: Generation of deletion constructs and diagnostic analysis using *Not I*

C

D

A Cloned region of HCaRG promoter and deletion constructs showing *Not I* cutting sites as used in diagnostic digest for all constructs. A second *Not I* site was located within the vector sequence. Region cloned included promoter upstream of TSS and 57 bp of the 73 bp exon 1. **B** HCaRG deletion mutants and 1 kb promoter construct digested with *Not I* to give 4484 bp in each case and 355 bp (HCaRG-93), 736 bp (HCaRG-474), 998 bp (HCaRG-701), and 1261 bp (HCaRG-999). **C** Cloned region of Nob1p promoter and deletion constructs showing *Not I* cutting sites as used in diagnostic digest for all constructs. Region cloned included promoter upstream of TSS and the entire 72 bp of exon 1. **D** Nob1p deletion mutants and 1 kb promoter construct digested with *Not I* to give 4484 bp in each case and 378 bp (Nob1p-71) 588 bp (Nob1p-312), 1001 bp (Nob1p-725), and 1342 bp (Nob1p-1066).

5.2.1 BASAL EXPRESSION FROM DELETION MUTANT CONSTRUCTS

Prior to investigating the drug-induced response for each of the deletion constructs it was necessary to analyse the basal function of each region of the HCaRG and Nob1p promoters. Constructs were transfected as previous into FaO cells (Section 2.4.4) and SEAP expression assayed after 48 hours incubation at 37°C in 5% CO₂. The results for the HCaRG deletion constructs (Figure 5.3a) showed a significant increase in SEAP expression compared to pSEAP empty vector for the initial 93 bp of proximal promoter, increasing the relative luminescence units over 1000-fold. This indicates not only the presence of one or more regulatory elements within the entire cloned region (as previously demonstrated in Chapter 4), but that the proximal 93 bp of the HCaRG proximal promoter contains sufficient regulatory elements to support a reasonable level of basal expression.

The HCaRG-474 bp construct showed a slight increase in SEAP expression over the HCaRG-93 bp construct indicating that the intervening 381 bp of promoter contains one or more regulatory elements exerting a further positive effect on HCaRG gene expression, but to a lesser extent than the element(s) within the initial 93 bp of promoter sequence. The HCaRG-701 bp construct induced a lesser increase in SEAP expression than the previous constructs, inducing 72-percent of the level of the previous construct. This suggests the presence of a negative regulatory element(s) within this additional 227 bp region of the HCaRG promoter as indicated in Figure 5.3a. The region between 701 and 999 bp upstream of the transcription start site induced a lower increase in SEAP expression compared to the previous construct, again indicating the presence of one or more negative elements within this region.

Overall the basal analysis of HCaRG promoter functionality indicates that the response element(s) located within the initial 474 bp of proximal promoter sequence are influential on positive regulation of SEAP expression, whereas the region 474 to 999 bp has a net negative effect on gene expression.

Figure 5.3: Basal expression of SEAP from HCaRG and Nob1p deletion constructs

Deletion constructs for HCaRG (A) and Nob1p (B) were transfected into FaO cells as described in section 2.4.4. Following incubation for 24 hours the growth medium was removed and assayed for SEAP as described in section 2.4.5. Deletion analysis of the HCaRG and Nob1p promoters indicated the presence of positive (+) and negative (-) response elements important for the basal regulation of gene expression as compared to the previous construct in each case. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance relative to previous construct (n=5) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The results for the Nob1p deletion constructs are shown in Figure 5.3b. As observed in Chapter 4 the Nob1p promoter produced approximately 10-fold lower activation of SEAP expression compared to the HCaRG regulatory region (Section 4.3.2). Inclusion of the initial 71 bp of proximal promoter produced an 8-fold increase in SEAP expression over the empty pSEAP plasmid alone, indicating the presence of one or more positive regulatory elements within this region. However, the region with the greatest positive influence on basal expression contained the first 725 bp of the proximal promoter, this region inducing a maximum 26-fold over pSEAP alone. The Nob1p-1066 bp construct induced SEAP expression to 60-percent of the Nob1p-725 bp construct indicating the presence of one or more negative regulatory elements within this 341 bp region. Overall the basal analysis of Nob1p promoter functionality indicates that the response element(s) located within the initial 725 bp of proximal promoter sequence are influential on positive regulation of SEAP expression, whereas the region 725 to 1066 bp has a net negative effect on gene expression.

5.2.2 EFFECT OF LIVER GROWTH AGENTS ON DELETION MUTANT CONSTRUCTS

Following analysis of the basal expression of SEAP induced by each of the promoter constructs it was necessary to investigate the effect of dosing with liver growth agents as this would allow identification of the region(s) of the HCaRG and Nob1p promoters that were responsible for the inhibition observed in response to dosing with liver growth agents: It is not necessarily the case that the same regulatory element(s) responsible for basal control of gene expression would also be involved in the response to xenobiotics.

The deletion constructs were transfected into FaO cells and then dosed on the following day with one of several liver growth agents for a 48 hour period. This had previously been shown to represent a period of maximal inhibition of gene expression (Section 4.3.3). The results for the dosing experiment are shown in Figure 5.4. The HCaRG-93 bp construct (Figure 5.4a) showed a significant decrease in SEAP expression in response to all three compounds. Dosing this construct with dexamethasone and CPA (both at 100 μ M) resulted in 30-percent SEAP expression compared to DMSO control. SEAP expression was reduced similarly for the HCaRG-474, -701 and -999 bp constructs in response to dexamethasone and CPA versus control. The response to CFA (100 μ M)

was reduced compared to dexamethasone and CPA, only achieving approximately 60-percent of control for the HCaRG-93 bp construct. Only the HCaRG-999 bp construct maintained the level of inhibition of SEAP expression observed for the -93 bp construct.

Figure 5.4a includes the level of significance calculated between constructs above the graph. For dexamethasone and CPA treatment there was no significant difference between each of the promoter constructs, indicating that the response element(s) responsible for the inhibition of SEAP expression are located within the initial 93 bp of proximal promoter. CFA treatment produced significant differences between the -474 bp and -93 bp constructs, and between the -999 bp and -701 bp constructs. However, there was no significant difference in SEAP expression between the -93 bp and -999 bp constructs, indicating that the response was again located to the initial 93 bp of the HCaRG promoter. Overall, as all liver growth agents caused a decrease in gene expression for all of the reporter gene constructs, including the construct containing the shortest regulatory region, then this is suggestive that the response elements responsible for their action are located within the first 93 bp of the HCaRG proximal promoter.

The Nob1p deletion constructs (Figure 5.4b) demonstrated a similar response to the HCaRG constructs. The Nob1p-71 bp construct showed the greatest degree of inhibition of SEAP expression for dexamethasone and CPA, resulting in 20-percent of the control level. There was no significant difference between the -71 bp, -312 bp, -725 bp and 1066 bp constructs for these two compounds, indicating that the initial 71 bp of the Nob1p promoter were important in the inhibition of gene expression observed. The Nob1p constructs demonstrated greater similarity between the three compounds than the HCaRG constructs. However, there was a significant difference in the level of inhibition of gene expression between the -71 bp and -312 bp constructs in response to CFA. The suppression of SEAP expression was at 70-percent of control for the -71 bp construct compared to 40-percent of control for the -312 bp construct.

Figure 5.4: HCaRG and Nob1p deletion construct analysis following dosing with liver growth agents

Deletion constructs for HCaRG (A) and Nob1p (B) were transfected into FaO cells as described in section 2.4.4. Following 24 hours incubation in growth medium the transfected cells were exposed to either dexamethasone, CPA, CFA (100 μ M) or DMSO control (0.1%) for 48 hours prior to assay of SEAP expression. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance relative to DMSO control for each construct (n=5) * p<0.05, ** p<0.01, ***p<0.001. A further one-way ANOVA with Bonferroni's post-hoc test was performed to assess statistical significance between constructs for each compound (above graphs).

This indicates that although the initial 71 bp of promoter contains one or more regulatory elements important for the response to xenobiotics, there is a further element(s) within the following 241 bp of promoter sequence that is required for the full extent of inhibition observed. This may be related to the basal activities of these two constructs (Figure 5.3b) where the -312 bp construct obviously contained one or more elements essential for the basal functioning of the promoter that were not contained within the initial 71 bp of promoter sequence. Overall a similar situation was seen for Nob1p as for the HCaRG reporter constructs, with all liver growth agents causing a decrease in gene expression across all of the Nob1p reporter gene constructs. This is therefore suggestive that the response elements responsible for their action are located within the first 71 bp of the Nob1p proximal promoter.

5.2.3 DISCUSSION

The results of the basal and drug-induced analysis of promoter functionality indicate that the initial 93 bp of proximal promoter sequence for HCaRG, and the initial 71 bp of the Nob1p promoter are largely responsible for the inhibition of gene expression observed. The response of the Nob1p-312 bp construct to CFA (Figure 5.4b) indicates the presence of other element(s) within the proximal promoter that are important for the full extent of inhibition. Further analysis of the proximal promoter regions of these two genes is now required to identify potential response elements located within them that are responsible for the inhibitory effects of the liver growth agents on gene expression.

5.3 INVESTIGATION OF THE KEY RESPONSE ELEMENTS IN THE CONTROL OF HCaRG AND NOB1P GENE EXPRESSION

5.3.1 *IN SILICO* ANALYSIS OF IMPORTANT REGULATORY REGIONS

Following identification of the regions of the HCaRG and Nob1p promoter sequences responsive to liver growth agents, it was necessary to identify the transcription factor(s) responsible for this response. *In silico* analysis allows regions of DNA sequence to be probed for potential response elements based on published consensus sequences for a variety of transcription factors. Several search facilities are available to probe the TRANSFAC database (Wingender *et al.*, 1996) that lists these consensus sequences based on published information. MatInspector (www.genomatix.de) (Quandt *et al.*, 1995) and MatchTM (www.gene-regulation.com) are two such tools, and interrogation using both tools allows a greater confidence that identified response elements are likely to be biologically relevant. However, experimental verification is always necessary to ensure that potential binding sites do exist, including techniques such as electromobility shift assay (EMSA) to prove the transcription factor binds to the response element, and site-directed mutagenesis to ablate the binding of the transcription factor and investigate its influence on gene expression.

Matrix searching was first proposed by Quandt and colleagues (1995) whereby the sequence is compared to a known consensus sequence for a transcription factor, and scored based on the matrix similarity and the core similarity to that consensus sequence. The matrix describes the individual binding profile for an individual or group of transcription factors (Wingender *et al.*, 1996). The matrix similarity describes the similarity between a matrix and a part of the input sequence, with the core similarity representing the similarity between the five most conserved nucleotides in the matrix and the input sequence. The search initially identifies regions with core similarities above a pre-defined threshold that can be set to minimise false positives or false negatives. The matrix similarity is calculated only for the regions with sufficiently high core similarity. For the analysis of the HCaRG and Nob1p promoters, relatively stringent values for core similarity (>0.70) and matrix similarity (>0.70) were used since the regions to be analysed were sufficiently small (93 and 71 bp respectively) to allow for the minimisation of false negatives. Despite this, approximately four hundred sites were identified using this method for each gene,

with the vast majority of these likely to be false positives, often due to identification of binding sites for transcription factors that are poorly expressed in the liver.

Initially, sites for transcription factors that were not expressed in the liver were removed as these would be unlikely to be responsible for a liver-specific effect. The remaining sites were further reduced by comparison between the HCaRG and Nob1p promoters: Since co-regulation has been observed, this would require the presence of the same response element(s) in both promoters. Of the remaining sites several interesting transcription factors were selected as being possible candidates in the regulation of HCaRG and Nob1p gene expression (Table 5.2). These included numerous response elements for members of the family of signal transducer and activator of transcription (STAT), for the retinoic acid receptor (RAR)-related orphan receptor alpha (ROR α), the estrogen receptor (ER) and for NF- κ B. However there were still in excess of ten potential transcription factors of interest and numerous potential response elements for each within the initial ~100 bp of proximal promoter of either gene. Due to the impossibility of mutating each site in a trial and error approach it was decided to utilise simple dosing experiments using the promoter constructs already generated in order to reduce the number of potential response elements implicated in the response under investigation.

Table 5.2: Potential transcription factor binding sites common to HCaRG and Nob1p proximal promoter regions

Transcription factor	HCaRG	Nob1p
AhR	-74, -68, -16	-65, +10
Nrf2	-28	+32
STAT1	-50, -29	-65
STAT6	-27	-4
NF- κ B	-87, -55, -33, -23	-39
ER	-62	-5
ROR α	-36, -24	-7

The 93 bp and exon 1 of the HCaRG proximal promoter, and 71 bp and exon 1 were searched against the TRANSFAC database using both MatInspector and the Match tool. A selection of the potential binding sites common to both promoters is shown. AhR: aryl hydrocarbon receptor, Nrf2: NF-E2-related factor 2, STAT: signal transducer and activator of transcription, NF- κ B: nuclear factor kappa B, ER: estrogen receptor, ROR α : RAR-related orphan receptor alpha.

5.3.2 DOSING WITH β -NAPHTHOFLAVONE

β -naphthoflavone (β -NF) is a synthetic flavone compound and an inducer of phase I detoxifying enzymes such as CYP1A1/2. β -NF is known to both increase the expression of the NFE2-related factor 2 (Nrf2) (Huang *et al.*, 2000) and to be an agonist of the aryl hydrocarbon receptor (AhR) (Sinal *et al.*, 1999). Activation of Nrf2 and AhR have been implicated in the control of the response to xenobiotics, activating a gene battery with particular affect on expression of phase I and phase II detoxifying enzymes (Kohle and Bock, 2006). Several potential Nrf2 and AhR response elements were identified as being present in both the HCaRG and Nob1p proximal promoter regions, hence a dosing experiment was performed to examine the involvement of these transcription factors. Cells were dosed following transfection of the HCaRG-93 bp and Nob1p-71 bp deletion constructs, along with the full 1 kb constructs to compare and allow confirmation that the effect was localised to the first ~100 bp of proximal promoter. β -NF was dosed in the range 1 to 100 μ M and the results were compared to the dosing experiments using liver growth agents. Similar results for dosing with β -NF and with the liver growth agents would indicate that the AhR and/or Nrf2 were involved in the mechanism leading to reduced expression of HCaRG and Nob1p during drug-induced liver growth.

Figure 5.5 shows the results of the β -NF dosing experiment. The results for HCaRG (Figure 5.5a) show that there was a significant inhibition of gene expression in response to 10 μ M β -NF that increased with the maximal inhibition of SEAP expression occurring in response to the 100 μ M dose, where a reduction in SEAP expression to 44-percent of control was observed. The similarity in inhibition elicited by β -NF for both the -93 bp and -999 bp promoter constructs indicates that the response is occurring in the initial 93 bp of promoter proximal. In agreement with this were the results for the Nob1p reporter gene constructs (Figure 5.5b) where a dose response was observed across the range tested, with inhibition of SEAP expression occurring to the greatest extent (36-percent of control) following dosing with 100 μ M β -NF. In addition, similarity between the -71 bp and -1066 bp constructs' response indicates that the response to β -NF is mediated via the initial 71 bp of promoter proximal to the TSS, and adds to the evidence that the HCaRG and Nob1p

promoters are controlled by the same transcription factor(s) and are co-regulated in response to liver growth stimuli.

The dosing experiment using β -NF highlighted the similarity in the response of the two promoters and more importantly, resulted in inhibition of SEAP expression to a similar extent to that resulting from liver growth agent administration. Therefore it is likely that this response is mediated through the action of the AhR, Nrf2, or a combination of both transcription factors.

Figure 5.5: Effect of β -naphthoflavone on SEAP expression controlled by HCaRG and Nob1p proximal promoter regions

HCaRG (A) and Nob1p (B) constructs containing the smallest and largest regions of the promoter were transfected into FaO cells as described in section 2.4.4. Cells were treated with β -NF in the range 1 to 100 μ M for 48 hours prior to assay for SEAP. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance relative to DMSO control for each construct (n=5) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.3 TRANSCRIPTION FACTORS EXHIBITING INFLUENCE ON HCaRG AND NOB1P

The identification of the involvement of Nrf2 and/or AhR in the response of HCaRG and Nob1p to liver growth agent administration greatly reduced the number of potential response elements requiring further investigation: Figure 5.6 illustrates the positions of the AhR and Nrf2 sites within the HCaRG and Nob1p promoters as identified using MatInspector and verified using the Match™ tool. Using both MatInspector and Match™ increased the likelihood of selecting genuine binding sites, especially as MatInspector can automate the selection of promoter regions, verifying the sequence obtained manually (as in section 4.3.1.1). Figure 5.6 also shows the core and matrix similarity scores (section 2.2.2.3) for each potential response element.

The region of interest for the HCaRG promoter contains a single Nrf2 site located 28 bp upstream of the TSS. This site shows a high core similarity (0.884) indicating that the most conserved nucleotides for the Nrf2 consensus sequence are present in the HCaRG promoter at this point. Three potential AhR response elements (termed dioxin-response elements) were identified at 16, 68 and 74 bp upstream of the TSS. All three show a core similarity of 0.747 indicating that the five most conserved nucleotides of the AhR consensus sequence are not necessarily present at these sites. However, this does not discount the possibility that these AhR sites are functional since there can be a degree of flexibility with regard to the exact consensus sequence. Often some of the core nucleotides are more crucial for transcription factor binding than others, or a change from one purine or pyrimidine to the other can sometimes occur without any detrimental effect.

The only Nrf2 site identified within the region of interest for the Nob1p promoter is located within the sequence of exon one. This region was cloned into the construct purely to improve optimisation of the initial PCR amplification. This potential Nrf2 site shows a core similarity of 1.000 indicating a perfect match to all five of the most conserved nucleotides of the Nrf2 consensus sequence. Figure 5.7 shows the positioning of the exons of the Nob1p mRNA (NM_199086), plus those exons that code for the untranslated regions (UTRs) and coding sequence (CDS) of the gene. The rat Nob1p gene possesses a short 5' UTR of nine base pairs (Figure 5.7), hence the potential Nrf2 site is located 23 bp within the coding region i.e. the region of the mRNA that is translated into protein; such a location would suggest that is not necessarily biologically

active, as response elements are traditionally associated with regulatory and, not coding regions. However, recent evidence has demonstrated that response elements can occur located upstream and downstream of the transcription start site. Downstream regulatory elements for genes transcribed by RNA polymerase II have been identified within proximal and distal exons for example within exon 1 of the elastin gene (Pierce *et al.*, 2006) and exon 6 of the keratin 18 gene (Neznanov *et al.*, 1997), and within introns for example intron 2 of the mouse thymidine kinase gene (Rotheneder *et al.*, 1991).

Figure 5.6: Location of potential Nrf2 and AhR response elements for HCaRG and Nob1p

Promoter and exon (dark grey) regions are shown for HCaRG and Nob1p. Potential sites of interest shown are **Nrf2** and **AhR** with the corresponding core similarity (CS) and matrix similarity (MS) scores (section 2.2.2.3) obtained by interrogation of each sequence using MatInspector (www.genomatix.com) to probe the TRANSFAC database of consensus sequences. Sites are positioned relative to the transcription start site (TSS) for each gene.

The Nob1p promoter also contains two potential AhR response elements, the first just within the first exon and the second located within the promoter, 65 bp from the TSS. Of the two sites identified it is the second that possesses the higher core similarity score (0.820 versus 0.747) indicating that this region of the DNA is potentially more suited to binding of the AhR/ARNT complex. Figure 5.6 also shows the position of a second potential Nrf2 response element in the Nob1p promoter region located 272 bp from the TSS. This region was included in the search due to the finding that the Nob1p-312 bp construct exhibited close to a 3-fold increase in SEAP expression versus the Nob1p-71 bp construct under basal conditions (Figure 5.3b). Therefore this second Nrf2 site may potentially be involved in achieving complete transactivation of the Nob1p gene.

Figure 5.7: Structure of mRNA for HCaRG and Nob1p

HCaRG

Nob1p

The mRNA structure for HCaRG and Nob1p are shown following a BLAST alignment between the mRNA (HCaRG - NM_139108; Nob1p - NM_199086) and the genome to highlight the positions of exon sequence. The location of the untranslated regions (UTR) are shown as determined by a BLAST alignment of the protein sequence (HCaRG - NP_620808; Nob1p - NP_954517) using tblastn (protein query versus the translated database) (Altschul *et al.*, 1997). The numbers shown are relative to the mRNA sequence in each case.

5.3.4 DOSING WITH BENZO(A)PYRENE AND 3-METHYLCHOLANTHRENE

Dosing with β -NF indicated the involvement of Nrf2 and/or AhR in signal propagation, but cannot distinguish between the activation of these two transcription factors. It was necessary to identify which of the two transcription factors was responsible for the inhibition of gene expression observed for HCaRG and Nob1p in response to liver growth agent administration. In order to attempt to distinguish this a dosing experiment was performed using the known AhR agonists 3-methylcholanthrene (3-MC) and benzo(a)pyrene: If the response to agonists of the AhR was similar to that elicited by liver growth agent and β -NF administration this would be indicative of AhR involvement in the control of HCaRG and Nob1p expression. A failure to respond to the AhR agonists would indicate that Nrf2 was more likely to control expression of these genes.

The HCaRG and Nob1p promoter constructs were initially dosed with the AhR agonist 3-MC. Dosing with 3-MC resulted in decreased SEAP expression to 70-percent of control at 25 μ M for the HCaRG-93 construct and at 0.1 μ M for the HCaRG-999 construct (Figure 5.8a) indicating the possible involvement of AhR in the regulation of this gene. The results for Nob1p show a more pronounced effect with expression of SEAP decreasing to 61-percent of control for the Nob1p-71 construct following dosing with 3-MC at 25 μ M. This was similar for the Nob1p-1066 construct with a significant inhibition of SEAP expression across the range 5 μ M to 100 μ M.

The inhibition observed was not of the same magnitude as that seen using either β -NF or the liver growth agents, despite the fact that 3-MC is a potent AhR activator. This may be merely a dose-dependent effect, a ligand-specific effect or be suggestive that the inhibition of HCaRG and Nob1p gene expression observed in response to liver growth agent administration is unlikely to be controlled solely by the AhR.

The results observed for 3-MC administration were confirmed using a second AhR agonist. Administration of benzo(a)pyrene failed to produce a significant inhibition of SEAP expression except at 500 μ M for the HCaRG-999 construct (Figure 5.9a). The effect on the Nob1p-71 construct was to inhibit SEAP expression to 80-percent of control at 25 μ M B(a)P (Figure 5.9b). A similar response occurred for the Nob1p-1066 construct between 10 μ M to 250 μ M B(a)P,

mirroring the difference observed between the 71 bp and 1066 bp constructs in response to dosing with 3-MC (Figure 5.8b). This difference in the sensitivity of the two constructs could indicate the presence of an influential AhR response element located between 71 and 1066 bp upstream of the transcription start site.

Figure 5.8: Dose response curve for 3-methylcholanthrene administration to FaO cells transfected with HCaRG and Nob1p promoter constructs

The effect of 3-methylcholanthrene on the HCaRG promoter constructs (A) and Nob1p promoter constructs (B) are shown. For each dose both constructs were transfected for comparison with the response by the initial ~100 bp of proximal promoter. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance relative to DMSO control for each construct (n=5) * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 5.9: Dose response curve for benzo(a)pyrene administration to FaO cells transfected with HCaRG and Nob1p promoter constructs

The effect of benzo(a)pyrene on the HCaRG promoter constructs (A) and Nob1p promoter constructs (B) are shown. For each dose both constructs were transfected for comparison with the response by the initial ~100 bp of proximal promoter. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance relative to DMSO control for each construct (n=5) * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To estimate whether a difference in potency between β -NF, 3-MC and benzo(a)pyrene was the cause of the differing response between the compounds their EC_{50} values were compared. The EC_{50} value of a compound relates to the molar concentration producing 50-percent of the maximal effect for that compound. Example EC_{50} values are shown in Table 5.3 for β -NF, 3-MC and

benzo(a)pyrene to allow comparison between the three compounds with respect to their ability to cause activation and nuclear translocation of the AhR. This is commonly measured by the activation of expression of the AhR target gene CYP1A1. The EC₅₀ values shown in Table 5.3 were not produced in the FaO cell line, but using a yeast reporter assay co-expressing human or mouse AhR/ARNT (Kawanishi *et al.*, 2003) or using the rat hepatoma cell line H-4-II E (Piskorska-Pliszczyńska *et al.*, 1986). As can be seen from Table 5.3, β-NF is the most potent activator of the AhR having the lowest EC₅₀ value. 3-methylcholanthrene has an EC₅₀ value approximately double that of β-NF (Kawanishi *et al.*, 2003), with benzo(a)pyrene being the weakest activator of the AhR, with an EC₅₀ value ranging from 20-fold to 45-fold greater than that of 3-methylcholanthrene (Kawanishi *et al.*, 2003). Hence it would be expected that double the concentration of 3-methylcholanthrene, and up to 150-fold higher the concentration of benzo(a)pyrene would be required to produce the same result as dosing with β-NF.

Table 5.3: EC₅₀ values for β-NF, 3-methylcholanthrene and benzo(a)pyrene

Organism	EC ₅₀ (nM)		
	β-NF	3-methylcholanthrene	Benzo(a)pyrene
Mouse ¹	14	30	600
Mouse ¹	8	14	700
Human ¹	6	20	900
Human ¹	5	20	600
Rat ²		28	

¹From experiments using yeast containing reporter constructs co-expressing mouse AhR and ARNT (Kawanishi *et al.*, 2003). ² Measured in rat hepatoma H-4-II E cells (Piskorska-Pliszczyńska *et al.*, 1986).

Given that B(a)P, like 3-MC, is a potent activator of AhR, albeit with an EC₅₀ 10-fold greater than that of 3-MC (Kawanishi *et al.*, 2003), this adds weight to the prediction that the AhR is not the sole transcription factor controlling HCaRG and Nob1p expression. This data is therefore consistent with a second factor, possibly Nrf2, being involved.

5.3.5 ASSESSMENT OF THE INVOLVEMENT OF NRF2 AND AHR IN THE CONTROL OF HCaRG AND Nob1P

As experiments using β -NF and the AhR agonists 3-MC and benzo(a)pyrene implicated the involvement of Nrf2 and AhR in the inhibition of HCaRG and Nob1p gene expression by liver growth agents, but failed to distinguish which receptor was more important, alternate strategies were required. First, the influence of Nrf2 was removed using treatment with the protein synthesis inhibitor cycloheximide, and second, a series of mutagenesis experiments were performed to remove the potential transcription factor binding sites identified in the previous section.

5.3.5.1 Dosing with cycloheximide

Cycloheximide (CHX) is a protein synthesis inhibitor that can be dosed for short periods to reduce the levels of labile transcription factors: Nrf2 is one such factor with a half-life of less than 20 minutes (Itoh *et al.*, 2003). Therefore a dosing experiment was performed using CHX at an appropriate concentration with or without dexamethasone (100 μ M) or β -NF (100 μ M), to study the effect on reporter gene expression when Nrf2 levels had been reduced. If Nrf2 mediates the inhibitory effect of these chemicals on HCaRG and Nob1p gene expression then dosing with CHX should remove or lessen this inhibition in response to dexamethasone or β -NF. This technique has been used previously by Ma *et al.* (2004) where CHX was dosed and the levels of Nrf2 and the downstream enzyme under investigation were assessed after 5 hours incubation using Northern Blotting. This experiment has two potential flaws in that CHX dosing for 24 hours is toxic to FaO cells, as visible by light microscopy, and that the reporter gene SEAP needs to be translated to be detected. To mitigate these potential issues dosing with CHX was limited to the first 5 hours post-dose, as performed in the experiments by Ma *et al.* (2004).

However, as can be seen from Figure 5.10, CHX exposure did have a significant effect on basal SEAP expression. Dosing with CHX (10 μ g/mL) for 5 hours caused decreased SEAP expression for both HCaRG-93 (Figure 5.10a) and Nob1p-71 (Figure 5.10b) constructs, reducing it to 25-percent for HCaRG and 30-percent for Nob1p. The effect of CHX on the expression of SEAP for HCaRG-93 and Nob1p-71 promoter constructs suggests that treatment with CHX has caused inhibition of SEAP expression and therefore prevented accurate determination of the effect of

Nrf2 depletion on HCaRG and Nob1p expression using this method. Dosing with CHX inhibits protein synthesis and hence only long-lived proteins synthesised prior to the time of dosing would remain active within the cell.

Figure 5.10: Effect of CHX dosing on SEAP expression from HCaRG-93 and Nob1p-71 constructs

The effect of dosing for 5 hours with CHX (10 µg/mL) on SEAP expression from HCaRG-93 (A) and Nob1p-71 (B) constructs is shown. Dexamethasone (100 µM) or β-NF (100 µM) was dosed with or without CHX to determine the effect of labile transcription factors on the inhibition of SEAP expression in response to liver growth agents observed previously. Data was analysed using one-way ANOVA with Bonferroni's post-hoc test for both liver growth agent versus control, and for CHX alone versus CHX in combination with liver growth agent (*** p<0.001).

To attempt to overcome this difficulty the experiment was repeated, but quantitative real-time PCR was used to measure genomic expression of Nob1p and HCaRG. Figure 5.11a shows the effect of exposure to CHX with and without liver growth agents on the genomic expression of HCaRG. Dosing with CHX alone produced a 9-fold decrease in HCaRG expression compared to untreated cells that were exposed to DMSO only, which could indicate that a labile protein is responsible at least in part for the basal level of expression of HCaRG. Dosing with CHX plus either dexamethasone, CPA or CFA resulted in a significant decrease in HCaRG expression compared to the respective liver growth agent alone. This could indicate that a labile factor is also involved in the response to liver growth agents, and that removal of this factor may limit the response. Treatment with CHX in combination with CPA was not sufficient to completely ablate the response to CPA, as shown by the significant difference versus CHX alone. This could indicate that a labile factor alone is not sufficient to cause the response to CPA.

Figure 5.11b shows the results for Nob1p expression that are similar to those for HCaRG. The effect of CHX in combination with dexamethasone, CPA or CFA versus CHX alone was less pronounced for Nob1p, decreasing expression approximately two-fold in each case. These results could indicate a similar situation as for HCaRG, with a labile factor being required for the response to liver growth agents, and the need for one or more factors unaffected by the incubation with CHX particularly in the response to CPA. However this experiment suffered two major drawbacks in that the suppression of HCaRG and Nob1p expression caused by CHX alone is potentially so large as to obscure the effects in combination with the liver growth agents. Also, due to the cytotoxic effect of prolonged CHX exposure on FaO cells it was not possible to continue to monitor the effect of CHX into the period of inhibition of gene expression i.e. 24 to 96 hours post-dose. Hence it would be necessary to use a more specific technique such as site-directed mutagenesis or gene silencing by RNA interference to assess the involvement of Nrf2 in the regulation of HCaRG and Nob1p.

Figure 5.11: Effect of CHX dosing on HCaRG and Nob1p expression in FaO cells by quantitative RT-PCR

HCaRG (A) and Nob1p (B) expression in FaO cells was determined by quantitative RT-PCR following a 5-hour incubation with CHX (10 µg/mL) with or without liver growth agent. Cells were seeded into 6-well plates at a density of 5 x 10⁵ cells/mL and incubated overnight at 37°C (5% CO₂) prior to dosing. Cells were exposed to CHX +/- dexamethasone, CPA or CFA (100 µM). Following dosing cells were lysed, RNA was extracted and cDNA synthesised as described in section 2.5. One-way ANOVA with Bonferroni's post-hoc test was used firstly to assess statistical significance between cells exposed to liver growth agent with and without CHX, and secondly between liver growth agent with CHX and CHX alone (n=3) * p<0.05, ** p<0.01, ***p<0.001.

5.3.5.2 Site-directed mutagenesis of AhR and Nrf2 regulatory elements

SDM is a technique that allows the introduction of point mutations into a DNA sequence. This is accomplished using PCR amplification with primers containing the mutated sequence. In this way several bases can either be changed/deleted to alter/remove a transcription factor response element. It is often useful when altering the sequence to change the base in question from a purine to a pyrimidine residue as this is most likely to impact upon transcription factor recognition of, and binding to, the response element. The steps involved in the generation of mutants are shown in Figure 5.12 and described in section 2.3.6.7.

Figure 5.12: Strategy for generation of mutant constructs by site-directed mutagenesis

A unique restriction enzyme cutting site for *Acc III* was introduced within the Nrf2 response element causing mutation of several nucleotides important for the binding of Nrf2 to the response element. PCR amplification from the HCaRG-93 bp construct using the sense 1 and antisense 1 primers produced the upstream fragment, with sense primer 2 and antisense primer 2 producing the downstream fragment. The two fragments were subsequently digested with *Acc III* and ligated together. The HCaRG-93 bp primers (Table 2.11) were then used to amplify the ligated fragment prior to digestion with *Acc65 I* and *Xho I* and ligation into pSEAP. This procedure was repeated for generation of the other mutant constructs using the primers shown in Table 2.13.

The *in silico* analysis of the HCaRG-93 and Nob1p-71 promoter regions identified the presence of both Nrf2 and AhR potential response elements in both cases. To attempt to address the hypothesis that one or both transcription factors were involved in the control of HCaRG and Nob1p, constructs containing mutated response elements for each of the potential binding sites were produced. Since the potential Nrf2 site identified in the Nob1p-71 bp construct was found within the exon 1 sequence, a second potential Nrf2 site located within the 241 bp between the Nob1p-71 bp and -312 bp constructs was also mutated. As both the response of the Nob1p deletion mutants under basal conditions (Figure 5.3b) and in response to CFA (Figure 5.4b) indicated that this 241 bp region was important it was also useful to mutate this potential Nrf2 site to assess its role in the response to the peroxisome proliferator. For each response element of interest, one mutation per construct was produced; hence the involvement of each binding site was determined separately. Wild-type and mutated constructs were transfected into FaO cells and the basal activities and the effect of dosing with liver growth agents determined. Unfortunately due to time constraints and difficulties with the initial PCR optimisation of the downstream fragments, the potential Nrf2 site in the Nob1p-71 bp and one of the potential AhR sites in the same construct could not be successfully mutated. The potential Nrf2 site in the HCaRG-93 bp was successfully mutated, along with the potential Nrf2 site in the Nob1p-312 bp construct and the second potential AhR site in the Nob1p-71 bp construct that had produced the higher core similarity score of the two AhR sites identified (Figure 5.6).

The basal expression of the wild-type and mutated constructs is shown in Figure 5.13. The expression of SEAP from the wild-type constructs observed in Figure 5.13 is in agreement with the previous basal analysis of these deletion constructs (Figure 5.3), with the Nob1p-312 bp construct showing greater than two-fold SEAP expression over the Nob1p-71 bp construct. With regard to the mutant constructs, of the three potential response elements mutated only the Nrf2 site in the HCaRG-93 bp construct appears to have any effect on basal expression, with its ablation eliciting a 1.7-fold increase in SEAP expression versus the wild-type construct; this suggests that the Nrf2 response element in the HCaRG-93 bp construct is a functional binding site, and under basal conditions it may act in an inhibitory fashion on HCaRG gene expression.

Figure 5.13: Basal expression of SEAP from HCaRG and Nob1p wild-type and mutant constructs

Constructs containing wild-type HCaRG and Nob1p promoter sequences were transfected into FaO cells (section 2.4.4) in parallel with constructs containing mutated sites for Nrf2 and AhR. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance of each mutation relative to the wild-type sequence (n=5) ***p<0.001.

Following analysis of response element ablation on basal expression, the effect of liver growth agent administration for a 48 hour period was examined. Figures 5.14 to 5.16 show the effect of mutagenesis on the inhibition of SEAP expression by dexamethasone, CPA, Wy-14,643 and β -NF. For all three cases, no consistent effect was seen upon mutation of the individual response elements, which in general had no effect on the inhibition of reporter gene expression by the three xenobiotics. However, some small, statistically significant effects were seen with Wy-14,643. Mutation of the potential Nrf2 response element in the HCaRG-93 bp construct influenced the response to Wy-14,643, with the Nrf2 mutant decreasing SEAP expression to 64-percent of the wild-type construct, indicating that removal of the influence of this potential Nrf2 element would cause further inhibition of HCaRG expression. Mutation of the potential AhR element in the Nob1p-71 bp construct also affected the response to dosing with Wy-14,643, resulting in an increase in SEAP expression to 130-percent of the wild-type construct. The significance of this

result is difficult to determine since dosing with this compound failed to significantly change SEAP expression for either the wild-type or mutated construct versus DMSO control.

Figure 5.14: Effect of mutation of the potential Nrf2 response element in the HCaRG-93 bp construct

The construct containing the wild-type promoter sequence were transfected into FaO cells (Section 2.4.4) in parallel with the construct containing the mutated site for Nrf2. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance of the effect of each compound versus DMSO control, and also the effect of the mutation relative to the wild-type for each compound (n=5) *p<0.05, **p<0.01, ***p<0.001.

Figure 5.15: Effect of mutation of the potential AhR response element in the Nob1p-71 bp construct

The construct containing the wild-type promoter sequence were transfected into FaO cells (Section 2.4.4) in parallel with the construct containing the mutated site for the AhR. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance of the effect of each compound versus DMSO control, and also the effect of the mutation relative to the wild-type for each compound (n=5) *p<0.05, **p<0.01, ***p<0.001.

Figure 5.16: Effect of mutation of the potential Nrf2 response element in the Nob1p-312 bp construct

The construct containing the wild-type promoter sequence were transfected into FaO cells (Section 2.4.4) in parallel with the construct containing the mutated site for Nrf2. One-way ANOVA with Bonferroni's post-hoc test was used to assess statistical significance of the effect of each compound versus DMSO control, and also the effect of the mutation relative to the wild-type for each compound (n=5) *p<0.05, **p<0.01, ***p<0.001.

5.4 DISCUSSION ON CO-ORDINATE REGULATION OF HCaRG AND NOB1P

A consistent finding throughout this study was the similarity in the response of HCaRG and Nob1p to the various compounds administered. This similarity in the response of the genes to liver growth agents that had been shown in Chapter 4 at the mRNA and promoter level was continued throughout the deletion analysis, the exposure to β -NF and the AhR agonists 3-MC and B(a)P, and the use of cycloheximide to remove the effect of Nrf2. This indicates a strong possibility that HCaRG and Nob1p are co-regulated and that the transcription factor(s) influencing their coordinated regulation are key to the response to compounds causing liver growth. The data presented in this chapter shows that the regulation is likely to be mediated through one of two factors, AhR and Nrf2, although we could not fully discriminate which is the central factor in the response.

In the analysis of gene promoters and the upstream factors governing their regulation the major difficulty is determining the key response elements from the thousands of potential sites located within the sequence. In the case of the two genes of interest in this study there was no information to assist in the selection of candidate transcription factors since the majority of the experiments performed to date have been interested in the downstream effects of the two genes. The analysis of deletion constructs performed in Section 5.2 successfully reduced the region of each promoter for further analysis to 93 bp for HCaRG and 71 bp for Nob1p.

In silico analysis of the potential binding sites revealed approximately 400 potential response elements within either region. Even following removal of sites that were not common to both sequences, and sites for transcription factors not expressed in liver, there were in excess of 20 potential sites for each gene. Dosing experiments were utilised to reduce the number of potential sites for further analysis. β -NF was selected since it was a known inducer of the expression of genes under the control of Nrf2 and AhR. Dosing with β -NF is known to induce expression of the phase 2 detoxifying enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) in mouse hepatoma cells via increased Nrf2 that can be blocked using the protein synthesis inhibitor cycloheximide (Ma *et al.*, 2004). β -NF also induced expression of the gamma-glutamylcysteine synthetase subunit genes in

HepG2 cells where a concomitant increase in the binding of Nrf2 to the ARE in the gene promoters was observed (Wild *et al.*, 1999). Similarly β -NF was found to induce expression of CYP1A1 in rodent white adipose tissue via the AhR, at the same time as inducing expression of the Nrf2 downstream target genes NQO1, glutathione S-transferase Ya (GST-Ya) and heme oxygenase-1 (HO-1) (Yoshinari *et al.*, 2006). Exposure of the HCaRG and Nob1p constructs to β -NF resulted in reduced expression of SEAP that was dose-dependent with a maximal two-fold decrease observed in response to 100 μ M in both cases (Figure 5.5). This was similar to the response observed for the same constructs following exposure to dexamethasone, CPA and to a lesser extent CFA (Figure 5.4). This indicated that the response of the HCaRG and Nob1p promoters to liver growth agents was mediated by Nrf2, AhR or a combination of both transcription factors.

The AhR agonists 3-MC and B(a)P were used to attempt to differentiate between the influence of Nrf2 and AhR in the control of expression of HCaRG and Nob1p. The two agonists are known to have different potencies with 3-MC being approximately 10 times more potent than B(a)P as evidenced by the ability of each compound to induce expression from a reporter construct containing multiple xenobiotic response elements upstream of the β -galactosidase gene (Kawanishi *et al.*, 2003). The response of the HCaRG and Nob1p constructs to the AhR agonists failed to discriminate between the AhR and Nrf2 since a partial response was observed for both gene promoters in response to both compounds (Figures 5.8 and 5.9). Therefore it is possible that the AhR is either co-operating with Nrf2 as suggested by Ma and colleagues (2004) in the control of NQO1, or that the AhR is capable of compensating for Nrf2, or possibly the AhR is involved upstream of Nrf2 as observed by Miao and colleagues (2005) who identified xenobiotic-response element (XRE)-like sequences in the Nrf2 promoter. In this last scenario, activation of AhR and nuclear translocation of the receptor would occur prior to transactivation of the Nrf2 gene via the XREs in the Nrf2 promoter, and Nrf2 would then directly affect the transactivation of the HCaRG and Nob1p genes. This may explain why the response to the AhR agonists was less pronounced, with activation of both the AhR and liberation of pre-existing Nrf2 from Keap1 being required initially, followed by increased expression of Nrf2 in response to the AhR. Without co-induction of

Nrf2 initially the response may be stunted, relying solely on AhR-induced up-regulation of Nrf2 expression to exert the effect on HCaRG and Nob1p expression.

Figure 5.11 appears to implicate a labile factor, such as Nrf2, in the control of HCaRG and Nob1p under basal conditions as shown by the decrease in expression of these two genes in the presence of CHX. Co-administration of liver growth agents with CHX decreased expression of HCaRG and Nob1p compared to liver growth agent administration alone. Therefore it is possible that a labile factor is also involved in altering the expression of both genes in response to liver growth agents. This appears to be the case at 5 hours post-dosing, but to allow monitoring of the effect of removal of Nrf2 beyond this point another technique such as gene silencing would be required in order to maintain the cells beyond the initial 24 hour dosing period whilst removing the effect of Nrf2. However since the basal levels of Nrf2 are very low (data not shown), dosing with a suitable agonist such as β -NF a few hours after transfection with the siRNA may improve the ability to optimise the gene silencing of Nrf2. Dosing with β -NF could be continued during the 48 hour period prior to basal analysis, thus increasing the expression of Nrf2 in the unsilenced cells to a level that would allow comparison with the silenced cells. If silencing of Nrf2 resulted in the continued reduction of the response to liver growth agents as observed during the first 5 hours during the period of elevation of HCaRG and Nob1p expression, this would indicate that increased transactivation by Nrf2 was responsible for the inhibition of expression observed.

The mutagenesis experiments (section 5.3.5.2) failed to discriminate between Nrf2 and AhR as the central mediator behind the liver growth agent effects. This can be explained by one of three possible scenarios: First, as not all the response elements were ablated it is possible that one of the other sites is responsible for the effects observed. Second, if more than one site acts in unison to propagate the effect then ablation of one site may not have a noticeable effect. Third, it is possible that ablation of a single site is compensated for by the activation of a second response element to propagate any signal. To discover which of these scenarios is true it will be necessary to undertake further investigation, ablating more sites and ablating multiple sites in a single construct.

6 GENERAL DISCUSSION

6.1 DISCUSSION

Many compounds are known to cause liver enlargement (hepatomegaly) in rodents, including a variety of compounds frequently exposed to humans. This exposure can be from the environment, such as herbicides, occupational, such as industrial solvents, or from pharmaceutical compounds such as the hypolipidaemic agent clofibrate. The finding that a potential therapeutic compound causes liver growth in pre-clinical testing results in the requirement for in-depth studies to understand the risk to humans, and indeed the potential for compound attrition at this point is high. Therefore it is advantageous to identify such compounds as early in development as possible, which can be made possible using *in vitro* screening for gene expression changes known to correlate with hepatomegaly. Similarly, it is important to further understand the species differences between humans and rodents, so that potentially useful drugs are not discarded based on irrelevant information. The major advantages of *in vitro* screening in this way are that human cell lines can be used allowing more relevant information about potential undesirable effects on humans, and that the analysis is more suited to high throughput than using *in vivo* experiments. Therefore reliable biomarkers of liver growth are necessary to identify compounds causing liver growth and the relevance to humans.

Hepatomegaly is caused by either an increase in the number of hepatocytes (hyperplasia) as caused by compounds such as the peroxisome proliferators and CPA, or an increase in the size of the hepatocytes (hypertrophy) as induced by dexamethasone. However these physiological processes are not mutually exclusive and it is common that a compound will cause hepatomegaly through a combination of hyperplasia and hypertrophy. Since the mechanisms controlling hyperplasia and hypertrophy within the liver are not well understood at present it can be difficult to assess the risk of potential novel therapeutic compounds that exhibit this effect in pre-clinical species.

This project aimed to examine the molecular mechanisms underlying xenobiotic-induced liver enlargement, and thus ultimately to address the difficulties in risk assessment of therapeutic compounds exhibiting this effect in pre-clinical species. The compounds chosen are known to cause hepatomegaly in rodents through different routes, hyperplasia, hypertrophy or both, allowing

us to examine general liver responses' to liver growth stimuli as opposed to a specific response to either hyperplasia or hypertrophy. The transcriptome profiles of the three structurally diverse compounds were compared with a view to identifying genes central to the process of liver enlargement. It is hoped that in the future such genes could then be used as biomarkers of liver enlargement directly, and that identification of the upstream factors responsible for the gene expression changes would allow greater understanding of the mechanism controlling liver enlargement.

The project identified several groups of genes exhibiting potential co-ordination in their expression profiles in response to the three compounds, and one of these groups was selected for further study: The details of this examination are summarised in Figure 6.1. In depth analysis suggested that rBACH and Me1 actually form a distinct group as they showed high similarity in the response to Wy-14,643, but failed to show similar changes in response to CPA and dexamethasone (Figures 4.7 and 4.8). These two genes were hence not investigated further as they appeared to be subject to a peroxisome-proliferator specific response. The resultant group of six known genes, plus one EST sequence that could not be linked to any known gene were hence examined in more detail.

Deletion analysis of the promoters of two of the group members, HCaRG and Nob1p, confirmed the high similarity in the response to peroxisome proliferators, CPA and dexamethasone observed previously (Figure 5.4). The region of the proximal promoter central to mediating this effect was localised to within the initial ~100 bp immediately upstream of the transcription start site in both cases, and *in silico* analysis combined with dosing experiments using inducers known to activate specific transcription factors allowed identification of two potential regulators. The polycyclic aromatic hydrocarbon β -NF produced a similar dose response to that of dexamethasone and CPA (Figure 5.5), and is known to act through activation of the Ahr and Nrf2, suggesting a role for these transcription factors in mediating the response to liver growth agents. β -NF induces expression of the cytochrome P450 isoenzyme CYP1A1 via activation of the AhR in a variety of rat tissues (Sinal *et al.*, 1999), and in numerous species (Martignoni *et al.*, 2006, Pesonen *et al.*, 1992). In addition β -NF affects other pathways involved in detoxification such as drug transport out of cells. For example, induction of mRNA expression of the Multidrug Resistance-Associated Proteins (Mrps)

has been reported in the mouse via activation of the AhR (Maher *et al.*, 2005). Mrp proteins are important for the efflux of conjugated and unconjugated compounds from cells, and the same Mrp family members were found to regulated by Nrf2 as by AhR (Maher *et al.*, 2005), indicating that these two factors were predominant in the regulation of drug efflux from hepatocytes. β -NF activation of Nrf2 occurs via release of the transcription factor from its inhibitory protein Keap1 (Itoh *et al.*, 1999) leading to activation of downstream genes such as NQO1, a gene that is under dual control of both the AhR and Nrf2 (Ma *et al.*, 2004).

Figure 6.1: Summary of findings for co-ordinate regulation in response to liver growth agents

A summary of the findings are illustrated including the initial gene expression changes analysed by DNA Microarray and confirmed by real-time quantitative PCR. Reporter gene assay results are shown for the HCaRG, Nob1p, Sp3 and LRCH4 promoters, and the subsequent findings for HCaRG and Nob1p with respect to their upstream regulation.

Nrf2 is a protective gene against toxic insult and oxidative stress, and hence would be unlikely to inhibit genes that were protective against cellular transformation. It is possible that the crucial effect of administration of liver growth agents is interference with Nrf2 at the protein level either with the mechanism of release of Nrf2 from its inhibitory protein Keap1, or with the rate of degradation of Nrf2 that is free to translocate to the nucleus. Similarly the AhR is important in the response to xenobiotics, increasing expression of Phase I and Phase II detoxifying enzymes such as CYP1A1 and NQO1, as well as increasing its own expression simultaneously (Brauze *et al.*, 2006). AhR agonists such as benzo(a)pyrene, a polycyclic aromatic hydrocarbon, are genotoxins that can initiate and promote carcinogenesis, which is known to occur via activation of the AhR as evidenced from studies using AhR knockout mice (Shimizu *et al.*, 2000). Since the liver growth agents used in this study are known to cause carcinogenesis through both non-genotoxic (Wy-14,643 and dexamethasone) and genotoxic (CPA) routes, it may be that differing extents of co-operation between the Nrf2 and AhR pathways is the fundamental mechanistic difference between non-genotoxic and genotoxic hepatocarcinogenesis.

Regulation of gene expression by Nrf2 is under intense investigation at present, with the increasing finding that this transcription factor co-operates with the AhR to complete its actions (Ma *et al.*, 2004, Nioi and Hayes, 2004, Maher *et al.*, 2005). This co-operation is rarely straightforward, with a number of scenarios being postulated such as (i) direct co-operation between Nrf2 and another factor via the formation of a composite element from the separate response elements in a gene promoter, (ii) induction of Nrf2 as a result of prior activation of the co-operating factor, or (iii) effects on the phosphorylation status of Nrf2 as a result of the action of the co-operating factor such as the generation of reactive oxygen species. A compound null mutant for Nrf2 and AhR has been described recently by Noda and colleagues (2003), which was generated due to the increasing number of genes regulated by Nrf2 and AhR where the exact mechanism is not understood. This could prove a useful model for the investigation of Nrf2/AhR interactions.

There is a link between agonists of AhR (and Nrf2) and increased cell proliferation. The role of AhR in proliferation appears to be complex with variation between cell types and the AhR agonist used. For example, Zatloukalova and colleagues (2007) used the agonists β -NF and 3'-methoxy-4'-

nitroflavone (3M4NF) in an *in vitro* model of liver “stem-like” and found increased AhR-dependent proliferation with down-regulation of proteins involved in cell-to-cell contact. Vondracek and colleagues (2005) tested a range of polychlorinated biphenyls (PCBs) in a rat liver epithelial cell line and found a correlation between increased cell proliferation and the ability of the PCBs to activate the AhR. However, in contrast to these findings sustained activation of the AhR has been shown to attenuate liver regeneration following 70-percent PH, whereby administration of AhR agonists such as TCDD disrupted progression through the G₁ phase of the cell cycle (Mitchell *et al.*, 2006).

The response of the HCaRG and Nob1p promoter constructs to β -NF (Figure 5.5) indicates that the inhibition observed in response to liver growth agent administration is regulated by either the AhR, Nrf2, or a combination of both transcription factors. Such a co-ordinated response has been observed previously in the regulation of NQO1 in hepatoma cells (Ma *et al.*, 2004) whereby the presence of AhR/ARNT was required for induction of NQO1, but functional Nrf2 was necessary for basal and inducible expression of the gene in response to the AhR ligands tetrachlorodibenzo-*p*-dioxin (TCDD) and tert-butylhydroquinone (tBHQ). The use of an Nrf2/AhR compound null mutant mouse (Noda *et al.*, 2003) confirmed that Nrf2 is essential for the induction of NQO1 expression observed in response to the AhR agonist 3-MC. Significantly it has been shown using electromobility shift assays that the AhR is part of an ARE-binding complex isolated from the nuclei of mouse Hepa-1 cells (Vasiliou *et al.*, 1995) and hence the AHR could participate in both a TCDD-induced XRE response and an oxidative stress-induced response via the ARE. This effect may also occur in extrahepatic tissue such as white adipose tissue, where the Ahr and Nrf2 pathways have been shown to be active, hence lipophilic compounds accumulating in this tissue could induce detoxification via these pathways (Yoshinari *et al.*, 2006). Whether the AhR and Nrf2 require a functional interaction is not known, and further analysis is required with regard to the importance of spacing between XRE and ARE elements in the cross-talk between the two transcription factors.

To attempt to differentiate the influence of the AhR and Nrf2 on HCaRG and Nob1p gene expression, FaO cells transfected with the HCaRG and Nob1p promoter constructs were exposed to the AhR agonists 3-MC (Figure 5.8) and benzo(a)pyrene (Figure 5.9). The response to these

compounds was reduced compared to dosing with either β -NF (Figure 5.5) or the liver growth agents (Figure 4.28), with benzo(a)pyrene only producing only a modest inhibition of SEAP expression for both promoter constructs. The response to 3-MC was similar to that observed with β -NF although the same extent of inhibition was not achieved. In explanation of this observation, benzo(a)pyrene is an AhR agonist and as such a monofunctional inducer, whereas 3-MC is known to be a bifunctional inducer of drug metabolism in that it can induce both Phase I and Phase II genes (Prochaska and Talalay, 1988). Kwak and Kensler (2006) showed that 3-MC induces expression of the proteasomal gene *Psm5* predominantly via activation of the ARE and not the XRE in the gene promoter. Hence, the diminished inhibition of the reporter gene constructs in response to the AhR agonists benzo(a)pyrene and 3-MC observed herein is indicative that whereas AhR does play a role in the inhibitory action, activation of the AhR alone is not sufficient to inhibit expression from the *HCaRG* and *Nob1p* genes.

A highly relevant finding by Miao and colleagues (2005) places the Nrf2/ARE pathway downstream of the AhR/XRE pathway. Analysis of the mouse Nrf2 upstream promoter region identified three potential AhR binding motifs that were each found to respond to treatment with the AHR ligand TCDD using a luciferase reporter assay. Furthermore binding of the AhR was shown for each of the XREs using chromatin immunoprecipitation (ChIP), and silencing of AhR expression ablated the induction of Nrf2 expression in response to TCDD. The implication of these findings is that activation of AhR leads to increased expression of phase I and phase II enzymes both through direct interaction with regulatory elements for these genes, but also via Nrf2 signalling, forming an integrated system for the detoxification of xenobiotics. This finding that signalling by the AhR/XRE pathway may be upstream of the Nrf2/ARE pathway may explain why dosing of the *HCaRG* and *Nob1p* promoter constructs with the AhR agonist benzo(a)pyrene failed to produce the full extent of response observed for β NF. Dosing with an AhR agonist may lead to decreased expression of downstream genes such as *HCaRG* and *Nob1p* via induction of the Nrf2/ARE pathway, however the direct activation of Nrf2 using a compound such as β -NF may increase this effect on *HCaRG* and *Nob1p* expression or cause an earlier response. Similarly the co-operation between Nrf2 and the AhR observed for many other genes may be required for the full

response of the HCaRG and Nob1p promoters to β -NF, with AhR agonists alone not being sufficient to produce the full extent of inhibition observed.

Another possible theory for the response of HCaRG and Nob1p to liver growth agent administration is alteration of the methylation pattern of the gene promoters (Section 1.3.3.1). It is possible that transactivation of these genes is repressed following exposure to liver growth agents due to hypermethylation of CpG islands in the HCaRG and Nob1p promoters. There is no current literature regarding the extent of methylation of these genes specifically, however there is evidence that binding of the AhR to the XRE can be inhibited by hypermethylation within the core sequence of the XRE (Takahashi et al., 1998). Mulero-Navarro and colleagues (2006) have recently identified the AhR promoter as being hypermethylated in tumour cells from a number of leukaemia patients, which resulted in very low level expression of the AhR in these cells. It was also shown that the mechanism for this down-regulation of AhR expression was impaired binding of Sp1 to the AhR promoter due to hypermethylation.

The data presented herein suggests, as discussed above that Nrf2 and AhR co-operate to produce the inhibition of gene expression observed by microarray analysis; one potential way of further dissecting this interaction was through the ablation of specific protein:DNA interaction sites within the proximal promoters of the HCaRG and Nob1p genes. The HCaRG-93 bp construct contained only one potential Nrf2 site and mutation of the central nucleotides for Nrf2 transactivation failed to remove the inhibition of SEAP expression observed in response to the compounds tested (Figure 5.14). It is possible that the mutation was not sufficient to prevent Nrf2 binding, and subsequent deletion of the region would be useful in determining whether the site was functional. However it could be possible that the multiple AhR sites in this region of the HCaRG promoter were able to compensate for the removal of the influence of Nrf2. Unfortunately it was not possible to determine the extent of involvement of the potential Nrf2 site located within the exon 1 sequence of Nob1p. Mutation of one of the two potential AhR response elements in the Nob1p-71 bp construct (Figure 5.15), and the potential Nrf2 site in the Nob1p-312 bp construct (Figure 5.16) similarly failed to ablate the response to liver growth agent administration. Further work would

include a more comprehensive series of mutagenesis experiments to examine the effect of multiple ablations within the proximal promoters of these genes.

For the majority of the dosing experiments performed using the HCaRG and Nob1p promoter constructs, the peroxisome proliferators clofibric acid and Wy-14,643 failed to produce the same extent of response as that of dexamethasone and CPA. This was most notable during dosing of the HCaRG-93 bp and Nob1p-71 bp deletion constructs (Figure 5.4) where treatment with clofibric acid (100 μ M) produced ≤ 50 -percent of the inhibition of SEAP expression observed for dexamethasone and CPA treatment. A possible explanation for this finding is that the peroxisome proliferators used in this study could act on HCaRG and Nob1p predominantly through induction of the AhR/XRE pathway as opposed to the Nrf2/ARE pathway. In support of this is the finding of Fallon and colleagues (2005) where the Caco-2 cell line was used to evaluate the effect of Wy-14,643 and 3-MC on induction of CYP1A1 expression, an enzyme largely under the control of the AhR. Exposure to Wy-14,643 (200 μ M) alone caused a two-fold increase in the expression of CYP1A1 over control. Co-administration of Wy-14,643 and 3-MC produced an additive effect on CYP1A1 expression at low doses of the peroxisome proliferators (30 μ M), and a synergistic effect was observed using a higher dose (200 μ M). This suggests that activation of PPAR α by Wy-14,643 led to both direct induction of the expression of CYP1A1 via the PPREs in the CYP1A1 promoter, but also enhanced the promoter's inducibility by AhR agonists via increasing AhR expression. The mechanism for the induction of AhR by PPAR α agonists is not known at present, but could be relevant to the response of HCaRG and Nob1p to peroxisome proliferators. Unlike the peroxisome proliferators used in this study, there is currently no evidence linking either dexamethasone or cyproterone acetate to activation of the AhR.

Based on the results obtained from the dosing experiments using AhR and Nrf2 agonists and from literature sources, the following model can be proposed (Figure 6.2). The predicted model for the regulation of HCaRG and Nob1p implicates a degree of cooperation between Nrf2 and AhR, as has been identified previously (Ma *et al.*, 2004), in the response to the liver growth agents dexamethasone, CPA and WY-14,643. It should be noted that basal expression of HCaRG and Nob1p could be under the principal control of another system, although the CHX experiments are

suggestive that this is a labile factor, which would be consistent with a role for Nrf2. However, following exposure to mitogenic stimuli, we propose that the release of Nrf2 from Keap1 coupled with activation and nuclear translocation of the AhR would combine to inhibit expression of the HCaRG and Nob1p genes.

Since Nob1p is essential for 26S proteasome synthesis (Tone *et al.*, 2002), decreased cellular levels of Nob1p could reduce the degradation of proteins such as cell cycle regulators and oxidatively-damaged proteins that are known to accumulate in various degenerative diseases (Davies and Shringarpure, 2006), and of Nrf2 itself, all of which rely on degradation by the 26S proteasome to regulate their cellular levels. Once activated and released from Keap1, Nrf2 translocates to the nucleus to activate various downstream genes via binding to ARE sequences in gene promoters (Itoh *et al.*, 1999). However, Keap1 can shuttle in and out of the nucleus to promote ubiquitylation of Nrf2 and target it for degradation by the 26S proteasome (Nguyen *et al.*, 2005). Reduced levels of Nob1p may impact on this ability to reduce cellular levels of activated Nrf2 through proteasomal degradation. This could lead to a circular amplification of the effect, through the removal of the degradation pathway, which may lead to further decreases in the expression of protective genes such as HCaRG that have been shown to cause G₂M cell cycle arrest and promote cellular differentiation (Devlin *et al.*, 2003). This in combination with potential effects on the other genes within this group (LRCH4 and Sp3) that may also act as transcription factors could lead to cellular transformation events that could ultimately result in hepatocarcinogenesis.

The inhibition of expression of Nob1p in response to liver growth agents observed herein suggests that the formation of fully functional 26S proteasomes may be impaired in hepatocytes exposed to such compounds. This in turn would potentially affect the degradation rates of numerous proteins including that of the Nrf2 protein itself. Contrary to this hypothesis was the finding by Kwak and Kensler (2006) that peptidase activities of the 26S proteasome and the expression of the catalytic core subunit PSMB5 were induced by 3-MC, and that this was controlled exclusively by the Nrf2/ARE pathway. Although this supports the idea that Nrf2 expression is a self-regulated system, it is not indicative of the reduced degradation of Nrf2 in response to compounds such as 3-MC and β -NF. Similarly, Meiners *et al.* (2002) showed that proteasome inhibition caused an increase

in the expression of many mammalian 26S proteasomal genes, suggesting that this inhibition could be averted by increased *de novo* synthesis of proteasomes. However this analysis was performed prior to the cloning of the human NOB1 gene (Zhang *et al.*, 2005) and hence the effect on this essential protein could not be assessed.

Figure 6.2: Proposed model for the pathway from liver growth agent administration to liver growth

Liver growth agents entering the cell may bind to the cytoplasmic Nrf2/Keap1 complex causing release of Nrf2 that can translocated to the nucleus. Similarly AhR may translocated to the nucleus in combination with its nuclear translocator protein ARNT. The combination of Nrf2 binding to the antioxidant response element (ARE) and AhR binding to the xenobiotic response element (XRE) in gene promoters may cause decreased expression of genes such as HCaRG and Nob1p. HCaRG can suppress the cell cycle via G₂M arrest (Devlin *et al.*, 2003) and inhibition of its expression may remove a protective influence against cellular transformation. Nob1p is essential for 26S proteasome synthesis (Tone *et al.*, 2002), hence decreased expression of Nob1p would affect proteasomal degradation of proteins. This could include the degradation of Nrf2 following promotion of its ubiquitylation by Keap1 that is thought to enter the nucleus to modulate Nrf2 levels (Nguyen *et al.*, 2005). This could result in positive feedback on Nrf2 levels that would amplify the situation. These effects in combination with those on other co-regulated genes could ultimately lead to carcinogenesis.

The work presented herein suggests that exposure of cells to compounds such as 3-MC could lead to decreased Nob1p expression and reduced levels of functional 26S proteasomes. In support of this hypothesis is the finding that treatment of thymocytes with dexamethasone reduces 20S and 26S proteasomal activity in cell extracts (Beyette *et al.*, 1998). Western Blotting using an antibody to the C2 subunit that is present in 20S and 26S proteasomes showed that this effect was not due to a change in the number of proteasomes in response to dexamethasone, hence it could be an alteration in the functional capacity of the proteasomes. Since Nob1p is thought to be a chaperone for joining of the 19S regulatory particle with the 20S catalytic core of the proteasome (Tone *et al.*, 2002), decreased Nob1p may cause disruption of this process and decreased activity of the resultant proteasomes.

A link between the coupling of the AhR and Nrf2 pathways and chemoprevention has been postulated previously, with disruption of the pathways leading to increased carcinogenesis. Cooperation between the AhR and Nrf2 was reviewed recently with respect to the role of dietary phytochemicals in chemoprevention (Kohle and Bock, 2006). In particular this indicated flavonoids (sourced from green tea) and dithiolthiones (from cruciferous vegetables) to be activators of both the AhR and Nrf2 pathways. Nrf2-deficient mice have been shown to display a reduced hepatic and gastric complement of the phase II detoxifying enzymes NQO1 and GST, and that the effect of the chemopreventative compound oltipraz (a dithiolthione) on increasing the expression of these enzymes was abrogated in these animals (Ramos-Gomez *et al.*, 2001). As such the incidence of gastric neoplasia in the Nrf2-deficient animals in response to treatment with benzo(a)pyrene was significantly greater than in controls (Ramos-Gomez *et al.*, 2001).

The role of Nrf2 and AhR in the regulation of HCaRG and Nob1p is still to be determined. Following characterisation of this regulation it would be possible to determine the extent of co-regulation between HCaRG, Nob1p and the other five genes identified as exhibiting potential co-ordinate regulation in Chapter 3. If it is the case that increased Nrf2/AhR binding to the promoters of HCaRG and Nob1p results in inhibition of these protective genes then it would be interesting to understand the reason for this inhibition, since the majority of pathologies result from an underlying physiological mechanism responding inappropriately to a new stimulus.

In summary, we have identified a group of genes exhibiting potential co-ordinate regulation in their expression, which has been confirmed for two of these genes. We are the first to postulate a link between these two genes, HCaRG and Nob1p, for which little information is currently available particularly with regard to their upstream regulation. Similarly neither gene has previously been implicated in the response to compounds causing liver growth. We have examined the promoter functionality of the proximal 1 kb gene promoters, and are the first to identify a role for the transcription factors Nrf2 and AhR in the regulation of HCaRG and Nob1p, both individually, and regarding their co-regulation. This work has also contributed to the increasing finding that Nrf2 and the AhR co-operate in the regulation of genes in response to xenobiotic stimuli.

6.2 FUTURE DIRECTIONS

The results obtained during the course of this project have highlighted an interesting group of genes with high similarity in their response to growth stimuli and which exhibit co-ordination in their regulation at the level of transcription. However, biological function is generated at the protein level and hence assessment at the transcript level alone is not sufficient to assume that the changes observed are biologically relevant. To address this it would be necessary to characterise the response at the protein level for example using Western blotting. Currently antibodies are commercially available for rCOUPg and Sp3 in the rat, and antibodies have been developed against human HCaRG (Solban *et al.*, 2000) and Nob1p in yeast (Tone *et al.*, 2000).

To characterise the regulation of HCaRG and Nob1p it would be useful to silence Nrf2 and AhR individually and jointly to assess the impact on the inhibition in response to liver growth agent administration, and at the same time to assess the effect on cellular proliferation (e.g. BrdU incorporation) and apoptosis (e.g. caspase activation). Initial optimisation using an siRNA for Nrf2 has been undertaken (data not shown) using β NF to increase the cellular levels of Nrf2. Following completion of the optimisation it would be possible to directly assess mRNA levels of HCaRG and Nob1p using real-time quantitative PCR. Alternatively, to produce a clearer signal the relevant promoter constructs could be transfected on the day following silencing of Nrf2, and subsequently the effect of dosing with liver growth agents could be assessed relative to a negative control siRNA.

Since mutation of individual Nrf2 and AhR response elements failed to identify the influence of either transcription factor, it would be useful to produce a compound mutant through repeated rounds of SDM. Similarly, it would be interesting to overexpress Nrf2 and AhR in FaO cells. This, in comparison to the silencing experiment, would identify whether it was increased or decreased transactivation of the HCaRG and Nob1p gene promoters by Nrf2 and/or AhR that was resulting in inhibition of gene expression. Ablation of the response to liver growth agents would indicate which possibility was true. Following silencing and overexpression of Nrf2 and/or AhR it would be possible to assess the potential co-ordinate regulation between HCaRG, Nob1p and the other genes namely Sp3, LRCH4, rCOUPg and OGFRL1, using real-time quantitative PCR to measure the effect on the transcript levels of all six genes. Binding studies using either an EMSA, supershift

assay or ChIP (subject to antibody availability) would prove whether Nrf2 and AhR do bind to the HCaRG and Nob1p promoters under basal and drug-induced conditions. This would increase the understanding of the regulation of these genes both physiologically and during drug-induced liver growth. Immunocytochemical staining of Nrf2 and AhR would also be useful in determining whether nuclear localisation of the transcription factors occurs in response to liver growth agent administration.

It would be interesting to pursue some of the other genes highlighted during the microarray analysis (Chapter 3) with a view to identifying further potential markers of liver growth. Finally it would be necessary to compare the response of the rat genes identified to that of the human orthologues to assess (i) the degree of evolutionary conservation for the pathway, (ii) whether the genes were involved in hepatomegaly in humans, and (iii) to what extent the genes would be useful as markers of liver growth for use in the pharmaceutical industry.

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8 APPENDIX

8.1 VECTOR MAPS

Figure 8.1: pSEAP plasmid

MCS: Multiple cloning site, SEAP: secreted alkaline phosphatase, SV40 poly A: SV40 polyadenylation, Amp^r: lactamase conferring resistance to ampicillin

Figure 8.2: TOPO® pCR®2.1 plasmid (Invitrogen)

lacZα: lacZ gene, f1 ori: origin of replication of the filamentous phage f1, Kanamycin: Kanamycin resistance gene, Ampicillin: lactamase conferring resistance to ampicillin, pUC ori: pUC origin of replication

Figure 8.3: pCR®-Blunt plasmid (Invitrogen)

lacZα: lacZ gene, ccdB: ccdB cytotoxic gene, Kanamycin: Kanamycin resistance gene, Zeocin: Zeocin resistance gene, pUC ori: pUC origin of replication

8.2 ORAL PRESENTATIONS OF THIS RESEARCH

British Toxicology Society (BTS) Annual Congress, University of Warwick, March 2006 - highly commended.

Drug Metabolism Discussion Group (DMDG), Heriot-Watt University, September 2006.

8.3 POSTER PRESENTATIONS OF THIS RESEARCH

British Toxicology Society (BTS) Annual Congress, Heriot-Watt University, March 2004.

British Toxicology Society (BTS) Autumn Meeting, University of Liverpool, September 2004.

Pfizer Drug Discovery, Sandwich, Kent, September 2004.

13th North American Meeting of the International Society for the Study of Xenobiotics (ISSX), Maui, Hawaii, October 2005 (see following page).

Introduction

- Many xenobiotics are known to cause augmentative liver growth in rodents, a process which may ultimately result in **carcinogenesis**
- Induction of liver growth in rodents results in the end of development of a candidate drug → advantageous to eliminate such compounds early in development
- Rodent hepatocytes proliferate rapidly in response to certain non-toxic liver growth agents (e.g. peroxisome proliferators (PPs))

↑ DNA synthesis and ↓ apoptosis (Roberts *et al.*, 1995)

- PP-IV increases hepatocyte DNA synthesis to >30% the basal level (Plant *et al.*, 1996)
- PP-IV reversibly suppresses TGF- β 1, CD95, and etoposide-induced apoptosis *in vitro* (Gill *et al.*, 1998)
- The mechanism controlling this coordinated response to liver growth agents is not well understood at present

Aims

- ❖ To identify genes exhibiting coordinate regulation during drug-induced liver growth
- ❖ To identify candidate regulatory elements responsible for this regulation and investigate their use as potential markers of liver growth

Results (DNA Microarray Analysis)

LIVER GROWTH AGENTS

Cyproterone acetate (CPA)	60 mg/kg
Dexamethasone (DEX)	100 mg/kg
WY-14,643 (WY)	50 mg/kg

Groups of male Sprague-Dawley rats ($n = 3$) received either drug or vehicle control

FRAGMENTS CLUSTERED MANUALLY INTO GROUPS
(DIRECTION OF CHANGE NOT MAGNITUDE OF CHANGE)

The group selected showed inhibition of gene expression in response to liver growth agents. The group contains six genes: nin one binding protein (*Nob1*); *Sp3*; *COUP-2* (*rCOUP-2*); hypertension-related, calcium-regulated gene (*HtCaRG*); opioid growth factor receptor-like 1 (*OGFR-L1*) and leucine-rich repeats and calcitonin receptor domain-containing 4 (*LRC44*).

Microarray results were confirmed using TaqMan™ Real-Time PCR on *in vivo* samples (*n* = 5) dosed as in the microarray experiment (data not shown) → profiles were maintained but with reduced fold-change

Results (Reporter Gene Assay)

^b regulatory regions for the six genes were obtained (NCBI database) and 1 kilobase of upstream promoter sequence cloned into an expression vector upstream of the secreted alkaline phosphatase (SEAP) gene. Negative control (pSEAP) and positive control (SV40 promoter upstream of SEAP gene) were analysed simultaneously.

Constructs were transfected into FaO rat hepatoma cells using Transfast™ reagent (Promega). Cells were used between passages 5 and 8. SEAP was assayed using the AURORA™ kit (MP Biochemicals).

One-way ANOVA with Bonferroni post-hoc test used to assess for statistical significance versus DMSO control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

HCaRG post-dose SEAP expression

The response to liver growth agents is similar between genes and between compounds. Therefore the 1 kb of promoter sequence adjacent to the transcription start site (TSS) must contain important regulatory elements. Deletion mutants were then produced for each gene and dosed with liver growth agents for 48 hours to pinpoint the region containing these regulatory elements:

HCaRG deletion mutants

- 158 bp

Dexamethasone and cyclo-oxygenase both repress gene transcription of the HCARG and Nob1p reporter genes. As both liver growth agents repress expression in all tested fragments, this demonstrates that the transcription factor(s) mediating this effect must bind within the first ~150 bp of the proximal promoters of these two genes.

III

Results (Promoter Analysis)

In silico analysis was undertaken to identify putative transcription factor binding sites that may mediate the observed effects (Krausspeiser²⁰). Within the first 150 bp of the H-CaRG and Nob-1p proximal promoters common putative binding sites are present that may mediate the drug-induced repression of gene expression.

IV

Results (NRF2 ligand)

To examine the potential role of NFE2-related factor 2 (NRF2) or the aryl hydrocarbon receptor (AhR) in the repression of gene expression, reporter constructs were exposed to activators of these two transcription factors. β -naphthoflavone (β -NF) is a ligand for NRF2 and AhR. Cells were transfected with the deletion mutant of interest and with the full 1 kb promoter constructs, and a dose response generated across 1 to 100 μ M.

β -NF produced a similar response to the liver growth agents indicating the involvement of either NRF2 or AhR in the response. An AhR ligand (benzo(a)pyrene) was dosed across a similar concentration range and failed to produce any change versus control (data not shown). Therefore NRF2 is the likely transcription factor causing the inhibition of expression of HCAR2 and Nob1 α .

Conclusions

Manipulation of initial microarray data yielded a group of genes exhibiting potential co-regulation. These show decreased expression in response to liver growth; agents → indicates a possible inhibitory effect on growth and/or carcinogenesis

Reporter gene assay using the 1 kb upstream promoters confirmed *in vivo* experiments, and deletion mutants showed the key regulatory element(s) to lie within 160 bp of the TSS for each promoter. Mainspector™ also identified many potential regulatory elements of which NRF-2 shows potential regulation. Proof of involvement is required using:

- ❖ Mutation studies to confirm input of individual regulatory elements (NR2) within gene promoters
- ❖ Electromobility shift assay (EMSA) to confirm TF-DNA interactions within gene promoters
- ❖ Gene silencing of the appropriate transcription factor using RNAi to assess the effect on proliferation

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